

Toxinology

P. Gopalakrishnakone *Editor-in-Chief*

Hidetoshi Inagaki

Carl-Wilhelm Vogel

Ashis K. Mukherjee

Tarek Rashed Rahmy *Editors*

Snake Venoms



SpringerReference

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Editor-in-Chief

P. Gopalakrishnakone

In recent years, the field of toxinology has expanded substantially. On the one hand it studies venomous animals, plants and micro organisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on human body. Given the huge impact of toxin-based deaths globally, and the potential of venom in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that collects all of the important biochemical, biomedical and clinical insights relating to toxinology.

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Editors

Snake Venoms

With 87 Figures and 32 Tables

 Springer

Editor-in-Chief

P. Gopalakrishnakone
Venom and Toxin Research Programme
Department of Anatomy
Yong Loo Lin School of Medicine
National University of Singapore
Singapore, Singapore

Editors

Hidetoshi Inagaki
Biomedical Research Institute
National Institute of Advanced Industrial
Science and Technology (AIST)
Tsukuba, Ibaraki, Japan

Carl-Wilhelm Vogel
University of Hawaii Cancer Center
Honolulu, HI, USA

Ashis K. Mukherjee
Department of Molecular Biology and
Biotechnology, Research and Development
ONGC-Centre for Petroleum Biotechnology
DBT Nodal Centre for Medical Colleges and
Biomedical Research Institutes of North-East
India (Unit of Department of Biotechnology
Govt. of India)
Tezpur University
Tezpur, Assam, India

Tarek R. Rahmy
Suez Canal University
Ismailia, Egypt

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Series Preface

The term TOXIN is derived from the Greek word *Τοεικον* and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On the one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology, etc. In addition, there is a fast developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally and the potential of venom in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy target of producing about 12 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

P. Gopalakrishnakone
M.B.B.S., Ph.D., F.A.M.S., D.Sc.
Editor-in-Chief

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I would like to sincerely thank the section editors of this volume, Hidetoshi Inagaki, Carl-Wilhelm Vogel, Ashis K. Mukherjee, and Tarek R. Rahmy for the invaluable contribution of their expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

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Singapore

P. Gopalakrishnakone
MBBS, PhD, FAMS, DSC
Editor-in-Chief

Volume Preface

As we see with the Rod of Asclepius in Greek mythology, ancient people believed that snakes have mystical powers to heal illness via their venoms, even though they did not know the essence of these powers. Since about 100 years ago, new technologies have been developed to analyze the components, structures, and physiological functions of snake venoms, and we have gradually figured out the essence of the powers. Furthermore, we can obtain various diagnostic and therapeutic agents from snake venoms.

Initially, because of the inadequate sensitivity of devices and other limitations, only major venom components of the dominant species were studied. However, in the last two decades, increased sensitivity of devices and the development of new techniques, such as transcriptomics and proteomics techniques, have permitted us to analyze the structures and functions of rare species and to identify novel minor components. As a result, the number of venom components identified is increasing dramatically and we can understand the physiological functions of each component more deeply.

The scope of this book provides the readers an updated and comprehensive presentation on snake venoms. The subjects are divided into six sections, starting with the overview of snake venom compositions in Malaysian species. We then describe the enzymes and enzyme inhibitors contained in snake venoms. We also discuss the effects of snake venom on cellular receptors, ion channels, blood, and lymph, as well as cytotoxic and antimicrobial molecules present in snake venoms. The last section covers the clinical consequences of envenomation and the management of snakebites using antivenins.

We would like to express our appreciation to all the authors for contributing to this project and providing their scientific knowledge. We are particularly indebted to Professor Gopalakrishnakone of the National University of Singapore, who started up and advanced this project, and allowed us to contribute. Finally, we would like to thank Meghna Singh, Sunali Mull, Sarah Mathews, and Audrey Wong of Springer, who patiently supported us on this project.



Statue of Asclepius, exhibited in the Museum of Epidauros Theatre, in Argolis on the Peloponnese peninsula, Greece (Photo by Michael F. Mehnert (Own work) (GFDL [<http://www.gnu.org/copyleft/fdl.html>] or CC BY-SA 3.0 [<http://creativecommons.org/licenses/by-sa/3.0/>]), via Wikimedia Commons)

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University of Hawaii Cancer Center
Honolulu, HI, USA

Carl-Wilhelm Vogel M.D., Ph.D.

Department of Molecular Biology and
Biotechnology, Research and Development
ONGC-Centre for Petroleum Biotechnology
DBT Nodal Centre for Medical Colleges and
Biomedical Research Institutes of North-East India
(Unit of Department of Biotechnology, Govt. of India)
Tezpur University
Tezpur, Assam, India

Ashis K. Mukherjee Ph.D.

Biomedical Research Institute
National Institute of Advanced Industrial
Science and Technology (AIST)
Tsukuba, Ibaraki, Japan

Hidetoshi Inagaki Ph.D.

Suez Canal University
Ismailia, Egypt

Tarek R. Rahmy Ph.D.

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Editor-in-Chief



P. Gopalakrishnakone

Venom and Toxin Research Programme
Department of Anatomy
Yong Loo Lin School of Medicine
National University of Singapore
Singapore, Singapore
antgopal@nus.edu

P. Gopalakrishnakone, M.B.B.S., Ph.D., F.A.M.S., D.Sc., is professor of anatomy and chairman of the Venom and Toxin Research Programme at Yong Loo Lin School of Medicine, National University of Singapore, where he has become an emeritus professor. Prof.

Gopal has also got a new appointment in the newest University in Singapore, Singapore Institute of Technology (SIT), as a professor of anatomy in the Health and Social Sciences Cluster. Prof. Gopalakrishnakone is also a consultant to the Defence Science Organization in Singapore; adjunct senior research scientist at the Defence Medical Research Institute; and an honorary principal fellow at the Australian Venom Research Unit, University of Melbourne, Australia.

His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employs include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of *Toxicon*, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000

Award in Singapore; and the Research Excellence Award from the Faculty of Medicine at NUS (2003).

His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Professor Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

Editors

**Hidetoshi Inagaki**

Biomedical Research Institute
National Institute of Advanced
Industrial Science and Technology (AIST)
Tsukuba, Ibaraki
Japan
h-inagaki@aist.go.jp

Hidetoshi Inagaki is Senior Researcher at the National Institute of Advanced Industrial Science and Technology, in Japan, since 2001, working on the bioactive peptides of venomous animals, such as snakes and ants. During that time, from April 2006 to March 2007, he worked at CSTP (Council for Science and Technology Policy) on the “Guidelines on the Derivation and Distribution of Human Embryonic Stem Cells” and other science policies. From April 1994 to March 2001, Inagaki was a researcher at the National Industrial Research Institute of Nagoya, Japan.

Inagaki received his Ph.D. from Nagoya University, Japan, in 1994, with time spent in Beth Israel Hospital, Boston, United States, from August 1991 to March 1992. He was also a graduate (April 1989–March 1991) and an undergraduate (April 1984–March 1989) at Nagoya University, Japan.

For last 5 years, his research group has been working on the directed evolution of bioactive peptides. Using directed evolution, the group could generate agonist- and antagonist-like molecules of receptors and enzyme inhibitors.

**Carl-Wilhelm Vogel**

University of Hawaii Cancer Center
Honolulu, HI
USA
cvogel@cc.hawaii.edu

Carl-Wilhelm Vogel received his M.D. degree and Ph.D. degree in Biochemistry from the University of Hamburg in Germany. For three-and-a-half years, Dr. Vogel was a postdoctoral research fellow in molecular immunology at Scripps Clinic and Research Foundation in La Jolla, California. He completed a medical internship in Germany and 4 years of residency training in clinical pathology at Georgetown University in Washington, D.C., and at Indiana University/Purdue University in Indianapolis. He is a licensed physician and a board-certified clinical pathologist both in the USA and Germany.

He was on the faculty of Georgetown University School of Medicine for approximately 9 years in the Departments of Biochemistry and Molecular Biology and Internal Medicine, and a member of the Lombardi Cancer Center, before assuming the chairmanship of the Department of Biochemistry and Molecular Biology at the University of Hamburg in 1990. In 1999, he became Director of the Cancer Research Center of Hawaii at the University of Hawaii, a position he held for about a decade. He currently is a full professor at the same institution.

His research interests have been in the area of basic biomedical research with particular emphasis on the immunological aspects of cancer as well as the development of novel therapeutic concepts for diseases with complement pathogenesis, based on the complement-depleting activity of cobra venom factor. His research has been supported continuously by peer-reviewed grants since 1983, mainly from the National Institutes of Health. He is the author of well over 100 publications and patents.

**Ashis K. Mukherjee**

Department of Molecular Biology and
Biotechnology, Research and Development
ONGC-Centre for Petroleum Biotechnology
DBT Nodal Centre for Medical Colleges and
Biomedical Research Institutes of North-East India
(Unit of Department of Biotechnology, Govt. of India)
Tezpur University
Tezpur, Assam, India
akm@tezu.ernet.in

Ashis K. Mukherjee is currently serving as Dean, Research and Development and Professor in the Department of Molecular Biology and Biotechnology at Tezpur University, Assam, India. Dr. A.K. Mukherjee received a Bachelor of Science in

Chemistry (Hons.) from Banaras Hindu University, Varanasi, India, in 1990 and a Master of Science in Biochemistry from the same university in 1992. He was awarded a Ph.D. degree in 1998 from the Department of Biochemistry, Burdwan Medical College, Burdwan University, India. His topic of Ph.D. research was on biochemistry and pathophysiology of Indian cobra and Russell's viper venom. The current research activity of Dr. Mukherjee broadly includes biochemistry and drug discovery from snake venom as well as microbial biotechnology. His research has been funded by several extramural research grants. In addition to receiving several other prestigious awards, he was awarded a DST-BOYSCAST fellowship from the Government of India to work on spider venom toxins at the University of Connecticut Health Center, Connecticut, USA (2002–2003), and the DBT-Crest Award (2011–2012) from the Government of India to work as a visiting scientist at the School of Biological Sciences, University of Northern Colorado, Colorado, USA, and National Bioscience Award for Career Development-2013 from the Department of Biotechnology, Govt. of India. He is the recipient of Best Researcher Award of Tezpur University in 2008. Dr. Mukherjee has published more than 90 research papers in peer-reviewed journals and contributed several book chapters, and in the editorial board member of many journals. He has guided eight graduate students for the Ph.D. degree and 32 students for the M.Sc. dissertation work. Dr. Mukherjee also teaches numerous graduate and undergraduate courses in biochemistry, enzymology, toxinology, and fermentation technology.



Tarek R. Rahmy

Suez Canal University

Ismailia

Egypt

tarekrah@hotmail.com

egysocnattox@yahoo.com

Tarek R. Rahmy, Ph.D., is currently the Vice President for Teaching and Student Affairs at Suez Canal University (SCU). He is a Professor of Histology and Cytology, Zoology Department, Faculty of Science; Head of the same department and Director of the University Teaching Development Center; and Dean of Fish Farming and Technology Institute at SCU. He graduated from the Zoology Department, Faculty of Science, SCU, in 1981, and subsequently worked as demonstrator in the same department, before going to Colorado State University (USA) where he obtained his Ph.D. in 1989 through a joint supervision program, before working as a lecturer, an associate professor, and a professor at the Zoology Department at SCU (2001). His main interests are in toxinology, especially in the pathogenesis of snake and scorpion envenoming as well as in the use of natural products to neutralize their effects. He is a member of the National Committee of Toxicology in Egypt, and the founder and chairman of the Egyptian Society of Natural Toxins (2003 to date). He had obtained many research grants from Egypt,

UAE, and Saudi Arabia, and organized six international conferences and four workshops on natural toxins. He is also the coeditor of the *Egyptian Journal of Natural Toxins* (12 volumes) and a peer reviewer for quality assurance at the Egyptian Council of Universities, has written and revised chapters of several books on vertebrate biology, and participated in the translation of the biological *Glossary of Campbell Biology*. He has conducted many field surveys in Egypt, USA, UAE, and Saudi Arabia on the arid ecosystems with focus on venomous snakes and published 62 scientific papers. He has taught 16 courses on different aspects of histology, cytology, cell biology, histochemistry and vertebrate biology, and natural toxins, as well as supervised more than 23 M.Sc. and Ph.D. students. He has participated in more than 50 conferences, seminars, and workshops, and is a member of national and international committees that deal with toxicology.

Contributors

Alejandro Alagón Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

Miryam Paola Alvarez-Flores Biochemistry Laboratory, Butantan Institute, São Paulo, Brazil

Satohiko Araki Department of Science, Nagoya University, Nagoya, Japan

Wasim Awal School of Medical Science, Griffith University, Gold Coast, QLD, Australia

Leslie Boyer Venom Immunochemistry, Pharmacology, and Emergency Response (VIPER) Institute, University of Arizona, Tucson, AZ, USA

J. D. Campeiro Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

Patrícia Cota Campos Laboratório de Enzimologia Aplicada, Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil

Dibakar Chakrabarty Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Zuarinagar, Goa, India

Chandrasekhar Chanda Department of Biological Sciences, Birla Institute of Technology and Science, Zuarinagar, Goa, India

J. P. Chippaux Institute of Research for Development (IRD – UMR 216 “Mother and Child Facing Tropical Diseases”), Cotonou, Bénin

Faculté de Pharmacie, University Paris Descartes, Sorbonne Paris Cité, Cotonou, France

Center for the Study and Research of Malaria Associated with Pregnancy and Childhood (Cerpage), Cotonou, Benin

Ana Marisa Chudzinski-Tavassi Biochemistry Laboratory, Butantan Institute, São Paulo, Brazil

C. Dal Mas Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

Sonia Aparecida de Andrade Biochemistry Laboratory, Butantan Institute, São Paulo, Brazil

Frédéric Ducancel CEA, iMETI, Joint Unit IMVA, CEA Fontenay-aux-Roses, Fontenay-aux-Roses, France

Fernanda Faria Biochemistry Laboratory, Butantan Institute, São Paulo, Brazil

D. Felix Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

Carlos Alexandre H. Fernandes Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil

Marcos Roberto M. Fontes Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil

Consuelo L. Fortes-Dias Laboratório de Enzimologia Aplicada, Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil

David C. Fritzinger University of Hawaii Cancer Center, University of Hawaii at Manoa, Honolulu, HI, USA

Mirian A. F. Hayashi Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

Hidetoshi Inagaki Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan

Dušan Kordiš Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

Igor Križaj Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia

Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Ljubljana, Slovenia

Yazine Mahjoub Pôle d'Anesthésie-Réanimation, CHU Amiens, Unité de réanimation chirurgicale, Amiens, France

Unité INSERM U1088, Amiens, France

Stéphanie Malaquin Pôle d'Anesthésie-Réanimation, CHU Amiens, Unité de réanimation chirurgicale, Amiens, France

M. P. Marinovic Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

A. Massougbojji Center for the Study and Research of Malaria Associated with Pregnancy and Childhood (Cerpage), Cotonou, Benin

G. G. Monte Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo, SP, Brazil

Ashis K. Mukherjee Department of Molecular Biology and Biotechnology, Research and Development, ONGC-Centre for Petroleum Biotechnology, DBT Nodal Centre for Medical Colleges and Biomedical Research Institutes of North-East India (Unit of Department of Biotechnology, Govt. of India), Tezpur University, Tezpur, Assam, India

Navin R. Niranjana Faculty of Science, University of Queensland, Brisbane, QLD, Australia

Selvanayagam Nirthanan School of Medicine, Griffith University, Gold Coast, QLD, Australia

School of Medical Science, Griffith University, Gold Coast, QLD, Australia

Menzies Health Institute Queensland, Gold Coast, QLD, Australia

Alexey V. Osipov Laboratory of Molecular Toxinology, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

Dayanira Paniagua Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

Gandhi Rádís-Baptista Laboratory of Biochemistry and Biotechnology, Institute for Marine Sciences, Federal University of Ceara, Fortaleza, Ceará, Brazil

Michela Rigoni Department of Biomedical Sciences and CNR Institute of Neuroscience, University of Padova, Padova, Italy

Debashree Saikia Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, Assam, India

Angshuman Sarkar Birla Institute of Technology and Science, Pilani, Zuarinagar, Goa, India

R. P. Stock Institute of Biotechnology, National Autonomous University of Mexico (IBt-UNAM), Cuernavaca, Mexico

Denise V. Tambourgi Immunochemistry Laboratory, Research Division, Butantan Institute, São Paulo, SP, Brazil

Choo Hock Tan Department of Pharmacology, University of Malaya, Kuala Lumpur, Malaysia

Nget Hong Tan Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

Rupamoni Thakur Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, School of Sciences, Tezpur University, Tezpur, Assam, India

Fiorella Tonello Department of Biomedical Sciences and CNR Institute of Neuroscience, University of Padova, Padova, Italy

Yuri N. Utkin Laboratory of Molecular Toxinology, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, Russia

Carmen W. van den Berg Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, Cardiff, UK

Irene Vergara Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

Carl-Wilhelm Vogel University of Hawaii Cancer Center, Honolulu, HI, USA

Part I

Composition of Snake Venoms

Toxinology of Snake Venoms: The Malaysian Context

1

Choo Hock Tan and Nget Hong Tan

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Abstract

Venomous snakes native in Malaysia include the elapids (cobras, king cobra, kraits, coral snakes, sea snakes) and crotalids (Malayan pit viper, Asian lance-headed pit vipers). The elapid venoms are typically neurotoxic, while the crotalid

C.H. Tan (✉)

Department of Pharmacology, University of Malaya, Kuala Lumpur, Malaysia

e-mail: tanchoochock@gmail.com; tanch@um.edu.my

N.H. Tan

Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia

e-mail: tanngethong@yahoo.com.sg

venoms are usually hemorrhagic, coagulopathic, and necrotizing. Among the elapids, cobra and king cobra venoms produce pain and tissue-necrotizing effect, whereas sea snake venoms can cause rhabdomyolysis and acute kidney injury. Venom compositions greatly vary from species to species, resulting in differences in the clinical presentation of envenomation by different species and the varied effectiveness of antivenoms on different venoms. In Malaysia, research activities on snakes have been carried out since the 1960s; through these years venoms have been subjected to multifaceted characterizations where knowledge on toxin variants and compositions, envenoming pathophysiology, venom pharmacokinetics-pharmacodynamics, as well as antivenom neutralization had been greatly advanced. The knowledge is indispensable for improving clinical protocol on snakebite assessment and to guide on the appropriate type of antivenoms to stock in local hospitals for clinical use. This is especially important for Malaysia, where antivenoms are supplied by manufacturers from overseas without a clear standardization for the selection of antivenom source. Until today, there are research gaps and repertoires for scientists to further explore. These include proteomic profiling of venoms and venom gland transcriptomics for local species, antivenomics, antivenom formulation optimization, and so on.

Keywords

Venom • Toxin • Antivenom • Malaysian snakes

Introduction

Backgrounds

Malaysia as a tropical country is a natural habitat to a wide variety of herpetofauna. In its land and coastal waters, there are at least 18 different species of venomous land snakes and more than 22 different species of sea snakes (Tweedie 1983), comprising 3 main subfamilies of snakes: Elapinae (cobras, king cobra, kraits, coral snakes), Hydrophinae (sea snakes), and Crotalinae (pit vipers). They are the venom source of local species, with medical significance both in snakebite envenomation and drug discovery.

Epidemiological studies of snakebite in Malaysia were carried out mostly on infrequent basis through retrospective data survey in individual hospitals. The findings were fragmentary over the years and were confined to the respective hospital, thus difficult to represent the true epidemiology of snakebite in Malaysia. Based on the three most recent retrospective surveys (Jamaiah et al. 2004, 2006; Chew et al. 2011), identification of the biting species was never satisfactory as in more than 50 % of the cases, the snakes were never identified. Species diagnosis is crucial in snakebite management as it facilitates the assessment of syndrome evolution and dictates the choice of antivenom to be administrated. Unfortunately, under-reporting and neglecting of the clinical significance of snakebite has led to a lack of

emphasis in the training of envenomation management for medical doctors and healthcare providers in this country.

On the other hand, scientific research on Malaysian snake venoms appears more consistent, although remaining a rather exotic field in local academia. In as early as the postcolonial era in the 1960s, the myotoxic action of sea snake venom was demystified, and anticoagulant arvin (ancrod from the Malayan pit viper venom) was discovered (Reid 1961; Hawgood 1998). More Malaysian snake venoms have been subsequently characterized biochemically and pharmacologically, propelling the understanding of the pathophysiology of envenomation in the country. Research efforts were also seen in the development of immunological assay and antivenom assessment to improve the management of snake envenomation. In recent years, with the advent of sequencing technologies, transcriptomic and proteomic studies have enabled researchers to gain deep insights into the compositional variation of snake venoms. Such advancement is believed to be beneficial in orchestrating an integrative approach to improve the understanding of venom pathophysiology and the efficacy of antivenom for snake envenomation (Williams et al. 2011).

Snake Venom Overview: Definition, Composition, and Characteristics

Venom is regarded as a complex secretion produced in a specialized gland, typically delivered via specialized systems that include the gland, dedicated fangs/stings, and a suite of specific behaviors allowing the introduction of the venom into recipient tissues, usually through injection (in contrast to ingestion, inhalation, or transdermal absorption in the case of poisoning) (Mackessy 2009). Snake venom contains mainly proteins (70–90 %, including enzymes and nonenzymatic proteins) with small amounts of metals, amino acids, peptides, nucleotides, carbohydrates, lipids, and biogenic amines (Tu 1977). The biochemical composition of venom toxins among snake species (or even within a species) can vary greatly, although in general, the dominance of major protein families in venoms often follows a broad taxonomic trend and is hence partially predictable (Mackessy 2009). The wide diversity of snake venom toxins is due to their mode of evolution, where diet has played the central role in the adaptive radiation of snakes (Daltry et al. 1996b). Predator–prey arms race (Heatwole and Poran 1995) contributes to repeated gene duplication that creates redundancy and allows a gene copy to be selectively expressed in the venom gland, where it subsequently undergoes neofunctionalization through positive selection and adaptive molecular evolution at an accelerated rate (Kini and Chan 1999; Kordis and Gubensek 2000). In envenomation, the multiplicity of venom toxins and actions result in (i) complex clinical syndromes and (ii) varied therapeutic response to antivenom treatment due to differences in antigenicity – this underlies the principle that antivenom’s action is usually species specific.

The clinical presentation of a patient represents a complex syndrome resulted from the body responses to the pharmacological actions of different components in

snake venom. Venom toxic effects have often been conveniently classified as neurotoxic, hemotoxic, cardiotoxic, nephrotoxic, myotoxic, etc. based on the predominant clinical effect of particular venom. Organ- or system-based descriptions as such receive criticism at times that the classification oversimplifies the complexity of venom effects and does not represent the interaction of various toxins on tissues of different organs. Nonetheless, the descriptions suggest the prominent clinical syndrome of particular snakebite and hence have a practical value in management, for instance, the preparation of intubation equipment, blood products for transfusion, and dialysis facility in anticipation of the likely pathological outcome from the bite of a particular species. On the other hand, laboratory characterizations of venom toxicities can never be oversimplified, and it demands multiple disciplinary approaches, involving both in vitro and in vivo techniques.

Medically important Malaysian snakes largely originate from the Elapidae family and the Crotalinae subfamily. Venoms of many elapid snakes (cobra, krait, and sea snakes) generally produce flaccid paralysis and respiratory difficulty leading to asphyxia. Elapid venoms mainly contain proteins/peptides of low to moderate molecular mass (<15 kDa), with one key group called the three-finger toxins (6–8 kDa), which include various neurotoxins and cytotoxins (or cardiotoxins). Crotalid venoms constitute moderate- to high-molecular mass proteins (>15 kDa), many of which are enzymes, e.g., thrombin-like serine proteases, metalloproteases, L-amino acid oxidases, etc. However, phospholipases A₂ present usually in substantial amount in both the elapid and crotalid venoms. The envenoming effect of elapid bites usually develops rapidly, and death can ensue within hours, whereas that of pit viper bites tends to develop more insidious and manifest more often as hemorrhage and coagulopathy in contrast to rapid neuromuscular paralysis by the elapids.

With the advancement in molecular phylogenetics in the last two decades, the taxonomy of many medically important snakes including those in Malaysia has been extensively revised (Wüster and Harvey 1996), making interpretation of findings from earlier works difficult. The importance of development in snake systematics, however, cannot be overlooked in the field of medical toxinology, as venom compositions often vary extensively even between very closely related species or subspecies, resulting in a diverse presentation of envenoming effects and inconsistent therapeutic response to antivenom therapy. The chapter reviewed the profiles of venoms and toxins of medically important snakes relevant to local occurrence, taking into account the recently revised snake systematics.

Elapids

Naja sp. (Cobras)

Two species of *Naja* (cobra) had been previously recognized in the Malayan Peninsula according to Tweedie (1983): the spitting Malayan cobra (formerly known as *NajaNaja sputatrix* or *Naja sputatrix*, now renamed *Naja sumatrana*) and the monocellate/monocled cobra (*Naja kaouthia*, formerly known as *NajaNaja kaouthia*).

Both species adapt well to a wide range of habitats, from natural to anthropogenically impacted environments, and may hence account for most of the snakebites in Malaysian population as rapid urbanization increases the risk of exposure when humans encroach into snakes' habitat (Jamaiah et al. 2006). Cobra bites can result in extensive local necrosis and fatal systemic envenoming due to neuromuscular paralysis or cardiac abnormality (Reid 1964; Jamaiah et al. 2006; Chew et al. 2011; Ismail et al. 2012). The local spitting cobra (*N. sumatrana*) venom is known to cause venom ophthalmia, with the ocular syndrome of intense pain, blepharospasm, increased lacrimation, erythema, and chemosis (Cham et al. 2006).

Systematics of Asiatic Spitting Cobra: The Malaysian Concern

The new systematics restricts the use of *Naja sputatrix* to the Javan spitting cobra in Java and the Lesser Sunda Islands, while the spitting cobra population in Malaysia has been renamed *Naja sumatrana* (equatorial spitting cobra) and the Siamese spitting cobra in Thailand *Naja siamensis* (Indochinese spitting cobra) (Wüster 1996). The term “Malayan cobra,” a common name used previously for the spitting cobra in Malaysia (formerly *Naja naja sputatrix*), is also no longer appropriate. Following the taxonomical revision, findings of previous studies on cobra venom samples of unknown geographic source become dubious: It would be difficult to ascertain whether the spitting cobra venom studied was from (i) the equatorial spitting cobra (labeled as *Naja Naja sputatrix* by commercial suppliers); (ii) the Javan spitting cobra, the true *Naja sputatrix*; or (iii) a confused sample with venom pooled by local folks from different spitting cobras in the region that might have included the Indochinese species *Naja siamensis*. Indeed, online literature search (e.g., PubMed) using the keyword *Naja sumatrana* yielded limited returns (<10) despite numerous early reports on the toxinological profiles of “spitting cobra” under the old nomenclature *Naja Naja sputatrix*. The section below strictly reviewed the literature which reported appropriate species identity according to the latest systematics and those which reported old nomenclature (*Naja sputatrix*) but clearly stated the geographic source and the type of cobra sampling.

***Naja sumatrana* (Equatorial Spitting Cobra)**

A comparative study on the lethal and enzymatic activities among the venoms of Southeast Asian cobras (*N. sumatrana*, *N. sputatrix*, *N. siamensis*, *N. kaouthi*, species identified according to latest systematics) was reported by Yap et al. (2011). *N. sumatrana* venom is distinct from the other cobra venoms based on its protein fractionation profile on ion exchange HPLC (Fig. 1) and i.v. LD₅₀ in mice (Table 1). All the four *Naja* venoms exhibit relatively low activities of protease, phosphodiesterase, alkaline monoesterase, 5'-nucleotidase, and L-amino acid oxidase activities, moderately high acetylcholinesterase and hyaluronidase, and high phospholipase A₂. The major lethal fractions of *N. sumatrana* venom were identified to be phospholipases A₂(PLA₂), neurotoxins (NTX), and cardiotoxins (CTX), with venom mass composition percentage as 24 %, 23 %, and 40 %, respectively. NTX is the most potent and lethal toxin of the venom, with an i.v. LD₅₀ of <0.1 µg/g, followed by PLA₂ (0.8–0.9 µg/g) and CTX (1.0 µg/g). The pathogenic role of these

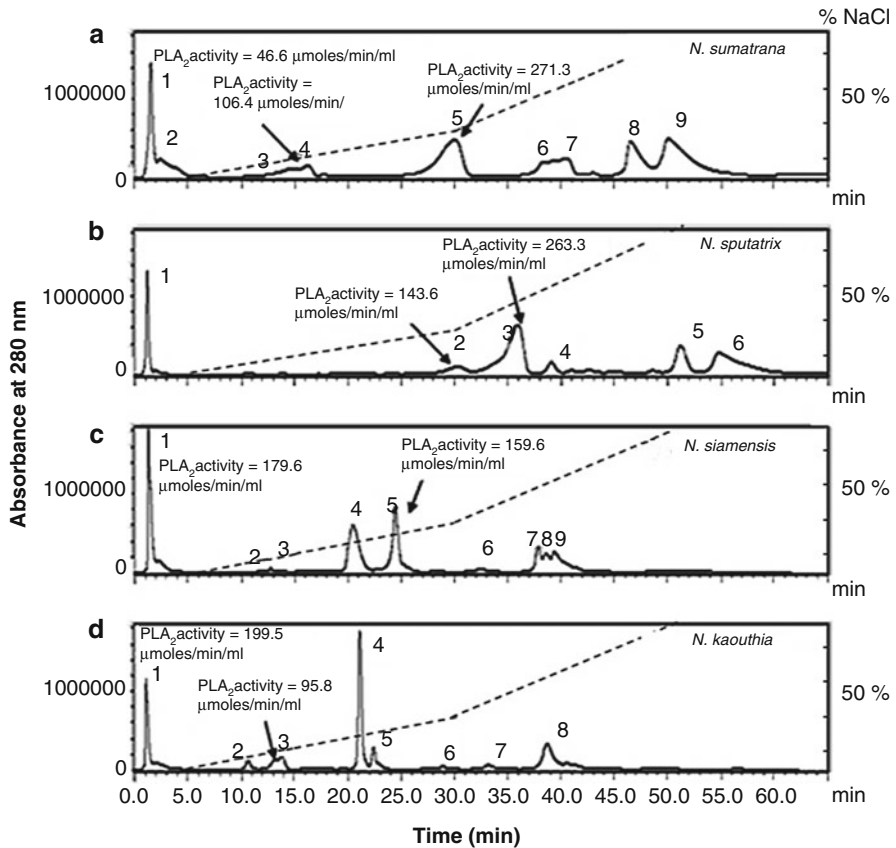


Fig. 1 Fractionation of four Southeastern Asian cobra venoms by Resource[®] S cation exchange chromatography. Ten milligrams of the venom was injected into the column equilibrated with 20 mM MES (identify), pH 6.0, and eluted by a linear gradient (0 to 0.8 M NaCl of 0–30 % from 5 to 30 min, followed by 30–100 % from 30 to 55 min). Flow rate was 1 mL/min. Dotted line: gradient of elution. The venoms were from: (a) *N. sumatrana*; (b) *N. sputatrix*; (c) *N. siamensis* and (d) *N. kaouthia* (Figure reproduced from Yap et al. (2011))

toxins in the venom's lethality was supported by a recent proteomic study which revealed the subtypes and high abundance of 3FTX and PLA₂ in *N. sumatrana* venom (Yap et al. 2014a).

Phospholipases A₂ (14 kDa)

Venoms of local *Naja* sp. usually exhibited much higher PLA₂ enzymatic activities with the range of 150–300 $\mu\text{mol}/\text{min}/\text{ml}$ as compared to <100 $\mu\text{mol}/\text{min}/\text{ml}$ in Malayan pit viper venom (Tan 1991). Armugam et al. (1997) reported the sequences of 3 cDNAs PLA₂-encoding from the venom gland of *Naja sumatrana* (though mislabeled as *Naja sputatrix*, the spitting cobra was captured in Peninsular Malaya). One of these encoded an acidic PLA₂ (APLA), while the other two encoded neutral

Table 1 Intravenous median lethal doses (LD₅₀) of medically important Malaysian snakes and the neutralization efficacy measurement of antivenom

Venom	<i>i.v.</i> LD ₅₀ (µg/g)	Recommended antivenoms	ED ₅₀ (mg/mL)	P (mg/mL)	Reference
<i>Naja sumatrana</i>	0.50 (0.40–0.62)	NPAV	2.30 (1.86–2.85)	1.84	Leong et al. (2012b)
<i>Naja kaouthia (Thailand)</i>	0.23 (0.15–0.34)	NPAV	1.18 (0.78–1.79)	0.94	Leong et al. (2012b)
<i>Naja kaouthia (Malaysia)</i>	0.89 (0.59–1.35)	NPAV	0.68 (0.62–0.75)	0.55	Leong et al. (2012b)
<i>Ophiophagus hamah</i>	1.00 (0.81–1.24)	NPAV	10.23 (6.74–15.54)	8.19	Leong et al. (2012b)
<i>Bungarus fasciatus</i>	1.67 (1.10–2.53)	NPAV	1.73 (1.14–2.62)	1.38	Leong et al. (2012b)
<i>Bungarus candidus</i>	0.11 (0.07–0.17)	NPAV	0.91 (0.60–1.38)	0.73	Leong et al. (2012b)
<i>Bungarus flaviceps</i>	0.175 (0.09–0.21)	NPAV	1.84 (1.21–2.80)	1.47	Leong et al. (2012b)
<i>Hydrophis schistosus (Malaysia)</i>	0.06–0.10	CSL	Not reported	Not reported	Tan CH (unpublished)
<i>Calloselasma rhodostoma (Malaysia)</i>	1.48 (0.92–2.56)	HPAV	9.09 (5.88–14.29)	7.27	Tan et al. (2011); Leong et al. (2014)
<i>Calloselasma rhodostoma (Indonesia)</i>	1.35 (0.78–2.06)	HPAV	12.5 (7.69–20.00)	10.00	Leong et al. (2014)
<i>Cryptelytropis purpureomaculatus</i>	1.1 (0.75–1.68)	HPAV	5.26 (2.04–14.28)	4.21	Leong et al. (2014)
<i>Tropidolaemus wagleri</i>	1.50 (1.37–1.64)	HPAV	Not effective	Not effective	Leong et al. (2014)
<i>Parias sumatranus</i>	1.90	Not tested	Not reported	Not reported	Tan and Tan (1989c)

Assays were conducted in mouse model. For LD₅₀ and ED₅₀, values in brackets are 95 % CI (where applicable). P is potency of the antivenom. Challenge dose 2.5 or 5.0 LD₅₀ of venom in 50 µL was premixed with 200 µL of antivenom and incubated for 30 min. The mixture was then injected into mice (*n* = 4, 20–25 g) and monitored for 48 h. ED₅₀: median effective dose, defined as the amount of venom (mg) neutralized per mL antivenom to confer protection in 50 % of the mice that received lethal challenge dose

P potency, defined as the amount of venom (mg) completely neutralized per mL antivenom. NPAV/Neuro polyvalent antivenom (Thai Red Cross Society), HPAV/Hemato polyvalent antivenom (Thai Red Cross Society), CSL Commonwealth Serum Laboratories (Australia)

PLA₂ (NPLA). The specific characteristics of the several isoforms were attributed to mutations at nt139 and nt328 from G to C and G to A, respectively. However, through ion exchange HPLC fractionation, Yap et al. (2011) demonstrated the presence of both acidic and basic PLA₂ in *N. sumatrana* venom, with the basic PLA₂ being lethal, comprising two fractions (7 % and 17 % composition) with i.v. LD₅₀ of 0.8 µg/g and 0.91 µg/g respectively.

Neurotoxins (6–7 kDa)

The neurotoxins (NTX) of the Malayan *Naja* sp. are of the α -neurotoxins which are antagonists of the nicotinic cholinergic receptor on the skeletal motor endplate (Tan 1983). They are generally divided into short-chain NTX (60–62 amino acid residues with 4 disulphide linkages) and long-chain NTX (66–74 amino acid residues with 5 disulphide linkages) (Endo and Tamiya 1991). The functional sites of long- and short-chain NTXs are not identical, accountable for their differences in specificities (Hodgson and Wickramaratna 2002).

Two variants of short-chain NTXs have been isolated from the venom of spitting cobra in Peninsular Malaysia and Singapore (Afifiyan et al. 1998). From the venom glands, the authors reported cloning of the cDNAs encoding two major groups of short neurotoxins based on point mutation (substitution) at ¹⁰E¹¹T and ¹⁰Q¹¹A (Table 2). These NTX variants shared 96 % homology with α -cobrotoxin from *Naja atra* and were all basic polypeptides (pI 7.89 and 8.13) of 62 amino acid residues, comparable to the findings by Tan (1983) and Chung et al. (1994). The classification of *N. sumatrana* short-chain NTXs into the two fundamental groups (E¹⁰T¹¹ and Q¹⁰A¹¹) unique to *N. sumatrana* is supported by phylogenetic analysis on molecular properties of the NTXs. The role of such point mutation and the functions of the NTX variants, however, remain to be elucidated.

The occurrence of one long-chain NTX (LNTX-7) gene and four variants of weak NTX (WNTX) genes in the venom glands of *N. sumatrana* were subsequently reported (Poh et al. 2002; Jeyaseelan et al. 2003). The LNTX-7 was lethal with an i.v. LD₅₀ of 0.14 µg/g, and shares 88 % homology with α -cobrotoxin (a long-chain α -NTX from *N. kaouthia*). While the SNTXs (short-chain NTXs) mainly bind to muscular nicotinic cholinergic receptor (AChR), the LNTXs (long-chain NTXs) bind with equal high affinity to both muscular (α 1) and neuronal (α 7) nAChRs. On the other hand, the WNTXs showed low affinity toward both receptor types and is nonlethal but dissociated slowly from the receptors (Nirthanan et al. 2003a, b). Phylogenetic study further implies that the neurotoxic genes have evolved from a common NTX ancestor with gene duplication followed by accelerated adaptive evolution on the new molecular surfaces, resulting in the three main clusters of NTXs (short-chain, long-chain, and weak NTXs) for evolutionary success (Jeyaseelan et al. 2003).

Cardiotoxins (6–7 kDa)

Cardiotoxins (CTX), or cytotoxins, constitute the major venom protein fraction of *N. sumatrana*, *N. sputatrix*, and *N. siamensis* (30–40 % of venom proteins) in several isoforms (Yap et al. 2011). They are basic polypeptides with 59–60 amino acid residues and molecular mass of approximately 7 kDa (Tan 1991). While CTX

Table 2 Amino acid sequences of some important toxins of *Naja sumatrana* venom

NPLA-1	NLYQFKNMIQCTVPNRSWWHFADYGCYCGRGGSTPVDLDRCCQIHDNCYNEAEKISRWCWPYFKTY SYECSSQGLTCKGGNACAAAVCDCLRAAICFAGAPYNDNNYNIDLKARCQ
NPLA-2	NLYQFKNMVQCTVPNRSWWHFADYGCYCGRGGSTPVDLDRCCQIHDNCYNEAEKISRWCWPYFK TYSYECSSQGLTCKGGNACAAAVCDCLRAAICFAGAPYNDNNYNIDLKARCQ
APLA	NLYQFKNMVQCTVPNRSWWHFADYGCYCGRGGSTPVDLDRCCQVHDNCYGEAEKISRWCWPYFKTY SYECSSQGLTCKGGNACAAAVCDCLRAAICFAGAPYNDNNYNIDLKARCQ
NTX-1	LECHNQSSSETPTTTCSSGGETNCKKSWRDHRGYRIERGCGCPSVKKGIEINCCTTDRCNN
NTX-2	LECHNQSSQAPTPTTTCSSGGETNCKKSWRDHRGYRIERGCGCPSVKKGIEINCCTTDRCNN
NTX-3	LECHNQSSSETPTTTCSSGGETNCKKGWDRDHRGYRIERGCGCPSVKKGIEINCCTTDRCNN
NTX-4	LECHNQSSQAPTPTTTCSSGGETNCKKGWDRDHRGYRIERGCGCPSVKKGIEINCCTTDRCNN
LNTX-7	TIRCFITPDV/TSIDCPNGHVCYTKTWCDGFCSSRRGRVELGCAATCPTVK PGVDIQCCSTDNCNPFTRP
WNTX-5	LTCLNCPMFCKGKFQTCRNGEKICFKLQQRPFSLRYIRGCAATCPGTPKPRDMVECCSTDRCNR
CTX-3	LKCNKLVPLFYKTCPAGKNLCYKMFMMVATPKVPVKRGCIDVCPKSSLLVKYVCCNTDRCN

*PLA*₂, Phospholipase A₂ (*NPLA* neutral *PLA*, *APLA* acidic *PLA*), Armugam et al. (1997)

NTX Neurotoxin, Affiyan et al. (1998)

LNTX Long neurotoxin, Poh et al. (2002)

WNTX Weak neurotoxin, Jeyaseelan et al. (2003)

CTX Cardiotoxin, Jeyaseelan et al. (1998)

lethality is generally lower than that of NTX and PLA₂ (1.0 µg/g, approx. ten-fold less lethal than NTX), it exhibits a wide multitude of pharmacological actions including potent cytolytic effect and disturbance in cardiac impulse transmission and contractility (Tan 1991; Hedge et al. 2009). CTX and PLA₂ were reported to interact synergistically (Tan and Armugam 1990; Fletcher and Jiang 1993), and the effects may be accountable for local tissue necrosis and venom ophthalmia (Ismail et al. 1993). Interestingly, heparin relieved the venom ophthalmia in few studies (Ismail and Ellison 1986; Cham et al. 2006), probably because heparin at high concentration antagonizes the binding of CTX to chondroitin-6-sulfate in the cornea (Vyas et al. 1998).

Jeyaseelan et al. (1998) showed the existence of four CTX isoforms in the venom and nine cDNA sequences encoding six isoforms of cardiotoxins (CTX 1-3, 4a, 4b, 5) from *N. umatraa* venom gland. One of these, CTX 3, is highly homologous to cardiotoxins of *Naja atra* and *NajaNaja*, suggesting its possible universal existence in the venoms of all *Naja* sp. Phylogenetic study showed that both α-NTXs and CTXs (both are homologous 3-finger toxins) evolve from a common ancestor protein likely by gene duplication. Hydropathy profiles revealed that the Asiatic cobras might be the modern descendants of African and Egyptian counterparts, who share the common hydrophilic CTX isoforms with Asiatic cobras.

Gene Expression Study

Study on accumulated mRNA level and protein synthesis rates for the three toxins of *N. sumatrana* showed that the amount of toxin proteins increases from day 1 to day 10 after milking and that the rate of increase is always greater for CTX, followed by PLA₂, and is the lowest for NTX, resulting in varying concentrations of CTX, PLA₂, and NTX in the ratio of 13:3:1 respectively. The more rapid venom production, as compared to that of viperids, could be due to the evolutionary adaptation of venom-spitting cobras, which need to expel enormous amounts of venom toward aggressors for self-defense (Lachumanan et al. 1999). The findings on the mRNA expression levels of the three main lethal toxins were in accord with toxin abundance result by venom HPLC fractionation (Yap et al. 2011).

Naja kaouthia (Monocled Cobra)

The venom of *Naja kaouthia* has been more extensively studied compared to that of *N. sumatrana*. It is well recognized that *N. kaouthia* has a much wider geographic distribution across parts of China, Indian subcontinent, Indochina, and Peninsular Malaysia. The venom consists mainly of moderate- to low-molecular mass proteins (Kulkeaw et al. 2007) including PLA₂ and three-finger toxins (NTXs, CTXs) (<20 kDa), in addition to a number of enzymes, e.g., proteases, acetylcholinesterase, hyaluronidase, and L-amino acid oxidase (Tan and Tan 1988; Yap et al. 2011). Geographic variations have been well documented (Kulkeaw et al. 2007).

The enzymatic characteristics of *N. kaouthia* venom are similar to those of most Southeast Asian *Naja* sp., although its PLA₂ activity is notably lower and nonlethal (Yap et al. 2011). The PLA₂, however, may be important in potentiating the effects of other vital toxins in the venom. Reali et al. (2003) showed that the venom's

postsynaptic neurotoxic activity was significantly reduced when PLA₂ in the venom was inhibited, while Mukherjee (2010) demonstrated that a noncovalent synergistic interaction of PLA₂ and kaouthiotoxin (KTX, a cytotoxin) enhanced the hemolytic potency of the venom. KTX possibly first destabilizes the target cell membrane followed by higher activity of PLA₂ on the phospholipid bilayers leading to membrane damage and cell lysis.

Postsynaptic NTXs (α -NTX), accounting for 40 % of the venom mass, are the major lethal toxin of the venom (Yap et al. 2011). Postsynaptic neuromuscular blockade, reversible with neostigmine and 4-aminopyridine, was demonstrated in rat left hemidiaphragm and chick biventer cervicis preparations (Reali et al. 2003). Its major α -neurotoxins are long-chain NTXs with 71 amino acid residues (Karlsson and Eaker 1972). Other neurotoxin variants include short-chain NTXs (Wei et al. 2003) and a tryptophan-containing weak NTX (Utkin et al. 2001). However, substantial intraspecific NTX variation has been noted between *N. kaouthia* of different localities (Yunnan and Zhejiang of China vs. Thailand) where the Chinese sample contains principally short-chain NTXs, while the Thai sample is predominated with long NTXs. This is likely due to the variations in climate, prey, and habitat (Wei et al. 2003).

In contrast to most Southeast Asian spitting cobras, *N. kaouthia* venom (Thailand) consists of lower amount of basic CTXs (18 % of the venom dry mass, Yap et al. 2011). Its CTXs also contribute to the lethal toxicity of the venom, with an i.v. LD₅₀ of 1.2 μ g/g – approx. 10-fold less lethal than most α -NTXs (Tan 1991). Other researchers reported that CTX activities of *N. kaouthia* venom include skeletal muscle contracture, hemolysis (Fletcher et al. 1991), cardiotoxicity (auricular block), and cytotoxicity (on human leukemic cell lines) (Debnath et al. 2010).

Chromatographic profiling and preliminary two-dimensional electrophoresis showed substantial differences in the physicochemical properties of the venoms of *N. sumatrana* and *N. kaouthia* (Vejayan et al. 2010; Yap et al. 2011). *N. sumatrana* venom differs from *N. kaouthia* venom in their compositions: *N. kaouthia* venom has a higher NTX and a lower CTX content. The basic PLA₂ of *N. sumatrana* venom is lethal to mice, while PLA₂ from *N. kaouthia* venom are not. Besides, the major neurotoxins from *N. kaouthia* venom are long-chain polypeptides (71 amino acid residues:

IRCFITPDITSKDCPNGHVICYTKTWCDAFCSIRGKRVDLG-CAATCPTVKTGVDIQCCSTDNCPFPTRKRP) that may be immunologically varied from the major NTX of short-chain type (62–63 amino acid residues) in *N. sumatrana* venom. Cross-neutralization potency of antivenom against the two common *Naja* species in Malaysia and Thailand can be enhanced by incorporating the toxins of both species as immunogens.

The i.v. LD₅₀ of Thai *N. kaouthia* (0.23 μ g/g mouse) is one of the lowest (very lethal) among those of common Southeast Asian cobras – lower than the LD₅₀ of *N. sumatrana* venom (Yap et al. 2011). As neuromuscular paralysis (with subsequent respiratory failure) is the principal fastest lethal effect of cobra envenoming, the higher content of long-chain NTX in *N. kaouthia* venom may explain for the lower LD₅₀ of this venom. Interestingly, Leong et al. (2012a) showed an intraspecific variation in the LD₅₀ of *N. kaouthia* sourced from Thailand (0.23 μ g/g) and Ipoh,

Malaysia (0.89 $\mu\text{g/g}$). The Malaysian *N. kaouthia* venom appears less lethal because the Malaysian *N. kaouthia* venom has a lower long-chain NTX content but a higher CTX content (Tan NH, unpublished).

***Ophiophagus hannah* (King Cobra)**

King cobra is believed to be “intelligent” and perceived not as aggressive as the *Naja* cobras, a fact that makes it a favorable pet and show animal and a common cause of bite among the snake wranglers and hobbyists.

The amount of venom that can be injected by an adult king cobra is enormous (>1 g dry venom based on milking experience). The venom has much richer enzyme content than venoms of *Naja* cobras. The major lethal toxins are the polypeptide neurotoxins, and the main systemic effect of king cobra venom bite is neurotoxic that leads to asphyxia (Ganthavorn 1971; Gold and Pyle 1998). Biologically active constituents of king cobra venom that have been characterized include neurotoxins (NTX), hemorrhagic and nonhemorrhagic proteases, phospholipases A₂ (PLA₂), L-amino acid oxidases (LAO), and alkaline phosphomonoesterases.

Three-finger toxins (3-FTX; with typical molecular mass of 6–8 kDa) constitute the major component of *O. hannah* venom (Chang et al. 2013). Earlier works on *O. hannah* venom sampled in Malaysia and Thailand showed that the venom contains two lethal postsynaptic NTXs (α -NTX) with 73 amino acid residues each (Joubert 1973; Tan and Saifuddin 1989). The two toxins are basic polypeptides with i.v. LD₅₀ of 0.2 $\mu\text{g/g}$ and account for 20 % of the venom dry mass. The amino acid sequences of these two toxins are homologous to postsynaptic NTXs from common cobra and krait venoms. Venom of king cobra in Southern China, however, contains more diverse (>4 types) postsynaptic NTXs (Sun et al. 1981). From the venom gland of *O. hannah* (China), cDNA cloning study indicated that the α -NTXs comprise seven long-chain NTXs, seven short-chain NTXs, one weak toxin, and one variant (He et al. 2004). Phylogenetic analysis revealed that the obtained cDNAs are highly homologous to other snake venom α -NTXs. More diverse group of 3-FTXs is further demonstrated by Li et al. (2006) from the cDNAs of *O. hannah* venom gland. Six types of 3-FTXs were identified based on the putative peptide sequences: long-chain NTX, short-chain NTX, cardiotoxin (CTX), weak NTX, muscarinic toxins, and a toxin with a free SH group. Interestingly, the recombinant long-chain α -NTXs were found to exhibit CTX-like cytolytic activity. Rajagopalan et al. (2007) reported the cDNA of a unique 3-FTX, termed β -cardiotoxin, which is expressed in low abundance into the venom and possesses beta-blockage activity on the heart. Another NTX was subsequently characterized from cDNA and venom and shown to be a homodimeric short-chain α -NTX (termed haditoxin). Haditoxin uniquely blocks neuronal $\alpha 7$ -nAChRs as well as muscle nAChRs, which is recognized by neither short-chain α -NTXs nor κ -NTXs (Roy et al. 2010). The findings to date reveal that within the 3-FTX group of *O. hannah* venom, despite the similar structural fold, there are remarkably diverse molecular targets and functions of different 3-FTX, which could also be further compounded by geographic variation

of the venoms. In addition, the molecular variations between the NTXs of king cobra and other elapid species are also the cause of distinct immunoreactivity of king cobra venom as antivenom formulated for local *Naja* cobra is essentially ineffective against king cobra venom.

The venom of *O. hannah* contains a substantial amount of proteolytic enzymes with molecular mass in the range of 30–70 kDa and are functionally diverse. At least five proteolytic enzymes of approximately 70 kDa have been identified, two of which are hemorrhagic proteases (Tan NH, unpublished data; Yamakawa and Omori-Satoh 1988). The proteases account for 7 % of the venom mass. All five proteases exhibit strong edema-inducing activity, indicating its proinflammatory property. The major hemorrhagic protease, termed hannah toxin, is a 66-kDa zinc-dependent metalloprotease with an isoelectric point of 5.3, and it accounts for 2 % of the venom mass (Weissenberg et al. 1987; Tan and Saifuddin 1990b). cDNA cloning of a metalloproteinase gene from the venom gland of a Chinese sample revealed the presence of a P-III snake venom metalloproteinase (with both disintegrin-like and cysteine-rich domain). Termed ohagin, the 50-kDa metalloproteinase was also isolated from the venom and has been shown to degrade α -chain of fibrinogen and to inhibit platelet aggregation induced by ADP, TMVA, and stejnulxin (Guo et al. 2007).

There are at least five forms of alkaline phosphomonoesterases with molecular mass of 95,100 Da (Qin and Wei 1986) and the venom has high alkaline phosphomonoesterase activity (Tan and Hj 1989). The PLA₂ isoenzymes account for 4 % of the venom mass. The two major isoforms of phospholipase A₂ enzymes have isoelectric points of 3.81 and 3.89, respectively (Tan and Saifuddin 1990b), and are not lethal to mice at a dose of 10 μ g/g but exhibit moderate anticoagulant and edema-inducing activities. Huang et al. (1997a) also demonstrated the presence of a single-chain acidic PLA₂ (OHV A-PLA₂, 13,719 Da) with “pancreatic loop,” a region with high degree of conformational flexibility on the surface of the molecule, and hence it may be a potential protein-protein interaction site that leads to toxic effects (cardiotoxicity, myotoxicity, and antiplatelet activities). OHV A-PLA₂ inhibited platelet aggregation (Huang et al. 1997b) and caused significant histological damages in the tissues of skeletal muscle, myocardia, and cardiac ganglia of mice within 24 h (Huang and Gopalakrishnakone 1996).

High content of L-amino acid oxidase (LAAO) in king cobra venom is a well-established fact, considering the fact that the flavin content in the LAAO gives the venom its intense yellow color. The major LAAO from the venom has been purified to homogeneity (Tan and Saifuddin 1989). It has a monomeric molecular mass of 68 kDa, an isoelectric point of 4.5, and an i.v. LD₅₀ of 5 μ g/g in mice. Unlike other venom LAAO, king cobra venom LAAO exhibits exceptional thermal and alkaline stability and is therefore a suitable source for the study of mechanism of action of this enzyme. The *O. hannah* LAAO has been shown to exhibit antiplatelet activity (Li et al. 1994), and the therapeutic potential of king cobra LAAO has been investigated notably as a potent antibactericidal (Lee et al. 2011) and antiproliferative against several solid tumors (Ahn et al. 1997).

Other pharmacologically active venom constituents that have been isolated from king cobra venom include ohanin (107 amino acid residues, does not show similarity

to any known venom protein families) that induces hypolocomotion and hyperalgesia in mice (Pung et al. 2005); an unusual dual Kunitz/BPTI serine proteinase inhibitor named OH-TCI (He et al. 2008), and a group of nonpeptidic toxins (OH-NPT1) with cardiotoxic activity and minimal lethal dose between 0.1 and 0.2 $\mu\text{g/g}$ (Saha et al. 2006).

It should be noted that *O. hannah* is a species that is widely distributed across Asia, with much variations in their morphology and perhaps distinct phylogenetic relationship too. Variations in the lethal potency and some toxin constituents have been reported in *O. hannah* venom sourced from different localities (Chang et al. 2013). Indeed, with the advancement of molecular technology, it is anticipated that more comprehensive genomic profilings will soon be available for king cobras of various localities. Currently, a comprehensive genomic profiling of an Indonesian (Balinese) king cobra is available, and the venom gland transcriptome as well as venom proteome of the specimen have revealed rapid expansion in the genes of principal toxins, especially 3FTX (primarily NTXs) (Vonk et al. 2013). The species may soon be subjected to taxonomic revision and further division of species, much a similar way as what happened to the Asiatic *Naja* species. A great extent of venom variations is expected for species from different geographic regions; clinically, the bites can manifest in diverse envenoming syndrome and suboptimal response to antivenom sourced from different localities. Further toxinological characterization and assessment of antivenom are therefore needed.

***Bungarus* sp. (Kraits)**

Kraits are generally nocturnal and less aggressive elapids (rarely bite) when compared to cobras; the bites, however, can deliver potent venoms which are fatally neurotoxic (Warrell et al. 1983). The following section reviewed the venom profiles of three krait species found throughout Malaysia.

***Bungarus fasciatus* (Banded Krait)**

The i.v. LD_{50} for *B. fasciatus* venom is 1.67 $\mu\text{g/g}$, approximately 10 times less lethal than venoms of other kraits (Table 1). The main toxic constituents of *B. fasciatus* venom include presynaptic and postsynaptic neurotoxins (NTX) and PLA_2 . At least three postsynaptic NTXs (α -NTX), four presynaptic NTXs (β -NTX), and a weak reversibly acting neurotoxin (fasciatoxin) have been identified from the venom (Kruck and Logan 1982; Jiang et al. 1986; Liu et al. 1989). Among these, a 6,500-Da basic (pI: 10) α -NTX was found to share immunological determinants with α -bungarotoxin (a commonly referred α -NTX isolated from *B. multicinctus* venom) but functionally less potent than the latter (Jiang et al. 1986). More recently, a presynaptic NTX (P-elapitoxin-Bf1a, a PLA_2 -containing complex) and a postsynaptic NTX (α -elapitoxin-Bf1b) were isolated from the venom of Malaysian *B. fasciatus* (Rusmili et al. 2014a, b). On isolated skeletal muscle preparation, these two NTXs were not neutralized by the monovalent *B. fasciatus* antivenom or the neuropolyvalent antivenom currently indicated for clinical treatment.

Nonetheless, this peculiar finding is perhaps of low impact on management as the whole venom of Malaysian *B. fasciatus* could indeed be neutralized effectively by the antivenoms on the isolated tissue preparation (Rusmili et al. 2014c) as well as in in vivo animal protective study (Leong et al. 2012b).

Lu and Lo (1981) and Liu et al. (1990) elucidated and revised the structures of three cardiotoxin-like proteins from the venom, which in fact are weak PLA₂ with i.v. LD₅₀s ranging between 0.2 and 4.8 µg/g (Chang et al. 1983; Gong et al. 1989). Ceruleotoxin, an irreversible postsynaptic nAChR antagonist unique to *B. fasciatus* venom, has been shown to be a dimeric PLA₂ with an i.v. LD₅₀ of 0.03–0.07 µg/g (Bon and Saliou 1983) (note: the toxin was named ceruleotoxin due to misidentification of the source of the venom during its discovery, not to be confused with toxins from another krait, *B. caeruleus*).

Banded krait venom acetylcholinesterase has also been purified to homogeneity (Kumar and Elliott 1973). This enzyme has a molecular mass of 126 kDa (a dimer) and accounts for less than 1 % of the venom mass. It does not exhibit lethal toxicity, although its role in envenoming is unclear. Other constituents purified from *B. fasciatus* venom include cathelicidin (Cathelicidin-BF), a very basic peptide of 30 amino acid residues with antibacterial activity (Wang et al. 2008), serine protease inhibitor (Bungaruskunin) with a conserved Kunitz domain (Lu et al. 2008), and an L-amino acid oxidase (BF-LAAO) which is cytotoxic and proinflammatory (Wei et al. 2009).

***Bungarus candidus* (Malayan Krait)**

While krait venoms are generally considered neurotoxic, sustained hypotension and impaired renal hemodynamics had been observed in a rabbit model following experimental envenoming by *B. candidus* (Chanhome et al. 2010). Clinically, parasympatholytic effects such as mydriasis, prolonged hypertension, and tachycardia following envenomation have been recorded in *B. candidus* envenomation, where the use of *Bungarus fasciatus* antivenom and plasma pheresis was not beneficial (Laothong and Sitprija 2001). Rapid improvement of signs and symptoms were only observed recently with the use of specific *B. candidus* antivenom (Leeprasert and Kaojarern 2007). The lack of cross-neutralization of venom between the two *Bungarus* species indicates the need of incorporating both venoms as immunogens in the production of a polyvalent antivenom for *Bungarus* envenoming in the region, which was well taken care of by the manufacturer for the region (Thai Red Cross Society, Queen Saovabha Memorial Institute) (see below).

Bungarus candidus venom is highly lethal with an i.v. LD₅₀ of 0.11 µg/g and exhibits high hyaluronidase, acetylcholinesterase, and PLA₂ activities (Tan et al. 1989a, b, c). There are at least four lethal polypeptide toxins (i.v. LD₅₀ of 0.02–0.83 µg/g); the most lethal one is a basic 21-kDa protein with PLA₂ activity (named Candidus toxin) and comprises 13 % of the venom mass. From a Thai sample of *B. candidus* venom, Khaw et al. (2003) identified three neurotoxic PLA₂ components, with amino terminal sequences highly homologous to those of A-chains (PLA₂) and B-chains (Kunitz-type protease inhibitor), resembling the heterodimers of β-bungarotoxin (BTX) from *B. multicinctus* venom. Being β-BTX

like, the neurotoxic PLA₂ in *B. candidus* venom is deduced to act presynaptically. The potential implications of a likely accelerated mode of molecular evolution of β -BTX genes for krait venom variability should therefore be considered when the production of specific antivenom is attempted.

Postsynaptic neurotoxin void of PLA₂ activity has been reported in the venom of *B. candidus* from Java by Kuch et al. (2003). The toxin is indistinguishable from α -BTX originated from *B. multicinctus* in its mass (7,983.75 Da), LD₅₀ (0.23 μ g/g mouse, i.p.), affinity to nAChR, and the 40 N-terminal amino acid residues. The entire mitochondrial cytochrome *b* gene of Javan *B. candidus* and Taiwanese *B. multicinctus* was sequenced for comparison, suggesting that these snakes are phylogenetically closely related. Nevertheless, despite the fact that α - and β -bungarotoxins are widely conserved in the *B. candidus* and *B. multicinctus*, Warrell et al. (1983) found that Taiwanese *B. multicinctus* antivenom was ineffective in neutralizing Thai *B. candidus* venom in mouse protection assay. This might be due to possible differences between the two venoms' antigenic properties and other toxins not shared by two, which also contribute synergistically to the lethal effects of *B. candidus* venom.

Kuhn et al. (2000) and Torres et al. (2001) reported the X-ray crystallographic and NMR structures of Bucandin, a novel NTX isolated from the venom of Balinese *B. candidus*, which has 63 amino acid residues. It enhances the release of acetylcholine from nerve terminals and belongs to a family of three-finger toxins that incorporate five disulphide bridges but with low sequence similarity with other three-finger toxins. Candoxin, isolated from *B. candidus*, is another nonconventional three-finger toxin present in elapid venoms that produces a novel pattern of blockade in isolated nerve-muscle preparations: unlike the irreversible postsynaptic neuromuscular blockade by some curaremimetic α -NTXs, it is rapidly and completely reversible with washing or by anticholinesterase neostigmine, and it blocks presynaptic nAChR that mediates a transmitter release via a positive feedback mechanism (Nirthanan et al. 2003a, b). It shares 30–40 % identity with short- and long-chain α -neurotoxins as well as other three-finger toxins, however, only 29 % identity to bucandin, a three-finger toxin structurally related to weak toxin.

***Bungarus flaviceps* (Red-Headed Krait)**

Human encounter with the red-headed krait is rare. The venom contains κ -flavitoxin, a 6,500-Da postsynaptic neurotoxin which is also a potent inhibitor of nicotinic transmission in autonomic ganglia (Chiappinelli et al. 1987). *B. flaviceps* venom has potent neurotoxicity and exhibits enzymatic activities similar to other *Bungarus* venoms: low protease but high acetylcholinesterase and PLA₂ activities. Like most krait venoms, it did not exhibit hemorrhagic, procoagulant, or anticoagulant activities (Tan et al. 2010). Khoo et al. (2002) purified the major lethal toxin (basic protein of 29 kDa, LD₅₀ ~ 0.013 μ g/g) from the venom. It has (i) comparable lethal activity as that of β -BTx, (ii) PLA₂ activity, (iii) two polypeptide constituents (18 and 7 kDa) held by disulphide bridges, indicating that the major lethal toxin is indeed a presynaptic neurotoxin. This was supported later with molecular cloning studies which demonstrated that the major lethal toxin from *B. flaviceps* is a novel isoform of

β -bungarotoxins with a unique pattern of cysteine residues different from other known β -bungarotoxins (Yanoshita et al. 2006).

The venom gland transcriptomics of a juvenile *B. flaviceps* indicates that the three-finger toxin is most abundant component of the venom (39 %) (Siang et al. 2010). Three groups of Kunitz-type serine protease inhibitors (SPIs) were identified, where one group is comparable to the classical SPIs, while the other two groups are comparable to B chain of β -BTx (with or without the extra cysteine) based on sequence identity. A natriuretic peptide was also first found for Asian elapids and is distantly related to Australian elapid natriuretic peptides. Its function has not been investigated but likely contributes to disturbances in diuresis and blood pressure control of the prey.

***Calliophis* sp. (*Maticora* sp.) (Asian Coral Snakes)**

The venoms of the exotic Southeast Asian (Old World) coral snakes represent an underexplored research repertoire. Although bites are extremely rare (and the snakes are seemingly incapable of biting humans), the venoms are thought to be highly potent in producing neurotoxic syndrome, in a way similar to many kraits and cobras. From the venom of the long-glanded blue coral snake, *Maticora bivrivata* (*Calliophis bivrivata*), Takasaki et al. (1991) identified two phospholipases A₂, four cytotoxin homologues (termed *maticotoxins A*), and a large amount (about 1 mg/specimen) of adenosine accompanied with smaller amounts of inosine and guanosine. The amino-terminal amino acid sequences of two PLA₂ and one maticotoxin suggest that *Maticora bivrivata* is closely related to *Hemachatus* and *Naja*. The venom gland of this snake is unusually elongated with unusual characteristics (Gopalakrishnakone and Kochva 1990), and the venom yield can be up to 50–100 mg per milking. The venom has an i.v. LD₅₀ of 0.7 μ g/g mouse, and the lethality was not neutralized by antivenoms raised against the venom of *B. candidus* and *B. fasciatus* but moderately by that of *B. multicinctus* of Taiwan (Tan CH, unpublished).

Sea Snakes

***Hydrophis schistosus* (Formerly Known as *Enhydrina schistosa*, Beaked Sea Snake)**

There are more than 18 species of sea snake in the coastal waters of Malaysia. The commonest species is *Hydrophis schistosus* (beaked sea snake), which is also the most aggressive species (Reid 1979). Other common sea snakes include *Hydrophis fasciatus*, *H. cyanocinctus*, *H. spiralis*, *H. klossi*, and *H. curtus* (synonym: *Lapemis hardwickii*).

The venom contains mainly polypeptides, and the content of enzymes is low. The biological constituents of the venom that have been characterized include postsynaptic NTXs, PLA₂ enzymes, and acetylcholinesterase. In laboratory animals,

H. schistosus venom is neurotoxic, acting mainly at the neuromuscular junction. In humans, however, the venom is primarily myotoxic (Reid 1979). The myonecrosis causes generalized intense myalgia, myoglobinuria (potentially leads to acute kidney injury), and much of the paresis in severe sea snake envenomation (Reid 1961).

Postsynaptic NTXs account for 70 % of the venom dry mass. The two major NTXs account for 14 % and 25 % of the venom proteins respectively and are polypeptides with 60 amino acid residues (Karlsson et al. 1972), and the sequences are homologous to the cobra neurotoxins (Fryklund et al. 1972). At a dose of 0.1 µg/g, the toxins (*schistosus* 4 and *schistosus* 5) produce respiratory paralysis and death in guinea pigs within 1–2 h after i.p. injection (Geh and Toh 1978). These toxins cause neurotoxic envenoming in animals. Human, however, is probably less susceptible to the neurotoxic action of these proteins.

The venom contains more than seven highly lethal basic PLA₂ toxins with i.v. LD₅₀ of less than 0.1 µg/g and two nonlethal acidic PLA₂ enzymes, which, however, are able to suppress muscle excitability to direct stimulation as well as the muscle response to nerve stimulation and to exogenous acetylcholine (Geh and Lin-Shiau 1987). The basic and acidic PLA₂ enzymes account for 13 % and 4 %, respectively, of the venom dry mass (Tan 1982). The major basic PLA₂ toxin, which accounts for 7 % of the total venom protein, caused death in animals through respiratory muscle paralysis, described as a neurotoxic effect of the venom (Fohlman and Eaker 1977). This was disputed subsequently by Geh and Toh (1978): when injected into guinea pigs, the sublethal low dose of basic PLA₂ caused breakdown of myofilaments and mitochondrial cristae with invasion of phagocytic cells 6 h following its injection while leaving the neuromuscular junction and myelinated nerves spared, although demyelination was observed at higher doses. A more complete study undertaken by Gopalakrishnakone (1984) demonstrated the progression of myonecrosis and regenerative events in mouse calf at intervals from 6 h to 14 days following the venom injection at sublethal dose. The work reported by Brook et al. (1987) indicated that the basic PLA₂ is predominantly myotoxic. This myotoxin (the basic PLA₂) when injected intramuscularly caused myonecrosis of almost every fiber in slow-twitch (red) muscle but selectively affect scattered single fibers in the fast-twitch (white) muscle, a finding comparable to the description mentioned by Marsden and Reid (1961) in human victim bitten by sea snake. The fiber selectivity in myonecrosis is possibly due to variation in the composition and structure of the plasma membranes of differing muscle fiber types (Leung et al. 1986). To mark the various venom PLA₂ with neuro- and/or myotoxicity on a spectrum in relation to its primary site of action, the basic PLA₂ of *H. schistosus* will be at one end opposite to that of the purely neurotoxic, presynaptic β-bungarotoxins, while notexin, taipoxin, and crotoxin, which act on both nerve and muscle, would be in an intermediate position of the spectrum (Brook et al. 1987).

Hydrophis schistosus venom acetylcholinesterase has been partially purified (Kanthimathi 1980). The enzyme, which accounts for less than 0.1 % of the venom mass, has a molecular mass of 77 kDa. Its toxic property has not been studied in depth.

A Note on the Tissue-Necrotizing Activity of Malaysian Elapid Venoms

The local and regional tissue damaging process following snakebite is often due to the presence of lytic enzymes and cytotoxins in the venoms. Local tissue damages can be further complicated by inflammatory responses, infections, and iatrogenic factors (Chippaux 2006), the sequel of which can be disastrous: amputation and crippling deformity that add to the toll of suffering of a poor family. The bites by Malaysian elapid snakes produce characteristic distinction on local responses: *N. sumatrana*, *N. kaouthia*, and *O. hannah* bites generate intense pain within minutes of bite, and the subsequent edema, tissue sloughing, and necrosis that can be deep and extensive, while bites from *Bungarus* sp. and most sea snakes are generally painless and easily go unnoticed by the patients – until eventually the manifestation of systemic symptoms. *Naja* sp. venoms are known to contain high levels of PLA₂ and cardiotoxins (cytotoxins); both seem to bind favorably to local tissue (Yap et al. 2014b), while *O. hannah* venom contains a variety of lytic enzymes including the PLA₂, cytotoxic L-amino acid oxidase, and metalloproteases, all capable of causing cell death and tissue necrosis at the bite site. The PLA₂s of *Bungarus* sp. and sea snakes appear more readily to exert systemic effects as in neuromuscular blockade and generalized myonecrosis. The subtle effect on bite wound following *Bungarus* and sea snake bites can cause a delay in seeking medical help; in some instances, victims died in a “mysterious way” before even realizing that there was a snakebite. The knowledge has practical implications from the public health point of view, where (1) awareness and sense of urgency to seek medical help should be instilled especially in rural and coastal community; (2) pressure-immobilizing bandage should be applied only if the biting species’s venom does not usually cause local tissue necrosis – in Malaysia, this will be a “rare” chance as most common biting snakes (cobras, king cobra, and pit vipers) do produce extensive tissue necrosis.

Viperids

The Malayan Peninsula (including Singapore) and the Borneo Island do not have Asiatic true vipers, e.g., *Daboia* sp. and *Echis* sp. as their native species. Both arboreal and terrestrial pit vipers (crotalids) can be found in these regions; however, the endemic occurrence of Malayan pit viper in Borneo is unconfirmed.

Calloselasma rhodostoma (Malayan Pit Viper)

Pit vipers (Crotalinae) are the most widely distributed subfamily of the Viperidae, with major radiations over 190 species (~75 % of viperid species allocated in 29 genera) (<http://reptile-database.org>). The origin of crotalids is firmly placed in Asia with the Asiatic genera *Calloselasma*, *Hypnale*, *Deinagkistrodon*,

Tropidolaemus, and *Garthius* seated at the basal-most position (Wüster et al. 2008). The Malayan pit viper, previously known as *Agkistrodon rhodostoma* or *Ancistrodon rhodostoma* from the *Agkistrodon* Complex (once thought to be monophyletic), is now placed within the monotypic genus *Calloselasma* (species: *rhodostoma*).

The recent revised i.v. LD₅₀ of *C. rhodostoma* venom was 1.48 µg/g mouse (Tan et al. 2011). The venom generally exhibits strong thrombin-like enzyme and hemorrhagic activities. Like most pit viper venoms, *C. rhodostoma* venom is rich in enzymes and biologically active compounds, e.g., thrombin-like enzymes, hemorrhagins, platelet aggregation inducers and inhibitors, fibrinogenase, L-amino acid oxidase, proteases, phospholipase A₂ enzymes, and arginine esterase (Tan 1991). Remarkable intraspecific variation in the composition and biological activities of the venom, however, has been noted (Daltry et al. 1996a). The age or developmental stage, geography, diet, season, and sex of the snakes are all possible sources for intraspecific venom variation (Mackessy 2009). As compared to the elapid venoms, crotalid venoms generally exhibit more complex actions due to the presence of many toxins/enzymes; each appears to play a significant role in the envenoming pathophysiology. The biochemical and functional aspects of major toxins in *C. rhodostoma* venom are reviewed in the following section.

Serine Proteases and Thrombin-Like Enzymes (TLE)

The venom contains several isoforms of TLEs. The major form, ancrod (or arvin), with a molecular mass of 55 kDa, accounts for approximately 7 % of the venom mass (Esnouf and Tunnah 1967), and it is heavily glycosylated (36 % by mass) with five N-linked glycosylation sites (Pfeiffer et al. 1993). Ancrod exhibits arginine ester hydrolase activity and enzymatic specificity toward several substrates of thrombin. It coagulates fibrinogen solution by catalyzing the release of fibrinopeptides A, AP, and AY. Clots that formed with ancrod are not cross linked and are susceptible to rapid lysis by plasmin. When injected into humans/animals, it causes continual microcoagulation of fibrinogen, but the resulting fibrin is virtually simultaneously disposed. Thus, although ancrod is a “coagulant” in vitro, the in vivo effect is a defect in coagulation due to low fibrinogen level. This is generally perceived as a mechanism of hemostatic dysfunction associated with consumptive coagulopathy commonly reported in viperid snakebites.

The cDNA sequence reveals that ancrod, a serine protease, is synthesized as a prezymogen of 259 amino acids. Au et al. (1991) showed that Ancrod contains 13 cysteine residues, to which 6 disulphide bridges can be predicted. Ancrod amino acid sequence (Table 3) exhibits a high degree of sequence similarity to many mammalian serine proteinases as well as reptilian fibrinogenases, most notably the catalytic triad and many conserved cysteine positions (Burkhart et al. 1992; Phillips et al. 2009). Figure 2 showed the consensus regions of TLE peptide sequences from several crotalid venoms.

Ancrod, manufactured under the brand name Viprinex, has been regarded as a unique antistroke agent with triple actions: a defibrinogenating agent, a fibrinolytic agent, and an anticoagulant, with clinical benefits in limiting clot propagation,

Table 3 Amino acid sequences of the mature chains of some major toxins from the Malayan Pit Viper venom

Ancrod (Thrombin-like Enzyme)	VIGDECNINEHRLVAAYEGTNWTFICGGVLHPEWVITAEHCARRRMNLVFGMHRHSEKFDDEQERYPKKRYFIRCNKTRTS WDEDIMLIRLNKPVNNSEHIALPSNPPIVGSDCRVMGWSINRRIDVLSDEPRCANINLHNFTMCHGLFRKMPKKGRVLCAG DLRGRRDSCNSDGGPLICNEELHGIVARGPNPCAQPNKPALYTSIYNYRDWVNNVIAGNATCSP
Rhodostoxin (Hemorrhagin) in blue; rhodostomin and/or kistrin in red	NHEIKRHVDIVVVDSRFCTKHSNDLEVIRKVFHEVVNHAIESKYMHFGISLVNLETWCNGDLINQEDSYETLKAFGKWRES LIKHVNHNSNAQFLM(T)DMKFIKNIIGKAYLDSICDPERSGVQVNYHGITLVNVAIMAH HEMGNH(N) LG V RHDGEYCTCYGSSECI MSSHISDPSPK YFSNCSYYQFWKYIENQNPOCILNKPLRTVSIPIVSGNEHLEAGKECDSSPENPCDAATC KLRPGAQCGEG LCC EQCKFSRA GK ICRIPRGDMPDDRCTGQ SAD CPRY HSHA *italic bold font: active site of ZnMc superfamily
Kistomin	QQESPQAKVYLVVADKSMVDKKHNGNIKKIEEQGHQMVNTMNECYRPMGIHIMAGIECWTTNDFFEVKSSAKETLYSFAKWR VEDLSKRKKPHNDAQFLTNKDFDGNITVGLAFVGGICNEKYCAGVVQDHTKVPLLMAIT MGHEIGHNLGMEHDE ANCKCKACV MAPEVNNNPTKKFSDCSRNYQKFLKDRKPECLFKKPLRTDTVTPVSGNEPLEVITMDDFYA *italic bold font: active site of ZnMc superfamily
Rhodocytin subunit α	GLEDCDFGWSPYDQHCYQAFNEQKTWDEAEKFCRAQENGAAHSLASIESNGEADFVSWLSQKDELADEDYVWIGLRAQNKEQQ CSSEWSDGSSVSYENLIDLHTKKCGALE KLTGFRKWVNYCYCEQMHAFCVKLLPY *sequence identical to that of aggreitin chain A; and rhodoaggreitin subunit α
Rhodocytin subunit β	DCPSGWSSYEGHCYKPFNEPKNWADAERFCKLQPKHSHLVSFQSAEEADFFVKLTPLRKANLVWMGLSNVWHGCNWQWSD GARLNYKDWQEQSECLAFRGVHTEWLNMDCSSTCSFVCKFKA *sequence identical to that of aggreitin chain B; and rhodoaggreitin subunit β

(continued)

Table 3 (continued)

L-amino acid oxidase	ADDRNPLAECFQENDYEEFLEIARNGLKATSNPKHVIVGAGMAGLSAA YVLAGAGHQVTVLEASERPGGRVVRTYRNEEAGW YANLGPMLRLEKHRIVREVIRKFDLRLNEFSQENDNAWYFIKNIKKVGEVKKDPGLLKYPVKPSEAGKSAGQLYEESLGKVVE ELKRTNCSYILNKYDTYSTKEYLKEGDLSPGAVDMIGDLLNEDSGYYVSFIESLKHDDIFAYEKRFEIIVDGMMDKLPTAMYRDIQ DKVHFNAQVIKIQQNDQKVTVVYETLSKETPSVTADYVIVCTTSRAVRLIKFNPPLLPKKAHALRSVHYRSGTKIFLTCTTKFWE DDGIHGGKSTTDLPSRFHYYPNHNFTNGVGVIIAYGIGDDANFFQALDFKDCADIVFNDLSLIHQLPKDKIQSFYCPSYVIQKWSLDK YAMGGITTFPTYQFQHFSDPLTASQGRIFYAGEYTAQAHGWIDSTIKS GLRAARDVYNLASENPSTGHIHLSNDNEL
Phospholipase A₂ H1E6	HLLQFETMIUKMTKQTGLFSYSFYGCYCGWGGHGRPDPTDRCCFVHDCCYGKVTNCDPKAAAYSYTIENGIVCGGDDPCKK QICECDRAAAMCFRDNLDYNYAKYWKFSKDCQEESDPC
Phospholipase A₂ G6K49	SLIELGKMIFQETCKNPVKNYGLYGCNCGVGNRGK PVDATDRCCFVHKCCYKKVTGCDPKKDRYSYSWENKAIVCGEKNPPCL KQVCECDKAVAICLRENLTGYDKKHRVTVKFLCKAPESC
Phospholipase A₂ W6D49	SMFNLWKMIMVMTGKEATKNYGMYGNCNCGPMKRGPKDATDQCCADHDCCYKKLTDCDPKKESYSYKFEKGELCGETNPC LNQACECDKAVATCFRDNLDYNNKKQQFNTGIFCSKAKAC

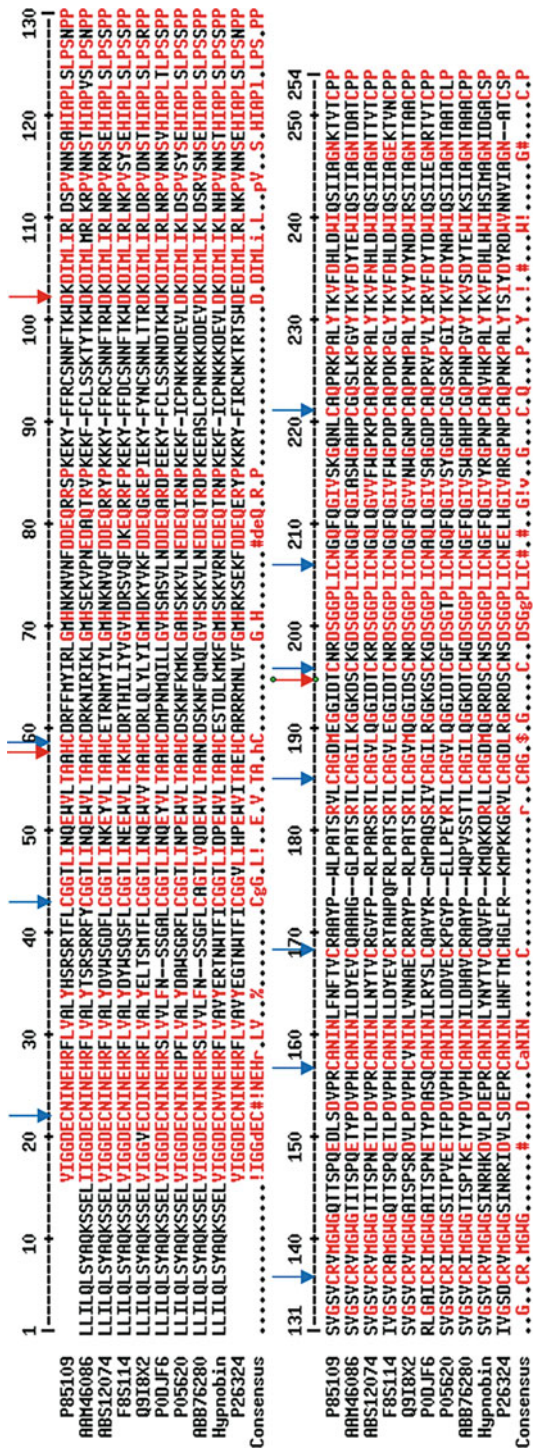


Fig. 2 Consensus regions of TLE peptide sequences from several crotalid venoms were shown in red and included the conserved cysteine residues (blue arrow) important for disulfide bonding and 3 residues (red arrow) of the catalytic triad of serine proteases. Numbering based on chymotrypsin. Alignment was done using mutalin® on-line software available from <http://multalin.toulouse.inra.fr/multalin/multalin.html>. P85109: kangshuanmei (*Gloydus brevicaudus*); AAM46086: thrombin-like enzyme (*Gloydus ussuriensis*); ABS12074 Thrombin-like serine protease 1 (*Cryptelyrops albolabris*); F85114.1 Thrombin-like enzyme crotalase (*Crotalus adamanteus*); Q918X2 Thrombin-like enzyme acutobin (*Detigakistrodon acutus*); P00JF6: Thrombin-like enzyme chitribisin (*Trimeresurus Cryptelyrops albolabris*); P05620 Thrombin-like enzyme flavoxobin (*Trimeresurus flavoviridis*); ABB76280.1 thrombin-like enzyme (*Bothrops asper*); P26324 Thrombin-like enzymecanrod (*Calloselasma rhodostoma*); Hypnobin: thrombin-like serine protease (*Hypnale hypnale*) (Tan CH, unpublished)

reducing plasma viscosity, improving microcirculatory flow, and activation of endogenous fibrinolysis (Liu et al. 2011). The clinical application, however, was hampered by the fact that the European Stroke Treatment with Ancrod Trial (ESTAT) and the Ancrod Stroke Program (ASP) showed no benefit in functional outcome for patients given ancrod within 6 h of stroke onset (Hennerici et al. 2006; Levy et al. 2009), leading to termination of the study (<http://clinicaltrials.gov/ct2/show/NCT00141011>). The suboptimal clinical effect in human stroke trials could be due to ancrod-induced microvascular thrombotic occlusion following local fibrin formation and secondary depletion of tissue-type plasminogen activator by binding to fibrin clot, although ancrod may still have a role in a subset of patients with stroke with elevated levels of fibrinogen (Liu et al. 2011). The therapeutic implication of ancrod and the clinical study design need to be revisited for further optimization of its potential application.

Metalloproteases: Hemorrhagin, Rhodostoxin, and Antiplatelet kistomin

Snake venom hemorrhagins are generally metalloproteinases that act by destruction of the collagenous basement membrane and other connective tissue collagens with consequent weakening of vascular walls causing hemorrhagic effect (Ohsaka 1979). The major *C. rhodostoma* venom hemorrhagin termed *rhodostoxin* is a zinc-dependent metalloproteinase with 203 amino acid residues (30 kDa) (Table 3) and a carbohydrate content of 15 %. The extended consensus active-site sequence of MXHEXGHNLGXXHD (residues 140 to 153) and the predicted secondary structure in the zinc-binding region of the hemorrhagin is similar to that of other snake venom metalloproteinases; rhodostoxin, however, is unique in that it contains four disulfide bridges (Ponnudurai et al. 1993; Tan 1998a). The structure of the oligosaccharides attached to the protein has also been examined (Chung et al. 1996), and deglycosylation causes changes in the interactions between rhodostoxin and the relevant substrate in the membrane. Epitope mapping of rhodostoxin using polyclonal antibodies showed that the antigenic sites of rhodostoxin include residues 45–76, 81–88, 111–120, and 165–177 and that the oligosaccharide moieties of rhodostoxin were not part of the antigenic determinants (Ponnudurai 1995).

Huang et al. (1993) isolated a metalloprotease, kistomin, and showed that it degraded fibrinogen and inhibited ristocetin-induced platelet agglutination, suggesting that it was a GPIb-cleaving protease. It prolonged the latent period of platelet aggregation and inhibited ATP secretion of human washed platelets stimulated by thrombin in a dose- and time-dependent manner, hence contributing to the venom hemorrhagic effect. cDNA sequence of kistomin revealed that kistomin is a P-I snake venom metalloprotease with 227 amino acids (25.7 kDa) (Hsu et al. 2007). It blocks vWF-induced platelet activation by specifically cleaving platelet GPIb α and vWF and exhibits in vivo antithrombotic effect, hence it is potentially useful in metalloproteinase-substrate interaction study and antithrombotic drug discovery.

Disintegrins: Rhodostomin, Kistrin, Rhodocetin

Disintegrins are a group of Arg (or Lys)-Asp-containing snake venom proteins which can inhibit platelet aggregation via the blockade of $\alpha_{IIb}\beta_3$ integrin. Rhodostomin, the first disintegrin isolated from *C. rhodostoma* venom, is a 68 amino acid residue polypeptide with a hairpin loop that presents the binding sequence RGD (Arg-Gly-Asp) at positions 49–51 (Huang et al. 1987; Chang et al. 1997). It inhibits platelet aggregation by blocking the binding of fibrinogen to the integrin $\alpha_{IIb}\beta_3$ of platelet. Moreover, Dennis et al. (1990) identified an RGD-containing peptide of 68 amino acids which was differently termed *kistrin* and shown to inhibit platelet aggregation by antagonism of the platelet GPIIb-IIIa-fibrinogen interaction (amino sequences shown in Table 3). Further work that produced recombinant rhodostomin has been found useful in studying cell adhesion via interaction of the RGD motif and integrins on cell surface (Chang et al. 1997; Tseng et al. 2004). The amino acid sequence deduced from the cDNA sequence indicates that the 68-amino acid sequence of rhodostomin is located at the carboxyl terminus of the precursor protein, which also contains the sequence of rhodostoxin, the major hemorrhagin (Ponnudurai 1995) (see above for inhibitory actions of the metalloprotease on hemostasis). It appears that rhodostomin and rhodostoxin share a common gene sequence, suggesting that these proteins may be synergistic in function.

Wang et al. (1999a) purified a novel platelet aggregation inhibitor, rhodocetin, from the venom and demonstrated that it has a heterodimeric structure with alpha (15,956.16 Da, 133 residues) and beta (15,185.10 Da, 129 residues) subunits. The authors classified rhodocetin as a Ca^{2+} -dependent lectin-related protein, which was disputed later by Eble et al. (2001) by demonstrating that its inhibitory effect bases on its strong and specific binding to $\alpha_2\beta_1$ integrin, suggesting that rhodocetin is a disintegrin. Rhodocetin blocks $\alpha_2\beta_1$ integrin as the major collagen receptor on platelets and therefore hampers collagen-induced, $\alpha_2\beta_1$ integrin-mediated platelet activation, leading to hemorrhages and bleeding disorders. Studies have also shown that rhodocetin is able to inhibit $\alpha_2\beta_1$ integrin-mediated adhesion of fibrosarcoma cells to type I collagen completely and antagonize stromal tumor invasion (Eble et al. 2001, 2002). The main difference of the biochemical moiety between rhodocetin with kistrin or rhodostomin is that rhodocetin lacks interference with RGD-dependent integrins.

C-type Lectins: Aggretin, Rhodocytin, Rhodoaggretin

Ouyang et al. (1986) isolated a 28-kDa platelet aggregation inducer from *C. rhodostoma* venom. Further characterization work by Huang et al. (1995) identified the component as a heterodimeric 29-kDa nonenzymatic protein termed *aggretin*. It acts as a glycoprotein Ia/IIa (integrin $\alpha_2\beta_1$) agonist to elicit platelet aggregation through the activation of endogenous phospholipase C, leading to subsequent hydrolysis of phosphoinositides and subsequent intracellular Ca^{2+} mobilization. The term “rhodocytin” came into picture when Shin and Morita (1998) isolated a disulfide-linked heterodimer of 18- and 15-kDa subunits, which induces

platelet aggregation independently of glycoprotein Ib (a transmembrane complex subunit bears the binding site for von Willebrand factor (vWF), α thrombin, leukocyte integrin $\alpha\text{M}\beta 2$, and P-selectin) – a function thought to be novel and distinct from known snake venom C-type lectins. The authors suggested that rhodocytin was not identical to aggretin based on their different relative elution volumes from the Sephadex G-75 column, although subsequent characterizations revealed striking similarities in the molecular masses and N-terminal sequences of the subunits (Chung et al. 1999; Wang et al. 2001). From cDNA cloning results, Chung et al. (1999) showed that the mature α and β chains of aggretin contain 136 (18 kDa) and 123 amino acid (15 kDa) residues, respectively, and with hyper-variable regions likely to be the counterparts directly interacting with different receptors or domains on the surface of platelet.

Some reports suggested that aggretin/rhodocytin induces platelet aggregation by interacting with the collagen receptor $\alpha_2\beta_1$ integrin (Navdaev et al. 2001; Suzuki-Inoue et al. 2001), but this was disputed by the finding that rhodocytin does not bind to a recombinant soluble form of $\alpha_2\beta_1$ integrin (Eble et al. 2001). Bergmeier et al. (2001) subsequently demonstrated that rhodocytin (as aggretin) could activate murine platelets lacking all three receptors ($\alpha_2\beta_1$ integrin, glycoprotein VI, and glycoprotein Ib α), confirming that the mechanisms are fundamentally different from those induced by collagen. It should be noted that the term *rhodocytin* has been increasingly used over aggretin in literature since then.

The exact mechanism of rhodocytin was subsequently elucidated. Using rhodocytin affinity chromatography and mass spectrometry, Suzuki-Inoue et al. (2006) discovered the novel receptor CLEC-2 (C-type lectin receptor 2) expressed on the surface of platelets that serves as the receptor for rhodocytin in the activation of platelet. The discovery of the CLEC-2 receptor has led to extensive studies on its structures, endogenous/exogenous ligands, and physiological/pathological roles (Suzuki-Inoue et al. 2011; Watson and O’Callaghan 2011; Bruserud 2013). Interestingly, the role of CLEC-2 receptor on platelet is beyond hemostasis and thrombosis: it is expressed on tumor cells and facilitates metastasis by inducing platelet activation through the endogenous ligand podoplanin (Suzuki et al. 2011; Lowe et al. 2012). Rhodocytin appears to be a useful ligand tool in studying CLEC-2 as a target protein for novel antiplatelet drugs and antimetastatic drugs.

Besides, rhodoaggretin, a dimer of C-type lectin heterodimers with its native molecular mass of 66 kDa, was identified by Wang et al. (2001). The didimer was proposed to exist as a 2($\alpha\beta$) complex, stabilized by noncovalent and disulphide linkages between the two heterodimers. Rhodoaggretin activates platelet aggregation; however, the mechanistic aspect including the target site of the toxin has not been well characterized.

Clinically, the in vivo effect of platelet aggregation induced by rhodocytin is accompanied by thrombocytopenia and microthrombosis that contribute to the systemic hemorrhagic activity of *C. rhodostoma* envenomation.

L-Amino Acid Oxidase

L-amino acid oxidase (LAAO) is a major constituent of the venom of Malayan pit viper, constituting up to 30 % of the crude venom mass (Tan 1998b). The enzyme is an acidic glycoprotein with a molecular mass of 132 kDa and consists of two subunits of equal molecular mass (Ponnudurai et al. 1994; Geyer et al. 2001). Kinetic studies suggest the presence of an alkyl side-chain binding site in the enzyme and that the binding site comprises at least four hydrophobic subsites, with characteristics differing slightly from those of king cobra venom L-amino acid oxidases. The enzyme's primary function in most snake venoms is believed to be of antimicrobial purpose; however, the huge amount of LAAO in *C. rhodostoma* venoms seems to suggest a more diverse pharmacological and toxinological property of the LAAOs. Hemorrhagic, inflammatory, platelet-disrupting, anticoagulant, and apoptotic activities have been reported in many snake venom LAAOs (Tan and Fung 2009).

Macheroux et al. (2001) reported from the cDNA-deduced amino acid sequence that *C. rhodostoma* venom LAAO comprises 516 amino acids, with its sequence closely related to other ophidian LAAOs, and displays highly conserved FAD-binding fold. The FAD-binding domain consists of three discontinuous regions of the structure: residues 35–64, 242–318, and 446–486 (Pawelek et al. 2000), and the complex structure reveals the substrate bound to the reduced flavin (FADred) (Moustafa et al. 2006). The LAAO also shares the remarkable freeze- and pH-induced inactivation characteristics with LAAOs isolated from *Crotalus atrox* and *Crotalus adamanteus* venoms. The inactivation processes are fully reversible and are associated with changes in the UV/visible range of the flavin absorbance spectrum (Macheroux et al. 2001).

Phospholipase A₂

The pit viper venom phospholipase A₂ (PLA₂) enzymatic activity is generally lower than that of *Naja* cobras (Tan 1991). A proteomic study of *C. rhodostoma* venom showed the presence of acidic, basic, and neutral PLA₂, predominated by the basic isoforms (Ali et al. 2013). Wang et al. (1999b) identified two subtypes of PLA₂ from *C. rhodostoma* venom based on their sixth amino acid residues: Glu 6 (E6-PLA₂) and Trp 6 (W6-PLA₂). The enzymatic E6-PLA₂ inhibits platelet aggregation induced by ADP and agonists, while the noncatalytic W6-PLA₂ possesses a heparin-binding property and edema-inducing effect. Complete amino acid sequences of the purified PLA₂s were subsequently deduced from their cDNA sequences (Tsai et al. 2000), and various geographic venom samples were shown to contain a major noncatalytic basic PLA₂ homologue and two or three acidic PLA₂s in different proportions (Tsai et al. 2001a). The basic PLA₂ contains both D49 and other unusual substitutions unique for the venom K49-PLA₂ subtypes isolated from many crotalid venoms (presence of L5, W6, Q11, N28, P31, mutations at G32/G33). The low catalytic activity is explained by an abnormal calcium-binding pocket, while the distinct Arg/Lys-rich and Met-rich regions at positions 10–36 of the PLA₂ homologue presumably are involved in its heparin binding and the cell membrane interference leading to edema and myotoxicity (Tsai et al. 2001b). The observation is in accord with

many reports on the strong membrane-damaging and myotoxic properties of these PLA₂ variants (Lomonte et al. 2003). Generalized myotoxicity has not been reported clinically in *C. rhodostoma* envenomations; however, the cytolytic activity of snake venom PLA₂s causing myonecrosis has been widely reported (Gopalakrishnakone et al. 1997; Lomonte et al. 2003). Therefore, in *C. rhodostoma* envenomation, the E6-PLA₂ and W6-PLA₂ may respectively contribute to hemorrhage (platelet dysfunction) and local myonecrosis in addition to pathological effects induced by other components in the venom. Besides, two variants, K49- and D49- PLA₂s, from *C. rhodostoma* venom have been shown lethal in mice, inducing myonecrosis and edema as well as causing cytolysis on cultured skeletal muscle tissues (Bonfim et al. 2006, 2008).

Other Enzymatic Activities

Other enzymatic activities detected from the Malayan pit viper venom include phosphodiesterase, alkaline phosphomonoesterase, 5'-nucleotidase, hyaluronidase, arginine ester hydrolase, arginine amidase, and fibrinogenase; many of the enzymes were reported to exist in isoforms based on the venom fractionation using ion exchange chromatography (Tan et al. 1986). The venom contains at least four different basic caseinolytic enzymes with molecular masses of approximately 25 kDa, all of which exhibit moderate edema-inducing activity (Tan NH, unpublished) and more than nine different forms of arginine ester hydrolases, five of which exhibit thrombin-like enzyme activity. Two minor forms of arginine ester hydrolase also exhibit arginine amidase activity (Tan 1984). However, significant intraspecific variation in the biological activities of the venom, including phosphodiesterase, alkaline phosphomonoesterase, L-amino acid oxidase, arginine ester hydrolase, 5'-nucleotidase, thrombin-like enzyme, and hemorrhagic activities, have been long noted (Daltry et al. 1996a), leaving a remarkable impact on the antivenom industry where manufacturers and researchers need to constantly address the inclusiveness of venom variety during venom sampling for the production and assessment of the antivenom.

The primary defect in systemic bleeding caused by *C. rhodostoma* envenoming is apparently hemostatic derangement due to defibrinogenation and thrombocytopenia, while the local swelling, hemorrhage, and necrosis are due to the actions of the hemorrhagins and various edema-inducing (inflammatory) venoms. Shock, which leads to multiple organ failure and death, is often a sequel to hypovolemia following massive blood loss and fluid extravasation into the bitten limb, with possible contribution from bradykinin release caused by autopharmacological actions of the venom. The nature of the autopharmacological factor of the venom has however not been investigated.

Trimeresurus sensu lato (Asiatic Lance-Headed Pit Vipers)

The taxonomy of *Trimeresurus* complex that largely contains the Asiatic arboreal pit vipers has been highly impacted by further morphological exploration and genetic

markers (Malhotra and Thorpe 2004). The genus *Trimeresurus* has been separated into seven genera, at least two of which are medically relevant in Malaysia. Bites from these crotalids, albeit less frequently reported, can cause local and systemic envenoming particularly on the coagulation system (Hutton et al. 1990; Rojnuckarin et al. 2007). Major toxins of medically important pit vipers of *Trimeresurus s. l.* are reviewed in the following section.

***Cryptelytropis purpureomaculatus* (Shore Pit Viper; Mangrove Pit Viper)**

The venom has an intravenous LD₅₀ of 0.9 µg/g. It exhibits moderate thrombin-like enzymes and strong hemorrhagic activities (Tan et al. 1989a). The lethal action of the venom appears to be due to synergistic action of several venom constituents (Tan and Tan 1988a). Biological constituents of the venom that have been characterized include thrombin-like enzymes, hemorrhagin, arginine ester hydrolases, phospholipase A₂ enzymes, and acetylcholinesterase.

The major form of thrombin-like enzyme, purpurase (35 kDa), exhibited both arginine ester hydrolase and arginine amidase activities. Its clotting mechanism appeared to involve the release of fibrinopeptide A from bovine fibrinogen. Double-sandwich ELISA revealed that rabbit antipurpurase IgG cross-reacted with venoms of few species from the *Trimeresurus* complex in a way that corresponded to the recent revision of the taxonomy (Tan 2010).

The major hemorrhagic toxin of the venom has been identified as maculatoxin (Tan and Fung 2002). It is a metalloprotease of 38 kDa. It is mildly acidic (pI 4.2), with an N-terminal sequence TPEQQRFPPTYIDLGIFVDHGMAT. Khow et al. (2002a) identified a metalloprotease hemorrhagin from the venom with a higher molecular mass of 72 kDa. The enzyme hydrolyzed all gelatin preparations of type I-IV collagens while only type IV native collagen was susceptible to the hydrolysis.

Purpureotin, a 30-kDa didimeric (αβ) C-type lectin-like protein, was purified from the venom and shown to dose-independently induce platelet aggregation without any cofactor, presumably through the binding of GPIb of platelet (Li et al. 2004). Besides, there are four PLA₂ isoenzymes in the venom with isoelectric points of 4.03, 4.5, 4.8, and 5.3, respectively. They are not lethal to mice at high dose but exhibit edema-inducing activity (Tan et al. 1989c). Unlike most other crotalid venoms, *C. purpureomaculatus* venom exhibits acetylcholinesterase activity. Interestingly, the enzyme is an acidic protein with kinetic properties similar to acetylcholinesterases isolated from cobra and krait venoms (Tan and Tan 1988b). It accounts for less than 0.3 % of the venom dry mass, and its pathophysiological role is uncertain.

The pharmacokinetics of this venom in rabbits has been investigated. It was shown best fitted to a multicompartmental model (Sim et al. 2013). The venom elimination half-lives on intravenous or intramuscular injection are not significantly different (~28 h), although the intramuscular administration significantly reduces the systemic bioavailability of the venom, presumably due to high affinity of some toxins to local tissues at the injection site. The pharmacokinetic profile of the venom appears similar to that of another Asiatic crotalid, *Hypnale hypnale*, which shows

consistency in the systemic pharmacokinetic behaviors of the venom regardless of intravenous or intramuscular injection, while systemic availability is greatly reduced on intramuscular administration (Tan et al. 2014).

***Tropidolaemus wagleri* (Speckled Pit Viper)**

The i.v. LD₅₀ of the venom is 0.9 µg/g in mice. Unlike most pit viper venoms, *T. wagleri* venom does not exhibit hemorrhagic activity (Minton 1968). The venom is feebly procoagulant, contains unique phospholipases A₂, and is rich in atypically low-molecular mass proteins (Tan and Tan 1989a; Wang et al. 1999b). Two basic polypeptides of approximately 7–8 kDa and considerable abundance from the venom were previously found to be lethal in mice with i.v. LD₅₀ values of 0.17 and 0.19 µg/g, respectively (Tan and Tan 1989a); these basic low-molecular mass proteins, however, have not been further characterized. Weinstein et al. (1991) subsequently identified from the venom two smaller lethal polypeptides, termed *waglerin I* (2,504 Da, murine i.p. LD₅₀ of 0.369 mg/kg) and *waglerin II* (2,530 Da, murine i.p. LD₅₀ of 0.583 mg/kg), respectively, with potential vasoactive or neurotoxic activity. Waglerins, however, constitute less than 1 % of total venom proteins (Weinstein et al. 1991). Considering that the venom has an i.v. LD₅₀ of 0.8 µg/g and is capable of inducing very rapid death in mice (Leong et al. 2014), the exceptionally low abundance of waglerins does not appear to support the toxins as the venom's lethal principle. The heat-stable waglerin I has been extensively studied: it is a 22–24 amino acid peptide with a proline-rich sequence, lethal for its neuromuscular paralytic effect by acting on both pre- and postsynaptic sites of mouse motor endplate (Lin et al. 1995; Tsai et al. 1995). It has high affinity and selectivity toward the muscle type of adult postsynaptic nicotinic cholinergic receptor where the γ-subunit is replaced by the ε-subunit (McArdle et al. 1999) and hence a potentially useful receptor-binding tool for probing the expression and function of nAChR (Teichert et al. 2008). Interestingly, waglerin I was also found to have potentiating effect on murine I (GABA) receptors, mimicking diazepam (the action was antagonized by flumazenil) (Ye and McArdle 1997). Phylogenetic study revealed that waglerin peptides were so distinct that they did not match with known proteins and may be derived from a uniquely reptilian peptide (Fry 2005).

The venom exhibits high hyaluronidase activity and moderate LAAO and PLA₂ activities, but the enzymes are nonlethal in mice (Tan and Tan 1989a; Tan 1991). The PLA₂ contains the conserved Glu-6 residue and has a higher molecular mass (15,319 Da) (Wang et al. 1999b) likely due to the presence of complex-type oligosaccharides at N14 (Tsai et al. 2012). It activated mouse platelet aggregation but inhibited human platelet aggregation, and the effect was unaltered by deglycosylation of the enzyme.

Other toxins identified from the venom include platelet aggregation inducers triwaglerin (66 kDa) and trowaglerix (a high-mass 212-kDa multimer of C-type lectin). Triwaglerin activated platelet independent of the formation of thromboxane A₂ and platelet-activating factor or the release of ADP (Teng et al. 1989), while trowaglerix was found to activate platelet through specific binding to GPVI leading to kinase-dependent exposure of functional α_{IIb}β₃ and matrix metalloproteinase-dependent GPVI shedding (Chang et al. 2008).

Interestingly, the species is known to display sexual dimorphism, where the body size and coloration between male and female adult snakes are remarkably distinct. Preliminary profiling of the venoms of both sexes using reverse-phased HPLC, however, showed no significant differences between the chromatograms of venoms from both sexes (Tan CH, unpublished). Molecular variations of toxins from both sexes await further investigation.

***Parias sumatranus* (Sumatran Pit Viper)**

Parias sumatranus venom has an i.v. LD₅₀ of 0.6 µg/g. It exhibits potent hemorrhagic but less thrombin-like activities. The major lethal components of the venom appear to be principally hemorrhagic (Tan and Tan 1989b).

Antivenom Studies for Malaysian Snake Venoms

Antivenom is the specific treatment for snake envenomation (WHO 2010). Unfortunately, the management of snakebite in many places is still confronted with issues pertaining to limited supply and suboptimal quality of antivenom products, ostensibly for financial reasons (Gutiérrez et al. 2006; Williams et al. 2011). Proper assessment and optimization of existing antivenoms are therefore important for expanding and sustaining the use of an effective product. It is hoped that this approach will benefit endemic areas where the local authority has failed to develop a specific antidote.

Preclinical assessment of antivenom efficacy principally relies on neutralization studies using animal models, both in vitro and in vivo, against the lethal effect of venom. Survival is perhaps the most direct, meaningful, and evidenced outcome to indicate the protection conferred by an antivenom. In brief, in vitro neutralization studies involved preincubation of venom (at 2–5 LD₅₀ as lethal challenge dose) and antivenom (at various doses) followed by injection of the mixture into mice, while in in vivo neutralization studies, animals were experimentally envenomed with a lethal dose of venom, followed by administration of antivenom at a later time, mimicking actual snakebite. The “efficacy” of an antivenom against a particular venom is then expressed in terms of “median effective dose” (ED₅₀) or “potency” (P), the standardization of which needs to be strictly adhered for comparison between antivenoms. Other assessment methods include neutralization tests for various toxicities; e.g., hemorrhagic, nephrotoxic, myotoxic, procoagulant, neuromuscular depressant activities (Tan et al. 2011, 2012; Leong et al. 2012a) are sometimes required to serve as corroborative evidence to support the lethality protection by an antivenom. Time course study of blood venom profile provides a more conclusive picture of how an antivenom can alter the pharmacokinetics of a venom in favor of hastening the venom elimination from the body; however, such studies remain lacking locally.

Malaysia depends greatly on antivenom supply from Thailand, notably products from the Thai Red Cross Society of the Queen Saovhaba Memorial Institute (QSMI), Bangkok, for the fact that many venomous snakes in Thailand are similar or closely

related to those in Malaysia (at least from Peninsular Malaysia). Recent studies revealed that polyvalent antivenom imported from India, raised against the Indian Big Four (*Naja Naja*, *Bungarus caeruleus*, *Daboia russelii*, and *Echis carinatus*), has weak or nil protective effect against the venom toxicities of Malaysian snakes, but effective cross-neutralization has been observed with the Thai antivenoms (Leong et al. 2012a, 2014). Two recent improved products from QSMI, Neuro polyvalent antivenom (NPAV) and Hemato polyvalent antivenom (HPAV), are equine F(ab)₂ refined through capric acid precipitation and pepsin digestion of the IgG (Khomvilai 2008). NPAV is raised against *N. kaouthia*, *O. hannah*, *B. candidus*, and *B. fasciatus* venoms, while HPAV is raised against *C. rhodostoma*, *C. albolabris*, and *D. siamensis*, all of Thai origin. These polyvalent antivenoms, usually indicated when the biting species is uncertain, showed higher neutralizing capacity in animal studies compared to their monovalent counterparts, presumably due to synergistic cross-neutralizing components in the polyvalent antivenom and technical enrichment during the manufacturing process.

Generally, NPAV effectively neutralizes venoms of many common Asiatic cobras (*Naja* sp.), *O. hannah*, and kraits (*Bungarus* sp.) from Southeast Asia including those of Malaysian origin but only moderately to weakly effective against venoms of *Naja* cobras from India and Africa. Studies with several venoms showed that the in vivo neutralization potency of the NPAV was comparable to the in vitro neutralization potency (Leong et al. 2012b). HPAV effectively neutralized venom lethality of selected Asiatic species from *Calloselasma*, *Cryptelytrops*, *Protobothrops*, *Popeia*, *Deinagkistrodon*, *Daboia*, except for *Tropidolaemus wagleri* venoms (Leong et al. 2014; Tan CH, unpublished data). In addition, the procoagulant, hemorrhagic, and necrotic effects of Malayan pit viper were effectively neutralized. It is also likely that HPAV can neutralize venoms of other species of pit vipers belonging to the *Trimeresurus* complex (Asian lance-headed vipers), as early works have demonstrated extensive cross-neutralization of specific antivenom against different species of the *Trimeresurus* complex (Tan et al 1994). The Australian Commonwealth Serum Laboratories (CSL) sea snake antivenom has remained thus far effective against a wide range of sea snakes including those in the coastal waters of Malaysia (White 2013).

Table 1 shows the i.v. LD₅₀ vales of some medically important Malaysian snake venoms and the ED₅₀ as well as potencies of appropriate antivenoms. Majority of the species in Malaysia are heterologous to those venoms which are used in immunizing horses for antivenom production. Cross-neutralization of venom is however possible when there are common toxin epitopes which can be bound and rendered inactive by a paraspecific antivenom. This may be largely reflected by the close phylogenetic relationship and geographic proximity of different snake species.

Conclusion and Future Directions

The venomous species inhabited in Malaysia are medically important. The vast variations in the toxin compositions and molecular structures in venoms of different species are accountable for the diverse pharmacological actions of the venoms as

well as the different responses to antivenoms. In the absence of a local Malaysian manufacturer for antivenom, the country relies mainly on antivenoms supplied by the Queen Saobhava Memorial Institute, Bangkok, for use in Malaysia. While being therapeutically useful, the cross-neutralization results may also contribute to the design of broad-spectrum polyvalent antivenoms for a wider regional application. Antivenomic study on local snake venoms represents another progress in venom research in the country; the findings are useful for the improvement of antivenom efficacy and neutralizing spectrum.

Cross-References

- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Snake Venom Toxins Targeted at the Nervous System](#)

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Part II

Snake Venom Enzymes and Enzyme Inhibitors

Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins

2

Fiorella Tonello and Michela Rigoni

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Abstract

Snake venoms contain a large amount of toxins endowed with phospholipase A₂ (PLA₂) activity. Despite an overall conserved structure, snake PLA₂s display a variety of pharmacological activities that are the result of different cell targets and mode of actions. Here follows an overview of the present knowledge on the mechanism of action of the two main classes of snake PLA₂s, myotoxins and neurotoxins, derived from in vivo, ex vivo, and in vitro models, along with a comparison with mammalian secreted PLA₂ (sPLA₂) homologues. In spite of many qualified efforts, several aspects of snake envenomation are still undefined, including the identification of specific receptors on nerve and muscle membranes. Further studies are required to elucidate the unclear molecular steps of snakebite intoxication that might contribute to shed light also on the mode of action of mammalian PLA₂ counterparts. There is a high degree of homology in terms of primary structure between snake and mammalian sPLA₂s, suggesting that snake PLA₂ receptors might be also candidates for mammalian sPLA₂s; this topic is of

F. Tonello (✉) • M. Rigoni (✉)

Department of Biomedical Sciences and CNR Institute of Neuroscience, University of Padova, Padova, Italy

e-mail: fiorella.tonello@gmail.com; rignonimic@gmail.com

high relevance, in the light of the emerging involvement of mammalian sPLA2s in many human disorders.

Keywords

Myotoxic snake PLA2s • Neurotoxic snake PLA2s • Mammalian PLA2s

Introduction: Snake Venom PLA2s

Toxic phospholipases A2 (PLA2) are frequently major components of animal venoms, particularly of snakes'. The UniProtKB/Swiss-Prot Tox-Prot program (Jungo et al. 2012) reports that 7 % of more than 5,000 animal toxins (release 2013_09) are phospholipases, and this percentage reaches 20 % in the case of the 1,799 annotated toxins from snake venoms.

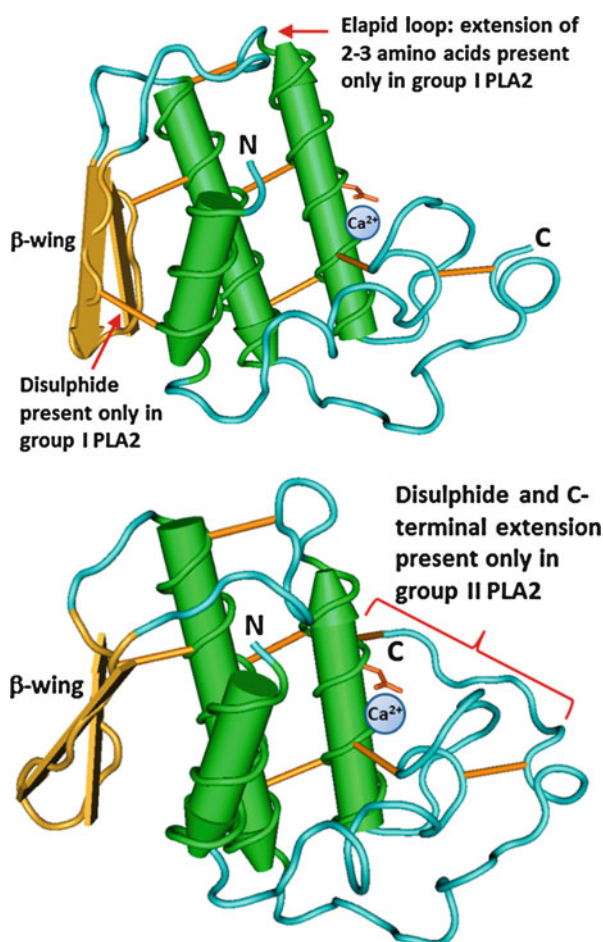
Phospholipases A2 are a superfamily of intracellular and secreted enzymes that hydrolyze the sn-2 ester bond of glycerophospholipids; they have been subdivided in different groups based on size, sequence, localization, function, and divalent cation requirements (Dennis et al. 2011). Snake phospholipases are secreted PLA2s (sPLA2s). sPLA2 from Old World snakes (group I) differ in their disulfide bond pattern from New World snakes (group II). PLA2s from Elapidae (group I) show a sequence similarity with mammalian pancreatic phospholipases, whereas PLA2s from Viperidae (group II) have an amino acid sequence similar to that of mammalian synovial phospholipases. PLA2s of both groups have similar molecular weight (13–15 kDa) and seven intrachain disulfide bonds, the location of one of them being variable (Fig. 1). The conserved structural fold, common to both groups, presents a short N-terminal α -helix, followed by a loop implicated in Ca^{2+} binding, a second long α -helix, a β -wing region, and a third long α -helix ending with a C-terminal loop. Group II PLA2s have a longer C-terminal loop (6–7 amino acids) and a disulfide bond connecting a C-terminal cysteine to the second α -helix; in group I a disulfide bond, not present in group II PLA2s, connects the central turn region of the β -wing to the first α -helix. Moreover, group I PLA2s present an insertion of 2–3 amino acids in the loop between the second α -helix and the β -wing, the so-called elapid loop (Dennis et al. 2011; Lomonte and Gutiérrez 2011).

Only PLA2s of group I are secreted with a pro-peptide that is removed during the maturation process, which probably involves snake venom serine proteinases. Sometimes the peptide is not removed at all, giving rise to PLA2s that lack the catalytic activity, similar to the precursor of pancreatic PLA2 enzyme (Doley and Kini 2009).

Many other sPLA2s were successively found in animals, plants, fungi, bacteria, and virus and have been divided in 17 groups (Dennis et al. 2011). Almost all sPLA2s contain a highly conserved calcium-binding loop (XCGXGG) and a catalytic motif (DXCCXXHD). The active site is composed by the dyad Hys/Asp, by a calcium ion coordinated by the conserved Asp49, and by the carbonyl oxygen of tyrosine and glycine in the calcium-binding loop.

Many natural variants of snake PLA2s have no enzymatic activity but are toxic. They present a mutation in position 49: the aspartic acid (D49) is substituted by

Fig. 1 Tridimensional structures of group I (*left*) and II (*right*) snake PLA₂s. Group I model was obtained from the notexin 1AE7 pdb atomic coordinate file, while group II model from the ammodytoxin A 3G8G file. The pictures were prepared with the Cn3D structure viewer from NCBI's Entrez Structure Database. The N- and C-termini of the proteins are indicated by the letters *N* and *C*, respectively; the three main α -helices are represented by *green* cylinders and the β -wing by *gold* barrels; the disulfide bonds are in *yellow*. Calcium ions, not present in the coordinate files, are represented by *gray* spheres close to the lateral chains of aspartic 49. The differences between group I and group II structures are evidenced



another amino acid (lysine in most cases, but also asparagine, serine, glutamine, and arginine have been described), unable to coordinate the calcium ion essential for catalysis. In some cases, the lack of PLA₂ activity of these natural variants also depends on additional amino acid substitutions. Despite the lack of catalytic activity, these PLA₂ homologues show a high myotoxic activity together with other toxic effects, such as edema, hyperalgesia, and release of proinflammatory cytokines (Lomonte and Rangel 2012).

The quaternary (IVD) structure of snake PLA₂s is highly variable: most of these proteins exist as monomers or non-covalent homodimers, but some of them form complexes through covalent and/or non-covalent interactions with other PLA₂s or with other proteins. The most complex neurotoxin is textilotoxin (group I), which consists of four different PLA₂ subunits (A, B, C, and D); originally described as a pentamer (Tyler et al. 1987), it was recently reported to be a hexamer, possibly indicating loose interactions with in vitro dissociation

(Aquilina 2009). Taipoxin (group I) is the only known heterotrimeric PLA2 constituted of three PLA2 subunits; many heterodimeric snake toxins are made of two PLA2 subunits; others have one PLA2 subunit (A chain) and a B chain homologous to the basic protease inhibitor (i.e., β -bungarotoxin, group I). In the case of multimers of different PLA2 subunits, usually only one of them is catalytically active, while the others are inactive or partially active (Doley and Kini 2009).

The isoelectric point of snake PLA2s is highly variable: the same venom contains both basic and strongly acidic PLA2s. Several acidic PLA2s are not toxic and have presumably a digestive role; in some cases, they are associated with basic PLA2 subunits in dimeric or multimeric assemblies. Toxicity is usually ascribed to basic PLA2s (Lomonte and Gutiérrez 2011).

Pharmacological Effects of Snake PLA2s

Snake venoms consist of a mixture of toxins and enzymes that have evolved to capture and digest the prey as well as for defense from predators. The major clinical effects of envenoming in humans include coagulopathy, neurotoxicity, myotoxicity, and renal impairment, depending on the snake species (Warrell 1996).

Snake venom PLA2s display different toxic effects, and for this, they have been classified in three main groups: neurotoxins, myotoxins, and hemostasis-impairing toxins.

Neurotoxins cause paralysis by affecting the pre- or postsynaptic element of the neuromuscular junction (NMJ); myotoxins target the skeletal muscle and can act locally and/or systemically, and hemostasis-impairing toxins include PLA2s with pro- or anticoagulant effects and others with hemolytic activity. Some phospholipases display more than one toxic activity: i.e., in group I some PLA2s are both neurotoxic and myotoxic, but myotoxicity is observed only when the toxins are intramuscularly injected. In vivo, they cause death of the prey by respiratory paralysis due to a presynaptic action (Montecucco et al. 2008). On the other hand, a presynaptic neurotoxic activity of some myotoxins of group II has been described only in vitro and ex vivo systems (mouse hemidiaphragm and chick biventer nerve-muscle preparations, Gallacci and Cavalcante 2010; Ponce-Soto et al. 2010). Crotoxin and crotoxin-like proteins (group II) are examples of toxins with both systemic neurotoxic and myotoxic activities. Additional toxic effects include edema, hypotension, and damage to other organs like the liver, kidney, lungs, testis, and pituitary gland (Kini 2003; Doley and Kini 2009).

For some snake PLA2s, other pharmacological effects reported comprise antibacterial, antiviral, antiparasitic, antitumoral, anti-inflammatory, immunomodulatory, and analgesic activities (Nevalainen et al. 2008; Rodrigues et al. 2009; Sampaio et al. 2010).

A detailed correlation between such different activities and primary sequence motifs is still lacking. The resolution of a higher number of PLA2 structures, together with a comparison of the amino acid lateral chains exposed in 3D models, will allow to identify 3D motifs crucial for a defined pharmacological activity.

Some recent papers are moving in this direction (Dos Santos et al. 2009; Cendron et al. 2012; Faure and Saul 2012; Salvador et al. 2013).

Figure 2 reports a statistical analysis of snake PLA₂s presently annotated by the UniProtKB/Swiss-Prot Tox-Prot program. From this analysis, it is possible to observe that:

- The number of group I PLA₂s is lower than that of group II.
- The majority of group I snake PLA₂s are D49, with two exceptions (a K49 and a N49), that are part of multimeric toxins containing other catalytically active PLA₂ subunit(s), leading to an enzymatically active complex. The members of this group mainly affect the nervous and hematic systems, with only five toxins reported to affect the skeletal muscle (three of them are both myo- and neurotoxins).
- In group II, hemostasis-impairing toxins prevail on myotoxins and neurotoxins. Many catalytically inactive phospholipases are present, mainly among myotoxins (75 %). Very few catalytically inactive PLA₂s are neurotoxins or hemostasis-impairing toxins, except for those that display both neurotoxicity and myotoxicity. K49 PLA₂s are traditionally considered myotoxins, though some neurotoxicity has been observed in few cases in vitro or ex vivo preparations (Gallacci and Cavalcante 2010).
- In both groups, the number of basic proteins exceeds that of acidic proteins. The percentage of acidic PLA₂s is higher among hemostasis-impairing toxins. Interestingly, almost all myotoxic PLA₂s are basic, which may suggest that membrane binding might be dictated by electrostatic interactions.
- The PLA₂s with the most complex IVD structure (heterohexameric, heterotrimeric) belong to the first group, and in both groups all heterodimeric proteins are neurotoxins.

Here follows a review on the mode of action of snake PLA₂ myotoxins and neurotoxins on different experimental models (in vivo, ex vivo, and in cultured cells), and a brief discussion about the contribution of these results to the understanding of the complex mechanism of action of snake PLA₂ toxins. Finally, analogies between snake and mammalian sPLA₂s will be analyzed, pointing out how the depiction of the molecular mechanism of action of snake PLA₂s could help to better understanding of the role and function of mammalian homologues. Many other authors published several important contributions (_____, _____, _____, _____)

Myotoxic Snake PLA₂s

Acute skeletal muscle damage is a frequent manifestation of snakebite; the most relevant and abundant muscle-damaging toxins present in animal venoms are PLA₂s. Myotoxic PLA₂s are mainly present in viperid venoms, whereas in elapids few myotoxins are described and they display additional activities (see Fig. 2).

PLA2G1	n	%	D49	K49	N49	S49	Q49	R49	Acidic	Basic	Heteromultimers
Myo	2	3,1	2							2	
Myo+Neuro	3	4,6	3						1	2	
Neuro	28	43,1	26	1	1				6	22	1 exa, 2 tri, 2 di
Neuro+Hemo	4	6,2	4						1	3	1 di
Hemo	26	40	26						17	9	
Myo+Hemo	1	1,5	1						1		
Myo+Neuro+Hemo	3	4,6	3							3	
Total	67		65	1	1				26	41	

PLA2G2	n	%	D49	K49	N49	S49	Q49	R49	Acidic	Basic	Heteromultimers
Myo	28	20,1	7	17	1			3	2	26	
Myo+Neuro	14	10,1	11	2		1			1	13	2 di
Neuro	23	16,5	23						9	13	17 di
Neuro+Hemo	9	6,5	9						1	8	5 di
Hemo	49	35,3	46	2		1			37	14	1 di
Myo+Hemo	14	10,1	4	8	2					14	
Myo+Neuro+Hemo	2		1				1			2	
Total	139		101	29	3	2	1	3	50	90	

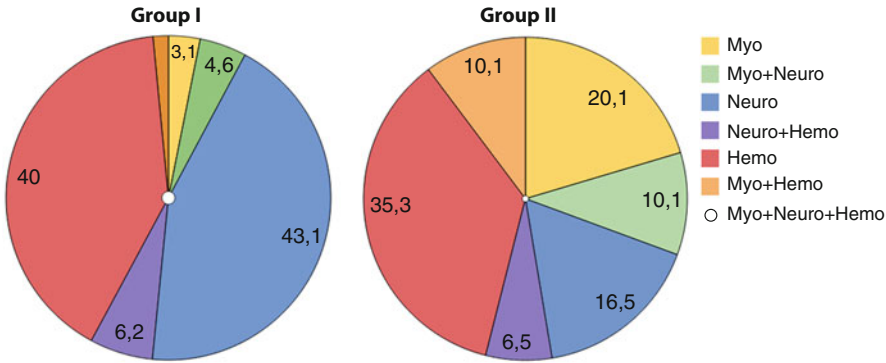


Fig. 2 Statistical analysis of snake PLA2 toxins manually annotated and reviewed by the UniProtKB/Swiss-Prot Tox-Prot program, release 2013_09 (Jungo et al. 2012). Snake PLA2s have been subdivided into groups I and II according to some structural features (see text). Within these groups, a further classification has been made according to their pharmacological properties (*myo* myotoxins, *neuro* neurotoxins, *hemo* hemostasis-impairing toxins), the amino acid at position 49, their acidic or basic properties, and their IVD structure (*di* dimeric, *tri* trimeric, *hexa* hexameric). A graphic representation of the distribution of the toxic properties of group I and II snake PLA2s is reported below

In most cases the major clinical manifestation is a prominent local myonecrosis at the site of snakebite. The venoms of a number of sea snakes, terrestrial elapids, and some viperid species also induce a systemic myotoxicity. In the latter case, envenomation is characterized by a widespread muscle damage, with consequent massive release of creatine kinase (CK) and myoglobinuria, often associated with kidney alterations.

The local effects on skeletal muscle have been mainly described by histological and ultrastructural studies: pathological alterations include plasma membrane disruption, wedge-shaped lesions at the periphery of muscle fibers, hyper-contraction of the myofibers, mitochondrial swelling, and nuclear pyknosis (Gutiérrez and Ownby 2003; Gutiérrez et al. 2009). The toxic activity is rather specific: satellite cells, blood vessel, and nerves are not affected, except in the case of toxins that display both neurotoxic and myotoxic activities. The common target of myotoxins appears to be the sarcolemma. Although the precise binding sites and/or targets of myotoxins have not been identified so far, the common belief is that myotoxic PLA₂s affect the plasma membrane integrity, with consequent rapid influx of calcium ions that triggers a series of degenerative events (Montecucco et al. 2008). In addition, different molecules released by damaged cells may act as danger signals, thus contributing to extend the damage to the surrounding tissues (Cintra-Francischinelli et al. 2010; Zornetta et al. 2012). The putative internalization of group II PLA₂s and their possible interaction with intracellular targets have not been yet explored. The mechanism of membrane damage by myotoxic PLA₂s has been initially ascribed to their enzymatic activity, but the discovery of catalytically inactive myotoxins and experiments with chemically modified PLA₂s (enzymatically inactive but still toxic) led to the concept that in snake myotoxins, enzymatic and toxic activities can be dissociated (Lomonte and Rangel 2012; Gutiérrez and Lomonte 2013). It was therefore proposed that the toxic activity might be due to the interaction with critical targets in tissues, followed by disorganization of plasma membrane due to phospholipid hydrolysis and/or ability to penetrate into the lipid bilayer. Systemic myotoxins are less diffuse and display also some neurotoxicity; differently from local myotoxins, which are monomers or homodimers, they are heterodimers, composed of a non-covalently linked basic and catalytically active PLA₂ subunit and an acidic nonenzymatic subunit. In crotoxin (a PLA₂ neurotoxin from the South American rattlesnake), the prototype of this class of myotoxins, the acidic subunit acts as chaperone that releases the enzymatically active part at the site of action. Accordingly, the recently solved 3D structure of crotoxin reveals that the acidic subunit blocks the substrate access to the catalytic dyad and masks several interfacial binding surface residues important for the interaction of the PLA₂ with phospholipids (Faure and Saul 2012). The ability of crotoxin to affect muscles far away from the site of injection has been explained by its high specificity and affinity for a muscle receptor, whereas local myotoxins, which induce myonecrosis only at very high doses, appear to interact with low-affinity receptors. Moreover, some local myotoxins also bind to and affect different types of cells, speaking in favor of non-muscle-specific receptors or an interaction with different low-affinity sites. Their high isoelectric points and positively charged surface suggest an interaction with negatively charged glycoproteins or phospholipids.

Besides the *in vivo* models, many *in vitro* systems have been employed to shed light on the specificity of action of these myotoxins and on their molecular partners (i.e., the C2C12 muscle cell line). As many local myotoxins display a quite broad toxicity, different kinds of cells that could be involved in the envenomation process

have been employed, including endothelial cells and immune cells (Gutiérrez et al. 2009). C2C12 myotubes appear more sensitive than myoblasts to *Bothrops asper* myotoxins I and II (Angulo and Lomonte 2005; Cintra-Francischinelli et al. 2009). In both kinds of cells, these toxins induce a calcium release from intracellular stores, followed by massive calcium entry from the extracellular medium only in myotubes and by a rapid cell lysis (Cintra-Francischinelli et al. 2009). These toxins also cause the release of K^+ and ATP (Cintra-Francischinelli et al. 2010). ATP release has been studied in details also in RAW274.7 and J744 macrophage cell lines intoxicated with the K49 PLA2-like myotoxin II of *Bothrops asper* (Mt-II). The peak of ATP release precedes the cell lysis, and different inhibitors of purinergic receptors protect cells from Mt-II-induced necrosis. ATP release induced by Mt-II seems to involve different signaling pathways such as those involving trimeric G-proteins, calcium signaling from intracellular stores, and the opening of anionic channels. The catalytically active D-49 myotoxin of *Bothrops asper* (Mt-I), together with other snake myotoxins that lack cytotoxicity toward macrophages, does not induce any ATP release, supporting an idea that ATP is a mediator of Mt-II-induced cell death mechanisms. Released ATP is rapidly hydrolyzed into ADP and AMP by extracellular ATPases; these hydrolysis products can contribute as autocrine and paracrine signals to the activation of purinergic receptors. It has been proposed that this signaling is functional to the insertion of the myotoxin in the plasma membrane, since ATP and its downstream purinergic pathways are involved in membrane composition changes and dynamics (Tonello et al. 2012). Moreover, many purinergic inhibitors protect C2C12 myotubes from the rapid cell necrosis caused by intoxication with *B. asper* myotoxins (Cintra-Francischinelli et al. 2010 and unpublished data), suggesting a mechanism similar to that of Mt-II in macrophages.

B. asper Mt-I and Mt-II, at nonlytic concentrations, and crotoxin activate peritoneal macrophages. Crotoxin increases the basal expression of COX-1 in a process that involves iPLA2 (Moreira et al. 2008). Macrophage activation by *B. asper* myotoxins (both catalytically active and inactive) involves different protein kinases and intracellular phospholipases, COX-2 expression, and prostaglandin production via a NF- κ B pathway and leads to the formation of intracellular lipid droplets (Moreira et al. 2008; Leiguez et al. 2011; Giannotti et al. 2013). The accumulation of lipids in cytoplasmic inclusions has been recently associated with inflammatory and metabolic pathologies (atherosclerosis, obesity, diabetes). Different human secreted PLA2s are involved in inflammation and atherosclerosis, and some of them share a very high homology with snake venom PLA2s (about 50 % of identity). Human sPLA2 activity is partially independent from the enzymatic activity and presumably involves the interaction with specific receptors (Dennis et al. 2011; Leiguez et al. 2011).

B. asper Mt-I activates mouse peritoneal neutrophils inducing COX-2 mRNA and protein expression, followed by production of PGE₂ (Moreira et al. 2011); Bthtx-I, a K49 PLA2 from *Bothrops jararacussu*, stimulates human primary neutrophils migration through the activation of GTP-binding proteins and PKC (Gambero et al. 2004).

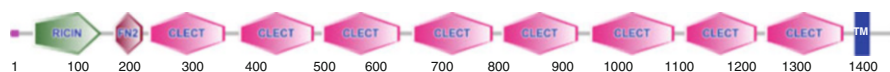


Fig. 3 PLA2R1 structure. PLA2R1 (1,461 amino acids) consists of a N-terminal ricin domain, a fibronectin domain (FN2), eight C-type lectin domains (CLECT), and a C-terminal single trans-membrane domain (TM). PLA2R1 interacts with different snake and human secreted PLA2s. The fifth CLECT domain is the binding domain of OS1 and OS2 of *Oxyuranus scutellatus*. A CLECT domain is present also in α -type PLA2 inhibitors from snake serum (α -PLIs). The picture has been obtained from SMART (a Simple Modular Architecture Research Tool, doi:10.1093/nar/gku949)

These data suggest an additional role for myotoxins. They can act as cytokines, modulating the immune response with a mechanism independent from their hydrolytic activity.

The search for the membrane interactors for snake myotoxins is still ongoing. One PLA2 receptor (PLA2R1) has been found in skeletal muscle, using as a bait OS2, a PLA2 from *Oxyuranus scutellatus* that displays both neurotoxic and local myotoxic activities (Lambeau et al. 1990). This receptor, a 180-kDa protein similar to mannose receptor (a member of C-type lectin family) (Fig. 3), was successively found not to be muscle specific and to interact also with other snake and mammalian PLA2s. Its primary role appeared to be the neutralization and clearance of sPLA2s (Murakami et al. 2011). No protein receptor that could explain the specific myonecrotic activity has been identified so far, and also few investigations on lipidic or glucidic acceptors gave no results (Lomonte and Gutiérrez 2011).

Neurotoxic Snake PLA2s

As shown in Fig. 2, a large number of sPLA2s belonging to both groups I and II are neurotoxins. The blockade of neurotransmission is a common result of the envenomation by elapids and some species of Viperidae and Colubridae. The first type of neurotoxins found in elapid venoms was called α -neurotoxins, nonenzymatic neurotoxins which block neurotransmission by binding with high affinity to the post-synaptic acetylcholine receptor at the neuromuscular junction (NMJ) (Chang and Lee 1963). The same authors isolated and characterized in the same venom the first β -neurotoxin, β -bungarotoxin (β -Btx, from the taipan snake *Bungarus multicinctus*). β -neurotoxins act presynaptically, causing neuromuscular paralysis. β -neurotoxins from elapid snakes are the main factors responsible for the death of the prey, despite a combination of different types of neurotoxins (e.g., ion channel blockers, receptor blockers, and PLA2 active enzymes) is present in the venom of a single species. Once injected in the prey, they rapidly diffuse systemically and reach, among others, respiratory muscles, whose paralysis leads to respiratory failure. However, these neurotoxins do not cross the blood-brain barrier, and if the patient is mechanically ventilated, he/she recovers with time (several weeks) to a very large extent.

The pioneering studies that paved the way to the understanding of the intoxication process by snake presynaptic PLA2 neurotoxins date back to the 1970s. A large

amount of information came from muscular twitch recordings on ex vivo nerve-muscle preparations and from electron micrographs. β -Btx, added to the mouse phrenic nerve-hemidiaphragm preparation, causes an initial increase in the frequency of miniature end-plate potentials (mepps) that then declines almost to zero, while the contractile response of the muscle to direct stimulation remains unaltered. A typical triphasic change of the twitch tension of mouse phrenic nerve-hemidiaphragm preparation has been described: (i) an initial transient decrease in twitch tension followed by (ii) a facilitation phase during which twitch height is greater than control and (iii) a progressive decline of neurotransmission. It is generally accepted that the initial reduction of twitch tension (phase I) is due to the binding of the toxin to the nerve terminal (Chang 1985), while PLA2 activity appears to be essential for phase II and III, that correspond to the toxin-induced exocytosis of synaptic vesicles and the consequent depletion of the terminal. The time required to induce paralysis is shortened by nerve activity (Chang et al. 1973). Cull-Candy and colleagues in 1976 showed that taipoxin (Tpx) and notexin (Ntx), from the elapids *Oxyuranus scutellatus* and *Notechis scutatus*, respectively, cause a similar blockade of the NMJ following in vivo administration, or to the nerve-hemidiaphragm preparation, suggesting that the three snake neurotoxins display a similar presynaptic mode of action. Ultrastructurally, the poisoned endplates appear altered in the presynaptic portion but not postsynaptically, suggesting a very specific site of action of the toxins. In an early stage of poisoning, the presynaptic membrane shows an increased number of Ω -shaped indentations coated with electron-dense material, suggesting aborted endocytosis, and altered electron-dense mitochondria. At later stages, large vesicles are observed, together with a great reduction in synaptic vesicles number (due to enhanced neurotransmitter release) and swollen or broken mitochondria. Axon degeneration appears to be limited to the nonmyelinated terminals.

Axon terminal damage caused by in vivo inoculation of β -Btx was first reported in detail by Dixon and Harris (1999), who performed an accurate analysis of the prolonged neuromuscular paralysis caused in rats by β -Btx by electron microscopy and immunocytochemistry and of the subsequent functional recovery of NMJ. Labeling of the NMJ with anti-synaptophysin and anti-neurofilament antibodies showed that the early onset of paralysis is associated with the depletion of synaptic vesicles from the motor nerve terminals (within 3 h from toxin addition) and followed by the complete degeneration of nerve terminals, while AChR labeling is retained during the whole intoxication process. Synaptophysin reactivity does not return until 5 days after the inoculation of the toxin. The reinnervation of the muscle fiber is associated with extensive pre- and post-terminal sprouting at 3–5 days, which stabilizes by 7 days and then declines.

Experimental models alternative to in vivo and ex vivo NMJ have been employed in the attempt to elucidate the mechanism of action of PLA2 neurotoxins at a molecular level. These models include primary cultures from the hippocampus, cortex, cerebellum, and spinal cord and primary chromaffin cells, as well as cell lines (i.e., NSC34 and PC12), and more recently even the yeast model. These systems are easier to manipulate and can be subjected to a variety of techniques,

but under several aspects are quite different from the physiological target of the toxins; therefore, these results have to be interpreted with caution. The intoxication of primary neurons with low concentrations of different PLA₂ neurotoxins allowed to get insights into some steps of the intoxication process: Ntx, β -Btx, Tpx, and textilotoxin (TeTx), elapid neurotoxins of increasing structural complexity, affect primarily the neurites of cultured primary neurons inducing membrane swellings which are hallmarks of intoxication. *Bulges* are sites on exo-endocytic unbalance (demonstrated by accumulation of the synaptic markers VAMP2 and synaptophysin I and by the permanent exposure on the plasma membrane of a luminal epitope of the vesicular protein synaptotagmin I) (Rigoni et al. 2004). A direct interaction of ammodytoxin (Atx, a group II sPLA₂ from *Vipera ammodytes*) with the endocytic protein amphiphysin has been recently shown in yeast (Mattiazzi et al. 2012). Once bound to the nerve terminal, snake PLA₂ neurotoxins begin to hydrolyze phospholipids of the external layer, and this hydrolysis leads to a rapid accumulation of lysophospholipids and fatty acids. The extent and kinetics of phospholipid hydrolysis by the four toxins are similar, in contrast to the very different values of PLA₂ activities obtained for the same toxins in in vitro PLA₂ assays (Paoli et al. 2009). This study helped to clarify the puzzling lack of correlation between the neurotoxicity in vivo and the PLA₂ enzymatic activity measured in vitro by different methods (Montecucco and Rossetto 2000). Lysophospholipids induce a membrane curvature which favors membrane fusion of synaptic vesicles and, at the same time, inhibits endocytosis, thus explaining the typical features shown by electron microscopy, i.e., synaptic vesicle depletion and Ω -shaped plasma membrane figures. Indeed all the morphological and functional alterations induced by these snake neurotoxins can be reproduced by the addition of a mixture of fatty acids and lysophospholipids (Rigoni et al. 2005; Caccin et al. 2006). High amounts of lysophospholipids, such as those produced by these snake neurotoxins, destabilize membranes, and it was found that *bulges* are sites of calcium influx from the extracellular medium in the cytosol, which is then accumulated inside mitochondria causing loss of their membrane potential (Rigoni et al. 2007, 2008). The increase of cytosolic calcium is well known to cause mitochondrial swelling, cytoskeleton fragmentation, and neuronal degradation (Nicotera et al. 1992); cytoskeleton alterations in primary neurons following neurotoxin addition have been recently associated with calpain activation (Duregotti et al. 2013). In this respect, presynaptic snake PLA₂ neurotoxins are similar to latrotoxin (Tedesco et al. 2009). These data on primary neurons reproduce the main features of NMJ intoxication (unbalanced exo-endocytosis, cytoskeleton disruption, mitochondria damage), thus validating primary neurons as useful models for investigating the molecular mode of action of presynaptic neurotoxins. Calcium toxicity, triggered by the PLA₂ activity of the toxins, appears to be the major determinant of nerve terminal failure and degeneration.

Notwithstanding the many efforts made by several laboratories to identify toxins receptor/acceptors on presynaptic membranes, this fundamental aspect remains ill known. β -Btx B-subunit (the binding part) was shown to interact with voltage-dependent K⁺ channels in synaptic plasma membranes of bovine cerebral cortex (Scott et al. 1990), but the exact role of such interaction in the intoxication process is

still undefined. For OS2, a neurotoxin from the Australian taipan snake *Oxyuranus scutellatus scutellatus*, proteins of different molecular weight were detected as receptors in rat synaptic membranes and termed N-receptors (Lambeau et al. 1989), but not further defined. It appears that the different β -neurotoxins have different receptors and that N-receptors may consist of different receptor sub-populations. Neuronal pentraxins 1 and 2 were also proposed as extracellular-binding proteins for taipoxin (Schlimgen et al. 1995), though these are soluble proteins.

In addition, several intracellular proteins have been described as binding partners at least for some neurotoxic PLA2s, implying toxin internalization and intracellular activity. Some interactors are cytoplasmic (i.e., calmodulin and 14-3-3 proteins); some are located in ER lumen (i.e., PDI and TCBP49), and also a mitochondrial protein (R25) was described (Sribar and Krizaj 2011). It appears difficult to extend the results obtained with a specific neurotoxin to other neurotoxic sPLAs, despite a common structure and intoxication mechanisms. The internalization of Tpx, Ntx, and two other elapid neurotoxins has been investigated by *live imaging* in primary neurons employing Alexa-conjugated toxins. A rapid uptake was observed, with a clear and neuronal-specific mitochondrial localization within few minutes from toxin addition (Rigoni et al. 2007). Electron microscopy of soleus muscle injected with a nanogold-conjugated mutant of Atx showed nanogold particles in the synaptic cleft, in particular in the presynaptic plasma membrane and also within the nerve terminal, in close association with mitochondria and cytoplasmic vesicles, in agreement with the results obtained in primary neurons (Logonder et al. 2009).

Conclusion and Future Directions

Snake PLA2s have been long known: the first snake PLA2 was isolated about 80 years ago and termed crotoxin. Despite many decades of research, several aspects of their mechanism of action are still undefined. Clearly the enzymatic activity is important for the block of neurotransmission at the presynapsis by neurotoxic PLA2s, as well as for the degeneration of the sarcolemma in the case of myotoxins. The PLA2 activity alone, however, does not explain the specificity of action of these toxins; moreover, some natural PLA2 variants deprived of catalytic activity nevertheless retain toxicity, mainly toward muscles.

In vitro cellular models allowed to unveil additional properties of snake PLA2s that can contribute to the composition of the puzzle regarding the toxic activity of these proteins: for example, besides their activity toward muscles, myotoxic PLA2s can act as cytokines on immune cells, stimulating the activation of macrophages and neutrophils.

Similarly to snake PLA2s, mammalian sPLA2s are involved in different cellular processes such as blood coagulation, inflammation, proliferation, and the development of many kinds of tumors (Dennis et al. 2011). Figure 4 shows a matrix of the sequence identity between small secreted human PLA2s and some representative neuro- and myotoxic snake PLA2s. Interestingly, the degree of homology between

	Ntx	OS-2	β-Btx	Mt-I	Mt-II K49	Ctx CBa2	AtxA	PLA2 G1b	PLA2 G2A	PLA2 G2D	PLA2 G2E	PLA2 G2F	PLA2 G5	PLA2 G10
Ntx	-	56	45	31	30	29	36	48	35	29	37	26	31	32
OS-2	56	-	46	36	33	36	36	47	37	30	40	26	32	34
β-Btx	45	46	-	38	33	39	36	44	36	27	39	24	32	31
Mt-I	31	36	38	-	58	63	66	34	44	43	46	32	39	39
Mt-II K49	30	33	33	58	-	48	59	30	50	46	43	32	41	36
Ctx A CBa2	29	36	38	64	48	-	60	33	48	46	46	31	43	33
AtxA	36	36	36	66	59	61	-	35	48	44	47	33	46	40
PLA2 G1b	48	47	44	34	30	33	35	-	34	34	34	26	28	28
PLA2 G2A	35	37	36	44	50	50	48	34	-	50	56	33	44	36
PLA2 G2D	29	30	27	43	46	49	44	34	50	-	40	41	43	39
PLA2 G2E	37	40	39	46	43	47	47	34	56	40	-	37	43	38
PLA2 G2F	26	26	24	32	32	31	33	26	33	41	37	-	32	29
PLA2 G5	31	32	32	39	41	44	46	28	44	43	43	32	-	37
PLA2 G10	32	34	31	39	36	33	40	28	36	39	38	29	37	-

Fig. 4 Matrix of identity between the primary sequence of some representative snake toxic PLA₂s and small secreted human PLA₂s. The degree of identity was calculated by the UNIPROT sequence alignment tool with standard setting. Pre- and pro-peptide sequences were not considered in the comparison. Ntx, notexin, a PLA₂ from *Notechis scutatus scutatus* that displays both neurotoxic and myotoxic activities (P00608); OS2, a presynaptic and myotoxic PLA₂ from *Oxyuranus scutellatus* (Q45Z47); β-Btx, β-bungarotoxin A1 chain from *Bungarus multicinctus* (P00617), which is part of a presynaptic heterodimeric toxin; Mt-I and Mt-II, myotoxins I (P20474) and II (P24605) from *Bothrops asper*; Ctx CBa2 CtxA, basic subunit of crotoxin (P24027), a heterodimeric systemic myotoxin and neurotoxin from *Crotalus durissus terrificus*; AtxA, ammodytoxin A, a presynaptic neurotoxin from *Vipera ammodytes ammodytes*

snake and human PLA₂s is comparable, and in some cases higher, with respect to the sequence homology among snake PLA₂s having the same toxic activities. The highest degree of homology with group II snake myotoxins is shown by mammalian PLA₂G2A, a PLA₂ highly concentrated in synovial fluids and tears, where it plays antibacterial and inflammatory roles. As other sPLA₂s of group II, it contributes to inflammation by releasing fatty acids, therefore initiating the synthesis of lipidic inflammatory mediators. As in the case of snake PLA₂s, also some mammalian

sPLA2s act independently from their enzymatic activity; in addition, one secreted mammalian PLA2 is catalytically inactive (PLA2G12B), and proteins containing enzymatically inactive PLA2-like motifs have been described (otoconin-90) (Dennis et al. 2011).

Among its different activities, mammalian PLA2G2A participates (together with PLA2G5 and PLA2G10) in the formation of atherosclerotic plaques, and different sPLA2s (including PLAG1B and PLAG2A) are involved in obesity and insulin resistance (Divchev and Schieffer 2008; Murakami et al. 2011). A direct activity of mammalian sPLA2s toward muscle cells has not been documented so far. Given the important role played by skeletal muscle in the regulation of glucose and lipoproteins levels in the blood and the homology between snake myotoxins and mammalian sPLA2s, skeletal muscle could be a putative target also for the mammalian enzymes.

In the central nervous system, PLA2G2A has been shown to play a role in mediating several important physiological processes, including regulation of neurotransmitter release, neuritogenesis, and neuronal apoptosis. In normal rat brain, low levels of PLA2G2A protein are detected in the brain stem and spinal cord and also in the mitochondria of cerebellar granule cells and cortical neurons. Studies using the rat model have demonstrated an upregulation of this enzyme in the brain in response to injury and inflammation, although the mechanism remains to be further investigated (Lee et al. 2011). Anti-inflammatory and neuroprotective effects of sPLA2 inhibition have been reported after cerebral cortex lesions, with inhibition of the recruitment and activation of macrophages/microglia and protection toward cortical neurons. A protection at the spinal cord level was also described (Chen et al. 2012).

There is increasing interest in the contribution of secreted phospholipase A2 enzymes to neurodegenerative diseases. In Alzheimer disease (AD) brains, sPLA2G2A immunoreactivity is predominantly associated with astrocytes, some of them surrounding AD plaques. Although each neurodegenerative disease has a separate etiology, under many aspects they recall some features of the intoxication by snake toxins, i.e., excitotoxicity, oxidative stress, and mitochondrial dysfunction. The definition of the molecular steps mediating snake PLA2 toxicity could contribute to the understanding of the mechanism(s) and physiological consequences of PLA2G2A upregulation in AD brain; these studies will enhance the development of novel therapeutics to inhibit the inflammatory responses and to retard the progression of the disease.

Notwithstanding the role of human secreted PLA2s in many pathologies, to date few human PLA2 interactors are known. The first mammalian PLA2 receptor, PLA2R1, has been identified by cross-linking experiments involving the snake PLA2 OS2 (Lambeau et al. 1990). PLA2R1 shares a common structural motif with a class of PLA2 inhibitors identified in snake blood. Other 2 classes of PLA2 inhibitors from snake blood have structural motifs in common with abundant mammalian surface proteins, i.e., human leucine-rich alpha2-glycoprotein and Ly-6 superfamily of proteins. These homologies could help in the research for snake PLA2 receptors that might act as well as mammalian sPLA2 receptors.

Cross-References

- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Secreted Phospholipases A₂ with \$\beta\$ -Neurotoxic Activity](#)

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Secreted Phospholipases A₂ with β -Neurotoxic Activity

3

Dušan Kordiš and Igor Križaj

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D. Kordiš (✉)

Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

e-mail: dusan.kordis@ijs.si

I. Križaj (✉)

Department of Molecular and Biomedical Sciences, Jožef Stefan Institute, Ljubljana, Slovenia

Department of Chemistry and Biochemistry, Faculty of Chemistry and Chemical Technology, University of Ljubljana, Ljubljana, Slovenia

Centre of Excellence for Integrated Approaches in Chemistry and Biology of Proteins, Ljubljana, Slovenia

e-mail: igor.krizaj@ijs.si

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Abstract

Natural toxins are a threat to health but have also turned out to be very useful. Among the most illustrative examples are the deadly botulinum toxins that are used today in numerous therapeutic applications. The essential condition for a toxin to be used as a medicament or a research tool is to understand about how it interferes with the victim's physiology. In this chapter, a group of snake venom toxins is described, the β -neurotoxins, whose mechanism of action at the molecular level is still not completely understood, leaving their medical potential yet to be exploited. As explained, these molecules have evolved by a process of accelerated evolution from harmless digestive enzymes, the secreted phospholipases A_2 , to potent toxins that block synaptic signaling in vertebrate skeletal muscles. To advance the insight into the molecular basis of action of β -neurotoxins, the results of the most recent investigations have been examined critically. A draft picture of how β -neurotoxins poison the nerve terminal is presented. Important details, including the identity of β -neurotoxicity-linked receptors, pathways by which β -neurotoxins cross plasma and mitochondrial membranes, and intracellular regulation of their enzymatic activity, are however still unknown. Unraveling these issues should provide considerable support for clarifying the functions and dysfunctions of mammalian secreted phospholipases A_2 in the nervous system.

Keywords

Snake venom • Secreted phospholipase A_2 • β -neurotoxin • β -neurotoxicity • Adaptive evolution • Neofunctionalization

Introduction

Secreted phospholipases A_2 (sPLA₂s; EC 3.1.1.4) are important components of many animal venoms. Besides their digestive function, these enzymes exert a variety of pharmacological activities, the best known being neurotoxicity, myotoxicity, anticoagulant activity, cardiotoxicity, and cytotoxicity (Kini 1997). The focus of this chapter is on the sPLA₂s that paralyze the neuromuscular junctions (NMJ) in vertebrate skeletal muscles, the so-called β -neurotoxic sPLA₂s (β -ntxs).

Secreted PLA₂s have been classified into eleven groups according to their structure (Murakami et al. 2011). Only some members of groups GI, GII, and GIII can block NM transmission (reviewed in Pungerčar and Križaj 2007; Šribar et al. 2014). Most abundantly, β -ntxs are found in the venoms of Elapidae and

Viperidae snakes. All these sPLA₂s have a molecular mass between 13 and 19 kDa, have 5–8 disulfide bonds, and require Ca²⁺ ions for enzymatic activity. While their primary structures are highly homologous, they differ substantially in their quaternary structure, being monomeric or non-covalent complexes of two, three, or five protein subunits. At least one subunit in a multimeric β -ntx is always an sPLA₂. β -bungarotoxins (β -Butxs), from the *Bungarus* family of snakes, are unique for being covalent dimers of an sPLA₂ and a bovine pancreatic trypsin inhibitor-like protein. Intriguingly, no matter what the structural type, the pathological result of the action of a β -ntxon NMJ scarcely varies. The crystal structures of a monomeric, a dimeric, and a trimeric β -ntx have been reported recently, those of ammodytoxin (Atx) from the venom of *Vipera ammodytes ammodytes* (Saul et al. 2010), crotoxin from the venom of *Crotalus durissus terrificus* (Faure et al. 2011), and taipoxin from the venom of *Oxyuranus scutellatus scutellatus* (Cendron et al. 2012), respectively.

In the first part of the chapter, we describe our current knowledge of the origin, evolution, and impact of novel functions of the snake venom sPLA₂s. The functional diversification of snakesPLA₂ genes is a key evolutionary process that is manifested in the gain of diverse pharmacological activities, including the β -neurotoxicity. In the second part of the chapter, we discuss the most recent insights in the process of β -neurotoxicity at the cellular and molecular level.

Molecular Evolution of Snake Venom sPLA₂s

The phenomenon of accelerated evolution in the snake sPLA₂ toxins was first observed in the cDNAs encoding sPLA₂ isozymes from *Protobothrops flavoviridis* venom gland (Nakashima et al. 1993). Since then, it has been reported in other Crotalinae, Viperinae, and Elapidae snakes (reviewed in Gubenšek and Kordiš 1997; Ohno et al. 1998; Kordiš and Gubenšek 2000; Kordiš et al. 2002). The extraordinarily high level of positive selection acting on snake venom sPLA₂ genes indicates that adaptive evolution has played an important role in the emergence, diversification, and refinement of numerous pharmacological functions and may contribute to niche differentiation following speciation (Lynch 2007). Venom sPLA₂ multigene families contain a variety of sPLA₂ paralogues with diverse pharmacological activities acquired during the course of evolution (Gubenšek and Kordiš 1997; Kordiš and Gubenšek 2000; Kordiš et al. 2002). The evolutionary history of snake venom sPLA₂ multigene families can be regarded as the evolution from a single, general purpose (digestive), ancestral sPLA₂ gene to multiple, increasingly diversified sPLA₂s (e.g., myotoxins and neurotoxins) through repeated gene duplications. The occurrence of a number of sPLA₂ genes in a single snake species indicates that several gene duplications have occurred during the evolution of sPLA₂s in most snake species following speciation (Kordiš and Gubenšek 1996; Gubenšek and Kordiš 1997; Kordiš and Gubenšek 2000; Kordiš et al. 2002).

Numerous Recruitment Events Have Led to Snake Venom sPLA₂ Toxins

Venom proteins have resulted from toxin recruitment events in which an ordinary protein gene, typically one involved in a key regulatory process, is duplicated, and the new gene is selectively expressed in the venom gland (Fry et al. 2009; Casewell et al. 2013). After their recruitment, venom proteins evolve rapidly by a “birth and death” model of evolution, in which frequent duplication of protein-encoding genes permits rapid functional and structural diversification alongside enhanced rates of sequence evolution (Kini and Chan 1999; Kordiš and Gubenšek 2000; Casewell et al. 2013). Although some genes were deleted from the genome or degenerated into pseudogenes, others have undergone neofunctionalization (Fig. 1), resulting in the generation of functionally diversified proteins (Kordiš and Gubenšek 2000; Kordiš et al. 2002; Lynch 2007; Fry et al. 2009). Toxin multigene families preserve the molecular scaffold of the ancestral protein, but key functional residues outside the core scaffold are often modified to acquire numerous novel pharmacological activities (Kordiš and Gubenšek 2000; Kordiš et al. 2002; Fry et al. 2009).

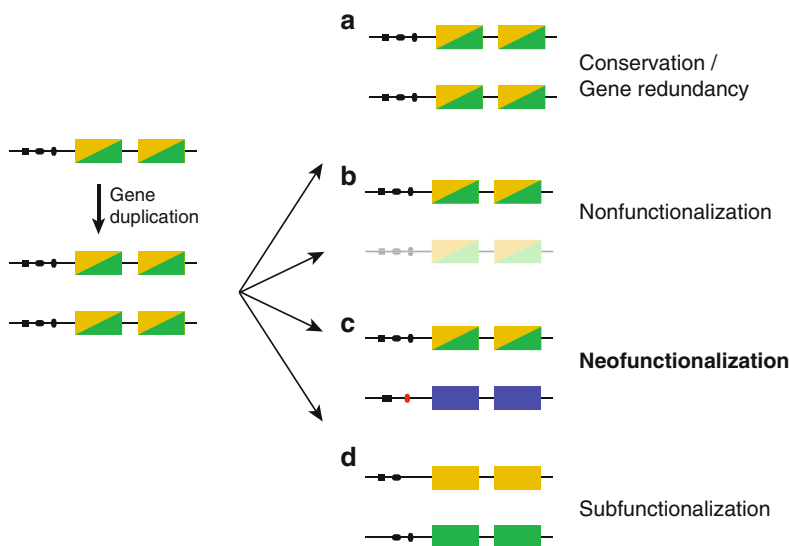


Fig. 1 Evolutionary fates of gene copies after duplication. On the *left side*, schematic representation of a gene undergoing gene duplication (exons are depicted as yellow/green blocks and regulatory elements as geometric forms). On the *right side*, the four major possible evolutionary fates of the copies are represented: **(a)** both copies remain essentially unchanged and functionally redundant (conservation/gene redundancy). **(b)** One of the gene copies is deleted from the genome (or pseudogenized), restoring the initial situation (nonfunctionalization). **(c)** One of the copies accumulates mutations in its coding sequence and/or associated regulatory elements (in red and blue), thereby acquiring new gene functions (neofunctionalization). **(d)** Coding sequences and regulatory elements may also evolve and be partitioned according to the specific roles played by each subfunctionalized gene copy (exons indicated as yellow or green only) (subfunctionalization).

Convergently recruited proteins share several conserved features: a secretory protein ancestor, functionally versatile protein ancestors with a fundamentally conserved basal activity, extensive disulfide cross-links, and stable molecular scaffolds. Once recruited, adaptive evolution generates a suite of novel isoforms with neofunctionalization (Fry et al. 2009; Casewell et al. 2013). Snake sPLA₂ toxins originated by two independent recruitment events (Fry et al. 2009), confirming the independent evolution of the toxins in elapid (GI sPLA₂s) and viperid venoms (GII sPLA₂s). The recruitment of GII sPLA₂s in viperids was linked closely to the evolution of advanced venom delivery systems and ambush feeding (Fry et al. 2009). In contrast to the snakes, GI sPLA₂s have undergone much more complex and dynamic evolution in invertebrates by numerous gene duplications, resulting in the largest diversity of GI sPLA₂s in invertebrate genomes (Razpotnik et al. 2010).

Neofunctionalization and Adaptive Evolution in Snake Venom sPLA₂ Toxins

Neofunctionalization in sPLA₂ toxins is defined as the emergence of a new toxic effect from an ancestral enzyme that did not possess that effect as its main toxin function (Lynch 2007). Although previous studies of sPLA₂ genes identified positive Darwinian selection in viperid GII sPLA₂s and elapid GI sPLA₂s genes (reviewed in Gubenšek and Kordiš 1997; Ohno et al. 1998; Kordiš and Gubenšek 2000; Kordiš et al. 2002), these studies focused only on one or two species and included relatively few genes and used methods that lack power to detect episodic adaptive evolution. Snake venom sPLA₂s are members of large multigene families with diverse pharmacological activities including neurotoxic, myotoxic, cardiotoxic, anticoagulant, and hemolytic effects (Kini 1997). These diverse activities evolved from an ancestral nontoxic sPLA₂s by a process of repeated gene duplication followed by functional divergence. Diverse pharmacological activities of snake venom sPLA₂s originated in sPLA₂ genes after gene duplications that occurred after they diverged from nontoxic ancestors. It has been found that increase in genomic complexity (through gene duplications) can lead to phenotypic complexity (venom composition) and that positive Darwinian selection is a common evolutionary force in snake venoms (Lynch 2007). Recently, it has been shown that positive Darwinian selection and neofunctionalization is common in snake venom sPLA₂ genes and is associated with the evolution of new toxin functions and speciation events, demonstrating that molecular adaptation has played a pervasive role in the evolution of snakes and their venom arsenal. The pattern of gene duplication and positive selection indicates that adaptive evolution occurs immediately after duplication events as novel functions emerge and continues as gene families diversify and are refined. Maximum likelihood models of coding-sequence evolution have been used to test the hypothesis that functional diversification of snake venom sPLA₂ genes was driven by positive Darwinian selection (Lynch 2007). Positive selection occurred in the stem lineage of 67 % of GI sPLA₂ functional groups and of 88 %

of GII sPLA₂ functional groups, indicating that positive selection played a pervasive role in the origin of novel toxin functions during the diversification of vipers and elapids and their venoms. Up to 65 % of sites in GI sPLA₂ genes and 27 % of sites in GII sPLA₂ genes have been identified to be under positive selection. This is strong evidence that diversification of snake venom sPLA₂ genes is driven by recurrent positive selection and suggest that venomous snakes are caught in a co-evolutionary arms race with their prey - as prey evolves resistance to the current venom arsenal snakes evolve ever more toxic venoms (Lynch 2007). The molecular evolution of GI sPLA₂ and GII sPLA₂ genes is characterized by the “birth and death” and “selective sieve” processes of gene duplication, divergence, and loss. Such a pattern suggests a scenario where dietary shifts following speciation run the sPLA₂ gene repertoire through a “selective sieve” – those genes that are no longer effective in subduing new prey species are lost, while genes that are still effective adapt to the new prey type and subsequently diversify (Lynch 2007).

Regions Under Positive Selection Are Located on the Surface of Snake Venom sPLA₂ Proteins and Produce a Wide Spectrum of Pharmacological Effects

Secreted PLA₂ toxicity is independent of enzymatic activity and is mediated through “pharmacological sites” on the protein surface that interact directly with ligands on the cell membrane (Kini 2003). The surface of sPLA₂s thus forms a scaffold for adaptive modification from which a diverse array of pharmacological effects has been generated through a process of neofunctionalization (Lynch 2007). It has been found that amino acid substitutions in GI sPLA₂ and GII sPLA₂ genes correlate with surface accessibility, suggesting that modifications of surface residues and positive selection play important roles in generating toxin diversity (Kini and Chan 1999). To investigate how functional diversity is generated in sPLA₂ enzymes, sites that were identified as being under diversifying selection were mapped on to the crystal structure of GI sPLA₂s and GII sPLA₂s (Lynch 2007). The vast majority of amino acids under diversifying selection were found to be located outside the α -helical central scaffold and in regions of the protein that form connecting loops. The scaffold was found to be more conserved in GII sPLA₂ than in GI sPLA₂ proteins. Functionally important residues, including cysteines that participate in disulfide bonds, the catalytic triad, the calcium-binding site, and the hydrophobic channel, were found to be under strong structural and functional constraint because their dN/dS ratios are close to zero. In contrast, several clusters of amino acids on the molecular surface are under intense diversifying selection in GI sPLA₂ and GII sPLA₂ proteins. These rapidly evolving regions were found to be similar to the pharmacological sites, suggesting that regions under positive selection on the protein surface are responsible for generating toxic functions (Lynch 2007).

High Rates of Gene Turnover in Snake Venom sPLA₂ Multigene Families

Key features of the rapid evolution of snake sPLA₂ genes, such as the targets of selection, rates of gene turnover, and functional diversity of toxins generated, have remained unclear, especially in closely related species with divergent diets. The evolution of sPLA₂ genes was therefore studied in the four closely related *Sistrurus* rattlesnake species that feed on different prey (Gibbs and Rossiter 2008). It was found that each taxon possesses four to seven distinct sPLA₂ sequences, and phylogenetic analysis suggested that these sequences constitute a rapidly evolving gene family consisting of both paralogous and homologous loci with high rates of gene gain and loss. Both gene gain and loss and protein sequence evolution by positive selection were found to be important evolutionary forces driving adaptive divergence in venom proteins in closely related species of venomous snakes (Gibbs and Rossiter 2008). In the closely related *Sistrurus* species, a “birth and death” mechanism has been shown to drive GII sPLA₂ gene family evolution over very short evolutionary timescales, providing the first estimates of the rates of gene gain (0.40 locus/species/myr) and loss (0.37 locus/species/myr) for a venom GII sPLA₂ gene family. Evolutionary turnover in sPLA₂ gene families through a “birth and death” process was found to be at least one order of magnitude greater than that found in most other gene families. This implies that gene gain and loss have been an important evolutionary force in the adaptive evolution of venom genes, even among closely related species of venomous snakes. The extremely high level of sPLA₂ gene turnover hints that a hitherto unknown genetic mechanism may allow unusually high rates of venom gene turnover to occur, leading to exceptionally rapid adaptive divergence through gene gain and loss when speciation occurs (Gibbs and Rossiter 2008).

Molecular Mechanisms That Cause the Accelerated Evolution of Snake Venom sPLA₂ Toxins and Mechanisms That Generate Their Molecular Diversity

The molecular mechanisms that cause accelerated evolution – a bias toward nucleotide mutations that lead to amino acid changes (nonsynonymous substitutions), as compared to those that do not (synonymous substitutions) – are not understood. Interestingly, nucleotide sequences appear to determine the accelerated rate of point mutations. The distribution of stable and unstable nucleotide triplets has been analyzed in sPLA₂ genes. It was found that it is correlated with, and may explain, the accelerated evolution of exons in sPLA₂ genes. The proteins expressed in the venom gland, compared with those expressed elsewhere in the snakes, have higher levels of unstable and less stable triplets in exons. It was suggested that specific nucleotide sequences in the toxin genes may determine the rate of evolution in the corresponding proteins (Kini and Chinnasamy 2010). Accelerated segment switch in exons to alter targeting (ASSET) may play an important role in generating the

molecular diversity in snake venom molecules (Doley et al. 2009). During ASSET, certain parts of exons are changed through accelerated segment switch and generate a functionally new toxin with a conserved structural fold. Such an accelerated segment switch in exons can lead to a change in surface properties and hence functional diversification. Comparison of the amino acid sequences of sPLA₂ toxins has revealed that the N-terminal region is undergoing exchange of a 13–14 amino acid long segment (forms helix B in several sPLA₂ toxins) that lies between the first helix and the calcium-binding loop. Differences in exchange within the same species appear to arise due to ASSET, since there is more than one amino acid replacement. It has been proposed that the residues in this short segment play a crucial role in some of the pharmacological effects of sPLA₂ enzymes. Accelerated point mutations result in fine modifications of the surface topology and/or electrostatic potential, whereas ASSET drastically alters the surface producing, virtually instantaneously, large-scale changes in the ligand interaction site(s) (Doley et al. 2009). Alternative splicing (AS) plays a crucial role in the diversification of gene function and regulation. The rarest AS event in vertebrates and invertebrates is intron retention, in which an intron remains in the mature mRNA transcript, accounting for less than 5 % of known AS events. The preponderance of retained introns in UTRs may indicate their regulatory role, for example, affecting RNA stability or translational efficiency (Keren et al. 2010). Comparison of the Crotalinae and Viperinae sPLA₂ genes has shown that Crotalinae sPLA₂s retain the first intron in their mRNAs (Kordiš and Gubenšek 1996). All splicing signals are conserved in the nearly 90 % identical retained intron of Crotalinae sPLA₂ genes. 40 bp from the 3' part of exon 1 in all Crotalinae sPLA₂ genes were deleted. The deleted segment is responsible for the retention of the subsequent intron within the 5'-UTR of their mRNAs that are, consequently, longer by about 120 bp. Analysis of the secondary structure of the pre-mRNA of the AtxC gene has shown that the first exon is able to form an intra-exon hairpin which is absent in Crotalinae sPLA₂ pre-mRNAs. It was demonstrated that 5' terminal exons and their secondary structures are involved in the aberrant pre-mRNA splicing, resulting in retention of the downstream intron (Kordiš and Gubenšek 1996). The full genome-wide functional implications and importance of 5' UTR retained introns in Crotalinae sPLA₂s needs to be further addressed.

First Insight into the sPLA₂ Gene Locus of Venomous Snakes

In an attempt to elucidate the multiplication mechanism of sPLA₂ genes, a 25 kb genome segment was obtained from Amami-Oshima *Protobothrops flavoviridis* that contains five sPLA₂ genes (three normal and two pseudogenes) (Ikeda et al. 2010). The sPLA₂ genes in this cluster are PfPLA 1(ψ), PfPLA 2 that encodes the strongly myolytic Lys49 sPLA₂ and is called BPfII, PfPLA 3(ψ), PfPLA 4 that encodes the neurotoxic Asp49 sPLA₂ and is called PLA-N, and PfPLA 5 that encodes the edema-inducing basic Asp49 sPLA₂ and is called PLA-B. PfPLA 1(ψ) and PfPLA 3(ψ) are pseudogenes. Truncated CR1 LINEs were found close to the 3' end of the sPLA₂ genes and were named sPLA₂ gene-coupled RT fragments (PcRTFs).

A recombination hotspot (37 bp long), named Scomb, was found 548 bp upstream of the TATA box of sPLA₂ genes. It was concluded that multiple gene duplications of PfPLA-PfCR1 occurred by unequal crossing over of the segment “Scomb-PfPLA-PfCR1-Scomb.” It was proposed that the *P. flavoviridis* sPLA₂ gene cluster originated 40–50 million year ago, after the split of Crotalinae and Viperinae snakes. The tandemly organized sPLA₂ gene cluster in *P. flavoviridis* genome was formed by consecutive gene duplication of an ancestral PfPLA-PfCR1 unit, as a result of unequal crossing over. FISH analysis of the chromosomes in metaphase, using the gene probes pgPLA 1a, pgPLA 1b, and BP11 labeled with biotin-16-dUTP, showed that all sPLA₂ isozyme genes are located on a pair of microchromosomes in the *P. flavoviridis* genome (Ikeda et al. 2010).

Influence of Diet on the Snake Venom sPLA₂ Toxin Profile

Venom toxins form multigene families that are unique bioweapons in the predator–prey arms race. The “birth and death” model best describes the evolution of these large multigene families. Such a mode of evolution generates a suite of toxins that allow predatory snakes to adapt to a variety of prey species (Casewell et al. 2013). The vast majority of venomous snakes possess sPLA₂ multigene families functionally diversified in response to diet preference (Kordiš and Gubenšek 2000). The variation in venom sPLA₂ proteins at inter- and intraspecific levels was found to be the result of diversification in sPLA₂ genes by positive selection. The composition of snake venom proteins is under strong natural selection for adaptation toward specific diets (Gibbs and Rossiter 2008; Casewell et al. 2013). Prey capture is considered to be a major biological imperative driving the venom toxin selection process. The adaptations to diet occur within venom toxin families rather than resulting from changes in expression levels of entire toxin families (Casewell et al. 2013).

Regulatory Evolution of Snake Venom sPLA₂ Toxins

The effects of gene regulation must now be considered as a potentially important explanation for the variation in abundance of different proteins in whole venom (Gibbs et al. 2009). To date, much attention has been focused on how structural differences in proteins, due to positive diversifying natural selection in relation to diet, contribute to inter- and intraspecific variation in whole venom and provide strong indirect evidence that such genetic effects are important (Kordiš and Gubenšek 2000; Gibbs and Rossiter 2008; Casewell et al. 2013). It was proposed that the abundant venom proteins can perform generic killing and digestive functions that are not prey specific, whereas less abundant proteins may be more plastic in either evolutionary or ecological timescales and serve to “customize” an individual snake’s venom to feeding on a particular prey, requiring a specific venom protein. Given the insights arising from studies of the molecular evolution of venom gene

coding regions, a direct study of inter- and intraspecific polymorphism in the promoter regions upstream of coding regions of specific genes would be useful in exploring the possible genetic basis for regulatory effects (Gibbs et al. 2009). In all Viperidae sPLA₂ genes, the proximal part of the promoter region is highly conserved, and all putative binding sites for transcription factors are in the same positions. These putative binding sites and the promoter regions are almost 90 % identical within all known sPLA₂ genes of the Viperidae family (Kordiš et al. 1998). Insights into whether promoter regions of sPLA₂ genes undergo selection could come from determination of levels of polymorphism in specific regulatory elements or from more general tests of whether the sequence in the promoter region is evolving under selection, by comparing levels of polymorphism in the promoter and intronic regions. Regulatory evolution should be considered as an important mechanism causing phenotypic variation in whole-venom composition, in which differential expression of venom loci is important in producing a species-specific venom that allows a predator to capture a specific prey (Gibbs et al. 2009).

β-Neurotoxicity at the Cellular Level

The natural target tissue of β-ntxs is the peripheral nervous system. The most relevant systems on which to study the action of β-ntxs are thus isolated NMJ. Different neuronal cell lines that are susceptible to β-ntxs are used as well, either primary from the central nervous system (CNS), for example, hippocampal, cerebellar granule and cortical neurons, or immortalized, such as PC12 and NSC34. Using model systems, it is important, however, to be aware of possible differences in the action of β-ntxs in such systems and at the NMJ.

Electrophysiological Insights

β-ntxs primarily block NM transmission, producing a flaccid paralysis of skeletal muscles in experimental animals. Under conditions for observing small changes in neurotransmitter acetylcholine (ACh) release, indirectly stimulated isolated vertebrate NM preparations, when exposed to β-ntxs, exhibit a characteristic three-phasic change in the evoked transmitter release, detected by recording the amplitude of the muscle twitch. Phase 1, which starts soon after the addition of a β-ntx and lasts for several minutes, is characterized by a transient inhibition of the muscle twitching. In phase 2, neurotransmitter release is facilitated, the maximal release of ACh being reached in 10–20 minutes. In phase 3, the indirectly evoked muscle twitching declines progressively until it stops completely in an hour or two, the time depending on factors that influence the phospholipase activity of the toxin. After complete blockade of NM transmission, the resting membrane potential of the muscle fibers remains unchanged, and the muscle is fully responsive to the neurotransmitter.

The following important observations have resulted from this kind of experiment.

(1) The amplitude of the miniature end plate potential does not change much,

indicating that synaptic vesicles (SVs) do not fuse significantly inside the nerve terminal and that the ACh transporter is not affected by the action of β -ntxs. (2) On lowering the experimental temperature, the first phase disappears but the two subsequent phases do not, which suggests that the former cannot just be a toxin binding step (Fathi et al. 2013). (3) Stimulation of the NM preparation is not required for β -ntxs to completely block the synaptic transmission (Prasarnpun et al. 2004; Logonder et al. 2008).

Insights Using Electron and Fluorescence Microscopy

Observations under the electron microscope of mammalian NMJ acutely poisoned with a β -ntx have generally reported the following alterations: up to 80 % reduced content of SVs inside the nerve terminal, formation of Ω -shaped invaginations in the presynaptic membrane, appearance of large vesicles in the nerve ending, and damaged mitochondria (reviewed in Pungerčar and Križaj 2007). Schwann cell processes enter the synaptic cleft, and the synaptic boutons detach from the post-synaptic membrane and finally degenerate completely. Quantitative assessment of the motor nerve terminal area of β -ntx-intoxicated (β -Butx and AtxA) and control NM preparations revealed insignificant differences (Prasarnpun et al. 2004; Logonder et al. 2008). The status of neuronal proteins participating in neuroexocytosis has been analyzed using immunocytochemistry. The two SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors), SNAP-25 and syntaxin, remained apparently unaffected in β -Butx-paralyzed rat phrenic nerve terminals. The fluorescence signal of synaptophysin, the marker for SV, was however substantially reduced. Some observations that differ from the standard ones pointed out above for β -ntxs on mammalian NM preparations are worthy of attention. They were obtained on mouse NMJ completely paralyzed by AtxA (Logonder et al. 2008). The reduction of SV content in terminal boutons was only 13.6 %, not large enough to cause a loss of immunolabeling of synaptophysin in poisoned nerve endings. This was consistent with another “peculiarity” detected – the absence of Ω -shaped profiles from the terminal boutons. Unlike the case of the β -ntxs from elapid venoms (e.g., notexin, β -Butx, taipoxin, and textilotoxin), which are GIA sPLA₂s, where drastic depletion of SVs is the primary factor responsible for the acute onset of paralysis, the loss of SVs clearly cannot be considered as the major reason for transmission failure in the case of AtxA, the GIIA sPLA₂ from viperid venom. The predominant reason in the case of AtxA may well be the de-energization of the nerve terminal resulting from mitochondrial degeneration, which impairs coupling between the action potential-induced depolarization of the nerve terminal and the evoked transmitter release. In accordance with this, clustering of SVs within the cytosol and at release sites in AtxA-poisoned nerve terminal was observed. Such clustering of SVs has not been observed in GIA β -ntx-poisoned nerve endings.

A characteristic effect observed following intoxication of nerve cells with β -ntxs is fragmentation of the neuronal cytoskeleton (reviewed in Pungerčar and Križaj 2007). This phenotype appears to be the result of activation of cytosolic proteases,

calpains, arising from the increase of Ca^{2+} concentration induced by β -ntxs (Tedesco et al. 2009). These calpains then participate significantly in nerve terminal degeneration, the final stage of intoxication of the NMJ by β -ntxs (Duregotti et al. 2013). Some results, but not all (Rigoni et al. 2007), suggest that at first, β -ntxs induce an influx of Ca^{2+} ions by the rather specific route of influencing the conductance of Ca^{2+} channels (Prasarnpun et al. 2004; Yagami et al. 2013). Later, with the accumulation of phospholipid hydrolysis products, the ion permeability of the plasma membrane and of the membranes of intracellular Ca^{2+} stores (mitochondria and ER) increases (Rigoni et al. 2007, 2008).

β -Neurotoxicity at the Molecular Level

Isolated NMJ are too complex to be studied at the molecular level by contemporary methods. Model systems, therefore, have to be used to unravel the molecular basis of β -neurotoxicity. Most frequently, biochemical experiments are performed on brain preparations. Cell lines, primary from CNS or immortalized, are also employed. *Saccharomyces cerevisiae*, the simplest eukaryotic cell, has proved a valuable experimental model for studying certain cellular and toxic effects of β -ntxs. Although most model cell lines are not cholinergic, all of them are susceptible to β -ntxs.

Enzymatic Activity and β -Neurotoxicity

All known β -ntxs catalyze deacylation of 1,2-diacyl-*sn*-3-phosphoglycerides at the *sn*-2 position, and this activity is fundamental for β -neurotoxicity. Membranes containing anionic phospholipids, phosphatidylserine, phosphatidic acid, or phosphorylated phosphatidylinositols, are the preferred substrates of β -ntxs. Such phospholipids are enriched in the cytosolic leaflets of subcellular organelles and plasma membrane of eukaryotic cells. β -ntxs bind to these membranes with much higher affinity than to the membranes composed exclusively of zwitterionic phospholipids that predominate in the extracellular leaflet of the plasma membrane. Importantly, the high-affinity association of an sPLA₂ with membrane also reduces its Ca^{2+} requirement for effective hydrolytic activity to concentrations close to that in the cytosol (Kovačič et al. 2009, 2010). The most recent confirmation of the necessity of phospholipase activity for β -neurotoxicity was provided by the protein engineering of a myotoxic ammodytin L (AtnL), an enzymatically inactive paralogue of the β -neurotoxic ammodytoxin A (AtxA) from *Vipera a. ammodytes* venom. AtnL was switched to a β -neurotoxin, endowing it with enzymatic activity, by replacing three key amino acid residues, His28, Asn33, and Ser49, by Tyr28, Gly33, and Asp49 (Petan et al. 2007; Vardjan et al. 2013). The relationship between the rate of hydrolysis and the β -neurotoxic potential of sPLA₂s has still not been established conclusively. When assaying phospholipase activity in vitro, no correlation between the hydrolytic power and the β -neurotoxicity of sPLA₂s is apparent.

Paoli et al. (2009), however, showed that the phospholipase activities of β -ntxs with substantially different lethalties do not differ so dramatically if assessed on a biologically relevant substrate. Moreover, enzyme activities measured on living neurons in culture were found to be much lower than those measured in vitro on synthetic substrates.

β -ntxs catalyze hydrolysis of phospholipids in biological membranes. The products of the phospholipase activity, lysophospholipids and fatty acids, have physico-chemical features (charge, geometry, diffusion ability) that differ from those of the parental phospholipids, resulting in different characteristics of the membranes – their curvature, fluidity, fusogenicity, and transitivity. Such alterations can trigger conformational changes in membrane proteins involved in neurotransmission. In addition, free fatty acids and their metabolites can affect the functioning of different ion channels and receptors (Davletov and Montecucco 2010). Mixtures of lysophospholipids and fatty acids added to NM preparations in an experimental bath have been shown to elicit effects similar to those elicited by β -ntxs (Rigoni et al. 2005, 2007; Megighian et al. 2007; Caccin et al. 2009). However, an experiment involving the action of β -Butx on motoneurons poisoned by botulinum toxin C (BoNT/C) revealed that the explanation of β -neurotoxicity is not so simple. Unimpeded access and unchanged enzymatic activity of β -Butx on the external leaflet of the axolemma was able to induce only very limited β -neurotoxic effects (Prasarnpun et al. 2004). Taken together with the results of the first quantitative assessment of hydrolytic products of β -ntxs acting on neurons in culture, which suggested that hydrolysis takes place mostly on the cell surface (Paoli et al. 2009), it can be concluded that the contribution of the intracellular phospholipase activity of β -ntxs to the β -neurotoxicity, although small in extent, is very important.

β -Neurotoxicity Relies on the Nerve Cell-Specific Binding of sPLA₂s

β -neurotoxicity does not depend solely on the phospholipase activity of a toxin. Many sPLA₂s are highly active enzymes but not at all β -neurotoxic. Although it has long been known that β -ntxs possess specific high-affinity binding sites on the presynaptic membrane, the β -neurotoxicity-related receptors have not been identified. Since some pairs of β -ntxs, but not others, together potentiate β -neurotoxicity, a heterogeneous population of presynaptic β -neurotoxicity-related receptors would be expected. A likely candidate for this function remains the neuronal or N-type sPLA₂ receptor which was discovered using OS₂, a β -ntx from *Oxyuranus s. scutellatus* venom, as a tracing ligand in rat brain synaptic membranes. Binding affinities of different sPLA₂s for this receptor correlate strongly with their lethalties. In the case of β -Butx, the β -neurotoxicity-related receptor may be a subpopulation of α -dendrotoxin-sensitive voltage-dependent K⁺ channels. Identification of the β -neurotoxicity-related receptor(s) on the extracellular side of the presynaptic membrane remains one of the most important tasks to be accomplished in order to explain conclusively the action of β -ntxs at the molecular level (reviewed in Pungerčar and Križaj 2007; Šribar and Križaj 2011).

β -ntxs Are Imported into Nerve Cells

There is now a convincing experimental evidence that relates to nerve cell internalization of some β -ntxs. First, the internalization of β -Butx and AtxA into the rat hippocampal neurons was demonstrated. While β -Butx was found localized preferentially to lysosomes, AtxA finally accumulated in the nucleus of these CNS neurons (reviewed in Pungerčar and Križaj 2007). Since then, internalization of AtxA by intact NSC34 cells, which are very similar to motoneurons, has been shown by fluorescence and nanogold labeling and by use of a photo-reactive derivative of this β -ntx (Jenko Pražnikar et al. 2008). AtxA rapidly translocates to the cytosol and to certain intracellular organelles of NSC34 cells, including SVs and mitochondria. In the same year, Rigoni et al. (2008) published evidence of the specific binding of Alexa-labeled notexin, β -Butx, and taipoxin to mitochondria of rat cerebellar granular neurons. Internalization of a β -ntx into the mammalian motor nerve terminal was demonstrated for the first time in 2009 (Logonder et al. 2009). Following subcutaneous injection into a mouse, the nanogold-labeled AtxA(N79C) accumulated selectively in the peri-synaptic regions at the NMJ and was internalized into the terminal boutons, as detected using transmission electron microscopy. Within the boutons, the accumulated toxin was commonly associated with mitochondria and cytoplasmic vesicles.

The pathway by which β -ntxs are imported into nerve cells is still unknown. There are however some suggestive experimental facts. β -ntxs may internalize into neurons using recycling SVs as “Trojan horses.” The blockade of SV exocytosis from motoneurons by BoNT/C, which cleaves two plasma membrane SNAREs, syntaxin and SNAP25, effectively reduced the toxic effects of β -Butx. However, the SV membrane translocation mechanism differs from that operating in the case of BoNTs, since inhibition of the V-type ATPase did not prevent poisoning of the NM preparation by β -ntxs (reviewed in Pungerčar and Križaj 2007). Another original idea appeared recently. According to this, by progressive hydrolysis of the plasma membrane, β -ntxs create high local concentrations of lysophospholipids and fatty acids able to mediate the passage of these toxins directly across the lipid bilayer (Montecucco et al. 2009).

Intracellular Activities of β -Ntxs

Specific internalization of β -ntxs into nerve cells is an experimentally confirmed fact. Several intracellularly localized proteins have been identified *in vitro* as specific binding targets for β -ntxs. These are the cytosolic proteins, calmodulin (CaM), 14-3-3 γ and ϵ proteins (14-3-3p), proteins from the endoplasmic reticulum (ER), crocalbin, taipoxin-associated Ca^{2+} -binding protein of 49 kDa (TCBP-49) and protein disulfide isomerase (PDI), and a hitherto unidentified protein from the mitochondria, R25 (reviewed in Šribar and Križaj 2011). A mitochondrial receptor may be implicated in the process of opening mitochondrial transition pores by β -ntxs, as revealed in spinal cord motor neurons and cerebellar granule neurons

(Rigoni et al. 2008). Importantly, the association of a β -ntx with CaM and 14-3-3p has also been demonstrated in vivo, in NSC34 cells (Jenko Pražnikar et al. 2008). Moreover, in PC12 cells, PDI and AtxA have been shown to co-localize substantially (J. Šribar, personal communication). This indicates that the interaction of β -ntxs with CaM, 14-3-3p, and PDI has physiological relevance. Whether or not a relation with β -neurotoxicity exists is, however, still a matter of speculation. For example, CaM, which has been shown to stabilize sPLA₂s in the complex and to boost their enzymatic activity substantially under cytosol-like conditions (Kovačič et al. 2009, 2010), may be crucial for β -ntxs to retain their high phospholipase activity in the reducing conditions of the nerve cell cytosol. The enzymatic activity of β -ntxs is additionally supported by the increased concentration of Ca²⁺ in the cytosol (Tedesco et al. 2009). 14-3-3p may be however implicated in targeting β -ntxs to sites on the presynaptic membrane, where endocytosis of the SV takes place. In a study of the molecular basis of the inhibition of vesicle endocytosis by AtxA in *S. cerevisiae*, the importance of the interaction of the toxin with Bmh2, the yeast 14-3-3p homologue, for effective blocking of the process of SV formation, was revealed (Mattiuzzi et al. 2012). These authors proposed that AtxA inhibits vesicle endocytosis then by hydrolyzing the inner leaflet of the plasma membrane. Due to phospholipolysis membrane curvature changes and this interferes with the activity of the membrane curvature-sensing protein amphiphysin, which function in vesicle endocytosis is very important. By association with the membrane of appropriate curvature, amphiphysin namely stabilizes the neck of the emerging vesicle and recruits the pinchase dynamin to pinch the vesicle off the membrane.

Conclusions and Future Directions

The last extensive description of β -ntxs was published 7 years ago (Pungerčar and Križaj 2007). In the present review, the experimental facts that have been established since then are surveyed and shown as to how they provide an improved insight at the molecular level into their mode of action.

The main steps, so far established, in the toxic action of β -ntxs are as follows (Fig. 2):

1. β -ntxs bind specifically to the presynaptic membrane at the NMJ. Different β -ntxs appear to bind to different β -neurotoxicity-related receptors.
2. On association with the presynaptic membrane, a β -ntx catalyzes the hydrolysis of phospholipids in the former's external leaflet. Accumulating products – lysophospholipids and fatty acids – promote the exocytosis of hemi-fused synaptic vesicles (SVs). The conductivity of certain Ca²⁺-channels is increased.
3. β -ntx is rapidly internalized into the nerve ending. Internalization probably depends on SNARE assembly but clearly not on the V-type ATPase activity. The translocation mechanism may include the recycling of SVs.
4. The β -ntx migrates into the cytosol and mitochondria.

they interact with mitochondria. Unraveling these details will not just clarify the phenomenon of β -neurotoxicity but will suggest new approaches to the prevention of intoxication with β -ntxs or accelerate the regeneration of the affected tissue. It should also lead to a very important insight into the action of mammalian sPLA₂s, especially concerning their functions and dysfunctions in the nervous system (Goracci et al. 2010; Yagami et al. 2014).

Evolutionary analyses of the sPLA₂ toxins in venomous snakes took place in the pre-genomic era, from the early to late 1990s, and were based on a small sample of taxonomic diversity and diversity within the sPLA₂ toxins. Since then, the number of representatives has increased significantly, largely due to the accumulation of snake venom transcriptomic resources, since the more numerous genomic data on sPLA₂ toxins in venomous snakes are nevertheless still very sparse (Vonk et al. 2013). The progress in the last decade has increased our understanding of the evolution of sPLA₂ toxins in venomous snakes. A significant increase in understanding the evolution of sPLA₂ toxins at the genome level can be expected in the near future.

Cross-References

- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins](#)
- ▶ [Snake Venom Toxins Targeted at the Nervous System](#)

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Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes

4

Debashree Saikia and Ashis K. Mukherjee

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D. Saikia

Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, Assam, India

e-mail: debasri@tezu.ernet.in

A.K. Mukherjee (✉)

Department of Molecular Biology and Biotechnology, Research and Development, ONGC-Centre for Petroleum Biotechnology, DBT Nodal Centre for Medical Colleges and Biomedical Research Institutes of North-East India (Unit of Department of Biotechnology, Govt. of India), Tezpur University, Tezpur, Assam, India

e-mail: akm@tezu.ernet.in; editor_tsi@yahoo.in

Abstract

Snake venom is a complex mixture of pharmacologically active proteins and polypeptides in addition to containing some minor nonprotein components. Snake venom plays an important role in incapacitating and immobilizing, as well as in digesting prey after being swallowed. Phospholipase A₂ (PLA₂) is one of the major toxic enzymes present invariably in all snake venoms. Despite their structural similarities, snake venom PLA₂ enzymes differ greatly in their pharmacological properties and biological activities. Venom PLA₂s most likely prolong the blood coagulation through hydrolysis of and/or binding to procoagulant phospholipids essential for clotting process. In addition to enzymatic mechanism of anticoagulant effect, anticoagulant PLA₂ enzymes strongly extend blood coagulation by a mechanism that is independent of phospholipids hydrolysis and is achieved mostly by inhibiting the blood coagulation factor(s). Studies on such anticoagulant PLA₂s may contribute to understand different “vulnerable” sites in the coagulation cascade which may further help to design novel strategies to develop anticoagulant therapeutics to treat cardiovascular disorder (CVD).

Further, most of the toxic effects of snake venom PLA₂s are exerted by hydrolysis of membrane phospholipids. Different isoforms of venom PLA₂ can display dramatically different affinities for bio-membranes, composed of different phospholipids polar head groups and fatty acyl chains, resulting in their differential membrane damaging activity. This differential affinity of PLA₂s towards phospholipids of different bio-membranes has been used extensively to explore the physical structure of phospholipids in biological membranes. Study of membrane damaging activities of venom PLA₂s will be helpful in exploring different membrane properties. It is exemplary to mention that only a few reports on membrane hydrolyzing property of snake venom PLA₂s are available. Therefore, more precise studies are required to understand the complete mechanism(s) of membrane damage and subsequent toxicity of PLA₂ enzymes from snake venom.

Keywords

Anticoagulant activity • *Daboia russelii* • *Naja* species • Membrane damage • Membrane domain • Phospholipase A₂ • Phospholipids hydrolysis • Snake venom

Introduction

Snakes have been fascinating the mankind since the ancient times. This intense fascination possibly arises from the deadly effect of snake venom, which when injected into the victim causes a variety of patho-physiological reactions such as paralysis, myonecrosis and often leading to death. On the other hand, in some of countries such as India, snakes are also worship as a symbol of deity and have shown profound respect by many communities.

Snakes use their venoms as offensive weapons in incapacitating and immobilizing their fast moving and/or big prey before being swallowed (the primary function), as defensive tools against their predators and occasionally against human (the secondary function) and also to aid in digestion of prey (Kang et al. 2011). Biochemically, snake venom is complex mixtures of pharmacologically active proteins and polypeptides. Venom from a single species of snake typically contains from 30 to over 100 protein/peptide toxins (Kang et al. 2011) many of which interact synergistically to enhance their toxicity (Mukherjee 2010). Some of these venom proteins exhibit enzymatic activities, whereas several others are nonenzymatic proteins and polypeptides. Based on their structures, they can be grouped into a small number of toxin super-families; the members in a single family despite of showing remarkable similarities in their primary, secondary and tertiary structures- very often show distinct pharmacological effects by different mechanisms of action (Kini 1997; Doley and Mukherjee 2003; Kang et al. 2011; Mukherjee and Mackessy 2013).

The different enzymes that have been identified in different snake venoms include proteases, phosphomonoesterase and phosphodiesterase, arginine ester hydrolase, acetylcholinesterase, collagenase, RNAase, DNAase, hyaluronidase, phospholipase A₂ (PLA₂), phospholipase B, phospholipase C, 5'-Nucleotidase; L-Amino acid oxidase, lactate dehydrogenase and adenosine triphosphatase etc. However, single venom does not contain all of these enzymes but these are present in different combinations in different venoms (Phui et al. 2004). Studies have shown that PLA₂ is typically present in almost all snake venoms and this enzyme greatly contributes the overall toxicity of the whole venom (Kini 1997; Mukherjee et al. 1998a) implying significance of characterization of its structure-function and pharmacological properties.

Phospholipase A₂ Enzymes from Snake Venom

Among the snake venom enzymes, phospholipase A₂ (EC: 3.1.1.4) are the most interesting group of proteins due to their crucial role in inducing various pharmacological effects on snakebite victims (Mukherjee et al. 2000; Doley et al. 2004; Kini 2005; Lomonte and Gutiérrez 2011). The first report on PLA₂ enzymes purification from venom of *Naja naja* and *Naja tripudians* was published in 1944 and the PLA₂s were named haemolysins due to their ability to indirectly haemolyse the red blood cells (De 1944). Since then, more than several hundreds of snake venom PLA₂ enzymes have been purified and characterized. Till date, amino acid sequence of over 300 PLA₂ enzymes have been reported from snake venom. PLA₂ enzymes share 40–99 % identity in their primary structures however, despite of sharing a significant similarity in their three-dimensional folding (Doley et al. 2009); they differ greatly in their pharmacological properties (Doley et al. 2004; Kini 2006; Mukherjee 2007; Saikia et al. 2011, 2012, 2013). Thus, the functional differences among PLA₂ enzymes cannot be easily correlated to their structural differences.

The structure-function relationships and the mechanism of this group of small proteins are subtle, complex and intriguing challenges to biochemists, molecular

biologists, toxinologists, pharmacologists and physiologists. In addition to the digestion of prey, PLA₂ exhibit wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrotic, anticoagulant, hypotensive, hemolytic, haemorrhage and edema inducing activities (Kini and Evans 1989; Doley and Mukherjee 2003; Doley et al. 2004; Mukherjee 2007; Saikia et al. 2011, 2012, 2013; Mukherjee 2014; Mukherjee et al. 2014). This diverse pharmacological profile has been acquired through an accelerated evolutionary process (Doley et al. 2009). Single venom may contain several isoforms of PLA₂ and according to their overall net charge they may be categorized as acidic, basic or, neutral PLA₂ enzymes (Jayanthi and Gowda 1988; Saikia et al. 2011, 2013). Each PLA₂ may exert different pathophysiological effects by different mechanisms of action in snakebite victims (Kasturi and Gowda 1989; Mukherjee 2007; Saikia et al. 2011, 2012). Till date, there is a dearth of knowledge on the actual biochemical basis of diversity among snake venom phospholipase A₂ enzymes.

Mukherjee et al. (2000) have reported that the majority of death from Russell's Viper envenomation in eastern India is attributed to prolong blood coagulation time of victims. Circulatory system is one of the physiological systems targeted by anticoagulant PLA₂s from snake venom (Kini 2006; Saikia et al. 2011, 2013). Venom PLA₂s most likely affect blood coagulation through hydrolysis of and/or binding to procoagulant phospholipids those are essential for blood clotting rendering them unavailable for initiating the coagulation process (Kini 2006; Saikia et al. 2011, 2013). In addition to the above mechanism, strongly anticoagulant PLA₂ enzymes also affect blood coagulation by mechanisms that are independent of phospholipids hydrolysis (Kini 2006; Saikia et al. 2011). However, the mechanism of anticoagulant action of snake venom PLA₂ enzymes has still remained incomprehensible. Studies on such anticoagulants contribute to understand different "vulnerable" sites in the coagulation cascade which may further help to design novel strategies to develop anticoagulant therapeutic agents for treating cardiovascular disorder (CVD) and new functional diagnostic test kits in the field of hemostasis (Kini 2006; Koh and Kini 2012). In the following sections, the mechanisms (reported till date) of anticoagulant action and membrane damaging activity of snake venom PLA₂ enzymes will be discussed; however, more emphasis will be given to describe the effect of cobra and viper venom PLA₂ enzymes.

A Brief Account on Structure and Catalytic Mechanism of Snake Venom PLA₂ Enzymes

Snake venom PLA₂ enzymes are small proteins (~13–14 kDa) containing on an average 115–133 amino acid residues. They have 14 conserved Cys residues that form seven disulfide bridges and stabilize the tertiary structure (Scott et al. 1991). The structural data indicate that snake venom PLA₂ enzymes share strong structural similarity to mammalian pancreatic as well as secretory

PLA₂ enzymes. PLA₂ enzymes consist of three major α -helices, a distinctive backbone loop that binds catalytically important calcium ions, and a β -wing that consists of a single loop of antiparallel β -sheet. The C-terminal segment forms a semicircular “banister,” particularly in viperid and crotalid PLA₂ enzymes, around the Ca²⁺-binding loop (Kini 2006). In addition, they have a similar catalytic function in hydrolysing phospholipids at the *sn*-2 position. However, in contrast with mammalian PLA₂ enzymes, many snake venom PLA₂ enzymes are found to be toxic and induce a wide spectrum of pharmacological effects (Kini and Evans 1989). Although some of the reports show nontoxic PLA₂ enzymes from snake venom (Doley and Mukherjee 2003; Mukherjee 2007; Saikia et al. 2012, 2013); however, studies have suggested that they may interact synergistically with another molecule of the venom to enhance the toxicity of PLA₂-protein complex (Mukherjee 2010).

Phospholipase A₂ preferentially catalyzes reaction at interfaces which is known as “interfacial catalysis.” In 2006, Winget and co-workers proposed a model for interfacial catalysis of venom PLA₂ enzymes. According to this model, the PLA₂ enzyme (E) binds to the substrate (S) at the interface as the E* form resulting in formation of a transient enzyme-substrate (E*-S) interface-bound form. At this stage, an additional anion activating step occurs where an anionic phospholipid(s) interacts at the interface of the enzyme, forming the (E*-S)[#] complex. Once activated, the PLA₂ can catalyze the formation of the enzyme-product (E*-P) complex and then release the products (lysophospholipids and fatty acids). After release of the product, the E* diffuses in a scooting mode to bind with another substrate (S*) to initiate the next cycle of catalysis (Winget et al. 2006).

The active site of the PLA₂ molecule consists of four residues: His48, Asp49, Tyr52 and Asp99 (Kang et al. 2011). A conserved water molecule is connected to the side chains of the active site residues His48 and Asp49 through hydrogen bonds and it plays an essential role in the catalysis (Kang et al. 2011). The mechanism of PLA₂ catalyze hydrolysis of phospholipids involves the highly specific interaction between the active site histidine of PLA₂, the Ca²⁺ cofactor, conserved water, and the glycerophospholipids substrate. During catalysis, His48, assisted by Asp99, polarize the bound water molecule, which then attacks the *sn*-2 bond of the bound phospholipid to form a tetrahedral oxyanion intermediate (Scott 1997; Doley et al. 2009). An alternative mechanism has also been proposed, whereby two more water molecules are involved in the formation and breakdown of the tetrahedral intermediate (Edwards et al. 2002). In both mechanisms, Ca²⁺ ion, coordinated by the oxygen atom of Asp99, serves as an electrophile during catalysis (Scott 1997; Edwards et al. 2002). Overall, catalysis by sPLA₂ enzymes can be summarized (Murakami et al. 2006) as follows:

1. Binding of Ca²⁺ and substrate;
2. General base-mediated catalysis;
3. Formation and breakdown of tetrahedral intermediate,
4. Release of the reaction products.

Classification of PLA₂ Enzymes from Snake Venom on the Basis of Their Anticoagulant Potency

The anticoagulant activity was first attributed to venom PLA₂ enzymes by Boffa et al. (1980). PLA₂ enzymes may be classified into the following three groups depending on their anticoagulant potency (Verheij et al. 1980; Fig. 1).

Strong anticoagulant PLA₂ enzymes: They inhibit blood coagulation at a low protein concentration preferably at a dose of $\sim 2.0 \mu\text{g ml}^{-1}$. The PLA₂s of this group are isolated from the venom of *Naja nigricollis* (basic), *N. m. mossambica* (CM-III), *Vipera berus orientale*, *D. russelli* (acidic), *D. russelli* (neutral), *Agkistrodon halys blomhoffi* (basic), and *Crotalus durissus terrificus* (basic subunit of crotoxin) enzymes (Kini 2005; Saikia et al. 2011, 2013).

Moderately anticoagulant PLA₂ enzymes: They show anticoagulant effect at a protein concentration between 3.0 and 10.0 $\mu\text{g ml}^{-1}$. The PLA₂s from venom of *Naja mossambica* (CM-II), *Naja nigricollis* (acidic), *Agkistrodon halys blomhoffi* (neutral), *Enhydrina schistosa* (myotoxin) and *Oxyuranus scutellatus* (taipoxin) belong to this group (Verheij et al. 1980; Kini 2005).

Weak or nonanticoagulant PLA₂ enzymes: This group of PLA₂ enzymes have little influence on blood clotting times even at concentrations higher than 15.0 $\mu\text{g ml}^{-1}$. This group of PLA₂s includes those isolated from venom of *N. m. mossambica* (CM-I), *N. naja*, *N. melanoleuca* (DEI and DEIII), *A. halys blomhoffi* (acidic), *Hemachatus hemachatus* (DEI), *Bitis gabonica*, *Crotalus admanteus*, *C. durissus terrificus* (crotoxin), *Vipera aspis* (*V. aspis* B), *Notechis scutatus* (notexin and II-5), and *Bungarus multicinctus* (β -bungarotoxin) (Verheij et al. 1980; Kini 2005).

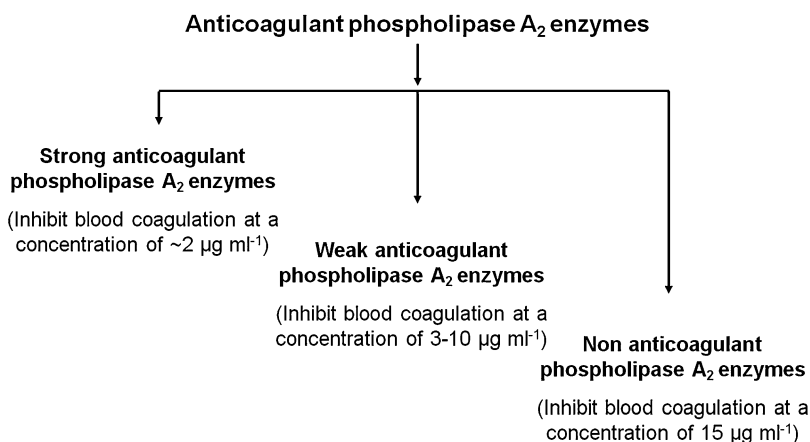


Fig. 1 Classification of anticoagulant PLA₂ enzymes of snake venom on the basis of their anticoagulant potency

Anticoagulant Portion in Snake Venom PLA₂ Enzymes

By a systematic and direct comparison of the amino acid sequences of strong, moderate and very weak anticoagulant PLA₂ enzymes, Kini and Evans (1987) identified the anticoagulant portion to be between amino acid residues 54 and 77 in the primary structure of PLA₂ molecule. This region is positively charged in strong anticoagulant PLA₂ enzymes. The major difference between the strong anticoagulant and moderate or very weak anticoagulant PLA₂ is the replacement of the residues in this anticoagulant portion. However, according to Kini and Evans (1987), not the overall basicity but the nature of the charge in the anticoagulant site determines the anticoagulant potency of PLA₂ enzymes. In the strong anticoagulant PLA₂ enzymes, the anticoagulant region is positively charged, but negatively charged in the moderate and weak anticoagulant enzymes. Chemical modification of PLA₂ enzymes (Kini 2006), site-directed mutagenesis studies (Mounier et al. 2000) and synthetic peptide studies (Mounier et al. 2000) have supported this prediction.

Anticoagulant Property of Snake Venom PLA₂s

There are various mechanisms by which snake venom PLA₂ enzymes exhibit their anticoagulant property. A simple “dissection approach” was used to identify the specific stage of the coagulation cascade that is inhibited by anticoagulant PLA₂ enzymes (Stefansson et al. 1989). Generally, the effect of anticoagulants on different stages of blood coagulation is determined by three commonly used clotting time assays viz., prothrombin time, Stypven (Russell Viper venom) time and thrombin time (Kini 2005; Table 1). The anticoagulant would prolong the normal clotting time of plasma when the cascade is initiated “upstream” of the inhibited step, whereas it will not interfere with the clotting time of plasma when the cascade is initiated “downstream” of the inhibition step. Since the above clotting assays specifically initiate the coagulation cascade at three different stages, it is easier to identify the specific step(s) that is (are) inhibited by the anticoagulant PLA₂ enzymes from snake venom (Stefansson et al. 1989; Kini 2005). The moderately anticoagulant PLA₂ enzymes prolong the prothrombin time but not the Stypven time and thrombin time clotting assays. In contrast, the strong anticoagulant PLA₂s delay prothrombin time and Stypven time, but not the thrombin

Table 1 Anticoagulant property of three types of PLA₂s

Property	Strong anticoagulant PLA ₂	Moderately anticoagulant PLA ₂	Weak anticoagulant PLA ₂
Concentration for inhibit clotting process	~2.0 µg ml ⁻¹	3.0 and 10.0 µg ml ⁻¹	>15.0 µg ml ⁻¹
Delay clotting assay	Stypven time and thrombin time	Prothrombin time	None

time clotting assays (Stefansson et al. 1989; Kini 2005). However, recent reports have shown the thrombin inhibitory property of *Naja Kaouthia*, *N. naja* and *Daboia russelii russelii* venom PLA₂ enzymes (Mukherjee 2014; Mukherjee et al. 2014; Dutta et al. 2015) indicating some of the strong anticoagulant PLA₂ enzymes may exert their anticoagulant action by interfering the thrombin time.

Mechanism of Action of Anticoagulant Activity of Snake Venom PLA₂ Enzymes

Most of the death from Russell's Viper envenomation is attributed to prolongation of blood coagulation time of victims (Mukherjee et al. 2000). Since plasma phospholipids play a crucial role in the formation of several coagulation complexes; therefore, it might be endorsed that the obliteration of phospholipids surface by snake venom PLA₂s could be the primary mechanism to account for their anticoagulant effect (Doley et al. 2004; Kini 2006; Saikia et al. 2011, 2013). In most of the cases, PLA₂s affect blood clotting either through the enzymatic hydrolysis of pro-coagulant phospholipids of plasma, or by nonenzymatically binding to the plasma phospholipids or blood coagulation factors resulting in their nonavailability to initiate the blood clot. The anticoagulant activity of some PLA₂s is shown to be dependent on the presence of phospholipids (Stefansson et al. 1989), whereas some other PLA₂s can inhibit blood coagulation process even in the absence of phospholipids (Saikia et al. 2011). Furthermore, some of the PLA₂s can bind to other blood coagulation factors (viz., Factor Xa, Factor Va, prothrombin, thrombin etc.) as well as show hydrolysis of plasma phospholipids and thus can inhibit the clotting of blood (Kini 2006; Saikia et al. 2011; Mukherjee 2014; Mukherjee et al. 2014; Dutta et al. 2015). Therefore, the mechanisms of inhibition of blood coagulation by different PLA₂ enzymes are different and complicated. The role of enzymatic activity and nonenzymatic mechanism to exert anticoagulant action by snake venom PLA₂ molecules are discussed below.

Correlation Between Enzymatic Activity and Anticoagulant Activity of PLA₂s

The issue of association/dissociation of enzymatic activity of PLA₂ with its anticoagulant potency is controversial and fragmentary data are presented to support this hypothesis (Kini 2005; Saikia et al. 2011, 2013). Some experiments suggest that catalytic activity is essential for anticoagulant effect. For example, in all snake venom PLA₂ enzymes, His48 is conserved and plays a crucial role in the hydrolysis of phospholipids. Modification of His48 by alkylation leads to the complete loss of enzymatic activity. PLA₂ enzymes from *N. nigricollis*, *H. hemachatus*, *N. atra*, and *N. Kaouthia* venoms upon alkylation using *p*-bromophenacyl bromide lost their

enzymatic activity with a concomitant loss of anticoagulant effect (Verheij et al. 1980; Condrea et al. 1981, 1983; Doley et al. 2004). On the other hand, few studies have suggested that nonenzymatic mechanism might play a key role in inhibiting the different blood coagulation factors by venom PLA₂ and therefore, nullifying the role of PLA₂ activity in anticoagulant process (Stefansson et al. 1990; Faure et al. 2007; Saikia et al. 2011).

Stefansson et al. (1990) have reported a basic PLA₂ from *Naja nigricollis* venom (CM-IV) that inhibits the prothrombinase complex in the absence of phospholipids. Further, RVVA-PLA₂-I, an acidic PLA₂ purified from Russell's Viper venom (RVV) exerts its anticoagulant effect by a dual mechanism of action by enzymatically hydrolyzing the plasma phospholipids as well as by binding with those phospholipids essential for the blood coagulation process (Saikia et al. 2011).

Nonenzymatic Mechanism of Anticoagulant Action: Interaction of PLA₂s With Coagulation Factor Xa and Plasma Phospholipids

Generally, strong anticoagulant PLA₂s from snake venom may inhibit blood coagulation cascade nonenzymatically by competing with clotting factors for the lipid surface and they bind to coagulation factor X and/or factor Xa and thereby, inhibiting the formation of prothrombinase complex (complex of factor Xa, factor Va, phospholipids and Ca²⁺) which is the most important step of the blood coagulation system (Fig. 2), or by inhibition of thrombin (Osipov et al. 2010; Mukherjee 2014; Mukherjee et al. 2014). For those PLA₂ enzymes, there is no correlation between the enzymatic activity and anticoagulant potency (Stefansson et al. 1989). For example, RVVA-PLA₂-I, an acidic PLA₂ from RVV interferes the blood coagulation process nonenzymatically by inhibiting the coagulation factor Xa, even in the absence of phospholipids/Ca²⁺, and thereby slowing down the process of thrombin formation from prothrombin by the action of factor Xa (Saikia et al. 2011).

One of the most important key factors influencing the anticoagulant potency is the penetrating property of PLA₂ enzymes (Kini 2006). Penetrability is the ability of the PLA₂ enzyme to insert itself into the phospholipids membrane followed by hydrolysis of membrane phospholipids (Kini 1997; Mukherjee 2007). Since phospholipids in plasma membranes are packed at a higher density as compared to the phospholipid vesicles, PLA₂ enzymes with higher penetrability cause more significant damage to the membrane as compared to those PLA₂ enzymes with lower penetrability (Kini 1997). Very low but specific plasma phospholipids hydrolysis is the characteristics feature of strongly anticoagulant PLA₂s, whereas non specific, nonanticoagulant PLA₂ enzymes randomly hydrolyze the plasma phospholipids (Kini and Evans 1989; Saikia et al. 2011). Therefore, higher amounts of phospholipids hydrolysis are required before nonspecific PLA₂s show any significant anticoagulant effect (Kini and Evans 1989).

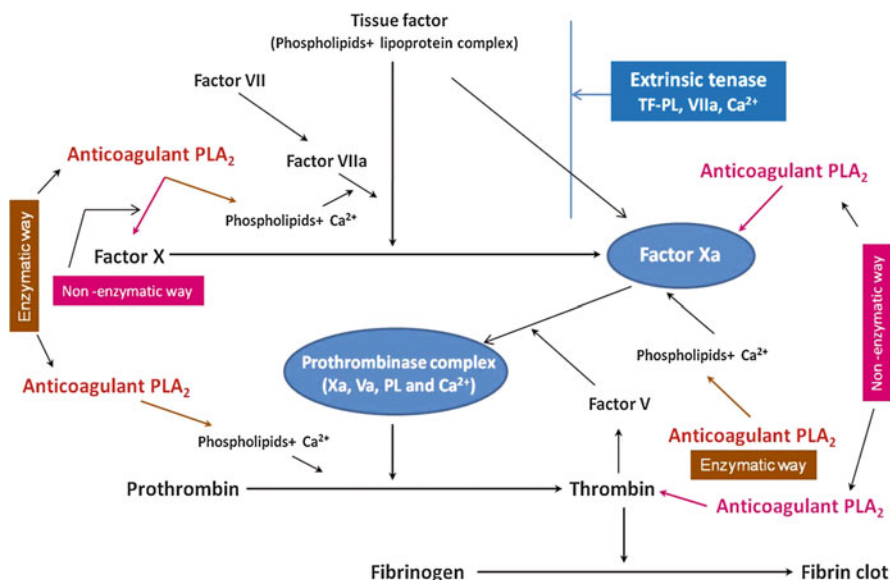


Fig. 2 Effect of snake venom anticoagulant PLA₂ enzymes in different stages of the extrinsic pathway of blood coagulation. Enzymatic way: Enzymatic way of anticoagulant mechanism of snake venom PLA₂s; Nonenzymatic way: Nonenzymatic way of anticoagulant mechanism of snake venom PLA₂s; Anticoagulant PLA₂: Anticoagulant PLA₂s of snake venom

Membrane Damaging Property of the Snake Venom PLA₂ Enzymes

Hydrolysis of cellular or subcellular membrane phospholipids by snake venom PLA₂ enzymes results in generation of phospholipids hydrolysis breakdown products which in turn cause extensive membrane damage (Kini and Evans 1989; Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). Kinetic studies of sPLA₂ in the scooting mode establish that these PLA₂ enzymes bind to the intact membrane surface as a prelude to loading of the active site with a single phospholipid molecule, more specifically they bind with the phosphatidylcholine (PC) present in the outer leaflet of the membrane for the lipolysis reaction (Sharp et al. 1991). It is becoming apparent that differential membrane damaging property of different isoforms of venom PLA₂ is attributed to their dramatically different affinities for biomembranes which are composed of different phospholipids polar head groups and fatty acyl chains (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). This specificity of phospholipases may be used extensively to explore the physical structure of phospholipids in biological membranes (Shukla and Hanahan 1982; Mukherjee 2007; Saikia et al. 2012).

Management of viper bite patients with massive intravascular hemolysis is a severe problem (Mukherjee et al. 1998a; Mukherjee et al. 2000). Therefore,

endeavour must be directed towards stabilizing the erythrocyte membrane against the hydrolytic action of this venom which is due to presence of PLA₂ (Mukherjee et al. 1997, 1998a). Venom PLA₂ enzymes can also damage lysosomes (Mukherjee et al. 1997) and encapsulated lysosomal enzymes may be released following venom toxicity, especially after *Vipera (Daboia) russelli* envenomation (Sahu et al. 1991). Lysosomes are a structurally heterogeneous group of organelles containing many hydrolytic enzymes concerned with the degradation of metabolites (Dowben 1971) or any other biological molecules. Russell's Viper venom PLA₂ is also reported to degrade the microsomal membrane as well however, to a different extent than the hydrolysis of lysosomal membrane (Mukherjee et al. 1997, 1998b).

Myonecrosis may be due to an indirect action as a consequence of vessel degeneration and ischemia caused by a direct effect of myotoxic phospholipase A₂s (PLA₂s) homologues from snake venom on plasma membranes of muscle cells (Samy et al. 2010). Some results have also shown the bactericidal property of snake venom PLA₂s enzymes (Buckland and Wilton 2000; Samy et al. 2010; Vargas et al. 2012) albeit other reports have shown that *N. Kaouthia* and *D. r. russelii* venom PLA₂ enzymes are devoid of bactericidal activity (Mukherjee 2007; Saikia et al. 2012).

Differential Hydrolysis by Snake Venom PLA₂s on Different Biomembrane Phospholipids may Support Membrane Domain Hypothesis

Different isoenzymes of PLA₂ present in snake venom show differential membrane damaging activity on different cell membranes (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). Membrane surface properties, including membrane fluidity, curvature, surface charge, and membrane-induced structural changes in the enzyme, determine the strength of interaction, cooperatively of membrane binding, and the extent of PLA₂ hydrolytic activity on membranes (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). For example, it is well known that mitochondrion membrane possesses much greater percentage of PC on its outer leaflet (40.9 %) than the outer leaflet of the erythrocyte membrane (19 %) (Karp 1996). Therefore, it may be presumed that mitochondrial membrane would be hydrolyzed to a higher extent as compared to the erythrocyte membrane by a snake venom PLA₂ enzyme. Two PLA₂ isoenzymes were reported from *Naja kaouthia* venom those showed preferential hydrolysis of mitochondrial outer membrane as compared to erythrocyte membrane as PC is the preferred substrate for both these PLA₂ enzymes (Doley et al. 2004). In a sharp contrast, an acidic PLA₂ (RVVA-PLA₂-I) purified from the venom of Indian Russell's Viper preferentially hydrolyzed the phospholipids of erythrocyte membrane compared to mitochondrion membrane though PC is its preferred substrate (Saikia et al. 2012). This may be due to the existence of significantly greater number of RVVA-PLA₂-I sensitive regions in erythrocytes membrane as compared to mitochondrial membrane which might throw a light on the relevance of higher degree of phospholipids hydrolysis of the former membrane as compared to the latter

membrane (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). These specific domains or venom PLA₂ susceptible region(s) may result from the presence of different fatty acids, more particularly the presence of short-chain fatty acids in these regions of the membrane (Mukherjee 2007). Therefore, the presence of a large number of venom PLA₂-sensitive phospholipid compositions, rather than only the PC content of that particular membrane, may determine the extent of membrane damage induced by a particular PLA₂ (Mukherjee 2007; Saikia et al. 2012).

It has been suggested that even if venom PLA₂ binds uniformly across the solid and fluid domains of membrane enriched in the long and short chain fatty acids respectively, enzyme shows a preference for short chain fatty acids, which are in fluid phase (Kaasgaard et al. 2001). This is due to the lower surface area density of the lipid head groups in short chain fatty acids that are present in fluid phase. Therefore, sPLA₂s display large variations in their affinities for membranes with differing surface properties (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012).

Furthermore, in case of some venom PLA₂ enzymes, an initial lag phase was observed for binding with a particular biomembrane (Saikia et al. 2012); while in case of some other PLA₂, no lag phase could be detected (Doley et al. 2004; Mukherjee 2007). RVVA-PLA₂-I (an acidic PLA₂ of RVV) could hydrolyze the erythrocyte membrane phospholipids within 15 min of incubation (Saikia et al. 2012) which is in sharp contrast to the reports on PLA₂s from *Naja Kaouthia* venom (NK-PLA₂-I and NK-PLA₂-II) which require a lag phase for binding with the erythrocytes membranes and subsequent membrane hydrolysis (Doley et al. 2004; Mukherjee 2007). It is to be noted that RVVA-PLA₂-I (Saikia et al. 2012) and NK-PLA₂-I (Doley and Mukherjee 2003) are acidic in nature, whereas NK-PLA₂-II is basic in nature (Doley et al. 2004). Therefore, the differences in overall net charge in a venom PLA₂ molecule may attribute to differential binding and subsequent hydrolysis of phospholipids of a particular membrane (Shukla and Hanahan 1982).

Evidences have been accumulating regarding the presence of nanometer range small-scale structures and lipid domains in the lipid bilayer and such organizational heterogeneity of lipids microdomains may have structural and functional significance (Mukherjee and Maxfield 2004). The phospholipids-binding domains with secondary binding sites for specific proteins are well known, and this binding is often tightly regulated (Lemmon 2008). The differences in the membrane phospholipids hydrolysis by a venom PLA₂ can also be supported by some observations that the phospholipids constituent of microsomal membranes are less hydrolyzed as compared to lysosomal membrane by the action of *Vipera (Daboia) russelli* venom basic PLA₂ (Mukherjee et al. 1997; Mukherjee et al. 1998b). Furthermore, it has been suggested that PLA₂ may be particularly active at domain interfaces which are the sites of structural defects and hence good points of attack for these enzymes (Doley et al. 2004).

The mechanism by which snake venom PLA₂ sensitive phospholipid domains are formed in mitochondrial or erythrocyte membrane is not very clear. Although no adequate description of the nature of such membrane lipid domains in terms of their abundance, composition or dynamics has been provided; however, it may be

assumed that the physicochemical properties of individual phospholipids, circumstantial effects of membrane proteins, phospholipids/cholesterol ratio, and vitamin E content of the membrane contribute significantly to the formation of such domains (Mukherjee et al. 1997; Doley et al. 2004; Mukherjee and Maxfield 2004; Mukherjee 2007; Siakia et al. 2012).

When the intact erythrocytes and mitochondrial membranes were treated with RVVA-PLA₂-I, an acidic PLA₂ from RVV and then the ratio of saturated / unsaturated fatty acids released from those two membranes was compared, it was noted that the above enzyme has a distinct preference for hydrolyzing phospholipids containing saturated fatty acids at *sn*-2 position in mitochondrial membrane (Saikia et al. 2012). Conversely, during the initial phase of attack the RVVA-PLA₂-I prefers hydrolysis of unsaturated fatty acids in erythrocyte membrane and therefore, the ratio of saturated/unsaturated fatty acids was less than 1.0 up to 30 min of incubation. A further increase in incubation time resulted in a greater hydrolysis of erythrocytes phospholipids containing saturated fatty acids compared to phospholipids containing unsaturated fatty acids at *sn*-2 position and the ratio of saturated/unsaturated fatty acids was higher than 1.0 post 60 min of incubation (Saikia et al. 2012). In a sharp contrast of the above result, NK-PLA₂-I from *N. Kaouthia* venom does not distinguish between the saturated and unsaturated fatty acids substrates whereas NK-PLA₂-II, another PLA₂ isoenzyme from the same venom shows a preference for unsaturated fatty acids during the initial phase of attack (Doley et al. 2004). These data indicate the differential hydrolysis of erythrocytes and mitochondrial membrane phospholipids by different PLA₂s possessing different overall net charge.

The difference in saturated and unsaturated fatty acids released from the above mentioned membranes may be explained as RVVA-PLA₂-I preferentially binds to one of the PLA₂-sensitive domains of the mitochondrial membrane resulting in quantitative increase in release of fatty acids from that membrane with respect to time (Saikia et al. 2012). However, RVVA-PLA₂-I attacks different phospholipids domains/ region(s) of erythrocyte membrane and therefore, this kinetics of erythrocyte membrane phospholipids hydrolysis favours the release of quantitatively as well as qualitatively different FAs with respect to time (Saikia et al. 2012). The ratio of release of saturated/unsaturated fatty acids from erythrocyte membrane may lead to presume that during the initial phase of attack, RVVA-PLA₂-I hydrolyzes a particular domain of erythrocyte membrane consisting of mostly the unsaturated fatty acids (Saikia et al. 2012). Slowly with an increase in time, the reaction products accumulate which may help in PLA₂ to bind and subsequent attack to another domain of the same membrane enriched in more saturated fatty acids than unsaturated fatty acids. Interestingly, this result contradicts with the finding of Shukla and Hanahan (1982) showing the acidic PLA₂ purified from the venom of *Agkistrodon halys blomhoffi* hydrolyses only one domain of PC in intact erythrocytes. These differences in erythrocyte membrane phospholipids hydrolysis pattern of acidic PLA₂s from two different snake venoms have demonstrated that not only the overall acidic charge, but the charge at a particular region, known as interfacial binding surface (IBS) of PLA₂, may determine its competence for binding with different domains of a membrane (Stahelin and Cho 2001). This emphasizes the presence of venom

PLA₂-specific sensitive regions in a particular membrane. It may be presumed that by virtue of possessing arrays of phospholipase A₂ isoenzymes, injected venom can induce greater damage to membranes and subsequent toxicity to the cells.

Furthermore, it was also found that an acidic PLA₂ of Russell's Viper venom (RVVA-PLA₂-I) did not hydrolyse HT-29 colon adenocarcinoma cell membranes even though PC is the most abundant phospholipid present in the outer cell membrane of HT-29 cells (Saikia et al. 2012). Amongst the tested membranes (i.e., mitochondrial, erythrocytes and HT-29 cell membranes), the least hydrolysis of HT-29 cell membrane by RVVA-PLA₂-I can again be explained on the basis that it is not the overall quantity of PC in a membrane but either the availability of PC in a PLA₂-sensitive membrane and/or, physicochemical properties of a membrane are the most important key elements in order to elicit the PLA₂-induced membrane damage (Doley et al. 2004; Mukherjee 2007; Saikia et al. 2012). It has been observed that some of the components of the membrane such as cholesterol/phospholipids ratio and vitamin E (α -tocopherol) content may influence its fluidity which in turn modulates the activity of venom PLA₂ enzymes (Mukherjee et al. 1997; Mukherjee et al. 1998a, b; Rangel et al. 2011).

It is noteworthy to mention that although the knowledge of the mode of hydrolysis of different membrane phospholipids by sPLA₂ has been increasing; till date there is a dearth of knowledge on membrane phospholipids hydrolyzing property of PLA₂s from different snake venoms. Such studies will advance current thoughts of the mechanism(s) of membrane damage and subsequent toxicity by PLA₂ enzymes from snake venom.

Conclusion and Future Direction

Study of snake venom PLA₂ enzymes is a great challenge to protein chemists as subtle and complex puzzles in structure-function relationships. Strongly anticoagulant PLA₂ enzymes exhibit their anticoagulant effect either by nonenzymatic way (Kini 2005; Mukherjee et al. 2014) or, in a combination of both enzymatic and nonenzymatic mechanisms (Saikia et al. 2011; Mukherjee 2014). Identifying the sites of interaction of the anticoagulants at the level of the specific amino acid residues of coagulation factors may eventually lead to the development of new peptide based therapy to treat cardiovascular diseases.

Further, the extent of membrane damage by different venom PLA₂s are dependent on the presence of PLA₂-specific membrane phospholipids domains in that particular membrane. Till date, some of the studies have provided enough evidences in support of membrane domain hypothesis; nevertheless, the exact nature of the membrane domain(s) responsible for binding with the PLA₂ enzymes from snake venom could not be identified. Further studies to identify the nature of these membrane domains and the target proteins (receptors) for venom phospholipase A₂s will help to unravel the details of the molecular mechanism underlying the various pharmacological activities of these enzymes, which might be useful for the development of novel drug delivery system targeting a particular tissue or an organ.

Cross-References

► Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins

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Phospholipase A₂ Inhibitors from Snake Blood (sbPLIs)

5

Consuelo L. Fortes-Dias, Patrícia Cota Campos,
Carlos Alexandre H. Fernandes, and Marcos Roberto M. Fontes

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Abstract

Several snake species have been naturally provided with protein inhibitors with the primary aim of self-protection against the eventual presence of venom toxins in their circulating blood. Phospholipase A₂ inhibitors are among the most

C.L. Fortes-Dias (✉) • P.C. Campos (✉)

Laboratório de Enzimologia Aplicada, Diretoria de Pesquisa e Desenvolvimento, Fundação Ezequiel Dias, Belo Horizonte, MG, Brazil

e-mail: consuelo.latorre@funed.mg.gov.br; consuelo_latorre@yahoo.com.br; patricia.cota@funed.mg.gov.br

C.A.H. Fernandes (✉) • M.R.M. Fontes (✉)

Departamento de Física e Biofísica, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Botucatu, SP, Brazil

e-mail: carlosalexandrebtu@gmail.com; fernandes@ibb.unesp.br; fontes@ibb.unesp.br

studied molecules in that group. Such molecules have been identified in venomous and nonvenomous snake species and are generally known as sbPLIs (which stand for snake blood phospholipase A₂ inhibitors). Based on the presence of known domains of mammalian proteins, sbPLIs may belong to any of three structural classes (alpha, beta, or gamma). All the domains are involved in protein-protein interactions. The sb α PLIs present a characteristic C-type lectin-like domain (CTLN) highly similar to the carbohydrate recognition domain (CRD) of Ca²⁺-dependent lectins, which includes mannose-binding and lung surfactants A and D (SP-A and SP-D) proteins. Beta-type inhibitors (sb β PLIs) are characterized by the presence of nine tandem leucine-rich repeats (LRRs), whereas sb γ PLIs share the characteristic three-finger motifs of the Ly-6 family, CD59 and the urokinase-type plasminogen activator (u-PAR). Despite of no crystallographic data of sbPLIs, several computational and biophysical techniques have been applied, in an effort to provide structural data for structure-function relationships between these inhibitors and svPLAs. This chapter will be devoted to a brief but not extensive review of general aspects, quaternary structures, molecular mechanisms, and applications of sbPLIs.

Keywords

Phospholipase A₂ • PLA₂ • PLA₂ inhibitor • sbPLI • Snake

Introduction

Phospholipases A₂ (PLA₂, EC 3.1.1.4) are part of a superfamily of enzymes compiled into 15 groups and distributed in four main types: secreted, cytosolic, calcium-independent iPLA₂, and platelet activating factor (PAF) acetyl hydrolase/oxidized lipid lipoprotein-associated (Lp) PLA₂s (Burke and Dennis 2009). Secreted PLA₂s are abundantly found in snake venoms (svPLA₂ for an acronym). In spite of sharing similar backbones, changes at their molecular surfaces driven by accelerated evolution ensured a great diversity of actions to svPLA₂ (Ohno et al. 1998; Kini and Chan 1999). Depending on the snake species, venom injection on preys or accidental victims may cause neurotoxic, myotoxic, hemolytic, edematogenic, hyperalgesic, pro-inflammatory, hypotensive, platelet-aggregation inhibitory, bactericidal, anticoagulant, and cytotoxic effects (reviewed by Gutierrez and Lomonte 2013). In many cases, even displaying more than one activity, a preponderant one may call into question the biological relevance of the others, such as the case of potent PLA₂ neurotoxins in certain venoms (Kini 2003).

It has long been known that several venomous and nonvenomous snake species are naturally resistant to the deleterious actions of homologous or heterologous venoms. Concerning svPLA₂s, the first proteins with inhibition ability were partially purified from the circulating blood of *Protobothrops flavoviridis* (previously named *Trimeresurus flavoviridis*) and *Vipera palaestinae* (Kihara 1976; Ovadia et al. 1977).

Generally called sbPLIs (an acronym for snake blood phospholipase A₂ inhibitors), these molecules co-evolved with svPLA₂s through accelerated evolution (Nobuhisa et al. 1997; So et al. 2011), with the primary aim to prevent any possible damage from toxins that might have reached the snake's bloodstream (Kochva 1978). In the last three decades, different sbPLIs have been described, and a categorization in three structural classes (α , β , γ) has been proposed, based on the presence of known protein domains in mammals (Ohkura et al. 1997). sbPLIs from a given structural class can be found in several species, independently of being venomous or not, and inhibitors belonging to distinct classes may coexist in given species, possibly ensuring the effective inhibition of different svPLA₂s (Ohkura et al. 1997; Lizano et al. 2000; So et al. 2011). When the target svPLA₂ are Lys⁴⁹ homologues or Asp⁴⁹ myotoxins, the sbPLIs have been particularly named MIPs (or myotoxin inhibitor proteins) (Quirós et al. 2007; Lizano et al. 2000; Oliveira et al. 2011). Up to date, sb α PLIs and sb γ PLIs were found more widely distributed than sb β PLIs. Interestingly, liver expression of sb α PLI and sb β PLI has been found to be upregulated by acidic svPLA₂ in *G. brevicaudus* (Kinkawa et al. 2010).

In general, native sbPLIs are homo- or hetero-oligomers of glycosylated and/or non-glycosylated subunits. The carbohydrate moiety is not essential to the in vitro functionality of most sbPLIs (Ohkura et al. 1999; Thwin et al. 2000; Soares et al. 2003; Fortes-Dias et al. 2014), and this dependence may vary according to the ionic nature of the target svPLA₂ (Okumura et al. 1999a). The next sections will be devoted to a brief overview of the main studies performed with sbPLIs. Although their classification has been based on structural domains, crystallographic structures are unavailable, to this date. Experimental difficulties may be explained by the observed heterogeneity of forming subunits, as well as type, number, and relative site occupancy of sugars in the carbohydrate portion of these glycoproteins. In addition, some sbPLIs may assume multiple oligomeric forms, therefore enhancing the heterogeneity of samples and hampering crystallization attempts (Fortes-Dias et al. 2014). Nonetheless, in silico computational and biophysical methods – circular dichroism, fluorescence spectroscopy, dynamic light scattering, and small angle X-ray scattering – have provided significant structural data on particular sbPLIs, in an effort to contribute to the elucidation of structure-function relationships between these kinds of inhibitors and svPLA₂s.

Alpha Class of Phospholipase A₂ Inhibitors (sb α PLIs)

The sb α PLIs are acidic glycoproteins that present a characteristic C-type lectin-like domain (CTLD) highly similar to the carbohydrate recognition domain (CRD) of Ca²⁺-dependent lectins, which includes mannose-binding and lung surfactant A and D (SP-A and SP-D) proteins (Inoue et al. 1991; Ohkura et al. 1993) (Table 1).

Other structural elements shared by sb α PLIs are 19-amino acid signal peptides, highly conserved hydrophobic cores at the C-terminus, and α -helical coiled-coil necks also called exon 3 segments (Estevão-Costa et al. 2016). Based on the crystal

Table 1 Identifiers for snake blood phospholipase A2 inhibitors (sbPLIs) at the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Only complete protein or nucleotide sequences deposited at protein and nucleotide databases have been included. PMIDs (PubMed identifiers) refer to unique reference numbers for PubMed citations at NCBI. Numbers or letters between parentheses, after the identifiers, refer to different subunits or chains in a given sbPLI

Snake		Alpha PLI			Beta PLI			Gamma PLI		
Family	Species or subspecies	Nucleotide	Protein	PMID	Nucleotide	Protein	PMID	Nucleotide	Protein	PMID
Colubridae	<i>E. climacophora</i>	AB462510	BAH47548	19673083	AB462511	BAH47549	19673083	AB462512 (A) AB462513 (B)	BAH47550 (A) BAH47551 (B)	19673083
	<i>E. quadringata</i>	AB030247	BAC53925 Q8AXS4	14658760	AB060637 AB060638	BAB86898 BAB86899	12485610	AB021425 (A) AB021426 (B)	BAA83078 (A) BAA83079 (B) Q9PW14 (A) Q9PW13 (B)	10377258
Elapidae	<i>N. n. kaouthia</i>	–	–	–	–	–	–	AAB32582 AAB32583	Q7LZ11 (A) Q7LZ12 (B)	7980598
	<i>N. ater</i>	–	–	–	–	–	–	AF211161 to AF211163 (α) AF211155 (β)	AAF23778 (α) AAF21046 (β)	10625636
	<i>N. scutatus</i>	–	–	–	–	–	–	AJ249827 AJ249828 (α) AJ249829 AJ249830 (β)	CAB56615 to CAB56617 (α) CAB56618 (β)	11575939
	<i>O. microlepidotus</i>	–	–	–	–	–	–	AF211167 AF211168 (α) AF211157 to AF211158 (β)	AAF23784 AAF23785 (α) AAF21048 AAF21049 (β)	10657250
	<i>O. scutellatus</i>	–	–	–	–	–	–	AF211164 AF211165 (α) AF211156 (β)	AAF23781 AAF23782 (α) AAF21047 (β)	10657250
	<i>P. textilis</i>	–	–	–	–	–	–	AF211166 (α) AF211160 (β)	AAF23783 (α) AAF21050 AAF21051 (β)	10657250

Pythonidae	<i>M. reticulatus</i>	–	–	–	–	–	–	–	AF232771	AAF73945	10924158
Viperidae	<i>A. nummifer</i>	DQ657241 DQ657242	–	–	–	–	–	–	–	–	–
	<i>B. alternatus</i>	EU421901 to EU421905	–	–	–	–	–	–	EU155166 EU155167	ABV91326 ABV91327	18620721
	<i>B. erythronelas</i>	EU421906 to EU421911	–	–	–	–	–	–	EU155168 EU155169	ABV91328 ABV91329	18620721
	<i>B. jararaca</i>	EU421912 to EU421915	–	–	–	–	–	–	EU155170 EU155171	ABV91330 ABV91331	18620721
	<i>B. jararacussu</i>	EU421916 to EU421919	–	–	–	–	–	–	EU155172 EU155173 EU574628	ABV91332 ABV91333 ACB70225	18620721 21682685
	<i>B. moojeni</i>	EU421920 to EU421925	–	–	–	–	–	–	EU155174 EU155175	ABV91334 ABV91335	18620721
	<i>B. newwiedi</i>	EU421926 to EU421929	–	–	–	–	–	–	EU155176 EU155177	ABV91336 ABV91337	18620721

(continued)

Table 1 (continued)

Snake	Alpha PLI		Beta PLI			Gamma PLI		
<i>C. d. terrificus</i>	EU421930 to EU421933	ABZ82347 to ABZ82350 B1A4Q6 B1A4Q8 B1A4Q9	26806211	–	–	U08289	AAA19162 Q90358	8195214
		AB026666 AB026667	10526227 8514730	AB007198	BAA31994 O93233	AB018372 (A) AB018373 (B)	BAA86970 (A) BAA86971 (B) P82144 (A) P82143 (B)	10791922
		EU421934 to EU421938	26806211	–	–	AY425346 AY425347	AAR04437 AAR04438 P60591 P60592	12782092
		AB559516 AB559517 (A) AB559512 to AB559515 (B)	21389624	–	–	AB559507 to AB559509 (A) AB559510 AB559511 (B)	BAJ14719 to BAJ14721 (A) BAJ14722 BAJ14723 (B)	21389624
<i>P. flavoviridis</i>	D87548 D87549 (A) D87550 (B)	BAF93271 BAF93272 (A) P21755 (A) BAF93273 (B) P21756 (B)	9210585 1985928 18243268 9210585 1985928 18243268	–	–	AB003472 AB003473 AB290845 (B)	BAA24502 BAA24503 O57690 BAF75726 (B)	9395334 18317831

structure of mammalian SPs, in silico models have been generated for sb α PLIs from *G. brevicaudus*, *Bothrops jararacussu* and *B. alternatus* (Okumura et al. 2005; Oliveira et al. 2008; Santos-Filho et al. 2011) (Table 2). These models confirmed the conservation of CRDs and α -helical necks in those sb α PLIs from viperid snakes (Fig. 1). Furthermore, circular dichroism assays with MIPs from three Latin American species – *B. alternatus*, *B. jararacussu*, and *B. moojeni* – have demonstrated that these inhibitors present characteristic curves of helical proteins, with well-defined peaks at 208 and 222 nm. Remarkably, the association of sb α PLIs with svPLA₂s does not induce significant secondary structure changes on those particular MIPs (Soares et al. 2003; Oliveira et al. 2008; Santos-Filho et al. 2011).

Differently from SP-As, sb α PLIs interact with group II-PLA₂s independently of calcium ions. Calcium ions have no effect on the formation of sb α PLI-svPLA₂ complexes, and the amino acids involved in the cation coordination are lacking in the inhibitors (Ohkura et al. 1993; Okumura et al. 2005; Santos-Filho et al. 2011). Since calcium ions are involved in carbohydrate binding, it can be inferred that sugars are not involved in PLA₂ binding or inhibition by sb α PLIs, in spite of conserved N¹⁰³ glycosylation sites in these molecules (Lizano et al. 2003; Santos-Filho et al. 2011; Estevão-Costa et al. 2016).

Quaternary Structure of sb α PLIs

Analytical size exclusion chromatography experiments have shown that sb α PLIs can be found in solution as homo- or hetero-oligomers of molecular masses between 75 and 120 kDa. The α -helical coiled-coil necks of the composing subunits in sb α PLI trimers form a central pore, which is actually the binding site of svPLA₂s (Okumura et al. 2005; Santos-Filho et al. 2011). The accurate assembly of that pore is dependent on the primary structure and matching of the α -helical coiled-coil necks in the forming monomers (Okumura et al. 2005; Shimada et al. 2008; Nishida et al. 2010). SvPLA₂ inhibition is impaired when the central pore is atypically or not assembled at all, such as observed for nonfunctional PLI α -like proteins from *Elaphe* snakes or for truncated mutants of *G. brevicaudus* sb α PLI (Okumura et al. 2003; Okumura et al. 2005; Shirai et al. 2009).

On the basis of in silico modeling of sb α PLI monomers from *G. brevicaudus*, Okumura et al. (2005) have reported that the α -helical necks stabilize the trimer, such as observed in the crystal structure of rat SP-A (PDB ID 1R13). Later on, Santos-Filho et al. (2011) have constructed a structural model of sb α PLI homotrimer from *B. alternatus* (DOI: ma-a4btt at ModelArchive). In the sb α PLI from *P. flavoviridis*, two different subunits A and B may associate, in a random way, to form four different trimer arrangements (Shimada et al. 2008). The sb α PLI-A homotrimer is more specific against acidic svPLA₂s, when compared to the sb α PLI-B homotrimer. However, the presence of different subunits and the heterotrimeric formation was only observed for sb α PLIs isolated from Asian *P. flavoviridis*.

Table 2 Structural data available for snake blood phospholipase A2 inhibitors (sbPLIs)

Inhibitor class	Snake species or subspecies	Technique	Reference
Alpha PLI	<i>A. nummifer</i>	Analytical size exclusion chromatography	Quirós et al. 2007
		Chemical cross-linking	
	<i>B. alternatus</i>	Circular dichroism spectroscopy	Santos-Filho et al. 2011
		Dynamic light scattering	
		Molecular modelling of monomer and trimer	
		Molecular modelling and structural comparisons	Estevao-Costa et al. 2016
	<i>B. jararacussu</i>	Analytical size exclusion chromatography	Oliveira et al. 2008
		Circular dichroism spectroscopy	
		Molecular modelling of monomer	
	<i>B. moojeni</i>	Analytical size exclusion chromatography	Soares et al. 2003
		Circular dichroism spectroscopy	
	<i>G. brevicaudus</i>	Analytical size exclusion chromatography	Ohkura et al. 1993
		Chemical cross-linking	
		Site-directed mutagenesis	Okumura et al. 2005
		Chimeric protein constructions	
		Molecular modelling of monomer	Nishida et al. 2010
		Site-directed mutagenesis	
		Chimeric protein constructions	
		Fluorescence spectroscopy	
	<i>P. flavoviridis</i>	Chemical cross-linking	Shimada et al. 2008
Beta PLI	<i>G. brevicaudus</i>	Chemical cross-linking	Okumura et al. 1998
Gamma PLI	<i>C. d. terrificus</i> (CNF)	Peptide arrays	Fortes-Dias et al. 2009
		Analytical size exclusion chromatography	Fortes-Dias et al. 2014
		Chemical cross-linking	
		Circular dichroism spectroscopy	
		Dynamic light scattering	
		Small angle x-ray scattering	
		Fluorescence spectroscopy	
		Molecular modelling of monomer	
	<i>C. d. collilineatus</i>	Circular dichroism spectroscopy	Gimenes et al. 2014
		Dynamic light scattering	
	<i>M. reticulatus</i>	Synthetic peptides	Thwin et al. 2010

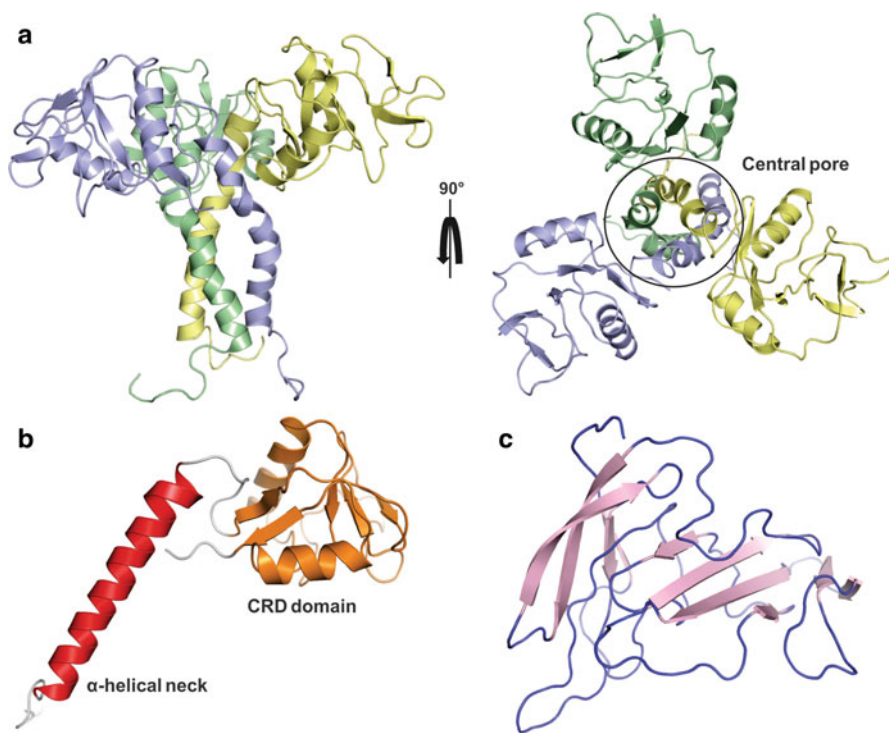


Fig. 1 Cartoon representation of *in silico* structural models of alpha and gamma snake blood phospholipase A₂ inhibitors (sbPLIs). (a) αBaltMIP, a sbαPLI from *Bothrops alternatus*, in trimeric form (each chain is represented by a different color) highlighting the central pore region; (b) αBaltMIP in monomeric form highlighting the CRD (orange) and the alpha-helical neck region (red); (c) CNF, a sbγPLI from *Crotalus durissus terrificus*, in monomeric form. The models are available at ModelArchive database under the following digital object identifiers: ma-a4btt, ma-a2iil and ma-avb44, respectively

Molecular Mechanism of svPLA₂ Inhibition by sbαPLIs

A monomer from *B. jararacussu* MIP has been reported to be active for a series of biological activities (Oliveira et al. 2008). Nevertheless, recent data reinforced the importance of trimer formation for svPLA₂ binding in a similar sbαPLI from *B. alternatus*, by showing that the central pore forms a highly positive charged area (~1,600 Å²) due to the presence of conserved basic amino acid residues (R⁵⁷, K⁷¹, R¹⁰⁸, and H¹⁰⁹) (Estevão-Costa et al. 2016). This positively charged area is compatible with negatively charged surfaces in the crystal structures (1,200 Å²) of acidic svPLA₂s. The trimer model accepts that one PLA₂ molecule binds to one central pore, in agreement with previous stoichiometrical data reporting molar ratios of 1:3 or 1:6 in svPLA₂-sbαPLI complexes with the inhibitor from *Cerrophidion godmani* (Lizano et al. 2000).

Whereas there is little doubt about the importance of trimer assembling for the functionality of sb α PLI, the amino acids required for trimer stabilization are not consensual. In contrast to previous data that proposed the involvement of N⁴⁵, K⁴⁷, and D⁴⁸ (Okumura et al. 2005), recent multiple alignment data indicated that these amino acids are not conserved in every sb α PLI (Estevão-Costa et al. 2016). Instead, the L³⁹Q⁴⁰(R/K)⁴¹E⁴²F⁴³ peptide on the alpha-helical neck is conserved in every sb α PLI analyzed. This pentapeptide undergoes drastic mutations in sb α PLI-like proteins from nonvenomous *Elaphe* snakes, suggesting that this segment may be essential to trimer stabilization (Okumura et al. 1999b). Concerning Y¹⁶⁵, another amino acid supposed to be important in svPLA₂ binding by *G. brevicaudus* sb α PLI (Okumura et al. 2005), in silico data of *B. alternatus* MIP do not support the location of this residue at the central pore of the trimer. Finally, the C-terminal hydrophobic core (L¹⁵⁸ to V¹⁶¹) may play a role in the structural integrity of the central pore of sb α PLIs (Estevão-Costa et al. 2016). In spite of a preferential binding to acidic svPLA₂s, sb α PLIs from *Bothrops* genus are able to bind and inhibit basic svPLA₂s. Since basic svPLA₂s are also positively charged at their surfaces, the ionic interaction at the central pore of sb α PLI trimers may be hampered by equally charged surfaces (dos Santos et al. 2009). Hence, the inhibition of basic svPLA₂s by sb α PLIs remains to be better understood.

Applications for sb α PLIs

Inhibition by sb α PLIs is generally evaluated through a number of known svPLA₂-induced actions, such as edema inducing, myotoxic, cytotoxic, and bactericidal, besides enzymatic action itself, with positive results for homologous and heterologous svPLA₂s (Soares et al. 2003; Oliveira et al. 2008; Santos-Filho et al. 2011). Preincubation of polyvalent (Crotalinae) antivenom with BaMIP – a sb α PLI from *B. asper* – reduced the myotoxic of the homologous venom in mice, in a dose-dependent way (Lizano et al. 2003). However, as raised by the authors, any commercial use of sbPLIs will be hindered by the amounts needed and subsequent ecological implications.

A reasonable alternative is the obtainment of functional recombinants in heterologous systems of protein production. Homologs or truncated forms of sb α PLIs from *P. flavoviridis* and *G. brevicaudus* have been successfully produced in *E. coli* (Nobuhisa et al. 1998; Okumura et al. 1999a), as well as *B. alternatus* sb α PLI in *Pichia pastoris* (Santos-Filho et al. 2014). However, these proteins have not been commercially available for the purpose of research and clinical use yet.

Beta Class of Phospholipase A₂ Inhibitors (sb β PLIs)

Beta-type inhibitors (sb β PLIs) are acidic, highly glycosylated proteins that are characterized by the presence of nine tandem leucine-rich repeats (LRRs) in their structure. Other main structural features are an N-terminal proline-rich region and

ten cysteine residues, eight of which are involved in disulfide bonds. The first sbβPLI in the literature has been purified from *G. breviceaudus* and extensively characterized as such. This sbβPLI displays 33 % sequence homology with human leucine-rich α₂-glycoprotein (LRG), a protein with unknown function, in which LRRs have been firstly described. Similar to LRG, *G. breviceaudus* sbβPLI contain nine tandem LRRs of 24 residues each, which encompass over two-thirds of the molecule (Ohkura et al. 1997; Okumura et al. 1998).

So far, sbβPLIs have been solely purified from three snake species: the venomous *G. breviceaudus* viperid (Ohkura et al. 1997; Okumura et al. 1998) and the nonvenomous *E. quadrivirgata* and *E. climacophora* colubrids (Okumura et al. 2002; Shirai et al. 2009) (Tables 1 and 2). *E. quadrivirgata* is supposed to prey upon venomous snakes, including *G. breviceaudus*, whereas *E. climacophora* is believed to be non-ophiophagous (Shirai et al. 2009). In an effort to elucidate the physiological function of such an inhibitor in a nonvenomous, non-ophiophagous species with apparently no need of protection against toxic svPLA₂, the presence of sbβPLI transcripts has been investigated in several organs and tissues of *E. climacophora* (Okumura et al. 2002). Except for the liver and lung, no transcripts have been detected in the spleen, pancreas, fat body, small intestine, gallbladder, testis, kidney, esophagus, or heart of that snake species. It has been suggested that lung tissue might contain endogenous group II basic PLA₂ to be specifically regulated by the sbβPLI. Although not applicable to *E. climacophora*, the authors suggested that sbβPLI might play a role in the local regulation of PLA₂-rich tissues, such as the venom glands of venomous snakes. In fact, a putative sbβPLI has been cloned from the venom glands of *L. muta* snakes (Lima et al. 2011), which venom is rich in PLA₂s including group II basic enzymes (Damico et al. 2005).

Quaternary Structure of sbβPLIs

Native sbβPLI are trimers of 170 kDa formed by monomers with approximately 50 kDa (Table 2). No higher multimers have been detected by cross-linking experiments (Ohkura et al. 1997; Okumura et al. 1998). Two different subunits, A and B, differ in 16 of 309 amino acids in the sbβPLI from *E. quadrivirgata*. Both subunits display 78.3 % of sequence identity with the sbβPLI from *G. breviceaudus* (Okumura et al. 2002). In *E. climacophora*, a single subunit has been detected that corresponds to the B subunit of the inhibitor from *E. quadrivirgata* snakes.

Molecular Mechanism of svPLA₂ Inhibition by sbβPLIs

sbβPLIs have been proved to be selective inhibitors against group II basic svPLA₂s. They did not inhibit group II acidic, group I, or group III svPLA₂s from several venoms tested (Okumura et al. 2002). By comparing the amino acid sequences of the selectively inhibited with non-inhibited svPLA₂s, Okumura et al. (1998) proposed the recognition and binding of sbβPLI to specific residues

in the group II basic svPLA₂s, namely, H¹, R⁶, E¹⁷, and W⁷⁰. These conserved residues are located in or close to the interfacial binding surface of the svPLA₂s, and it has been suggested that the inhibitor binding to that surface might cover the enzyme catalytic site. Based on the high conservation of amino acids and same inhibition specificity of the sbβPLIs, the N-terminal flanking region and the following LRR₁ and LRR₂ have been suggested to be involved in the binding site to group II basic svPLA₂s (Okumura et al. 2002; Shirai et al. 2009).

Applications for sbβPLIs

No specific application has been devised for the sbβPLIs yet. Interestingly, the *G. breviceaudus* sbβPLI has been found to bind to Cyt *c*, similar to the LRG (Shirai et al. 2010). This has been the first description of another ability of a sbβPLIs, differently from svPLA₂ binding and inhibition. Due to its ability to neutralize the autologous Cyt *c* released from dead cells in the circulatory system, an ancestral function has been suggested for that particular sbβPLI as a Cyt *c*-binding protein prior to svPLA₂ inhibition.

Gamma Class of Phospholipase A₂ Inhibitors (sbγPLIs)

Gamma class of phospholipase A₂ inhibitors (sbγPLIs) comprise the major number of sbPLIs, identified in four different snake families (Table 1). The sbγPLIs are acidic glycoproteins that are characterized by a tandem pattern of cysteine residues, typical of three-finger motifs, as found in the Ly-6 family, CD59, ThB (mouse thymocyte B), and urokinase-type plasminogen activator (u-PAR) (Ohkura et al. 1994). In fact, the in silico model of *Crotalus* neutralizing factor (“CNF” available with DOI: [ma-avb44](https://doi.org/10.1016/j.sbspro.2014.03.004) at ModelArchive database), a sbγPLI from *Crotalus durissus terrificus*, was built based on the crystal structure of urokinase-type plasminogen activator and confirms the prevalence of three-finger motifs in its tertiary structure (Fig. 1) (Fortes-Dias et al. 2014). The proposed model presents high stereochemical adequacy, as expressed by a high percentage of residues (97.7 %) in the allowed regions of the Ramachandran plot, as well as an overall good quality (Z-score equal to −4.2).

Circular dichroism assays with CNF, as well as with sbγPLIs from *C. d. collilineatus* and *Python sebae*, have demonstrated that these inhibitors present characteristic curves of β-sheet rich proteins, with a well-defined peak around 210–217 nm (Donnini et al. 2011; Fortes-Dias et al. 2014; Gimenes et al. 2014). The arrangement of β-sheets and loops/disordered secondary structure elements in well-ordered three-finger domains has been confirmed in the in silico structure of CNF.

Based on predicted structural homology, a classification into groups 1 and 2 has been proposed for the sbγPLIs. Inhibitors with higher homology to u-PAR have been

placed in class 1, whereas those most homologous to Ly-6 and CD-59 remained in class 2 (Hains et al. 2001).

Quaternary Structure of sbγPLIs

SbγPLIs, similar to alpha and beta sbPLIs, assemble in oligomeric structures of 90–160 kDa. The number of forming subunits remains undetermined for most sbγPLIs, with the exception of the inhibitors from *Laticauda semifasciata* (Ohkura et al. 1994) and *Naja naja kaouthia* (Ohkura et al. 1999) elapid snakes, which have been identified as trimers. For other sbγPLIs, the number of monomers in the oligomers has been mostly inferred from analytical size exclusion chromatography, which has been shown to be inappropriate for this purpose. By taking CNF as an example, the estimated molecular mass of the native molecule was 127.1 ± 39.2 kDa on the average, with a variation coefficient of 30.8 %. Since the oligomers are formed by 20 kDa (unglycosylated) and 24 kDa (glycosylated) monomers, the number of subunits in native CNF could vary from four to eight, depending on the used column. For this particular sbγPLIs, additional biochemical and biophysical approaches – namely, chemical cross-linking, fluorescence spectroscopy, dynamic light, and small angle X-ray scatterings – have been employed (Table 2). The whole data set has indicated tetramers to be the prevailing quaternary structure of CNF in solution. Based on fluorescence spectroscopy experiments and molecular modeling of the monomer, tyrosine residues have been proposed to play an important role in the oligomerization of CNF (Fortes-Dias et al. 2014).

SbγPLI oligomers may be homo- or heteromeric, depending on the type of forming subunits (Table 1). Typically, heteromeric sbγPLIs are composed of two subunits (A and B) under 2:1 ratio, where only A subunit is glycosylated (Lizano et al. 2000). Further classification of sbγPLIs in subclasses I and II, depending on the homo- or heterogeneity of oligomer formation, has been proposed (Lizano et al. 2003). According to the authors, subclass I comprises sbγPLIs isolated from Elapidae, Colubridae, and Hydrophiidae families, as well as from the Old World viperid *G. breviceaudus* (Table 1). On the other hand, sbγPLIs belonging to class II are formed by single subunits, such as in *Python*, American viperid, and Old World *P. flavoviridis* species (Table 1). Nonetheless, the recent identification of putative second subunits or subtypes in *P. flavoviridis*, *P. elegans*, and *C. d. terrificus* (CNF) inhibitors challenged the homomeric concept of sbγPLI-IIs (So et al. 2008, 2011; Fortes-Dias et al. 2014). The contribution of putative subunit B to the functionality of sbγPLI-IIs remains to be clarified, since homogenous and functional sbγPLIs have been purified from American *C. godmani* and *B. jararacussu* using affinity chromatography with target svPLA₂s (Lizano et al. 2000; Oliveira et al. 2011).

Isoforms have been reported for both A and B subunits in heteromeric sbγPLIs in a number of species (Table 1). According to Dunn and Broady (2001), the combination of more and less conserved subunit isoforms observed in Australian species could introduce subtle structural changes to sbγPLIs, which might modify the recognition site of the molecule to allow interaction with slightly different svPLA₂s,

thus increasing the binding repertoire of these inhibitors. More recently, the frequency of amino acid substitutions has been mathematically proved to be higher for isoforms of B subtype than in A subtype, in the sb γ PLI from *P. elegans*. It has been suggested that sb γ PLI-As derived from ancestral sb γ PLI-B and that particular loops in the three-finger motifs evolved in an accelerated manner to possibly support specific inhibitory functions (So et al. 2011).

Anyhow, the homomeric idea persists for PIP, a sb γ PLI from *Malayopython reticulatus* (Boidae) formed by six identical subunits of 23 kDa (Thwin et al. 2000). Interestingly, a structural sb γ PLI homolog highly similar to PIP and with poor PLA₂ inhibition effect has been isolated from *P. sebae* (Donnini et al. 2011). The PIP-like molecule is composed of two subunits in a 1:1 stoichiometric ratio and forms heterotetramers in equilibrium with heterohexamers, in solution. Both subunits are highly similar to PIP monomer and differ among each other in 8 out of 182 amino acid residues, with no homology with B subunits described for the heteromeric sb γ PLIs.

Molecular Mechanism of svPLA₂ Inhibition by sb γ PLIs

Differently from sb α PLIs, a general mechanism is unavailable for the inhibition of svPLAs by sb γ PLIs, although it has been related to the formation of a stable, non-covalent complex with the target enzymes.

Concerning CNF, the target svPLA₂ in the homologous venom is crotoxin, a heterodimeric β -neurotoxin formed by an enzymatically inactive subunit (CA) and a PLA₂ counterpart (CB). These subunits are strongly bound by non-covalent interactions in the crotoxin complex (CA.CB) (Habermann and Breithaupt 1978). Addition of CNF to crotoxin leads to the displacement of CA and consequent formation of a stable CNF.CB complex, which is also sustained by non-covalent interactions. This nontoxic complex (CNF.CB) seems reminiscent of the interaction of crotoxin with its receptor at the presynaptic site of the neuromuscular junctions (Fortes-Dias et al. 1994; Perales et al. 1995). Peptide array results have suggested two segments at the N-terminal and beta-wing of CB to be involved in CNF and CA interactions, in their respective complexes with CB (Fortes-Dias et al. 2009). Possible interaction segments at CA and CNF in these complexes remain to be determined.

Applications for sb γ PLIs

As aforementioned for sb α PLIs, the inhibition by sb γ PLIs is also evaluated by testing their interference on svPLA₂-induced actions. Preincubation of polyvalent antiothropic serum with a sb γ PLI from *B. jararacussu* (γ BjussuMIP) reduced the myotoxic of the homologous venom in mice (Oliveira et al. 2011), as observed before for the sb α PLI from *B. asper*, BaMIP (Lizano et al. 2003).

Regarding protein expression attempts, a recombinant inhibitor with identical chromatographic and biochemical behavior of PIP – the sb γ PLI from python – has

been successfully expressed in *E. coli*, but not scaled-up (Thwin et al. 2000). Instead, the authors reported main advances with synthetic PIP-derived peptides and highlighted their therapeutic potential in inflammatory PLA₂-related diseases and as antivenom-like bioactive molecules for snake venom neutralization (Thwin et al. 2010; Samy et al. 2011). For instance, besides strongly reducing the PLA₂ activity of beta-neurotoxins, a 17-mer peptide (P-NT.II) has been shown to be a selective inhibitor of human secreted sPLA₂-IIA and to modulate joint destruction in a clinically relevant transgenic mouse model of inflammation (Thwin et al. 2010). In vitro screening of a number of synthetic PIP-derived peptides with single amino acid changes highlighted the strong inhibition of the 18-mer linear peptide corresponding to the amino acid sequence 59–76 of PIP against *C. adamanteus* and human synovial PLA₂s (Samy et al. 2011). Recently, PIP-18 has been reported to possess higher antimicrobial activity than classical antibiotics (chloramphenicol, ceftazidime, and streptomycin) (Samy et al. 2015). Within the same snake genus, the sbPLI homolog from *P. sebae* showed remarkable in vitro and in vivo antitumor effects (Donnini et al. 2011).

Conclusion and Future Directions

Because groups I and II-svPLA₂s share structural similarities with human secretory PLA₂s, the sbPLIs have been considered potential molecular models for the development of new, selective therapeutic agents in inflammatory-related diseases, besides snake envenoming. Although significant progress has been achieved in the last decades, further elucidation of structure-function relationships within the sbPLIs and in sbPLI-svPLA₂ complexes is essential to full understanding and use of those molecules to treat human health problems. Crystallization data on sole or complexed sbPLIs, in a near future, may provide missing structural data and help to clarify those issues.

Cross-References

- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins](#)
- ▶ [Secreted Phospholipases A₂ with β-Neurotoxic Activity](#)

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A Brief Appraisal on Russell's Viper Venom (*Daboia russelii russelii*) Proteinases

6

Rupamoni Thakur and Ashis K. Mukherjee

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Abstract

Russell's viper (*Daboia russelii*) is an important member of the Viperidae family which is widely distributed across Southern Asia including India. Russell's viper venom (RVV) is indisputably a potent mixture of various toxic and nontoxic components that have evolved to interfere with vital physiological processes like coagulation and fibrinolysis. Many of these components are metallo- or serine proteinases. Venoms from the snakes of Viperidae family are reported to be rich in

R. Thakur

Microbial Biotechnology and Protein Research Laboratory, Department of Molecular Biology and Biotechnology, School of Sciences, Tezpur University, Tezpur, Assam, India

e-mail: rupat@tezu.ernet.in

A.K. Mukherjee (✉)

Department of Molecular Biology and Biotechnology, Research and Development, ONGC-Centre for Petroleum Biotechnology, DBT Nodal Centre for Medical Colleges and Biomedical Research Institutes of North-East India (Unit of Department of Biotechnology, Govt. of India), Tezpur University, Tezpur, Assam, India

e-mail: editor_tsi@yahoo.in; akm@tezu.ernet.in

proteinases which contributes to hemostatic alterations in the victims and is also responsible for tissue-specific hemorrhagic effects. However, till date, not many proteinases have been reported from RVV. In this brief review, the currently available data on the structural and functional attributes of RVV proteinases have been focused and summarized. The pathophysiological significance and the therapeutic/ diagnostic application(s) of the identified RVV proteinases have also been discussed. It is relevant to note that a more detailed analysis of the entire complexity of RVV proteinases will contribute to the better understanding of their role in Russell's viper envenomation and subsequent antivenom therapy. Most importantly, novel RVV proteinases can also be explored for their usefulness in the development of diagnostic/therapeutic agents to deal with life-threatening diseases.

Keywords

Blood coagulation • Pathophysiology • Proteinases • Russell's viper • Snake venom

Introduction

Snakebite is an important but a neglected health hazard in the tropical countries of Southeast Asia like India, Sri Lanka, Bangladesh, Pakistan, Myanmar, Thailand, etc. (WHO). Estimates show that annually approximately 421,000 cases of envenomation occur resulting in around 20,000 deaths throughout the world (Monteiro et al. 2012). India is one of the countries of the Southeast Asia that has recorded the highest incidence of envenomation (81,000 per year) and the maximum number of deaths (11,000 per year) due to snakebite (Kasturiratne et al. 2008; Monteiro et al. 2012), albeit this does not reflect the true magnitude of the problem because most of the snakebites occurring in the rural areas of the country remain unreported. Russell's viper (*Daboia russelii*), an important member of the Viperidae family of snakes, is widely distributed over large areas of Asia from Pakistan to Taiwan and the Lesser Sunda Islands (Thorpe et al. 2007). Russell's viper (RV) is one of the most frequently encountered snake species responsible for the majority of snakebite morbidity and mortality in many Southeast Asian countries including Thailand, Myanmar, China, and Indonesia as well as the Indian subcontinent (Warrell 1989). Therefore, RV is regarded as one of the members of "Big Four" venomous snakes of India whose bite is fatal and requires immediate medical attention. RV envenomation is an occupational hazard and a grave problem particularly for the farmers and rice cultivators in Southeast Asia. This species of snake is particularly abundant in forested plantations and farmlands (e.g., rice fields) because of the easy availability of its primary dietary preference which includes rodents (rats/mice) which thrive near these areas (Warrell 1989). In India, RV is abundant in Punjab and is common along the West Coast and its hills in Southern India and up to Bengal. It is uncommon to rare in the Ganges valley, Northern Bengal, and Assam.

On the basis of its geographical distribution, *Daboia russelii* has been categorized into a number of subspecies (Warrell 1989; Thorpe et al. 2007). The species inhabiting the Indian subcontinent (India, Pakistan, and Bangladesh) has been classified as *Daboia russelii russelii*. The other subspecies of RV include *Daboia russelii nordicus* pertaining to Northern Indian region, *Daboia russelii pulchella* found in the Sri Lankan region, and *Daboia russelii formosensis* and *Daboia russelii limitis* are abundant in Taiwan and in Indonesia, respectively (Warrell 1989; Thorpe et al. 2007). Recently, on the basis of morphological characteristics and mitochondrial DNA data analysis, RV has been categorized into two subspecies, namely, *Daboia russelii russelii* which is abundant in the Indian subcontinent and *Daboia russelii siamensis* reported in regions other than the Indian subcontinent (Thorpe et al. 2007).

Compositional Analysis of Russell's Viper Venom (RVV) from Different Geographical Regions and Corresponding Differences in Clinical Manifestations After RV Bite

Studies related to the clinical manifestations of RV envenomation and compositional analysis of RVV of a particular geographical location have suggested that due to differences in geographical origin, RVV varies in its composition and illustrates differences in the clinical manifestations following envenomation (Jayanthi and Gowda 1988; Prasad et al. 1999; Mukherjee et al. 2000). The major clinical manifestations of RV envenomation are systemic coagulopathy and tissue hemorrhage (Warrell 1989). Symptoms such as neurotoxicity and intravascular hemolysis are predominantly noticed in RV bite victims from Sri Lankan regions, whereas complications such as pituitary hemorrhage and increased capillary permeability are observed in envenomation by the same species from regions of Burma and Southern India (Warrell 1989; Prasad et al. 1999). The composition and lethal potency of RVV is also reported to show region-wise variation within India (Jayanthi and Gowda 1988). In the eastern region of India, RVV exhibits procoagulant effects (at low doses) and anticoagulant effects (at higher doses) and correspondingly RV bite is characterized by spontaneous hemorrhage (Prasad et al. 1999; Chakrabarty et al. 1993). On the contrary, RVV of all the other regions exhibit procoagulant effects (Prasad et al. 1999). In addition, RV bite in South India is characterized by neurotoxic and myonecrotic symptoms (Kasturi and Gowda 1988; Prasad et al. 1999), while RVV from Western India is mainly hemorrhagic (Prasad et al. 1999). RVV from the eastern region of India is reported to lack of neurotoxicity in patients (Mukherjee et al. 2000) but contributes to intravascular hemolysis and hemorrhage (Mukherjee et al. 2000).

Proteinases are important constituents of snake venom, and RVV is also enriched in different proteinase enzymes (Mukherjee et al. 2000; Chen et al. 2008; Mukherjee 2008; Mukherjee and Mackessy 2013). Proteolytic enzyme profiling in addition to comparison of levels of proteolytic activity of these enzymes from different subspecies of RVV has thrown light on the differences in their level of clinical

manifestations (Faiz et al. 1996). Snake venom proteinases have been concluded to be responsible for some of the characteristic local symptoms of snake bite such as hemorrhage, necrosis, and muscular degeneration (Gutierrez and Rucavado 2000), besides interfering with the important physiological processes like coagulation and fibrinolysis. There are reports of RVV proteinases which bring about hemostatic alterations in the victims along with symptoms of tissue necrosis, besides local, dermal, and systemic hemorrhage. Understanding the roles of various RVV components, particularly those of RV proteinases, may not only serve in understanding their contributions to RV envenomation but may also serve in exploring their therapeutic and diagnostic values.

RVV Proteinases: Structural and Functional Characteristics

Proteinases or proteolytic enzymes function as molecular knives incising long amino acid sequences into fragments, a process that is essential for the synthesis of all proteins, controlling their size, composition, shape, turnover, and ultimate destruction (Shen and Chou 2009). Proteinases are highly selective and show varying substrate specificity. According to their catalytic mechanisms, proteinases are classified into the following six types: aspartic, cysteine, glutamic, metallo, serine, and threonine (Rawlings et al. 2010). Each of these proteinase types has been characterized to possess specific amino acids in their catalytic sites. Functionally, aspartic (e.g., pepsin), glutamic (e.g., G1 proteases), and metallo (e.g., RVV-X)-proteinases activate a water molecule which performs a nucleophilic attack on the peptide bond to hydrolyze it, whereas serine (e.g., trypsin), threonine (e.g., ornithine acetyltransferase), and cysteine proteinases (e.g., TEV protease) use a nucleophilic residue in their catalytic triad to covalently link the proteinase to the substrate protein. The next step involves the hydrolysis of the covalent acyl-enzyme intermediate by activated water to complete the catalysis, thereby releasing the product and regenerating the free enzyme. Among various lethal and nonlethal enzymatic components, proteinases contribute significantly to the overall composition of most of the snake venoms including RVV. The venom proteinases that have been studied so far exhibit vivid pharmacological properties and mostly belong to either the serine or the metalloproteinase group (Matsui et al. 2000). RVV comprises a number of nonenzymatic and enzymatic components of which proteinases make an important contribution. A brief portrayal of the proteinases identified so far from RVV along with their functions has been listed in Table 1.

RVV Metalloproteinases

Snake venom metalloproteinases (SVMPs) play a significant role in venom-induced pathogenesis by damaging the local tissues and causing tissue necrosis (Chakrabarty et al. 1993, 2000; Mukherjee 2008; Chen et al. 2008). Structurally, SVMPs are monozinc endopeptidases that are classified under the subfamily of “reprolysins”

Table 1 List of proteinases identified from Russell's viper (*Daboia russelii*) venom and their biological functions

Nature	Name of proteinase	Molecular weight (kDa)	UniProt Accession no.	Biological Functions	References
Serine proteinase	RVV-V	27.2	P18964 P18965	Activation of factor V	Kiesel (1979) Tokunaga et al. (1988)
	Russelobin	51.2	–	Thrombin-like proteinase causes defibrinogenation	Mukherjee and Mackessy (2012, 2013)
	RV-FV/P α RV-FV/P β RV-FV/P γ RV-FV/P δ	32.9 33.36 33.35 34.59	–	$\alpha\beta$ -fibrinogenases with factor V-activating properties	Mukherjee (2014)
	RVV-X	79.0	Q4PRD1 Q7LZ61	Activation of factor X	Furie and Furie (1976) Takeya et al. (1992)
	VRR-73	73.0	–	Fibrinolytic proteinase with hemorrhagic and esterolytic properties	Chakrabarty et al. (2000)
	VRH-1	22.0	–	Causes severe lung hemorrhage	Chakrabarty et al. (1993)
Metalloproteinase	RVBCMP	15.0	–	α -Fibrinogenolytic procoagulant proteinase Causes distinct liver hemorrhage	Mukherjee (2008)
	Daborhagin-M Daborhagin-K	65.0 65.0	P0DJH5 B8K1W0	Fibrinogenolytic proteinase Causes severe hemorrhage	Chen et al. (2008)

along with the ADAMs (“a disintegrin and metalloproteinase” proteins) (Bode et al. 1993; Hite et al. 1994; Gutierrez and Rucavado 2000; Fox and Serrano 2009), as they share a common overall domain organization (Hite et al. 1994; Moura-da-Silva et al. 2011). These reprolysins are classified as part of the “metzincin” superfamily of zinc-dependent metalloproteinases which exhibit zinc-binding environments besides possessing the consensus sequence “HEXXHXXGXXH” and a methionine turn (Bode et al. 1993; Gutierrez and Rucavado 2000; Fox and Serrano 2009). After numerous speculations, SVMPs have finally been classified into three main groups based on the differences in their domain structure *per se* (Moura-da-Silva et al. 2011):

- (i) P-I class of SVMPs are the simplest class of proteinases comprising only the metalloproteinase domain.
- (ii) P-II class of SVMPs possesses a disintegrin-like domain in addition to the metalloproteinase domain. This class of SVMPs is further classified into six different subclasses based on the position and number of cysteinyl residues in their protein sequence (Fox and Serrano 2005). In the first subclass of P-II SVMPs, there are characteristic cysteinyl residues found at positions 222 and 241, which are not found in these positions in the other P-II SVMPs (Fox and Serrano 2005). The second subclass of P-II SVMPs is characterized by the lack of cysteinyl residues at positions 233 and 235 (Fox and Serrano 2005). The third subclass is characterized by having a cysteinyl residue at position 168, but lacking the cysteinyl at position 164 (Fox and Serrano 2005). The other possible structural groups in the PII class are represented by atrolysin E, ussurin, and insularinase (Fox and Serrano 2005).
- (iii) P-III SVMPs in addition to the metalloproteinase and disintegrin-like domains also contain cysteine-rich domains. The P-III SVMPs are further divided into four subclasses (P-IIIa, P-IIIb, P-IIIc, P-IIId) based on their distinct posttranslational modifications such as homo-dimerization (P-IIIc) or proteolysis between the metalloproteinase and disintegrin-like domains (P-IIIb) (Takeda et al. 2012). It is important to note that previously classified heterotrimeric P-IV SVMPs that contains an additional snake C-type lectin-like (snaclec) domain is now included in the P-III group as a subclass (P-IIId), since no P-IV gland transcriptome has been established to date. Consequently, the P-IIId class of SVMPs is considered as a posttranslationally modified derivative of the P-IIIa subclass of the P-III SVMP (Fox and Serrano 2009; Takeda et al. 2012).

SVMPs exhibit a large variation in molecular weights ranging from 15 kDa to over 100 kDa (Hite et al. 1994; Bjarnason and Fox 1994; Mukherjee 2008). Crystallographic studies of many of the SVMPs have helped the researchers to understand the structure-function properties of this class of proteinases in a much better way (Bode et al. 1993; Gutierrez and Rucavado 2000; Fox and Serrano 2009). In addition to their pathological significance, SVMPs also remain relevant targets for the development of novel therapeutic agents as they have been shown to interact at several stages of the blood coagulation cascade causing changes in the coagulopathy through either the stimulation

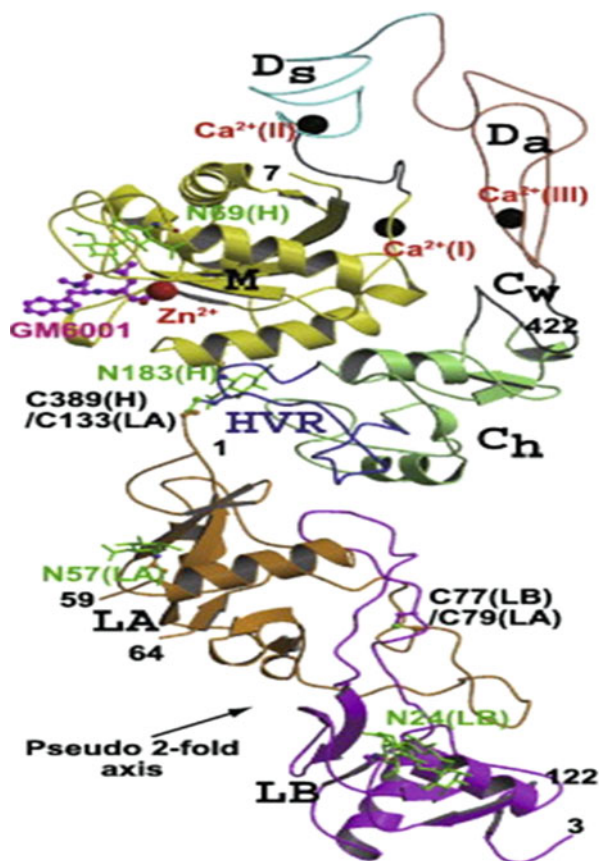
or prevention of fibrin formation (Markland 1998; Howes et al. 2005). Viperid snake venoms are rich sources of metalloproteinases and many of these metalloproteinases have been identified and characterized from RVV. A brief review on each of the identified RVV metalloproteinases is summarized below.

RVV-X

One of the major metalloproteinases identified from RVV is RVV-X. RVV-X is a unique 79 kDa heterotrimeric glycoprotein containing a disintegrin and metalloproteinase (ADAM)-like heavy chain (57.6 kDa) and two heterogenous C-type lectin-like light chains (19.4 kDa and 16.4 kDa), which are covalently held together by disulfide bonds (Kisiel et al. 1976; Takeya et al. 1992; Gowda et al. 1994; Takeda et al. 2012). RVV-X contains 13 % carbohydrate by mass which includes 6.0 % hexose, 1.7 % N-acetylneuraminic acid, and 5.3 % galactosamine (Kisiel et al. 1976). On the basis of its structural arrangements, RVV-X has been classified under P-IIIId class of SVMPs. Crystallographic studies of RVV-X indicated that RVV-X adopts a “hook-spanner-wrench”-like structure; the metalloproteinase/disintegrin portion constitutes a mobile “hook,” whereas the lectin-like domains and the rest of the heavy chain comprise the “handle” (Takeda et al. 2012). The lectin-like domains form an intertwined dimer with high structural similarity to anticoagulant factor X-binding proteins (Takeda et al. 2012). Recently, the crystal structure of RVV-X has been elaborated by Takeda and his colleagues (Takeda et al. 2012) (Fig. 1). It has been postulated that the structure of RVV-X displays a 6.5 nm separation between the catalytic zinc atom and a putative gamma carboxyl glutamic acid (Gla) domain-binding exosite, which are involved in factor X activation by RVV-X (Takeda et al. 2012).

RVV-X, a potent activator of factor X (Furie and Furie 1976), specifically activates factor X via a single cleavage at the same internal Arg¹⁹⁴-Ile¹⁹⁵ bond in factor X as done physiologically by factors IXa and VIIa during the process of blood coagulation (Furie et al. 1974). Activation of factor X by RVV-X is not inhibited by serine proteinase inhibitors, viz., diisopropyl fluorophosphate (DIFP) and phenylmethylsulfonyl fluoride (PMSF), but significantly inhibited by metal chelator, viz., ethylene diamine tetra acetic acid (EDTA), thereby suggesting that RVV-X is not a serine proteinase but a metalloproteinase (Furie and Furie 1976). Moreover, activation of factor X by RVV-X is strictly dependent on the presence of Ca²⁺-ions. RVV-X does not show esterase activity toward benzoyl-Phe-Val-Arg p-nitroanilide or benzoylarginine ethyl ester and is highly specific for factor X (Furie and Furie 1976). Neutralization study of RVV-X with commercial anti-RV antivenom revealed that the factor X activator activity of purified RVV-X toward its specific chromogenic substrate (S-2765) could be completely neutralized when taken in the ratio of 250:1 (w/w) (Suntravat et al. 2011). Moreover, RV antivenom could also effectively prevent the D-dimer formation caused by purified RVV-X, under in vivo conditions when injected intravenously prior to RVV-X administration (Suntravat et al. 2011). Further, no apparent histopathological changes in kidneys and lungs were observed in rats receiving RV antivenom along with purified RVV-X (Suntravat et al. 2011).

Fig. 1 Ribbon structure of RVV-X in stereo. Bound calcium and zinc ions are represented by black and red spheres, respectively. The carbohydrate moieties (in green) linked to asparagine residues and GM6001 (in magenta) are shown in ball-and-stick representations (Image courtesy: Takeda et al. 2007; Reproduced with permission from the publisher)



VRR-73

VRR-73, a hemorrhagic metalloproteinase specific to the skin and muscle, was isolated from venom of RV of Eastern Indian origin by Chakrabarty and his colleagues in 2000. VRR-73 contains Mg^{2+} in a mole per mole ratio in contrast to a large number of hemorrhagins where the associated metal is found to be Zn^{+2} or Ca^{+2} (Meier and Stocker 1995). This 73 kDa metalloproteinase was reported to exhibit strong fibrinolytic activity that is independent of its hemorrhagic activity. Biochemical characterization revealed that VRR-73 did not cause any significant proteolysis of basement membrane proteins such as laminin and collagen, albeit it showed hydrolysis of $N\alpha$ -benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA), a chromogenic substrate for trypsin, with a specific activity of 3,000 U/mg. Trypsin, on the other hand, under the identical experimental conditions showed the specific activity of 6,500 U/mg (Chakrabarty et al. 2000). VRR-73 did not show phospholipase A_2 or any other enzyme activity and appeared to activate plasminogen to produce plasmin which in turn causes fibrinolysis (Chakrabarty et al. 2000). Neutralization study of VRR-73 with commercial bivalent antiserum

revealed that intradermal or intravenous administration of antiserum after 2 min of intravenous administration of VRR-73 to laboratory inbred rats did not show any visible sign of inhibition of VRR-73-induced hemorrhage (Chakrabarty et al. 2000). However, when the antiserum was introduced simultaneously with VRR-73, a complete inhibition of hemorrhagic activity of VRR-73 was observed. This suggested that binding of VRR-73 to its site of action was a very rapid event and it occurred virtually in an irreversible manner (Chakrabarty et al. 2000).

Though the detailed structural and functional analysis of VRR-73 is warranted, studies conducted by Chakrabarty and his colleagues (2000) suggested that VRR-73 is likely to be consisted of multiple domains from where its activities are operated independently. Therefore, by applying reversible thermal denaturation to VRR-73, it is possible to restore its fibrinolytic activity almost completely while losing the hemorrhagic activity permanently (Chakrabarty et al. 2000). This unique property of VRR-73 makes the metalloproteinase a potential candidate for the development of a fibrinolytic agent for the treatment of thrombosis-associated disorders.

VRH-1

Yet another proteinase contributing to the overall toxic effects of RV envenomation is VRH-1 (Chakrabarty et al. 1993). VRH-1 is identified to be a Mg^{2+} containing metalloproteinase which shows organ-specific hemorrhagic property (Chakrabarty et al. 1993). Structurally, VRH-1 has been suggested to be a globular protein of a single polypeptide chain of 22 kDa which is not asymmetric in shape (Chakrabarty et al. 1993). However, further structural details of VRH-1 have not been elucidated till date. This hemorrhagic metalloproteinase was reported to be devoid of esterase activity and does not affect the process of blood coagulation (Chakrabarty et al. 1993). Though the complete characterization of VRH-1 as well as studies pertaining to its neutralization by RV antivenom is still warranted, the contribution of VRH-1 to the overall hemorrhagic effect of RVV cannot be neglected. The hemorrhagic effects of VRH-1 have been discussed in greater detail in a different section (see section “[Pharmacological Properties and Toxicity of RVV Proteinases](#)”).

RVBCMP

Studies on the composition of venom of RV from Eastern India origin has been carried out extensively by Mukherjee et al. (2000). A 15 kDa metalloproteinase named RVBCMP (Russell's viper basic coagulant metalloproteinase) showing hemorrhagic and potent in vitro procoagulant activities was purified and characterized from RVV (Mukherjee 2008). In fact, this is the smallest metalloproteinase reported from RVV. Further, the carbohydrate content of RVBCMP was reported to be about 20 % of total protein mass, which is much higher than the carbohydrate content of the many of the coagulant glycoproteins isolated from RVV (Mukherjee 2008). Inhibition of the procoagulant activity of RVBCMP posttreatment with EDTA resulted in abolition of its proteinase (catalytic), coagulant, as well as liver hemorrhagic activities indicating RVBCMP is a metalloproteinase isolated from RVV (Mukherjee 2008). RVBCMP was reported to be an α -fibrinogenase as it specifically degraded the A α -chain of fibrinogen in addition to showing tissue (liver)

hemorrhagic activity (Mukherjee 2008). Investigations such as neutralization studies with commercial antivenom and medicinal plants revealed that RVBCMP contained distinct perhaps overlapping sites for rendering coagulant and tissue hemorrhagic effects (Mukherjee 2008).

Daborhagins

Two P-III class hemorrhagic metalloproteinases identified from RVV are daborhagin-M and daborhagin-K (Chen et al. 2008). Daborhagin-M was isolated from the RVV of Myanmar origin (*Daboia russelii siamensis*), whereas daborhagin-K was isolated from RVV of Indian origin (*D. r. russelii*). Both daborhagin-M and daborhagin-K are glycosylated zinc metalloproteinases with an apparent molecular mass of 62 and 66 kDa under nonreducing and reducing conditions, respectively (Chen et al. 2008). Peptide mass fingerprinting analysis of daborhagin-M and daborhagin-K suggested that they both had 11 and 23 unique peptides, which matched the predicted sequence from daborhagin-K cDNA (Chen et al. 2008). Further, the N-terminal sequence of daborhagin-M was also reported to be identical to that of daborhagin-K, except for the replacement of the Pro⁶ with Arg⁶ (Chen et al. 2008).

Functionally, both the enzymes were suggested to share common epitopes and they show potent in vitro fibrinogenolytic activity by cleaving the alpha-chain of fibrinogen (Chen et al. 2008). Besides, daborhagin-M also hydrolyzed the beta-chain of insulin at Ala¹⁴-Leu¹⁵ and Tyr¹⁶-Leu¹⁷ followed by His¹⁰-Leu¹¹ and Phe²⁴-Phe²⁵ positions as well as type IV collagen in vitro (Chen et al. 2008). Daborhagin-M has been reported to bind to a single zinc and calcium ion per subunit. Daborhagin-K on the other hand binds to single zinc and two calcium ions per subunit (Chen et al. 2008). Both daborhagins are monomeric proteinases and are readily inhibited by EDTA, ethylene glycol tetra acetic acid (EGTA), and 1,10-phenanthroline (Chen et al. 2008). Addition of Mg²⁺ or Ca²⁺ increased the rate of casein hydrolysis (proteolytic activity) of both these proteinases, suggesting they were activated in the presence of these divalent cations (Chen et al. 2008). However, the neutralization potency of RV antivenom toward these daborhagins has not been reported till date.

RVV Serine Proteinases

Serine proteinases are characterized by the presence of a typically highly reactive “catalytic triad” in their active site, which comprises of serine¹⁹⁵, histidine⁵⁷, and aspartic acid¹⁰² (Barrett and Rawlings 1995). The catalytic triad is a highly conserved and coordinated structure. In the event of enzyme catalysis, each of the key amino acids of the catalytic triad has a specific role to play. The catalysis of a peptide cleavage is characterized as a “ping-pong” catalysis which involves many intermediates. Briefly, the hydroxyl group of serine acts as a nucleophile and attacks the carbonyl carbon of the scissile peptide bond of the target substrate. The histidine nitrogen coordinates with serine by accepting the hydrogen from the hydroxyl group of serine. Finally, the carboxyl group of aspartic acid forms hydrogen bonds with histidine leading to the release of the peptide molecule (Barrett and Rawlings 1995).

Serine proteinases are reported to contribute significantly in the pharmacological attributes of venoms of Viperidae, Crotalidae, Elapidae, and Colubridae families of snakes (Serrano and Maroun 2005). Snake venoms serine proteinases (SVSPs) belong to the MEROPS peptidase family S1, subfamily S1A (chymotrypsin-A subfamily), and are reported to originate from the glandular kallikrein that later progress to obtain coagulation and fibrino(geno)lytic activities (Rawlings et al. 2010). The enzymatic properties of these proteinases are influenced significantly by the peptide moiety adjacent to the scissile bond in their substrates. It has also been elaborated that SVSPs illustrate rigid macromolecular substrate specificity in contrast to the lesser specific activity of trypsin (Serrano and Maroun 2005). Despite possessing a high degree of similarity in their amino acid sequences and three-dimensional structures, SVSPs exhibit different substrate specificity and biological functions (Serrano and Maroun 2005; Mukherjee and Mackessy 2013; Mukherjee 2014). It has been suggested that a relatively minor surface residue changes can result in significant differences in the preference for hydrolyzing a particular macromolecular substrate by SVSPs (Serrano and Maroun 2005; Mukherjee and Mackessy 2013). Furthermore, the degree of glycosylation in SVSPs also plays a significant role in modulating the enzyme activity, stability, and protection of these SVSPs against neutralization by host endogenous proteinase inhibitors (Serrano and Maroun 2005; Mukherjee and Mackessy 2013; Mukherjee 2014). Single venom may contain several proteinase isoenzymes which are encoded by multigene family and during the process of evolution; duplication followed by variation of a single ancestral gene resulted in formation of several proteinase isoenzymes in single venom (Deshimaru et al. 1996). Majority of the SVSPs have been reported to affect the hemostasis of victim/prey owing to their procoagulant or anticoagulant nature in addition to demonstrating proteolytic cleavage of fibrin (ogen), kininogen, factor V, protein C, or plasminogen as well as platelet aggregation (Matsui et al. 2000; Kini 2005; Serrano and Maroun 2005; Mukherjee and Mackessy 2012, 2013).

Though SVSPs are abundant in Viperidae venoms, not many serine proteinases from RVV have been identified so far, and there is still a dearth of knowledge on different proteinases present in RVV. In fact, only a few of serine proteinases have been functionally characterized from RVV, the properties of which are discussed below.

RVV-V

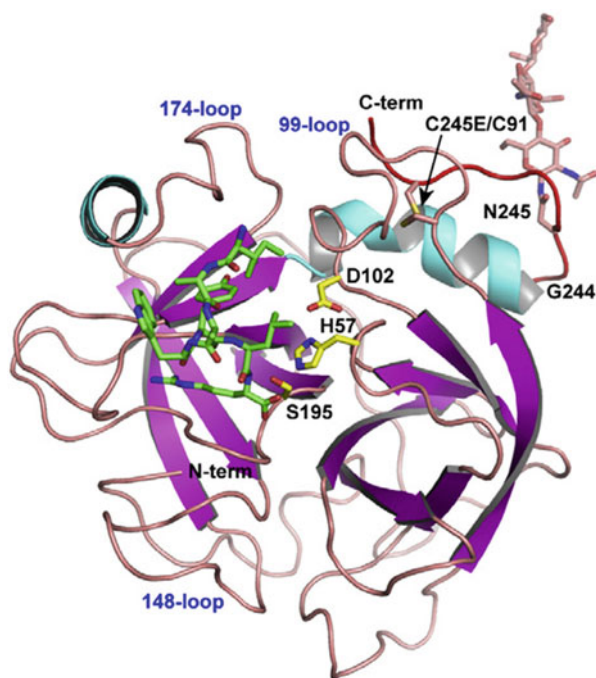
One of the earliest identified serine proteinases from RVV (of unknown geographical region) is a thrombin-like factor V-activating proteinase, RVV-V (Kisiel et al. 1976; Tokunaga et al. 1988). RVV-V was purified to homogeneity and was characterized to be a monomeric glycoprotein containing 6 % carbohydrate by mass with an apparent molecular weight of 29,000 Da (Kisiel et al. 1976; Kisiel and Canfield 1981). RVV-V is made up of 236 amino acids and is found to be abundant in RVV as three different isoforms designated as RVV-V α , RVV-V β and RVV-V γ (Tokunaga et al. 1988). The enzyme is composed of a single polypeptide chain possessing an amino-terminal sequence of Val-Val-Gly-Gly-Asp-Glu-Cys-Asn-Ile-Asn-Glu-His-Pro-Ile. Tokunaga and his colleagues (1988) reported that the sequences of the RVV-V

isoproteins exhibited 62 % identity with that of batroxobin, a thrombin-like enzyme present in *Bothrops atrox* venom (Itoh et al. 1987), and 33 % identity with that of human thrombin B-chain. The structural difference between RVV-V and other trypsin-like serine proteinases was also elaborated (Tokunaga et al. 1988). It has been suggested that the conservative Ser²¹⁴-Trp²¹⁵-Gly²¹⁶ sequence, which is considered as the site of antiparallel β -sheet formation between the protein substrate, in most of the serine proteinases, is replaced by the corresponding sequence Ala-Gly-Gly in RVV-V (Tokunaga et al. 1988).

Recently, the structural basis of coagulation factor V recognition for cleavage by RVV-V was shown by Nakayama et al. (2011) (Fig. 2). In the study conducted by Nakayama and his colleagues (2011), the crystal structure of RVV-V in complex with the FV14 peptide (residues 1533–1546 of human factor V) revealed multiple interactions between RVV-V and the seven residues, Ile¹⁵³⁹ (P7)–Arg¹⁵⁴⁵ (P1), of the cleaved substrate (Nakayama et al. 2011). A comparison with substrate-free structures revealed conformational changes of the RVV-V loops upon substrate binding, suggesting that the multiple interactions were mediated by an induced-fit mechanism (Nakayama et al. 2011). These results provided an explanation for the narrow substrate specificity of RVV-V (Nakayama et al. 2011).

RVV-V activates factor V to the same extent as the physiological enzyme α -thrombin and mediates factor V activation by a single peptide bond cleavage at Arg¹⁵⁴⁵–Ser¹⁵⁴⁶ bond in factor V, generating the heavy and light chains of factor Va (Tokunaga et al. 1988), unlike α -thrombin which results in the cleavage of three

Fig. 2 Structure of the RVV-V/ factor V14 (residues 1533–1546 of human FV) peptide complex. The FV residues Ile¹⁵³⁹–Arg¹⁵⁴⁵ and the side chains of the active site residues in RVV-V are shown in green and yellow, respectively. RVV-V has a unique C-terminal nine-residue extension (residues 244, 245, and 245A–245G shown in red) that contains an extra disulfide bond (Cys91–Cys245E) and an N-glycosylation site linked to Asn245, which is not found in thrombin or other mammalian serine proteinases (Image courtesy: Nakayama et al. 2011; Reproduced with permission from the publisher)



peptide linkages in factor V releasing a large connecting fragment (Segers et al. 2006; Suzuki et al. 1982). RVV-V demonstrated a narrow range of specificity with no apparent proteolytic activity toward other coagulation factors such as fibrinogen, plasminogen, prothrombin, factor VIII, and factor XIII (Segers et al. 2006). It is reported that RVV-V exhibits arginine esterase activity and is sensitive to the serine proteinase inhibitor diisopropyl fluorophosphates (Tokunaga et al. 1988). RVV-V was also inhibited by D-Phe-Pro-Arg-chloromethyl ketone (FPRCK) (97 %), PMSF (76 %), and benzamidine (50 %) but remained unaffected by bovine pancreatic trypsin inhibitor (BPTI), antithrombin, and EDTA (Segers et al. 2006). RVV-V has established immense biomedical application and is used for the routine assay of factor V by virtue of its selective activation of factor V as well as for studying the structural aspects of factor V. However, the in vivo pharmacological property of RVV-V is yet to be characterized.

Russelobin

Recently, another thrombin-like serine proteinase named Russelobin has been identified from *Daboia russelii russelii* venom of Pakistan origin (Mukherjee and Mackessy 2012, 2013). This 51.2 kDa glycosylated, monomeric protein is a new thrombin-like enzyme from RVV which has a molecular mass greater than the typical masses of most of the thrombin-like serine proteinases purified from snake venom (Mukherjee and Mackessy 2012, 2013). The N-terminal amino acid sequence of Russelobin was reported to be VVGGECDNINEHRSL which showed 87–100 % sequence homology with snake venom thrombin-like enzymes. Peptide mass fingerprinting (PMF) analysis of Russelobin clearly demonstrated the novelty of the thrombin-like serine proteinase from RVV. Studies conducted by Mukherjee and Mackessy (2013) suggested that Russelobin shares considerable sequence homology with a serine β -fibrinogenase-like protein precursor (28 kDa) from *D. r. siamensis* venom although Russelobin is predominantly α -fibrinogenase.

Russelobin was reported to be extensively glycosylated, because of which it remains protected from endogenous proteinase inhibitors such as α_2 -macroglobulin, thereby demonstrating prolonged in vivo efficacy (Mukherjee, and Mackessy 2012, 2013). Russelobin was found to be significantly inhibited by thrombin inhibitors such as heparin and weakly inhibited by antithrombin-III unlike other thrombin-like snake venom proteinases such as batroxobin, cerastocytin, cerastotin, and contortrixobin (Mukherjee and Mackessy 2013). The difference in the active site of Russelobin compared to other SVSPs was more prominent due to the fact that Russelobin was not inhibited by tosyl phenylalanyl chloromethyl ketone (TPCK) and tosyl lysine chloromethyl ketone (TLCK) (Mukherjee and Mackessy 2013), whereas other SVSPs were inhibited by TPCK as well as TLCK (Marrakchi et al. 1997; Amiconi et al. 2000). Additionally, Russelobin hydrolyzed the insulin B-chain in a slow rate, showing that venom serine proteinases hydrolyze insulin B-chain much more slowly than venom metalloproteinases. Russelobin demonstrated preferential slow rate of hydrolysis of insulin B-chain at Val¹⁵ and Leu¹⁶ and at several other minor cleavage sites which are mostly not shown by other venom proteinases (Mukherjee and Mackessy 2013). In view of its distinct cleavage

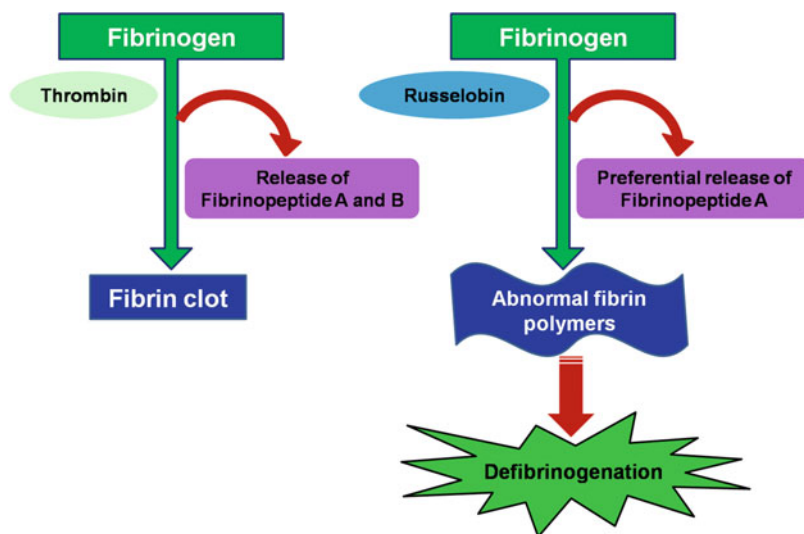


Fig. 3 Proposed mechanism of action of the thrombin-like enzyme Russelobin purified from RVV and comparison of its activity with that of physiologically active enzyme thrombin

sites in insulin B-chain, Russelobin appears to be distinct from most of the other venom serine proteinases reported till date.

Russelobin demonstrated a high degree of selectivity toward fibrinogen without detectable proteolysis of other plasma proteins, suggesting that fibrinogen is the primary physiological substrate for Russelobin. Russelobin hydrolyzed both the α - and β -chain of fibrinogen without releasing any detectable quantities of either fibrinopeptide A or B at a concentration of 40 nM Russelobin (Mukherjee and Mackessy 2013). However, at a concentration higher than this, Russelobin showed dose-dependent preferential release of fibrinopeptide A from fibrinogen (Fig. 3). The release of fibrinopeptide B from fibrinogen by Russelobin was also reported however, to a much slower rate, and the γ -chain of fibrinogen remained intact (Mukherjee and Mackessy 2013). Russelobin is therefore found to be an α - β -fibrinogenase with high specificity toward fibrinogen, both in vitro and in vivo.

In addition to possessing in vivo defibrinogenolytic property, Russelobin was also found to be nontoxic and nonlethal in experimental mice (Mukherjee and Mackessy 2013). Taken together, Russelobin may be ventured for the treatment of hyperfibrinogenemia-associated diseases such as vascular ailments. Further characterization and structural analysis of Russelobin will throw light valuable for therapeutic applications of this RVV serine proteinase.

RV-FVP

Of late, four new RVV serine proteinase isoenzymes, namely, RV-FVP α , RV-FVP β , RV-FVP γ , and RV-FVP δ of molecular masses corresponding to 32901.044 Da, 333631.179 Da, 333571.472 Da, and 34594.776 Da, respectively,

have been characterized from RVV (Mukherjee 2014). The N-terminal residues of these isoenzymes were shown to have significant sequence homology with the N-terminal sequences of snake venom thrombin-like and factor V-activating serine proteinases. Interestingly, these proteinase isoenzymes were reportedly different from previously identified factor V activators isolated from snake venoms. These proteinases demonstrated significantly different fibrinogenolytic, BAEE-esterase, and plasma clotting activities, but they did not show fibrinolytic, TAME-esterase, or amidolytic activity against the chromogenic substrate for trypsin, thrombin, plasmin, and factor Xa. On the basis of the fibrinogen degradation pattern, these procoagulant isoenzymes have been classified as $\alpha\beta$ -fibrinogenases.

These RVV isoenzymes were found to be extensively glycosylated with 42–44 % of N-linked carbohydrates by mass. The partially deglycosylated enzymes showed significantly less catalytic activity as compared to native enzymes. As such, the studies suggested that the varying degree of glycosylation tends to play a significant role in physiological substrate recognition and/or enhancement of catalytic activity of the proteinase isoenzymes. In vitro studies suggested that the RV-FVP isoenzymes induced blood coagulation through factor V activation, whereas in vivo RV-FVP isoenzymes provoked dose-dependent defibrinogenation and anticoagulant activity in the mouse model (Mukherjee 2014). Moreover, it was shown that monovalent antivenom against RVV exerted dose-dependent neutralization of the proteinase, esterase, and plasma clotting activity of RV-FVP isoenzymes (Mukherjee 2014).

Pharmacological Properties and Toxicity of RVV Proteinases

RVV proteinases and their isoforms are the key components likely to be responsible for generating hemostatic alterations and severe tissue hemorrhage. The various RVV proteinases identified so far (Table 1) are shown to affect the mammalian coagulation cascade at different steps (Fig. 4). On the basis of their effect on the coagulation pathway, RVV proteinases are found either to prolong the clotting time of blood (procoagulant nature) or they exert anticoagulant activity by inhibition of blood coagulation (Table 1). Besides, RVV metalloproteinases are found to affect the basement membrane proteins of different tissues, thereby leading to microvessel damage and symptoms such as tissue necrosis, internal tissue, and dermal hemorrhage (Chakrabarty et al. 1993, 2000). As reported by Suwansrinon and his colleagues (2007), RVV fractions are known to affect the systemic and renal hemodynamics in dogs. They suggested that the changes in hemodynamics were possibly caused by the presence of some proteolytic enzymes in the RVV although the RVV proteinase(s) actually responsible for this pharmacological effect was not identified by them. Later, it was reported that RVV-X is the key component that causes renal dysfunction and coagulopathy in experimental animals (Suntravat et al. 2011). Changes in renal hemodynamics and renal functions such as decreased glomerular filtration rate (GFR), effective renal plasma flow (ERPF), effective renal

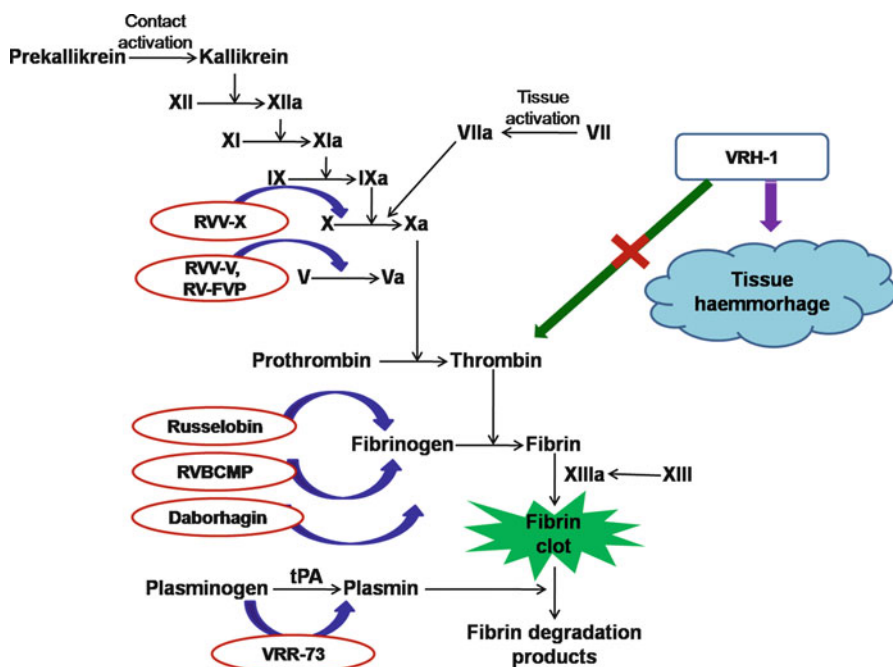


Fig. 4 Diagrammatic representation of RVV proteinases affecting the blood coagulation cascade

blood flow (ERBF), and increased renal vascular resistance (RVR) suggested the formation of renal lesions due to RVV-X treatment in experimental animals (Suntravat et al. 2011). In addition to renal dysfunctions, RVV-X-treated animals also demonstrated intravascular clots and increased D-dimer level (Suntravat et al. 2011). Also, a synergistic interaction between RVV-X and a trypsin inhibitor was reported to significantly increase the toxicity of RVV-X (Jayanthi and Gowda 1990).

In addition to RVV-X, other metalloproteinases from RVV are also reported to demonstrate appreciable tissue damage and induce hemorrhage in experimental animals (Chakrabarty et al. 1993, 2000; Chen et al. 2008). For example, VRR-73 showed a minimum hemorrhagic dose (MHD) of 0.5 mg in mouse skin as compared to 5.0 mg for whole RVV (Chakrabarty et al. 2000). VRR-73 produced hemorrhagic spots in the abdominal muscle wall and adjacent skin near the site of intradermal introduction with very little hemorrhage in the peritoneal cavity or in the intestine (Chakrabarty et al. 2000). Systemic hemorrhage was not detected in animals injected with VRR-73 (both i.p. and i.v.), and the LD₅₀ of VRR-73 was calculated to be 2.5 mg/20 g in mouse (Chakrabarty et al. 2000). Another metalloproteinase, VRH-1, was also reported to exert tissue-specific hemorrhage and caused severe lung

hemorrhage upon intradermal administration in mice with little hemorrhagic effect on the skin. It was also postulated by Chakrabarty and his colleagues (1993) that VRH-1 exerted its hemorrhagic effects as a result of an intermediate formation which was otherwise not recognized by anti-whole RVV antiserum, thereby the difficulty to neutralize this toxin (Chakrabarty et al. 1993).

Pharmacological characterization of the procoagulant metalloproteinase RVBCMP suggests that the identified proteinase lacks edema-inducing properties and is nonlethal and nontoxic to experimental mice (~30 g) at a dose of 5.0 µg (Mukherjee 2008). In view of the pharmacological properties of RVBCMP, it is suggested that RVBCMP contributes to consumption coagulopathy in treated mice and is likely to play a significant role in RV envenomings. The other metalloproteinases, daborhagin-M and daborhagin-K are also reported to display high levels of hemorrhagic activity with a MHD of 0.86 µg and 0.82 µg, respectively, when subcutaneously injected into mice (Chen et al. 2008). It has been suggested that the RVV from Myanmar contains six to seven times more daborhagin than that from Eastern India (Chen et al. 2008).

None of the serine proteinases identified from RVV are accounted to be lethal in nature. RVV-V exerts its procoagulant effect by cleaving FV to FVa which in turn accelerates prothrombin activation by FXa (Tokunaga et al. 1988). As reported by Nakayama et al. (2011), RVV-V undergoes substantial changes in the substrate specificity pockets but only minimal changes both in the catalytic triad and in the oxyanion hole upon substrate/inhibitor binding (Nakayama et al. 2011). Their results suggest that the catalytic activity of RVV-V is regulated by induced-fit substrate binding mechanism which explains the narrow specificity of RVV-V toward its substrates (Nakayama et al. 2011).

Another nonlethal, factor V-activating proteinase, RV-FVP and its isoenzymes have been reported to demonstrate in vitro procoagulant effects but in vivo anticoagulant effects by virtue of its fibrinogenolytic and factor V-activating properties (Mukherjee 2014). The studies on RV-FVP clearly suggested the suitability of these isoenzymes for clinical application as cardiovascular drugs (Mukherjee 2014). Russelobin on the other hand is an anticoagulant SVSP which does not show in vivo lethality and toxicity in NSA strain experimental mice or house geckos at a dose of 5.0 mg/kg (Mukherjee and Mackessy 2013). The lack of histologically detectable changes to several tissues and lethality or cytotoxicity on mammalian cells underpins the nontoxic nature of Russelobin supporting its suitability for clinical applications (Mukherjee and Mackessy 2013). Studies conducted by Mukherjee and Mackessy (2013) showed that Russelobin forms a tenuous clot by preferentially releasing fibrinopeptide A and slowly releasing fibrinopeptide B from fibrinogen leading to formation of non-crossed linked, labile (soft) clot which is easily degraded by plasmin. This indicates that Russelobin is a potent defibrinogenating agent in in vivo, possibly contributing to uncontrolled bleeding seen after RV envenomation (Mukherjee and Mackessy 2013).

Biomedical Applications of Russell's Viper Venom Proteinases

Not many RVV proteinases have established themselves in the field of therapeutics and diagnostics (Table 2). However, RVV-X (see section “RVV-X” for details) and RVV-V (see section “RVV-V” for details) are two milestones isolated from RVV which have carved a niche for themselves in the field of diagnostic applications. Both RVV-V and RVV-X are marketed by Pentapharm Ltd. (Basel, Switzerland) and are regularly used to diagnose factor-deficient blood for routine assay of coagulation factors and as diagnostic reagents for studying both coagulation factors and hemostasis in general.

RVV-V (Pefakit[®] Pentapharm) is used in the laboratory for the estimation of level of factor V in human plasma (Kisiel and Canfield 1981). In addition, RVV-V also serves as a useful tool in the study of factor V activation (Marsh and Williams 2005). RVV-V is also used for studying the resistance of factor Va inactivation by activated protein C caused by the factor V Leiden mutation (Marsh and Williams 2005).

Like RVV-V, RVV-X is also used extensively in the diagnosis of quantitative conversion of the zymogen factor X into factor Xa (Marsh and Williams 2005). The enzyme has become widely used for determination of factor X as well as for distinguishing between factor VII and FX deficiency in plasma (Quick 1971) and lupus anticoagulant assay (Thiagarajan et al. 1986). The clotting time of plasma using RVV-X is known as the Stypven timeTM. A normal Stypven time used in conjunction with a prothrombin time (PT) suggests FVII deficiency, whereas a prolonged Stypven time indicates FX deficiency in the plasma (Quick 1971). RVV-X enzyme is also used to assay the platelet factor 3 (Jy et al. 1995), and this may perhaps have some advantages over the routine coagulation tests in discriminating between thrombotic patients and healthy controls (Jy et al. 1995). In view of the exclusive characteristics, RVV enzymes and proteinases have been used

Table 2 Diagnostic application of RVV proteinases

Name of proteinase	Application(s)	Marketed by	References
RVV-V	Measurement of factor V level in plasma study of structural aspects of factor V	Pentapharm	Kisiel (1979) Tokunaga et al. (1988)
RVV-X	Measurement of factor X level in plasma to assay platelet factor 3	Pentapharm	Farie and Farie (1976) Takeya et al. (1992) Jy et al. (1995)
VRR-73	Probable application as a fibrinolytic agent for the treatment of thrombotic disorders	—	Chakrabarty et al. (2000)
Russelobin	Probable application as a defibrinating agent for the treatment of cardiovascular disorders	—	Mukherjee and Mackessy (2012, 2013)

regularly for the improvement in the detection of von Willebrand disease (Marsh and Williams 2005).

Therapeutic defibrinogenation with snake venom thrombin-like enzymes has been endeavored to treat peripheral arterial occlusion diseases in which surgical revascularization could not be satisfactorily achieved (Furukawa and Ishimaru 1990). Potential nontoxic thrombin-like enzymes from RVV such as Russelobin can be considered effective candidate for application as a clot buster. Under controlled clinical setting, Russelobin is likely to result in defibrinogenation which would benefit in the treatment of thrombus and cancer-associated pathologies such as deep vein thrombosis, myocardial infarctions, and other cardiovascular disorders. In view of the nontoxic nature of Russelobin, the therapeutic application of this proteinase is a welcome measure to deal with hyperfibrinogenemia-associated disorders and vascular ailments (Mukherjee and Mackessy 2012, 2013).

Conclusions and Future Directions

RVV has always charmed researchers across the globe in terms of its diverse array of components. Proteinases are major components of RVV, and their molecular and biochemical characterizations have thrown immense light on understanding of their mechanism of pathogenesis in patients after RV envenomation. However, when the overall lethality of RV envenomation is concerned, there is still a gap in the knowledge on all the proteinase isoforms in RVV. In addition, understanding the mechanism of interaction of these proteinases with other components from the same venom and their structure-function relationships and identifying their target site as well as in vivo pharmacological properties can help researchers as well as clinicians in developing better antivenin strategies. This may reduce the chances of mortality and avert the morbidity generated as a consequence of RV envenomation. Further studies on RVV proteinases will also help in identification of potential diagnostic and therapeutic agents which might be developed in the near future to resolve many hardcore medical emergencies such as severe thrombosis-associated ailments like cardiovascular diseases.

Cross-References

► [Snake Venom Disintegrins](#)

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Endothelial Cell Toxicity of Vascular Apoptosis-Inducing Proteins from Hemorrhagic Snake Venom

7

Satohiko Araki

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Abstract

Vascular apoptosis-inducing protein (VAP) is a protein family in hemorrhagic snake venom which induces apoptosis in vascular endothelial cells. It belongs to snake venom metalloprotease (SVMP) and therefore to a disintegrin and metalloprotease (ADAM) family. There is a large amount of VAPs in hemorrhagic snake venom. In addition to their hemorrhagic activity, VAPs have some special activities on vascular endothelial cells including the apoptosis-inducing

S. Araki (✉)

Department of Science, Nagoya University, Nagoya, Japan

e-mail: saraki@bio.nagoya-u.ac.jp

effect. One of the special activities is cell fragmentation in apoptosis. VAPs also induce detachment of cell-cell contacts and morphological changes with protrusion in vascular endothelial cells. Although the receptor or target molecule of VAPs in endothelial cells is not well known, VAPs are supposed to have some important functions against cells. VAPs may play roles in resolving the hemorrhagic snake venom mechanism, as well as resolving the vascular endothelial cell functions in mammalian body.

Keywords

Snake venom • Hemorrhage • Endothelial cell • Metalloprotease • ADAM

Introduction

In snake venom, there are many cell effectors for which the mechanisms of action are unknown, such as the cytotoxic phospholipases (Lomonte and Rangel 2012; Gutiérrez and Lomonte 2013). Vascular apoptosis-inducing protein (VAP), a metalloprotease-type cell effector, is also one of the effectors for which the action mechanism to cell is unknown. VAPs are defined by their apoptosis-inducing activity on vascular endothelial cells (Masuda et al. 1997, 1998). And VAPs have hemorrhagic activity (Paine et al. 1992; Kikushima et al. 2008). However, the target molecule of the cells is not well known. VAPs have some special activities on vascular endothelial cells including the apoptosis-inducing effect. One of the special activities is cell fragmentation activity in apoptosis (Maruyama et al. 2005). The activity is seldom seen in general physiologically active substances. Additionally, it is interesting that the amount of VAPs is large in hemorrhagic snake venom (Calvete et al. 2009). From these things, VAPs are expected to have some important function against cells. VAPs belong to a disintegrin and metalloprotease (ADAM) family (Wolfsberg et al. 1995; Masuda et al. 1997). Although mammalian ADAMs are considered to be involved in various intercellular signaling, the target molecules of most ADAMs on the cells are unknown (Edwards et al. 2008; Nyren-Erickson et al. 2013; Tripathi et al. 2014). Also for understanding cell signaling of mammalian ADAM, the cellular effects of snake toxin VAP are very interesting.

Family of VAP

Various snake venom metalloproteases (SVMPs) are reported to induce apoptosis on vascular endothelial cells (Masuda et al. 1997). Most are derived from hemorrhagic snake venom. The difference of primary or tertiary structure between SVMPs with and without VAP activity is unknown. Disulfide-linked dimer types of full-length SVMP have often been reported as VAP; however, single molecule types and small molecule types have also been reported (Table 1).

Table 1 SVMP with apoptosis-inducing activity on vascular endothelial cells

Toxin	Origin	Reference	Type ^a
VAP1	<i>Crotalus atrox</i>	Masuda et al (1997)	P-IIIb
VAP2	<i>Crotalus atrox</i>	Masuda et al (1998)	P-III
HV1	<i>Trimeresurus flavoviridis</i>	Masuda et al (2001)	P-IIIb
Graminelysin I	<i>Trimeresurus gramineus</i>	Wu et al (2003)	P-I
Halysase	<i>Gloydius halys</i>	You et al (2003)	P-IIIb
Agkistin	<i>Agkistrodon halys</i>	Wang et al (2003)	P-III
VLAIP	<i>Vipera lebetina</i>	Trummal et al (2005)	P-IIIb
BaP1	<i>Bothrops asper</i>	Díaz et al (2005)	P-I
Jararhagin	<i>Bothrops jararaca</i>	Schattner et al (2005)	P-III
TSV-DM	<i>Trimeresurus stejnegeri</i>	Wan et al (2006)	P-IIIb
Stejnitin	<i>Trimeresurus stejnegeri</i>	Han et al (2007)	P-II
BnP1	<i>Bothrops neuwiedi</i>	Baldo et al (2008)	P-I
VLH2	<i>Vipera lebetina</i>	Hamza et al (2010)	P-III
VaH3	<i>Vipera ammodytes ammodytes</i>	Sajevic et al (2013)	P-IIIb

^aPrimary structure type is shown with the classification by Fox and Serrano (2005)

Structure of Vascular Apoptosis-Inducing Protein

Primary Structure

VAPs belong to SVMP and therefore to a disintegrin and metalloprotease (ADAM) family with metalloprotease domain, disintegrin domain, and cysteine-rich domain (Takeya et al. 1993; Weskamp and Blobel 1994; Wolfsberg et al. 1995; Masuda et al. 1997). However, some VAPs such as graminelysin I, BaP1, BnP1, and stejnitin lose the cysteine-rich domain and/or disintegrin domain. Many VAPs like VAP1 form homodimer or heterodimer with a sulfhydryl connection and are classified to P-IIIb of SVMPs (Masuda et al. 1997; You et al. 2003; Fox and Serrano 2005). There are also many monomer-type VAPs such as VAP2, agkistin, jararhagin, and VLH2, although some VAPs like VAP2 are observed as a dimer without a sulfhydryl connection in crystal (Masuda et al. 1998; Igarashi et al. 2007) (Fig. 1).

Tertiary Structure

VAP1 is the first protein in which the 3D domain architecture of ADAM was clarified (Takeda et al. 2006). Among VAPs, tertiary structure is determined in VAP1 and VAP2 (Takeda et al. 2006; Igarashi et al. 2007). VAP forms a C-shape, in which each tip is a metalloprotease and cysteine-rich domain (Fig. 2). The hypervariable region (HVR) of the cysteine-rich domain has variable amino acid sequence and chain length among SVMP and ADAM and has some hydrophobic

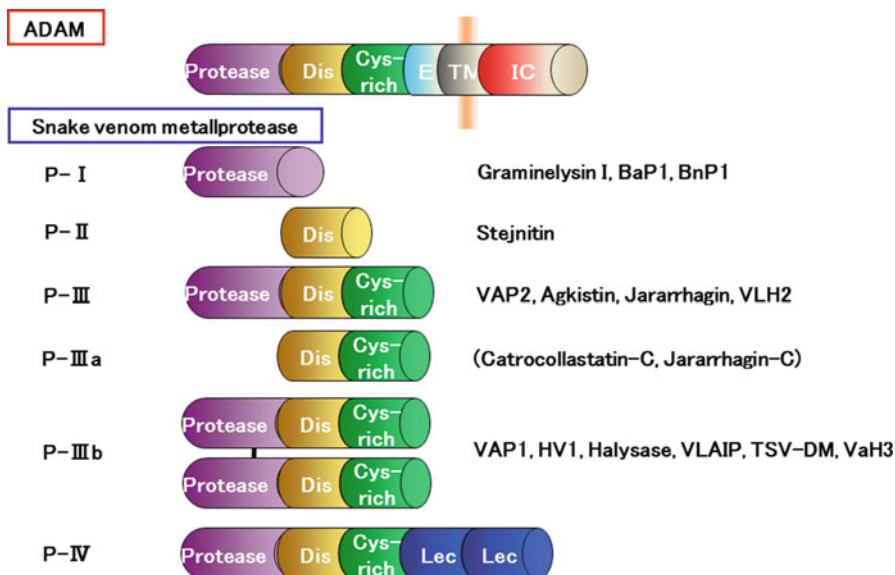


Fig. 1 Primary structure of VAPs. ADAM consists of metalloprotease domain, disintegrin domain, cysteine-rich domain, EGF domain, transmembrane domain, and intracellular domain. SVMP is classified from P-I to P-IV with the domain architecture (Fox and Serrano 2005). P-IIIb is disulfide-linked homodimer or heterodimer of P-III-type peptides

amino acid side chains on its surface. Therefore the region is suggested to be a binding region for each binding target (Takeda et al. 2006; Pinto et al. 2007; Tanjoni et al. 2010).

Biochemical Property of VAP

Basic Characteristics

Almost all VAPs have metalloprotease activity and are inhibited by peptide-mimic inhibitors such as batimastat and GM6001 (Escalante et al. 2000; Díaz et al. 2005; Takeda et al. 2006; Villalta-Romero et al. 2012). Most VAPs are also inhibited by chelating agents like EGTA and 1,10-phenanthroline (Kamiguti et al. 1996; Masuda et al. 1998; Clissa et al. 2001). Protease activity of some VAPs is reported to be inhibited by metalloprotease inhibitor proteins (antihemorrhagic factors) in snake serum such as BJ46a and SSP1-HSF (Valente et al. 2001; Shioi et al. 2013). They are mentioned in section “[Binding Substrates](#)” again. Interestingly, a plasma proteinase inhibitor, alpha2-macroglobulin, is not able to inhibit jararrhagin (Kamiguti et al. 1994a; Escalante et al. 2003). Jararrhagin is reported to be inhibited by triacetyl p-coumarate, an active compound isolated from root bark of *Bombacopsis glabra* vegetal extract (Mendes et al. 2013). Electrostatic character of VAPs is

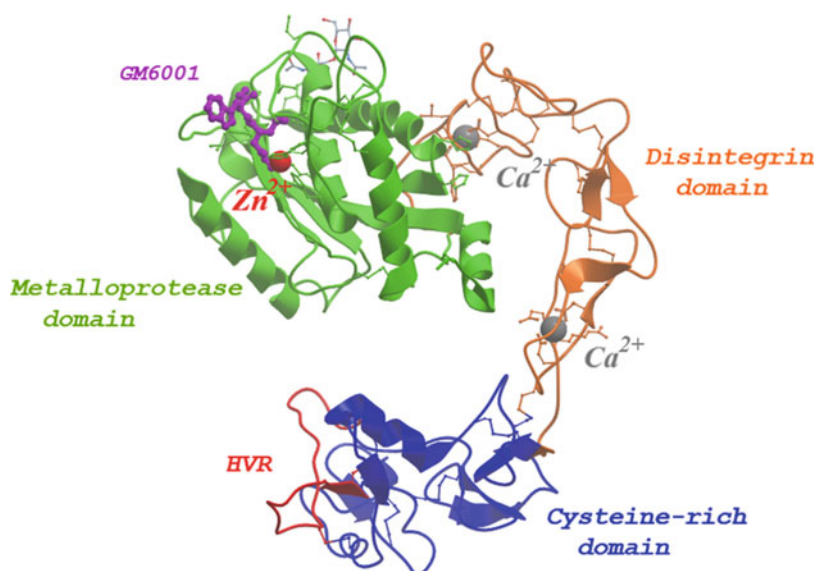


Fig. 2 Tertiary structure of VAP1. VAP consists of metalloprotease domain, disintegrin domain, and cysteine-rich domain. GM6001 is peptide-mimetic inhibitor and indicates substrate-binding pocket. Zn²⁺ ion is the active center of the metalloprotease. Ca²⁺ ions contribute for maintaining the arm structure of disintegrin domain. Hypervariable region (HVR) is the most variable region among the structure of SVMP and ADAM (Takeda et al. 2006)

various. For example, VAP1 is basic and VAP2 is acidic. VAPs are generally stable with heat and pH and not sensitive to self-cleavage (Masuda et al. 1998). However, some VAPs cleave themselves in limited conditions such as alkaline pH and high salt concentration and in the presence of small doses of EGTA or SDS (Moura-da-Silva et al. 2003; Masuda et al. 2007).

Cleavage Substrates

VAPs are reported to have various substrate selectivities from narrow to wide. Most VAPs including VAP1, VAP2, and HV1 cleave fibrinogen alpha chains well like other SVMPs (Kamiguti et al. 1994b; Masuda et al. 1997, 2001; Wu et al. 2003; Trummel et al. 2005; Baldo et al. 2008). They more slowly cleave fibrinogen beta chain. VAPs such as VAP1, VAP2, HV1, and BnP1 show narrow substrate selectivity (Araki et al. 2002; Baldo et al. 2010). They could not cleave type 4 collagen, denatured collagen, or insulin beta chain. VaH3 rapidly cleaved some basement membrane and extracellular matrix (ECM) proteins, such as collagen IV, fibronectin, and nidogen in spite of its narrow proteolytic specificity (Sajevic et al. 2013). Jararhagin also rapidly cleaves collagen IV and fibril collagen (Baldo et al. 2010). It is also reported to cleave integrin beta1 subunit (Kamiguti et al. 1996). VLAIP shows somewhat wide substrate selectivity and cleaves casein and insulin B peptides

(Trummal et al. 2005). VAP2 and jararhagin cleave themselves in limited conditions such as alkaline pH and high salt concentration and in the presence of small doses of EGTA or SDS (Moura-da-Silva et al. 2003; Masuda et al. 2007). And they lose the metalloprotease domain to produce C-terminal peptide consisting of disintegrin and cysteine-rich domains as jararhagin-C and catrocollastatin-C, a truncated form of each jararhagin and VAP2B/catrocollastatin (Usami et al. 1994; Shimokawa et al. 1997; Moura-da-Silva et al. 2003; Masuda et al. 2007). The truncated form exists in great quantity in the snake venom. Catrocollastatin-C represents at least 0.5 % of the total protein in *Crotalus atrox* venom (Shimokawa et al. 1997).

Binding Substrates

Some VAPs are able to bind with collagen (Zhou Q et al. 1996; Moura-da-Silva et al. 2008). The binding of jararhagin to collagens I and IV is suggested to occur through a disintegrin subdomain located opposite to the catalytic domain (Moura-da-Silva et al. 2008). Among SVMs, the collagen-binding activity does not correlate with inhibition activity of collagen-induced platelet aggregation. So the collagen-binding activity is considered to allow accumulation of toxins at the site of injection to induce a local hemorrhage. The cysteine-rich domain and/or disintegrin domain of jararhagin is reported to bind integrin $\alpha 2/\beta 1$, a collagen receptor, and inhibit collagen-induced platelet aggregation (Kamiguti et al. 2003; Tanjoni et al. 2010). On the other hand, metalloprotease domain of jararhagin and its small peptide also reported to bind to integrins $\alpha 2$ I-domain and $\alpha 1$ I-domain (Ivaska et al. 1999; Nymalm et al. 2004; Lambert et al. 2008). VAP1 action against endothelial cells is inhibited by antibodies against integrins $\beta 1$, $\alpha 3$, and $\alpha 6$ (Araki et al. 2002). It is well known that disintegrins or its disintegrin loop peptides interact specifically with particular integrins (Kini and Evans 1992; Blobel and White 1992; Jia et al. 1997; Zhu et al. 2000). However, in the full-length SVM, the disintegrin loop is packed by the cysteine-rich domain in the tertiary structure (Takeda et al. 2006). Therefore, the disintegrin loop is inaccessible for binding to integrins. It is interesting that VAPs nevertheless interact with integrins. Surprisingly, catrocollastatin-C is reported to bind to the VWA domain of von Willebrand Factor (vWF) (Serrano et al. 2006, 2007). Integrin I-domain is a member of the VWA domain family. Jararhagin is also suggested to bind to VWA domain because catrocollastatin-C inhibits jararhagin ability to cleave vWF (Serrano et al. 2007). The VWA binding site of jararhagin is mapped near HVR in the cysteine-rich domain (Pinto et al. 2007). Furthermore, they also bind to collagen XIV, collagen XII, and matrilins 1, 3, and 4 which have VWA domain (Serrano et al. 2006, 2007). Because cobra toxin kaouthiagin and human ADAMTS-13 are known to bind VWA domain of vWF, it is hypothesized that certain members of the expanded ADAM family target VWA domain-containing proteins (Hamako et al. 1998; Matsui and Hamako 2005; Dong et al. 2003; Serrano et al. 2006). HV1 binds to small serum protein-1 (SSP1) derived from the serum of *Trimeresurus flavoviridis* (Aoki et al. 2007b; Shioi et al. 2013). Small serum proteins (SSPs), belonging to

antihemorrhagic factor in the snake serum, are associate proteins of habu serum factor (HSF) which is a protease inhibitor (Omori-Satoh et al. 1972; Yamakawa and Omori-Satoh 1992; Deshimaru et al. 2005). So the complex of SSP1 and HSF inhibits the protease activity and apoptosis-inducing activity of HV1 (Shioi et al. 2013). SSP1 alone or HSF alone does not inhibit HV1. It is suggested that HSF binds to the catalytic region, while SSP1 binds to an exosite like the HVR of HV1 (Aoki et al. 2007a; Shioi et al. 2013). Antihemorrhagic factor BJ46a is able to bind and inhibit jararhagin (Valente et al. 2001). It indicates BJ46a binds to jararhagin at the catalytic site directly. Jararhagin also binds antihemorrhagic factors DM40, DM43, and PO41 derived from the serum of opossums which are resistant to snake venom (Neves-Ferreira et al. 2000; Jurgilas et al. 2003). In mouse serum, jararhagin is reported to bind some high molecular proteins (Escalante et al. 2003).

Cell Biological Activity of VAP on Endothelial Cells

Apoptosis-Inducing Activity

Crude hemorrhagic snake venoms induce apoptosis in cultured vascular endothelial cells, while crude neurotoxic snake venoms did not induce apoptosis, but necrosis in higher concentration (Araki et al. 1993). VAPs are purified with the apoptosis-inducing activity from hemorrhagic snake venom. VAPs induce apoptosis, cell death with DNA fragmentation, and apoptotic bodies (Masuda et al. 1997). The apoptosis occurs within periods of from 30 min to 2 days. Caspase 3 and some caspases are activated (Wu and Huang 2003; You et al. 2003; Díaz et al. 2005; Maruyama et al. 2005; Tanjoni et al. 2005). 1 µg/ml of VAP1 is able to induce apoptosis in vascular endothelial cells such as human umbilical vein endothelial cell and human aortic endothelial cell. However, the immortalized endothelial cell line ECV-304 is resistant (Masuda et al. 1997; Wan et al. 2006). Human skin fibroblasts and rat smooth muscle cells and some cell line cells are also resistant (Masuda et al. 1997). Cell death is inhibited by a protein synthesis inhibitor (cycloheximide, Masuda et al. 1997) and a phospholipase inhibitor (D609, Zhao et al. 2004). Several pro-inflammatory genes such as E-selectin, VCAM-1, IL-8, CD69, Ang-2, and MMP-10 are reported to be induced in jararhagin-induced apoptosis (Lopes et al. 2012). And the genes of Fas ligand/TNF-alpha receptor apoptotic pathway are upregulated (Gallagher et al. 2003). However, the mechanism in inducing apoptosis has not been clarified. The receptor of VAPs is unknown. In apoptosis induced by some VAPs, it has been suggested that the cell detachment from the extracellular matrix (ECM) is responsible for the apoptosis, and hence the apoptosis is “anoikis” (Díaz et al. 2005; Tanjoni et al. 2005). In other VAPs, there is a morphological difference between the VAP-induced apoptosis and anoikis (Maruyama et al. 2005). In this case, VAP1 does not influence the ECM-attachment ability of endothelial cells nor collagen coated on the dish (Araki et al. 2002). So there may be another apoptosis-inducing mechanism than anoikis.

Cell Fragmentation-Inducing Activity

VAP1 induces severe cell fragmentation in endothelial apoptosis (Masuda et al. 1997; Maruyama et al. 2005). Cell fragmentation in apoptosis is rare in cultured endothelial cells because the cells show round-up shape in apoptosis caused by growth factor deprivation, anoikis, and other factors (Araki et al. 1990; Meredith et al. 1993). In vivo, there are mainly two types of apoptosis, the fragmented type and the round-up type (Yamaguchi et al. 2011). In cultured cells, some cells show the fragmented style and other cells show the round-up style. However, each cell shows one apoptosis style independent of the cause (Orlando et al. 2006). The mechanism of apoptosis type selection is unknown. Interestingly except for VAPs, it is seldom that physiological factor is reported to be able to change the apoptosis morphology type. Then VAPs may induce a unique cell signal. In the fragmented type of apoptosis induced by VAP, caspase inhibitors are able to inhibit cell fragmentation; however, they cannot inhibit the cell death (Maruyama et al. 2005). Protein synthesis inhibitors also inhibit cell fragmentation.

Other Activities on Endothelial Cells

VAPs induce morphological change in vascular endothelial cells in several tens of minutes. The cells show a spindle shape and protrude some pseudopodia (Masuda et al. 2000). VAPs also induce detachment of cell-cell contact (Masuda et al. 2000; Wu et al. 2003). However, whether this is the direct result of cleavage of cell-cell junctional proteins or some other cause is unknown. Graminelysin is reported to cleave and activate matrix metalloprotease type 2 to cleave adherens junction proteins such as VE-cadherin and beta- and gamma-catenin (Wu et al. 2003).

Cell Biological Activity of VAP on the Other Cells

Some VAPs are reported to have inhibition activity of platelet aggregation. B-peptide of VAP2 was first reported as “catrocollastatin,” which inhibits collagen-induced platelet aggregation (Zhou et al. 1995; Masuda et al. 2007). Interestingly, a truncated form which lose the metalloprotease domain, catrocollastatin-C, also has platelet inhibition activity (Shimokawa et al. 1997). Similarly, jararhagin and jararhagin-C are reported to inhibit platelet aggregation with their cysteine-rich domain (Mourada-Silva et al. 1999; Kamiguti et al. 2003). VLAIP also inhibits ADP- and collagen-induced platelet aggregation (Samel et al. 2012). Several mechanisms of the platelet aggregation inhibition are proposed. In a case, VAPs cleave a platelet collagen receptor, integrin $\alpha 2/\beta 1$, to inhibit the platelet aggregation (Kamiguti et al. 1996). And another case, VAPs competitively inhibit collagen binding to platelet receptor integrins by binding to both integrin $\alpha 2$ and collagen (Lambert et al. 2008; Tanjoni et al. 2010). VLAIP induces detachment of PC3, a human

prostate cancer cell line, while it had no significant effect on LNCaP and K-562 cells (Samel et al. 2012). And jararhagin induces morphological alterations in fibroblasts and melanoma cells (Corrêa et al. 2002; Klein et al. 2011; Maria et al. 2014). In this case, jararhagin upregulates genes TP53, CDKN1A, CDKN2A, CASP3, CASP5, CASP6, CASP8, and E-CDH in the melanoma cells and genes ITGB6, ITGB7, CASP3, TP53, and CDKN1B in fibroblasts (Klein et al. 2011). Interestingly, the disintegrin-like and cysteine-rich domain of jararhagin, jararhagin-C, is reported to induce pro-inflammatory response with the leukocyte rolling and release of cytokines (Clissa et al. 2006). There may be some common VAP receptors on endothelial cells and other cells.

Hemorrhagic Activity of VAP

VAPs have hemorrhagic activity against mice (Moura-da-Silva et al. 2003; Kikushima et al. 2008). The hemorrhagic activity of VAPs is moderate in comparison with toxins like HR2b with a strong hemorrhagic activity (Takahashi and Osaka 1970). The minimum hemorrhagic dose of VAP1, VAP2, and jararhagin is 8 µg, 15 µg, and 1.5 µg for intracutaneous injection in mice (Moura-da-Silva et al. 2003; Kikushima et al. 2008). The responsible factor of snake venom-induced hemorrhage is known to be the metalloproteases which belong to a disintegrin and metalloprotease family (ADAM) (Takahashi and Osaka 1970; Takeya et al. 1990, 1993). However, the mechanism of hemorrhage induced by the metalloprotease is not clear. There are several hypotheses concerning the mechanism. One of them is that the metalloprotease dissolves the extracellular matrices of the vascular basement membrane (Gutiérrez et al. 2005; Escalante et al. 2006; Moura-da-Silva et al. 2008; Herrera et al. 2015). Another is that it dissolves the intercellular junction of the vascular endothelial cells (Wu et al. 2003). And the other one is that it stimulates vascular endothelial cells to increase fragility of the vascular system (Gallagher et al. 2003; Clissa et al. 2006; Kikushima et al. 2008; Lopes et al. 2012). Just as there are various anti-hemostatic effects in snake venom, all of the known activity of the metalloprotease may be important. And VAPs may play a role in vascular endothelial cells in the snake venom-induced hemorrhage. Because some VAPs interact with integrins and collagen, their collagen cleavage at the focal adhesion of endothelial cells may cause cell anchorage disruption and vascular fragility (Moura-da-Silva et al. 2008; Baldo et al. 2010, 2015). VAPs induce apoptosis from several hours, whereas they induce hemorrhage from several minutes (Masuda et al. 1997; Kikushima et al. 2008). So the endothelial cell apoptosis may be not involved directly in the initial hemorrhage. On the other hand, VAPs induce cell-cell contact detachment and morphological change in several minutes (Masuda et al. 2000; Wu et al. 2003). Graminelysin is reported to act in concert with cleaved and activated MMP-2 to cause cleavage of adherens junction proteins such as VE-cadherin, beta cadherin, and gamma cadherin (Wu et al. 2003). These effects may be also involved in VAP-induced hemorrhage.

Conclusion

Whereas VAPs have hemorrhagic activity, VAPs show narrow substrate selectivity and unique activity in endothelial cells. Therefore VAPs are expected to have some important function against cells. If the receptor or target of VAPs in the endothelial cells is found in the future, VAPs may play some role in resolving the hemorrhagic mechanism of snake venom, as well as resolving the vascular endothelial cell functions in mammalian bodies.

Cross-References

- ▶ [A Brief Appraisal on Russell's Viper Venom \(*Daboia russelii russelii*\) Proteinases](#)
- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Snake Venom Protease Inhibitors: Enhanced Identification, Expanding Biological Function, and Promising Future](#)

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Snake Venom Protease Inhibitors: Enhanced Identification, Expanding Biological Function, and Promising Future

8

Hidetoshi Inagaki

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Abstract

Homeostasis of the body's biochemical reactions is maintained by the balance of specific proteases and their counterparts, protease inhibitors. Snake venom protease inhibitors disrupt this balance and are thereby utilized by snakes as weapons to prey on other animals. Snake venom protease inhibitors primarily disrupt blood coagulation and blood pressure regulation. The resulting blood loss, blood clotting, or hypotension leads to immobilization or death of the prey.

This review describes the protease inhibitors that are thought to be distributed among many snake species, Kunitz-type protease inhibitors, bradykinin-potentiating peptides, and cystatins. In addition, as many components of snake venoms have diverse functions, the protease inhibitors that share motifs other

H. Inagaki (✉)

Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Ibaraki, Japan

e-mail: h-inagaki@aist.go.jp

than the protease inhibitory domains, i.e., phospholipase A₂-like proteins and three-finger toxins, are described. The majority of these toxins function as enzymes and neurotoxins, respectively. Finally, recent studies including genome, transcriptome, and proteome analyses have identified protease inhibitor-like proteins as new components of snake venoms; however, none of these have yet been proven to exhibit protease inhibitory activities. In this regard, waprin and Kazal-type protease inhibitor-like proteins will be introduced.

Keywords

Kunitz • Cystatin • Kazal • BPP • Three-finger toxin • PLA₂

Introduction

Protease inhibitors are defined by their ability to inhibit the proteolytic activities of proteins and peptides. After the discovery of bovine pancreatic trypsin inhibitor (BPTI), a mammalian Kunitz-type protease inhibitor, in the 1930s, a large number of protease inhibitors have been identified in microorganisms, plants, and animals from various bioresources. The purification and characterization of certain sanguine protease inhibitors or protease inhibitor-like molecules were initiated in the 1970s. According to the protease inhibitor database MEROPS (<http://merops.sanger.ac.uk/inhibitors/index.shtml>), 93 families of protease inhibitors have been reported to date. Five of these, Kazal (family I1), Kunitz (I2), waprin (I17), cystatin (I25B), and bradykinin-potentiating peptide (BPP) (I25C), are found in snake venoms. A simple keyword search of UniProtKB displayed 29 Kazal-type protease inhibitors, 195 Kunitz-type protease inhibitors, 61 waprins, 35 cystatins, and 59 B.P. in snake (UniProt release 2015_05), although protein inhibitor-like molecules, which have different functions other than protease inhibitory activities (e.g., dendrotoxin), might also be included among these molecules. Currently, the members of each family of snake venoms continue to increase.

The sites of action of snake venom protease inhibitors are predominantly within the systems of blood coagulation reaction and blood pressure regulation. Some protease inhibitors act on specific proteases in each system, and many of these have been co-opted for pharmacological, physiological, and pathological use. In the era before molecular biology, venom protease inhibitors were valuable tools for biological discoveries in lieu of specific antibodies and recombinant proteins, and even now, some remain as indispensable tools for basic science. Certain protease inhibitors have also become lead compounds for drug discovery, one of the most famous examples being captopril, an angiotensin-converting enzyme (ACE) inhibitor, developed from BPP.

The taxonomic distribution of venomous snakes is primarily within Viperidae and Elapidae including Hydrophiidae; they are also found occasionally in Colubridae, but rarely in other snake families (Brahma et al. 2015). Snake venoms consist of various components, which are acquired or deleted during the evolution.

The compositions and properties of the venoms depend on the snake species with some reflection of the growth environment including with feed type. Results from recent comprehensive studies have led to our ability to outline snake venom composition. In some species, protease inhibitors are one of the major venom components. Proteomics analysis of *Dendroaspis polylepis* (Elapidae) has indicated that Kunitz-type proteins including dendrotoxin (61.1 % of the total protein content) are predominant in the venom of this species (Laustsen et al. 2015). Transcriptomic profiling of the venom glands of eight venomous snakes in Viperidae has shown that BPP transcripts account for 8.3–23.6 % and Kunitz transcripts account for 0–0.2 % of total venom-related transcripts (Durban et al. 2011). On the other hand, protease inhibitors might be a minor component in Hydrophiidae. For example, reversed-phase HPLC-based analysis of *Pelamis platura* venom reveals that there are no protease inhibitors therein (Lomonte et al. 2014).

The focus of this review will be on the structure and function of protease inhibitors from snake venoms. Firstly, as well-studied protease inhibitors in snake venom, Kunitz, BPP, and cystatin will be described. Secondly, wapirins and Kazal will be introduced. Although these are both well-known protease inhibitors in nonvenomous animals, their functional characterizations as protease inhibitors in snake venoms have not yet been reported. Lastly, three-finger toxins (3FTxs) and phospholipase A₂s (PLA₂s) will be discussed as atypical protease inhibitors.

Well-Studied Protease Inhibitors in Snake Venom

Kunitz-Type Proteins

A History of Kunitz-Type Proteins

Kunitz-type protease inhibitors consist of approximately 60 amino acid residues with three distinctive disulfide bridges. The first well-studied Kunitz-type protease inhibitor was identified as bovine pancreatic trypsin inhibitor (BPTI) by Kunitz and Northrop in 1936 (Kunitz and Northrop 1936). Although Kunitz-type protease inhibitors are found in many animal species, the amino acid sequences are variable except for conserved six cysteine residues in venomous animals, a result of accelerated evolution. These variations are reflected in a wide variety of pharmacological properties aside from protease inhibition, such as blockage of potassium, sodium, and calcium channels (Harvey 2001), and TRPV1 agonism (Andreev et al. 2008).

Kunitz-type proteins from snake began to be reported in the 1970s. Initially, dendrotoxins (DTXs) I and K of *Dendroaspis polylepis polylepsis* were reported as potassium channel blockers, and a Kunitz-type protein of *Daboia russelii* was identified as a protease inhibitor of kallikrein, plasmin, and trypsin (Takahashi et al. 1974; Takahashi et al. 1972). To date, about two hundred Kunitz-type protease proteins have been recorded in UniProtKB. From these, we have selected 31 Kunitz-type proteases for which complete biological data are available, and

their origins, names, primary structures, and target molecules including ion channels are listed in Table 1. While Kunitz-type protease inhibitors were originally identified as being serine protease inhibitors, two act on metalloproteases. In addition, although Kunitz-type cysteine protease inhibitors have not yet been identified from snake venoms, several sea anemone Kunitz-type protease inhibitors are known that act on cysteine proteases including papain, cathepsin B, and cathepsin L (Lenarcic et al. 1997). However, even the utilization of peptidome and transcriptome approaches has not yet identified Kunitz-type proteins in Hydrophiidae.

Certain Kunitz-type proteins in sea anemone (Schweitz et al. 1995), spider (Yuan et al. 2008), and scorpion (Chen et al. 2012) are bifunctional protease inhibitors and ion channel blockers. Among snake Kunitz-type proteins, only BF9 from *Bungarus fasciatus* has been proven to be bifunctional to date (Chen et al. 2001). However, since some of the Kunitz-type protease inhibitors have not been evaluated for their effects on ion channels, these properties might have been overlooked. For example, DTX-E (DpE) of *Dendroaspis polylepis polylepis* (Joubert and Strydom 1978) and DaE of *Dendroaspis angusticeps* (Tytgat et al. 2001) have displayed ion channel and protease inhibitory activities, respectively. Because DTX-E and DaE differ by only a single amino acid residue (at position 55) that is not critical for ion channel and protease inhibitory activities, they might also be bifunctional molecules.

Some Kunitz-type proteins also form complexes with other molecules. The MitTx complex of *Micrurus tener tener*, a heterodimer without an intermolecular disulfide bridge, composed of a Kunitz-type protein and a PLA₂-like protein, activates acid-sensing ion channels (Bohlen et al. 2011). β -bungarotoxin of *Bungarus multicinctus*, a heterodimer with an intermolecular disulfide bridge, composed of a Kunitz-type protein and a PLA₂-like protein, blocks neural transmission (Kwong et al. 1995). Taicatoxin of *Oxyuranus scutellatus*, which is composed of four Kunitz-type proteins, one neurotoxin (three-finger toxin), and one PLA₂-like protein, blocks Ca²⁺ channels (Possani et al. 1992).

To avoid confusion regarding the nomenclature of Kunitz-type proteins, a Kunitz-type protein that has protease inhibitor activity is defined as a Kunitz-type protease inhibitor, and a Kunitz-type protein that has activities other than protease inhibitor activity is defined as a Kunitz-type toxin in this chapter.

Structure of Kunitz-Type Proteins

The three-dimensional structures of BPTI and BPTI with serine proteases have been extensively studied by crystallography in the 1970s (Deisenhofer and Steigemann 1975; Huber et al. 1970) and 1980s (Chen and Bode 1983). BPTI consists of two antiparallel β -sheets (amino acid residues 16–24 and 29–35) and an α helix (amino acid residues 49–52). A loop extended from a disulfide bond (amino acid residues 13–18) contacts serine proteases (at amino acid residues 214–216 and 40–41 in kallikrein). Another segment (amino acid residues 34–39) stabilizes the complex formation with the protease. The P1 position at the tip of the loop interacts with S1 pockets of the proteases; therefore, the P1 position is important for protease inhibitor specificity (Fig. 1a).

Table 1 Major Kunitz-type proteins of snake venoms

Species	Name (Uniprot KB)	Amino acid sequence	Target molecule	Reference
<i>Dendroaspis polylepis polylepis</i>	DTX-1 ^c (P00984)	LQHRITFKLPAEPGR[R]SIPAFYNNWAAKKQLFHYGGCKGNANRSTIEKCHRA[C]VG	Trypsin, Chymotrypsin	Joubert and Strydom (1978)
	CBPT1-1 ^a (A877N4)	QDRPKFCNLAPESGR[R]GHLRRIYNNPDSNKCEVFFYGGCGGNDNNFETKKCRQTGAPRKGRPT	Trypsin, Plasmin, FXIa Activated protein C	Cheng et al. (2012)
<i>Daboia russelii siamensis</i>	CBPT1-2 ^b (A877N5)	HDRPTFCNLAPESGR[R]HLRRIYNNLESNKCEVFFYGGCGGNDNNFSSWDECHRT[C]VGK	Trypsin	Guo et al. (2013b)
	CBPT1-3 (A877N6)	HDRPKFCYLPAIDGHE[R]AYIRSFYYDSIESKCKEKFHYGGHGNANNPTTRDKCRQT[C]RGK	Chymotrypsin	Guo et al. (2013b)
	BBPT1-1 (A877P1)	HDRPKFCYLPAIDGEC[R]AHMRSFYDYDSIESKCKEKFHYGGHGNANKFPRDKCRQT[C]GK	Chymotrypsin	Guo et al. (2013a)
<i>Daboia russelii russelii</i>	DnKn-II ^b (H6YC06)	HDRPTFCNLAPESGR[R]HLRRIYNNLESNKCEVFFYGGCGGNDNNFSTWDECHRT[C]VGK	Plasmin, Trypsin, FXa, FXIa	Cheng et al. (2014)
<i>Hemachatus haemachatus</i>	Venom basic protease inhibitor 2(P00985)	RPDFCELPAETGL[R]AYIRSFHYNLAQAQCLQHFYGGCGGNNANRFTIDECHRT[C]VG	Trypsin, Chymotrypsin, Kallikrein, Plasmin	Hokama et al. (1976)
<i>Naja nivea</i>	NNV inhibitor II (P00986)	RPRFCELPAETGL[R]ARIRSFHYNRAAQCL[E]FYGGCGGNNANRFTIDECHRT[C]VG	Trypsin	Hokama et al. (1976)
<i>Vipera ammodytes ammodytes</i>	cVamT1-I (P00991)	HPKFCYLPAIDGR[R]AHIPRFYDASNKNCKFYGGCPGNANNEKFTWDECRQT[C]GASAMGRPT	Trypsin, Chymotrypsin, Kallikrein, Plasmin	Ritonja et al. (1983a)
	cVamChi (P00992)	DRPKFCYLPAIDGR[R]AYMPSFYDASNKNCKFYGGCRGNANNEKFTWDECHRT[C]VASGIQPRIASN	Chymotrypsin , Trypsin, Plasma kallikrein	Ritonja et al. (1983b)
<i>Bungarus fasciatus</i>	BF9 (P25660)	KNRPTFCNLLPETGR[R]ALIPAFYNNSHLHKQKFNKYGGCGGNANNEKFTIDECHRT[C]CAAKYGRSS	Chymotrypsin, K channel	Liu et al. (1983)
	Bungaruskuini (B2KTG1)	RPGEFLPAAGKL[R]HKPAFYNNKDSHRCQKHYGGCGGNNANRFTIDECHRT[C]VG	Chymotrypsin, Trypsin, Elastase	Lu et al. (2008)
<i>Naja naja</i>	Trypsin inhibitor (P20229)	RPGEFLPAAGKL[R]HKPAFYNNKDSHRCQKHYGGCGGNNANRFTIDECHRT[C]VG	Trypsin	Shafiqat et al. (1990)
<i>Naja atra</i>	NAC1 (Q5ZP17)	RPRFCELAPSAGS[R]AFVTSYYNNQYSNTCHISFTYGGCGGNNANRFTIDECHRT[C]VG	Chymotrypsin	Zhou et al. (2004)
<i>Ophiophagus hannah</i>	Oh1-I-1 (P82966)	GRPKFCFLPPEPGL[R]ARKTFFYYSLHSHAQKFNKYGGCGGNNANNEKFTIDECHRT[C]VG	Chymotrypsin	Chang et al. (2001)
	OH-TCI (B6RLX2)	GRPKFCFLPANSGF[R]AYIPSFYNNPDASAQKFNKYGGCGGNNANNEKFTIDECHRT[C]VG	Chymotrypsin, Trypsin	He et al. (2008)

(continued)

Table 1 (continued)

<i>Bungarus multicinctus</i>	PILP-1 (B4SA2)	KDRPSCDKAPDTERK ~~Q~~ RVVAFYYNPSARDCLQFYVGGDNGKHF ~~R~~ SKALCLFHCH ~~R~~	Trypsin	Chang et al. (2008)
	PILP-3 (B4SA4)	BKRHFQCNVPEPGRC ~~Q~~ ANVRAFYYNPRLRK ~~Q~~ IEFSYGGCGGNANFNKSRGECKRTCAE	Metalloprotease2	Chou et al. (2010)
<i>Oxyuranus scutellatus</i>	TSPI (B7SAN9)	KDRPKFCHLPKPGPC ~~Q~~ AI ~~Q~~ PRFYYNPHSKQCEKHYGGCHGNANSHFKTPDECNV ~~T~~ CLGVSLPK	Kallikrein, Trypsin, Plasmin, Elastase, Factor Xa, α -factor XIIa	Earl et al. (2012)
<i>Macrovipera lebetina transmediterranea</i>	P1VL (I2G9B4)	QDRPKFYLPADPAEC ~~Q~~ YVMPRFYD ~~S~~ ASNNK ~~Q~~ EFYGGGRGNANFNKRAE ~~R~~ HTCVASRKGIO ~~Q~~ PR	Trypsin	Morjen et al. (2013)
	Textilinin-1 (Q90WA11)	KDRPDC ~~F~~ ELPADTGR ~~Q~~ R ~~Q~~ RPFSFYYNPD ~~E~~ KK ~~Q~~ LEFYGGEGGNANFNKTEEC ~~E~~ STCAA	Trypsin, Plasmin	Earl et al. (2011)
<i>Pseudonaja textilis textilis</i>	Textilinin-2 (Q90WA0)	KDRPEL ~~F~~ ELPD ~~T~~ GR ~~Q~~ R ~~Q~~ RPFSFYYNPD ~~E~~ QK ~~Q~~ LEFYGGEGGNANFNKTEEC ~~E~~ STCAA	Plasmin	Masci et al. (2000)
<i>Pseudochis australis</i>	<i>Pr</i> -mullin 1 (E7FL11)	KDRPR ~~F~~ CELPA ~~D~~ RGPC ~~Q~~ GLFQAFYYNPVQRK ~~Q~~ CLKFRYGGCKGNPN ~~T~~ FKTIEE ~~C~~ KRTCAA	Metalloprotease 2	
	<i>Pr</i> -mullin 2 (E7FL12)	KDRPH ~~F~~ CHLPHD ~~R~~ GPC ~~Q~~ ENFQAFYHPVRR ~~T~~ CL ~~E~~ FHYGGCGQGNPN ~~N~~ FKTIDE ~~C~~ KRTCAA	Trypsin, Chymotrypsin, Plasmin	Inagaki et al. (2012)
	<i>Pr</i> -mullin 3 (E7FL13)	KDRPK ~~F~~ CELPA ~~D~~ SGSC ~~Q~~ ENFQAFYHPVHR ~~T~~ CL ~~E~~ FHYGGEGNDNN ~~N~~ FKTIDE ~~C~~ KRTCA ~~T~~	Trypsin, Plasmin	
<i>Dendroaspis polylepsis polylepsis</i>	DTX-1 (P00979)	QPLRKL ~~C~~ ILHRNPGR ~~C~~ YQKIPAFYYNQKK ~~Q~~ CEGFTW ~~S~~ GGCGGNSNRFK ~~T~~ IEE ~~C~~ RR ~~T~~ CR ~~K~~	K ⁺ channel	Harvey (2001)
	DTX-K (P00981)	AAKY ~~C~~ KLPLRGPK ~~R~~ KIPSFY ~~Y~~ KKWKA ~~Q~~ CLP ~~F~~ DYSGGGGNANRFK ~~T~~ IEE ~~C~~ RR ~~T~~ CVG	K ⁺ channel	Owen et al. (1997)
	α -DTX (P00980)	QPRRL ~~C~~ ILHRNPGR ~~C~~ YQKIPAFYYNQKK ~~Q~~ CE ~~R~~ FDW ~~S~~ GGCGGNSNRFK ~~T~~ IEE ~~C~~ RR ~~T~~ CG	K ⁺ channel	Harvey (2001)
	δ -DTX (P00982)	AAKY ~~C~~ KL ~~P~~ VRYGPCK ~~K~~ KIPSFY ~~Y~~ KKWKA ~~Q~~ CLP ~~F~~ DYSGCGGNANRFK ~~T~~ IEE ~~C~~ RR ~~T~~ CVG	K ⁺ channel	Owen et al. (1997)
<i>Dendroaspis angusticeps</i>	Calcichlidine (P81658)	WOPPWYCKEP ~~V~~ RGSCQK ~~Q~~ SSFYFKWTAK ~~Q~~ CLP ~~F~~ SGCGGNANRFQ ~~T~~ IGECRR ~~K~~ CL ~~G~~ K	Ca ²⁺ channel	Schweitz et al. (1994)
	DaE ^c	LQHR ~~T~~ CKLPAEPGPCK ~~Q~~ ASIPAFYYNWAAKK ~~Q~~ CLFHYGGCKGNANRFSTIEK ~~C~~ RRAC ~~V~~ G	K ⁺ channel	Tytgat et al. (2001)

Kunitz-type proteins without available mature peptide amino acid sequences or inhibitory activity data, and Kunitz-type proteins, which are peptide subunits of polymeric molecules such as β -bungarotoxin, were excluded from this list. P1 positions are highlighted in black, and cysteine residues are highlighted in gray

^aThe amino acid sequences of CBPTI-1 from *Daboia russelii siamensis* and DrKIn-I from *Daboia russelii russelii* are identical

^bThe amino acid sequences of CBPTI-2 and DrKIn-II are identical except for position 49 (Ser in CBPTI-2, Thr in DrKIn-II)

^cThe amino acid sequences of DTX-E and DaE are identical except for position 55 (His in DTX-E, Arg in DaE)

The three-dimensional structures of DTX-K (Berndt et al. 1993), α -DTX (Skarzynski 1992), BF9 (Chen et al. 2001), NACI (Lin et al. 2013), and textilinin-1 (Millers et al. 2009) from snake venom have been elucidated. Their overall structures are similar to that of BPTI. Serine proteases, which have a serine residue at their active site, share a catalytic triad consisting of Ser, His, and Asp residues. The substrate recognition sites around the active site modify the protease specificities. At these sites, positively charged, small hydrophobic, and large hydrophobic residues are preferred by trypsin-, chymotrypsin-, and elastase-like proteases, respectively.

Kunitz-type protease inhibitors with P1 Lys or Arg tend to inhibit trypsin, while those with P1 Leu, Met, Phe, Tyr, or Trp tend to inhibit chymotrypsin (Laskowski and Kato 1980). Although the P1 position is a key residue in protease inhibitors, other residues around the P1 position determine the potencies and specificities of the inhibitory activities. Another feature of Kunitz-type protease inhibitors is that most Kunitz-type protease inhibitors have a small hydrophobic amino acid residue

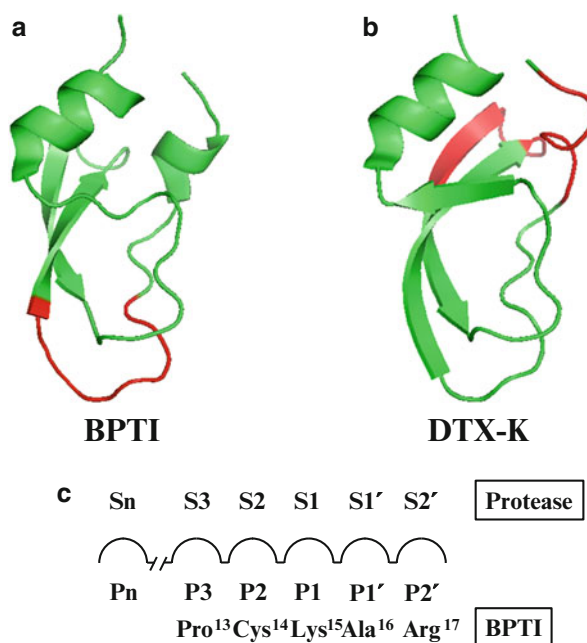


Fig. 1 Three-dimensional structure of (a) bovine pancreatic trypsin inhibitor I (BPTI) (PDB: 1PIT) and (b) dendrotoxin K (DTX-K) (PDB: 1DTK). The interfaces with the target molecules are highlighted in red. (c) The relative positions of protease and BPTI. The second Cys residue of all Kunitz-type protease inhibitors is located at the P2 position. The N- and C-terminal sides of the amino acid residues at the cleavage site in the substrate have been defined as Pn and Pn', respectively. Applying this definition to the competitive inhibitors, the N- and C-terminal sides of the amino acid residues at the site corresponding to the substrate cleavage site were defined as Pn and Pn', respectively, in this chapter. For the protease, the positions that interact with Pn and Pn' were defined as Sn and Sn', respectively

(Ala and Gly) at the P1' position next to the carboxyl terminal side of P1. However, the properties of some the protease inhibitors may not fit for these tendencies.

Substitution of Lys to Arg at the P1 position of BPTI has been shown to change the association constant for the protease. Inhibition of plasmin, kallikrein, thrombin, factor Xa, and XIIa was enhanced, while inhibition of trypsin and plasmin did not significantly change (Grzesiak et al. 2000). Substitution of Arg to Lys at the P1 position of Alzheimer's amyloid β -protein precursor I (APPI) improved the inhibition constant of plasmin (Van Nostrand et al. 1995). Following random mutation around the P1 position of APPI, mutants of His, Asn, and Phe at the P1 position were selected and were found to inhibit chymotrypsin (Scheidig et al. 1997). Furthermore, mutants of Ser at the P3 position of BPTI were found to significantly increase its activity against factor XIIa. However, some protease inhibitors do not share these properties.

Tick anticoagulant peptide (TAP), identified from the blood-sucking soft tick, *Ornithodoros moubata*, is an exception to this rule. TAP, a factor Xa inhibitor, binds to the target molecule using two steps. The initial binding sites of TAP to factor Xa consist of amino acid residues 40–54, followed by residues 1–3. Both sites differ from those used by the typical Kunitz-type protease inhibitor to bind to its counterpart (Wei et al. 1998). No snake venom Kunitz-type protease inhibitors that exhibit this mode of action, however, have been reported to date.

Although Kunitz-type protease inhibitors were originally identified as serine protease inhibitors, recently protease inhibitor-like protein 3 (PILP3) (Chou et al. 2010) and *Pr*-mulgin 2 (Inagaki et al. 2012), which have Asn residues at their P1 positions, were reported as metalloprotease 2 inhibitors. Swapping experiments between PILP-2 and PILP-3 suggested that the amino acid residues 49–52 of PILP3 are involved in protease binding. This site is different from that used by the typical Kunitz-type protease inhibitor to bind to its counterpart. Further studies are needed to elucidate the structure-function relationships of these molecules.

On the other hand, the interfaces between Kunitz-type toxins and potassium channels differ from those between Kunitz-type protease inhibitors and proteases (Harvey et al. 1997; Imredy and MacKinnon 2000; Smith et al. 1997; Wang et al. 1999). Chemical modification and mutation analyses of DTX toxins revealed that Lys³ (the numbering refers to DTX-K, P00981) is a key residue for potassium channel binding. Other important residues for binding are concentrated in the N-terminus (amino acid positions 3–10 in DTX-K) and the β -turn region (amino acid positions 24–28 in DTX-K), although some differences are observed among DTX proteins (Fig. 1b). Results from Ala-scanning approaches using huwentoxin (HWTX)-XI of spider toxin (Yuan et al. 2008) and the Hg1 peptide (Chen et al. 2012) of scorpion toxin support this tendency.

To further verify the importance of various residues to target binding, the Liang group estimated the non-synonymous/synonymous mutation rates (ω) for two interfaces of Kunitz-type proteins with serine proteases and potassium channels in spider and snake. A high ω ratio is an index of accelerated evolution at the respective sites. In snake, the ω values of amino acid residues in both interfaces were higher than those of other regions (Yuan et al. 2008).

Gene Structure of Kunitz-Type Proteins

Most Kunitz-type proteins are encoded in a single transcript. However, cloning of the Kunitz-type protein cDNAs of Australian elapid snakes and the red sea turtle revealed that two Kunitz-type proteins or a single Kunitz-type protein with one waprin (EC 3.1) is encoded in a single transcript. Some waprins share a signal peptide with Kunitz-type proteins in snake. Together, these findings suggest the common evolution of Kunitz-type proteins and waprin gene families (Jackson et al. 2013).

Physiological Functions and Applications

Target molecules of Kunitz-type protease inhibitors include factors Xa and XIIa, activated protein C, thrombin, and plasmin, which are involved in the coagulation reaction. Inhibitors of factors Xa and XIIa and thrombin promote bleeding directly. Because activated protein C retards coagulation through inactivation of factors Va and VIIIa, the inhibition of activated protein C results in fibrinogen consumption and eventually promotes bleeding. One of the best-studied Kunitz-type protease inhibitors in terms of the physiological aspects of its envenomation is DrKIn-I, which was identified from the Russell viper *Daboia russelii russelii*. DrKIn-I inhibits activated protein C and promotes coagulation in collaboration with other venom components with eventual coagulopathy, leading the envenomed patients to bleed (Cheng et al. 2012).

Another important target of Kunitz-type protease inhibitors is kallikrein, which produces bradykinin from kininogen. Because bradykinin downregulates blood pressure, kallikrein inhibition results in upregulation of the blood pressure. Since kallikrein also activates factor XII, kallikrein inhibitors promote bleeding (Fig. 2).

BPTI, another name for aprotinin, has been widely used for clinical application as an antifibrinolytic agent to reduce blood loss associated with surgery because of its plasmin inhibitory activity. However, after unexpected side effect of aprotinin, including increased risk of renal, cardiac, or cerebral events during the surgery, became apparent, textilinin-1 (Q8008), a plasmin inhibitor identified from *Pseudonaja textilis textilis*, became a candidate as an alternative for aprotinin use (Earl et al. 2011).

BPPs

BPPs as ACE Inhibitors

BPPs consist of 5–13 amino residues, are rich in proline residues, and function as ACE inhibitors. Although variations exist in amino acid composition and length, typical BPPs have pyroglutamate and proline residues at the N- and C-termini, respectively. ACE, a metalloprotease that hydrolyzes the C-terminal dipeptide of its substrate protein, is a key player in the control of blood pressure (Camargo et al. 2012). After the discovery of bradykinin in 1949 (Silva et al. 1949), bradykinin-potentiating factor was reported in 1965 as a pharmacologically active peptide fraction prepared from *Bothrops jararaca* venom (Ferreira 1965). From this

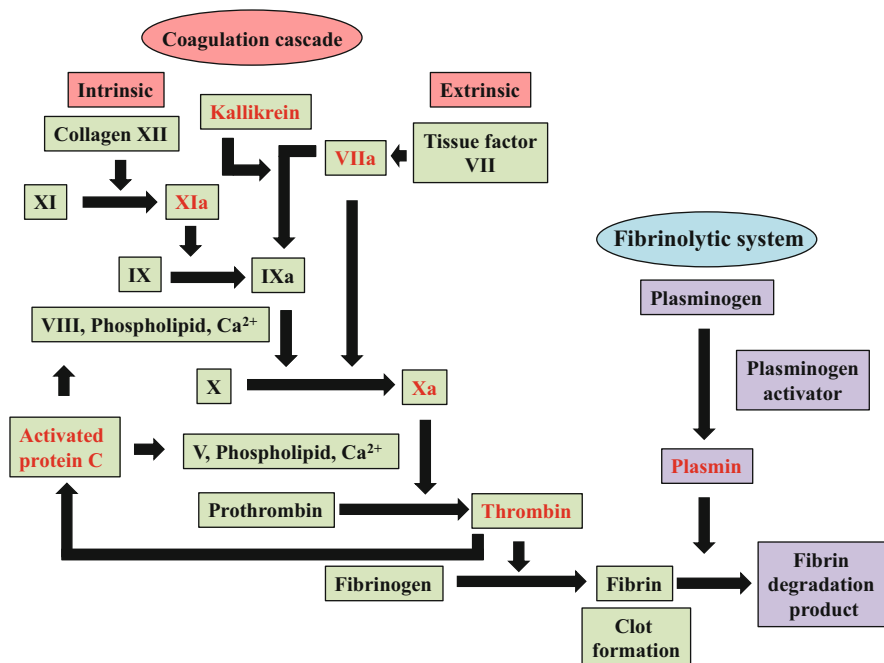


Fig. 2 Targets of Kunitz-type protease inhibitors in the coagulation cascade and the fibrinolytic system. The coagulation cascade is a chain reaction of various proteases in plasma, which finally leads to clot formation. Most of the factors are produced by the liver and function as proteases (Factors VII, IX, X, and thrombin) in this reaction after activation. The fibrinolytic system consists of the degradation of fibrin by plasmin, which is activated by plasminogen activators from endothelial cells. Targets of the Kunitz-type protease inhibitor are indicated in red

fraction, BPPs were identified in 1970 (Ferreira et al. 1970). Subsequently, BPPs were identified from other snake species including *Agkistrodon halys blomhoffii* (Kato and Suzuki 1971).

Cloning of BPP cDNAs from *Bothrops jararaca* revealed that seven BPPs were encoded in a single transcript in a so-called rosary-type structure along with a C-type natriuretic peptide (CNP) (Murayama et al. 1997). In this transcript, BPP-10a, BPP-13a, BPP-10c, BPP-11b, BPP-11c, and BPP-5a were encoded (Fig. 3). After translation, the BPPs and CNP are cleaved off by proteases. Each BPP has a slightly different amino acid sequence with a distinctive potency.

The development of captopril, based on structure-activity studies of BPPs, is one of the early success stories of rationalized drug design. Among the BPPs, BPP-9a (teprotide) was first introduced into clinical studies in the 1970s (Gavras et al. 1975). Because BPP-9a was not suited for oral administration, further approaches to reduce the size of BPP were utilized. Extensive structure-activity studies of synthetic BPP analogs indicated that the optimal amino acid sequence for binding to the active site of ACE was Phe-Ala-Pro. Although the structural information of sACE was not available in the 1960s and 1970s, it was predicted that

ACE would exhibit similarities to carboxypeptidase A, a zinc-containing exopeptidase, because the activities of ACE were sensitive to metal chelating agents. Captopril was obtained in 1977 by adding a zinc-binding succinyl group to Pro and then replacing its carboxyl group with a sulfhydryl group (Cushman and Ondetti 1999).

Involvement of BPPs in Two Systems for Blood Pressure Regulation

The Rennin-Angiotensin System

The rennin-angiotensin system is one of the important systems utilized to regulate blood pressure. Initially, low blood pressure stimulates the secretion of the 40 kDa renin protein by juxtaglomerular cells of the kidneys. Renin is released into the plasma and processes angiotensinogen to produce angiotensin I. Then, angiotensin I is converted to angiotensin II by ACE molecules located on endothelial cells. Angiotensin II has a strong vasoconstrictive effect by way of the AT₁ receptor; the half-life of angiotensin II in plasma is 1–2 min.

The Kallikrein-Kinin System

Another system utilized to regulate blood pressure is the kallikrein-kinin system.

Tissue and plasma kallikreins are serine proteases that cleave kinins from kininogen. One of these, bradykinin, has a vasodilatory effect mediated by the G protein-coupled receptors (B₁ and B₂) of smooth muscle cells. ACE removes the C-terminal dipeptides from bradykinins, transforming them into inactivated forms prior to receptor binding. Therefore, the inhibition of ACE activities lowers blood pressure, following vasodilation through disruption of both systems (Fig. 4).

Structures and Functions of BPPs and ACE

There are two isoforms of ACE, somatic (sACE) and testicular (tACE) types. Both are metalloproteases with zinc-binding motifs (His-Glu-X-X-His) in the active domains. sACE has two active domains (N- and C-domains) and tACE has one. The N- and C-domains of sACE primarily regulate the degradation of Ac-SDKP, a peptide regulator of hematopoietic stem cells, and Ang II production, respectively (Rousseau et al. 1995). Both domains inactivate bradykinin. Because of different substrate specificities and affinities to inhibitors among the domains, nonselective



Fig. 3 Multiple alignments of the amino acid sequences of C-type natriuretic peptide (CNP) precursors from humans and seven snake species. The amino acid sequences of CNP precursors were aligned using the ClustalW program. The protein accession numbers are given to the right of each name. Bradykinin-potentiating peptides (BPPs) and putative bradykinin-related peptides are highlighted in gray. Some of the BPPs have several intermediate products for which the translated products have not yet been confirmed. CNPs, bradykinin inhibitory peptides, and azemiopsin are highlighted in red, yellow, and blue, respectively. Proline residues in each bioactive peptide are highlighted in black

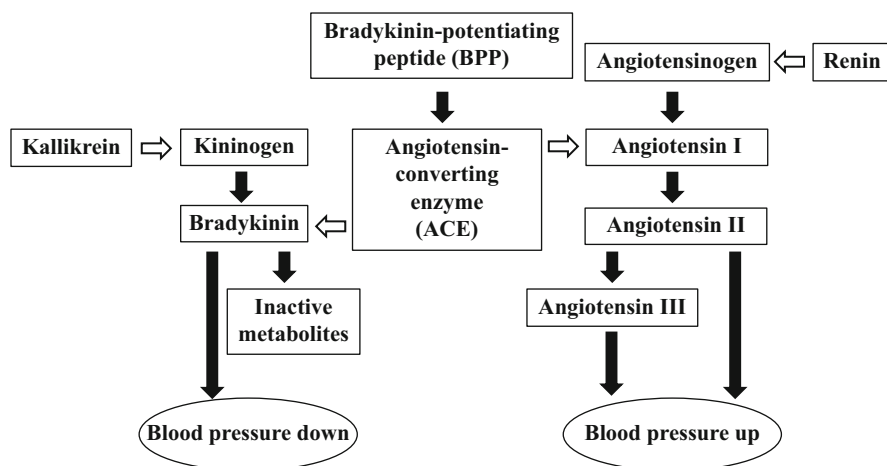


Fig. 4 Regulation of blood pressure by the renin-angiotensin and kallikrein-kinin systems. Somatic angiotensin-converting enzyme (sACE) regulates both the renin-angiotensin and kallikrein-kinin systems by processing angiotensin and bradykinin to reduce blood pressure. Protease reactions are indicated by open arrows

inhibitors of these domains lead to unexpected side effects; therefore, the development of C-domain-specific inhibitors is desirable.

Several crystal structures of ACE with various inhibitors have been elucidated over the past two decades. For example, utilizing co-crystallization techniques, structural models of captopril or lisinopril complexed with human tACE have been reported (Natesh et al. 2003). In addition, a model of a BPP complexed with a *Drosophila* ACE homologue has also been reported (Akif et al. 2012). From these structures, the crystal structures of the human C-domain of sACE complexed with Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and BPP-b (pGlu-Gly-Leu-Pro-Pro-Arg-Pro-Lys-Ile-Pro-Pro) (Kato and Suzuki 1971) were elucidated by Masuyer et al. as examples (Masuyer et al. 2012). Comparison between these indicated that they partially share common structures. The two C-terminal Pro residues (P1' and P2') of BPP-b interact at the S1' and S2' subsites, respectively. The Pro residue at P2' interacts with Tyr⁵²⁰, Lys⁵¹¹, and Gln²⁸¹ of sACE. The C-terminal Pro/His residues (P2') of Ang II anchor at the S2' subsites with the same amino acid residues through hydrogen bonds and hydrophobic interactions. The backbone interactions at S2 are the same in both complex structures and are formed by Ala³⁴⁰ of sACE and the main chain of BPP-b or Ang II (Fig. 5).

In contrast, the binding of BPP-b to ACE led to the exclusion of the zinc ion from the active site and subsequent significant conformational changes. On the other hand, the zinc ion was retained at P1 position, which is stabilized by His³⁸³, His³⁸⁷, and Tyr⁵²³, following binding to Ang II. Overall, because of the unique properties of the Pro residue, BPPs might have many possible *cis* and *trans* stereoisomers. Hence, the three-dimensional structures of BPP alone have not yet been elucidated.

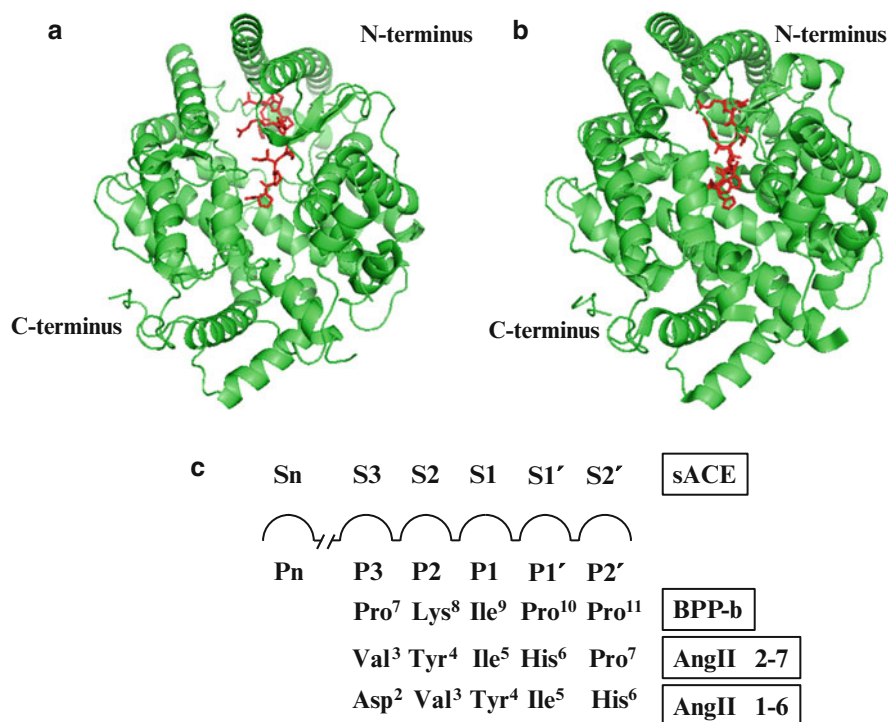


Fig. 5 Overall view of the structure of the somatic angiotensin-converting enzyme (sACE) C-domain interacting with angiotensin II (Ang II) (PDB 4APH) (a) or bradykinin-potentiating peptide b (BPPb) (PDB 4APJ) (b). sACE is displayed in *green*. Ang II and BPPb are displayed in *red*. The relative positions of the enzyme and BPPb, Ang II (Andreev et al. 2008; Banerjee et al. 2005; Barrett 1987; Berndt et al. 1993; Bohlen et al. 2011; Brahma et al. 2015), and Ang II (Akif et al. 2012; Andreev et al. 2008; Banerjee et al. 2005; Barrett 1987; Berndt et al. 1993; Bohlen et al. 2011) (c). It has been previously predicted that Ang II presents in two alternate (“sliding”) conformations when complexed with sACE

Biological Activities Other than Protease Inhibition

Low-dose administration of BPPs to spontaneously hypertensive rats (SHRs) results in potent and long-lasting reductions of mean arterial pressures. Affinity chromatography using BPP showed that the target molecule of BPPs is arginino-succinate synthase. BPPs increase plasma arginine levels by promoting arginino-succinate synthase activation; nitric oxide produced from arginine in endothelial cells then leads to the relaxation of smooth muscle cells (Guerreiro et al. 2009).

Additional targets of BPPs are ligand-gated ion channels. Since BPP-10c alleviates (+)-dizocilpine-induced nicotinic acetylcholine receptor (nAChR) inhibition in differentiated PC12 cells, BPP-10c is thought to interact with nAChR (Nery et al. 2008). In addition, BPP-5a promotes vasodilatation mediated by both bradykinin B2 and M1 muscarinic AChR (mAChR) (Moraes et al. 2011).

Gene Structure of BPP Precursor Proteins

Comprehensive transcriptome analyses have led to the isolation of many bradykinin-related peptides (BRPs) and BPP precursor cDNAs from various snake species (Higuchi et al. 1999; Higuchi et al. 2006; Soares et al. 2005).

Precursors of these peptides in snake venoms contained CNPs in their C-terminal portions. Two to seven BPPs or BRPs, depending upon the snake species, were found to be encoded in the N-terminal portion of the precursors. By comparison of the relative conservation of the CNP primary structures, it was found that the N-terminal portions of the precursor proteins were interchangeable among snake species and that the peptides in this portion might accordingly acquire new functions. For example, the proline-rich peptides of *Azemiops feae*, venomous snakes in Southeast Asia, which are encoded in the CNP precursor proteins, act as antagonists of nAChR (Utkin et al. 2012); Bradykinin inhibitor peptides, which are thought to act as antagonists of the bradykinin B2 receptor or other receptors, are encoded in the BPP precursor proteins of *Lachesis muta muta* (Soares et al. 2005) and *Trimeresurus gramineus* (Higuchi et al. 1999).

However, the ancestral origin of BPP peptides remains unknown, even though it has been found that BPP precursor transcripts are expressed in the snake brain as well as in its venom gland (Hayashi and Camargo 2005).

Cystatin-Like Proteins

Cystatins, which are cysteine protease inhibitors, are divided into 3 subfamilies based on their structural features. All cystatin-like proteins that have been identified from snake venoms belong to subfamily 2, which consists of molecules composed of approximately 120 amino acid residues with two disulfide bonds (Fig. 6) (Kordis and Turk 2009).

Studies of cystatin were initiated using egg white cysteine protease inhibitors (ovocystatins) in 1968, and the amino acid sequence of egg cystatin was reported in 1984 (Barrett 1987). It has been believed that cysteine protease inhibitors are widely distributed in snake venoms because several snake venoms inhibit the activity of papain, a cysteine protease (Mashiko and Takahashi 2002). The first cystatin-like protease inhibitor was identified from the African puff adder (*Bitis arietans*) in 1987 (Evans and Barrett 1987) and the second from the Taiwan cobra (*Naja naja atra*) in 1998 (Brillard-Bourdet et al. 1998). Following isolation of a cystatin cDNA from *Bitis gabonica gabonica* (Ritonja et al. 1987), twelve novel cystatin-like protease inhibitor cDNAs have since been isolated from ten Australian, one Asian, and one Papuan elapid snake species in 2011 (Earl et al. 2012). Immunoblot analysis in 22 snake species of Australian, Asian, and Papuan elapid snake species using an anti-*Austrelaps superbus* cystatin antibody revealed that cystatin was widely distributed in elapid snake venoms. Currently, following the results of comprehensive analyses, cystatin-like proteins are considered to be distributed among a wide variety of snake species. However, the amount of cystatin in snake venoms vary. Cystatin-like protein of *Bitis gabonica gabonica*

Fig. 6 Three-dimensional structure of *Austrelaps superbus* cystatin (AsCystatin). The three-dimensional structure of AsCystatin was modeled using the protein structure homology-modeling server (SWISS-MODEL) with the structure of human cystatin M (PDB: 4N6L) as a template. The putative regions interfacing with target molecules are displayed in red



represents 9.8 % of the total venom proteins, whereas that of *Bitis arietans* constitutes only 1.7 % of the total protein (Calvete et al. 2007).

The inhibitory spectrums of snake cystatins are different from those of nonvenomous animals. As is typical for subfamily 2 cystatins, AsCystatin of *Austrelaps superbus* inhibits cathepsin L, papain, and cathepsin B with K_i values of 0.9, 1.7, and 0.69 nM, respectively, and has no or low inhibitory effects on calpain 1 and legumain (asparaginyl endopeptidase). The activities of *Bitis arietans* and *Naja naja atra* cystatins are lower than that of ovocystatin (Table 2).

In addition to single cystatins, cathelin-like domains were identified in the leader sequence of the antimicrobial peptides, cathelicidins, from snakes (Falcao et al. 2014). Cathelin, a member of the cystatin family of cysteine protease inhibitors, was named after cathepsin L inhibitor. However, despite the inhibition of cathepsin L activity by porcine cathelicidin precursors, the inhibitory activities of the snake cathelicidin precursors have not been reported.

Snake venom metalloproteinase inhibitor (SVMPI), Habu serum factor (HSF), and BJ46a represent additional members of the cystatin family that are found in the serum of venomous snakes (Perales et al. 2005). SVMPI, HSF, and BJ46a are natural counteractives and inhibitors of metalloproteinases. However, because these protease inhibitors are not snake venom components, they are out of scope of this chapter.

In humans, some cystatins are expressed in fluid secretions and are considered to contribute to oral health. Conversely, the physiological significances of snake cystatins remain unclear. One possibility is that they exert protective effects on

Table 2 Inhibition constants of chicken and snake venom cystatins

Enzyme	<i>K_i</i> (pM)			
	Ovostatin (<i>Gallus gallus domesticus</i>)	Cystatin (<i>Bitis arietans</i>)	Cystatin (<i>Naja naja atra</i>)	AsCystatin (<i>Austrelaps superbus</i>)
Papain	5	100	190	1.74
Cathepsin L	19	n.d.	100	0.89
Cathepsin B	1700	2700	2500	690
Cathepsin S	n.d.	n.d.	1200	n.d.
Calpain	>1 × 10 ⁷	n.d.	n.d.	>1.4 × 10 ⁵
Legumain	<5.0 × 10 ³	n.d.	n.d.	>6.6 × 10 ⁵
Reference	(Barrett 1987; Chen et al. 1997)	(Evans and Barrett 1987)	(Brillard-Bourdet et al. 1998)	(Richards et al. 2011)

n.d. not determined

venom components. Brillard-Bourdet et al. reported that the relatively high concentration of cobra cystatin protects the venom proteins from proteolytic inactivation by victim proteases (Brillard-Bourdet et al. 1998).

Protease Inhibitors that Carry Protein Motifs Other than Protease Inhibitory Domains

PLA₂-Like Proteins

PLA₂ hydrolyzes glycerophospholipids at the sn-2 position of the glycerol backbone (EC 3.1.1.4). In addition to its possible role during digestion, PLA₂ is involved in a wide variety of pharmacological reactions such as the inflammation reaction through the release of arachidonic acid following its enzymatic actions.

Six and Dennis proposed that PLA₂s were divided into 11 groups based on their structural features (Six and Dennis 2000). Groups I and II were identified from snake venoms. The molecular weights of group I and II PLA₂s are approximately 13 kDa, and they contain around 14 conserved cysteine residues. Snake venom PLA₂s have gained more specialized functions than have the PLA₂s of nonvenomous animals, including neurotoxic, myotoxic, hemolytic, edema-inducing, and anticoagulant functions by both enzymatic and nonenzymatic actions.

Individual snake PLA₂s, which inhibit coagulation reactions, have specific target molecules and modes of actions. In these, the formation of a protease-phospholipid (PL) Ca²⁺ complex during the cascade reaction promotes the coagulation reactions. Although the protease activation in this cascade is dependent upon the respective phospholipid, the significance of PL hydrolysis on the anticoagulation activities of PLA₂s has been reported to vary and is contradictory (Kini 2006).

PLA₂s of *Naja nigricollis* are one of the well-studied examples of snake venom PLA₂s. Evans et al. purified three PLAs (CM-I, CM-II, and CM IV) with

anticoagulation activities from *N. nigricollis*. CM-I and CM-II have relatively weak anticoagulation activities and inhibit the extrinsic tenase complex by enzymatic action. On the other hand, CM IV exhibits a strong anticoagulation activity and inhibits the extrinsic tenase and prothrombin complex by both enzymatic and nonenzymatic actions. Furthermore, CM IV binds to factor Xa through a specific binding site, thus blocking the formation of the prothrombin complex (Kini 2005, 2006) (Fig. 7).

Because the effect of phospholipid hydrolysis on coagulation reactions is beyond the focus of this chapter, an example from *Naja haje*, thrombin inhibitor from *Naja haje* (TI-Nh), can also be used to exclude the effects of enzymatic actions of PLA₂, an archetypal protease inhibitor. TI-Nh has low PL hydrolysis activity but was shown to inhibit thrombin activities as evaluated by thrombin clotting time and amidolytic enzyme assay using a synthetic substrate; TI-Nh did not inhibit trypsin or other serine proteases of the coagulation cascade (Osipov et al. 2010).

Three-Finger Toxins (3FTxs)

In spite of the structural similarity among the 3FTxs, they have a variety of target proteins, including nAChR and mAChR (Kini and Doley 2010; Tsetlin et al. 2009). Some 3FTxs also have anticoagulation activity; one of which is dendroaspin, from *Dendroaspis jamesoni*, which interferes with platelet aggregation. Others are hemextin A and hemextin B from *Hemachatus haemachatus*, which form a heterotetrameric complex. This complex specifically inhibits the TF-FVIIa complex in the coagulation cascade, with a K_i value 50 nM. Kallikrein activates factor XII in an early step of the coagulation cascade. The hemextin A and B complex also moderately inhibits kallikrein function during this step (Banerjee et al. 2005) (Fig. 7).

Using a directed evolution technique, the specificities of neurotoxic 3FTx were changed to those of serine protease inhibitors. This illustrated that the structures of 3FTxs are potentially well suited for protease inhibitory activities (Cai et al. 2014).

Potential Protease Inhibitors with As-Yet-Unidentified Inhibitory Activities

To date, several protease inhibitor-like peptides and proteins have been found by the snake venom peptidome and snake venom gland transcriptome projects. However, although the physiological functions of the orthologs in nonvenomous animals have been previously reported, the functions of some snake protease inhibitor-like peptides and proteins have not yet been confirmed. Future studies will likely elucidate their physiological functions as potential protease inhibitors in envenomation. Here, waprin and Kazal-type protease inhibitors are described as potential protease inhibitors while recognizing the controversy over the inhibitory activities of these molecules.

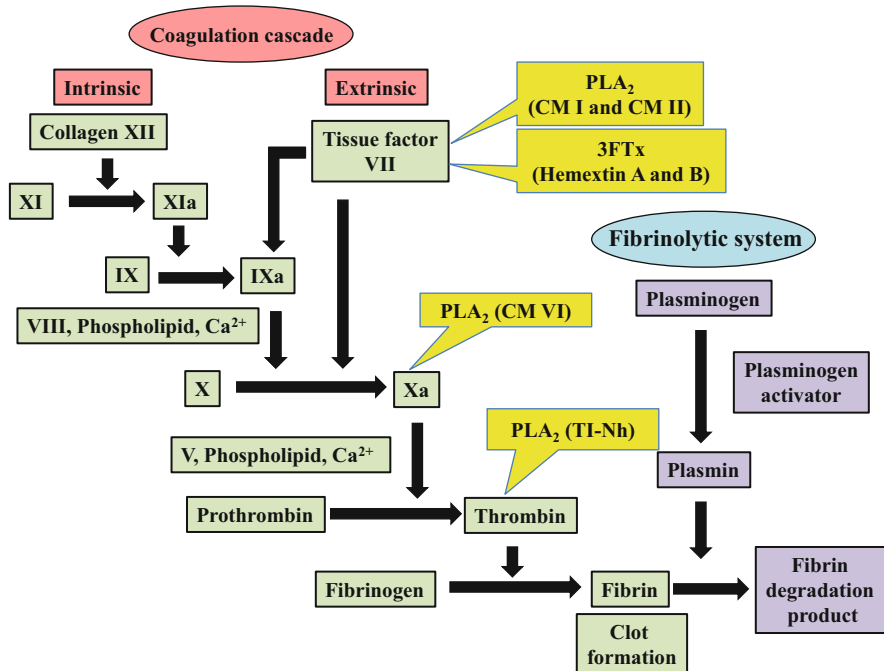


Fig. 7 Targets of phospholipase A₂s (PLA₂s) and three-finger toxins (3FTxs) in the coagulation cascade and the fibrinolytic system. Several plasma proteases involved in the coagulation cascade, a protease chain reaction that eventually leads to clot formation, are modified and thereby activated by various PLA₂s and 3FTxs, as shown in yellow boxes. This, in turn, indirectly affects the availability of fibrin for degradation by plasmin (the fibrinolytic system)

Waprans (Proteins Containing a WAP Domain)

Proteins containing a WAP domain have been well characterized as elastase inhibitors (elafins) (Wiedow et al. 1990); the protein has also been found in the milk of the tammar wallaby (*Macropus eugenii*) and termed whey acidic protein (WAP) (Simpson et al. 2000). WAP domains are composed of approximately 50 amino acid residues cross-linked by four disulfide bonds. Four-disulfide proteins, later termed waprins, have also been independently identified in snake venoms (Drenth et al. 1980).

Proteins containing a WAP domain have diverse functions (antimicrobial, protease inhibitory, mineral crystallization, and Na⁺/K⁺-ATPase inhibitory) and are present in amphibians, fish, insects, and abalone (Smith 2011). Waprin transcripts encoding one or two WAP domain proteins with or without a Kunitz-type protein have also been reported in elapid and colubrid snake venoms (Jackson et al. 2013). One of these, omwaprin, displayed antimicrobial but not protease inhibitory activities despite extensive analyses. The three-dimensional structure of nawaprin

(*Naja nigricollis*) has been determined and demonstrated that the binding regions of elafin to elastase, which are located at loops 1 and 2, seemed to differ from those of nawaprin (Torres et al. 2003; Tsunemi et al. 1996). To date, no reports regarding the protease inhibitory activities of waprin in the snake have been published.

Kazal-Type Protease Inhibitors

The Kazal-type protease inhibitor is named after the first discoverer, Kazal, of a pancreatic secretory trypsin inhibitor, which carries this domain (Kazal et al. 1948). Kazal-type protease inhibitors have several units composed of approximately 40–60 amino acid residues cross-linked by three disulfide bonds and are found in both vertebrates and invertebrates (Rimphanitchayakit and Tassanakajon 2010).

Using a venom gland transcriptome approach, only two of eight Costa Rican snake species, *Bothriechis schlegelii* and *Bothriechis lateralis*, were shown to express Kazal-type protease inhibitor-like proteins, and a Kazal-type protease inhibitor-like protein has been confirmed in *B. schlegelii* using a proteomics approach (Durban et al. 2011). However, to date there have been no reports about the protease inhibitory activities of these proteins, and the physiological functions of Kazal-type protease inhibitors in envenomation are likewise unclear.

In addition, some of the Kazal-type protease inhibitors in leech and tick salivary glands work as anticoagulation factors during blood sucking (Campos et al. 2004; Fink et al. 1986); therefore, snake Kazal-type proteases might possess similar activities, on the assumption that Kazal-type protease inhibitors exhibit inhibitory activities against serine proteases. Further studies are required to investigate this issue.

Conclusion

Following recent comprehensive analyses, a large number of protease inhibitor-like molecules have been identified from many snake species. A simple search of the UniProtKB database currently yields about two hundred snake venom Kunitz proteins. Although it is not clear whether this number reflects the results of all comprehensive analyses, they can be taken as a minimum number; however, the functional properties of most of these proteins have not been studied well or at all. The milieu of Kunitz-type proteins is similar to that of other protease inhibitors in snake venoms.

In general, because the orthologs and paralogs of a single protein tend to share similar biological functions, most have not been further characterized, following determination of their primary structures. However, in this regard the research of proteins in venom should differ from that of nonvenomous animals. For example, the Kunitz-type protein was originally identified as a protease inhibitor from nonvenomous animals, but subsequently certain orthologs from venomous animals

displayed additional blocking activities against ion channels. Such results of Kunitz-type protein studies imply the likelihood of future discoveries of novel inhibition spectra of protease inhibitors against proteases and novel functions beyond those of protease inhibitors within proteins exhibiting well-studied protease inhibitor motifs.

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Part III

Snake Venom Effects on Receptor and Ion Channel

Snake Venom Toxins Targeted at the Nervous System

9

Alexey V. Osipov and Yuri N. Utkin

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A.V. Osipov (✉) • Y.N. Utkin

Laboratory of Molecular Toxinology, Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry,
Russian Academy of Sciences, Moscow, Russia

e-mail: osipov@mx.ibch.ru; utkin@mx.ibch.ru; yutkin@yandex.ru

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Abstract

One of the main venom targets in a prey organism is the nervous system. The disturbance of this system kills or paralyzes a prey effectively. To achieve this task, snake venoms contain an array of peptide and protein toxins called neurotoxins which belong to several structural types and possess diverse biological activities. The most abundant neurotoxin groups are three-finger toxins and phospholipases A2, while other less represented groups include dendrotoxins of BPTI–Kunitz-type family, CRISPs (cysteine-rich secretory proteins), acetylcholine esterase, and peptide toxins. Neurotoxins affect different departments of the nervous system including both the central nervous system (CNS) and the peripheral nervous system (PNS). The toxins impairing the somatic PNS are acting at presynaptic site (β -neurotoxicity) or postsynaptic site (α -neurotoxicity); there are neurotoxins active inside a synaptic cleft as well. Effectors of sympathetic and parasympathetic systems of the autonomic PNS are also found in the venoms. Snake venom contains also neurotrophins and blockers of several types of ion channels, including effectors of sensory systems. Acting at different sites of the nervous system and being complementary, neurotoxins produce a cumulative effect resulting in very efficient oppression of the prey or predator.

Keywords

Channel blocker • CNS • Kunitz-type • neurotoxin • PNS • phospholipase A2 • phospholipase A2 • three-finger toxin

Introduction

All modern snakes belong to the suborder Serpentes, part of the order Squamata, and they have no legs or wing and move comparatively slow. In the course of evolution to defend themselves from the predators, some snake developed special system for production of venom which is a very potent weapon. On the other hand, snakes are predators themselves that feed on higher animals, mostly on vertebrates. They use the venom to kill or at least to paralyze the prey. The venom is synthesized in specialized venom gland and injected through the fangs possessing grooves for the venom delivery.

The nervous system controls and regulates (directly and non-directly) all the most important functions in higher animals, from a variety of cellular responses to complex behavior. It is not a surprise, therefore, that the nervous system has become one of the main targets for snake venom.

The venoms are complex mixtures of substances, mostly of polypeptide nature. Many of them affect different departments of the nervous system. Interactions between snake venoms and the nervous system are very diverse, and the goal of this chapter is a general overview of these interactions.

The Nervous System and Snake Venom

Nervous System Overview

One of the main features differentiating animals from other kingdoms is the presence of the nervous system (Fig. 1) that coordinates the actions of the animal and transmits signals between different parts of its body.

The main objects of hunting or active defense for most of snakes are vertebrates and, rarely, arthropods. The nervous system in these animals is subdivided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS of vertebrates contains the brain and spinal cord. The CNS is well protected inside the skull and vertebral column and by the blood–brain barrier (BBB) that hampers envenomation. Nevertheless, some snake components can affect the CNS.

PNS is essentially the nerves, which run through most of the tissues of the body and ganglia. Unlike the CNS, the PNS is not protected by BBB, being exposed to action of snake toxins. The PNS is divided into the somatic nervous system and the autonomic nervous system. The somatic nervous system mediates voluntary movement.

The autonomic nervous system, comprising the sympathetic nervous system and the parasympathetic nervous system, regulates involuntary functions (mainly, smooth muscle contraction and glandular secretion). Sometimes the enteric nervous system is distinguished; it represents a large semi-independent part of the autonomic nervous system, the function of which is to control the gastrointestinal system. In some cases the sensory system is identified as well.

The nervous system contains two main categories or types of cells: neurons and glial cells. Neuron's most fundamental property is that they communicate with other

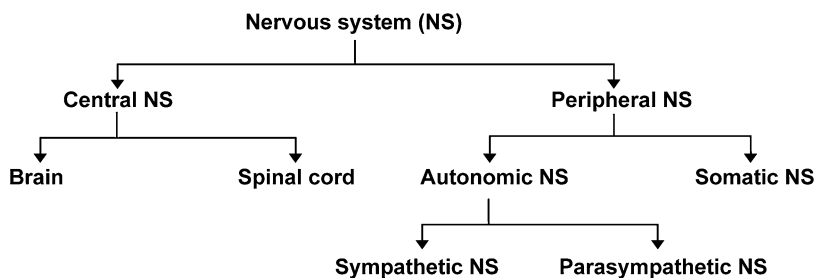


Fig. 1 Outline of the nervous system

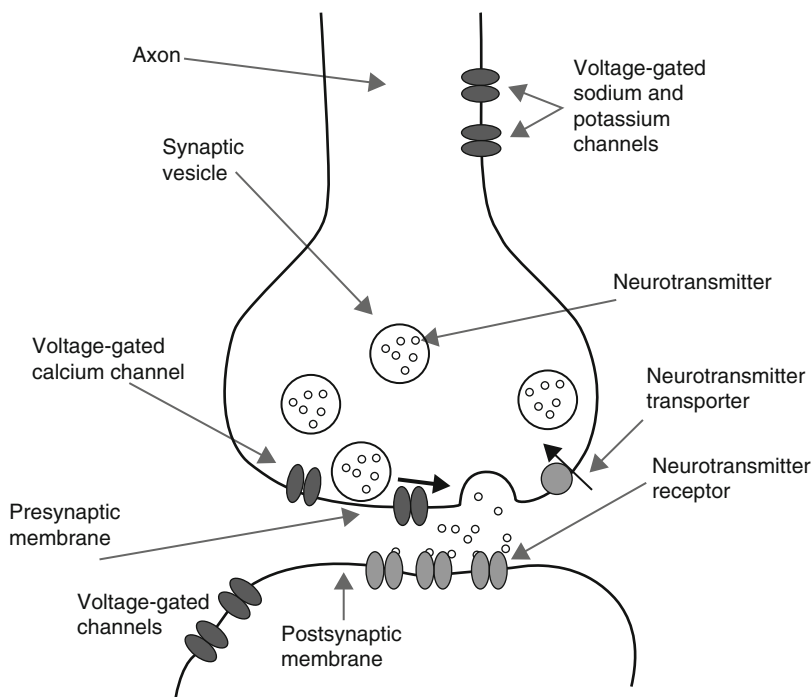


Fig. 2 Schematic outline of chemical synapse

cells via synapses (Fig. 2), which are membrane-to-membrane junctions containing molecular machinery that allows rapid transmission of signals, either electrical or chemical. Many types of neuron possess an axon, a protoplasmic protrusion that can extend to distant parts of the body and make thousands of synaptic contacts.

The signal generated by neuron is propagated through the axon to the synapse, voltage-gated ion channels being the main parts of signal propagation machinery. At the synapse electric signal activates neurotransmitter release from presynaptic membrane (Fig. 2). Neurotransmitter travels to postsynaptic membrane and activates receptors located there, thus transmitting signal to the receiving cell.

The basic efferent route of nerve signals begins in the upper motor neurons within the primary motor cortex in the brain. The upper motor neurons transmit the stimuli through release of neurotransmitter acetylcholine from their axon terminal knobs; the acetylcholine activates the sensory nicotinic acetylcholine receptors (nAChR) at postsynaptic site of lower alpha motor neurons of the brainstem and spinal cord. In turn, alpha motor neurons release acetylcholine from their axon terminal knobs at neuromuscular synaptic clefts that is received by postsynaptic nAChR of muscles, converting thereby the stimuli to contraction.

The sympathetic nervous system has the first synapse (preganglionic neuron to postganglionic neuron in a ganglion) which is mediated by nAChR. The next junction formed by axon of the postganglionic neuron is generally mediated by

adrenergic receptors activated by either noradrenaline (norepinephrine) or adrenaline (epinephrine). The parasympathetic nervous system utilizes acetylcholine as a mediator which acts on nAChR of postganglionic neurons in ganglia and on the muscarinic acetylcholine receptors (mAChR) of a target organ.

At the sensory systems, voltage-gated and acid-sensing (ASICs) ion channels are generally considered as players in the pain pathway; however, transient receptor potentials (e.g., TRPV1), purinoceptors (e.g., P2Y), kinin B2, and prostaglandin EP2 receptors are also critically involved in pain sensation.

Voltage-gated ion channels constitute a class of transmembrane ion channels that are activated by changes in electrical potential difference near the channel; these types of ion channels are especially critical in neurons but are common in many types of cells. They have a crucial role in excitable neuronal and muscle tissues, allowing a rapid and coordinated depolarization in response to triggering voltage change. Found along the axon and at the synapse, voltage-gated ion channels directionally propagate electrical signals.

Two main superfamilies of receptors are involved in nervous system functioning. These are ligand-gated ion channels and G-protein-coupled (or metabotropic) receptors. Receptors of both these superfamilies are affected by snake toxins. The main targets of snake neurotoxins are acetylcholine receptors both nicotinic and muscarinic. Recently, neurotoxins that affect adrenergic receptors and ASIC have been found.

nAChR, a pentameric transmembrane ion channel, is presented by several isoforms depending on a subunit composition. The so-called muscle-type nAChR is composed of two $\alpha 1$, one $\beta 1$, one γ (or ϵ), and one δ subunits. Neuronal nAChR may be composed of either α subunits only (homooligomeric receptors) or combination of α and β subunits (heterooligomeric). For example, nAChRs at mammalian ganglionic synapses are formed mostly by $\alpha 3$ and $\beta 4$ subunits.

Adrenergic and muscarinic receptors are metabotropic transmembrane G-protein-coupled receptors presented in mammals by several subtypes too: $\alpha 1$ - $\alpha 2$, $\beta 1$ - $\beta 3$, and M1-M5, respectively. All subtypes of muscarinic receptors are found in the CNS, while PNS comprises M2 and M3 subtypes that control, for example, the tonus of muscle cells in the heart, lung, intestine, and bladder.

Snake Venom Overview

Snake venoms are the complex mixtures of proteins and peptides belonging to several structural types and exerting strong physiological effects. Different components of snake venom serve to damage the functions of various systems in a prey organism; for example, some of them evoke severe disturbance of the hemostatic system, while others interfere with cell adhesion or the complement system. However, the main task of the venom is to paralyze or kill the prey efficiently, and this can be done easier by disturbing the function of the nervous system. All snake venoms very tentatively may be classified into “neurotoxic” (Elapidae family: Elapinae, Hydrophiinae, Australian elapids) and “hemotoxic” (Viperidae family: Viperinae,

Crotalinae); however, venoms of practically all snake genera contain substances disturbing nervous system function. They are usually called neurotoxins. At the same time, venoms comprise components, which affect the nervous system, but their toxic effects are not mediated through the nervous system itself, and therefore, these substances cannot be referred to as neurotoxins.

Snake venom toxins evolve more rapidly than other proteins through accelerated changes in the protein coding regions. This phenomenon, called “accelerated segment switch in exons to alter targeting” (ASSET), plays an important role in functional diversity within each structural type of snake toxins. In this phenomenon, short sequences in exons are radically changed to unrelated sequences (Doley et al. 2009). Hence, toxins arising from one ancestor and having one structural type may exert various effects on the nervous system. Furthermore, not all toxins thus generated within one particular structural type might display the well-defined biological effect.

Depending on snake species, neurotoxins can be represented in venom by different structural and functional classes. Some of them possess enzymatic activity while others do not. Their molecular masses cover the range from about two to more than fifty kilodaltons. They may contain several subunits bound either by disulfide bridges or non-covalently. No matter how amazing it seems, toxins belonging to most structural types presented in snake venoms may affect the nervous system or its parts.

To achieve the main task of the venom, a toxin should impair functions of the somatic nervous system: paralysis developed will deprive a prey of a chance to escape and capability to breathe. Indeed, toxins of different structural types isolated from different snake venoms have been found to disturb the somatic part of PNS at diverse stages of the nerve signal transmission. However, it has been shown also that several types of snake toxins affect the autonomic part of PNS.

Structural Types of Snake Venom Toxins

Considering all snake venoms, the most abundant components are, undoubtedly, phospholipases A2 (PLA2s). The toxins of so-called “three-finger toxin” family also form very abundant group. They prevail in “neurotoxic” venoms of Elapidae snakes. Furthermore, venoms contain polypeptides of Kunitz type that are homologous to bovine pancreatic trypsin inhibitor (BPTI). Toxins from the above structural types show diverse effects on the nervous system. Venoms of snakes, especially of Viperidae family, are also rich in proteases (serine proteases and metalloproteases), but no direct effects on the nervous system were found for these proteins. Other enzymes are also common for snake venoms; however, among these enzymes only acetylcholinesterase (AChE) is involved in the interaction with the nervous system. Toxins of other structural types that have been shown to affect the nervous system are represented by cysteine-rich secretory proteins (CRISPs), growth factors (nerve growth factor, NGF), and small peptides. Some structural types have been found in 1–2 copies only, for example, wesprins (ohanin) and prokineticins (protein A).

Phospholipases A2

PLA2s (phosphatide 2-acylhydrolase, EC 3.1.14) represent a superfamily of lipolytic enzymes which specifically hydrolyze the ester bond at the sn-2 position of glycerophospholipids resulting in the generation of fatty acid (usually arachidonic acid) and lysophospholipids. There are four main types of PLA2s: secreted, cytosolic, Ca^{2+} -independent, and lipoprotein-associated PLA2s (Kang et al. 2011). The PLA2 superfamily consists of about 20 groups which are further subdivided into several subgroups basing on subunit composition and substrate preference. Structurally PLA2 can be a monomeric protein or be composed of one, two, three, or five homologous subunits in non-covalent association, at least one of them having the phospholipolytic activity. Secreted PLA2s are very abundant in snake venoms. The venoms contain enzymes of groups I and II only. Group I consists of mammalian pancreatic PLA2s and PLA2s from venoms of Elapidae snakes. They are monomers of 12–17 kDa with 7 disulfide bridges (Fig. 3a).

Group II comprises PLA2s normal in venom of Viperidae snake family. PLA2s of group II are characterized by the shift of a disulfide bridge from N-terminus to the C-terminal extension. Some of them composed of two homologous subunits in non-covalent association (Fig. 3c). As a rule, the enzymatically active basic subunit of the PLA2 is toxic and binds to a specific membrane receptor, while the nontoxic component remains in solution. The acidic subunit behaves as a “chaperon” preventing a nonspecific binding of the enzyme to other substrates and potentiates the toxicity. For example, the acidic component of viperotoxin F potentiates the neurotoxicity of the basic subunit but reduces its enzymatic activity, while that of vipoxin plays a multifunctional role. It stabilizes the neurotoxic component of the complex preserving the toxicity for a long time and decreases the neurotoxicity of the basic PLA2 and its catalytic activity (Kang et al. 2011). In the whole, the role of enzymatically inactive PLA2 subunits is in targeting the toxin to specific binding sites.

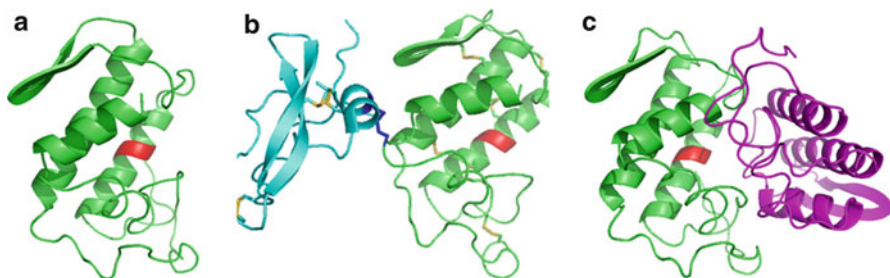


Fig. 3 Crystal structures of phospholipases A2 possessing neurotoxicity: (a) agkistrotoxin (PDB code 1A2A), (b) β -bungarotoxin (1BUN), (c) vipoxin (1JLT). Active site residues (His and Asp) are shown in red. In (b) BPTI-like subunit is shown in cyan and inter-chain disulfide in blue. In (c) enzymatically inactive subunit is shown in magenta

One of the most studied PLA2 effects is presynaptic neurotoxicity. In addition to PLA2s, this activity is manifested by heterodimeric toxin β -bungarotoxins. One subunit of these toxins (chain A) is a PLA2, and other is homologous to Kunitz-type inhibitors (Fig. 3b). In β -bungarotoxins, the structure of the enzymatically active subunit is similar to that of class I PLA2s. These toxins are believed to recognize sites at K^+ -channels of the plasmatic membrane. Interactions between the subunits at the interface region create conformational changes in both chains. A protein structurally resembling β -bungarotoxins is an agonist of ASIC receptor.

PLA2s may also exert postsynaptic neurotoxicity acting either on nAChR, e.g., bitanarin, or mAChR. Bitanarin from puff adder venom seems to be a unique PLA2 as it has molecular mass of 27.4 kDa and contains 28 cysteine residues forming 14 disulfide bonds within a single polypeptide chain (Vulfius et al. 2011).

Hydrolysis of phospholipids occurs at the active site of PLA2 molecule that involves His48 and Asp49 residues. However, neurotoxic activity of PLA2 depends weakly, if at all, on the enzymatic activity. The affinity of PLA2s to phospholipids covers the micromolar range, while PLA2s exert most of its activities in the nanomolar range of concentrations. “Target model” proposed by Kini and Evans (1989) explains that a “pharmacological site” in the PLA2 molecule recognizes a complementary “target site” in target cell or tissue, which may be a protein that is called “target protein.” To date, two types of PLA2 receptors are known: N (“neuronal”) and M (“muscular”) but their biochemical characterization is scarce.

There is unlikely to be a distinct “presynaptic neurotoxic site” on a PLA2 molecule similar to the enzyme active site. Instead, different parts of the toxin molecule are involved in different stages of the complex process of neurotoxicity that contribute to the final outcome (Pungercar and Krizaj 2007).

Three-Finger Toxins

Three-finger toxins (TFTs) have been found in venom of Elapidae and Colubridae. The polypeptide chain of these compounds consists of 58–81 amino acid residues stabilized by 4–5 disulfide bridges and has a three-finger fold of spatial structure. This typical fold comprises three loops (fingers) with five β -strands forming a twisted β -sheet, emerging from a small hydrophobic core containing four conserved disulfides (Fig. 4). The same fold is characteristic to proteins of Ly-6/uPAR superfamily found through animal kingdom.

Within the family, TFTs differ by length of their polypeptide chain and a number of disulfide bridges. Thus, toxins of one group which includes short-chain α -neurotoxins, β -cardiotoxin, cytotoxins, fasciculins, mambalgins, calciseptine, etc. consist of 57–62 amino acid residues bound by four disulfide bridges (Fig. 4a). Toxins of other group, consisting of long-chain α -neurotoxins (Fig. 4c), γ -neurotoxins, hannalgesin, κ -neurotoxins, and nonconventional or “weak” toxins (Fig. 4c), have additional 2–9 residues at the C-terminus and consist of 66–74 residues. They have an extra fifth disulfide. Muscarinic and muscarinic-like toxins are intermediate in length with 63–66 amino acid residues and 4 disulfide bonds.

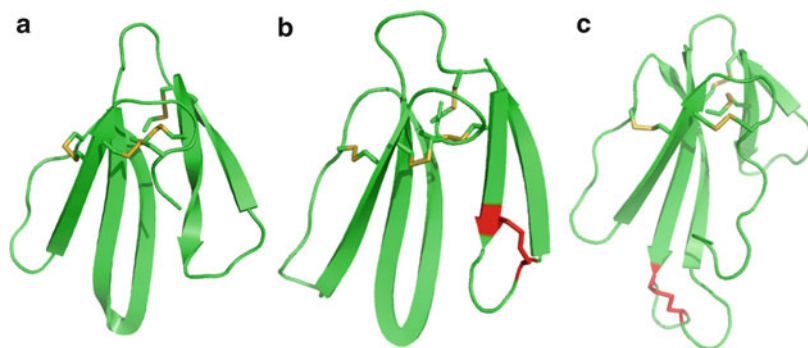


Fig. 4 The spatial structures of three-finger toxins: (a) short-chain toxin erabutoxin a (PDB code – 5EBX), (b) weak (nonconventional) toxin candoxin (1JGK), (c) long-chain α -cobratoxin (2CTX). Extra disulfide bridges in candoxin and α -cobratoxin are shown in red

α -Neurotoxins (both short chain and long chain) block muscle-type nAChR. Long-chain α -neurotoxins and κ -neurotoxins have an additional disulfide bond in their central loop II that gives rise to a turn and a short helical segment at the tip of the loop. This feature determines their capability to bind “neuronal” nAChRs (Kini 2011). In contrast, “weak” toxins and γ -bungarotoxin have an additional disulfide at the N-terminal loop I that twists and draws the loop in an orthogonal position. As in “weak” toxins, an additional disulfide in loop I is also present in the endogenous nAChR modulators lynx1 and SLURPs belonging to the Ly6/uPAR superfamily (Chang et al. 2002; Mordvintsev et al. 2009).

Within groups, along with the conserved motives, there are some differences in the toxin structures that result in very diverge biological activities. Thus, fasciculins inhibiting AChE contain a cationic cluster and an arginine residue in the area of loop I, while cardiotoxins (cytotoxins) which are structurally similar to fasciculins do not contain these elements. The main effect of cytotoxins is unspecific cell lysis. The binding of these toxins to the same enzyme (AChE) appears to be different: in contrast to fasciculin, cardiotoxins are bound with loop II to the peripheral site of AChE (Ranaei-Siadat et al. 2004). Moreover, if compared to the site by which fasciculins bind to AChE with the site by which α -neurotoxins bind nAChR, they are found at the opposite sides of the molecule.

Although most TFTs are monomers, some of them exist as dimers. Thus, κ -neurotoxins from krait venom, which are similar structurally to long-chain α -neurotoxins, exist as non-covalent homodimers; haditoxin (from King cobra venom) is a non-covalent homodimeric short-chain α -neurotoxin. Thailand cobra venom contains disulfide-bound homodimer of α -cobratoxin and heterodimers formed by α -cobratoxin with cytotoxins from the same venom. Dimerization seems to be crucial for the ability of these toxins to bind neuronal $\alpha 3\beta 2$ nAChR (Osipov et al. 2012).

The detailed information about TFTs can be found in many reviews, e.g., more recent one by Kini (2011).

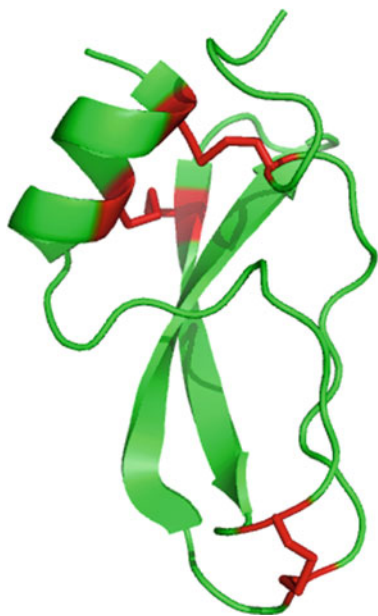
The three-finger fold is characteristic of snake toxins acting at different stages of nerve impulse transmission in different departments of the nervous system. Thus, the cholinergic system is impaired by long-chain and short-chain α -neurotoxins and their dimers, γ -neurotoxins, κ -neurotoxins, “weak” toxins, and muscarinic toxins, blocking different types of AChR, as well as by fasciculins, interacting with AChE. The adrenergic system is disturbed by β -cardiotoxin and other adrenoblockers. Calcium channels are blocked by calciseptine, ASIC – by mambalgins. Hannalgesin is an effector of opioid and nitric oxide systems.

Kunitz-Type Polypeptides

This fold is characteristic of small-size polypeptide inhibitors of serine proteases like trypsin and chymotrypsin. Indeed, some venom polypeptides of Kunitz type have shown such an activity. However, toxins of this structural type found in snake venoms manifest also some other biological properties. For example, dendrotoxins and calcicludine are channel blockers.

Dendrotoxins are blockers of K^+ -channels, preferably of neuronal subtypes. They are presented by several homologues (α -, β -, γ -, and δ -dendrotoxin and toxins I and K) exclusively in venom of mambas. Their single polypeptide chain contains 57–60 amino acid residues cross-linked by three disulfide bridges (Fig. 5). Dendrotoxins are homologous to Kunitz-type serine protease inhibitors, such as aprotinin or

Fig. 5 Crystal structure of α -dendrotoxin. PDB code – 1DTX. Disulfide bridges are shown in red



bovine pancreatic trypsin inhibitor (BPTI), although they have little or no antiprotease activity. For example, dendrotoxin- α from green mamba venom shares 35 % sequence identity with BPTI, including Cys residues. On the other hand, usually the protease inhibitors do not block K^+ -channels. Dendrotoxins are also related to kaliclidines from sea anemones, another K^+ -channel blockers of the Kunitz type (Harvey 2001).

Calcicludine from *Dendroaspis angusticeps* mamba venom is a 60-amino acid polypeptide homologous to dendrotoxins. It specifically blocks most of the high-threshold Ca^{2+} -channels (L, N, or P type), especially the L-type component of the Ca^{2+} current (K_i 0.2 nM in rat cerebellar granule neurons), and does not block K^+ -channels (Schweitz et al. 1994).

In β -bungarotoxin described above one subunit (chain B) is a Kunitz-type polypeptide.

Peptide Neurotoxins

There are two main groups of neurotoxic peptides found in snake venom: waglerins and sarafotoxins. Waglerins from Wagler’s pit viper are selective blockers of muscle-type nAChR. Waglerin is proline-rich peptide; its polypeptide chain consists of 22–24 amino acid residues with a single disulfide bond Cys9-Cys13 (Fig. 6). The toxin molecule has no ordered structure with the exception of the small fragment within the disulfide loop. The disulfide and N-terminal part of the molecule are important for functional activity. C-terminus does not contribute to waglerin toxicity (Hsiao et al. 1996). Azemiopsin from the venom of the viper *Azemiops feae* is a 21-amino acid peptide. Structurally, it is related to waglerins; however, it has no disulfide bridge. Azemiopsin exerts the same biological effect as waglerins, being a blocker of muscle-type nAChR with some preference for mature receptor form (Utkin et al. 2012).

Sarafotoxins are 21-amino acid peptides from burrowing asp and are related to mammalian endothelin-1. They are endothelin-B receptor antagonists, and their neuroprotector and other effects on the nervous system seem to be nonspecific. Centrally administered, it produces a transient increase in blood pressure accompanied by sympathetic nerve activity (Kumar et al. 1997).

Summarizing the data described above, one can say that the snake toxins of very diverse structural groups are involved in the interaction with the nervous system. The details of these interactions will be considered in the following sections.

Fig. 6 Amino acid sequences of azemiopsin, waglerin, and sarafotoxin

DNWWPKPPHQGPR-PPRPRPKP	AZEMIOPSIN
GGKPDLRPCHPPCHYIPRPKPR	WAGLERIN-I
PLCTCNDMTDEECLNFCHQDVIW	SARAFOTOXIN

Snake Toxins Interacting with CNS

As discussed above CNS is protected by BBB. This barrier greatly diminishes the effects of snake toxins on this department of the nervous system. All interactions of snake toxins with CNS can be classified into three main groups:

- I. Direct toxin interactions with CNS or its structures in vivo
- II. Toxin interaction with cells or with subcellular fractions of CNS in vitro
- III. Indirect toxin effects on CNS

The details of these interactions were considered in recent review (Osipov and Utkin 2012); therefore, only main points will be discussed here.

Direct Interaction of Snake Venom Polypeptides with CNS or Its Structures In Vivo

Some snake toxins may penetrate BBB and, therefore, induce effects observed after snake bite, while others need to be injected directly into the brain to affect the CNS. In general, the effective doses are much lower at the latter way of application. Snake venom toxins influence diverse CNS functions, one of them being pain sensation.

Thus, TFT α -neurotoxins, key components of elapid venoms, recognize and bind nAChRs, which are widely expressed in CNS including brain capillary endothelial cells, the main constituent of the BBB. The interaction with endothelial cells may facilitate α -neurotoxin penetration through the BBB. Several α -neurotoxins have been shown to exert analgesic effect mediated by CNS, long-chain hannalgesin (Pu et al. 1995) and α -cobratoxin, as well as short-chain cobrotoxin being among them. At intracerebroventricular injection, cobrotoxin and α -cobratoxin produce an antinociceptive response in mice (Chen et al. 2006; Cheng et al. 2009). The effect of α -cobratoxin is blocked by systemic administration of atropine, but not by methylatropine or naloxone suggesting the involving central cholinergic neurons. Najanalgesin from *N. atra* venom, highly homologous to TFT cyto-/cardiotoxins, exerts the antinociceptive effect in the rat neuropathic pain model when administered intrathecally. This effect is partly inhibited by intrathecal injection of naloxone or atropine suggesting the involvement of opioid and muscarinic receptors (Liang et al. 2009). One of central effects of snake venom PLA2s along with convulsions is an analgesia shown for crotoxin. Crotamine from rattlesnake venom, a small basic myotoxin belonging to the β -defensin-like superfamily, induces an analgesic effect which is inhibited by naloxone (Mancin et al. 1998).

On the contrary, ohanin, a 107-amino acid polypeptide from King cobra venom, shows dose-dependent hyperalgesic effect in a pain threshold assay as well as produced statistically significant and dose-dependent hypolocomotion in mice. It is several orders of magnitude more active when injected intracerebroventricularly as

compared to intraperitoneal injection. Ohanin belongs to toxin family vespryns that is related to PRY and SPRY domain proteins (Pung et al. 2005). Hyperalgesia was also observed after intracerebroventricular injection of a few micrograms of protein A (Mollay et al. 1999). This nontoxic polypeptide isolated from black mamba venom consists of 81 amino acid residues with 5 disulfide bonds and belongs to the AVIT (prokineticin) family.

Dendrotoxins from mamba venom, showing potent convulsant activities, cross the BBB and provoke an increase in the activity at monoaminergic terminals. Intrahippocampal infusion of 35 pmol of dendrotoxin K and α -dendrotoxin was associated with elevated extracellular aspartate and glutamate (Richards et al. 2000); transient functional change was consistent with an action on presynaptic K^+ -channels.

Fasciculins block acetylcholinesterase of locus coeruleus that results in significant increase of noradrenaline level, and they also block acetylcholine-evoked release of dopamine in rat striatum (Abo et al. 1989; Dajas-Bailador et al. 1996).

Muscarinic toxins may serve either as a selective agonist or a selective antagonist for M1 as well as a selective antagonist for M4 neuronal mAChR. They modulate learning and memory. The role of muscarinic toxins in the study of muscarinic neurotransmission is summarized in the recent review (Servent and Fruchart-Gaillard 2009).

Interaction of Snake Toxins with Cells or with Subcellular Fractions of CNS In Vitro

Due to their high affinity and selectivity, snake venom toxins are widely used as biochemical tools for the localization and for the functional characterization of corresponding receptor (ion channel) sites in CNS. So, α -neurotoxins are used for investigation of $\alpha 7$ nAChR; κ -bungarotoxin, for $\alpha 3$ -containing nAChR; and muscarinic toxins MT1 and MT4, for M4 and M1 subtypes of mAChR in the brain.

Dendrotoxins α -dendrotoxin, toxin K, toxin I, and γ -dendrotoxin have been used for the investigation of potassium channels containing Kv1.1, Kv1.2, and Kv1.6 subgroups (Harvey 2001), and calcicludine has been applied for localization of high-threshold Ca^{2+} -channels in the CNS (Schweitz et al. 1994). Sarafotoxin S6b was employed for the characterization of a neuronal form of endothelin receptor (Kumar et al. 1997).

Often researchers study interactions of snake venom components with cellular membranes obtained from the whole brain or from different structures of the CNS. As a rule snake venom toxins are used in the form of radioactive derivatives. These studies allow the determination of the binding parameters as well as specificity and selectivity of binding sites.

In general, α -neurotoxins, muscarinic toxins, dendrotoxins, and PLA2s are used to characterize their binding sites in the CNS.

Snake Venom Components Indirectly Affecting the CNS

Such compounds may disturb the immune or hemostatic systems or impair the integrity of the capillary wall that, in turn, can result in the dysfunction, sometimes severe, of the CNS. More details can be found in the recent review (Osipov and Utkin 2012).

Interestingly, the venoms and toxins may serve as labels for prey relocation by the predatory snake, which can be regarded as indirect influence on snake CNS. For example, as judged by discrimination trials of vomeronasal responsiveness (Saviola et al. 2013), rattlesnake predatory behavior responsible for effective relocation of an envenomated prey depends on the presence of the injected nonenzymatic disintegrins crotatroxins 1 and 2 in the prey organism. Disintegrins are polypeptides of 7–8 kDa found in Viperidae venom. They contain RGD motif essential for the binding to integrin receptors that results in the disruption of cell–cell and cell–extracellular matrix interactions.

Snake Toxins Impairing the Somatic PNS

Presynaptic Toxins

Toxins with presynaptic action are referred to as β -neurotoxins. Three distinct phases in the response to β -neurotoxins are distinguished (Pungercar and Krizaj 2007). Typically, there is an initial transient inhibition of evoked transmitter release (phase 1) followed by facilitation of transmitter release (phase 2) and a final phase during which there is a progressive fall in evoked release until transmission is blocked completely. This last phase of the neuromuscular response to β -neurotoxins is strictly dependent on phospholipase activity. Miniature end-plate potential frequency is similarly affected, although spontaneous release tends to occur at a low frequency after the failure of evoked neuromuscular transmission. As the miniature end-plate potential amplitude does not change significantly, synaptic vesicles do not fuse extensively, if at all, inside the nerve terminal, and the acetylcholine loading apparatus is not impaired by β -neurotoxins (Pungercar and Krizaj 2007).

β -Bungarotoxins

In β -bungarotoxins, an enzymatically active basic PLA2 (A subunit) is covalently bound to Kunitz-type module (B subunit) by an inter-subunit disulfide bond (Fig. 3b). In β -bungarotoxins, the covalently linked Kunitz-like subunit confers the target specificity by binding to voltage-dependent potassium channels (Kang et al. 2011; Pungercar and Krizaj 2007).

PLA2s

Presynaptic neurotoxicity has been found only for the members of groups IA, IIA, and IIIA. Their pathophysiological action appears to be closely similar. The main

neurotoxic PLA2 targets are cholinergic neurons. However, β -neurotoxins do not act exclusively on cholinergic neurons as well as certain cholinergic neurons are not affected by β -neurotoxins. In both CNS and PNS tissues, N-type PLA2 receptors were detected with several neurotoxic PLA2, but other binding proteins may exist as well. It should not be excluded that the toxin has to be internalized into a presynaptic site to complete its action as it can bind intracellular calmodulin and two isoforms of protein 14-3-3 (Pungercar and Krizaj 2007).

Dendrotoxins

Dendrotoxins belong to the same Kunitz-type proteins as Kunitz module of β -bungarotoxins. Dendrotoxin from green mamba venom initially was described as a prejunctional effector that facilitated the release of acetylcholine at a nerve–muscle synapse. At mouse and frog neuromuscular junctions, these toxins increase quantal content and induce single nerve action potentials to cause short bursts of repetitive activity. It has been suggested that they block a rapidly activating K current that is important for the control of the excitability of motor nerve terminals. To date, the facilitatory effects of the dendrotoxins are explained by blockade of some neuronal K channels (Harvey 2001).

Postsynaptic Toxins

In the somatic nervous system, their biologic effect is a block of nAChR at postsynaptic membrane of the neuromuscular synapse. Thus, they imitate the effects of the alkaloid curare and are called sometimes as curare-mimetic toxins. The best studied and the most active postsynaptic toxins are α -neurotoxins.

α -Neurotoxins

They belong to the TFT structural type. Short-chain α -neurotoxins block selectively nAChR of muscle type, while long-chain ones are capable to interact with neuronal $\alpha 7$ and $\alpha 9$ types as well. Affinity of α -neurotoxins to their targets is in a nanomolar range. Binding of long-chain α -neurotoxins to nAChR is practically irreversible; they stay bound to the receptor for several days. Short-chain α -neurotoxins dissociate from receptor easier. Due to their high affinity and selectivity, α -neurotoxins remain so far the best instruments for the study of several nAChR types.

“Weak” Toxins

Usually, nonconventional or “weak” toxins have lowered toxicity, justifying their name. These toxins also bind in vitro with muscle-type and $\alpha 7$ nAChRs virtually irreversible but with lower affinity than α -neurotoxins (Utkin et al. 2001). Injection of WTX, a “weak” toxin from Thailand cobra venom, into mice and rats produces signs of intoxication consistent with WTX interactions with both nAChRs and mAChRs. WTX shows an orthosteric interaction with nAChR and an allosteric interaction with mAChRs (Mordvintsev et al. 2009). Similar activities were found for γ -bungarotoxin (Chang et al. 2002).

Waglerins

Peptide neurotoxins waglerins from Wagler's pit viper are selective blockers of muscle-type nAChR. They block receptors, which contain ϵ subunit, with affinity by 2–3 order higher than those containing fetal γ subunit (Schmidt and Weinstein 1995). A novel peptide, azemiopsin from the venom of *Azemiops feae* viper, is also a blocker of muscle-type nAChR with some preference for the receptor containing ϵ subunit (Utkin et al. 2012). Waglerins and azemiopsin have affinities for nAChR in nanomolar range.

PLA2

Unusual PLA2 bitanarin from puff adder venom competes with [^{125} I]iodinated α -bungarotoxin for binding to human $\alpha 7$ and *Torpedo californica* nAChRs, as well as to acetylcholine-binding protein from *L. stagnalis*, the IC_{50} being 20 ± 1.5 , 4.3 ± 0.2 , and 10.6 ± 0.6 μ M, respectively. It also blocks reversibly acetylcholine-elicited current in isolated *L. stagnalis* neurons with IC_{50} of 11.4 μ M. The receptor-blocking ability of bitanarin does not seem to depend on its high phospholipolytic activity (Vulfius et al. 2011).

Toxins Acting Inside a Synaptic Cleft

Acetylcholinesterase

AChE is an enzyme which cleaves acetylcholine into choline and an acetic acid, thereby terminating the chemical impulse within synapses. AChE (EC 3.1.1.7) is a member of the cholinesterase family of hydrolases. AChE from snake venom exists in the form of soluble, hydrophilic monomers of 62–65 kDa, in contrary to the synaptic enzyme that is a tetrameric membrane-anchored glycoprotein or, more rarely, a water-soluble dimer. N-terminal catalytic domains of the venom enzymes exhibit a high level of homology. All six cysteines, four glycosylation sites, and the catalytic triad (Ser200, Glu327, and His440) are conserved. Aromatic residues lining the active site cleft of the AChE including the tryptophan residue binding to the quaternary ammonium group of acetylcholine are conserved as well (Kang et al. 2011).

AChE is abundant in the venoms of elapid snakes, except mambas. At the same time, venom of mamba contains fasciculins, which are inhibitors of AChE. Interestingly, AChE is one of the fastest enzymes known; moreover, snake venom enzymes are yet more active than *Torpedo* and mammalian AChEs in hydrolyzing acetylcholine. Despite the key role of junctional AChE in cholinergic transmission, it is assumed at present that venom AChE does not contribute to toxic action (Kang et al. 2011). The role of AChE in venoms remains to be uncovered.

Fasciculins

The toxins that block AChE are named fasciculins as they induce fasciculations of muscles due to accumulation of acetylcholine at the synapse. They bind to the peripheral site of AChE and block the entry of acetylcholine into the active site of

the enzyme, thereby preventing its hydrolysis. Fasciculins inhibit AChE in sub-nanomolar concentration range (summarized in Kini 2011).

Interestingly, some cobra venom cardiotoxins having secondary and tertiary structures similar to fasciculins have an opposite effect once they interact with AChE. Fasciculin seems to stabilize nonfunctional conformation, but cardiotoxins seem to stabilize the functional conformation of the enzyme (Ranaei-Siadat et al. 2004).

Calcium Channel Blockers

While calcicludine belonging to Kunitz type has been described as a blocker of L-type Ca current in CNS, a group of TFTs from mamba venom, including calciseptine and FS2, has shown to be blockers of L-type Ca channels in the heart, skeletal muscle, and other organs. The predicted Ca^{2+} -channel binding site also is present in closely related toxins, $\text{C}_{10}\text{S}_2\text{C}_2$ and S_4C_8 (Kini 2011). Calciseptine binds to a 1,4-dihydropyridine recognition site of the L-type calcium channel of rat synaptosomal membranes and physically block the calcium currents as well as allosterically modulates the binding of other L-type Ca^{2+} -channel ligands (Yasuda et al. 1993).

Snake Toxins Affecting the Autonomic PNS

Similarly to somatic PNS, ganglia of autonomic PNS contain cholinergic synapses, transmission at which is mediated by nAChR. However, the pharmacological properties of the nAChR at mammalian ganglion synapses are most attributable to $\alpha 3\beta 4$ type. Therefore, α -neurotoxins, being the blockers of muscle-type and $\alpha 7$ nAChRs, cannot exert a noticeable effect at the ganglion level. Among nAChR blockers, κ -bungarotoxins, which bind efficiently to neuronal $\alpha 3\beta 2$ type, can interact also with $\alpha 3\beta 4$ type albeit with lower affinity. However, a little is known about the interaction of κ -bungarotoxins with PNS. So, κ -bungarotoxin blocks nicotinic transmission in chick lumbar and rat superior cervical sympathetic ganglia at concentration of 40 nM (Chiappinelli and Dryer 1984) as well as in the chick ciliary (parasympathetic) ganglion at concentration of 75 nM, while α -bungarotoxin does not (Chiappinelli 1983).

At the same time, snake venoms contain some relatively selective and strong effectors of autonomic PNS.

Effectors of the Parasympathetic System

So far TFTs and PLA2s are found to affect parasympathetic PNS. Thus, muscarinic toxins of TFT family isolated from venoms of mambas (*Dendroaspis* spp.) interact with different types of mAChRs possessing the highly variable extracellular loop

regions. These toxins are highly homologous proteins but exhibit wide functional differences, being competitive antagonist, allosteric modulator, or even agonist of muscarinic receptors. These toxins display distinct pharmacological profiles at their interactions with the different muscarinic receptor subtypes, and some of them show strong preferences for specific receptor subtypes. So, MT1 and MT2, muscarinic toxins from *D. angusticeps* venom, produce the contraction of the longitudinal muscle from the guinea-pig ileum (M_3 receptors), but their effects are via different mechanisms. MT2 is a partial agonist at the M_3 receptors, while MT1 acts at presynaptic muscarinic receptors, which lead to increased acetylcholine release and subsequent contraction. However, muscarinic toxins usually preferably interact with “neuronal” M1 or M4 subtypes of muscarinic receptors. Only MT α and MT β , isolated from *D. polylepis* venom, show a slightly higher preference for the M_3 subtype (K_d of 3 and 140 nM for MT α and MT β , respectively) (Servent and Fruchart-Gaillard 2009).

Toxins with up to 50 % sequence identity with above muscarinic toxins have been identified in the *Naja kaouthia* venom; however, despite this high degree of sequence identity, their apparent affinity for mAChRs is very low. One of them, MTLP-1, competed weakly with radioactive ligand for binding to all mAChR subtypes, with the most pronounced effect (IC_{50} of about 3 μ M) on M_3 subtype (Kukhtina et al. 2000).

“Weak toxin” WTX from *N. kaouthia* venom displays intoxication symptoms similar to those of muscarinic agonists. It has induced a dose-dependent decrease in blood pressure and an increase in heart rate in rats and mice (Ogay et al. 2005). More detailed studies show that this toxin interacts with orthosteric site in nAChR and allosteric site in mAChRs (Mordvintsev et al. 2009). γ -Bungarotoxin from *B. multicinctus* venom exhibits M_2 mAChR-blocking activity but has a weak activity toward nAChR (Chang et al. 2002). Long-chain neurotoxin BM14 (82 amino acid) from *B. multicinctus* krait venom blocks M_2 mAChR subtype. It contains ten cysteines with unusual distribution through the polypeptide chain (Chung et al. 2002).

Among other structural toxin types, monomeric PLA2 toxins purified from *Naja naja sputatrix* and *Naja atra* venoms as well as dimeric PLA2 from *Crotalus atrox* display affinities for various mAChRs subtypes with affinities ranging from several nanomoles to 1 μ M, the highest affinity being to M_5 . Their inhibition properties are not correlated with the enzymatic activity (Servent and Fruchart-Gaillard 2009). PLA2 from Chinese cobra venom induces a weak contraction of the guinea-pig ileum that is inhibited by atropine suggesting a partial agonist effect on mAChRs (Huang et al. 2008).

Effectors of the Sympathetic System

A cytotoxin-like toxin from King cobra venom, called β -cardiotoxin, blocks β -adrenergic receptors with a binding affinity (K_i) of 5.3 and 2.3 μ M toward β_1 and β_2 subtypes, respectively. It induces a dose-dependent decrease of heart rate, in

contrast to classical cytotoxins (cardiotoxins) from snake venom that increase the heart rate in animals (Rajagopalan et al. 2007).

Although the general effect of classical cardiotoxins is low-specific cytolysis (forming ion pores in the lipid membranes), their above effect allows cautious suggestion of a protein target in cardiac myocytes that has not yet been identified.

Earlier reports have pointed the ability of muscarinic toxins to interact with adrenergic receptors. Recently it has been found that some muscarinic toxins can block adrenergic receptors with nanomolar and even sub-nanomolar affinity. Muscarinic toxins MT β and CM-3 from African mamba venom have sub-nanomolar affinity for the α 1A subtype and to a less extent for α 1B and α 1D subtypes of adrenergic receptors; at the same time, they show very weak affinity for muscarinic receptors (Blanchet et al. 2013). Furthermore, two novel toxins, ρ -Da1a and ρ -Da1b, have been isolated from green mamba venom: they more closely resemble muscarinic toxins than each other and display only 35 % amino acid sequence identity to β -cardiotoxin. However, ρ -Da1a has shown an affinity of 0.35 nM for the α 1A adrenoreceptor, and ρ -Da1b has displayed affinities between 14 and 73 nM for the three different α 2 adrenergic receptors (Maïga et al. 2012). Cytotoxins/cardiotoxins and muscarinic toxins are of TFT family.

It is worth to note that bradykinin-potentiating peptide BPP-10c alleviates MK-801-induced inhibition of nicotinic acetylcholine receptor activity in the PC-12 pheochromocytoma cell line (Nery et al. 2008). However, whole-cell recording did not reveal receptor activation by BPP-10c or potentiating of carbamylcholine-provoked receptor activity. Pheochromocytoma cells originate from neuroendocrine chromaffin cells that are structurally similar to postsynaptic sympathetic neurons. Therefore, the effect found can be considered as BPP-10c action on sympathetic nervous system.

Sensory Systems

K Channel Blockers

Dendrotoxins (α -, β -, γ -, and δ -dendrotoxin and toxins I and K) block different potencies both slowly inactivating and non-inactivating voltage-dependent K⁺ currents in rat peripheral sensory neurons. δ -Dendrotoxin is relatively selective for non-inactivating voltage-dependent K⁺ currents with IC₅₀ of 0.24 nM, and α -dendrotoxin is more active on slowly inactivating voltage-dependent K⁺ currents (IC₅₀ of 1 nM) (Hall et al. 1994; Harvey 2001).

ASIC Effectors

Mambalgins, a separate group of TFTs purified from the black mamba venom, block heteromeric channels composed of ASIC1a and ASIC2a subunits in central neurons and of ASIC1b-containing channels in nociceptors thus exerting the analgesic effect

as strong as that of morphine. This effect is, however, resistant to naloxone, and mambalgins cause much less tolerance than morphine and no respiratory distress (Diochot et al. 2012). Recently a toxin called MitTx was purified from the Texas coral snake *Micrurus tener tener* venom. It is heteromeric toxin composed of Kunitz–BPTI and PLA2-like proteins that together function as a potent, persistent, and selective agonist for ASICs, showing equal or greater efficacy as compared to acidic pH. MitTx is highly selective for the ASIC1 subtype at neutral pH; under more acidic conditions (pH < 6.5), MitTx massively potentiates (>100-fold) proton-evoked activation of ASIC2a channels. Purified MitTx elicits robust pain-related behavior in mice by activation of ASIC1 channels on capsaicin-sensitive nerve fibers (Bohlen et al. 2011).

CRISP

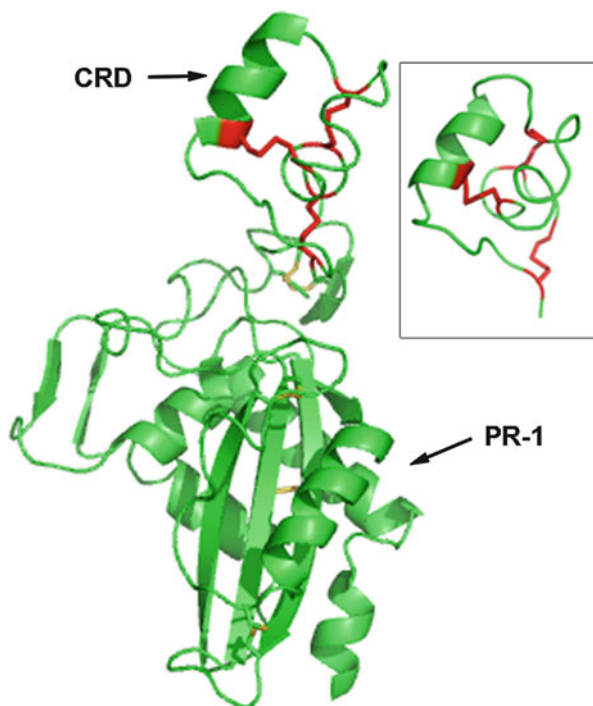
CRISPs from snake venom are single-chain proteins composed of about 220 amino acid residues. They are related structurally to the family of CRISP which participate in the processes of fertilization and maturation in mammals. With several exceptions snake venom CRISPs are blockers of different ion channels outside the nervous system. Their molecule consists of two structurally separate domains: N-terminal domain PR-1 (pathogenesis-related proteins of group 1), of about 150–160 amino acid residues, and C-terminal domain CRD (cysteine-rich domain), of about 40 residues (Fig. 7). These two domains are linked by a compact hinge region (of about 20 residues). CRD domain has been found structurally similar to K⁺-channel blockers (Fig. 7, inset) and may play an important role in the interaction of CRISP with different ion channels. Domain PR-1 comprises a putative Na⁺ ion-binding site and two conserved His residues forming a divalent cation-binding site which can bind Cd²⁺ and Zn²⁺ (Suzuki et al. 2008).

The CRISP targets in PNS are cyclic nucleotide-gated (CNG) ion channels that play pivotal roles in sensory transduction by retinal photoreceptors and olfactory neurons. The CRISP toxins pseudechetoxin and pseudecin from *Pseudechis australis* (Australian elapid) snake venom are the only known protein blockers of CNG channels. The apparent K_i of pseudechetoxin is 15 nM, while the affinity of pseudecin is 460 nM for olfactory channels composed of CNGA2 subunits, and K_i of pseudechetoxin is 70 nM and of pseudecin 1,000 nM for the CNGA1 subunit from rod photoreceptors (Yamazaki et al. 2002).

Crotalphine

Potent antinociceptive peptide crotalphine (pyroEFSPENCQGESSQPC) was isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Konno et al. 2008). Further studies have shown that depending on pain model different signaling pathways may be involved in crotalphine effects. Thus, in neuropathic pain crotalphine induces a potent and long-lasting opioid

Fig. 7 The crystal structure of CRISP triffin (PDB code – 1WVR). The spatial structure of sea anemone toxin Bgk, acting as voltage-sensitive K⁺-channel blocker, is shown in *inset*. Cysteine residues are shown in red



antinociceptive effect that surpasses that observed with standard analgesic drugs (Gutierrez et al. 2008). Peripheral δ -opioid and κ -opioid receptors, the nitric oxide–cyclic GMP pathway, and ATP-sensitive K⁺-channels are involved in this effect (Gutierrez et al. 2012). In prostaglandin E₂-induced hyperalgesia crotoalphine-induced antinociception involves peripheral CB₂ cannabinoid receptors and local release of dynorphin A, which is dependent on CB₂ receptor activation (Machado et al. 2014).

Neurotrophins (Growth Factors)

NGF and venom endothelial growth factor are the only growth factors found in snake venom; only NGF has shown to impact the nervous system. NGF is a member of neurotrophin family that belongs to growth factor superfamily. NGFs have been characterized from the venoms both of Viperidae (Trummal et al. 2011) and Elapidae snakes including Australian elapids (Earl et al. 2006). NGFs are glycosylated (Viperidae and Australian elapid venom) or non-glycosylated (Elapinae venom) non-covalently bound dimers. Only *B. arietans* NGF is a covalently bound non-glycosylated dimer. Molecular masses of subunits are in the range from 12.5 to 22 kDa, with few exceptions (34 kDa for NGF from *Bungarus caeruleus* venom and 35 kDa for NGF from *Agkistrodon bilineatus* venom) (Trummal et al. 2011).

Such a mass variation is explained by different degrees of glycosylation that does not seem to affect the biological activities of NGF.

NGF is a classical neurotrophin whose main function is support of neuronal maintenance and survival. Most of biological activities of NGF have been established on mammalian NGF. Concerning snake NGF, it can reduce the degeneration of nerve fibers and enhance the peripheral nerve regeneration and functional recovery from injury of sciatic nerves in mammals (cats) *in vivo* (Chen et al. 2004). Bioassays with cultured chick embryo ganglia and rat pheochromocytoma PC-12 cells reveal a promotion of fiber outgrowth by cobra NGF (Li et al. 1999). NGF exerts action through the two specific receptors, TrkA, a member of tropomyosin-related kinase subfamily of receptor tyrosine kinases, which regulate cell proliferation, and p75 neurotrophin receptor, a member of the tumor necrosis factor receptor superfamily that regulates apoptosis.

The physiological function of NGF in snake venom is not completely understood. Thus it was shown that venom NGF potently inhibited metalloproteinases isolated from the same or different venom and specifically bound to purified metalloproteinase immobilized on agarose beads (Wijeyewickrema et al. 2010). This fact was viewed as an indication of NGF role in prevention of venom autolysis (Wijeyewickrema et al. 2010). The other hypotheses suggest that NGF could act as cytotoxic proapoptotic factors, facilitate the efficient absorption of other venom components, or violate the normal immune response (Sunagar et al. 2013). Mammalian NGF was shown to play a key role in the generation of acute and chronic pain and in hyperalgesia in diverse pain states (McKelvey et al. 2013). Snake venom NGF may also possess this property and produce pain sensation in a victim. So, NGF may be involved in prey intoxication through several possible mechanisms.

Conclusion and Future Direction

Overall, snake venom toxins can severely impair the functions of the nervous system. They are isolated from different snake venoms, belong to diverse structural types, and display various biological activities. Thus, some neurotoxins affect voltage-gated ion channels. Dendrotoxins acting on potassium channels, calciseptine, calcicludine, and other calcium channel blockers should be mentioned among them. The action of β -neurotoxins might be at least partially explained by interaction with ion channels. Toxins interacting with ASICs were also found in snake venoms. A large group of neurotoxins block ligand-gated ion channels. These are α -, γ -, and κ -neurotoxins, weak toxins, and waglerins inhibiting nAChRs. Other large neurotoxin group includes effectors of metabotropic receptors. Muscarinic toxins, toxins affecting adrenergic receptors, NGF, and sarafotoxins belong to this group. Some snake venom proteins possess enzymatic activity. However, only acetylcholinesterase activity is directly involved in the action on the nervous system. Although PLA2s are enzymes also, their neurotoxicity only partially and not always depends on the phospholipolytic activity. Neurotoxic enzyme inhibitors were also

found in snake venoms. These are fasciculins, which block AChE at cholinergic synapses.

In any way, the action of all snake neurotoxins is directed to execute the main task of the venom – to paralyze or kill the prey. Obviously, neurotoxicity is an indispensable property of both “neurotoxic” and “hemotoxic” snake venoms.

Many of the snake venom neurotoxins described in this chapter have been successfully used and are used now as important biochemical tools in investigations of CNS and PNS as well as their components. The list of toxins affecting the nervous system is constantly replenishing, and new neurotoxins are still waiting for discovery.

Cross-References

► Secreted Phospholipases A₂ with β -Neurotoxic Activity

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S. Nirthanan (✉)

School of Medicine, Griffith University, Gold Coast, QLD, Australia

School of Medical Science, Griffith University, Gold Coast, QLD, Australia

Menzies Health Institute Queensland, Gold Coast, QLD, Australia

e-mail: n.nirthanan@griffith.edu.au

W. Awal (✉)

School of Medical Science, Griffith University, Gold Coast, QLD, Australia

e-mail: wasim.awal@griffithuni.edu.au

N.R. Nirranjan (✉)

Faculty of Science, University of Queensland, Brisbane, QLD, Australia

e-mail: navin.nirranjan@uq.net.au

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Abstract

α -Neurotoxins from snake venoms act on postsynaptic nicotinic acetylcholine receptors at the neuromuscular junction to produce clinically significant skeletal muscle paralysis following envenomation. From the seminal discovery almost 50 years ago of α -bungarotoxin, the prototypical α -neurotoxin, our knowledge of the composition of snake venoms, snake envenomation and its treatment, physiology of the neuromuscular junction, structure and function of nicotinic acetylcholine receptors, protein-protein interactions, and pathophysiological mechanisms that underpin diseases like myasthenia gravis has grown exponentially. The potential for the discovery of therapeutic lead compounds from snake venoms has also been advanced. This review looks back at the historical milestones of this research; highlights the challenges of nomenclature pertaining to the increasing number of novel toxins; provides an overview of nicotinic acetylcholine receptors and their subtypes; discusses the classification of snake α -neurotoxins; describes the three-finger protein fold that is the characteristic scaffold of most snake α -neurotoxins, while noting the unique exceptions; details the pharmacology of snake α -neurotoxins including their mechanisms of neuromuscular blockade, reversibility of blockade, and differential binding affinities for the two different acetylcholine-binding sites on the muscle nicotinic receptor; and lastly, delivers an updated and in-depth analysis of the structure-function relationships of the α -neurotoxin-nicotinic acetylcholine receptor interaction, including information gleaned from mutational, computer modeling, and structural studies of complexes formed between α -neurotoxins and receptor components or homologues. These new facets in toxinology have significantly broadened the scope of α -neurotoxins in scientific discovery as well as their therapeutic potential.

Keywords

α -Neurotoxin • Three-finger toxin • Structure-function • Cholinergic neurotransmission • Nicotinic acetylcholine receptor

Introduction

An extensive array of terrestrial and aquatic animals is capable of producing venoms comprising a cocktail of toxins aimed at incapacitating prey, molded through the evolutionary predator–prey dichotomy over millennia (Naamati et al. 2009). Often, these protein and peptide toxins are united by distinct structural motifs but diverge significantly in their mechanisms of action. Together, the combination of toxins in venom produce a plethora of potentially fatal biological effects such as peripheral and central neurotoxicity, cytotoxicity, hemotoxicity, and myotoxicity (Da Silva et al. 2004; Kini and Fox 2013). The phenomenal effectiveness of venom toxins can be attributed to their ability to bind to their molecular targets with high affinity and precision selectivity and in many instances with synergistic activities between different toxins. Their lethality notwithstanding, these toxins have been exploited to provide an array of biomolecules that are integral tools in scientific discovery and offer therapeutic potential as drug leads (Lewis and Garcia 2003; Harvey 2014).

Snake venoms may contain over a hundred different proteins, most of which belong to a relatively small number of structural superfamilies with a similar three-dimensional structure but diverse physiological targets and pharmacological effects (Ménez 1998; Kini and Fox 2013). Snake venom protein families are broadly categorized into enzymatic and nonenzymatic proteins. The enzymatic proteins include phospholipases A₂, serine proteases, snake venom metalloproteases, acetylcholinesterases, and phosphodiesterases, many of which contribute to the overall toxicity of snake venom, particularly of *Viperidae* species (see reviews Bailey (1998) and Kang et al. (2011)). The predominant nonenzymatic snake venom proteins include three-finger toxins (3FTXs), C-type lectin-related proteins, serine proteinase inhibitors, disintegrins, helveprins, waprins, sarafotoxins, vespryns, and natriuretic peptides (for a recent review see McCleary and Kini (2013)). The 3FTx subfamily constitutes the most abundant of the nonenzymatic snake venom proteins and has been found in the venom, primarily of *elapidae* and *hydrophiidae* as well as *colubridae* and *viperidae* species. Their name is derived from their distinctive protein scaffold characterized by three finger-like loops extending from a hydrophobic core that is stabilized by four disulfide bridges. However, in spite of their common protein scaffold, they exhibit diverse pharmacological activity by targeting a variety of receptors, ion channels, and other physiological processes (Kini and Doley 2010).

This chapter will focus on a specific subset of 3FTxs, the three-finger α -neurotoxins (3F α -NTxs), that act on postsynaptic nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction to produce skeletal muscle paralysis of clinical significance in human envenomation (Nirthanan and Gwee 2004; Barber et al. 2013; Utkin 2013; Tsetlin 2015). From the first breakthrough discovery almost 50 years ago of α -bungarotoxin, the prototypical α -neurotoxin, toxinology has seen influential revelations regarding the composition of snake venoms, snake envenomation, and its treatment as well as discovery of therapeutic lead compounds. However, the greatest contribution has arguably been the crucial role of α -bungarotoxin

as a pharmacological probe in the characterization and localization of nAChRs, making them one of the best studied receptors to date (Chang 1999; Dutertre and Lewis 2006; McCleary and Kini 2013; Utkin 2013).

Historical Perspectives of Snake Neurotoxin Research

The dawn of animal venom research was initially fuelled by the need for understanding the symptoms caused by envenomation and their remedies, in particular antivenoms. This was justifiable, given the burden of snake bites, estimated to cause over 400,000 amputations and between 20,000 and 125,000 deaths worldwide every year (Williams et al. 2010).

As early as 1781, Abbé Felice Fontana, the founder of modern toxinology, studied the myotoxic and coagulative properties of snake venom (Hawgood 1995). While the first neurotoxic compounds were crudely isolated from cobra venom by Toyosaku Sasaki in 1957 and from the sea snake by Carey and Wright in 1960, Chang and Lee were the first to use zone electrophoresis on starch to effectively isolate and characterize three distinct neurotoxins from the venom of the Taiwanese many-banded krait (*Bungarus multicinctus*) in 1963 (Lee 2012). The slowest moving fraction was named α -bungarotoxin, later found to have postsynaptic inhibitory activity on nAChRs; and the faster moving fractions were denoted with β and γ prefixes respectively and were shown to have presynaptic activity (Chang 1999). Consequently, thereafter, any toxin derived from animal venoms from various phyla and found to exhibit postsynaptic activity affecting cholinergic transmission was given the “ α ” prefix and referred to as α -neurotoxins (Chang 1999). The plant alkaloid d-tubocurarine, a known competitive antagonist of the muscle nAChR and precursor of muscle relaxant drugs in current clinical use, was found to prevent the binding of α -bungarotoxin, suggesting that α -bungarotoxin occupied the same binding sites on the nAChR (Nirthanan and Gwee 2004; Barber et al. 2013; Tsetlin 2015). Furthermore, α -neurotoxins also became referred to as “curaremimetic” toxins as they mimicked the pharmacological activity of d-tubocurarine (Nirthanan and Gwee 2004; Barber et al. 2013).

Following these discoveries, Eaker and Porath (1967) sequenced the amino acid residues of “toxin α ” from the spitting cobra (*Naja nigricollis*), leading to further studies on the primary sequences of toxins from cobras, kraits, and sea snakes (Endo and Tamiya 1987). This allowed for chemical modification experiments in the 1970s and subsequent determination of the crystal structure of erabutoxin-b from *Laticauda semifasciata* aimed at elucidating the function and three-dimensional structure of neurotoxins (Endo and Tamiya 1987). Further analyses including nuclear magnetic resonance (NMR) and X-ray diffraction of other neurotoxins such as α -bungarotoxin and α -cobratoxin have advanced our knowledge on the conformational aspects of toxins, immunology of neurotoxins, and the characterization of the nAChR (Ménez 1998; Barber et al. 2013; Tsetlin 2015).

Snake Toxins Affecting Cholinergic Neurotransmission

A variety of neurotoxins from snake venoms target cholinergic neurotransmission (see Fig. 1). Neuromuscular paralysis is the primary form of neurotoxicity elicited during envenomation by *elapidae* (kraits, cobras, Australian elapids, and coral snakes), *hydrophiidae* (sea snakes), and some true *viperidae* species (Lewis and Gutmann 2004; Ranawaka et al. 2013). The toxins from these snakes act primarily on the neuromuscular junction, preventing acetylcholine-mediated neurotransmission through various methods including presynaptic and postsynaptic activity (Nirthanan and Gwee 2004; Barber et al. 2013). Collectively, these effects result in flaccid paralysis of skeletal muscles including the diaphragm and other muscles of respiration ensuing in death through respiratory failure (Barber et al. 2013; Ranawaka et al. 2013; Kularatne and Senanayake 2014).

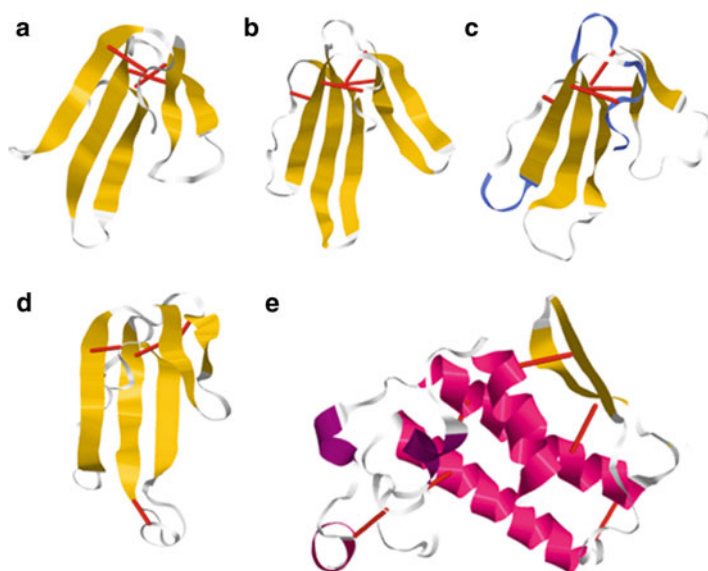


Fig. 1 Snake toxins affecting cholinergic neurotransmission (a) postsynaptic α -neurotoxin: **erabutoxin-a** (*Laticauda semifasciata*) (1ERA); (b) muscarinic acetylcholine receptor toxin: **muscarinic toxin 1** (*Dendroaspis angusticeps*) (4DO8); (c) acetylcholinesterase inhibitor: **fasciculin-2** (*Dendroaspis angusticeps*) (1FAS); (d) neuronal nicotinic acetylcholine receptor antagonist, k-neurotoxin: monomer of **k-bungarotoxin** (*Bungarus multicinctus*) (1KBA); and (e) presynaptic neurotoxic phospholipase A_2 : **notexin** (*Notechis scutatus*) (1AE7). The structures are α -carbon solid ribbon representations and presented with the aid of JSmol (Jmol: an open-source Java viewer for chemical structures in 3D. <http://www.jmol.org/>). Notable secondary structures include disulfide bridges depicted in red, β -sheets in yellow, and α -helices in red. The snake species from which each toxin was isolated and the respective Protein Data Bank (Research Collaboratory for Structural Bioinformatics) accession number for stated toxin structure are indicated in parentheses. The toxins are not shown to scale

While postsynaptic neurotoxicity is the hallmark effect of α -neurotoxins that competitively inhibit nAChRs at the motor end plate, presynaptic activity is typically produced by toxins such as β -bungarotoxin (*Bungarus multicinctus*) and notexin (*Notechis scutatus*) (reviewed in Rowan (2001), Montecucco et al. (2009), and Sribar et al. (2014)). Snake presynaptic toxins are larger protein complexes with phospholipase A₂ activity (Fig. 1e) that catalyzes the hydrolysis of phospholipids and interferes with membrane processes involved in the storage and release of acetylcholine at the motor nerve terminal (Kini and Evans 1989; Dixon and Harris 1999).

Another novel class of snake neurotoxins that affect cholinergic neurotransmission are fasciculins, found in the venom of mambas (*Dendroaspis spp.*) and which act by potentially inhibiting acetylcholinesterase, the enzyme which produces rapid hydrolysis of the neurotransmitter acetylcholine resulting in the termination of impulse transmission at cholinergic synapses. Fasciculin belongs to the 3FTx family (Fig. 1c) and utilizes amino acid residues in its first and second loops to bind to the peripheral anionic site of acetylcholinesterase and occlude the narrow gorge leading to the active site of the enzyme thereby preventing access to acetylcholine. Consequently, fasciculin produces generalized and long-lasting fasciculation of skeletal muscles due to the accumulation of acetylcholine in the synaptic cleft (Bourne et al. 1995; Rowan and Harvey 2011).

Other cholinergic targets of snake toxins include peptides that target muscarinic acetylcholine receptors (mAChRs). Muscarinic toxins from snake venoms, largely the mambas (*Dendroaspis spp.*), interact with high affinity and selectivity with mAChRs and are invaluable tools for studying the physiological role, structural organization, and pharmacology of the five subtypes of these G-protein coupled receptors (reviewed in Jerusalinsky et al. (2000), Karlsson et al. (2000), and Servent et al. (2011)). Despite the similarity in their primary and tertiary structures consisting of the 3FTx scaffold (Fig. 1b), snake muscarinic toxins exhibit distinct pharmacological selectivity profiles for the different subtypes of mAChRs and may also act as competitive antagonists, allosteric modulators, or agonists at these receptors (Servent et al. 2011).

Our knowledge of the diversity of neuronal nAChR subtypes have benefited greatly from the discovery of highly selective neurotoxins from animal venoms, particularly from cone snails (Lewis et al. 2012; Akondi et al. 2014; Lebbe et al. 2014) and snakes (Tsetlin 2015). k-Neurotoxins, of which k-bungarotoxin (*Bungarus multicinctus*) is a prototypical example, represent another group of 3FTxs (Fig. 1d) that are distinguished from other postsynaptic α -neurotoxins by their ability to bind to and inhibit $\alpha 3$ and $\alpha 4$ subunit-containing neuronal nAChRs (see review Chiappinelli et al. (1996)). The clinical significance of these k-neurotoxins in envenomation is unclear since most venomous snakes have a propensity for interfering with peripheral cholinergic transmission as it is difficult for large peptides to breach the blood-brain barrier (Ranawaka et al. 2013). Furthermore, some α -neurotoxins that interact with muscle nAChRs also extend their pharmacological selectivity by targeting subtypes of neuronal nAChRs (Barber et al. 2013;

Tsetlin 2015) and even other homologous cys-loop ligand-gated ion channels such as GABA_A receptors (McCann et al. 2006; Hannan et al. 2015; Kudryavtsev et al. 2015).

α -Neurotoxins

α -Neurotoxins bind to postsynaptic nAChRs at the neuromuscular junction as competitive antagonists of the neurotransmitter acetylcholine (Nirthanan and Gwee 2004; Barber et al. 2013; Tsetlin 2015). They exhibit strong affinity – approximately 15- to 20-fold greater than d-tubocurarine, with a K_d value between 10^{-9} and 10^{-11} M – while being poorly reversible in their binding in contrast to d-tubocurarine, the precursor to clinically used neuromuscular blocking drugs. While predominantly found in elapid and hydrophiid snake venoms, as well as in some viperid species, α -neurotoxins have also been identified in venoms of *Colubridae*, which, while being the largest snake family, has not been deemed medically important with respect to human envenomation (Pawlak et al. 2009; Barber et al. 2013).

The tertiary structure of most snake α -neurotoxins conforms to the conserved 3FTx protein scaffold, and these will be the focus of this review. Two notable exceptions to this convention need mention here. Waglerins were isolated from the venom of Wagler's pit viper (*Tropidolaemus wagleri*) and contain 22–24 residues with a single disulfide bridge. Interestingly, waglerins, which heralded the unique discovery of an α -neurotoxin from a viperidae venom, display an almost 2000-fold greater affinity for the adult muscle-type nAChR ($\alpha 1\beta 1\epsilon\delta$) over the fetal one ($\alpha 1\beta 1\gamma\delta$) (Molles and Taylor 2002). A recently discovered peptide α -neurotoxin, azemiopsin (*Azemiops faea* viper), which consists of just 21 residues and is devoid of any disulfide bridges, is another example of a structurally unique snake α -neurotoxin that does not conform to the 3FTx structure. Azemiopsin contains a C-terminal hexapeptide that is homologous to waglerins and also displays a modestly higher binding affinity for the α - ϵ over the α - γ and α - δ interfaces (Utkin et al. 2012). The three-dimensional structures of azemiopsin and waglerin have not been reported yet.

It should also be noted that α -neurotoxins are not exclusive to snakes as venomous marine snails are a rich source of a variety of α -conotoxins from marine cone snails which are typically disulfide-rich compact molecules that are 12–30 residues long. α -Conotoxins which bind with high selectivity to postsynaptic muscle nAChRs can also in some instances distinguish between the different subunit interfaces that constitute acetylcholine binding sites (Azam and McIntosh 2009). A large number of novel conotoxins have also been isolated and characterized that have high affinity and selectivity for a variety of neuronal nAChR subtypes (Lebbe et al. 2014) as well as other ion channels including sodium, potassium, and calcium channels (reviewed in Olivera and Teichert (2007), Akondi et al. (2014), and Prashanth et al. (2014)).

Screening for α -Neurotoxin Bioactivity

α -Neurotoxins are potent competitive antagonists of postsynaptic nAChRs ($\alpha 1\beta 1\gamma\delta$ or $\alpha 1\beta 1\epsilon\delta$) at the skeletal muscle neuromuscular junction. Isolated skeletal muscle–nerve preparations from the chick (chick biventer cervicis muscle (Ginsborg and Warriner 1960)) and mouse (mouse phrenic nerve-hemidiaphragm (Bulbring 1946)) provide simple but reliable in vitro organ bath assays for the examination of the effects of venoms and toxins on neuromuscular transmission (Harvey et al. 1994; Hodgson and Wickramaratna 2002; Nirthanan and Gwee 2004; Barber et al. 2013). This can be very effective for bioassay-driven chromatographical purification of α -neurotoxins as well as for their pharmacological characterization (Nirthanan and Gwee 2004). The presynaptic, postsynaptic, or myotoxic effects of α -neurotoxins can be effectively distinguished by the chick biventer muscle in particular, since it contains both focally and multiply innervated muscle fibers which, respectively, mediate twitch responses evoked by electrical nerve stimulation (via the presynaptic mechanism) and contractile responses evoked by exogenous nAChR agonists such as acetylcholine and carbachol (direct activation of postsynaptic receptors) (Nirthanan and Gwee 2004; Barber et al. 2013). Whereas a presynaptically active neurotoxin would abolish nerve-evoked twitches without affecting contractile responses to exogenous nAChR agonists, postsynaptically acting α -neurotoxins would block the responses to nAChR agonists as well as nerve stimulation. In both instances, contractions evoked by depolarization of the skeletal muscle by potassium chloride would not be affected, and only myotoxins that result in the destruction of muscle architecture would be expected to result in the abolition of the potassium chloride response (Harvey et al. 1994).

Other methods for assessing the bioactivity of α -neurotoxins include conventional binding experiments in which the competitive binding of a radio-labeled toxin (e.g., ^{125}I -labeled α -bungarotoxin) to nAChR-rich membranes from *Torpedo* electric organs is assayed (Ménez 2002). However, binding assays, while revealing an interaction between the toxin and receptor, do not provide evidence of functional block (Barber et al. 2013). Electrophysiological approaches where the ability of α -neurotoxins to inhibit currents or ion fluxes induced by agonists in nAChRs expressed in the oocyte expression system are very valuable to provide insight into the functional effects of the toxin as well as screening for selectivity against a variety of nAChR subtypes (Ménez 2002). While traditional two-electrode voltage clamping approaches may be time consuming and labor intensive, newer automated, high-throughput screening systems are routinely being employed for this purpose (Schnizler et al. 2003; Hogg et al. 2008).

Three-Finger Toxins

Three-finger toxins are the largest family of nonenzymatic polypeptides found primarily in venoms of elapid and hydrophiid species as well as colubrid and viperid snakes. Despite their significant functional diversity, they are characterized by a

common protein scaffold consisting of three β -stranded loops converging at a globular, hydrophobic core resembling three fingers of the outstretched hand (Nirthanan and Gwee 2004; Kini and Doley 2010; Tsetlin 2015). The 3FTx scaffold is composed of 60–77 amino acid residues and is typically held in place by four disulfide bridges in the core region, with some conserved amino acid residues such as Tyr²⁵ and Phe²⁷ contributing to structural stability (Nirthanan and Gwee 2004; Kini and Doley 2010). The 3FTx is essentially a flat “leaf-like” molecule with a slight concavity, the plane being determined by the extensive multistranded β -structure (β -sheet) that is the predominant feature. Furthermore, in most instances, the side chains of amino acids that are important for function are placed protruding from the concave side of the toxin (Ménez 1998; Nirthanan and Gwee 2004).

The 3FTx structure is engineered to expose different combinations of critical amino acids that constitute the various functional sites, thereby enabling venomous animals to utilize a small number of structural templates to bind to a multitude of molecular targets (Ménez 1998; Kini 2011). Hence, variations in the β -strands, size and shape of loops, and carboxy-terminal or amino-terminal extensions give rise to extraordinary functional diversity. As a result, 3FTxs are capable of a wide range of pharmacological actions including neurotoxicity, cytotoxicity, cardiotoxicity, coagulotoxicity, proteinase activity, and acetylcholinesterase inhibition (Tsetlin 2015). α -Neurotoxins that adopt the 3FTx structure are discussed in detail below.

Three-Finger α -Neurotoxins

3F α -NTxs are a primary toxic component of snake venoms known to produce significant peripheral neurotoxicity following envenomation (Nirthanan and Gwee 2004; Barber et al. 2013). They maybe loosely classified into four *groups* based on their structure: short-chain, long-chain, nonconventional, and dimeric α -neurotoxins (Table 1, Fig. 2) (Nirthanan and Gwee 2004; Tsetlin 2015). This classification is largely based on the structural characteristics of 3F α -NTxs. Diversity in nAChR selectivity and differences in binding affinities to various receptors is clearly evident from a functional perspective even among members of the same 3F α -NTx groups.

Generally, all 3F α -NTxs inhibit the muscle-type nAChRs with varying affinities, while long-chain and nonconventional α -neurotoxins also inhibit certain subtypes of neuronal nAChRs (Mordvintsev et al. 2005; Kini 2011; Rajesh and Sivaraman 2011). The known examples of dimeric 3F α -NTxs show that they may exhibit unusual receptor selectivity that differ from their constituent monomeric 3F α -NTx group. For example, haditoxin, a dimeric 3F α -NTx composed of two short-chain 3F α -NTx monomers, is active against both muscle and neuronal nAChRs (Roy et al. 2010) while short-chain 3F α -NTxs per se are not effective in inhibiting neuronal nAChRs.

Interestingly, α -bungarotoxin and other long-chain 3F α -NTxs were also recently shown to inhibit the GABA_A receptor at high concentrations (McCann et al. 2006; Hannan et al. 2015; Kudryavtsev et al. 2015). This was also reported for waglerin-1 (Ye and McArdle 1997). Although GABA_A and nAChRs are structurally

Table 1 Structural classification of snake three-finger neurotoxins that interact with muscle nicotinic acetylcholine receptors

	Major structural characteristics	Primary molecular target	Other molecular targets	Source and examples
Short-chain α-neurotoxins	Three-fingered monomers of 60–62 amino acid residues with four conserved disulfide bonds	High affinity for muscle or <i>Torpedo</i> ($\alpha 1\beta 1\gamma\delta$) nAChR ($K_d \sim 10^{-9}$ – 10^{-11} M)		<i>Elapidae</i> and <i>Hydrophidae</i> species. Typical examples include erabutoxin-a (<i>Laticauda semifasciata</i>), toxin- α (<i>Naja nigricollis</i>)
Long-chain α-neurotoxins	Three-fingered monomers of 66–74 amino acid residues with four conserved disulfide bonds and an additional fifth disulfide bond at the tip of loop II	High affinity for muscle or <i>Torpedo</i> ($\alpha 1\beta 1\gamma\delta$) nAChR ($K_d \sim 10^{-9}$ – 10^{-11} M)	High affinity ($K_d \sim 10^{-8}$ – 10^{-9} M) antagonists of the neuronal $\alpha 7$ nAChR	<i>Elapidae</i> species. Typical examples include α -bungarotoxin (<i>Bungarus multicinctus</i>), α -cobratoxin (<i>Naja kaouthia</i>)
Atypical long-chain neurotoxins	Three-fingered monomers of 69 amino acid residues and four conserved disulfide bonds	High affinity for <i>Torpedo</i> ($\alpha 1\beta 1\gamma\delta$) nAChR ($K_d \sim 10^{-11}$ M)		Toxins Lc-a and Lc-b, isolated exclusively from <i>Laticauda colubrina</i> (<i>Hydrophidae</i>)
Nonconventional neurotoxins	Three-fingered monomers of 65–67 amino acid residues with four conserved disulfide bonds and an additional fifth disulfide bond at the tip of loop I	Variable affinities have been reported for the muscle nAChR. WTX (<i>Naja kaouthia</i>) showed low affinity ($K_d \sim >10^{-6}$ M) and in electrophysiological studies, candoxin (<i>Bungarus candidus</i>) inhibited muscle ($\alpha 1\beta 1\gamma\delta$) nAChRs with high affinity ($IC_{50} = 10$ nM)	Candoxin (<i>Bungarus candidus</i>) is a high affinity antagonist of neuronal $\alpha 7$ nAChRs ($IC_{50} = 50$ nM)	<i>Elapidae</i> species. Examples include candoxin (<i>Bungarus candidus</i>), WTX (<i>Naja kaouthia</i>), Wntx-5 (<i>Naja sputatrix</i>)
Dimeric neurotoxins (see Table 2)	Covalently or noncovalently linked dimers composed of three-fingered subunits belonging to the short-chain, long-chain, or nonconventional neurotoxin types. Contain 65–77 residues and four or five disulfide bridges. They may be homodimeric (Haditoxin, <i>Ophiophagus hannah</i>) or heterodimeric (Iriditoxin, <i>Boiga irregularis</i>)	Variable affinities and species specificity have been reported for the muscle nAChR. Haditoxin, in electrophysiological studies, inhibited muscle ($\alpha 1\beta 1\gamma\delta$) nAChR ($IC_{50} = 0.5$ μ M). Iriditoxin inhibited avian muscle ($\alpha 1\beta 1\gamma\delta$) nAChR with high affinity ($IC_{50} = 10$ nM) but was three orders of magnitude less effective at mammalian muscle nAChRs	Haditoxin inhibited neuronal $\alpha 7$ ($IC_{50} = 0.2$ μ M), $\alpha 3\beta 2$ ($IC_{50} = 0.50$ μ M), and $\alpha 4\beta 2$ ($IC_{50} = 2.60$ μ M) nAChRs	<i>Elapidae</i> and <i>Colubridae</i> species. Examples include haditoxin (<i>Ophiophagus hannah</i>), iriditoxin (<i>Boiga irregularis</i>)

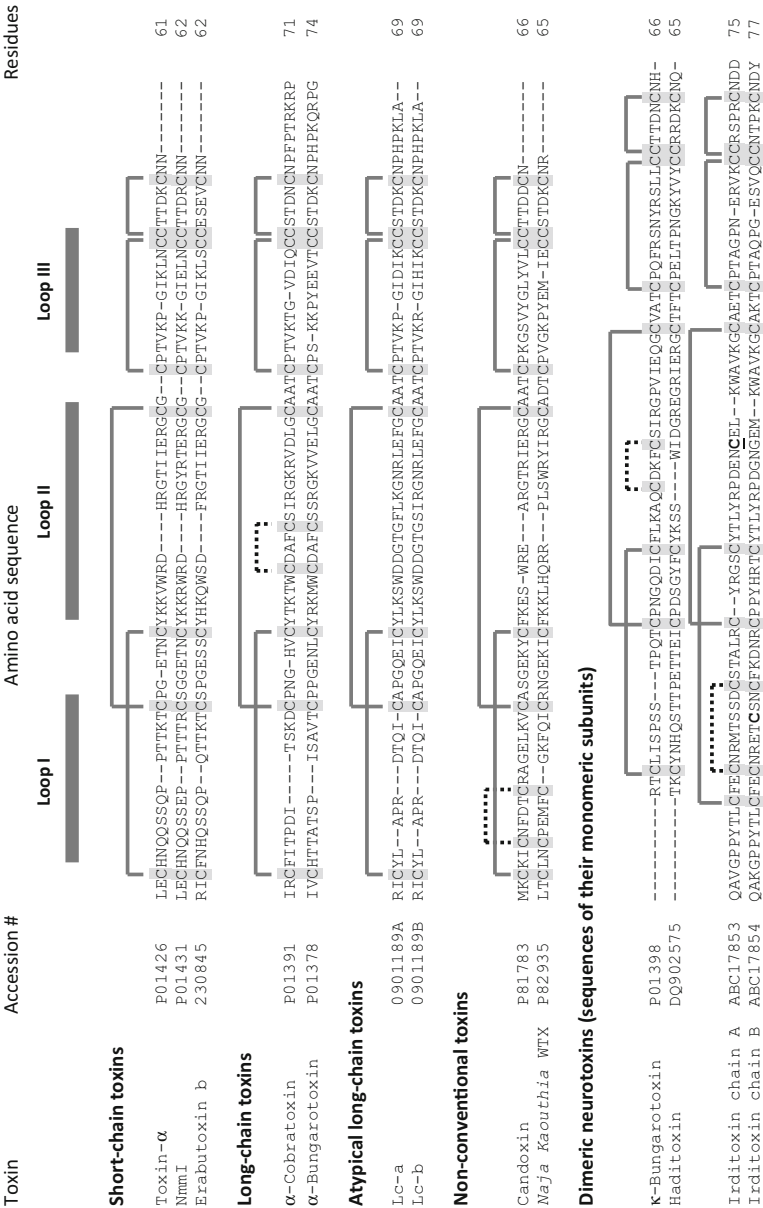


Fig. 2 Amino acid sequences of three-finger neurotoxins that interact with nicotinic acetylcholine receptors. The cysteine residues are shaded in gray and the disulfide linkages and the segments contributing to the three loops are outlined. The number of amino acid residues in each sequence is indicated at the end of the respective sequence. The species names are as follows: toxin- α (*Naja nigricollis*; Elapidae), Nmml (*Naja mossambica mossambica*; Elapidae), erabutoxin-b

homologous and belong to the “Cys-loop” family of ligand-gated ion channels, the significance and extent to which this occurs in vivo is unclear, bringing to the fore that 3F α -NTxs are not a functionally homogenous group. Thus, it is possible that other unknown molecular targets exist, especially for the less well-characterized nonconventional 3F α -NTxs which exhibit widely variable receptor selectivity and affinity (Nirthanan et al. 2003a), and some examples (such as bungaridin from *Bungarus candidus*) still do not have a clearly established molecular target (Torres et al. 2001).

Limitations in adhering to rigidly defined categories of 3F α -NTxs based on their primary structural characteristics is evidenced by examples of atypical toxins that are potent inhibitors of muscle nAChRs, such as pseudonajatoxin-a (*Pseudonaja textilis*; Elapidae), which has 117 amino acid residues and seven disulfide bonds (Barnett et al. 1980). This strongly suggests that there could be other classes of α -neurotoxins yet to be discovered (Barber et al. 2013).

Another notable outlier to the structure-function convention of 3F α -NTxs that are competitive antagonists of nAChRs has been reported recently. Ω -Neurotoxins, of which one example – Oh9-1 from king cobra (*Ophiophagus hannah*) venom has been characterized in detail, belong to the 3FTx family but conspicuously lack all the characteristic functional residues of α -neurotoxins for interaction with nAChRs (Hassan-Puttaswamy et al. 2015). Oh9-1 showed reversible postsynaptic neurotoxicity in the micromolar range with selectivity for muscle and neuronal $\alpha\beta 2$ nAChRs. Phylogenetic analysis revealed that the functional organization of Ω -neurotoxins is independent of α -neurotoxins, placing it in a new class of 3FTxs (Hassan-Puttaswamy et al. 2015).

Short-Chain and Long-Chain α -Neurotoxins

Based on the length of their polypeptide chains, 3F α -NTxs are distinguished as being either short-chain 3F α -NTxs with a molecular mass of 6–7 KDa, 60–62 amino acid residues, and four conserved disulfide bridges or long-chain 3F α -NTxs with a molecular mass of 7–9 KDa, 66–75 residues, a longer carboxy-terminal tail, shorter loop I region, and an additional fifth disulfide bond at the tip of loop II between



Fig. 2 (continued) (*Laticauda semifasciata*; Hydrophiidae), α -cobratoxin (*Naja kaouthia*; Elapidae), α -bungarotoxin (*Bungarus multicinctus*; Elapidae), Lc-a and Lc-b (*Laticauda colubrina*; Hydrophiidae), cadoxin (*Bungarus candidus*; Elapidae), WTX (*Naja kaouthia*; Elapidae), haditoxin (*Ophiophagus hannah*; Elapidae), k-bungarotoxin (*Bungarus multicinctus*; Elapidae), irditoxin A and B (*Boiga irregularis*; Colubridae). For dimeric neurotoxins, the sequences of the monomeric subunit of haditoxin and k-bungarotoxin (which are both homodimers) and monomeric subunits (chains A and B) of irditoxin (which is a heterodimer) are shown. In irditoxin, the cysteine residue in each chain A and B that contributes to the formation of the covalent bond linking the two subunits is bolded and underlined. Protein Data Bank (Research Collaboratory for Structural Bioinformatics) accession numbers are also stated for each toxin. The International Union of Pure and Applied Chemistry one-letter notation for amino acids is used (1968)

Cys³⁰ and Cys³⁴ (see Table 1 and Fig. 2) (Nirthanan and Gwee 2004; Barber et al. 2013).

Despite differences in structure, the two groups of 3F α -NTxs bind with high affinity ($K_d \sim 10^{-10}$ – 10^{-11} M) to muscle $\alpha 1\beta 1\gamma\delta$ as well as to its structurally homologous *Torpedo* (electric ray), nAChRs (Servent and Ménéz 2002). The notable difference in pharmacology is that long-chain, but not short-chain 3F α -NTxs, are capable of binding with high affinity to neuronal $\alpha 7$ nAChRs ($K_d = 10^{-9}$ – 10^{-8} M) (Antil-Delbeke et al. 2000; Nirthanan and Gwee 2004; Barber et al. 2013) as well as to $\alpha 9$ and $\alpha 9/\alpha 10$ nAChRs (McIntosh et al. 2009). High affinity interaction of long-chain 3F α -NTxs, with neuronal $\alpha 7$ nAChRs, has been attributed to the existence of the fifth disulfide bridge in loop II of its structure (Antil-Delbeke et al. 2000). However, it has subsequently been demonstrated that some 3F α -NTxs which do not have the fifth disulfide bridge in loop II (e.g., candoxin, a nonconventional 3F α -NTx) also inhibit neuronal $\alpha 7$ nAChRs, possibly by utilizing other molecular determinants (Nirthanan et al. 2002).

Two unusual examples of long-chain 3F α -NTxs need mention here. Based on sequence length and homology to long-chain 3F α -NTxs, the 69 residues long neurotoxins isolated from the sea snake *Laticauda colubrina* (Lc-a and Lc-b) (Kim and Tamiya 1982) were also classified as long-chain 3F α -NTxs. Like other prototype 3F α -NTxs, both Lc-a and Lc-b, which differed from each other at only five positions in their sequences, showed high affinity for the *Torpedo* nAChR ($K_d \sim 10$ – 11 M). However, Lc-a and Lc-b, unlike the typical long-chain 3F α -NTxs, retain only the four core conserved disulfide bridges and lack the fifth disulfide bridge at the tip of loop II (Kim and Tamiya 1982). Consequently, Lc-a and Lc-b bind poorly to neuronal $\alpha 7$ nAChRs (Servent et al. 1997).

Nonconventional Three-Finger α -Neurotoxins

Nonconventional 3F α -NTxs have 62–68 amino acid residues and five disulfide bridges like long-chain α -neurotoxins, with the fifth disulfide bridge located in loop I instead of loop II (Nirthanan et al. 2003b). Since nonconventional 3F α -NTxs (with some exceptions) usually have an LD₅₀ of 5–80 mg/kg, relative to the 0.04–0.3 mg/kg of some lethal α -neurotoxins, they are referred to as “weak toxins” (Nirthanan et al. 2003a). Despite this, some examples within this group, such as γ -bungarotoxin, are still highly toxic (Mackessy 2009) underscoring significant diversity in function among members of this group of 3F α -NTxs (Nirthanan et al. 2003b). For example, WTX (Utkin et al. 2001) and Wntx-5 (Poh et al. 2002), from *Naja kaouthia* and *Naja sputatrix* respectively, are two well-characterized nonconventional 3F α -NTxs that have demonstrated a poorly reversible and weak affinity for muscle ($\alpha 1\beta 1\gamma\delta$) and neuronal ($\alpha 7$) nAChRs at micromolar concentrations. In sharp contrast, candoxin, another nonconventional 3F α -NTx from the Malayan krait, exhibited potent reversible inhibition of the muscle nAChRs at nanomolar concentrations (Nirthanan et al. 2002, 2003a). Interestingly, in further highlighting the pharmacological potential of nonconventional 3F α -NTxs, WTX has

also been shown to interact with muscarinic nAChRs (Mordvintsev et al. 2009) while some examples such as bucandin from *Bungarus cadidus* have currently no known molecular targets (Torres et al. 2001; Nirthanan et al. 2003b).

Unusual examples of nonconventional 3F α -NTxs have been discovered in *Colubridae* venoms, which are typically longer than 75 amino acid residues and contain five disulfide bonds and have molecular masses of 8.5–10.7 kDa. These include α -colubritoxin (*Coelognathus radiates*), the first 3F α -NTx identified in colubrids, which contains 79 residues (Fry et al. 2003a) and denmotoxin (*Boiga dendrophila*) which has 77 residues (Pawlak et al. 2006).

Dimeric Neurotoxins

The concept of snake neurotoxins existing as dimers is not new. k-Neurotoxins exemplified by the typical example k-bungarotoxin (*Bungarus multicinctus*), which bind with high affinity to neuronal $\alpha 3\beta 2$ nAChRs as well as to a lesser extent $\alpha 7$ and $\alpha 4\beta 2$ nAChRs, are composed of two identical monomeric units held together by noncovalent interactions due to hydrogen bonds (Oswald et al. 1991; Chiappinelli et al. 1996). More recently a variety of dimeric 3F α -NTxs have been discovered and characterized that revealed novel structural differences and distinct receptor specificities that distinguishes them from k-neurotoxins (see Table 2). These include noncovalently bound dimers as well as toxins bound covalently by one or more disulfide bridges and have demonstrated a plethora of new pharmacological selectivity for nAChR subtypes not displayed by the original monomeric toxins that form these dimers (Roy et al. 2010; Osipov et al. 2012).

For instance, haditoxin (*Ophiophagus hannah*) is a noncovalent homodimeric 3F α -NTx that shares a similar quaternary structure as k-bungarotoxin with the dimeric interfaces held together by extensive hydrogen bonding (Roy et al. 2010). However, unlike k-bungarotoxin in which each monomeric unit belongs to the long-chain 3F α -NTx group, each monomer in haditoxin is structurally homologous to short-chain 3F α -NTxs. Interestingly, haditoxin was effective in inhibiting a range of nAChR subtypes including the muscle ($\alpha 1\beta 1\gamma\delta$) and neuronal ($\alpha 7$, $\alpha 3\beta 2$, and $\alpha 4\beta 2$) receptors. This is in sharp contrast to monomeric short-chain 3F α -NTxs which act with low nanomolar affinity at muscle but are ineffective at neuronal nAChRs (Roy et al. 2010). A covalently linked homodimeric α -cobratoxin (α CT- α CT) (*Naja kaouthia*), where the monomeric units of long-chain 3F α -NTxs were bound together by two intermolecular disulfide bridges, also revealed how dimerization not only retained α -cobratoxin's ability to inhibit muscle ($\alpha 1\beta 1\gamma\delta$) and $\alpha 7$ nAChRs but in addition gained the capacity to interact with $\alpha 3\beta 2$ nAChRs (Osipov et al. 2008, 2012).

Another covalently linked dimeric 3F α -NTx is irditoxin, a heterodimer composed of two nonconventional 3F α -NTx monomers linked by a single intermolecular disulfide bond (Pawlak et al. 2009). Irditoxin exhibited taxa-specific neurotoxicity towards avian muscle ($\alpha 1\beta 1\gamma\delta$) nAChRs, which was three orders of magnitude greater than that for mammalian muscle nAChRs. This was not surprising since

Table 2 Some examples of dimeric snake three-finger neurotoxins that interact with nicotinic acetylcholine receptors

Dimeric toxin	Dimeric linkage	Monomeric subunit	nAChR activity	Source	References
k-Bungarotoxin	Noncovalent Hydrogen bonds	Long-chain 3F α -NTxs	$\alpha 3\beta 2$ (IC ₅₀ = 3 nM) $\alpha 7$ (weak inhibition) $\alpha 4\beta 2$ (weak inhibition)	Taiwanese multi-banded krait <i>Bungarus multicinctus</i> (Elapidae)	Chiappinelli et al. 1996; Oswald et al. 1991; Osipov et al. 2008
α-Cobratotoxin dimer	Covalent Two intermolecular disulfide bridges	Long-chain 3F α -NTxs (or long-chain 3F α -NTxs in combination with cytotoxins)	<i>Torpedo</i> ($\alpha 1\beta 1\gamma\delta$) (IC ₅₀ = 10 nM) $\alpha 7$ (IC ₅₀ = 0.2 μ M) $\alpha 3\beta 2$ (IC ₅₀ = 0.15 μ M)	Monocellate cobra <i>Naja kaouthia</i> (Elapidae)	Osipov et al. 2008, 2012
Haditoxin	Noncovalent Hydrogen bonds	Short-chain 3F α -NTxs	$\alpha 7$ (IC ₅₀ = 0.2 μ M) Muscle ($\alpha 1\beta 1\gamma\delta$) (IC ₅₀ = 0.5 μ M) $\alpha 3\beta 2$ (IC ₅₀ = 0.50 μ M) $\alpha 4\beta 2$ - (IC ₅₀ = 2.60 μ M)	King cobra <i>Ophiophagus hannah</i> (Elapidae)	Roy et al. 2010
Irditoxin	Covalent One intermolecular disulfide bridge	Nonconventional 3F α -NTxs	Avian muscle ($\alpha 1\beta 1\gamma\delta$) (IC ₅₀ = 10 nM) Rat muscle ($\alpha 1\beta 1\gamma\delta$) (IC ₅₀ > 10 μ M)	Brown tree snake <i>Boiga irregularis</i> (Colubridae)	Pawlak et al. 2009

iriditoxin was isolated from the arboreal brown tree snake *Boiga irregularis*, and it is likely that its venom components are evolved to target its natural avian prey (Pawlak et al. 2009). Therefore, dimeric 3F α -NTxs constitute a structurally heterogeneous group of toxins exhibiting novel structural conformations and possibly unique modes of interaction with target receptors which enables them to diversify their receptor specificity (Osipov et al. 2008; Tsetlin 2015).

A Note on Nomenclature

With the number of peptide toxins being discovered from animal venoms, including snakes, ballooning exponentially, King et al. (2008) highlighted the inconsistencies and confusion caused by the naming of α -neurotoxins using trivial names – often based on the amalgamation of the word “toxin” with a derivative of either the genus, species, or both of the snake from which the toxin was isolated (King et al. 2008). They proposed a more systematic, rational, and unified nomenclature that, for the example of α -neurotoxins, uses the prefix “ α ” to denote nAChR antagonistic activity; followed by the snake’s family (e.g., *elapitoxin* if from the Elapidae family); the genus and species are then indicated using single letters (e.g., Bm for *Bungarus multicinctus*); and lastly, numbers designate whether the isolated α -neurotoxin is a short-chain (number 1), long-chain (number 2), or k-neurotoxin (number 3), while any isoforms of the toxin would then be designated sequentially with alphabets (e.g., a,b,c) (King et al. 2008; Barber et al. 2013). In this schema, α -bungarotoxin (from *Bungarus multicinctus* of the family *Elapidae*) would be referred to as α -*Elapitoxin*-Bm2a.

A Closer Look at Structure-Function Relationships of Three-Finger α -Neurotoxins

Nicotinic Acetylcholine Receptors

Due in large part to the discovery of α -bungarotoxin about 50 years ago and to an abundant and accessible source of receptor from the electric ray (*Torpedo* spp.), the nAChR is arguably the best characterized receptor or ion channel to date. The nAChR is a pentameric transmembrane allosteric protein (MW ~290 KDa) that evokes fast ionic responses to acetylcholine (see excellent reviews Hurst et al. (2013), Unwin (2013), and Cecchini and Changeux (2015)). The nAChR belongs to the large family of “cysteine-loop” ligand-gated ion channels that also include a number of other neurotransmitter receptors such as GABA_A, 5HT₃, and glycine (see the thematic review Stephenson (2012)). Five homologous subunits associate symmetrically around a central ion channel in a plane perpendicular to the membrane in the nAChR (Fig. 3). Each subunit comprises of a large amino-terminal domain that contributes to the formation of the binding sites for acetylcholine as well as other agonists and competitive antagonists. The acetylcholine binding sites are

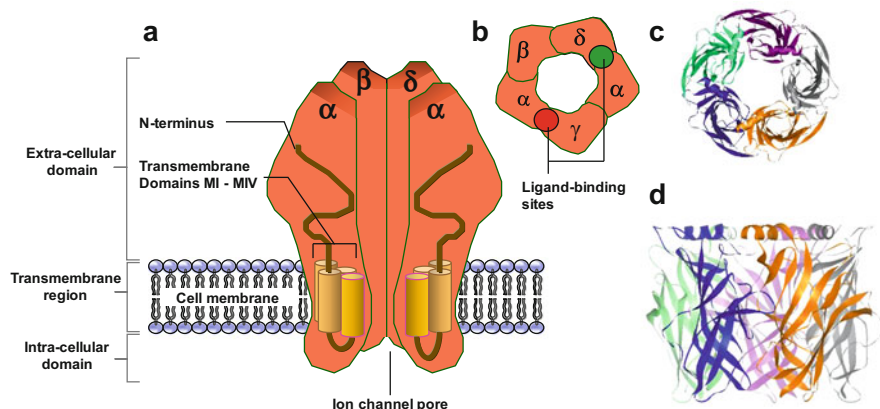


Fig. 3 The structure of the muscle type of nicotinic acetylcholine receptor. **(a)** The nAChR is a heteropentamer composed of five homologous subunits. The muscle receptor of the stoichiometry $(\alpha 1)_2\beta 1\gamma\delta$ is represented in this cartoon. For clarity, the γ subunit is not shown. Each subunit is composed of four helical transmembrane domains (MI, MII, MIII, MIV). **(b)** Top view of the heteropentameric receptor, viewed along the fivefold axis, showing the association of the five subunits. The extracellular amino-terminal domain of the $\alpha 1$ -subunit and the adjacent subunit (δ or γ) cooperate to form two distinct binding pockets for acetylcholine and other ligands at the interface between the subunits. **(c, d)** The molluscan acetylcholine-binding protein, a structural homologue of the nicotinic acetylcholine receptor ligand-binding domain. The 2.7 Å resolution crystal structure of the acetylcholine-binding protein homopentamer from the snail (*Lymnaea stagnalis*) glial cells (PDB accession # 119B) (Sixma and Smit 2003). **(c)** As viewed from the top, along the fivefold axis. Each subunit (depicted in different colors) is a single domain protein. The cavity or pocket at each interface constitutes the ligand-binding site. **(d)** As viewed perpendicular to the fivefold axis

located between subunit interfaces with contributions from both counterparts – typically the α subunit and a non- α subunit (depending on the subtype of receptor) (Pedersen and Cohen 1990). The transmembrane region of each subunit comprises of four transmembrane α -helical regions, of which the second (M2) of each subunit lines the ion channel pore (Unwin 2005).

Although the combinatorial assembly of various nicotinic receptor subunits ($\alpha 1$ to $\alpha 10$, $\beta 1$ to $\beta 4$, δ , γ , or ϵ) generates a wide diversity of receptor subtypes with a variety of attributes of pharmacological, physiological, and clinical significance, nAChRs can be broadly classified into either the muscle or neuronal types. The well-characterized muscle receptor consists of a combination of $\alpha 1$, $\beta 1$, δ , and γ or ϵ subunits in the stoichiometry of $(\alpha 1)_2\beta 1\gamma\delta$ or $(\alpha 1)_2\beta 1\epsilon\delta$ in the fetal or adult receptor, respectively. These are densely distributed on the postsynaptic membrane of the neuromuscular junction and mediate intercellular communication between the nerve ending and skeletal muscle. At the neuromuscular junction, acetylcholine, released into the synaptic cleft from the nerve terminal following depolarization, binds to the two binding sites located at the interfaces between $\alpha 1/\delta$ subunits and $\alpha 1/\epsilon$ (adult) or γ (fetal) subunits of the postsynaptic nAChR. In order to activate the receptor, acetylcholine must occupy both binding sites. This triggers sequential conformational changes in the receptor resulting in the opening of the associated ion channel and

increased permeability to cations, leading to depolarization of the motor end plate and eventually contraction of the muscle (Cecchini and Changeux 2015).

A homologue of the muscle-type receptor ($\alpha 1$)₂ $\beta 1\gamma\delta$ is also found in abundance in the electric organ of the *Torpedo* ray, and for this reason, this source has been widely used in the study of nAChRs (Cohen et al. 1972). Neuronal nAChRs comprise of various pentameric combinations of $\alpha 2$ - $\alpha 10$ and $\beta 2$ - $\beta 4$ subunits and play significant physiological roles in the central nervous system including cognition, memory, and addiction as well as in extraneurological locations where they have been implicated in key cellular signaling pathways (see reviews Dineley et al. (2015), Fasoli and Gotti (2015), and Zdanowski et al. (2015)).

The complex ligand-binding site located at the subunit interface with contributions from both subunits is composed of six polypeptide loops, three apiece. The principal part of the binding pocket is formed by $\alpha 1$ -subunit contributing to loops A, B, and C, whereas the neighboring subunit (δ , γ , or ϵ in muscle nAChR) contributes to loops D, E, and F that form the complementary part of the binding pocket. Specific amino acids, with a preponderance of aromatic residues, contributing to the acetylcholine binding site have also been identified – these include Tyr⁹³ (Loop A), Trp¹⁴⁹ (Loop B), and Tyr¹⁹⁰, Cys¹⁹², Cys¹⁹³, and Tyr¹⁹⁸ (Loop C) from the $\alpha 1$ subunit as well as Asp¹⁸⁰ and Glu¹⁸⁹ (Loop D), Thr¹¹⁹ (Loop E), and Trp⁵⁷ (Loop F) (Cecchini and Changeux 2015).

Thus, the muscle nAChR would contain two ligand-binding sites ($\alpha 1/\delta$ and $\alpha 1/\gamma$ or $\alpha 1/\epsilon$) which are structurally different at the amino acid level with consequent differences in affinities for various ligands (Pedersen and Cohen 1990). An excellent insight into the structure of nAChRs, and ligand-gated ion channels in general, was made possible by the discovery and structural characterization of an acetylcholine-binding protein (AChBP) from the snail *Lymnaea stagnalis* (Fig. 3c, d) which is a remarkable homologue of the amino-terminal extracellular domain of the nAChR. The crystal structure of the AChBP revealed that the ligand-binding pocket is located as a small cavity of about 10–12 Å diameter, primarily formed by aromatic residues contributed by the participating subunits (Sixma and Smit 2003). The refined structure of the *Torpedo* nAChR has also been detailed by state-of-the-art electron microscopy, which corroborates the structure of the aromatic box (Unwin 2005).

Distinguishing the Different Binding Site Interfaces in the Muscle Acetylcholine Receptor

The differences in the two binding sites of the muscle nAChR have been attributed to the influence of the complimentary subunits (i.e., γ , δ or ϵ) on the conformation of the binding sites at their respective interfaces with the $\alpha 1$ -subunit (Pedersen and Cohen 1990). Several agonists and antagonists that bind to the muscle nAChR show some degree of subunit selectivity that results in preferences for either the $\alpha 1/\gamma$ or $\alpha 1/\delta$ or $\alpha 1/\epsilon$ binding interfaces (Taylor et al. 1998, 2000). For example, in the

mouse muscle nAChR, conotoxin MI (*Conus magus*) showed a 10,000-fold preference for the $\alpha 1/\delta$ site (Sugiyama et al. 1998) and waglerin (*Tropidolaemus wagleri*) had a 2000-fold greater affinity for the $\alpha 1/\epsilon$ site (Molles et al. 2002); while azemiopsin (*Azemiops faea*) also showed a modest preference for $\alpha 1/\epsilon$ site (Utkin et al. 2012).

Although the ability of snake 3F- α NTxs to exhibit a preference for a specific binding site in the muscle nAChR is not widely established, a short-chain 3F- α NTx, Nmml (*Naja mossambica mossambica*) is reported to show an order of magnitude higher affinity for the $\alpha 1/\gamma$ or $\alpha 1/\delta$ interfaces ($K_d = 140$ pM) than for the $\alpha 1/\epsilon$ site ($K_d = 130$ nM), and this selectivity has been attributed to two residues (Pro¹⁷⁵ and Glu¹⁷⁶) in the γ subunit of the nAChR (Osaka et al. 2000). Less conclusively, pharmacological studies on the nonconventional 3F- α NTx, candoxin (*Bungarus candidus*) also suggest that it may have differential affinity for the $\alpha 1/\gamma$ or $\alpha 1/\delta$ interfaces of the muscle nAChR (Nirthanan et al. 2003a). Furthermore, site-directed mutagenesis studies on α -cobratoxin revealed that the mutation of Lys²³ and Lys⁴⁹ to Glu²³ and Glu⁴⁹, respectively, caused a differential lowering of binding affinity at the two binding sites of the muscle ($\alpha 1$)₂ $\beta 1\gamma\delta$ nAChRs (Antil-Delbeke et al. 2000). This observation was verified for Nmml, whereby the mutation of Lys²⁷ to Glu²⁷ affected binding at the $\alpha 1/\gamma$ site more than the $\alpha 1/\delta$ site (Ackermann and Taylor 1997). Interestingly, position 29 in candoxin (homologous to Lys²³ in α -cobratoxin and Lys²⁷ in Nmml) is occupied by a glutamic acid instead of a lysine suggesting a possible role for Glu²⁹ in conferring differential subunit selectivity (Nirthanan et al. 2003b).

Reversibility of α -Neurotoxin Action

The mimicry of tubocurarine by “curaremimetic” snake α -neurotoxins ends abruptly when it comes to the reversibility of neuromuscular blockade produced by most α -neurotoxins, especially the long-chain 3F α -NTxs which undergo almost irreversible binding to skeletal muscle nAChRs (Lee 1972). This contrasts sharply with the interaction of many α -conotoxins from marine cone snails that are well known to produce reversible postsynaptic neuromuscular blockade in vitro and in vivo (e.g., α -conotoxin MI and GI) (Marshall and Harvey 1990). Short-chain 3F α -NTxs have been reported to show considerable variability in reversibility of their neuromuscular blockade (Barber et al. 2013) and some examples such as CM10 and CM12 (*Naja haje annulifera*) and S5C10 (*Dendroaspis jamesoni*) as well as toxin LSIII (*Laticauda semifasciata*) have also been found to produce neuromuscular blockade that is almost completely reversible (Harvey and Rodger 1978). Studies on the pharmacology of a nonconventional 3F α -NTx, candoxin (*Bungarus multicinctus*) details a quick onset of neuromuscular blockade followed by rapid and complete reversal of neuromuscular blockade (Nirthanan et al. 2003a).

Generally, short-chain 3F α -NTxs have been reported to associate six- to seven-fold faster, and dissociate five- to ninefold faster, compared to long-chain 3F α -NTxs

at the nAChR (Chicheportiche et al. 1975). It has been proposed that amino acid residues in the α 182-198 region of the nAChR α -subunit is incapable of binding to contact regions of short-chain 3F α -NTxs as efficiently as it would to long-chain 3F α -NTxs (Ruan et al. 1991). It has also been suggested that short-chain 3F α -NTxs have a higher proportion of hydrophobic amino acid residues like valine or alanine in their primary sequences that underpins their reversible interaction with nAChRs (Lee et al. 1972).

It must be noted that the reversibility or irreversibility of neuromuscular blockade produced by 3F α -NTxs cannot be simply the consequence of their weak or high binding affinity to the nAChRs. This will be evident from electrophysiological studies which showed that α -bungarotoxin ($IC_{50} \sim 5$ nM) produced an irreversible block of muscle (α 1 β 1 γ δ) nAChRs, whereas candoxin ($IC_{50} \sim 10$ nM) produced a fully reversible blockade of the same receptor (Nirthanan et al. 2002). Furthermore, WTX (*Naja kaouthia*), a nonconventional 3F α -NTx that is structurally similar to candoxin but a 1000-fold weaker antagonist of muscle (α 1 β 1 γ δ) nAChRs, is almost irreversible in its action (Utkin et al. 2001). Therefore, the reversibility of 3F α -NTx action at the neuromuscular junction may perhaps be associated with a specific area of interaction on the toxin molecule, distinct from the receptor recognition site (Harvey and Rodger 1978). For instance, in contrast to most 3F α -NTxs, an aspartate (a hydrophilic residue) at position 31 is absent in many of the 3F α -NTxs including candoxin which have reported to be reversible in their action (Nirthanan et al. 2003a). It is likely that the absence of Asp³¹ (a polar/hydrophilic amino acid) may be associated with easy reversibility of neuromuscular blockade produced by 3F α -NTxs (Nirthanan and Gwee 2004), a hypothesis that is in agreement with the observations of Lee et al. (1972) regarding the higher prevalence of hydrophobic residues in reversible 3F α -NTxs.

Invariant Residues in Three-Finger α -Neurotoxins that Offer Structural Stability

The three-finger protein scaffold is a remarkably stable molecule that offers versatility for snake toxins and mammalian proteins of the Ly6 family to adapt for a variety of diverse pharmacological activities (Kini 2011; Tsetlin 2015). There are a number of conserved amino acid residues that are “structurally invariant” in these three-finger proteins in that they do not directly contribute to their interaction with their target receptors but, rather, contribute to the correct folding of the polypeptide chain and structural integrity of the backbone (Nirthanan and Gwee 2004; Kini and Doley 2010). Among the most important are the eight cysteine residues that form the four conserved disulfide bridges located in the core region of the three-finger protein scaffold. Menez et al. (1980) demonstrated that without these disulfide bridges, the three-finger scaffold would collapse, rendering the polypeptide dysfunctional. An aromatic residue, Tyr²⁵ (numbering of amino acid residues is as per the primary sequence of erabutoxin-a) (or a homologous residue Phe²⁷) is also conserved in most toxins that adopt the three-finger structure given its necessity for the proper folding

of the polypeptide chain and stability of the antiparallel β -sheet structure (Torres et al. 2001; Kini and Doley 2010).

Mutational and structural studies done on 3F α -NTxs showed residues Gly⁴², Pro⁴⁴, and Pro⁴⁸ to be essential for maintaining their spatial conformation, whereas Gly⁴⁰ has been found to be responsible for the close packing of the protein (Nirthanan and Gwee 2004; Pawlak et al. 2009; Roy et al. 2010). Likewise, Ser⁸ in erabutoxin-a, also conserved in many 3F α -NTxs, is deemed crucial in stabilizing the geometry of side chains in the adjacent loop II (Pillet et al. 1993). Some charged amino acid residues, including Arg³⁹ in erabutoxin-a and Asp⁶⁰ in α -cobratoxin, have also been reported to stabilize the native conformation of the three-finger protein, interestingly, by forming salt links with the carboxy or amino terminus of the toxin (Endo and Tamiya 1991; Roy et al. 2010).

A Functional Role for the Core Region of Three-Finger Toxins

While the disulfide bridge-rich core region of 3F α -NTxs has been considered a structural lynchpin from which the three loops extend, a functional role for the core region has also been proposed – suggesting that it interacts with the membrane lipid bilayer surrounding the nAChR (Lesovoy et al. 2009). Evidence for this, stemming from a study on neurotoxin II (*Naja oxiana*), showed the toxin undergoing a process of “membrane catalysis” whereby the hydrophilic and positively charged side chains from the core region formed hydrogen bonds with the polar head groups of the lipid bilayer (Lesovoy et al. 2009; Langelaan and Rainey 2010). It is proposed that this interaction with the lipid membrane effectively positions loop II of 3F α -NTxs in an orientation favorable for subsequent binding to the receptor (Lesovoy et al. 2009; Barber et al. 2013).

Conformational Determinants of Three-Finger α -Neurotoxin Function

From the perspective of the overall three-finger fold, the primary differences between short-chain and long-chain 3F α -NTxs include the presence of an extra disulfide bridge positioned at the tip of loop II, a longer carboxy-terminal tail and a relatively shorter loop I in long-chain 3F α -NTxs. These obvious, as well as other subtle, variations in the architecture of the three-finger protein scaffold often reflect varying degrees of functional significance (Ménez 1998; Nirthanan and Gwee 2004). For instance, structural variations in loop II of the scaffold has been attributed to the high affinity binding of long-chain 3F α -NTxs, but not short-chain 3F α -NTxs, to neuronal $\alpha 7$ nAChRs (Servent et al. 1997; Fruchart-Gaillard et al. 2002). Although the predominantly β -sheeted structure of 3F α -NTxs is generally assumed to be devoid of α -helices, a cyclical “helix-like” conformation at the tip of loop II that consists of two sequential turns is established by the fifth disulfide bridge present in loop II of long-chain 3F α -NTxs (Fig. 4b, c). This spatial

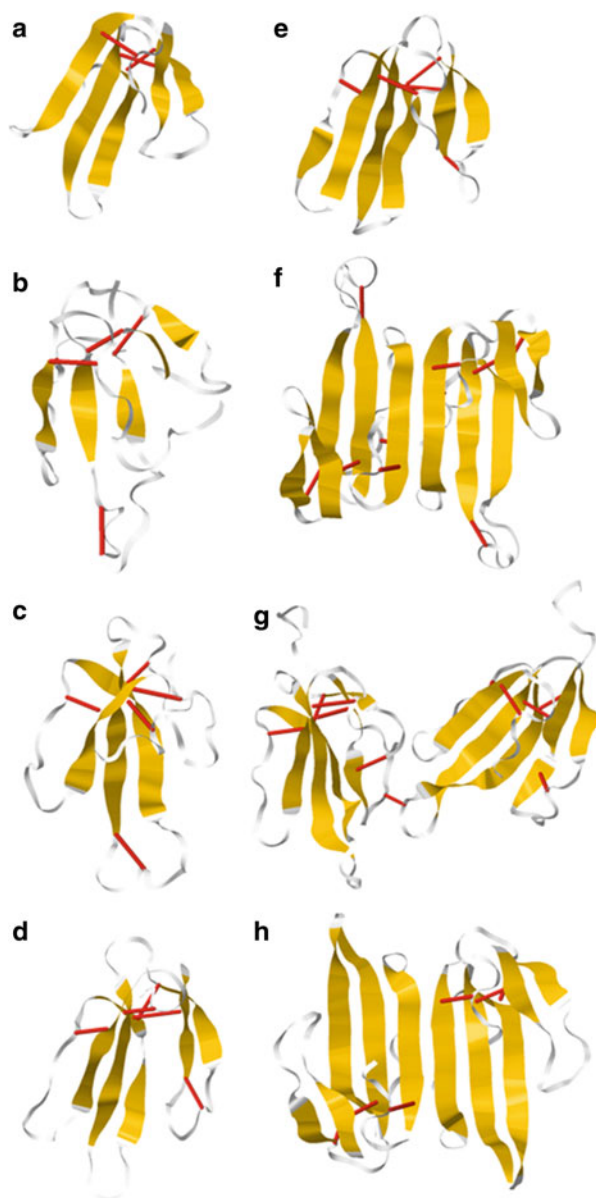


Fig. 4 Three-finger neurotoxins which interact with nicotinic acetylcholine receptors (a) short-chain 3F α -NTx: **erabutoxin-a** (*Laticauda semifasciata*) (1ERA); (b) long-chain 3F α -NTx: **α -bungarotoxin** (*Bungarus multicinctus*) (2ABX); (c) long-chain 3F α -NTx: **LSIII** (*Laticauda semifasciata*) (1LSI); (d) nonconventional 3F α -NTx: **candoxin** (*Bungarus candidus*) (1JGK); (e) nonconventional 3F α -NTx: **bucandin** (*Bungarus candidus*) (1F94); (f) noncovalently linked homodimeric long-chain 3F α -NTx: **k-bungarotoxin** (*Bungarus multicinctus*) (1KBA); (g) covalently linked heterodimeric nonconventional 3F α -NTx: **irditoxin** (*Boiga irregularis*) (2H7Z); and

conformation has been described as being vital for long-chain 3F α -NTxs to bind to neuronal nAChR targets (Maslennikov et al. 1999). Furthermore, NMR studies of free α -bungarotoxin and α -bungarotoxin in complex with short cognate peptides mimicking the nAChR toxin-binding region have revealed that this cyclic conformation of the tip of loop II was a dynamic entity which rendered flexibility to loop II in order for long-chain 3F α -NTxs to accommodate substantial structural deviations and adopt alternate conformations in order to bind to different receptor subtypes such as the muscle and neuronal nAChRs, accounting for the wider receptor selectivity seen among long-chain 3F α -NTxs (Moise et al. 2002a).

Consequently, short-chain 3F α -NTxs and atypical long-chain 3F α -NTxs (Lc-a, Lc-b from *Laticauda colubrina*), which lack this fifth disulfide bridge, as well as nonconventional 3F α -NTxs – WTX (*Naja kaouthia*) and Wntx-5 (*Naja sputatrix*), both of which have the fifth disulfide bridge in loop I (and lack a similar helical conformation in the structure), have weak affinity, in micromolar concentrations ($K_d \sim 3\text{--}22 \mu\text{M}$) at best, for the neuronal $\alpha 7$ nAChR (Servent et al. 1997, Nirthanan et al. 2003a). Interestingly, another nonconventional 3F α -NTx, candoxin (*Bungarus candidus*), is the first reported example that lacks this critical helix-like conformation in loop II but blocks neuronal $\alpha 7$ nAChRs in low nanomolar concentrations, suggesting that it likely utilizes other, yet undetermined, functional determinants in this interaction (Nirthanan et al. 2002). Other atypical interactions of 3F α -NTxs with nAChRs using unique functional determinants have also been described more recently, as reported by the discovery of a distinct class of 3FTxs, Ω -neurotoxin (*Ophiophagus hannah*), which was shown to inhibit muscle nAChRs without the involvement of the tip of loop II as established for most 3F α -NTxs (Hassan-Puttaswamy et al. 2015).

Molecular Interactions Between α -Neurotoxins and the Nicotinic Acetylcholine Receptor

Mutational Studies on Three-Finger α -Neurotoxins

Comprehensive mutational analysis by Menez and coworkers have established that short- and long-chain 3F α -NTxs utilize a *common binding core* of critical amino acids to interact with key invariant residues in the nAChR, while other residues specific for either short-chain or long-chain 3F α -NTxs determine ability to bind to



Fig. 4 (continued) **(h)** noncovalently linked homodimeric short-chain 3F α -NTx: **haditoxin** (*Ophiophagus hannah*) (3HH7). The structures are α -carbon solid ribbon representations and presented with the aid of JSmol (Jmol: an open-source Java viewer for chemical structures in 3D. <http://www.jmol.org>). Disulfide bridges are depicted in *red* and β -sheets shown in *yellow*. The snake species from which each toxin was isolated and the respective PDB accession number for stated toxin structure are indicated in parentheses

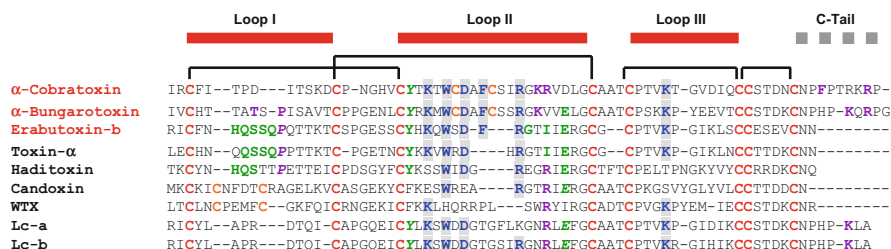


Fig. 5 Functionally invariant residues in three-finger neurotoxins that interact with muscle nicotinic acetylcholine receptors. Amino acid sequences of representative short-chain, long-chain, atypical long-chain, and nonconventional 3Fα-NTxs showing the distribution of residues critical for binding to muscle ($\alpha 1\beta 1\gamma\delta$) nAChRs. The core cysteine residues are in **red** bold lettering and the disulfide linkages and the segments contributing to the three loops and carboxy-terminus tail are indicated. Cysteine residues that contribute to the formation of the additional (fifth) disulfide bridges in long-chain (in loop 2) and nonconventional 3Fα-NTxs (in loop 1) are bolded in **orange**. The residues experimentally found to be critical for both short-chain and long-chain 3Fα-NTxs to bind to the muscle nAChRs are bolded in **blue** and **shaded**. Other residues experimentally found to contribute to binding to muscle nAChRs that are specific for short-chain 3Fα-NTxs or long-chain 3Fα-NTxs are bolded in **green** and **purple**, respectively. The figure is based on experimental data for the short-chain 3Fα-NTx, erabutoxin-a (Tremeau et al. 1995; Ducancel et al. 1996; Teixeira-Clerc et al. 2002) and long-chain 3Fα-NTxs, α-cobratoxin (Antil-Delbeke et al. 2000; Bourne et al. 2005) and α-bungarotoxin (Zeng et al. 2001; Moise et al. 2002; Huang et al. 2013). Since functionally important residues that are conserved are likely to have comparable binding function among 3Fα-NTxs, the putative functional residues in the short-chain 3Fα-NTx, toxin-α; nonconventional 3Fα-NTxs, candoxin and WTX; in the short-chain 3Fα-NTx subunit of the haditoxin homodimer; and the atypical long-chain 3Fα-NTxs, Lc-a and Lc-b, are also shown. The species names and PDB accession numbers are as for Fig. 2

nAChR subtypes (Ménez 2002). The common binding core of amino acids for the recognition of *Torpedo* or muscle ($\alpha 1\beta 1\gamma\delta$) nAChRs by 3Fα-NTxs are a group of positively charged and aromatic residues Lys²⁷, Trp²⁹, Asp³¹, Phe³², Arg³³, and Lys⁴⁷ (erabutoxin-a numbering) (Figs. 5 and 6) (Servent and Ménez 2002). The functional significance of Lys²⁷, Arg³³, and Lys⁴⁷ of loop II was also independently verified by mutagenesis studies on another short-chain α-neurotoxin NmmlI (*Naja mossambica mossambica*) (Ackermann and Taylor 1997) and by structural studies on the long-chain α-bungarotoxin (Moise et al. 2002a).

In particular, the highly conserved arginine residue at the tip of loop II (Arg³³ in erabutoxin-a and α-cobratoxin, and Arg³⁶ in α-bungarotoxin) is critical for toxin binding to nAChRs due to its positively charged guanidinium group which supposedly mimics the action of the natural neurotransmitter acetylcholine (Samson et al. 2002). The functional importance of Arg³³/Arg³⁶ has been conclusively validated by crystallographic structural data showing this amino acid in α-cobratoxin/α-bungarotoxin forming hydrophobic and aromatic interactions with key aromatic residues of the ligand-binding site of the AChBP (Bourne et al. 2005)/ chimera of AChBP and α7 nAChR (Huang et al. 2013), in complexes formed between these structures and the respective toxins.

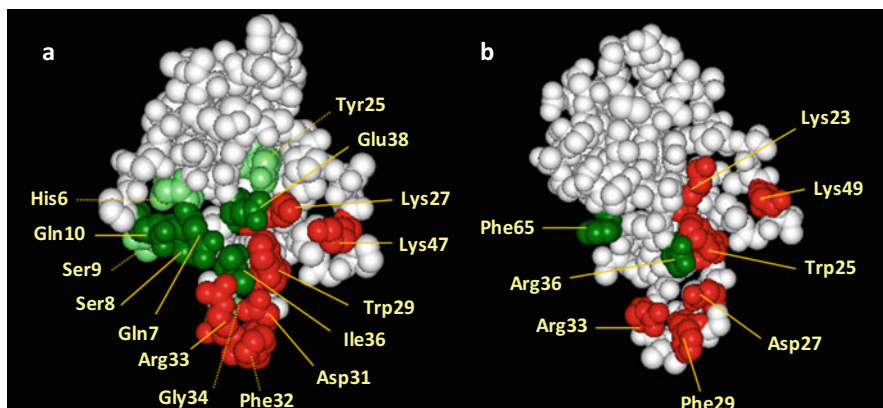


Fig. 6 Critical amino acid residues in short-chain (erabutoxin-a) and long-chain (α -cobratoxin) α -neurotoxins for binding to the *Torpedo* (or muscle) nicotinic acetylcholine receptor. (a) The 2.0 Å crystal structure of erabutoxin-a (PDB accession code: 5EBX) and (b) the 2.4 Å crystal structure of α -cobratoxin (PDB accession code: 2CTX), showing the residues by which they interact with the *Torpedo* ($\alpha 1\beta 1\gamma\delta$) receptor. Models are based on data for erabutoxin-a (Tremeau et al. 1995; Ducancel et al. 1996) and α -cobratoxin (Antil et al. 1999). The concave faces of the toxins, which have the side chains of all the functionally important residues, are shown. The residues constituting the common binding core of critical amino acids in both short-chain and long-chain α -neurotoxins are shown in red. The important residues specific for each class of toxin binding to *Torpedo* receptors are shown in dark green. In erabutoxin-a, the specific residues that have supplementary roles (whereby their mutations resulted in <10-fold decrease in receptor affinity) in the toxin-receptor interaction are shown in light green. The Corey-Pauling-Koltun space-filling molecular representation is presented

It has also been established that all the functionally important residues in both, short-chain and long-chain 3F α -NTxs for the $\alpha 1\beta 1\gamma\delta$ nAChRs are located on the *concave surface* of the molecule, with the most critical residue Arg³³ located at the very tip of loop II (Teixeira-Clerc et al. 2002). Further evidence that corroborates these findings comes from the crystal structure of the AChBP in complex with α -cobratoxin, where a critical segment of the AChBP (loop C) that binds to 3F α -NTxs is almost entirely wrapped around by the concave face of α -cobratoxin (Bourne et al. 2005).

In addition to the “*binding core*,” short-chain or long-chain 3F α -NTxs also utilize specific residues for receptor recognition (Fig. 6), which are, in erabutoxin-a (short-chain), His⁶, Gln⁷, Ser⁸, Ser⁹, and Gln¹⁰ of loop I and Tyr²⁵, Gly³⁴, Ile³⁶, and Glu³⁸ of loop II (Tremeau et al. 1995; Ducancel et al. 1996; Teixeira-Clerc et al. 2002) and in α -cobratoxin (long-chain), Arg³⁶ of loop II and Phe⁶⁵ of the carboxy-terminal tail (Antil et al. 1999).

With respect to amino acid residues in the characteristically long carboxy-terminal tail of long-chain 3F α -NTxs, Phe⁶⁵ (in α -cobratoxin) (Antil-Delbeke et al. 2000) and Lys⁷⁰ and Arg⁷² (in α -bungarotoxin) were found to be important for interaction with muscle nAChRs (Zeng et al. 2001). However, another long-chain 3F α -NTx, LSIII (*Laticauda semifasciata*), which has an unusually short

carboxy-terminal tail (Fig. 4c) also displayed high affinity binding to muscle nAChRs, suggesting a minimal functional role for the tail region of this particular long-chain toxin, which is likely compensated for by other additional functional determinants (Servent et al. 1997).

Another important consideration (and a point of contention with new structural data) noted in mutational studies is that none of the residues in loop I of the long-chain α -cobratoxin were found to be functionally important (Antil et al. 1999) in contrast to the involvement of loop I in short-chain erabutoxin-a, suggesting that the considerably shorter loop I in α -cobratoxin makes it inaccessible to the receptor surface and restricts its ability for interaction (Servent et al. 2000). That said, NMR studies of another long-chain nAChR, α -bungarotoxin, have found that Thr⁸ and Pro¹⁰ in loop I interacted with short cognate peptides containing the putative neurotoxin-binding site of *Torpedo* nAChRs, and it was proposed that loop I of α -bungarotoxin, which is longer than that of α -cobratoxin by two residues, could be sufficiently long to be involved in receptor interaction (Zeng et al. 2001).

More recent structural studies have concluded that loop I and carboxy-terminal tail of both α -bungarotoxin and α -cobratoxin do form interactions within the ligand-binding site of the receptor (Bourne et al. 2005; Huang et al. 2013). These structural studies of toxin-receptor complexes have all noted that loop II as well as other parts of the toxin display significant flexibility and reorientate to make contact with the receptor upon binding. For instance, loop I and the carboxy-terminal tail showed reconfiguration in relation to the main body of the toxin while the tip of loop II simultaneously changed the conformation of its shape and curvature (Zeng et al. 2001; Moise et al. 2002b).

Extrapolation of Data from Mutational Studies on Three-Finger α -Neurotoxins

By extrapolating the data on functionally important residues in long- and short-chain 3F α -NTxs, plausible explanations for the wide variability in affinities shown by nonconventional 3F α -NTxs (which have not undergone the same scrutiny with regard to their structure-function relationships) is possible. Just three functionally invariant residues (Lys²⁷, Lys⁴⁷, Arg³⁷) are present in homologous positions in nonconventional 3F α -NTxs from cobras [e.g., WTX (*Naja kaouthia*); Wntx-5 (*Naja sputatrix*)] which are reported to interact weakly with muscle nAChRs. In contrast, critical residues Trp²⁹, Arg³³, Arg³⁶, Glu³⁸, and Gly³⁴ are present at homologous positions in the primary sequence and in similar spatial disposition in the tertiary structure of candoxin. Since highly conserved residues most likely have comparable function among neurotoxins (Poh et al. 2002), this may explain the nanomolar affinity of candoxin for muscle and neuronal $\alpha 7$ nAChRs, not previously reported for any other nonconventional 3F α -NTx (Nirthanan et al. 2003a). Likewise, the atypical long-chain 3F α -NTxs, Lc-a and Lc-b (*Laticauda colubrina*), retain most of the functionally important residues described above (Fig. 2), and accordingly these toxins bound with high affinity to muscle/*Torpedo* $\alpha 1\beta 1\gamma\delta$ nAChRs (Servent et al. 1997).

Studies Using Synthetic Peptides of α -Neurotoxin-Binding Regions of the Receptor

Synthetic peptides representing the putative binding sequences of the $\alpha 1$ -subunit of muscle nAChRs have been used to further our understanding of the mechanisms of interaction of 3F α -NTxs with nAChRs (Zeng et al. 2001; Moise et al. 2002a; Katchalski-Katzir et al. 2003). Collectively, these studies identified receptor segments corresponding to loop C of the $\alpha 1$ -subunit of the muscle nAChR, and a homologous region on the neuronal $\alpha 7$ nAChR, as the primary α -neurotoxin-binding site (McLane et al. 1991; Moise et al. 2002a). This region includes conserved aromatic residues Tyr¹⁹⁰ and Tyr¹⁹⁸ and the unique Cys¹⁹²-Cys¹⁹³ disulfide bridge (Moise et al. 2002b). Complexes formed between α -bungarotoxin and receptor peptides revealed that the overall three-finger fold of α -bungarotoxin was preserved in the complex, with the tips of loops I and II and the carboxy-terminal tail showing conformational rearrangements (Moise et al. 2002b; Katchalski-Katzir et al. 2003) to enable a snug fit of the receptor peptide to the toxin (Harel et al. 2001).

Studies Using Combinatorial Phage-Display Library Peptides that Inhibit α -Neurotoxin Binding

Since the advent of α -bungarotoxin as a pharmacological probe to dissect the neurobiology of the nAChR, a number of 3F α -NTx derivatives including radio-labeled, fluorescent, and biotinylated conjugates have been developed to localize, label, and study nAChRs as well as to characterize the pharmacology of other ligands for nAChRs (Ménez 2002). A new facet in toxinology evolved with the identification of a 13-residue long lead peptide from a combinatorial phage-display library that inhibited the binding of α -bungarotoxin with low-micromolar affinity (Balass et al. 1997). Stemming from this, a new peptide library was designed to optimize the toxin-receptor interaction by systematic substitution of single residues as well as multiple residue replacements of amino acids in the library lead peptide (Katchalski-Katzir et al. 2003). Interestingly, some of these designer peptides were better inhibitors of α -bungarotoxin binding to muscle nAChRs ($IC_{50} \sim 2$ nM) and were capable of neutralizing the toxicity of α -bungarotoxin in vivo in rodents (Katchalski-Katzir et al. 2003). Working on this concept, it was demonstrated that a neuronal $\alpha 3$ subunit of the nAChR, normally insensitive to α -neurotoxin blockade, could be converted to an α -neurotoxin-sensitive receptor ($IC_{50} \sim 20$ – 40 nM) by the exchange of just five residues in its loop C with the corresponding residues from the α -bungarotoxin-sensitive neuronal $\alpha 7$ nAChR (Levandoski et al. 1999). This strategy worked successfully even with non- α subunits of the nAChR (Moise et al. 2002a). More generally, the introduction of α -bungarotoxin-sensitive sites (“pharmatopes”) into receptors and membrane proteins not usually the target of these toxins could see the use of various α -neurotoxin tags to localize and isolate and characterize new proteins (Moise et al. 2002b).

Studies on Receptors from α -Neurotoxin-Resistant Species

An interesting angle on research into the interaction of 3F α -NTxs with nAChRs came from the comparison of muscle nAChRs of animal species such the mongoose and cobra that are inherently resistant to the toxicity of α -neurotoxins with those from the mouse and *Torpedo* ray which are sensitive (Barchan et al. 1995; Kachalsky et al. 1995; Takacs et al. 2001). Collectively, these studies led to the definition of two subsites important for nAChRs to interact with 3F α -NTxs: an aromatic subsite which includes two aromatic residues at positions 187 and 189 and a proline subsite that includes Pro¹⁹⁴ and Pro¹⁹⁷, in loop C of the ligand-binding domain of the α 1-subunit of the nAChR (Kachalsky et al. 1995). Barchan et al. (1992) propound that substitution of proline residues may trigger conformational changes sufficient to interfere with the binding of large proteins such as 3F α -NTxs, since prolines predispose to the formation of β -bends in polypeptides and tend to flank segments of protein-protein interaction sites (Kini 1998). Amino acid substitutions at these key positions (Barchan et al. 1995; Kachalsky et al. 1995) as well as n-linked glycosylation of loop C residues (e.g., Phe¹⁸⁹) (Kreienkamp et al. 1994; Takacs et al. 2001) are thought to confer the ability to resist α -neurotoxin binding to the muscle nAChRs. These studies verified the importance of loop C of the nAChR in binding to 3F α -NTxs.

Structural Studies on α -Neurotoxin-Acetylcholine Binding Protein Complexes

The discovery of the molluscan AChBP, a remarkable structural and functional homologue of the amino-terminal ligand-binding domain of the nAChR, was an unprecedented milestone in the research of both the nAChR as well as the binding of a multitude of ligands with the diverse subtypes of nAChRs (Sixma and Smit 2003; Rucktooa et al. 2009). From the perspective of snake 3F α -NTxs, it enabled crystallography studies of complexes formed between toxins and the AChBP (Bourne et al. 2005; Huang et al. 2013) as well as the construction of simulated models of α -neurotoxins docked with the nAChR ligand-binding domain, created on the template of the AChBP (Fruchart-Gaillard et al. 2002). These data showed that 3F α -NTxs bound perpendicularly to the receptor axis, with the tip of loop II plugging into the acetylcholine-binding site of the receptor at the interface between two subunits (Bourne et al. 2005) (Fig. 7). Finer analysis of the complexes also revealed that the carboxy-terminal tail and loop I of long-chain 3F α -NTxs made adjacent additional interactions at the receptor surface. From the receptor side, major contacts were established with the toxin principally via loop C, assisted by loops A and B (of the principal α subunits) as well as loops D and F (of the complementary subunits) (Moise et al. 2002a; Bourne et al. 2005; Huang et al. 2013).

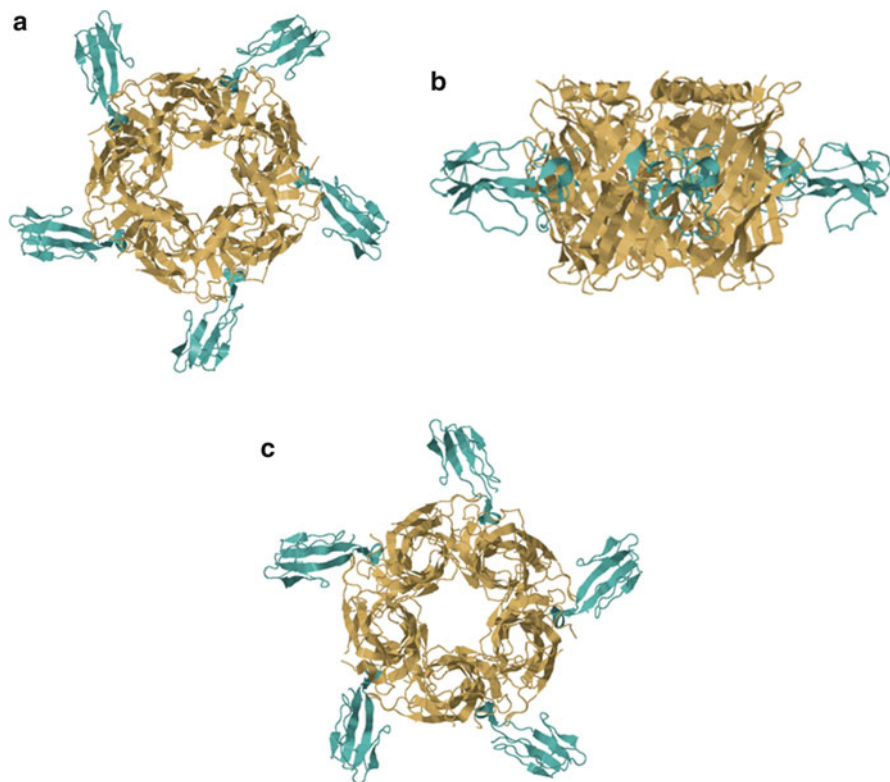


Fig. 7 Structure of the α -cobratoxin bound to acetylcholine-binding protein (AChBP) The pentameric AChBP – α -cobratoxin complex is viewed (a) from the *top*, along the fivefold axis of the AChBP; (b) perpendicular to the fivefold axis of the AChBP; and (c) from the *bottom*, along the fivefold axis of the AChBP. The model shows α -cobratoxin molecules (*cyan*) bound at the subunit interfaces of the pentameric AChBP (*yellow*). PDB accession number of this structure is 1YI5 (Bourne et al. 2005) and it is presented with the aid of JSmol (Jmol: an open-source Java viewer for chemical structures in 3D. <http://www.jmol.org/>)

Reconciling the Structural Data of the Different α -Neurotoxin: Receptor Complexes

There are differences in the conclusions arising from the major studies that examined complexes formed between 3F α -NTxs and short cognate peptides of the nAChR binding site (Zeng et al. 2001; Moise et al. 2002a), with the α 1-subunit of the nAChR (Dellisanti et al. 2007), with the AChBP (Bourne et al. 2005), and with a AChBP- α 7 nAChR chimera (Huang et al. 2013). These differences can be attributed to a multitude of factors, including limitations in resolution (4.2 Å) of the α -cobratoxin-AChBP complex structure (Bourne et al. 2005), difficulty in

extrapolating information gleaned from short peptide segments to the intact nAChR (Zeng et al. 2001; Moise et al. 2002b), and absence of the complementary subunit in the formation of the complex in the case of the α -bungarotoxin – $\alpha 1$ subunit of the nAChR (Dellisanti et al. 2007). Nonetheless, all these studies agreed on the critical points of toxin-receptor interactions, which have also largely been substantiated by early mutational analysis. Importantly, the role of critical aromatic amino acids (e.g., Tyr¹⁸⁴, Tyr¹⁹⁰) as well as residues that are their direct neighbors, at the interface of the nAChR ligand-binding site, has been verified in the binding of 3F α -NTxs. In particular, the role of loop C from the $\alpha 1$ subunit as the primary element essential for binding 3F α -NTxs has been established. A multi-point attachment of the 3F α -NTx to the receptor has also been observed, with the 3F α -NTx binding perpendicularly to the receptor axis. The primary interaction of the toxin occurred via the tip of loop II, with Arg³³/Arg³⁶ playing the most crucial role, while loop I and the carboxy-terminal tail also contributed to contacts with the receptor surface. Significant flexibility of the carboxy-terminal tail and loop I as well as flexibility and conformational changes of the tip of loop II were also noted, which is proposed to have functional ramifications, allowing for dynamic interactions with previously inaccessible parts of the toxin and receptor.

Conclusion and Future Directions

The past 50 or more years, since the advent of α -bungarotoxin as a pharmacological probe to study the nAChR (Chang 1999), have seen remarkable progress made in our understanding of the structure and function of both α -neurotoxins and the nAChR as well as the interaction between the two at an atomic level (Bourne et al. 2005; Huang et al. 2013). The arsenal of known snake α -neurotoxins has grown exponentially, along with the emergence of structurally and functionally novel classes of α -neurotoxins such as nonconventional 3F α -NTxs (Nirthanan et al. 2003b) and dimeric 3F α -NTxs (Roy et al. 2010). Significant advancements in our knowledge of the atomic structure, subtypes, and physiology of the nAChR, aided by the discovery of the molluscan acetylcholine-binding protein (Sixma and Smit 2003) as well as higher resolution electron micrographs of *Torpedo* nAChRs (Unwin 2013), have provided invaluable insight into the binding sites of a variety of ligands (Nirthanan et al. 2005, 2008). The amino acid residues by which the 3F α -NTxs bind to the nAChR, and the sites by which the receptor interacts with the toxin, have largely been delineated (Fruchart-Gaillard et al. 2006). These data divulge the complexity and extraordinary plasticity of the three-finger toxin fold (Kini 2011) that has optimally evolved to interact with specific molecular targets with precision that discerns subtle differences in just a few amino acids between receptor subtypes and high affinity that is often many folds greater than the affinity of the natural neurotransmitter for that receptor (Tsetlin 2015). The study of 3F α -NTxs also addresses important questions about protein-protein interactions as well as an interesting molecular basis about the evolution of protein scaffolds (Fry et al. 2003b). Furthermore, conclusions reached from the study of 3F α -NTxs and nAChRs can also

be extrapolated to other members of the “cys-loop” ligand-gated ion channel superfamily including GABA_A, serotonin, 5HT₃, and glycine receptors, giving broad appeal to this field of study (Changeux 2012). Biogenic molecules from animal venoms, including 3F α -NTxs, have already established themselves as strategic players in scientific discovery and medicine as ultra-selective pharmacological probes as well as lead compounds for drug discovery (Harvey 2014). With the introduction of new technologies and discovery of novel toxins, it is perfectly reasonable to envisage even more ambitious strategies for protein engineering and design of scientific probes and therapeutic molecules by manipulating the natural potential of animal toxins.

Cross-References

- [Secreted Phospholipases A₂ with \$\beta\$ -Neurotoxic Activity](#)
- [Snake Venom Toxins Targeted at the Nervous System](#)

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Abstract

Sarafotoxins (SRTXs) are extracted solely from the venom of snakes belonging to the *Atractaspis* genus, a burrowing asp from Africa and Middle East. They share a high-sequence homology with endothelins, potent vasoconstrictors produced by

Y. Mahjoub (✉)

Pôle d'Anesthésie-Réanimation, CHU Amiens, Unité de réanimation chirurgicale, Amiens, France

Unité INSERM U1088, Amiens, France

e-mail: mahjoub.yazine@chu-amiens.fr

S. Malaquin

Pôle d'Anesthésie-Réanimation, CHU Amiens, Unité de réanimation chirurgicale, Amiens, France

F. Ducancel

CEA, iMETI, Joint Unit IMVA, CEA Fontenay-aux-Roses, Fontenay-aux-Roses, France

mammalian endothelial cells and implicated in vascular tone regulation and cell growth. SRTXs and endothelins have a common core of 21 amino acids and two conserved disulfide bridges between cysteines +1/+15 and +3/+11 which constitute a unique and typical “signature” among natural bioactive peptides: Cys₁-X-Cys₃...Cys₁₁-X-X-X-Cys₁₅. SRTXs bind to endothelin receptors ET-A and ET-B situated on the membrane of numerous cells especially endothelial cells and smooth muscle cells. Recently long SRTXs have been discovered in the venom of *A. microlepidota microlepidota* and *A. irregularis*. These long SRTXs have three or four additional amino acids on their C-terminal domain. The affinity of long SRTXs for endothelin receptors is dramatically decreased compared to short SRTXs. Nevertheless, they still have a high toxicity as tested on mice. Combining invasive and echocardiographic procedures, recent experiments showed that short and long SRTXs have very different in vivo hemodynamic effects. Short SRTXs impair left ventricular function while long SRTXs impair right ventricular function and increase airway pressures with no effect on left ventricular function. Further experiments are needed to explain this discrepancy. The hypotheses of a new type of endothelin receptors (non-A non-B) cannot be discarded. Thus, the C-terminus extension seems to play a major role in defining the in vivo effect of these peptides.

Keywords

Atractaspis • Sarafotoxin • Endothelin • C-terminal domain • Vasoconstriction activity • Right ventricle

Introduction

Snake venom sarafotoxins (SRTXs) and mammalian endothelins (ETs) form a structurally and functionally related family of potent vasoconstrictor peptides. Endothelins are synthesized by mammalian endothelial cells and are released in very small amounts (plasma concentration in picomolar range).

SRTXs have been discovered in the early 1980s in the venom of *Atractaspis engaddensis*, a snake belonging to the Atractaspididae family (Kochva et al. 1982). The name “Sarafotoxin” comes from the Hebrew name of the snake: Saraf Ein Geddi. Saraf means dragon and “Ein Geddi” is the name of the oasis where *A. engadensis* has been first described. Atractaspididae are a single genus family of oviparous burrowing asps from Africa and Middle East. They are of medium length between 70 and 120 cm. These snakes have lateral small fangs that can be seen even when their mouth is closed (Fig. 1). Hence, to the contrary to other asps they do not bite but they can stab their prey mouth closed, thus they are sometimes known as side-stabbers. They feed essentially from small reptiles, amphibians, and rodents. “Bites” from *Atractaspis* snakes are responsible of pain, local edema and ischemia or necrosis, general weakness, nausea and vomiting, bradycardia, T-wave inversion, atrioventricular block, and may lead to cardiac arrest (Kurnik et al. 1999; Wolleberg et al. 1988; Lee et al. 1986).



Fig. 1 Head of *Atractaspis engaddensis* with a visible fang protruding from closed mouth

Recently, sarafotoxins extracted from other snakes belonging to the atractaspididae family have been described (Hayashi et al. 2004; Quinton et al. 2005). These sarafotoxins have a longer C-terminus extension but have the same 3-D structure as SRTXs from *A. engaddensis* and endothelins (Mourier et al. 2012). After presenting the structural diversity of SRTXs, and the effect of agonist propriety of SRTXs on endothelin receptors, this review will explain the functional diversity related to the structural diversity of SRTXs (short vs. long) based on recent experimental in vivo studies.

Sources, Structure, and Toxicity

Short Sarafotoxins from *A. engaddensis*

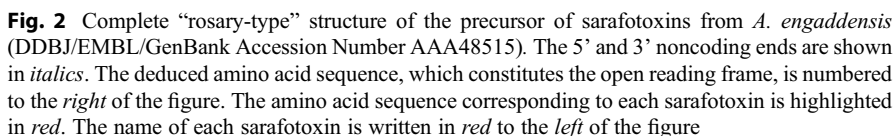
In 1988, the primary sequences of three peptides extracted from the venom of *A. engaddensis* and named SRTX-a, SRTX-b, and SRTX-c have been described (Takasaki et al. 1988). The same year, Yanagisawa et al. discovered a potent vasoconstrictor peptide synthesized by mammalian endothelial cells: endothelin-1 (ET-1) (Yanagisawa et al. 1988). Later, two other isoforms of endothelins have been discovered (ET-2, ET-3 and VIC) (Inoue et al. 1989; Saida et al. 1989) while another SRTX called bibrotoxin (Btx) was identified in the venom of *Atractaspis bibroni* (Becker et al. 1993).

Endothelins and SRTXs share a high sequence homology (about 60 %). They have a common core of 21 amino acids and two conserved disulfide bridges between cysteines +1/+15 and +3/+11 which constitute a unique and typical “signature” among natural bioactive peptides: Cys₁-X-Cys₃...Cys₁₁-X-X-X-Cys₁₅. Sequence homology between SRTXs from *A. engaddensis* and ET-1 is more than 60 %, while SRTX-b and BBTX differ only from one amino acid in

position +4. Three-dimensional structures analyses of these peptides are consistent with a similar overall structure: SRTXs and endothelins adopt a cysteine-stabilized alpha-helical motif characterized by an extended structure of the first three or four residues, a beta-turn structure between position +5 and +8, an alpha-helical conformation of the segment Lys₉-Cys₁₅, and the absence of conformation of the C-terminal domain (Mourier et al. 2012). Complementary DNA (cDNA) encoding SRTXs in *A. engaddensis* comprise 1948 base pairs (bp) including one open reading frame of 1629 pb coding for a long pre-pro-polypeptide of 543 amino acids which starts with a methionine that initiates translation followed by a hydrophobic peptide characteristic of signal sequence (Ducancel et al. 1993). This long open reading frame deduced for this precursor has an original repetitive structure comprising one sequence of 39 amino acids followed by 11 sequences of 40 residues. Each of these contains a SRTX sequence preceded by an invariant spacer peptide of 19 amino acids. The last copy of SRTX is followed by a peptide that differs the previous one both in terms of its sequence and length (13 amino acids). In all, five different isoforms of SRTXs are encoded by this single precursor: SRTX-a, SRTX-a1, SRTX-b, SRTX-c, and SRTX-e. A sixth isoform called SRTX-b1, differing from SRTX-b by one amino acid at position +7, was also identified during sequencing of an incomplete cDNA (Ducancel et al. 1993). So, six isoforms present in the venom of *A. engaddensis* are produced from 2 precursors. This so-called rosary-type organization is unique in terms of its regularity in precursors of snake toxins with disulfide bridges (Fig. 2). Thus “three-fingered” toxins, such as neuromuscular toxins, muscarinic toxins, fasciculins and type A2 phospholipases for which numerous isoforms have been identified, are all produced from mono-cistronic precursors (Ducancel et al. 1991). This peculiar organization seems to constitute a simple, economical, and effective way of amplifying the production of SRTXs in the venom of *A. engaddensis* (Ducancel 2005).

Long Sarafotoxins from *A. microlepidota microlepidota*

Through exhaustive study of the venom of *A. m microlepidota*, another species of the genus *Atractaspis*, a second poly-cistronic precursor encoding SRTXs have been described by Hayashi et al. (Hayashi et al. 2004). Using mass spectrometry analysis of the venom composition and molecular cloning of the precursors encoding SRTXs, these authors identified a new family of longer SRTXs, since displaying three additional residues at their C-terminus extremities. A cDNA fragment containing seven 144-nucleotide repeats, each coding for a peptide link followed by a SRTX sequence, has been identified. This cDNA fragment displays a poly-cistronic organization as in the case of precursor encoding SRTXs from *A. engaddensis*. Five different isoforms named SRTX-m, SRTX-m1, SRTX-m2, SRTX-m3, and SRTX-m4 are encoded by this precursor. These isoforms (except SRTX-m4) successively present downstream of the invariant tryptophan at position +21, an aspartate, glutamate, and a proline. Comparison of the primary structures of the five isoforms reveals between 54 % and 96 % homology: SRTX-m1 and -m2 differ from SRTX-m



SRTX-b	C	S	C	K	D	M	T	D	K	E	C	L	Y	F	C	H	Q	D	V	I	W				
SRTX-c	C	T	C	N	D	M	T	D	E	E	C	L	N	F	C	H	Q	D	V	I	W				
Btx	C	S	C	A	D	M	T	D	K	E	C	L	Y	F	C	H	Q	D	V	I	W				
SRTX-m	C	S	C	N	D	I	N	D	K	E	C	M	Y	F	C	H	Q	D	V	I	W	D	E	P	
SRTX-i3	C	S	C	T	D	M	S	D	L	E	C	M	N	F	C	H	K	D	V	I	W	V	N	R	N
ET-1	C	S	C	S	S	L	M	D	K	E	C	V	Y	F	C	H	L	D	I	I	W				
ET-2	C	S	C	S	S	W	L	D	K	E	C	V	Y	F	C	H	L	D	I	I	W				
ET-3	C	T	C	F	T	Y	K	D	K	E	C	V	Y	Y	C	H	L	D	I	I	W				
VIC	C	S	C	N	S	W	L	D	K	E	C	V	Y	F	C	H	L	D	I	I	W				

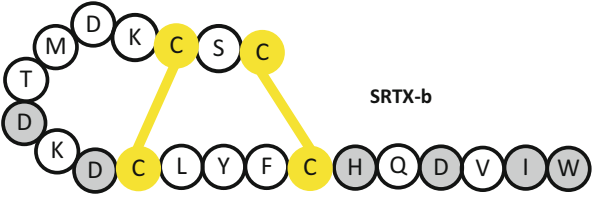


Fig. 3 Amino acid sequences of endothelins (ET1, 2, 3 and VIC), short-sarafotoxins (SRTX-b, -c and Btx) and long-SRTXs (SRTX-m and -i3). Cysteines responsible for invariant disulfide bridges are in yellow. All peptides possess two disulfide bonds: between C1 and C15 and between C3 and C11 (as shown for SRTX-b). Conserved amino acids are in gray. Btx bibrotoxin

only by one substitution, when SRTX-m4 is the most distant isoform with eight substitutions in the +1/+21 sequence, as well as a different C-terminal extension (Fig. 3). NMR studies showed that despite its longer C-terminal extension, SRTX-m displays a typical SRTX/ET three-dimensional structure. Nevertheless, unlike short-SRTXs and ETs, the conformation of the long C-terminus tail is restricted. NOE connectivities are observed between the end of the alpha-helix and the beginning of the C-terminus tail. As a result, the residues H16 to V19 are loosely looped back onto hydrophobic patch, restricting large amplitude motion (Mourier et al. 2012).

Long Sarafotoxins from *A. irregularis*

In addition to the previous work done on *A. m.m.*, a molecular study on a specimen of *A. irregularis*, another species of the genus *Atractaspis*, has been carried out (Quinton et al. 2005). Direct analysis by nanospray-Fourier-Transform ion cyclotron resonance of the crude venom of this snake revealed about 60 distinct molecular mass in the range 0.6–15.0 kDa, half of which is between 2.0 and 3.5 kDa, which corresponds to peptides between 20 and 30 amino acids, a characteristic length for SRTXs. In parallel, the precursors of *A. irregularis* SRTXs were cloned by homology, and de novo sequencing by mass spectrometry of the reduced crude venom was carried out. The presence of a SRTX sequence at the C-terminus of the 118-amino-acid residue precursor protein clearly establishes that it corresponds to an *A. irregularis* SRTX precursor. However, in the case of *A. irregularis*, only one

copy of mature SRTX emerges from each precursor when several tandem copies of mature SRTXs are encoded per molecule of the rosary-type precursors in the two previously studied *Atractaspididae* snakes. In total, three isoforms of SRTX emerged from this analysis: SRTX-i1, SRTX-i2, and SRTX-i3. These SRTXs are 25 amino acid long peptides (Fig. 3) with four additional residues at their C-terminus domain. SRTX-i1 and SRTX-i3 (the most abundant isoform) differ only by one amino acid: a valine for SRTX-i3 and an isoleucine for SRTX-i1 at position +22 following the conserved tryptophane at position +21 (Quinton et al. 2005).

NMR structure characterization showed that SRTX-i3 have the typical three-dimensional structure of SRTXs and endothelins. Unlike SRTX-m, the longer C-terminus extension of SRTX-i3 is found flexible and disordered without any interaction with the folded N-terminus CSH motif. This is due to the absence of NOE connectivities between these two regions and in agreement with the small dispersion of amide proton chemical shifts for residues H16 to N25 (Mourier et al. 2012).

Toxicity

SRTX-b from *A. engaddensis* is among the most lethal toxins ever described. It causes death in mice within minutes. The mean lethal 50 (LD₅₀) doses of SRTX-b are similar to that of ET-1 and are about 15 ug/kg (Kochva et al. 1993). SRTX-c is about 30 times less toxic with a LD₅₀ of 300 ug/g. Toxicity of SRTX-m is close to that of SRTX-b with an LD₅₀ of 32 ug/g while SRTX-i3 is slightly less with a LD₅₀ of 115 ug/g (Hayashi et al. 2004; Mourier et al. 2012).

Pharmacological Properties of SRTXs

Endothelin Receptors

Several data confirmed that SRTXs and ETs acts the same receptors called the endothelin receptors. Two main subtypes have been described: ET-A and ET-B (Ducancel 2002). They are members of the hepta-helical G-protein coupled receptor superfamily and range from 45 to 50 kDa in size. Human ET-A is formed by 426 amino acids and is encoded by a gene located on chromosome four. Human ET-B is formed by 442 amino acids and is encoded by a gene situated on chromosome 13. They share about 60 % homology and are highly preserved across mammalian species. Each subtype receptor consists of a long extracellular amino-terminal domain, seven loops of membrane-spanning domains, and an intracellular C-terminal domain. The C-terminal tail and the third cytoplasmic loop contain several putative phosphorylation sites. The Asp-Arg-Tyr motif in the second cytoplasmic loop is highly conserved and is possibly involved in coupling G-proteins. Moreover, the tyrosine on position +129 situated on the second transmembrane domain seems to play a major role in receptor selectivity (Krystek et al. 1994).

ET-A has a high affinity for ET-1 and ET-2 and a 100 times less affinity for ET-3. ET-B has the same affinity for all 3 ET isoforms (Barton and Yanagisawa 2008).

Affinity and Agonist Property of Short and Long SRTXs

The affinity of SRTXs for the endothelin is significantly different between short and long SRTXs. On one hand, endothelins and short SRTXs have high affinities for ET-B with affinity constants in the picomolar range, and on the other hand, long SRTXs have low affinities for ET-B receptors with constant affinity in the micromolar range. Hence, the truncated forms of long SRTXS have similar affinity that of short SRTXs. Moreover, the addition of an extended tail of a long SRTX to a short SRTX is responsible of a drop in affinity. These results are confirmed to a lesser extent for ET-A receptors (Mourier et al. 2012).

Mourier et al. have investigated the agonist property of SRTXs on ET-B receptors. They used ET-B receptors stably expressed at the surface of CHO cells and measured the fluorescence of a calcium dye that varies according to the intracellular calcium concentration. They studied SRTX-b, ET-1, and SRTX-c but also truncated forms of long SRTXs (SRTX-m and SRTX-i3 without their extended C-terminus tail) and chimeric long SRTXs (SRTX-b with the extended tail of SRTX-m or SRTX-i3). They measured the dose–response curves and highlighted the full agonist property of all SRTXs but also the high potency of short SRTXs (EC_{50} in the 10 nanomolar range) and the low potency of long SRTXs (EC_{50} in the micromolar range) (Mourier et al. 2012).

Mechanism of Action

Effect of Binding to ET-A

ET-A subtype receptor is present on the surface of smooth muscle cells but not on endothelial cells. ET-A binding stimulates a phospholipase C linked to a G-protein. Phospholipase C hydrolyzes phosphatidyl inositol 4,5 biphosphate to form the water-soluble inositol triphosphate (IP3) and the neutral diacylglycerol (DAG). IP3 and DAG are second messengers. IP3 induces calcium mobilization from the sarcoplasmic reticulum leading to an increase of intracellular calcium concentration that provokes the opening of store-dependent calcium channels (SOC) that further increase cytosolic calcium. DAG opens receptor-dependent calcium channels (Horinouchi et al. 2013). The intracellular calcium elevation activates various ions channels resulting in calcium entry across the plasma membrane and the sustained increase of intracellular calcium concentration. Intracellular calcium concentration leads to smooth muscle cell contraction and contributes to growth-promoting effects through induction of proto-oncogenes like c-fos, c-jun, and c-myc.

Effect of Binding to ET-B

ET-B subtype receptors are present on the surface of endothelial cells and on the surface of smooth muscle cells. ET-B binding on smooth muscle cells has similar effect to ET-A binding while ET-B binding on endothelial cells has different effects. On endothelial cells, ET-B binding activates the phosphoinositide 3 kinase (PI3/Akt) that stimulates phosphorylation of eNOS with subsequent NO production (Liu et al. 2005). ET-B binding also activates cyclooxygenase 2 that induces the synthesis of prostacyclin, resulting in the activation of adenylate cyclase that leads to a decrease in calcium concentration. This phenomenon is responsible for smooth muscle cells relaxation. Moreover, after binding on ET-B, ET-1 is rapidly internalized and degraded by endosomes. This is the main way of endothelin clearance especially in lungs and kidneys (Mazzuca and Khalil 2012).

Biological Activity: Differences Between Short and Long Sarafotoxins

In mammals, endothelins are the most potent endogenous vasoconstrictors to date (Yanagisawa et al. 1988), when SRTXs display various vasoconstriction potencies. Thus, SRTX-b has the same potent vasoconstriction activity than ETs, while SRTX-c is a very weak constrictor and shows at high doses a vasodilatory effect (Wollberg et al. 1989). Han et al. have shown on isolated perfused heart that SRTX-b and ET-1 had potent positive inotropic and negative chronotropic effects (Han et al. 1990). Animal studies have shown that intravenous injection of ET-1 is responsible of an increase of blood pressure due to a peripheral vasoconstriction while intracoronary injection provokes a coronary vasoconstriction (Konrad et al. 2005). Recently, an in vivo study on anesthetized rats under mechanical ventilation, combining invasive catheterization of the left ventricle and Doppler echocardiography, has investigated the precise cardiovascular effect of short and long SRTXs (Mahjoub et al. 2015). These authors showed that intravenous infusion of SRTX-b decreases cardiac output and impairs left ventricular systolic and diastolic function (negative lusitropic effect) while increasing total peripheral resistance. Hence, SRTX-b (the most potent vasoconstrictor among SRTXs) impairs left ventricular function in vivo by increasing left ventricular afterload. Concerning long SRTXs, the authors have shown that SRTX-m has no effect on left ventricular afterload or contractile function. Interestingly, these authors have shown that SRTX-m induces an acute right ventricular dysfunction while SRTX-b has no effect on the right ventricle. SRTX-m seems to have also a significant effect on bronchial smooth muscle cell as intravenous injection of SRTX-m significantly increases airway pressure. Moreover, a truncated form of SRTX-m (SRTX-m without its longer C-terminus extension) has exactly the same effects as SRTX-b (Mahjoub et al. 2015).

Together, these results pave the way for several hypotheses. First, an in vivo maturation of the long SRTXs by an endogenous enzyme may liberate a short-like SRTX. The previous results do not support this hypothesis as SRTX-m and SRTX-b

have different in vivo effects. Second, one may consider the involvement of another subtype of receptors (non-A, non-B endothelin receptors). Some authors have postulated this hypothesis: Zeng et al. have cloned and characterized a human gene that encodes a putative “human ET-B like protein” from a human hippocampus tissue cDNA library. The deduced amino acid sequence of this protein is 52 % similar and 26.7 % identical to human ET-B, but ligands and physiological significance of this protein remains unknown (Zeng et al. 1997). Moreover, Bdolah et al. have found that to the contrary to ET-1, SRTX-b failed to induce contraction of Egyptian mongoose (*Herpestes ichneumon*) aortal preparation. These authors also found ET-1 and SRTX specific binding sites in the mongoose brain. Thus, they made the hypothesis that some structural features of ET/SRTX receptors in the mongoose enable them to differentiate between the two peptides (Bdolah et al. 1997). Further studies remain necessary to confirm this hypothesis especially for other mammalian species. Finally, a hetero-oligomerization process may modulate the pharmacology of interacting ligands. Fukuroda et al. provided evidence that the combined treatment with ET-A and ET-B antagonists antagonized the contraction of human isolated bronchi induced by ET-1 but no significant inhibition was observed with the use of the respective antagonist alone suggesting collaboration in the recognition of ET-1 between ET-A and ET-B receptors (Fukuroda et al. 1996). Harada et al. have studied binding properties of ET-A and ET-B receptors of rat pituitary glands. They conclude that although there are ET-A and ET-B receptors with a functional binding capability for ET-receptor–ligands, the ET-B receptor does not independently recognize ET-1 without the aid of the ET-A receptor. They made the hypothesis that ET-1 can bridge between the two receptors to form an ET-A-ET-B receptor heterodimer (Harada et al. 2002). The phenomenon of hetero-oligomerization of G-protein-couple receptors has been described for functional opioid receptors (κ and δ) (Jordan and Devi 1999). Further studies remain mandatory to investigate these hypotheses.

Nevertheless, these results emphasize the major and elaborated role “played” by the C-terminus extension in defining the in vivo effects of SRTXs.

Conclusion and Future Directions

SRTXs extracted from the venom of atractaspididae snakes belong to the endothelin-like peptide family. They are potent vasoconstrictors. Short SRTXs have high affinity for endothelin receptors especially for ET-B and high toxicity. Long SRTXs have an extended C-terminal domain with to three or four additional amino acids. This extended C-terminal domain is responsible for a dramatic decay in agonist affinity but did not impair significantly the toxicity of these peptides. In vivo experiments have shown that the cardiovascular effects of short and long SRTXs are completely different. Long SRTXs impair right ventricular function and provoke bronchoconstriction while short SRTXs impair left ventricular function with no effect on the right ventricle. Some authors have made the hypothesis for new subtype receptors or hetero-oligomerization process. Further experiments are necessary to characterize the role of the C-terminus extension in defining the in vivo toxic

effect of these toxins. In addition, other *in vivo* studies, using chimeric SRTXs and/or truncated SRTXs, are needed to precise the role of the C-terminus extensions found in long-SRTXs from *A. microlepidota microlepidota* or *A. irregularis*. Addition of SRTX extensions to ETs would be of interest too! Finally, labeling of long SRTXs will help to localize their binding sites.

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Crotamine: Function Diversity and Potential Applications

12

M. P. Marinovic, C. Dal Mas, G. G. Monte, D. Felix, J. D. Campeiro, and Mirian A. F. Hayashi

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M.P. Marinovic • C. Dal Mas • G.G. Monte • D. Felix • J.D. Campeiro • M.A.F. Hayashi (✉)
Departamento de Farmacologia, Universidade Federal de São Paulo (UNIFESP/EPM), São Paulo,
SP, Brazil
e-mail: marcelo.marinovic@hotmail.com; carolinedalmas88@gmail.com;
gabrielaagmonte@gmail.com; nunes.df@bol.com.br; joanacampeiro@gmail.com;
mhayashi@unifesp.br; mirianhayashi@yahoo.com

Abstract

The snake venom is composed by a complex combination of components (namely, proteins and/or enzymes, peptides, nucleic acids, among others) known as toxins, which are designed in principle to act essentially on prey's cardiovascular system or on specific tissues as, for instance, muscles (skeletal or smooth muscles) and central nervous system (CNS). Crostamine is one of the most abundant components of the venom of the South American rattlesnake *Crotalus durissus terrificus*, corresponding to about 12–25 % of the dry weight of the crude venom. Several data reported by the group in the last decade suggested that this polypeptide may also be a promising tool for biotechnological and biomedical applications. In addition, it may also represent a potential structural model for the development of new drugs. Most significant evidences of crostamine versatility for diverse applications will be discussed in the present work, including: (1) the ability to translocate biological membranes and penetrate into highly proliferative cells; (2) the specificity for intracellular compartments, particularly nucleus and lysosomes; (3) the ability to carry nucleic acids, and other molecules, into cells, which could make it useful as a transfection vector; (4) the antimicrobial property with remarkable activity against some yeasts and fungus; and (5) the cytotoxic activity against cancer cells with the ability to stop the growth of certain tumors in vivo. In addition, other recently described biological activities of crostamine as the antiparasitic (antimalarial and anthelmintic) activity, as well its action on CNS, are described here.

Keywords

Crostamine • Snake toxin • Biotechnological application • Cell-penetrating peptide • Cationic peptide

Introduction

Crostamine was first described as a myotoxin, originally isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Laure 1975). Since then, different biological applications and functions for crostamine have been described (Fig. 1). In fact, the potential applications of natural peptides in drug development, diagnosis, and/or biotechnology are well recognized and widely described (Hayashi et al. 2012a).

The geographic distribution of *Crotalus durissus terrificus* and the correlation with the presence of crostamine in its venom was first proposed by Gonçalves and Vieira (1950), who suggested a geographic determination for crostamine expression after analyzing pools of venom from Northern, Central, and Southern Brazil, actually evaluated only in three localities (Morro Agudo, Franca, and Ituverava). Later on, the geographic determination for crostamine expression was also reinforced by

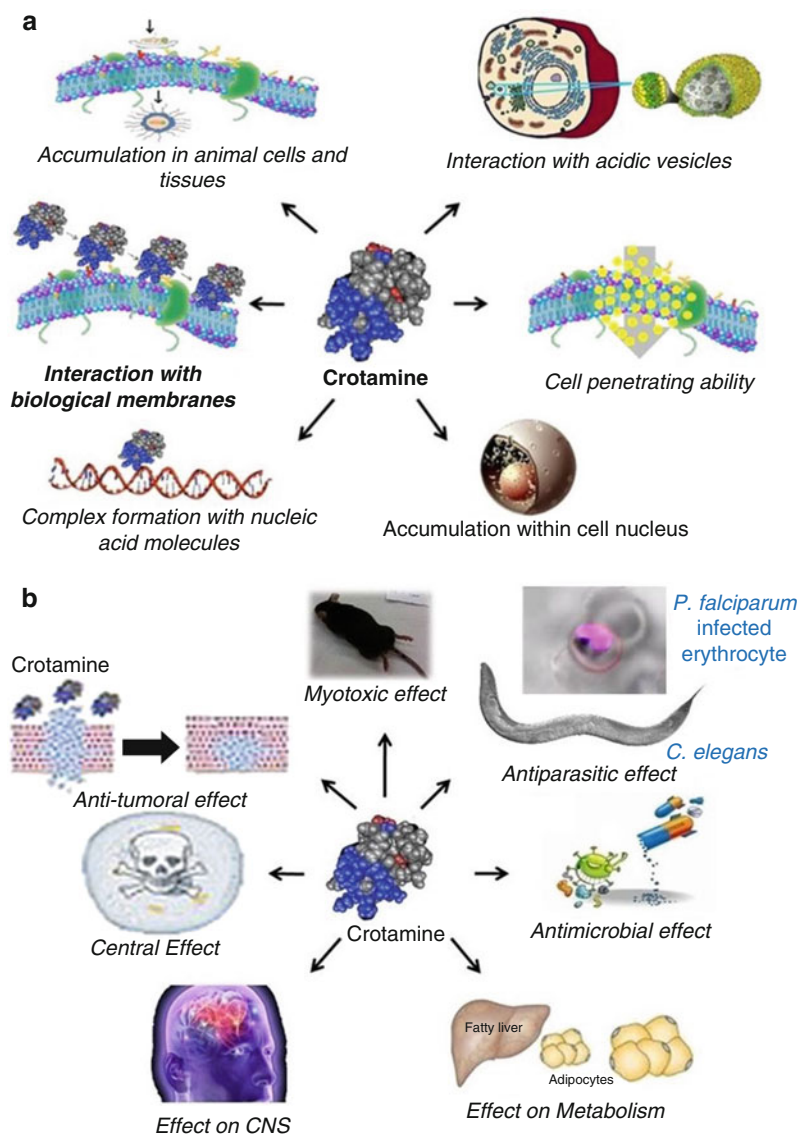


Fig. 1 Biological functions and properties of crotamine. (a) crotamine's properties are accumulation in animal cells and tissues, cell penetrating ability, affinity for acid vesicles, interaction with nucleic acids, function as vector for cargo transport, and interaction ability with lipid membranes with capability of permeabilization; in addition, (b) crotamine's biological functions are antitumoral/cytotoxicity, myotoxic, antimicrobial, antiparasitic (antimalarial and anthelmintic activities), and the effects on metabolism and on the central nervous system (CNS) will be described herein

Schenberg (1959). However, Vanzolini (Vanzolini and Calleffo 2002) and also other authors, including our group, consider the geographical specification insufficient, and therefore this matter remains not fully understood yet.

In fact, the intraspecific variation of the crotamine gene was demonstrated more recently (Oguiura et al. 2009). The genus *Crotalus* belongs to the Viperidae family and its karyotype is composed by 36 chromosomes, with eight pairs of macrosomes and ten pairs of microsomes (Radis-Baptista et al. 2003). Fluorescence in situ hybridization (FISH) studies revealed that crotamine genes are located at the end of the long arm of chromosome 2 and showed differences between homologous chromosomes, suggesting a variable number of copies of crotamine gene in each chromosome, which was proposed to be a possible explanation for the variable quantities of crotamine found in the venom of each individual *Crotalus* (Radis-Baptista et al. 2003).

In addition, other factors as climate, gender, age, and diet, as well as the captivity period and venom extraction frequency should be considered with attention, as it was described to influence the venom content in the same way as described for other snakes (Lameu et al. 2013).

Molecular and Structural Characteristics of Crotamine

Crotamine is a low molecular weight (4889.8 kDa) peptide, composed by 42 amino acid residues [YKQCHKKGGHCFPKEKICLPSSDFGKMDCRWRWKCKK-GSG], in which six are cysteine (C, Cys) residues forming three disulfide bonds, which guarantee a molecular tridimensional (3D) structure not very far from a spherical shape (Nicastro et al. 2003; Fadel et al. 2005). The presence of nine lysine (K, Lys) and two arginine (R, Arg) residues confers to this molecule a basic character and a liquid positive net charge (Nicastro et al. 2003; Fadel et al. 2005). In addition, the presence of two histidine (H, His) residues provides potential extra positive charge to this molecule, depending on the pH of the medium. Interestingly, crotamine lacks alanine (A, Ala), valine (V, Val), and threonine (T, Thr) residues, and its molecular formula, according to the isoform 1, is $C_{340}H_{530}N_{90}O_{82}S_8$ (UniProtKB/Swiss-Prot: Q9PWF3.1, LOCUS MYC2_CRODU).

Crotamine isoforms for several species of *Crotalus* are described, although the main difference among the most common isoforms were first described to be the substitution of leucine (L, Leu) to isoleucine (I, Ile) residue in the position 19 of the full-length native crotamine (Radis-Baptista et al. 1999). However, several other substitutions are also observed in different species of *Crotalus* genus (Fig. 2). Moreover, many isoforms of crotamine within the same species were also described (Fig. 3) (Toyama et al. 2000, 2003; Ponce-Soto et al. 2007).

Several studies were based on the studies conducted with the snake myotoxins from different species that due to the similarity of structure would also apply for

Crotalus durissus terrificus

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sp|Q9PWF3.1|MYC2_CRODU YKQCHKKGGHCFPKEKICLPSSDFGKMDCRWRWKCCKKGS 42
sp|O73799.1|MYC3_CRODU YKQCHKKGGHCFPKEKICIPSSDFGKMDCRWRWKCCKKGS 42
sp|O57540.1|MYC1_CRODU YKQCHKKGGHCFPKEKICIPSSDFGKMDCRWRWKCCKKGS 42
sp|P24332.1|MYX2_CRODU YKRCHIKGGHCFPKEKICIPSSDFGKMDCPWRRRKLKGSG 42
sp|P24331.1|MYX1_CRODU YKRCHIKGGHCFPKEKICIPSSDFGKMDCPWRRRKLKGSG 42
sp|P24333.1|MYX3_CRODU YKRCHIKGGHCFPKEKICIPSSDFGKMDCPWRRRKLKGSG 42

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Crotalus oreganus helleri

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sp|Q9PWF3.1|MYC2_CRODU YKQCHKKGGHCFFPKEKICLPPSSDFGKMDCRWKWKCKCKGSG 42
gb|AEU60011.1| YKRCLHKKGGHCFPPKTVICLPPSSDFGKMDCRWKWKCKCKGVS 42
sp|P01477.2|MYXC_CROOH YKRHHKKGGHCFPPKTVICLPPSSDFGKMDCRWKWKCKCKGSV 42
gb|AEU60013.1| YKRCHKKGGHCFPPKTVICLPPSSDFGKMDCRWKWKCKCKGSG 42
gb|AEU60014.1| YKQCHKKGGHCFFPKEKICI PPSSDFGKMDCRWKWKCKCKGSG 42
gb|AEU60009.1| YKRHHKKGGHCFPPKEKICI PPSSDFGKMDCRWKWKCKCKGSV 42
gb|AEU60015.1| YKRCHKKGGHCFPPKEKICI PPSSDFGKMDCRWKWKCKCKGSG 42
*****
*****
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Crotalus viridis viridis

sp|Q9PWF3.1|MYC2_CRODU YKQC**H**KKG**G**HCF**P**KEICLPPSSDFG**KMD**C**RW****W**K**CC**K**K**GSG 42
 sp|P63176.1|MYX3_CROVV YKR**C**H**K**KGGHCF**P**KT**V**I**C**LPPSSDFG**KMD**C**RW****K****W**K**CC**K**K**G**S**V 42
 sp|P63175.1|MYX2_CROVV YKR**C**H**K**KGGHCF**P**KT**V**I**C**LPPSSDFG**KMD**C**RW****K****W**K**CC**K**K**G**S**V 42
 sp|P01476.1|MYX1_CROVV YK**C**H**K**KGGHCF**P**KEICLIPPSSDLG**KMD**C**RW****K****W**K**CC**K**K**GSG 42

Fig. 2 Multiple sequence alignments of crotonamine isoforms from each *Crotalus* species, showing the sequence variation observed in each single species and also among different species. The alignment was created using ClustalW, and the codes are displayed with NCBI (*gi*) and Swissprot identifiers. The asterisks (*) indicate identical amino acid residues, while conserved substitutions are indicated by the symbol “:”. Cysteine residues are indicated by *red letters*, and the basic residues are in *blue*, while the histidine residues are in *green*.

Crotalus durissus terrificus YKQCHKKGGHCFPKEK-ICLPSSDFGKMDCKWKWKCKCKKSGK43
Crotalus durissus cumanensis YKQCHKKGGHCFPKEVLICIPSSDFGKMDCKWKWKCKCKKSG—42
Crotalus odamanteus YKQCHKKGGHCFPKIV-ICLPSSDFGKMDCKWKWKCKCKKSG—41
Crotalus oregonus helleri YKKCHKKGGHCFPKEK-ICIPSSDFGKMDCKWKWKCKCKKSG—41
Crotalus oregonus concolor YKKCHKKGGHCFPKEK-ICTPPSSDFGKMDCKWKWKCKCKKSG—41
Crotalus viridis viridis YKQCHKKGGHCFPKEK-ICIPSSDLGKMDCKWKWKCKCKKSG—42
Crotalus durissus ruruima YKQCHKKGGHCFPKEK-ICIPSSDFGKMDCKWKWKCKCKKSG—42
 ** * * * * * ** * * * * *

Fig. 3 Multiple sequence alignment of one single isoform of crotonase from each different *Crotalus* species. The alignment was created using ClustalW, and the codes are displayed with NCBI (*gi*) and Swissprot identifiers. The asterisks (*) indicate identical amino acid residues, while conserved substitutions are indicated by the symbol “:”. Cysteine residues are in *red*, and the basic residues are in *blue*, while the histidine residues are in *green*.

crotamine (Li et al. 1993; Cameron and Tu 1978), as in fact myotoxins may represent variable sequences of crotamine isoforms (Fadel et al. 2005; Fox et al. 1979).

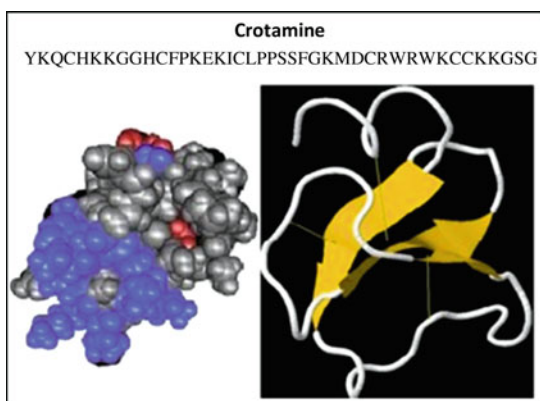
Crotamine Conformation

First studies proposed different disulfide bonds pairing, as the one conducted by Laure, in 1975, who proposed the pattern Cys⁴-Cys³⁷, Cys¹¹-Cys³⁶, and Cys¹⁸-Cys³⁰, while in 1979 (Laure 1975), Fox proposed the Cys⁴-Cys³⁶, Cys¹¹-Cys³⁰, and Cys¹⁸-Cys³⁷ pairings (Fox et al. 1979).

The determination of the three-dimensional (3D) structure of crotamine by proton nuclear magnetic resonance (NMR) spectroscopy, several years later, showed that, in fact, this toxin is composed by a short N-terminal α -helix (involving the residues 1-7), two stranded antiparallel β -sheets (residues 9-13 and 34-38), and two β -turns (residues 14-16 and 27-34), stabilized by three disulfide bonds (Cys⁴-Cys³⁶, Cys¹¹-Cys³⁰, and Cys¹⁸-Cys³⁷) (Nicastro et al. 2003; Fadel et al. 2005), which is in accordance to the description by Fox and colleagues (Fox et al. 1979). Consequently, it was suggested that the toxin is arranged in $\alpha\beta 1\beta 2$ topology, in which the first and second strands run antiparallel to each other (Fig. 4). Therefore, with these structural attributes, crotamine is characteristically highly compact, with a surface mainly positively charged and with a high ability to interact with negatively charged molecules and biological surfaces (Coronado et al. 2013; Costa et al. 2014).

Crotamine also presents 3D structural similarity with human β -defensins (Fadel et al. 2005; Coronado et al. 2013; Costa et al. 2014; Kerkis et al. 2014), which constitute a class of peptides with antimicrobial activity (Dhople et al. 2006). This similarity directed the studies to evaluate the antimicrobial activity of crotamine and its potential action on ion channels, as it will be discussed later on here. It is worth to mention that this structural similarity is not remarkable for the primary sequence, but the three disulfide bonds have exactly the same pattern in both crotamine and human

Fig. 4 Primary sequence and three-dimensional (3D) structure topology of crotamine. Color code (structure at left): negative charged residues are represented by red balls, while positive charged residues are in blue, and uncharged residues are in gray. Crotamine 3D structure showing the $\alpha\beta 1\beta 2$ structure and the disulfide bonds (structure at right)



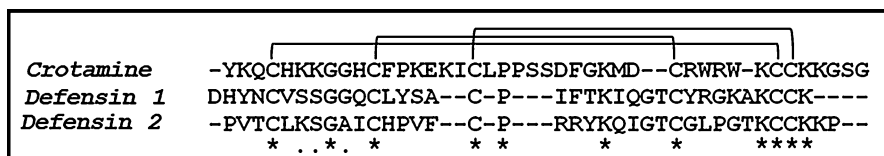


Fig. 5 Primary sequence of crotonamine and human β -defensins. Alignment of crotonamine and human β -defensin 1 e 2, created by using EMBL-EBI Clustal Omega (<http://www.ebi.ac.uk/Tools/services/web/toolresult.ebi?jobId=clustalo-I20151023-165518-0750-52590877-pg>), and slightly modified manually to align the cysteine residues. The disulfide bonds of crotonamine [Cys⁴-Cys³⁵, Cys¹¹-Cys²⁹, Cys¹⁸-Cys³⁶], and human β -defensins [Cys⁵-Cys³⁴, Cys¹²-Cys²⁷ and Cys¹⁷-Cys³⁷] are indicated by the bars on top of the sequences alignment

β -defensins (Fig. 5). These intramolecular disulfide bonds provide for both molecules a characteristic 3D structure that is also found in some scorpion toxins with biological activity on Na⁺ channels (Nicastro et al. 2003) and in some snake toxins acting on K⁺ channels (Harvey 1997).

Crotonamine Properties

Cell Penetrating Ability

Structural characteristics as the (a) cationic profile, (b) small size (usually up to 40 amino acid residues), (c) low molecular weight, and (d) high content of basic amino acids as arginine (R, Arg) and lysine (K, Lys) residues are all together the common features generally found in peptides with the capacity to cross membrane lipid bilayers of various cell types. These peptides are known as cell-penetrating peptides (CPPs), protein transducer domains (PTD), or membrane transport sequences (MTS).

In addition to the ability to cross the biological membranes, the CPPs are also able to mediate the internalization of bioactive compounds as drugs, nucleic acid molecules (namely, RNA and DNA, or short RNA or oligonucleotides), peptides, peptide nucleic acids (PNAs), proteins, imaging agents, antigenic cargos for vaccine production, and nanoparticles (Boisguerin et al. 2015; Jiang et al. 2014; Kang et al. 2014; Farkhani et al. 2014; Shiraishi and Nielsen 2014; Papadopoulou and Tsiftoglou 2013; Trabulo et al. 2013; MacEwan and Chilkoti 2013). The mechanism involved in the internalization of cationic CPPs by cells still generates discussion, and some authors have suggested that this mechanism is dependent of endocytosis, similarly to that described for toxins from bacteria and plants (Raagel et al. 2010). Another suggestion is the induction of endocytosis mechanism with a possible involvement of electrostatic interaction with the extracellular matrix of the cells, which depends upon the transmembrane negative potential (Raagel et al. 2010; Bechara and Sagan 2013; Reissmann 2014), as also described for crotonamine (Costa et al. 2014; Nascimento et al. 2007).

In addition, the cationic property of crotonamine allows its interaction with cargos with negative charge and also with negatively charged proteoglycans present in cellular membranes, which was shown to be essential for crotonamine translocation by endocytosis mechanism (Nascimento et al. 2007). Therefore, crotonamine suffers compartmentalization into endosomes, followed by permeabilization of these vesicles, which results in leakage of the vesicular content and release of crotonamine into the cell cytoplasm (Nascimento et al. 2007; Hayashi et al. 2008).

Therefore, crotonamine was also recognized as a CPP family member, although not presenting any obvious primary sequence similarity with other known CPPs (Kerkis et al. 2006). Moreover, crotonamine still stands out for its unique specificity for cells with high division rate, from which also emerged the motivation to study crotonamine as a tumor-targeting compound (Nascimento et al. 2007, 2012).

Marker of Cell Cycle Phases

As mentioned, accumulated evidences suggested that crotonamine is selectively translocated by actively dividing cells, and the penetration at the G1/S phase of cell cycle, followed by its binding to the centrosomes and nuclear chromosomes, was demonstrated both *in vitro* and *in vivo* (Kerkis et al. 2004). Once in the cytoplasm, free crotonamine interacts with centrosomes during the G1 phase. In S phase, when the duplication of centrosomes and chromosomes starts, crotonamine shows a limited perinuclear location. In M phase, crotonamine accompanies the duplication of centrioles and the separation in the cell poles. In prophase, crotonamine follows the separation of chromosomes and mitotic progression, and then, it is excluded from the nucleus and its presence was observed to be restricted to the cell cytoplasm (Nascimento et al. 2007; Kerkis et al. 2004). These evidences allowed suggesting crotonamine as a potential specific marker of cell cycle phases, but that still need further studies and future exploitations.

Accumulation of Crotonamine in Animal Cells and Tissues

There are several methods for snake venom detoxification, and the gamma irradiation is a potential tool for this process. Gamma rays are forms of ionizing radiation that interact with biomolecules in solution through two processes: in the direct process, the radiation reaches the target molecules, while in the indirect process, the water radiolysis products interact with the target molecules. The irradiation of aqueous solutions could induce changes in chemical and structural patterns of proteins and peptides, attenuating and/or abolishing the biological activities of the protein (Oliveira et al. 2015).

Several changes was described in irradiated crotonamine, including: (a) quantum yield decrease of about 25 % in comparison to the native form, suggesting an increase in the accessibility of tryptophan (W, Trp) residues due to unfolding of the polypeptide chain; (b) changes in residues distribution; (c) transition of a small

portion of the random coil regions towards an α -helical conformation; (d) partial denaturation of the molecule induced by the high temperatures achieved during the irradiation process, and (e) changes in the secondary and tertiary structures of this toxin (Oliveira et al. 2015). However, taken together, it seems that the main direct influence of these changes on the biodistribution of the radiolabeled irradiated form of crotamine was limited to a time course difference in the pharmacokinetics, with no significant difference in the tissue targeting or quantitative distribution (Boni-Mitake et al. 2006). This might suggest that there is some hope for conducting structure–function analysis studies for crotamine-derived linear peptides, as the 3D structure does not seem to be fundamental or to interfere in the overall biodistribution of this molecule in the irradiated form, although one might need to keep in mind the eventual and probable limitations due to the obviously expected lower stability of linear peptides imposed by their higher susceptibility to proteases action compared to the compact 3D structured form of native crotamine.

A study conducted using crotamine radiolabeled with iodine-125 (I^{125}) in the N-terminal tyrosine (Y, Tyr), either in its native form or in its irradiated form (with no toxicity), showed the accumulation of crotamine in the organs of a rodent (Boni-Mitake et al. 2006). The labeled crotamine was injected via intraperitoneal (ip) route in rats and the highest accumulation was observed in the kidneys after 0.5–4 h for the native crotamine and 24 h for the irradiated crotamine, showing a time course difference for the pharmacokinetics of structured crotamine compared to the irradiated form. The highest radioactivity levels detected in the rodent kidneys suggests that this toxin, besides its possible metabolites and the iodine, may be mainly eliminated by renal filtration or, in other words, that the protein may have a preferential renal clearance.

Main effects of a rattlesnake bite include neurotoxicity, myotoxicity, and renal toxicity (Cruz et al. 2009), but the maximum peak concentration of crotamine in brain, skeletal muscle, and kidney was shown to be 1000, 500, and 100 time less than that in the liver of mice after ip injection (Boni-Mitake et al. 2006). Moreover, the native and the irradiated crotamine were both less found in the skeletal muscles than in blood. Lower affinity for lungs, spleen, and heart compared to skeletal muscle was also observed for the native crotamine. In addition, it was also suggested that orally delivered crotamine may suffer hepatic metabolism and, after 30–60 min, higher concentrations of native molecule found in blood could indicate a plasmatic peak of the crotamine in biological system. Furthermore, the highest concentration of crotamine in the blood was observed at 1–1.8 h after the ip injection, which then decreased in the subsequent periods, up to 24 h after injection, when the background levels were achieved. This indicates that the clearance of crotamine is expected in the interval of 24 h after ip injection (Boni-Mitake et al. 2006).

Specificity for Actively Proliferating Cells In Vitro and In Vivo

The affinity of crotamine for actively proliferating cells was first described by Kerkis and colleagues (Kerkis et al. 2004), as demonstrated by 5-bromo-2'-deoxyuridine

(5-BrdU) cell proliferation assay, commonly used in the detection of proliferating cells in living tissues (Russo et al. 1984). The internalization of crotamine by actively proliferating cells in cultured differentiating mice embryonic stem (ES) cells was first analyzed using a Cell Proliferation Kit (Amersham Biosciences, Buckinghamshire, UK) and fluorescently labeled crotamine (Cy3-crotamine), which was analyzed by confocal microscopy at 5 min, 1 h, 3 h, 24 h, and 48 h after treatment. The incorporation of 5-BrdU into DNA was monitored during cell replication by anti-BrdU antibody immunostaining, and the colocalization with Cy3-crotamine in areas with actively proliferating ES cells was observed (Kerkis et al. 2004). In these cells, crotamine and 5-BrdU showed nuclear localization, although with no complete overlapping that allowed to suggest possible different sites of interaction for these compounds in the DNA structure (Kerkis et al. 2004).

Crotamine *in vitro* uptake in human primary fibroblasts, lymphoblastic cells, murine embryonic stem (pluripotent and differentiated), and endothelial s-vec cells was also described after 1 h of incubation with crotamine, as well as a strong accumulation of Cy3-crotamine within islands composed by rounded up juxtaposed pluripotent murine ES cells was demonstrated (Kerkis et al. 2004). Crotamine internalization was observed within the first 5 min after its addition into cultured cells dishes, and the number of labeled cells did not increased with time, although the maximum accumulation in cells could be observed about 3 h after treatment, while 16–24 h after the removal of the labeled Cy3-crotamine from the culture medium, no detectable fluorescence was observable in cultured cells (Kerkis et al. 2004). The dependence of temperature for this internalization was also demonstrated (Kerkis et al. 2004) and confirmed later on (Nascimento et al. 2007).

According to Kerkis and colleagues (Kerkis et al. 2004), crotamine strongly labels bone marrow, spleen, and peritoneal liquid cells of mice *in vivo*, 3 h after ip injection of Cy3-crotamine. The *in vivo* uptake into mice tissues and cells was also confirmed using labeling with other different fluorescent group (FITC-crotamine), showing its accumulation in liver, bone marrow, conjunctive tissues adjacent to muscle fibers, and brain cells with neuron-like morphology (Hayashi et al. 2008). Significant fluorescent signal was also observed in other tissues as kidney, spleen, and peritoneal liquid cells (Hayashi et al. 2008), in a similar way as previously described by Kerkis and colleagues using Cy3-crotamine (Kerkis et al. 2004).

Later on, aiming to demonstrate the specific accumulation of crotamine in tumor in living mice, a noninvasive optical imaging procedure that permits real-time monitoring of fluorescent labeled compounds *in vivo* was employed to show that labeled crotamine injected by ip route can efficiently target remote subcutaneous tumor engrafted in nude mice as early as 15 min, and with a maximum accumulation 3 h after injection. This fluorescence signal lasted for more than 5 h in tumor tissue, with a rapid clearance from nonproliferating tissues (Nascimento et al. 2012). Similar crotamine accumulation rate was also observed for remote tumor formed by either murine melanoma B16F10 or breast tumor TS/Apc cells, as determined by 2D-FRI analysis in both living animal or excised tumor tissues. Moreover, the analysis of these tumor nodules by cryostat sections showed a preferential accumulation of crotamine into potentially proliferating cells localized

in the peripheral region of the tumor mass with no detectable presence in the inner region of the tumor tissue (Kerkis et al. 2006).

Complex Formation with Nucleic Acid Molecules and Potential of Crotamine as Transport Vector for Cell Transfection

Interestingly, crotamine has some unique characteristics that distinguish it from most CPPs, among which stands out its electrostatic ability to form complexes with DNA (as plasmid vector carrying reporter gene) and other nucleic acid molecules as oligonucleotides and siRNAs/shRNAs (Hayashi et al. 2012b).

In addition, the selectivity of crotamine for actively proliferating cells is not lost even when this toxin is forming complexes with nucleic acid molecules (Nascimento et al. 2007). A practical example of the selectivity of crotamine for cells under division and of its ability to load reporter genes was confirmed by ip injection of crotamine/pEGFP complexes in living C57BL/6 mice, showing that 10–20 % of bone marrow cells were observed by the presence of a strong green fluorescence signal from the enhanced green fluorescent protein (EGFP) reporter gene expression. The green fluorescence signal from the reporter protein was also found in other cells as in the sinusoidal epithelium, bronchial epithelium, peritoneal fluid, spleen cells, in different areas of the skeletal muscle, heart, and also in the CNS (Hayashi et al. 2008).

Crotamine capacity of forming complexes with nucleic acid molecules, leads to its precipitation in solution (Chen et al. 2012), and the ability of penetration into the cell is not lost even when forming complexes (Nascimento et al. 2007; Hayashi et al. 2008). There are many ways that nucleic acids could bind to the surface of crotamine, but one region, which comprises the residues 31–35 of native crotamine and corresponds to Arg-Trp-Arg-Trp-Lys (RWRWK) sequence, was suggested to be potentially involved in the binding with DNA (Chen et al. 2012). In fact, studies to determine the affinity of crotamine to the DNA using oligonucleotides, which permits better biochemical and biophysical analysis, showed that when the complex is formed, five amino acid residues are occluded in crotamine, while 5 and 2.5 nucleotides residues are involved in the complex, for single- and double-strand DNA, respectively (Chen et al. 2012).

The crotamine-DNA complex formation was also demonstrated to be dependent of both the length (50–75 bp) and the concentration (250–500 μ M) of the DNA. In addition, this aggregate was effectively broken up by sodium dodecyl sulfate (SDS), but not by the presence of salt (Chen et al. 2012). On the other hand, the decreased affinity of crotamine to the DNA was shown to be directly proportional to the increase of the ionic strength, determined by increasing the salt concentration in the medium, although the salt was not able to completely disrupt the crotamine-DNA complexes, even at concentrations as high as 0.1 M of NaCl, although partial reversal was observed under some conditions (Chen et al. 2012). Moreover, despite no general preference could be observed for single- versus double-strands DNA sequence, composition, and structure, all these features seems to affect the affinity for crotamine (Chen et al. 2012).

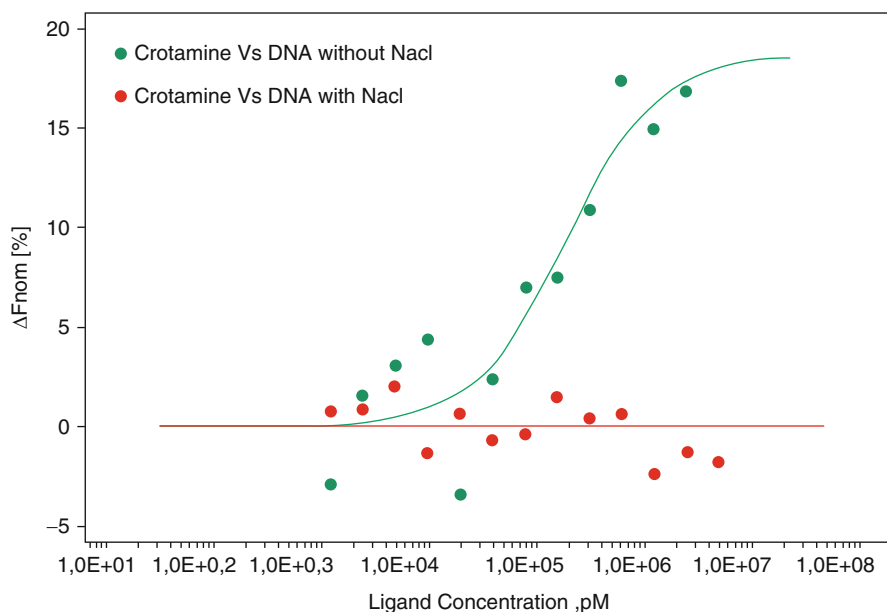


Fig. 6 Interaction of crotonamine with DNA. Comparison of the results obtained when the mixture was incubated in presence of 50 mM NaCl (*red*) and when the NaCl was added after incubation of crotonamine with DNA (*green*)

Moreover, thermophoresis measurements performed with crotonamine covalently attached to fluorescent label (Cy.5) (at 40 nM stock concentration) with a dilution series of DNA from 5 μ M to 306 pM, with addition of 50 mM NaCl only after a short incubation, also confirmed the interaction of crotonamine to DNA with a K_d value of 139 ± 16 nM. Same experiments performed in the presence of the same 50 mM NaCl did not show any interaction (Fig. 6). For these measurements, the samples were loaded into Microscale Thermophoresis (MST) NT.115 standard glass capillaries and the MST analysis was performed using the Monolith NT.115 (NanoTemper Technologies GmbH, Germany). MST is a method based on the thermo diffusion of molecules in a microscopic temperature gradient, and the directional movement of molecules on this gradient is sensitive to many kinds of binding-induced interaction such as molecular size, charge, hydration shell, and conformation (Seidel et al. 2013). As molecules moves, they reach a steady state, which will depend on the parameters already mentioned (size, charge, and hydration shell) (Duhr and Braun 2006). The steady state reached will depend on the fraction of molecule bound to the ligand, and it allows the determination of the K_d (dissociation constant) in solution. A deeper description of the instrument and theory can be found in several publications (Seidel et al. 2013; Duhr and Braun 2006; Wienken et al. 2010) and also in the company homepage [<http://www.nanotemper-technologies.com/>].

A detailed analysis of the salt dependence of binding indicates that as many as three ionic interactions occur between each crota mine polypeptide and its binding site (Chen et al. 2012). Interestingly, it was also estimated that the nonionic contribution to the interaction of crota mine with the negatively-charged polysaccharide, as heparin, is also significant (Nascimento et al. 2007).

Ideally, aiming eventual gene therapy, the perfect complex between crota mine and DNA must possess a link strong enough to permit the transport throughout the animal body until it can achieve the target tissue and cross the cell membrane, but weak enough to permit the liberation of DNA in the interior of the target cell (Hayashi et al. 2012b). Studies conducted by the group in the last few years, aiming to increase the evidences of the feasibility of using crota mine for the delivery of therapeutic genes to the target cell nucleus *in vivo*, have shown promising results.

Activity of Crota mine on Lipid Membranes

Among the variety of biological functions described for native crota mine, the antimicrobial as well as the cytotoxic activities have been postulated to be due to the membrane permeabilization properties of the polypeptide. The antimicrobial activity of crota mine was characterized by the group (Yamane et al. 2013), and studies to understand the mechanism of action underlying this activity suggested that lipid membrane permeabilizing activity of crota mine is not preceded by the formation of stable pores (Costa et al. 2014). Conversely, Sieber and coworkers showed that crota mine forms stable monolayers due to its amphipathic structure, and crota mine would then be incorporated in lipid bilayers inducing defined pores in this cell structure (Sieber et al. 2014). The tendency of crota mine to form oligomers in water was described previously (Hampe 1989), leading to the hypotheses that crota mine may form stable pores, with low cationic selectivity, in bacterial and other cell membranes, as crota mine structure does not allow the formation of monomeric pores (Sieber et al. 2014). However, it is also worth to mention that Sieber and colleagues (Sieber et al. 2014) performed their work at high ionic condition, and this might potentially explain the differences in the observations described by them, compared to the study conducted by us under low ionic strength (Costa et al. 2014).

Another important characteristic of crota mine is its affinity for negatively charged membranes, as demonstrated by its low or no interaction with neutral or cholesterol containing membranes (Costa et al. 2014). This specific association could be explained by an electrostatic interaction of the positively charged surface of crota mine with the negatively charged membranes of microorganisms (Yamane et al. 2013). As it was previously demonstrated, a possible mechanism of interaction is that positively charged crota mine initially binds to the lipid bilayer, and then, it would exert anionic lipid clustering on the membrane surface, resulting in the accumulation of crota mine on a limited membrane region, causing its destabilization. The assemblage would then create a macropore that compromises the membrane integrity, leading to its rupture and vesicle bursting (Costa et al. 2014).

Biological Activities of Crotamine

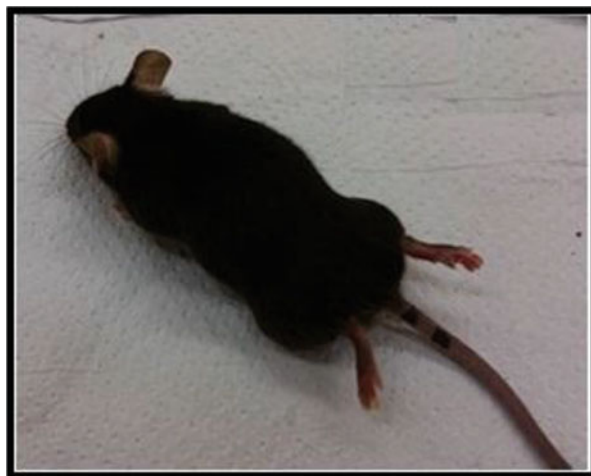
Toxicity and Myotoxin-like Activity of Crotamine

As reviewed and described by Boni-Mitake and colleagues (Boni-Mitake et al. 2006), the reported LD₅₀ values for crotamine range from 0.07 to 6.00 mg/kg for ip injection (Boni-Mitake et al. 2001; Giglio 1975), 1.5–3.0 mg/kg for intravenous (iv) and 0.46 mg/kg for subcutaneous (sc) injections (Cheymol et al. 1971; Brazil et al. 1979), although some authors described that 6.3 mg/kg by sc route was not lethal for C3H mice (Allen et al. 1986). Interestingly, the influence of sodium acetate enhancing the toxic effects of crotamine is also described (Allen et al. 1986). It remains to be explored the effect of the co-injection of sodium acetate described by these authors (Allen et al. 1986), as some other substances may also have the potential to increase the toxicity of crotamine injected in rodents (under study by the group).

Although the most accepted explanations for these various reported LD₅₀ values for crotamine were the purity and the employed methods of toxin purification, in assays performed by the group, the same discrepancy in the myotoxic effects (characteristic hind limb paralysis, as shown in Fig. 7) for the same batch of purified crotamine was observed.

Interestingly, gamma radiation was shown to reduce the toxicity of crotamine in about twofold range, although not completely abolishing the ability of inducing the hind limb paralysis in mice, as described by some authors (Boni-Mitake et al. 2006). As mentioned above, the ionizing radiation has been often employed to attenuate animal toxins by imposing molecular structure alterations, reducing the venom toxicity and maintaining the immunogenicity aiming to increase the production of antiserum (Kerkis et al. 2004; Clissa 1999; Ferreira Junior et al. 2005). At least for crotamine, it was shown that irradiation induced mainly a transition of a small

Fig. 7 Demonstration of the myotoxic effect of crotamine characterized by the animal hind limbs paralysis. Single dose crotamine (100 µg/animal) was administered ip, and after 1 h 30 min the paralysis of the animal hind legs was observed, as shown in the picture. The animals started to recover 10 min after crotamine administration, and after 1 h, they were completely recovered



portion of the random coil regions towards an α -helical conformation, with changes in the secondary structure and calorimetric profile, that could potentially explain the decrease in myotoxic activity of irradiated crotamine (Boni-Mitake et al. 2006).

The myotoxin- α (that was first isolated from the venom of the prairie rattlesnake), crotamine, myotoxin I, and myotoxin II are among some examples of myotoxins described to promote vacuolization and tissue destruction. And these effects have been explained by their action on ions fluxes, producing an imbalance in electrolytes and membrane potentials (Baker et al. 1993).

Some hypothesis found in the literature to explain the biological effects of myotoxins is mainly based on the promotion of vacuolization that is dependent of the action on sodium and potassium channels (Hong and Chang 1985). The sodium channels are fundamental for cell depolarization and some authors described that crotamine [50 μ M] is able to increase the cell depolarization peak in 50 % and also the maximum sodium conductance in 58 %, suggesting that this mechanism could be responsible for the vacuolization effect (Matavel et al. 1998).

On the other hand, it was also proposed that crotamine can act as a voltage-dependent potassium channel blocker based on 3D structure analysis and docking studies (Yount et al. 2009). Later on, Peigneur and colleagues (Peigneur et al. 2012) demonstrated the activity of crotamine on 12 different cloned voltage-gated potassium channels and 4 cloned voltage-gated sodium channels, confirming that 3 μ M of crotamine showed no effect on sodium channels, although a selective blockage of $K_v1.3$ potassium channel was observed at this same concentration (Peigneur et al. 2012).

Cytotoxic Activity

After the myotoxic activity, the most studied biological activity of crotamine is its cytotoxic and antiproliferative property, thanks to its selective toxicity against cells with activated proliferative machinery, as demonstrated by a significant more evident effects against several types of tumor cells with high rate of cell division compared to normal nontumoral cells (Yamane et al. 2013; Pereira et al. 2011). At concentrations around 5 μ g/mL, crotamine decreased the viability of tumor cell lines such as: (a) SK-Mel-28 (human melanoma cells) and (b) Mia PAC2 (human pancreatic carcinoma cells), while it was nontoxic to normal cells at this same concentration (Pereira et al. 2011).

In order to confirm the specificity of crotamine for highly proliferating cells *in vivo*, nude mice bearing subcutaneous tumor were injected ip with fluorescently labeled crotamine (Alexa680-crotamine), and the distribution and accumulation in tumor cells was assessed in real time *in vivo* and also in slices of the tumor tissue by confocal microscopy (Nascimento et al. 2012). Once confirmed the crotamine specific and main accumulation in tumor tissues, the antitumor effect of crotamine was also assessed *in vivo* by treating C57BL/6 mice bearing subcutaneous melanoma tumor with low concentrations of the toxin, showing a clear increase in the survival rate of treated animals compared to the control group, with a clear remission of the

tumor tissue growth, also suggesting that crotonamine is able to kill tumor cells *in vivo* at concentrations that has no cytotoxic effect on embryonic cells *in vitro* (Pereira et al. 2011).

The exact mechanism involved in the crotonamine cytotoxicity is still not completely clear, although couple of possible explanations for this activity was hypothesized. One hypothesis suggests that the electrostatic interaction between the negatively charged membrane components of cancerous cells and the positively charged residues of crotonamine were to participate strongly on this peptide binding and anchoring on cell membrane surface, with consequent selective damage to tumor cells (Nascimento et al. 2007), as also described for other peptides (Papo and Shai 2005).

Another mechanism proposed for crotonamine cytotoxicity involves its internalization by the tumor cells through endocytosis, followed by accumulation in the endosome/lysosomes (Nascimento et al. 2007; Hayashi et al. 2008). The high concentration of crotonamine within these cellular structures can result in the release of lysosomal enzymes, as cathepsin, to the cell cytosol, leading to the cell death process, presumably by mechanisms involving caspases activation and apoptosis (Nascimento et al. 2007; Hayashi et al. 2008). Another effect of the lysosomal disruption should be the increase of intracellular free calcium (Ca^{2+})_i. The release of Ca^{2+} , from intracellular stores as the endoplasmic reticulum, mitochondria, and lysosomes, among others, might be due to crotonamine effects on the membrane of these organelles (Costa et al. 2014; Nascimento et al. 2007, 2012). Increased concentrations of Ca^{2+} can also lead to activation of caspases (a family of proteases underlying the apoptosis process), which subsequently determine the cell death (Fig. 8).

It is worth mentioning that despite the several listed hypotheses to explain the mechanisms by which crotonamine performs its cytotoxic activity in tumor cells, systematic studies in order to clarify the fundamental issues imposed on this topic still need to be further carried on.

Genotoxicity on Human Lymphocytes

The objective of introducing crotonamine, as well as its recombinant or synthetic analogues as a tool for treatment or biotechnological applications, has increased the importance of understanding the toxicology of the crotonamine, including in the immune cells like lymphocytes (Marcussi et al. 2011). Therefore, the mutagenic potential and genotoxic effects of crotonamine, and also of the *Crotalus durissus terrificus* crude venom and its isolated toxins, were evaluated by others on human lymphocytes using the micronucleus and comet assays (Marcussi et al. 2011). In the comet assay, it was shown that all rattlesnake toxins (e.g., crotoxin, gyroxin, convulxin, and also crotonamine) presented genotoxic activity, and besides the significant damage of DNA determined by crotoxin and crotopotin, basic phospholipase A2 and crotonamine did not present any mutagenic potential as evaluated by micronucleus test (Marcussi et al. 2011).

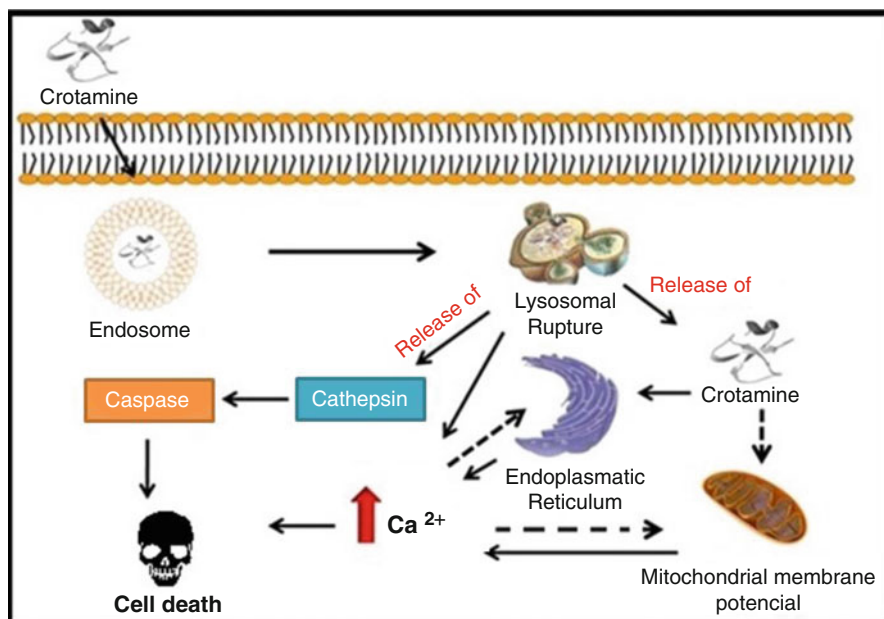


Fig. 8 Schematic representation of studied pathways underlying the cytotoxic effects of crotamine, which includes the crotamine endocytosis, followed by its release into the cell cytosol due to the lysosomal rupture, and consequent cathepsin and free intracellular calcium (Ca^{2+}) release, which increases the free intracellular calcium concentration that may contribute to the cell death by activating the caspases pathways

Although crotoxin at relative high concentrations ($7.5 \mu\text{g/mL}$) did not induce cell death, DNA damage was observed. On the other hand, crotamine and crotapotin induced a damage that was lower than that for the control drug doxorubicin, which in turn was greater than that observed for the lymphocytes culture without treatment. These results allowed suggesting that *Crotalus durissus terrificus* crude venom and also its isolated toxins have the potential to induce DNA breakage, but with no mutagenic potential, as the DNA damage observed may be corrected by the cell repair system during the cell checkpoints (Marcussi et al. 2011). Therefore, the full correction of the DNA breakage detected by comet assay should be ideally complemented by the micronucleus tests, which allows observing these DNA breakages after two generations of cells treatment (Araldi et al. 2015). In fact this assay suggested the nonmutagenic potential of crotamine (Marcussi et al. 2011).

Effects of Crotamine on CNS

As mentioned above, distribution study of native crotamine labeled with I^{125} injected ip in Swiss mice showed highest levels of radioactivity in the kidneys and liver of this rodent, with the lowest concentration in the brain (Boni-Mitake et al. 2006). In

other words, at the maximum concentration peak in brain, crotamine presence in this tissue was about 1,000 times lower than that found in the liver (Boni-Mitake et al. 2006), and this very low accumulation of crotamine in brain might explain the limited literature on central effects of this toxin.

Recently, a group demonstrated that a single intrahippocampal infusion of a crotamine (1 μ L/side) isolated from *Crotalus* crude venom induced acute and chronic alterations in hematological and cardiac parameters and also promoted subacute oxidative stress in plasma and brain of adult rats euthanized 24 h or 21 days after (Gonçalves et al. 2014). More specifically, 24 h after, crotamine infusion promoted increase of urea, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatine-kinase (CK), and platelets values ($p \leq 0.01$), while red blood cells and leukocytes values were decreased ($p \leq 0.01$). Additionally, the authors also describe that 21 days after infusion of crotamine, the animals showed increased creatinine, leukocytes, thiobarbituric acid reactive substance (TBARS) test for lipoperoxidation (plasma and brain), carbonyl (plasma and brain), and micronucleus compared to the saline-group ($p \leq 0.01$), showing that crotamine infusion alters hematological parameters and cardiac markers, as well as oxidative parameters, not only in the brain but also in the blood, indicating a systemic proinflammatory and toxicological activity (Gonçalves et al. 2014). After 24 h, the intrahippocampal infusion of crotamine also decreased the leukocytes number, which was increased after 21 days after the infusion, as well as the platelet number, suggestive of crotamine-induced inflammatory process and increase in the oxidative stress parameters (Gonçalves et al. 2014).

Interestingly, this same group also suggested that crotamine improves the persistence of object recognition and aversive memory in rats, locomotor and exploratory activities, and anxiety or pain threshold (Vargas et al. 2014), probably by inducing the release of acetylcholine and dopamine in the striatum of rats, as previously demonstrated also by others after infusion of 5 μ M of crotamine or crotoxin (Camillo et al. 2001). Although the well-known ability of dopamine and cholinergic system for positive modulation of memory shows persistence (Robinson et al. 2011; Rossato et al. 2009), unfortunately the content of these or other neurotransmitters was not measured at any CNS tissues of these animals pretreated with crotamine (Camillo et al. 2001; Robinson et al. 2011).

It is also worth to mention here that the classical symptoms well described for *in vivo* administration of crotamine, including the muscle spasms and spastic paralysis (Fig. 7), spasticity, and convulsive states (Habermann and Cheng-Raude 1975), were not observed after intrahippocampal administration of crotamine (Vargas et al. 2014). The influence of the administration route and consequent differences in the biodistribution was suggested as a possible explanation, but to the authors view, further studies are still necessary to clarify these several unclear points.

Taken together, considering the important proinflammatory and toxicological effects observed after infusion of crotamine into the CNS, and also the lack of experimental demonstration of neurotransmitter release alterations, the effects on modulation of memory persistence should be interpreted with caution.

Antimicrobial and Antifungal Activities

Infectious diseases are one of the main causes of morbidity and mortality worldwide. Nowadays, many infections are often caused by multiresistant microorganisms resulting in several difficulties in the treatment, and consequently, substantial increase in healthcare costs. Research in the field of natural products has demonstrated significant contributions of the nature in the discovery of compounds with antimicrobial activity. In fact, nature is a generous source of compounds with the potential to treat diseases, including infectious diseases. Among the well-known sources of natural compounds with valuable antimicrobial activity, it was highlighted the medicinal plants, and marine and terrestrial organisms, including fungi and bacteria (Hayashi et al. 2013).

Antimicrobial peptides (AMPs) are small cationic peptides (ranging in size from 6 to 60 amino acids) responsible for adaptive immunity, usually found in the outer surface of the skin. AMPs are a unique class of natural peptides composed by a variety of molecules grouped into subgroups based on amino acid composition and structure, classified into three major groups: (a) peptide with an α -helix conformation, (b) cyclic and open-ended cyclic peptides with pairs of cysteine residues (defensins and protegrin), and (c) peptides with an overrepresentation of some amino acids (as proline- and/or histidine-rich sequences) (Boulanger et al. 2004).

Each peptide may act in different pathways and the exact mechanism needed to be individually elucidated. In fact, pore formation is not the only mechanism for microbial killing, as simple translocation of peptides can also alter cytoplasmic membrane septum formation, inhibiting cell-wall synthesis, nucleic acid synthesis, protein synthesis, or enzymatic activity (Brogden 2005).

In contraposition to the wide range spectrum activity of the human beta-defensins, crotamine has a narrow and restricted spectrum of activity against most microbial strains studied with an almost insignificant activity against bacteria (Yamane et al. 2013). Although crotamine minimum inhibitory concentrations (MIC) for Gram-negative (i.e., *Escherichia coli* (12.5-25 $\mu\text{g/mL}$) and *Pseudomonas aeruginosa* (>200 $\mu\text{g/mL}$), and Gram-positive (i.e., *Staphylococcus aureus* (>200 $\mu\text{g/mL}$) and *Micrococcus luteus* (1-2 $\mu\text{g/mL}$) bacteria was determined, only the activity against several fungi of *Candida* spp., in particular against *C. albicans*, was remarkable for crotamine (Yamane et al. 2013), however no activity of crotamine on biofilms formed by this strain could be observed (Costa et al. 2014). The evidence of crotamine effects on microorganisms opens new perspectives for the use of this venom component in biomedicine, more specifically for infectious disease treatments, as crotamine does not show hemolytic activity (Yamane et al. 2013). Therefore, although there is lack of validation in the *in vivo* model, in the authors view, crotamine could be included in the class of natural compounds with recognized antimicrobial activities (Yamane et al. 2013).

Aiming to evaluate crotamine action on *Candida albicans* and *Candida krusei* infection treatment, the nematode *Caenorhabditis elegans* was used as a living host for an *in vivo* model of infection. The treatment with this peptide resulted in the discovery of a potential anthelmintic effect, but which masked the eventual effects on fungus infection (Dal Mas et al. 2015).

Antiparasitic Activity

Several small polypeptides with high content of positively charged amino acids shown to act as antimicrobial and/or antitumor molecules, and with basic moiety that may contribute to cell-penetrating property, have unique affinity for acidic vesicles. Uptake, accumulation, and action in the acidic compartments of the worms may determine anthelmintic activity for amphipathic peptides as crotamine, which in fact was confirmed by the group in studies using the nematode *Caenorhabditis elegans* as animal model (Dal Mas et al. 2015). Consistent with the ability of crotamine to target acidic vesicles in other species (Nascimento et al. 2007; Hayashi et al. 2008), the accumulation of the peptide in the lysosomes of the worms was confirmed (Fig. 9) (Dal Mas et al. 2015).

Anthelmintic drugs used to control human and veterinary infections caused by worms usually act on targets such as the nicotinic acetylcholine receptor, GABA receptor, glutamate-modulated chloride channels, voltage-dependent Ca^{2+} channels, β -tubulin, mitochondrial complexes I and II, and cathepsin B (Rana and Misra-Bhattacharya 2013; Holden-Dye and Walker 2014). Identification of a new class of anthelmintic molecules capable of overcoming the drug resistance problems described for these pesticides (Matthews 2014; Geary et al. 2010) is desirable, and the action on different pathways of commercially available anthelmintic drugs may represent a good solution for this worrying challenge.

The *Plasmodium* life cycle involves the arthropod mosquito vector and the vertebrate host, for the sexual and asexual stages, which is the main target for the antimalarial studies. The increased permeability of the erythrocyte membrane to different metabolites during the asexual stage favors the entrance of several inhibitors with potential as antimalarial drugs (Bagnaresi et al. 2012; Melo et al. 2014; Tokumasu et al. 2014). The search for new antimalarial compounds is crucial, since drug resistance is spreading quickly in the existing parasite population (Dondorp et al. 2009).

Interestingly, it was recently showed that *Plasmodium falciparum* development is inhibited by crotamine in a dose-dependent manner [IC_{50} value of 1.87 μM], and confocal microscopy analysis showed a selective internalization of fluorescent-labeled crotamine into *P. falciparum* infected erythrocytes, with no detectable fluorescence in uninfected healthy erythrocytes (El Chamy Maluf et al. 2016). The mechanism underlying the antiplasmodial activity may involve the disruption of parasite acidic compartments H^+ homeostasis, as crotamine promoted a reduction of parasites organelle fluorescence loaded with the lysosomotropic fluorochrome acridine orange (AO), in a similar way as previously observed and described for mammalian tumor cells (Hayashi et al. 2008). The selectivity for infected red blood cells (iRBCs) compared to uninfected RBCs is in good agreement with the previous findings of the group showing no hemolytic activity for crotamine against human erythrocytes for concentrations up to 100 μM (Yamane et al. 2013), and the importance of the negative charge on membranes for the selective affinity and activities of native crotamine (Costa et al. 2014). In addition to the ability of crotamine to carry cargos into the cells (Nascimento et al. 2007; Hayashi

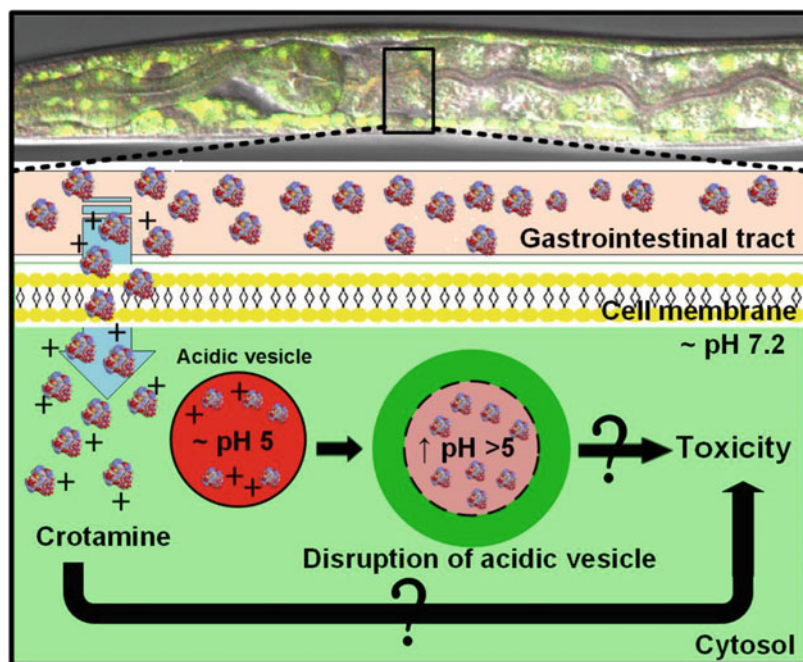


Fig. 9 The schematic representation suggests that the main route of absorption of crotamine is oral swallowing. Possibly the mechanism of action underlying the anthelmintic activity of crotamine involves the disruption of acidic vesicles, and activation of toxicity or cell death pathways, as demonstrated by confocal microscopy analysis of *C. elegans* worms stained with the fluorescent cationic metachromatic dye Acridine Orange (AO), which emits green fluorescence at a basic pH and red in acid pH, and treated with native crotamine [20 μ M]. The crotamine is mainly absorbed from the gastrointestinal worm tract and enters the cells and accumulates in the lysosomes (pH 5 – red), causing rupture of these vesicles and extravasation of the acid contents to the cytoplasm (pH 7.2 – green), and activating several cellular toxicity pathways, not fully identified yet (Dal Mas et al. 2015)

et al. 2012b), the antiplasmodial activity described for crotamine could also be further potentiated by its combination with lethal genes specific for malaria parasites, aiming eventual future therapeutic interventions.

A potential antileishmanial activity was also described for crotamine by others but with no mention or discussion on the molecular mechanism of action underlying this effect (Passero et al. 2007).

Crotamine in the Treatment of Myasthenia

Myasthenia gravis (MG) is an autoimmune disease of the neuromuscular junction in which skeletal muscle weakness is resulted from a reduction in the number of nicotinic acetylcholine receptors in the neuromuscular endplate (Hernandez-Oliveira

e Silva et al. 2013). Crotonamine was first considered for this application due to its actions in skeletal muscle, increasing the contraction tone in mammals (Hernandez-Oliveira e Silva et al. 2013). This led to the suggestion that at very low doses of crotonamine (50 ng), beneficial effects in the symptomatic treatment of MG, with a longer action and perhaps fewer side effects, would be expected (Hernandez-Oliveira e Silva et al. 2013).

The explanation for this crotonamine effect has been associated to its facilitatory effect on neurotransmission (Toyama et al. 2003), increasing the open time of sodium channels, although the eventual action of this toxin on potassium channels could better explain the suggested neurotransmission effect. When acetylcholine is released at the neuromuscular endplate, the channels pore opens to allow the influx of sodium ion and efflux of potassium ion, generating a local endplate potential (Hernandez-Oliveira e Silva et al. 2013). In this case, the participation of crotonamine could be more representative by blocking the potassium channels and increasing the resting membrane potential (Yount et al. 2009; Peigneur et al. 2012).

Crotonamine Sources

Purification from Snake Crude Venom

Since its first description, crotonamine has been mainly purified from the pooled dry crude venom of rattlesnake as described, although the absence of crotonamine in some venom may interfere in the final yield of the purification process (Hayashi et al. 2012b). Usually employing simple two-step purification protocol, about 50-100 mg of crotonamine per gram of dry weight crude venom can be obtained (Hayashi et al. 2012b).

In the final phases, the protein concentration of crotonamine in solution is usually determined by measuring the absorbance, in which OD_{280nm} of 0.62, using 1 cm quartz cuvette, corresponds to crotonamine concentration of 0.25 mg/mL. The concentration and also purity are, in general, further evaluated by amino acid composition analysis, in which the absence of alanine (A, Ala), valine (V, Val), and threonine (T, Thr) residues confirms the purity of the crotonamine preparation, besides allowing the identification of the eventual presence of crotonamine isoform with the isoleucine (I, Ile) residue at position 19 of crotonamine. For storage, the dialyzed (using benzoylated dialysis tubing) native purified crotonamine is then lyophilized after freezing, and kept at 4 °C or -20 °C until use (Hayashi et al. 2012b).

At the first moment, even if the access to the same rattlesnake specimen should be the best option to assure the homogeneity of the native purified crotonamine, one needs also to consider its isoforms with variable sequences reported. In the same way, other factors as climate, gender, age, and diet, as well as the captivity period and venom extraction frequency may influence the expression of this specific toxin, and therefore, they need also to be considered with attention, as mentioned above (see section [Molecular and Structural Characteristics of Crotonamine](#)). However, although the captive maintenance of rattlesnake is already a reality [<http://www.cbsnews.com/video/watch/?id=50145985n>], and this approach would be helpful to overcome the

limitations imposed by the biodiversity laws that protect the natural compounds from deleterious exploitation, it is important to keep on mind that eventual modifications in the amount and sequence of produced crotonamine in the venom may still represent a concern for its commercial use.

Recombinant Expression and Chemical Synthesis

Besides its purification from the *Crotalus* venom, crotonamine can also be obtained by other methods as recombinant expression or chemical synthesis (Hayashi et al. 2012b), and the use of these strategies to get crotonamine may allow possible improvement in the functionalities of this molecule. The refolded full-length synthetic crotonamine showed a circular dichroism (CD) spectrum very similar to the native crotonamine suggesting an overall similar secondary structure compared to native crotonamine, although the recombinant crotonamine expressed in bacteria demonstrated a profile more characteristic of a disordered peptide chain, suggesting that refolding may also be necessary (Yamane et al. 2013). However, several attempts to refold the recombinant crotonamine in fusion with a $6 \times \text{His}$ tag were unsuccessful up to now (Yamane et al. 2013).

The crotonamine expression performed by Yamane and colleagues (Yamane et al. 2013) using prokaryotic system was first designed to express crotonamine in fusion with $6 \times \text{His}$ tail, which allow purifying the protein by simple single-step affinity chromatography. The expression in the bacteria cytoplasm determined a low yield of expression besides the low recovery of the crotonamine fusion protein under the employed conditions due to the low solubility and high tendency to form aggregates. Moreover, as mentioned, structural analysis evaluated by CD revealed that this product was not structured (Yamane et al. 2013).

Considering the potential cytotoxicity towards eukaryotic and yeast cells, the efficient production for biomedical applications does not seem to be the best option in contraposition to the low expression and insolubility problems observed for the expression in prokaryotic cells (Hayashi et al. 2012b).

Other group described that the expression of crotonamine in *Escherichia coli* with maltose-binding protein (MBP), protein disulfide bond isomerase (PDI), and N-utilization substance protein A (NusA) was able to increase the solubility of the expressed fusion crotonamine (Vu et al. 2014). In particular, higher solubility at 20°C was demonstrated compared to other evaluated tags (Vu et al. 2014). Interestingly, after cleavage of the fusion protein using TEV protease to release the full-length recombinant crotonamine allowed the authors to demonstrate that the obtained recombinant crotonamine might have adopted the correct protein folding, as it was able to inhibit the $\text{hK}_{v1.3}$ channel (but not the $\text{hK}_{v1.5}$ channel), in a dose-dependent manner, as well as the fusion protein with MBP also possessed this inhibitory action on potassium channels (Vu et al. 2014). However, to the authors view, the correct folding still need to be confirmed by CD, NMR, and/or other biophysical analysis, as to the author's knowledge, the necessity or not of 3D structure of crotonamine for the inhibitory action on potassium ion channels was not clarified up to now.

Additionally, the full-length crotonamine chemical synthetic analogue produced on Boc-Gly-OCH₂-PAM resin, using an in-house chemistry on an ABI 433A peptide synthesizer, followed by a productive oxidative folding of synthetic crotonamine purified to homogeneity was achieved under thiol-disulfide exchange conditions, and the obtained peptide presented antimicrobial activity similar but not exactly identical to that described for the native crotonamine (Yamane et al. 2013), suggesting possible differences in the final 3D structure, but that still needs NMR analysis of this synthetic analogue for final confirmation.

Crotonamine-derived Small Peptides

Crotonamine-derived linear peptides were also evaluated by the group for different aspects including the ability to make complexes with DNA, to interact with lipid membranes, to carry nucleic acid cargos, and also due to their potential antimicrobial and/or cytotoxic effects. The first produced linear peptide analogues were designed based on the simple idea of splitting the total positive charges in two parts. Cysteine is susceptible to rapid oxidation, which can negatively influence the cleavage of protecting groups during synthesis and the subsequent peptide purification. To avoid this, cysteine was replaced with serine. Consequently, the crotonamine-derived linear peptides from 2–18 and 27–39 position of the full-length native crotonamine were the basis for the synthesis of a 16 and 12 amino acid residues long linear peptides, named as P1 [KQSHKKGGHSFPKEKIS] and P2 [KMDSRWKSSKK] (Fig. 10) (Yamane et al. 2013). Substitution of Cys by Ser residues is often employed to avoid their oligomerization and the potential formation of intra- and intermolecular disulfide bonds of synthetic peptides. Nevertheless, similar activities were observed for these peptides compared to the native crotonamine, including: (a) the ability of making complexes with DNA and (b) the interaction with the lipid membranes with affinity for negatively charged membranes, although these peptides were not as efficient as the native crotonamine on disrupting model lipid vesicles. Now, for the delivery of nucleic acid cargos into the cells, the linear peptide (both P1 and P2) showed to be as efficient as native crotonamine, indicating the viability of using this synthetic analogue as a transfection vector, with several advantages for these

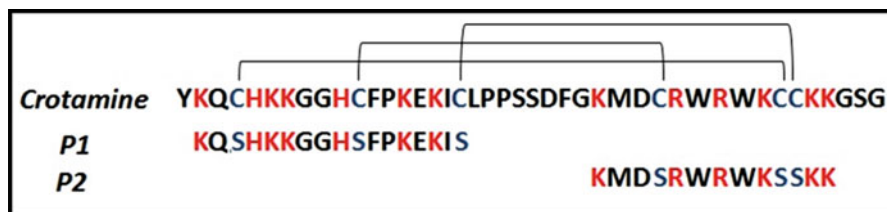


Fig. 10 Alignment of the primary structures of the native crotonamine and derived peptide fragments P1 and P2. Cysteine/serine residues are aligned and highlighted in blue. The basic amino acids are in red (adapted from Yamane et al. 2013)

peptides, including the lack of antimicrobial and cytotoxic activity (Yamane et al. 2013). It is also important to consider that crota mine was shown to be highly resistant to degradation in biological medium and fluids (Hayashi et al. 2012b), and this might be most probably due to the compact 3D structure (Nicastro et al. 2003; Fadel et al. 2005). However, for the linear short peptide analogues, stability of the molecule in biological conditions, especially for *in vivo* assays, is an essential concern to be considered.

Conclusion and Future Directions

According to the abovementioned description, it can be inferred that crota mine has several properties that allow suggesting diverse biological applications. However, it is fundamental now to clarify in more detail the mechanisms involved in each biological activity, in order to define the possible interactions with other interventions and better understand and predict the potential adverse effects.

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Part IV

Cytotoxic Effects

Viperacidins, Snake Venom Cathelicidin-Related Peptides, in the Milieu of Reptilian Antimicrobial Polypeptides

13

Gandhi Rádis-Baptista

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Abstract

After the “antibiotic age” we are experiencing a “post-antibiotic era”, in which our current antimicrobial arsenal is expiring. In addition, drug-resistant infectious diseases have emerged and reemerged. Antimicrobial peptides (AMPs) arose as an alternative to classical antibiotic drugs. AMPs are selective membrane-active compounds with a wide spectrum of action against bacteria, fungi, parasites, and viruses. Due to their properties, AMPs are also effective as anticancer peptides and some AMPs can connect the innate and acquired immunity. To date, thousands of sequences have been described from a wide range of phyla. In reptilians, the predominant classes of AMPs that have been found until now encompass β -defensins and the cathelicidins. Cathelicidin-related antimicrobial peptides (CRAMPs) have been characterized from Asian elapids and South American pit vipers. Viperacidins from rattlesnakes and jararacas and elapid CRAMPs from cobra and kraits consist of a signal peptide, a conserved cathelin domain, and variable carboxyl-terminal sequences of linear α -helical peptides, from where the

G. Rádis-Baptista (✉)

Laboratory of Biochemistry and Biotechnology, Institute for Marine Sciences, Federal University of Ceara, Fortaleza, Ceará, Brazil

e-mail: gandhi.radis@ufc.br

antimicrobials are released. Full and short synthetic versions of viperidins and elapid CRAMPs have been prepared and possess a distinct efficacy toward microbial and transformed malignant cells. Although not belonging to the class of the AMPs, venom polypeptides with biocide activity comprise enzymatic toxins (e.g., PLA2) and nonenzymatic waprins. Altogether, animal venom constitutes a rich source for the disclosure of AMPs with diverse sequences and multiple functions. Given the current knowledge, venom-derived AMPs offer a multitude of possibilities for understanding the evolution of this immune-effector molecule and for generating engineered peptides by de novo design.

Keywords

Viperidins • Elapid CRAMPs • Cathelicidin-related peptides • Snake venom • Antimicrobial peptides • Antimicrobial proteins • Molecular toxinology • Toxin evolution

Introduction

After the golden age of antibiotic discovery (“the antibiotic age”) in the twentieth century, in which most of the antibacterial compounds were discovered, the number of novel chemotherapeutic agents active against pathogenic microbes that have left the laboratories and reached the clinics has drastically reduced. For example, in just 20 years (from the 1940s to the 1960s), the major classes of antibiotics in use (e.g., aminoglycosides, macrolides, tetracyclines, quinolones, vancomycin, etc.) were discovered. In contrast, in the last decade, no more than ten new antibiotics were approved in the United States. Among these antibiotics was a synthetic cyclopeptide (the macrocycle POL7080) that was developed against Gram-negative bacteria (*Pseudomonas*). Another example that gained FDA approval in more than 40 years of endeavor from the antibacterial drug pipeline was the new compound bedaquiline, which specifically fights tuberculosis (Torrice 2013; Jarvis 2014). Authorities in the field agree that a “post-antibiotic era” is starting, in which our arsenal of antimicrobial compounds is running out and the battle to outcome infection diseases is pending in favor of the biochemically smart bugs and superbugs, despite intensive effort of the pharmaceutical industry to counteract (Jarvis 2014). Even large screening programs to find new antibiotic molecules with novel mechanism of action based on high-throughput in vitro assays using libraries of synthetic molecules and genomic sciences for selective target-driven screening have resulted in either very few hits or failure (Torrice 2013; Jarvis 2014). In contrast, infectious diseases, including opportunistic and tropical diseases, have reemerged in endemic zones and reached territories never or rarely affected by such health problems (Harrus and Baneth 2005). Several factors seem to contribute to this phenomenon of emergence and reemergence of infectious diseases, such as the misuse and overuse of antibiotics that culminate with the appearance of superbugs; the direct and indirect association with human development, such as the level of urbanization; the intensification of global trade and travel; and environmental

factors, such as global warming, pollution, agricultural management practice, etc. (Ooi and Gubler 2009; Bell 2014; Forum on Microbial Threats et al. 2014; Merker et al. 2015). For example, anthropogenic and environmental factors have contributed to the emergence and reemergence in Asia of arthropod-borne viruses (arboviruses), such as dengue virus, Japanese encephalitis, and West Nile virus (Dash et al. 2013). In addition, virulent strains of multidrug-resistant *Mycobacterium tuberculosis* appeared in Asia from the west after successive spreading waves that coincide with major human social events that occurred over 200 years of history (Merker et al. 2015). In this scenario, in which the antibacterial drug pipeline is experiencing a decline and more resistant pathogens are spreading worldwide, new strategies are demanding and urgent. Thus, to maintain the pace to combat epidemics and reemerged microbial diseases, strategies possibly including the development of “single-pathogen narrow-spectrum antibiotics,” antibiotics that act on more than one distinct bacterial target, and therapeutic proteins, such as antibodies, that neutralize not the microbes themselves but their virulent factors (e.g., endotoxins), have been in consideration by biotech and pharma companies (Jarvis 2014).

A class of natural compounds with intrinsic selectivity, a tunable spectrum of action, and amenability to rational design encompass antimicrobial peptides (AMPs). By possessing selectivity, peptides can exert their biological effect based on Paul Ehrlich’s principle of “magic bullets” (drugs – “therapeutic peptides” in this case – that go straight to the right target). Detaining a tunable spectrum of action, a wide-spectrum and a “single-pathogen narrow spectrum” peptides can be found from different organism reservoirs from diverse biomes (major ecological communities). Being amenable to peptide engineering, natural peptides can be designed to increase their beneficial properties (e.g., target specificity and stability in biological fluid), decrease their eventual toxicity toward host cells and tissues, and reduce their size, among other advantages. Antimicrobial peptides are essential components of the innate (or natural) immune system of organisms as diverse as invertebrates, plants, and animals (from nematodes to mammals), acting as the immediate effectors in defense against invading microbes (bacteria, fungi, parasites, and enveloped viruses). Thus, to evolve and thrive in an environment that is dominated by an uncountable number of microorganisms and to cope with microbial assault and infection, all living organisms invariably produce a certain class of AMPs. To date, thousands of different structures have been isolated and characterized from protists to higher animals, from which information has been compiled in dedicated databases, such as ADP (<http://aps.unmc.edu/AP/main.php>) and LAMP (<http://biotechlab.fudan.edu.cn/database/lamp>). One distinguished feature of the AMPs is their ability to recognize the biological membranes of microorganisms, which contain in their assemblage specific components (e.g., peptidoglycan and lipoteichoic acid in Gram-positive bacteria and lipopolysaccharides in Gram-negative bacteria). By interacting with these components, most antimicrobial peptides can insert into the lipid membranes and disrupt these membranes by a soap-like mechanism, killing the bacteria by forming transient pores and causing the leakage of metabolites and cytoplasm (Brogden 2005). Due to the discriminatory capacity in recognizing certain lipid membrane components that are associated with the

physicochemical characteristics, such as amphipathicity and cationicity, AMP sequences have a tremendous biological versatility. Thus, several AMPs have demonstrated antibacterial, antiviral, antifungal, antiparasitic, antiprotozoal, and anticancer activity. Therefore, the differential composition of lipid membrane of microbes and their intracellular constituents appear as effective targets for antibiotic activity exerted by these natural and synthetic therapeutic peptides (Otvos 2005; Sharma et al. 2015; Tsomaia 2015). Interestingly, some classes of antimicrobial peptides share structural scaffold and tridimensional topology with animal peptide toxins, for example, the cysteine-stabilized α -helical and β -sheet peptides, such as human β -defensins and snake venom crotoxin (Torres and Kuchel 2004; Rádis-Baptista and Kerkis 2011), and the plant knottins and arthropod ion channel toxins (Padovan et al. 2010; Bende et al. 2015). For this reason, animal venom and the venom gland, in addition to being a rich source for finding dozens of biologically active polypeptide families that meet applications in medical biotechnology, are also unique sources for the isolation of novel AMPs and functionally related membrane-active peptides. Poisonous animals are among the lineage of the most ancient extant living creatures on Earth. For example, cnidarians, such as the sea anemone, that produce deadly neurotoxins and cytolytic membrane active peptides, have a natural history on the Earth that date back 600–500 million years ago (mya) – the period of the Cambrian Explosion when the ancestors of the modern phyla appeared for the first time. Hymenopterans, such as bees, wasps, and ants, that produce a cocktail of biologically active peptides, including AMPs, first flew and stepped on primordial forest 400 mya. Amphibians and reptilians that secrete a repertoire of pharmacologically active organic compounds and bioactive polypeptide in their skin, in their saliva, and in their venom and have developed an efficient venom inoculation apparatus (in case of snakes) that emerged approximately 400–300 mya. The fact that primitive creatures, ancestors of modern animals, were equipped with a collection of host-defense effectors that were inherited, transmitted, and concomitantly evolved into efficient peptide components of the innate immunity system suggests the potential for success in the screening for novel antimicrobial peptides to treat multidrug-resistant bacteria, parasites, and viruses, which might contribute to the current antimicrobial drug arsenal.

Animal Antimicrobial Peptides

In contrast to organic antibiotics, such as aromatic polyketides (griseofulvin and macrolide antibiotics), that are biosynthesized by sequential enzymatic steps using simple building blocks that are originated from the primary metabolism (Dewick 2009), antimicrobial peptides (AMPs), no matter from plants or animals, are codified by genes and synthesized either constitutively or inductively in the ribosomes (Tossi and Sandri 2002). Classical examples of AMPs include the cecropins from the pulp of silkworm, *Bombyx mori* (Morishima et al. 1990; Boman 1998), the magainin from the skin of the amphibian *Xenopus laevis* (Zasloff 1987), and the mammalian α -defensins from neutrophils (Ganz et al. 1985, 1990). To date, thousands of natural

and synthetic antimicrobial peptide sequences from invertebrates to higher vertebrates have been maintained in dedicated databases from which current information about organism source, spectrum of activity, minimal inhibitory concentration (MIC), toxic and cytotoxic behavior, mechanism of action, and putative or known molecular targets can be rapidly consulted and retrieved (Wang et al. 2009; Zhao et al. 2013). Exceptions to the large class of gene-encoded AMPs are the nonribosomal peptides (NRPs). NRPs encompass antibiotic substances, such as β -lactams (penicillins and the cephalosporins) and daptomycin (a cyclic lipopeptide antibiotic), but also group together separate classes of medically important natural compounds, such as cyclosporins (anti-inflammatory and immunosuppressant undecapeptides), glycopeptides (vancomycin and teicoplanin), quinoxalines (the antitumor echinomycin and thiocoraline and the antiviral luzopeptins and quinoxapeptins), capreomycins (antituberculosis cyclic pentapeptides), and bleomycins (glycosylated peptides displaying anticancer activity), among others (Felngale et al. 2008). NRPs, instead of being biosynthesized directly by a gene sequence, are enzymatically built up by large multimodular nonribosomal peptide synthetases that can successively condensate nonproteinogenic, proteinogenic, and chemically modified amino acids as result of the secondary metabolism of fungi and bacteria (Felngale et al. 2008; Strieker et al. 2010).

Considering the physiochemical properties of the gene-encoded natural AMPs, they are predominantly cationic and short, displaying variable levels of amphipathicity, helicity, hydrophobicity, and solubility, which are determinants for the mode of action, spectrum of activity, target specificity, and toxicity of a given peptide (Bahar and Ren 2013). Formerly classified according to their basic structural types, examples from nature include linear α -helical (cathelicidins, cecropins, dermaseptins, and magainins), linear non- α -helical (proline-rich AMPs, histatins), one-disulfide (S-S) bond (amphibian epithelial AMPs and insect thanatin), two S-S bonds (scorpion androctonins, pig protegrins, and horseshoe crab tachyplesin), three S-S bonds (human α -defensins and β -defensins, primate θ -defensin and insect sapecins A), and four S-S bonds (*Drosophila* drosomycin and human hepatic hepcidin) (Andreu and Rivas 1998; Zasloff 2002). In Table 1, examples of animal AMPs are listed.

Posttranslational modifications of the basic structures of AMPs are recurrently observable and in several cases are critical for modulating antimicrobial activity, cytotoxicity, and peptide stability (Andreu and Rivas 1998; Wang 2012; Bahar and Ren 2013). Examples include amidation of aurein (Dennison et al. 2012) and PMAP-23 (Kim et al. 2011), glycosylation (Salazar et al. 2014), and reduction (Schroeder et al. 2011). Cyclization, i.e., the covalent linkage between the N- and C-termini, forming a head-to-tail backbone with a knot motif, occurs naturally almost exclusively in cyclic antimicrobial peptides (cyclotides) from plants (Craik 2006). However, the Rhesus θ -defensin (RTD-1) represents a particular example of a mini-cyclotide from mammals with antiviral activity (Lehrer et al. 2012). However, artificial cyclization has been applied to create a head-to-tail cyclic peptide aiming to improve the peptide stability to proteases of several noncyclic peptides, preserving the respective antimicrobial activity (Rink et al. 2010; Molhoek et al. 2011;

Table 1 Examples of antimicrobial peptides from animals (invertebrates and higher vertebrates) based on structural classes

Structural Class	Antimicrobial peptide	Organism	Tissue	References
Linear α-helical	Cathelicidins Cecropins Dermaseptins Magainins	Vertebrates Insect Amphibian Amphibian	Blood cells, epithelia Hemolymph, epithelia Skin Skin	(Wong et al. 2013; Zaiou and Gallo 2002) (Imler and Bulet 2005; Morishima et al. 1990; Nicolas and Amri 2009; Amiche et al. 1999; Nicolas and Amri 2009; Zasloff 1987, 2002)
Linear non-α-helical	Proline-rich Histatins	Arthropods and Vertebrates Human	Blood cells, epithelia Salivary gland	(Scocchi et al. 2011) (Melino et al. 2014; Kavanagh and Dowd 2004)
One S-S bond (β-hairpin)	Bactenecin Thanatin	Cow Insect	Blood cells Hemolymph	(Romeo et al. 1988) (Mandard et al. 1998)
Two S-S bonds	Androctonin Protegrins Tachyplesin	Scorpion Pig Horseshoe crab	Hemolymph Blood cells Hemolymph	(Hetru et al. 2000) (Kokryakov et al. 1993) (Nakamura et al. 1988)
Three S-S bonds	α -Defensins β -Defensins, Sapcins A	Vertebrates Vertebrates Amphibians	Blood cells, epithelia Blood cells, epithelia Skin	(Lehrer and Lu 2012; Ganz 2003) (Ganz 2003)
Four S-S bonds	Drosomycin Hepcidins	Insect Vertebrate	Hemolymph Liver	(Zhang and Zhu 2009) (Alvarez et al. 2014; Hao et al. 2012)
Cyclic	θ -Defensin	Nonhuman primates	Blood cells	(Lehrer and Lu 2012; Lehrer et al. 2012)

Chan et al. 2013; Arias et al. 2014; Sikorska and Kamysz 2014). Although the great majority of AMPs possess a net positive charge, being cationic compounds with hydrophobic patches, thus featuring amphipathic structures, a small but not less important number of anionic AMPs (AAMPs) play a role in the innate immunity of higher vertebrates, invertebrates, and plants (Harris et al. 2009). For example, enkelytin (FAEPLPSEEEGESYSKEVPEMEKRYGGFM) is a linear α -helical, aspartic acid-rich, anionic AMP that is proteolytically released from proenkephalin, displaying after phosphorylation a potent activity against Gram-positive bacteria, such as *Staphylococcus aureus* (Metz-Boutigue et al. 2003). Interestingly, enkelytin is an effector molecule that is intrinsically associated with opioid peptides, thereby connecting antibiosis, immune activation, and analgesia (Stefano et al. 1998).

The underlying mechanisms by which AMPs exert their effect involve not only a direct killing by the permeabilization and disruption of microbial membranes, culminating with cell lyses, but also interaction with intracellular targets, such as

nucleic acids and proteins. In some cases, as for buforin II from frog stomach and indolicidin from neutrophils, the biosynthesis of nucleic acids and proteins is impaired. Physically, the disruption of the lipid membrane function and integrity caused by AMPs occurs generally by pore formation, following different modes of membrane interaction, such as the carpet (detergent)-like model, barrel-stave model, and toroid pore model (Brogden 2005). The aggregation of peptides into the lipid bilayer to form transient channel-like pores and membrane thinning contribute to the mode of membrane dysfunction and the microbicidal activity of AMPs (Bahar and Ren 2013). These aspects of the mechanism of action as well as the physicochemical properties of AMPs can be explored to generate by *de novo* design fully active peptides that are devoid of disadvantageous cytotoxicity (Yamamoto and Tamura 2014). The mediation of pharmacological signaling by means of the chemoattraction of surveillance cells and the release of immunomodulators is observable for several AMPs, imparting to these peptides the property of being multi-effector host-defense molecules that connect immediate innate (natural) and long-lasting adaptive (acquired) immune systems. For example, mammalian defensins participate in host adaptive immunity, overlapping their function with chemokines and thereby exhibiting synergic microbicidal and immunoenhancing effects. Additionally, cathelicidins and defensins are chemotactic for human monocytes, T-cells, and immature dendritic cells (Yang et al. 2002). In addition, human cathelicidin, i.e., LL-37, is able to directly activate mast cells, inducing cell migration and degranulation, followed by histamine release and the production of proinflammatory cytokines. Thus, cathelicidin not only has a direct effect in causing bacterial killing but also plays a role in the antibacterial inflammatory response (Bąbolewska and Brzezińska-Błaszczyk 2015). In Table 2, the mechanism of action of some examples of antimicrobial peptides is presented.

Noteworthy, in addition to being effective against a wide number of bacteria, fungi, parasites, and viruses, a handful of AMPs display antitumor activity against several aggressive types of cancer both in vitro and in vivo by provoking rapid cell death through membrane disruption and metabolic disturbance. For example, natural AMPs displaying antitumor activities include α -helical peptides (e.g., bovine and human cathelicidins, insect cecropin, and venom bee melittin) and cysteine-stabilized α -helix and β -sheet peptides (e.g., vertebrate defensins) (Hoskin and Ramamoorthy 2008). Several peptides that have been identified from bee venom (namely, melectin, lasioglossins, halictines, and macropin) were also characterized as efficacious microbicides to Gram-positive and Gram-negative bacteria and fungi display anticancer activity (Slaninová et al. 2012). The list of AMPs with dual microbicidal and antitumor activities is growing and can be referenced from the antimicrobial databases and in the current literature (Hoskin and Ramamoorthy 2008; Schweizer 2009; Gaspar et al. 2013). Overall, the anticancer activity of these peptides involves the disruption of cancer cell membranes, but the membranolytic effect is not the only anticancer activity that is exerted by AMPs. The synergic mechanism of cytotoxicity against cancer cells additionally entails the interaction with intracellular targets; the disruption of cell signaling and circuitry, including calcium homeostasis; and the induction of cell death as mediated by

Table 2 Mechanism of action of some known antimicrobial peptides

AMP structural classes ^a	Mechanism of action	References
α-helical cathelicidins	Membrane disruption via toroidal peptide-lipid pores Neutralization of endotoxins released by bacteria Direct activation of mast cell and release of mediators of inflammatory response	(Zanetti 2005) (Bucki et al. 2010) (Babolewska and Brzezinańska-Błaszczak 2015)
Linear α-helical other than cathelicidins	Non-lytic, cell penetration, and mitochondrial dysfunction with loss of ATP Formation of pores in cell membrane and leakage of intracellular components Membrane disruption by pore forming and induction of bacterial apoptosis-like death Binding to nucleic acids and inhibition of DNA and/or RNA synthesis	(Kavanagh and Dowd 2004) (Lee et al. 2015) (Lee and Lee 2014) (Park et al. 1998)
Linear non α-helical	Bacterial filamentation and inhibition of protein synthesis	(Subbalakshmi and Sitaram 1998)
Anionic peptide	Flocculation of intracellular components and protein inactivation	(Brogden et al. 1996)
Defensins	Insertion in the cell membrane and formation of “channel-like” dimer pores Membrane perturbation (induced depolarization) and cell wall lyses Binding to the viral capsid and inhibition and occlusion of proteolytic cleavage Cell penetration and induction of membrane bleb, cellular elongation, and clumping Adjuvant of immunological function (chemoattraction of leukocytes and induction of chemokine release)	(Zhang et al. 2010) (Sahl et al. 2005) (Wiens and Smith 2015) (Chileveru et al. 2015) (Yang et al. 2002)
Pro-rich peptides	Membrane disruption and intracellular target inhibition Non-lytic deregulation, cell penetration, endotoxin binding, and immune modulation Binding to intracellular heat shock proteins	(Paulsen et al. 2013) (Li et al. 2014; Scocchi et al. 2011) (Otvos et al. 2000)
Nonribosomal peptide	Formation of transient ion channel and pores via barrel stave mechanism	(Krauson et al. 2012; Brogden 2005; Payne et al. 1970)

^aAntimicrobial peptides in a structural class that display the exemplified mechanism of action are from the correspondent references at the right column

mitochondrial dysfunction, as observed in the case of frog skin temporin-related peptide (Wang et al. 2013a). A reason for the susceptibility of tumor cells to AMPs relies on the fact that the cytoplasmic membrane of these cells possess, similar to the microbial lipid membrane, a high content of negatively charged components (e.g., heavily glycosylated proteins and anionic lipids). These integral anionic membrane macromolecules permit a selective electrostatic interaction of cationic AMPs by which their killing action efficiently initiates. Importantly, the negative potential of

membrane on the outside surface of cancer cells also contributes to the specific and selective cytotoxic activity of several cationic AMPs (Hoskin and Ramamoorthy 2008; Gaspar et al. 2013). Worthy of mention, based on the selectivity and cytotoxicity of natural AMPs, researchers have explored these capabilities to design short synthetic peptides and hybrid sequences with distinct potency, improved efficacy, and lower toxicity toward nonmalignant cells (Huang et al. 2010; Yang et al. 2013; Chen et al. 2014). In summary, given the spectra of activities and properties (i.e., microbiocide, antitumor, and immunoenhancing) that are displayed by the diverse sequences of AMPs, associated with their target specificity and selectivity, peptides in this class are versatile and provide tunable templates for peptide engineering. Moreover, these peptides represent good candidates for the development of diagnostic probes, therapeutic peptides, and adjuvant substances for clinical use in combination with the conventional antimicrobial and anticancer chemotherapeutic arsenal.

Reptilian Antimicrobial Peptides

In a recent work by van Hoek (2014), current knowledge about AMPs that have been characterized by genomic and proteomic studies from four orders of reptilians, namely, Crocrodilia (crocodilians), Sphenodontia (tuataras), Squamata (lizards and snakes), and Testudines (turtles and tortoises), was expanded. The main classes that were categorized in this study included the β -defensins (e.g., gallinacin-like), the linear α -helical cathelicidins, the β -defensin-folded crotamine-like peptides, hepatic hepcidin and LEAP-2, and enzymes with microbicidal activity, such as lysozymes. Some of these AMPs are not exclusively expressed in the venom (of snakes) and saliva (of lizards) but are detectable in nonsecretor tissues, such as blood cells, liver, and epithelia. For example, turtle β -defensin 1 (TBD-1) is found in leukocytes of the European pond turtle *Emys orbicularis* and skin and subdermal granulocytes of the soft-shelled turtle *Apalone spinifera* (Alibardi 2013b; Stegemann et al. 2009; van Hoek 2014). In crocodilians, apart from the description of hepatic hepcidin, liver-expressed peptide-2 (LEAP-2), and leucrocins – these latter probable nonribosomal peptides from leukocytes, the presence in the blood of AMPs as generated by the fragmentation of α - and β -chains of hemoglobin (van Hoek 2014) is remarkable. Originally identified in the serum of the American alligator (*Alligator mississippiensis*), which rarely gets infected, hemoglobin and chemically cleaved hemoglobin fragments have microbicidal activity (Parish et al. 2001). Thus, hemoglobin-derived peptides represent additional members of the antimicrobial arsenal of the innate system of crocodilians (Srihongthong et al. 2012) and even of humans, in which they display a synergistic effect with α - and β -defensins, cathelicidin, and lysozymes (Mak et al. 2007). As one of the most antique group of AMPs, cathelicidins are multifunctional host-defense effector peptides that evolved after the divergence of craniates (Uzzel et al. 2003) and, as probed, are expressed in different tissues of all of the vertebrates that have been investigated so far, including reptiles (Wang et al. 2008; Alibardi 2014). More detailed aspects about

cathelicidins will be discussed in the next section regarding snake venom cathelicidins. Together, cathelicidins and β -defensins are the most prevalent classes of AMPs found in reptiles, as exemplified by crocodilian gallinacin-like peptides (van Hoek 2014), lizard defensin- and cathelicidin-related peptides (Alibardi 2013a, 2014), and turtle β -defensins (Stegemann et al. 2009; Alibardi 2013b; Benato et al. 2013). In Table 3, examples of reptilian AMPs are listed.

In snakes, β -defensins have been evidenced by means of the molecular phylogenetic analysis of dozens of South American Crotalinae species (Correa and Oguiura 2013) and by the immunological cross-reaction of *Natrix natrix* granulocytes with antibodies that have been developed against lizard β -defensin 15 (Alibardi 2013a). Correa and Oguiura (2013) identified 13 β -defensin-related sequences from the liver, scale, and blood of 12 species of South American Crotalinae snakes, including *Bothrops*, *Crotalus*, and *Lachesis*. The mature Crotalinae β -defensin-related peptides have predictable sizes of approximately 4.5 kDa – a relative molecular weight that is expected for AMPs of this class and length, net charges ranging from +2 to +11, and, importantly, the same pattern of cysteine residues that usually form the β -defensin fold. Despite the limited number of known β -defensin AMPs in reptiles, the β -defensin motif configures a scaffold that is present in several membrane-active and neurotoxic peptides from animal venom, such as crotamine, from rattlesnake venom, and anthopleurins, from the sea anemones *Anthopleura xanthogrammica* and *A. elegantissima* (Torres and Kuchel 2004; Kerkis et al. 2010). Crotamine-like and β -defensin-related peptides were identifiable and described from the salivary glands of lizards (Fry et al. 2006) and from the venom of the primitive egg-laying venomous mammal platypus *Ornithorhynchus* (Whittington et al. 2008), accounting for venom-related peptides with the typical β -defensin fold and iterative convergent biological activity. In fact, the β -defensin fold features the archetypal γ -core signature – a central structural motif that appears in some class of host-defense peptides, such as AMPs, venom neurotoxic peptides, and microbicidal chemokines. Interestingly, the compositional assemblage of α -helix and β -sheet modules at the N- and/or C-termini to the γ -core scaffold – a sort of structural decoration – gives rise to the distinct structural classes of β -defensin peptides and their respective congruent function (Yeaman and Yount 2007). One example of a multifunctional and versatile snake venom peptide that is structured by the central γ -core and the canonical β -defensin fold is crotamine (Kerkis et al. 2010; Rádis-Baptista and Kerkis 2011). Initially characterized from the venom of South American rattlesnake *Crotalus durissus terrificus* and described as a myotoxin with the ability to interfere with Na^+ -channel function, crotamine was demonstrated to possess multiple molecular functionalities, such as selective in vitro and in vivo anticancer cytotoxicity (Hayashi et al. 2008; Pereira et al. 2011; Kerkis et al. 2014), biocide activity against bacteria and fungi (Oguiura et al. 2011; Yamane et al. 2013), selective interaction with the eukaryotic voltage K^+ channel (Yount et al. 2009; Peigneur et al. 2012), translocation across lipid bilayers and penetration into eukaryotic cells (Kerkis et al. 2004), and interaction with nucleic acids and delivery of genes and probes (Nascimento et al. 2007; Chen et al. 2012). These properties equip crotamine with a unique versatility to be explored for the development of diagnostic probes and

Table 3 Representative cathelcidin-related peptides from different species of vertebrates

Structural class ^a	Peptide	Peptide sequence	Cathelcidin source
α-helical	LL37/hCAP18	LLGDFRKSKEKIGKEFKRIVQRIKDFLRNLLVPRTES	Human
α-helical	mCRAMP	GLLRKGGEKIGEKLLKIGQKIKNFFQKLVQPQE	Mouse
α-helical	Crotalidicin	KRFKKFFKVKKSVKKRLKKIFKKPMVIGVTIPF	Snake
α-helical	Batroxidicin	KRFKKFFKLLKNSVKKRVKKFFRKPRVIGVTFPF	Snake
α-helical	Bf-CRAMP	KRFKKFFRKLKKSVKKRAKEFFKKPRVIGVSIPF	Snake
α-helical	Nh-CRAMP	KRFKKFFKLLKNSVKKRAKKFFKKPKVIGVTFPF	Snake
α-helical	Oh-CRAMP	KRFKKFFKLLKNSVKKRAKKFFKKPRVIGVSIPF	Snake
α-helical	Fowlicidin-1	RVKRVWPLVIRTVIAGYNLYRAIKKK	Chicken
α-helical	Fowlicidin-3	RVKRFWPLVPVAINTVAAGINLYKAIRRK	Chicken
α-helical	Fowlicidin-2	LVQGRFGFRLRKIRFRPKVTTTIQGSARF	Chicken
α-helical dimeric	CAP11	[GLRKKFRKTRKRIQKLGRKIGKTGRKVVWKAWREYQIPYPCR] ₂	Guinea pig
β-hairpin	Bactenecin	RLCRIVVIRVCR	Cow
β-hairpin dimeric	Bactenecin	[RLCRIVVIRVCR] ₂	Synthetic
β-hairpin	Protegrin-1	RGGRLCYCRRRFCVVCVGR-amide	Pig
Linear	MgCATH37	GWFKKAWRKVKHAGRRVLDTAKGVGRHYLNNWLNRYRG	Hagfish
Linear	MgCATH29	GWFKKAWRKVKNAGRVLKGVGIHYGVGLIG	Hagfish
Linear gly-rich	Cathelcidin-AL	RRSRGRGGRRGGSGRGGGGGSGAGSSIAGVGSRRGGGGGRHYA	Frog
Linear gly-rich	Cathelcidin-NA iso2	RRSRNGRGGGGRRGGGGSGRGGSGSGSSIAGGGGGGRMNGYA	Frog
Linear Pro-rich	Bac 5	RFRPPIRRPPRPFPYPFPPFRPPPIRPPPIRPPPLGPFP-amide	Pig
Linear Pro-rich	PR-39	RRRPRPPYLPRPRPPPPFPFPLPRIPPGFPPRFPFRFP-amide	Pig
Linear Trp-rich	Indolicidin	ILPWKWPWWPWRR-amide	Cow
Linear Trp-rich	Tritrpticin	VRRFPWWPFLRR	Pig

therapeutic peptides. In fact, short crodamine-derived synthetic peptides were designed that maintained most of these properties and improved others, such as reduced sized, efficient cell uptake and cell selectivity (Rádis-Baptista et al. 2008, 2012; Rodrigues et al. 2012), and facilitation to functionalize particles and proteins (Rodrigues et al. 2011). Crodamine-derived short-sized peptides consist of splice structures in which the first nine N-terminal amino acid residues are covalently linked to the last five C-terminal residues, displaying a high tendency to form α -helices. Thus, synthetic minimized crodamine peptides are constructed without the canonical γ -core motif of the β -defensin fold.

Viperidins and Snake Venom Cathelicidin-Related Peptides

The gallinacins, β -defensins of chicken (*Gallus gallus*) leukocytes, are ancient antimicrobial peptides that have existed for more than 250 mya, appearing before the evolutionary divergence of birds and mammals (Harwig et al. 1994). In fact, gallinacin-related peptide sequences are found in turtles and crocodilians; the latter are primitive animals that, together with birds, are living descendants of dinosaurs (stepping onto land 250–200 mya) as previously mentioned. However, the isolation of cathelicidins from the intestinal myeloid cells of the Atlantic hagfish (*Myxine glutinosa*) – the oldest-living jawless craniate that descends from a common ancestral creature that appeared 450 mya (Uzzel et al. 2003) – placed this class of AMPs among the most basal components of the innate immunity system of vertebrates. Thus, the presence of cathelicidins seems to predate the origin of the adaptive immune system that supposedly initiated around the same timescale of ~450 mya but evolved following the divergence of craniates from chordates (Uzzel et al. 2003). First characterized from bovine neutrophils, cathelicidin-related sequences (CRAMPs) have been subsequently isolated from several species of domesticated animals as well as mice and rats (Brogden et al. 2003; Zanetti 2005). Humans have a single cathelicidin peptide that is mainly expressed by neutrophils and epithelial cells (Zanetti 2005). With a molecular weight of 18 kDa and a size of 37 residues of amino acids, in which the first two N-terminal residues are leucine, human cathelicidin is known as hCAP18/LL-37. The disclosure of a cathelicidin in the frog skin of *Amolops loloensis* and sequence comparison with cathelicidins from several vertebrate species have allowed researchers to ascertain the maintenance of this class of antimicrobial peptide throughout the course of evolution and the diversification of aquatic and land-living animals. The presence of cathelicidins in basal organisms, such as the primitive jawless fish and amphibians, as well as their distribution in modern higher vertebrates, seems to indicate the evolutionary derivation of cystatins – cysteine protease inhibitors that primordially served as defensive components before the origin of adaptive immunity (Zhu 2008). The protease inhibitor domain of cathelicidins, which shares similarity to cystatins, is related to the cathepsin L inhibitor (cathelin). The highly conserved cathelin sequence constitutes the prodomain of cathelicidins. Cathelicidins are biosynthesized as

Table 4 Examples of antimicrobial peptides and proteins from snake venom and reptilian tissues

Antimicrobial peptide	Organism	Source	References
β -defensin and crotamine-like peptide	Pit viper snakes Lizards Crocodilians Turtles	Venom, venom gland Skin, oral gland In silico predicted Skin	(Correa and Oguiura 2013; Van Hoek 2014) (Alibardi 2013a; Dalla Valle et al. 2012; Fry et al. 2006) Van Hoek (2014) (Alibardi 2013a, 2014; Benato et al. 2013; Chattopadhyay et al. 2006)
α -helical cathelicidin	Elapid snakes Pit viper snakes Lizards Crocodilians Turtles	Venom, venom gland Venom, venom gland Epithelia In silico predicted In silico predicted	(Song et al. 2015; Xia et al. 2015; Zhao et al. 2008) (Falcao et al. 2014) (Alibardi 2014) (Van Hoek 2014) (Van Hoek 2014)
α -helical hemoglobin fragment (hemocidin)	Crocodilians	Blood	(Srihongthong et al. 2012; Parish et al. 2001)
Nonribosomal peptide (leucrocin)	Crocodilians	Blood cells	(Pata et al. 2011)
Cysteine-rich (hepcidin)	Crocodilians	Liver	(Hao et al. 2012)
Antimicrobial protein			
L-amino acid oxidase	Elapids Pit vipers and vipers	Venom	(Lee et al. 2011; Samel et al. 2008) (Rima et al. 2013; Guo et al. 2012)
Phospholipase A ₂	Elapids Pit vipers	Venom	(Wen et al. 2012) (Muller et al. 2014; Cecilio et al. 2013; Samy et al. 2012, 2014; Perumal Samy et al. 2007)
WAP-domain proteins	Elapids	Venom	(Nair et al. 2007)

prepropeptide precursors with an N-terminal leader sequence (signal peptide), followed by the conserved cathelin-like domain (proregion or prosequence) and a heterogenic and hypervariable C-terminal segment containing the antimicrobial peptide (Zanetti 2004, 2005). The AMPs that are proteolytically released from their respective cathelicidin precursor include linear α -helical and amphipathic peptides, a cysteine-stabilized structure, and peptides that are rich in certain amino acids. In Table 4, examples of cathelicidin-related antimicrobial peptides from several species of vertebrates are shown.

The structural and functional diversification of cathelicidin-related antimicrobial peptides seems to be a consequence of gene organization, gene duplication, accelerated molecular evolution, and rapid divergence (Zhu 2008; Zhu and Gao 2009). Functionally, despite the direct action on microbes via the disruption of membrane integrity and endotoxin neutralization (Bucki et al. 2010), cathelicidins, similar to human hCAP18/LL-37, modulate the immune response and mediate inflammation, displaying angiogenic, mitogenic, and chemotactic activity. Therefore, cathelicidins are considered multifunctional effector molecules connecting innate and adaptive immunity (Zaiou and Gallo 2002; Zanetti 2005). A recent study demonstrated that fowlicidin, a cathelicidin from chicken, also possesses the capability to stimulate and regulate the innate and adaptive immune by acting as an antimicrobial peptide, attracting neutrophils and activating macrophages (Bommineni et al. 2014). Therefore, such convergent functionalities appear to be recurrent between divergent AMPs of this class. The connection between innate and acquired immunity as mediated by AMPs is optimal for an efficient response against microbial assault, as observed for hCAP18/LL-37. However, in other situations, such molecular interplay comprises a double-edged sword, being the exacerbation of inflammation as provoked by a high content of cathelicidin or the absence of AMP expression associated with several inflammatory disorders. Diseases of the human skin, such as psoriasis, rosacea, and dermatite contact, are based on the immunopathogenesis of the overexpression or increased levels of LL-37, whereas atopic dermatitis and Crohn's disease are associated with the downregulation of LL-37 and α -defensin expression (Méndez-Samperio 2013). In psoriasis, cathelicidin and other AMPs, which are collectively attributed the name of alarmins, prime immune cells, induce the production of proinflammatory mediators and chemically attract leukocytes (Batycka-Baran et al. 2014). Interestingly, the physiopathological mechanism of psoriasis, an autoimmune disease, relies on the formation of complexes (aggregates and condensed structures) between human cathelicidins and host (self) DNA. DNA-peptide complexes are taken up by dendritic cells via Toll-like receptors, resulting in the massive production of type 1 interferon, the augmentation of an inflammatory response, and disease progression (Nakagawa and Gallo 2015; Lande et al. 2007). The involvement of antimicrobial peptides in immune regulation indicates that such a class of pharmaceutical peptides can be converted in vaccine adjuvants and modulate of the immune and inflammatory responses in different disorders.

In snakes, cathelicidin-related antimicrobial peptides (CRAMPs) were discovered for the first time in Asian elapid (cobra and kraits) venom and tissues (Wang et al. 2008; Zhao et al. 2008) and subsequently in South American pit viper (jararacas and rattlesnakes) venom glands (Falcao et al. 2014). The elapid CRAMPs encompass cathelicidin-related peptides from the banded krait *Bungarus fasciatus* (named BF-CRAMP or BF-30), from the Chinese cobra *Naja atra* (Na-CRAMP), and from the king cobra *Ophiophagus hannah* (Oh-CRAMP). The viperidins consist of cathelicidin-related precursors from the venom glands of *Bothrops atrox* (batroxicidin), *Bothrops lutzi* (lutzicidin), *Crotalus durissus terrificus* (cotralicidin),

and *Lachesis muta rhombeata* (lachesicidin). Two additional elapid CRAMPs have been identified from the venom gland of *Pseudonaja textilis* (Pt_CRAMP1 and Pt_CRAMP2), increasing the number of elapid CRAMPs (Falcao et al. 2014). In both groups of snake cathelicidins (elapid CRAMPs and viperidins), the overall structural features that are typical of all vertebrate cathelicidin precursors are maintained, including gene-encoded prepropeptide precursors with a signal peptide (~20 amino acid residues), followed by a highly conserved prosequence (the cathelin-like domain) and a hypervariable carboxyl-terminal in which the cationic AMP sequences are contained and proteolytically released (Fig. 1). A distinct hallmark of snake CRAMP precursors is the presence of an acidic signature at the carboxyl-terminal end of the cathelin-like domain that is rich in negatively charged aspartic acid and uncharged residues (Fig. 1). Elapid CRAMPs and viperidins are extremely conserved sequences; even their natural reservoirs – elapids and pit vipers – diverged for more than 40 mya and inhabit different geographical regions on Earth (Asia and South America). Figure 2 represents the phylogenetic relationship of cathelicidin prepropeptide sequences from hagfish, amphibians, snakes, turtles, and crocodilians.

The evolutionary conservation in the primary structures of snake cathelicidins is also observed in their function. These peptides are broad-spectrum antimicrobial peptides that are active against clinical and standard strains of bacteria and pathogenic fungi, including antibiotic-resistant isolates (Wang et al. 2008, 2011, 2013c; Zhao et al. 2008; Falcao et al. 2014; Xia et al. 2015). Interestingly, in a study using a mouse model of endotoxemia as induced by an intraperitoneal injection of lipopolysaccharide, *Bungarus fasciatus* cathelicidin precluded the damage and the cell death that were provoked by LPS to the intestinal epithelium. Moreover, cathelicidin BF inhibited the expression and secretion of tumor necrosis factor α (TNF- α) in mouse macrophages (Song et al. 2015).

Advanced research about the multiple actions of snake cathelicidins has revealed that apart from displaying an efficacious killing effect against microbes, elapid CRAMPs (Tian et al. 2013; Wang et al. 2013c) and viperidins (unpublished personal data) are cytotoxic for different types of tumor cells. The mechanisms by which snake venom cathelicidin kills bacterial and tumor cells are also conserved. These peptides are membrane-active peptides that in a synergic mode disrupt the integrity of the lipid bilayer, penetrate the cell cytoplasm, interact with DNA molecules, and prevent cellular processes, causing rapid and efficient cell death (Zhou et al. 2011; Hao et al. 2013; Wang et al. 2013b). Synthetic analogues of elapid CRAMPs and viperidins in their full length or short versions have been prepared, and their structure-activity relationship has been studied (de Latour et al. 2010; Chen et al. 2011b; Falcao et al. 2014). From these studies, snake venom cathelicidin-derived peptides with similar or better therapeutic index, i.e., selective versus unspecific biological activity, than the natural full-size peptides have been characterized. For example, de Latour and coworkers (2010) produced a series of very short peptides that were derived from the *Naja atra* cathelicidin, containing the so-called ATRA-motif [KR(F/A)KKFFKK(L/P)K], and demonstrated their low toxicity to host cells and high-potent antimicrobial activity against Gram-negative bacteria.

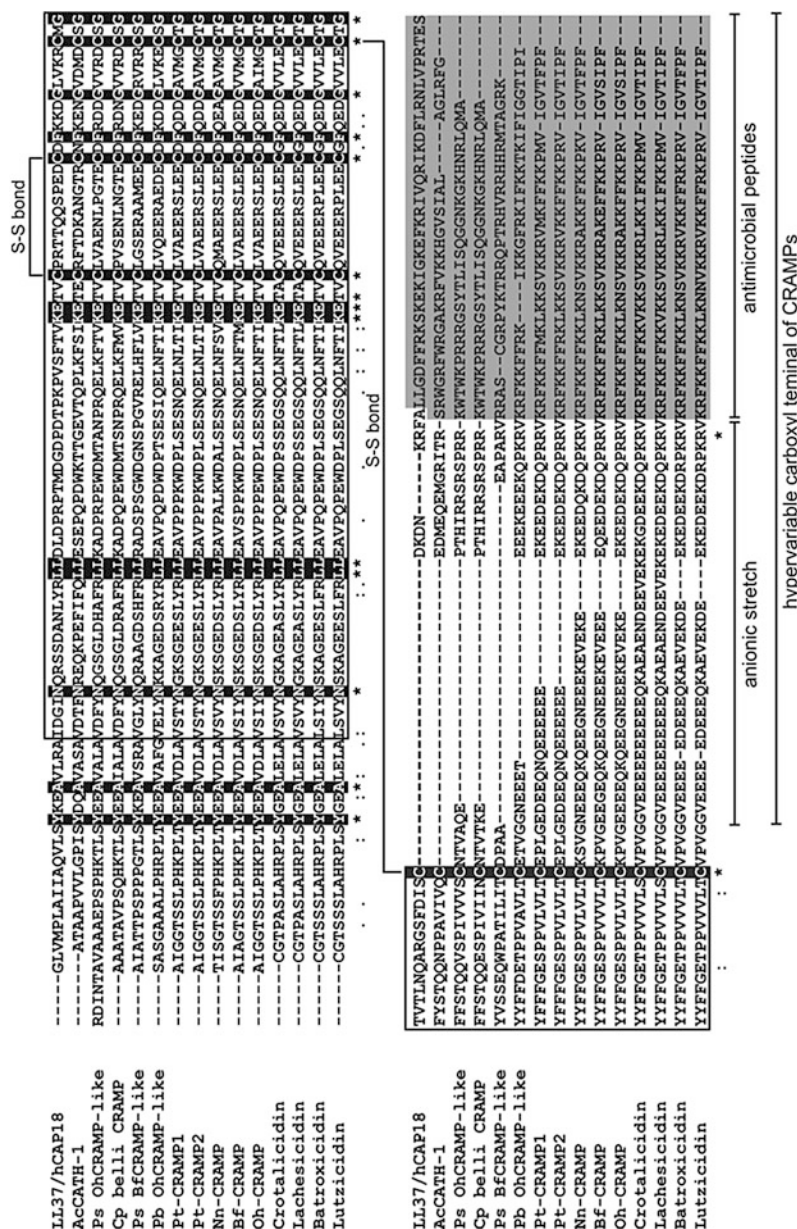


Fig. 1 Alignment of cathelicidin-related peptides from snakes, lizards, turtles, and human. The aligned cathelicidin-related presequences are identified as follows: LL37/hCAP18, human cathelicidin antimicrobial peptide preprotein, gene identification (gi) number 348041314 (gi|348041314); AcCATH-1,

The efficacy test of short *d*-enantiomer peptides that were derived from ATRA-motif indicates that these isomeric peptides exhibit varied potencies against bacterial strains and that helicity propensity (conformational helix) depends on the anionic content of the membranes (Juba et al. 2013). The modulation of the structure and function of snake venom cathelicidin can also be ascertained by BF-15, a 15-mer peptide that was derived from cathelicidin-BF (BF-30) and that maintains an amphipathic α -helix and potent antimicrobial activity against antibiotic-resistant bacteria but exhibits reduced hemolytic activity (Chen et al. 2011b). The positional replacement of amino acid residues in cathelicidin BF-30 converted the original peptide into a lysine-16 mutant that selectively inhibited in vitro non-small lung cancer cell proliferation in the detriment of metastatic melanoma B16F10 cells (Tian et al. 2013). Short synthetic derived of crotalicidin with a distinct potency and spectrum of action toward malignant human cells, pathogenic fungi, and clinical isolates of Gram-positive and -negative bacteria have also been prepared and studied (personal communication). For instance, in a recent work, Wang and collaborators (2015) have demonstrated that an encrypted nonapeptide (KRFKKFFKK), that is repeatedly encoded in tandem within viperidins sequences, displays toxic and cytotoxic activities in vivo and in vitro models only when the peptide's N-terminus was chemically conjugated to rhodamine B.

Taken together, the identification of snake venom cathelicidins and the preparation of the synthetic version and short peptide analogues demonstrate that elapid CRAMPs and viperidins are amenable to de novo design to generate tunable and customized peptides for the development of alternative antimicrobials, anticancer, and immunomodulator agents from a natural resource: snake venom.

Fig. 1 (continued) green lizard *Anolis carolinensis* cathelicidin, gi|674969844; Ps OhCRAMP-like gi|558192385 and Ps BfCRAMP-like gi|558122459, predicted cathelicidin-OH and cathelicidin-BF antimicrobial peptide-like, respectively, from the Chinese softshell turtle *Pelodiscus sinensis*; Cp belli CRAMP, predicted cathelicidin antimicrobial peptide from the painted turtle *Chrysemys picta bellii*, gi|530606957; and Pb Oh-CRAMP-like, predicted cathelicidin-OH antimicrobial peptide-like from the python snake, *Python bivittatus*, gi|602675584; Pt-CRAMP1 and Pt-CRAMP2, CRAMP isoform precursors from the elapid *Pseudonaja textilis*, gi|528320163 and gi|528320161, respectively. Nn-CRAMP, Bf-CRAMP, and Oh-CRAMP are CRAMP precursors from Chinese cobra *Naja atra*, gi|193792525, banded krait *Bungarus fasciatus*, gi|206944351, and the king cobra *Ophiophagus Hannah*, gi|193792529, respectively; Crotalicidin gi|528320151, lachesicidin gi|528320159, batroxocidin gi|528320155, and lutzicidin gi|528320157 are viperidins (pit viper CRAMPs) from *Crotalus durissus terrificus*, *Lachesis muta rhombeata*, *Bothrops atrox*, and *Bothrops lutzi*, respectively. The cathelicidin preprosequences were aligned using the algorithm MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>). The conserved cathelin domain is marked with an open box. Conserved amino acid and cysteine residues in the cathelin domain are labeled in black. The two disulfide bridges in the cathelin prosequences are indicated by connecting lines. The cathelicidin-related antimicrobial peptide sequences are enlightened with a gray box. The anionic stretch in the hypervariable-carboxyl terminal of CRAMPs, specifically observable in snake CRAMPs, is also indicated

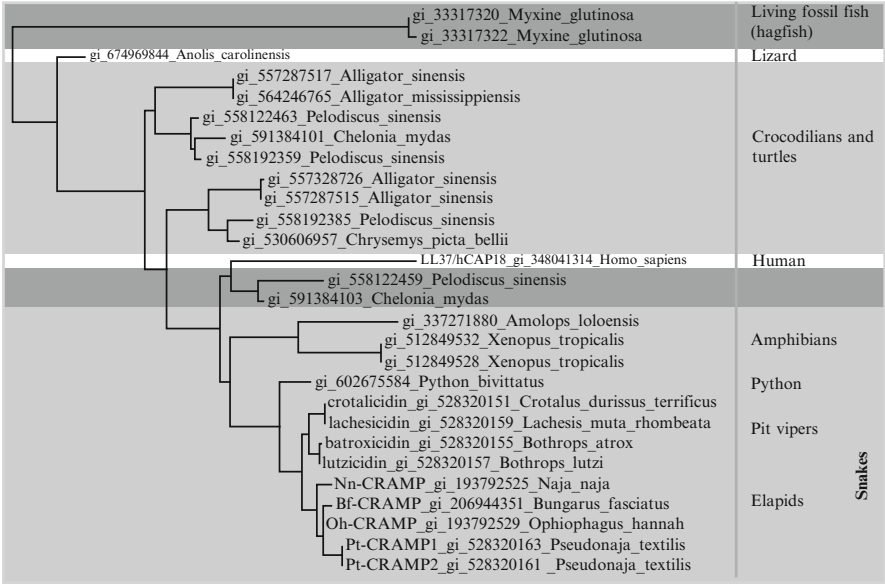


Fig. 2 Phylogenetic relationship of cathelicidin-related prepropeptides from amphibians, crocodilians, hagfish, turtles, and snakes. The numbers in the branch's tip of phylogenetic tree correspond to the gene identifier (gi) in the gene and protein database (<http://www.ncbi.nlm.nih.gov>). The phylogenetic tree was built online using the bioinformatics tools available at <http://phylogeny.lirmm.fr/phylo.cgi/index.cgi>. The species names are included in the figure. Snake venom CRAMPs (viperidicidins and elapid CRAMPs) group together and distant from ancient hagfish CRAMPs

Miscellaneous Polypeptide Toxin Classes with Antimicrobial Activity

In addition to antimicrobial peptides that are found widespread in different kingdoms of life and therefore comprise important resources for the discovery of novel sequences with multitask functionalities in host defense, snake venom contains several classes of toxins that possess microbicidal properties. In these categories are included the membrane-active phospholipases A₂ (Samy et al. 2012) and cardiotoxins (Chen et al. 2011a), the free radical-producing enzymes L-amino acid oxidase and LAAO (Samel et al. 2008; Guo et al. 2012), and mixed-function omwaprins (Nair et al. 2007). Venom phospholipases A₂ (PLA₂s) consist of one of the most abundant classes of toxin component in the secretion of poisonous animals. Essentially, PLA₂s are enzymes with a low molecular weight (~14 kDa) that catalyze the hydrolysis of glycerophospholipids at the sn-2 position, releasing lysophospholipids and free fatty acids, such as arachidonic acid (AA) – a precursor of bioactive eicosanoids. Based on their structural features, biological activity, and pattern of expression, PLA₂s are grouped into different subfamilies that are distinctly

predominant in a given tissue and biological compartment (Schaloske and Dennis 2006). In venom, type II-secreted PLA₂s are found in the acidic and basic forms as well as in monomeric, homodimeric, or heterodimeric toxin structures (Guarnieri et al. 2009). Snake venom PLA₂ exhibits antibacterial (Perumal Samy et al. 2007; Samy et al. 2012) and antiviral activity (Muller et al. 2014). For example, the basic svPLA₂ from the venom of the eastern diamondback rattlesnake (*Crotalus adamanteus*) – CaTx-II – is a potent non-cytotoxic bactericide that kills pathogenic *Staphylococcus aureus*, *Burkholderia pseudomallei*, and *Enterobacter aerogenes* by inducing pore formation and membrane disruption (Samy et al. 2014). Compared to CaTx-II, the antimicrobial activity exclusively against Gram-negative bacteria resides in the β -chain of the heterodimeric neurotoxic svPLA₂ (β -bungarotoxin) from the Taiwan banded krait *Bungarus multicinctus* (Wen et al. 2013). In another study about the antimicrobial activity of snake venom PLA₂s, Cecilio and collaborators (2013) characterized two PLA₂s from the venom of the white-tailed jararaca (*Bothrops leucurus*), namely, B/K-PLA₂ and B/D-PLA₂, in which the first contains a lysine residue at position 49 and the second has an aspartic acid in the same position. These PLA₂s are active against dengue virus serotypes 1–3 and demonstrated a synergic antiviral effect of both PLA₂s in vitro in a model of viral replication using rhesus monkey kidney epithelial (LLC-MK2) cells. Importantly, in a previous report about the mechanism of anti-HIV infection, Fernard and colleagues (1999) generated experimental evidence that the inhibition of HIV infection by svPLA₂ was not due to the direct virucidal effect or cytotoxic effect on host cells but to the prevention of viral entry, the blockade of viral capsid uncoating, and the dissociation of host cell membranes. Thus, the blockade of virus disassembly, independent of the catalytic activity and virus phenotype, was the main mechanism of cell protection against HIV that was intermediated by svPLA₂s, such as taipoxin (from *Naja mossambica mossambica*) and nigexine (from *N. nigricollis*). However, this mechanism that was observed for taipoxin and nigexine against HIV viruses contrasts to that of *Crotalus durissus terrificus* svPLA₂ against dengue and yellow fever viruses, in which a disruptive mechanism of the viral envelope is involved (Muller et al. 2014). Surely, structural differences between the diverse svPLA₂s as well as the compositional variation between virus envelopes and capsids influence the distinct mechanisms of antiviral action. Given the diversity of PLA₂ structures in the venom of animals, particularly, in snake venom, the spectrum of antimicrobial action of this class of polypeptide is broad and deserves the full attention of the research community and drug developers. Another major component of snake venom consists of the L-amino acid oxidases (svLAAOs). The svLAAOs that were isolated from the venom of *Montivipera bornmuelleri*, a viper inhabiting Lebanese lands, exhibit efficacious antimicrobial activity against Gram-negative bacteria, specifically *Morganella morganii* and *Klebsiella pneumonia* – two infectious agents to humans – but was devoid of cytotoxicity to human erythrocytes (Rima et al. 2013). In contrast, the heat-stable LAAO – an unusual form of snake venom LAAO toxin enzyme from the venom of the king cobra *Ophiophagus hannah* – showed moderate antimicrobial activity against Gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas aeruginosa*, and *K. pneumonia*) but high effectiveness against

Gram-positive bacteria, such as *Staphylococcus aureus* and *S. epidermidis*. This type of selectivity and discrimination by *O. hannah* LAAO depends on the membrane binding and production of hydrogen peroxide (Lee et al. 2011; Li-Lee et al. 2014). The revision of the multiple biological activities of snake venom LAAO reported that members of this group of animal toxins possess, in addition to the bactericide effect, antiparasitic and antiviral activity (Guo et al. 2012). An additional example of the venom component that is an antimicrobial is omwaprin – a cationic polypeptide that is found in the venom of the inland taipan *Oxyuranus microlepidotus*. Omwaprin belongs to the waprin family of proteins that structurally contain the whey acidic protein (WAP) domain and include proteins with diverse functions, such as inhibitor of Na^+ - K^+ -ATPase, protease inhibitors, and single WAP-domain proteins, both with antimicrobial activity. Omwaprin exhibits membrane-selective antibacterial activity against Gram-positive bacteria that depends on the structural intra-chain disulfide bond constraints (Nair et al. 2007). Prepared by means of total solid peptide synthesis, the *d*-enantiomer of omwaprin was achieved, and the activity against pathogenic *Bacillus* was confirmed. *d*-Omwaprin was in a racemic mixture with the *l*-form facilitating the crystal-structure resolution of this class of antimicrobial protein from snake venom (Banigan et al. 2010).

Conclusion

In this chapter, one of the aims was to focus on the antimicrobial peptides that are expressed in the venom gland and in the venom of snakes. The number of known reptilian and snake venom antimicrobial peptides is small when compared to the myriad of AMP sequences in the database from other organisms. Two of the most predominant classes of AMPs – β -defensins and cathelicidins – have been explored by nature for millions of years and presently by biological chemists to produce potent, wide-spectrum, stable, membrane-active sequences that cope with drug-resistant microbes and malignant cells. The underlying synergic mechanisms that connect the immediate innate and long-lasting adaptive immune response by these peptides reveal efficiency in defending the host against pathogenic microbes and parasites. However, an excessive amount of circulating AMPs in the host body induces a severe inflammatory response and contributes to severe chronic diseases. In this respect, human cathelicidin has been implicated in the exacerbation of skin diseases and arthritis. Whether the high expression level of antimicrobial peptides in snake venom is for good or for bad requires further investigation, that is, do snake venom cathelicidins protect the venom gland against infection, or do they contribute to aggravate the clinical symptoms of envenomation? Despite of this questioning, it is becoming evident that snake venom AMPs and related toxin peptides serve as templates to study the evolution of host-defense peptide effectors and to develop customized structures by de novo design and peptide engineering.

Cross-References

- ▶ [Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes](#)
- ▶ [Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins](#)
- ▶ [Toxinology of Snake Venoms: The Malaysian Context](#)

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D. Chakrabarty (✉)

Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Zuarinagar,
Goa, India

e-mail: diba27@yahoo.com

A. Sarkar (✉)

Birla Institute of Technology and Science, Pilani, Zuarinagar, Goa, India

e-mail: asarkar12@gmail.com

Abstract

Venomous snake bites are known to cause large number of fatalities in many countries in Africa and Asia. However, apart from mortalities, a venomous bite of a member of *Viperidae* or *Elapidae* families also cause a number of severe local tissue damage which frequently leads to amputation of the bitten limb to save the life of the victim. Most of the victims of venomous snake bites are rural farmers of these developing or poor countries. Many families lose their livelihood as the bread earning member loses his active hand or leg. The major damages caused by these snakes include severe muscular damage and vascular damage, eventually causing gas gangrene. The cytological changes caused by some elapid snakes, like spitting cobras have been studied in some detail. However, the mechanisms of cytotoxicity caused by snake envenomations are far from clear. Present information indicates that metalloproteinases, lectins and phospholipases play important roles in cytotoxic damage in snake envenomation. Recent advances in cell biology and proteomics have started to reveal some facts related to their modes of actions. More in depth research on the cytotoxins will help us in at least two ways: first by better therapeutic intervention in snake envenomation and second, by developing the future arsenal against life threatening diseases by exploiting specificity of snake venom cytotoxins.

Keywords

Snake venom • Cytotoxicity • Metalloproteinase • C-type lectin • Phospholipase • LAAO

Introduction

Snake bite claims about one million deaths around the world every year. Most of the victims belong to the farmers of South-Asia, South East-Asia and Sub-Saharan Africa (Kasturiratne et al. 2008). However, more chilling fact about snake-bite is probably not the fatality, but the morbidity it causes. It is known that a huge number of people rather than dying of snake envenomation, live a partially or totally unproductive life for many years due to the severe local reactions of snake venoms. This has immense impact on the economic status of the farmer community in Asian and African countries. Most of the farmers live at the edge of the lowest income group and a morbid bread earning member becomes the reason for economic devastation of the entire family. Therefore, it is extremely important to understand the local pathological consequences of snake envenomation.

Envenomation by viperid and elapid snakes cause severe local reactions apart from systemic damage. These local reactions include extensive tissue injuries and necrosis (cell death following injury) in the bitten area and the damage may go very deep into the muscle. In severe cases, muscle tissue is destroyed to reveal the bones over a few days. The local reactions may be stopped only with administration of appropriate dosage of antivenom serum (AVS) within a short time

following the bite. Cytotoxicity may lead to neurotoxic symptoms, changes in cell morphology or cell death. The nature of cytotoxicity depends on the chemistry of the toxins. Mechanisms for cytotoxicity may range from depolarization of cardiac myocytes, inhibition of platelet aggregation, lysis of RBC, interference with membrane bound enzymes, cytological changes and apoptosis (programmed normal cell death without injury). The snake is benefitted by the cytotoxins during feeding, as these toxins start the digestion process even before the prey is completely swallowed. It is definitely of great concern to block the cytotoxic actions of snake venom in the patient. However, the nature of cytotoxins may also be of advantage in finding cure for many life threatening diseases including cancer. This chapter is an effort to provide a brief overview of the cytotoxic molecules in snake venoms and their future scopes.

Chemistry of Cytotoxins

Snake Venom Metalloproteinases (SVMPs)

SVMPs are responsible for significant local and systemic damage in the victims of snake bite (Table 1). The major effects of SVMPs include myonecrosis, blistering, coagulopathy and hemorrhage. The in vivo effects depend on the protease activities of the individual toxins. Treatment of venoms with catalytic inhibitors could completely inhibit the pathological consequences. SVMPs have been classified on the chemical structure as follows (Fox and Serrano, 2005).

Classification of SVMPs

P-I

Members of SVMP family falling under this group contains a signal sequence followed by a pro-domain and a metalloproteinase domain. However, some P-II group members undergo proteolytic processing and may appear as mature proteins having only a metalloproteinase domain. Most P-I SVMPs are not glycosylated. The nascent protein does not show protease activity, as a Cys switch mechanism at the pro-domain inhibits protease activity. The mature and active SVMP is generated by cleaving the pro-domain and contains a Zn^{2+} binding sequence.

P-II

Precursors of these SVMPs contain a pro-domain followed by a metalloproteinase domain, which remains connected to a disintegrin domain by a short spacer sequence. However, proteolytic processing of the precursor may release the disintegrin domain from the rest of the toxin. Therefore, doubt arises on some SVMPs classified as P-I, as they may have been simply P-II SVMPs after proteolytic digestion.

Table 1 List of some SVMPs with biological activities

Sl. No.	SVMP	Source	Class	Activity
1	Catrocollastatin/VAP2B	<i>Crotalus atrox</i>	P III	Apoptotic; inhibition of collagen induced platelet aggregation
2	Bothropasin	<i>Bothrops jararaca</i>	PIII	Hemorrhagic, Fibrinolytic
3	Adamalysin	<i>Crotalus adamanteus</i>	P I	Hemorrhagic
4	Acutolysin A	<i>Agkistrodon acutus</i>	P I	Hemorrhagic
5	Atrolysin A	<i>C. atrox</i>	P III	Hemorrhagic, inhibition of collagen and ADP dependent platelet aggregation, Non-RGD disintegrin
6	Kaouthiagin	<i>Naja kaouthia</i>	P III	Cleavage of vWF, Inhibition of platelet aggregation.
7	BmoMP α -I	<i>Bothrops moojeni</i>	P I	Fibrin(ogen)olytic, Coagulant
8	Jerdonitin	<i>Trimeresurus jedonii I</i>	P II	Inhibition of ADP dependent platelet aggregation
9	Jararhagin	<i>B. jararaha</i>	P III	Hemorrhagic; inhibition of platelet aggregation.
10	Ecarin	<i>Echis carinatus</i>	P III	Activation of prothrombin
11	Multactivase	<i>Echis multisquamatus</i>	P III	Activation of prothrombin
12	Graminelysin-I	<i>Trimeresurus gramineus</i>	P I	Apoptotic
13	Jerdonitin	<i>Trimeresurus jedonii I</i>	P II	Inhibition of platelet aggregation
14	Carinactivase	<i>E. carinatus</i>	P III	Activation of prothrombin
15	RVV-X	<i>Daboia russelli</i>	P III	Activation of factor X
16	BaP1	<i>Bothrops asper</i>	P I	Activation of factor X
17	VLFXA	<i>Vipera lebetina</i>	P III	Activation of factor X
18	Berythracivase	<i>Bothrops erythromelas</i>	P III	Activation of prothrombin
19	FII	<i>A. acutus</i>	P I	Fibrin(ogen)olytic
20	TM-3	<i>Trimeresurus mucrosquamatus</i>	P I	Fibrinogenolytic
21	VRR-73	<i>Vipera russelli russelli</i>	Not fully characterized	Fibrinolytic, Hemorrhagic and esterolytic
22	NKV 66	<i>Naja kaouthia</i>	Not fully characterized	Fibrinogenolytic, Inhibitor of platelet aggregation, Anti-adhesive to A459 cells

P-III

These SVMP precursors are comprised of a pro-domain, a metalloproteinase domain, a disintegrin-like domain followed by a cysteine rich domain. Most members of this class of SVMPs are monomers. Like other groups, these toxins are known to undergo proteolytic digestion, producing toxins having one or two of the domains.

P-IV

These are toxins having the domains seen in P-III group members plus one additional C-type lectin like domain attached to the main peptide chain with disulfide bonds. Viper venoms are known for their rich content of c type lectins. These toxins are capable of causing a wide variety of pathological changes, because of their ability to bind to a number of substances. The C-type lectin chain is coded by a different cDNA and post-translational oligomerization attaches the same to the main peptide through disulfide bridges.

Pathophysiological Consequences**Hemorrhage**

The viperid venoms are known for their dramatic hemorrhagic activities in the victims. The P-I, P-II and P-III classes of SVMPs are mainly responsible for the hemorrhagic activity. The P-I class brings about hemorrhage by their proteolytic activities on capillary basement membrane proteins. However, it has been observed that SVMPs under the P-III class are much more potent hemorrhagic toxins than those under the P-I class. The P-III class differs from P-I in housing a disintegrin like domain and a cysteine rich domain. The disintegrin domains compete with basement membrane protein integrin receptors and destabilize the capillary endothelium. This leads to the so called ‘dramatic hemorrhage’ (Chakrabarty et al. 2000). On the other hand, it is suggested that the cysteine rich domain can cause weakening of the basement membrane sites by binding with Von Willebrand factor domain A of various proteins like FACIT and matrylins (Serrano et al. 2006). Some of the P-III SVMPs can also inhibit platelet aggregation by binding with $\alpha_2\beta_1$ integrin present on platelet membranes. Chakrabarty et al. (2000) reported a hemorrhagic protein of 73 kDa from Indian Russell’s viper venom. This toxin, named VRR-73 caused both fibrinolysis and hemorrhage with probably different mechanisms, as different inhibitors could reduce either of the activities with different degrees. For example, EDTA could completely stop *in vivo* hemorrhage in swiss mice, but had much less inhibitory activity on fibrinolysis. On the other hand, a bivalent antiserum did not

have any effect on hemorrhagic activity, but significantly inhibited fibrinolysis. Most interestingly, heat inactivated VRR-73 could regain its fibrinolytic and esterolytic activities. But hemorrhagic activity was lost forever following exposure to heat at 100 °C for 10 min. It was found to be a very uncommon property among large proteins of vertebrate origin (Chakrabarty et al. 2000).

Myonecrosis and Blisters

SVMPs are also responsible for severe myonecrosis in bite victims. Although, the actual mechanism of myonecrosis is yet to be revealed. It is believed by some researchers to be secondary to ischemia caused by impairment of circulation to the muscle cells. In addition to myonecrosis, muscle regeneration is also inhibited by SVMPs. It is known that muscle damage is followed by activation of muscle satellite cells for regeneration of the damaged muscle. This is the normal physiological process behind muscle building by exercise. This physiological process requires a normal blood supply to bring several growth factors required in the process. However, the SVMPs can block this by damaging the microvasculature in the muscles. Gutierrez et al. (1984) have shown that injection of myonecrotic phospholipase A₂ causes severe myonecrosis. However, microcirculation is not affected. In this case, the regeneration process is normal. Injection of SVMPs on the other hand cause myonecrosis as well as block regeneration of damaged muscles.

Local Inflammation

Viperid snake envenomations cause characteristic inflammation. Local edema and increase in vascular permeability are two most common signs of SVMPs in viper venoms. Wei et al. (2006) reported appearance of various mediators of inflammation on exposure to such metalloproteinases. SVMPs also activate complement system which produce a strong chemoattractant for leukocytes. Apart from complement activation, the SVMPs through their cysteine rich and disintegrin like domains can attract neutrophils. Contribution of cytokines in dermonecrosis has been demonstrated by cleavage of precursors of TNF α by jararahagin and absence of jararahagin induced dermonecrosis in mice knocked out for TNF 1 and 2 receptors. Jararahagin has also been found to help expression of various genes of inflammatory substances.

Phospholipase A₂ (PLA₂)

Phospholipase A₂s are abundant enzyme components of many cells and they are involved in various physiological activities. However, in snake venoms the PLA₂s have been recruited as a major toxin group. These toxins exhibit a wide variety of

pharmacological actions in the victim. These enzymes are also known to aid pre-digestion of the prey. The pharmacological actions of PLA₂s include neurotoxicity, myotoxicity (including necrosis), hemotoxicity, hemolysis, hemorrhage and tissue damage. Individual PLA₂s may exhibit one or multiple pharmacological activities. Surprisingly, the high level of homology (40–90 %) in structures of PLA₂s does not match with their wide variation in pharmacological actions (Kini 2003). All PLA₂s catalyze the cleavage of glycerophospholipids at the *sn*2 position and give out free fatty acids and lysophospholipids. PLA₂s require Ca²⁺ as cofactor for the enzymic activity. Ca²⁺ binds to a specific binding loop in the enzyme during catalytic action.

The PLA₂s may be divided into fourteen groups on the basis of primary structure, 3D structure, enzyme activity and protein expression. These groups are expanding rapidly with discovery of new PLA₂s in different venoms and other sources. Depending on the degree of homology in structure and functions, they are also divided into various subgroups. Most elapid and colubrid venom PLA₂s fall under Group I and those from viperid venoms generally fall under Group II. The Group II PLA₂s differ from those of Group I by having an extended C-terminal end. Some of the PLA₂s make complexes through both covalent (e.g., β -bungarotoxins) and non-covalent (e.g., Kaouthiatoxin) bonds with other proteins to produce maximum pharmacological effects.

Mechanism of Action

Secretary PLA₂s (sPLA₂) are water soluble enzymes capable of hydrolyzing phospholipids through a Ca²⁺ mediated pathway to produce lysophospholipids and free fatty acids. These enzymes have been observed to increase their catalytic ability on micellar substrates than on monomeric phospholipids. These enzymes possess a unique ability to bind to the lipid-water interface of micelles and then carryout the hydrolysis reaction followed by release of the reaction products. A conserved histidine at the 48th position constructs a catalytic site along with water and Ca²⁺ (as cofactor) to act on glycerophospholipids. It is suggested that Asp at 99th position and two Tyr residues, at 52nd and 73rd positions also take part in the construction of the catalytic framework (Scott 1997).

The pharmacological effects of the PLA₂s do not always depend on the enzymatic activity. However, in case of enzyme action dependent activities, either digestion of the phospholipids or the products released by the enzymatic hydrolysis may cause the effect (Kini and Evans 1989). Digestion of phospholipids in the membranes may alter membrane characteristics and affect permeability to ions and other materials. Products of phospholipid digestion, like lysophospholipids and free fatty acids may also produce a wide range of pharmacological activities, like inflammation, platelet aggregation, hemolysis, etc. The major factor however for the toxic efficacy in vivo is penetrability of the toxin. Penetrability depends on density of phospholipids at the target tissue (Kini 1997).

L-Amino Acid Oxidase (LAAO)

LAAOs are flavoenzymes abundantly available in snake venoms, particularly in the pit viper venoms. Malayan pit viper venom contains as much as 30 % LAAO in its venom (by weight) (Tan 1998). This enzyme helps in oxidative deamination reaction of L-amino acid to produce a ketoacid and ammonia. This group of enzymes is capable of causing several pharmacological effects in vivo including coagulopathy, inhibition of platelet aggregation and cytotoxicity. Because of its multiple pharmacological effects, LAAOs are considered to be next generation therapeutic tools against HIV and cancer. X-ray studies of the LAAO from *Calloselasma rhodostoma* (Malayan pit viper) venom reveal a complex protein with 15 α -helices and 22 β -strands. Functionally, this protein is a homodimer with three distinct domains – one for FAD binding, one for substrate binding and another helical domain which constructs one side of the entrance to the active site (Pawelek et al. 2000).

Catalysis

LAAO oxidizes the L-amino acid through generation of one intermediate α -imino acid which reacts with water to form the final product α -keto acid (Bright and Portar 1975). The reductive half reaction in this catalytic process probably takes place through a direct hydride transfer mechanism.

Pharmacological Activity

LAAO probably does not contribute significantly to the lethality of the whole venom, as LD₅₀ of the enzyme is generally higher than the venom (Tan and Saifuddin 1989). However, LAAO has attracted the attention of researchers for its wide pharmacological activities like apoptosis, edema formation, hemorrhage, blood coagulation and inhibition or induction of platelet aggregation.

Apoptosis Induction

Apoptosis or programmed cell death is characterized by breakage of nuclear DNA. The apoptotic activity may be completely inhibited by H₂O₂ scavenging agents. Therefore, the oxidative stress seems to be a major factor in LAAO induced apoptosis. It is proposed that the H₂O₂ generated by LAAO action produce the oxidative stress and thereby activate the Heat-Shock Proteins (HSPs) (Tempone et al. 2001). These proteins have multiple functions, including assistance in correct folding of the incorrectly folded proteins in stressed conditions and interactions in the signaling pathways. However, there are differences of opinion on the mechanism of LAAO action, as some researchers showed a difference in the mechanism of H₂O₂-induced apoptosis (Suhr and Kim 1996). A possible important step in

apoptosis is binding with cell surface molecules followed by internalization of the LAAO through the glycan moiety. Jian et al. (2014) showed that LAAO from *Crotalus adamanteus* venom induced apoptosis in NUGC-3 gastric cancer cells without involving the caspase dependent pathway.

Hyaluronidase

Hyaluronidase with its several isoforms is widely distributed in snake and other venoms, apart from many other tissues. Hyaluronidases are also known as the *spreading factor* as it helps in spreading of the venom by degrading the extracellular matrix and connective tissues around the blood vessels. This group of enzymes did not attract the attention of many toxinologists, hence the number of hyaluronidases from snake venom are few.

Mechanism of Action

Hyaluronidases digest hyaluronan, a high molecular weight glycosaminoglycan (GAG) found in high quantities in the extracellular matrix of soft tissues. Hyaluronan is involved in many physiological processes, like wound healing, cell migration, notochord formation and also in malignancy (Toole 2004). Being a negatively charged GAG, hyaluronan can hold a number of cations and water. Hyaluronan acts as a glue to give stability to the extracellular matrix. Hyaluronidases from snake venoms digest the hyaluronan on extracellular matrix and aid spreading of the venom toxins. Hyaluronidase is not listed among the toxic components of snake venoms and most of its toxic effects are indirect. However, Girish and Kemparaju (2007) suggest that the degradation products arising out of venom hyaluronidase once getting entry into the circulatory systems may cause life-threatening problems. The rate of digestion of hyaluronan by hyaluronidase may be the major cause of insufficient protection against local tissue damage by antivenoms.

C-Type Lectins

These are proteins found in many different types of tissues and are mainly involved in adhesion and signaling related to inflammation and immunity. Members of this family of proteins share homology in their carbohydrate recognition domain. The C-type lectins may not actually bind to carbohydrates. However, all of them contain a so called **C-type lectin fold** with a high variation in amino acid sequence. The family members include selectins, endocytic receptors, proteoglycans and some reptilian venom toxins (Cummings and McEver 2009). A Ca^{2+} dependent asialoglycoprotein receptor protein isolated from hepatocyte plasma membrane fractions by affinity chromatography was identified as the first C-type lectin. Till date more

than a thousand proteins have been found to have C-type lectin structure. As noted earlier, the characteristic C- type lectin fold may be possessed by proteins not involved in sugar binding. This domain is however used by some proteins for binding other proteins, lipids or other inorganic molecules. This fold is also in use by antifreeze glycoproteins for binding ice! The characteristic C-type lectin fold can accommodate –OH groups of sugars only in presence of Ca^{2+} . The fold consists of a domain made up of 110–130 amino acids. These are grossly arranged in two anti-parallel β -sheets connected by two α -helices and another three stranded anti-parallel β -sheet. This domain houses two conserved disulphide bonds and multiple Ca^{2+} binding sites.

Snake Venom C-type Lectins

C-type lectins exist in two different types in snake venoms – (1) C-type lectin proteins (CLP) and (2) Sugar binding snake lectins (SSL). These two types share some homology in structure. SSLs generally are about 28 kDa homodimeric proteins, which are capable of agglutination of RBCs through binding the carbohydrate side chains on their surface. Whereas, CLPs are generally heterodimeric proteins. The CLPs affect the hemostatic system in envenomation victims through actions on several plasma components or blood cells, particularly platelets. Because of their conservative and generally homologous structure, CLPs are useful tools in biochemical characterization of several proteins and helped the study of platelet signal transduction pathways.

Functions

Platelets are integral components of hemostasis. Platelet aggregation constitutes the first step in blood coagulation cascade. The aggregation process depends on interaction of many platelet surface receptors for ADP, Collagen and other factors. Some snake venom components interfere with this interaction to cause uninterrupted bleeding in bite victims. C-type lectins from snake venoms (CLPs) are known to target some glycoprotein receptors or integrins to either block or aid platelet aggregation. In spite of the wide variety of biological action on platelets, most of the CLPs have a common target, the GPIb (Lu et al. 2005). GPIV, another platelet surface glycoprotein receptor is involved in collagen-platelet interaction. Activation of platelet has been observed with a number of snake venom CLPs. However, there is no report on any snake venom toxin binding and inhibiting GPIV. Collagen like activation of GPIV has been reported with a toxin, Convulxin isolated from *Crotalus durissus terrificus* venom (Leduc and Bon 1998). Snake venom derived C-type lectins also bind to several integrins, von Willebrand Factor, Thrombin and some unidentified platelet surface receptors have been reported.

Therapeutic Use of Cytotoxins

The cytotoxic agents of snake venoms are as useful to the snake as to the modern medicine. However, till date none of the snake venom toxins could be used as medicine. Although, some of the toxins have been tried, used for trials and then withdrawn before releasing in the market due to some reason or other. The specificity of actions of these toxins may be exploited to treat many life threatening diseases. The anti-cancer potential of snake venom has been described by many studies. Cancer is still a major challenge to our society and anti-cancer therapy is one of the important areas of modern biological research. Therefore, exploring the use of proteins, peptides and enzymes originating from animals of different species for therapeutic purpose are of recent interest. Reports suggest that some of these proteins or peptides from snake venoms may bind specifically to cancer cell membranes, affecting the migration and proliferation of these cells. This biological property may be helpful in limiting metastasis. It has been observed that some cancer cells are more sensitive to the toxic effects of these agents than are normal cells. Certain cytotoxins purified from the venoms of a few cobra species penetrate smoothly into the A549 human lung adenocarcinoma cells and also accumulate in the lysosomes. However, the mechanism of their entry into the cytosol is still debatable, although it is thought that the disruptive action of these toxins on membrane phospholipids is the most plausible mechanism. On the other hand, cytotoxins can completely disturb ATP dependent metabolic processes in the cell. They have also been observed to enter nucleus and produce apoptotic effects through binding with DNA. It has been reported that whole Russell's viper venom treatment reduced the expression of HSPs in A549 cells in vitro (Pathan et al. 2015). HSPs constitute the anti-apoptotic system in living cells and cancerous cells express a high level of HSPs to avoid the apoptotic pathway. Therefore, the toxins involved in reduction of HSP expression in cancerous cells perhaps hold a key to kill the cancer cells.

Conclusion and Future Directions

Cytotoxicity including myonecrosis is mostly prevalent in viperid and elapid snake envenomations. Researchers for good reasons have studied the responsible toxins in isolation. However, recently Herera et al. (2016) argued that sometimes we might miss the holistic effects of the venoms in relation to muscle tissue damage, if the toxins are studied in isolation. They made extensive studies on *Bothrops asper* venom induced muscle damage through histological studies of the damaged muscle. Further, the team of researchers also included proteomic analyses of the damaged muscle exudates and detection of liberated extracellular matrix proteins. The authors claimed this study gives a clear understanding of the time course of muscle damaging activity of the *B. asper* venom and also possibly of other viperid venoms. The major outcome of this study is the identification of key bio-markers in muscle tissue damage and also that of the repair mechanism. Acquired knowledge from this study

will possibly help us design novel therapeutic measures against tissue damage caused by disease.

A huge diversity of shapes exists in animal cells ranging from columnar epithelial to highly complex and branched neuron. It is known by now that mammalian cells have hundreds of GTPases which are the mediators for various important aspect of cell biology. Rho gene was discovered in 1985, though the scientific knowledge about it started to pour out from different laboratories since 1992. The Rho family of guanine nucleotide (GTP)-binding proteins consist of three different groups, namely Rho, Rac and Cdc42 sub-families. They were originally found during search for proteins homologous to Ras proto-oncoprotein (Satoh et al. 1992). The proteins exist in two interconvertible forms: the GDP-bound inactive and GTP-bound active forms. Active GTPases interact with their specific downstream targets and perform their cellular functions, whereas GTP-hydrolysis and liberation of phosphate inactivate the GTPases. (Etienne-Manneville and Hall 2002). Small GTPases of the Rho family control cell growth, morphogenesis, cell motility, cytokinesis, trafficking and organization of cytoskeleton. These proteins contribute to multiple cellular processes like formation of lamellipodia (by activation of RAC), formation of stress fibres (due to the activation of RHO) and formation of filopodia (through CDC42 activation). They are also involved in oncogenesis (Jaffe and Hall 2005). It was shown that this activation could also affect cancer progression. High level of activated Rho is also responsible for the cytoskeletal alterations in epithelial cells. Recent attention is focused on the intracellular molecules involved in the enhancement of motility of cancer cells. Increased cell motility, manifested by the formation of membrane protrusions like filopodia, lamellipodia, and pseudopodia are key cellular attributes which facilitate tumor invasion and metastasis (Abraham et al. 2001). Rho proteins follow a regulatory mechanism common to all RasGTPases that depend on their ability to cycle between an active GTP bound state and an inactive GDP bound state (Boettner and Aelst, 2007).

Rho-like proteins have been reported to contribute to most steps of oncogenesis and progression including the acquisition of unlimited proliferation potential, survival and evasion from apoptosis, tissue invasion and the establishment of metastases (Aznar and Lacal 2001). Although initial discovery of Rho-like proteins as regulators of cytoskeleton dynamics implied that they are most likely to contribute to cancer cell migration and invasion, it is still not clear whether the function of Rho-like proteins is restricted to these events or they can affect tumor cells through modulation of gene transcription, cell division and survival, intracellular transport of signaling molecules or by altering the interaction of cancer cells with surrounding stromal cells. Therefore, the study of Rho family proteins and dissecting the mechanisms involved during oncogenesis is one of the important objectives for the scientific community throughout the world.

The major difference between Rho and RasGTPases (in case of cancer) is the absent of dominant point mutations that is responsible in the binding to GTP which results in its downstream signaling cascade. They also promote G1 to 'S' transition either as an independent molecule or as a partner of other pro-tumorigenic signaling molecules (Aznar et al. 2004). It is also very well known that all forms of cell

migration need a certain degree of actomyosin force which also affects tumorigenesis. Particularly, contractility of actomyosin is mainly driven by Rho signaling cascade and it is one of the major factors in controlling tumor dissemination (Sanz-Moreno and Marshall 2010). Li et al. (2006) had shown that stimulation of actomyosin contractility induced Rho/ROCK molecules in endothelial cells as happens in lung cancer. A most recently published review by Rodriguez-Hernandez et al. (2016) has not only pointed out towards the involvement of small GTPases like Rho, Cdc42 and ROCK molecules in actomyosin contractility and their further signaling events in colonization and metastasis but also using these molecules as possible drug targets Li et al. (2006).

Way back in 1988, Higashijima et al. reported a toxin, Mastoparan from wasp venom can mimic the receptors by regulating through the G Proteins. They had also shown that Mastoparan increases the GTPase activity at the rate of G proteins which are basically responsible for coupling of cell surface receptors to intracellular mediators. Available data also claims specific involvement of Pertussis toxins in catalyzing the ADP rybosylation of the α - subunit of several G protein coupled receptors and endorse their ability to hydrolyze GTP.

Study of some of the snake venom cytotoxins might reveal molecules capable of specifically affecting the above-discussed system of metastasis. This has been observed in case of Russell's viper venom at the authors' laboratory (Pathan et al. 2015). However, specific toxins responsible for this action has not been reported yet. It is not surprising to find many such toxins in other snake venoms also. Study of cytotoxins from snake venoms in this direction is expected to light up the future of cancer therapeutics.

Lectins have been utilized widely in histochemical tests to depict changes in tumor and malignant cell surface (Kumar et al. 2012). *BJcuL*, a lectin isolated from *Bothrops jararacussu* snake venom is involved in inhibiting tumor cell and endothelial cell growth progression (de Carvalho et al. 2001). Sarray et al., 2004 demonstrated that lebelectin, a novel C-type lectin isolated from *Macrovipera lebetina* venom, has anti-integrin activity. Lebelectin inhibited integrin-mediated attachment of various tumour cell lines to different adhesion substrata. This C-type lectin also completely blocked cell migration towards fibronectin in haptotaxis assays and prevented invasion of fibrin gels by tumour cells. Another interesting observation by Nunes et al. (2012) showed that, a galactoside-binding lectin isolated from *Bothrops leucurus* venom has cytotoxic activity on tumor cells and apoptosis in human myleoid leukemia cells. Further multi-disciplinary research is required to utilize the specific cytotoxic actions for therapeutics and solve the problems related to usability of venom toxins as medicines.

Cross-References

- ▶ Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes
- ▶ Cellular Mechanisms of Action of Snake Phospholipase A2 Toxins

- Snake Venom Disintegrins
- Toxinology of Snake Venoms: The Malaysian Context

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Part V

Snake Venom Effects on Blood and Lymph

Cobra Venom Factor: The Unique Component of Cobra Venom That Activates the Complement System

15

Carl-Wilhelm Vogel and David C. Fritzinger

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C.-W. Vogel (✉)

University of Hawaii Cancer Center, Honolulu, HI, USA

e-mail: cvogel@cc.hawaii.edu

D.C. Fritzinger

University of Hawaii Cancer Center, University of Hawaii at Manoa, Honolulu, HI, USA

e-mail: dfritzin@mac.com

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Abstract

Cobra venom factor (CVF) is an unusual venom component in the venom of many elapid snakes. Although CVF occurs in venom, it is not a toxin but a protein component of venom that facilitates the entry of the toxic venom components into the bloodstream by inducing complement activation at the site of envenomation. This manuscript reviews the structure of CVF and how it interacts with the complement system. The high degree of structural and functional homology of CVF to complement component C3 is described. The review also describes recombinant CVF. CVF has been used for over four decades as a research tool to deplete serum complement in animals in order to delineate the biological functions of the complement system and its role in the pathogenesis of many diseases. CVF has also been used as an experimental tool to target complement activation. Lastly, CVF has served as a lead substance for the generation of human C3 derivatives with CVF-like functions as an experimental therapeutic for complement depletion in diseases with complement pathogenesis (humanized CVF).

Keywords

Cobra venom factor • CVF • Complement depletion • Humanized cobra venom factor • C3 • Chimeric proteins

Introduction: Over 100 Years of Research on Cobra Venom Factor

Cobra venom factor (CVF) has been the object of scientific investigation for well over a century. The anticomplementary activity of cobra venom was first described in 1903 (Flexner and Noguchi 1903). At the time, complement was defined as the heat-labile, nonspecific component of serum which, together with the heat-stable, specific component of serum (the amboceptor, now known as antibody), was responsible for the hemolytic and bactericidal activities of serum. A decade later, the anaphylatoxin-generating activity of cobra venom was described (Friedberger et al. 1913). There followed a relative dormancy of CVF research until the 1960s when investigators purified the anaphylatoxin-generating activity (Vogt and Schmidt 1964) and the anticomplementary activity (Müller-Eberhard et al. 1966; Ballow and Cochrane 1969; Müller-Eberhard and Fjellström 1971) from cobra venom. Once it was shown that the anaphylatoxins are derived from complement proteins C3 and C5 (Dias da Silva et al. 1967; Dias da Silva and Lepow 1967; Cochrane and Müller-Eberhard 1968), it became apparent that the two activities of cobra venom were caused by the same protein, CVF. Subsequently, the mode of interaction of CVF with the

complement system became understood, and CVF was an important tool to unravel the biochemical reaction sequence of the alternative pathway of complement activation (Götze and Müller-Eberhard 1976). CVF activates complement, releasing the anaphylatoxins and eventually leading to complement depletion, thereby exhibiting its anticomplementary activity. CVF is therefore referred to as both the complement-activating protein and the complement-depleting protein in cobra venom.

The complement system is an intrinsic part of the immune system of all vertebrates, with important functions in both innate and adaptive immunity (Fujita 2002; Gros et al. 2008; Rother et al. 1998; Müller-Eberhard 1988; Walport 2001a, b). The classical pathway is activated by an antibody bound to an antigen, whereas both the alternative and lectin pathways are part of the innate immune system, and activation is spontaneous. The three activation pathways share a similar molecular architecture: an initial recognition event is amplified by a succession of proteolytic enzymes, the C3 and C5 convertases, leading to the formation of the membrane attack complex (MAC) which causes membrane damage and lysis. Multiple activation products exhibit important biological functions including opsonization (C3b), B-cell activation (C3d), and inflammation (anaphylatoxins C3a and C5a). Figure 1 shows a schematic representation of the three activation pathways of complement.

As described further below, complement depletion of laboratory animals by CVF has served for almost 50 years as an important tool to understand the biological functions of complement as well as its role in the pathogenesis of many diseases. In a recent review article describing the interaction of animal toxins with the

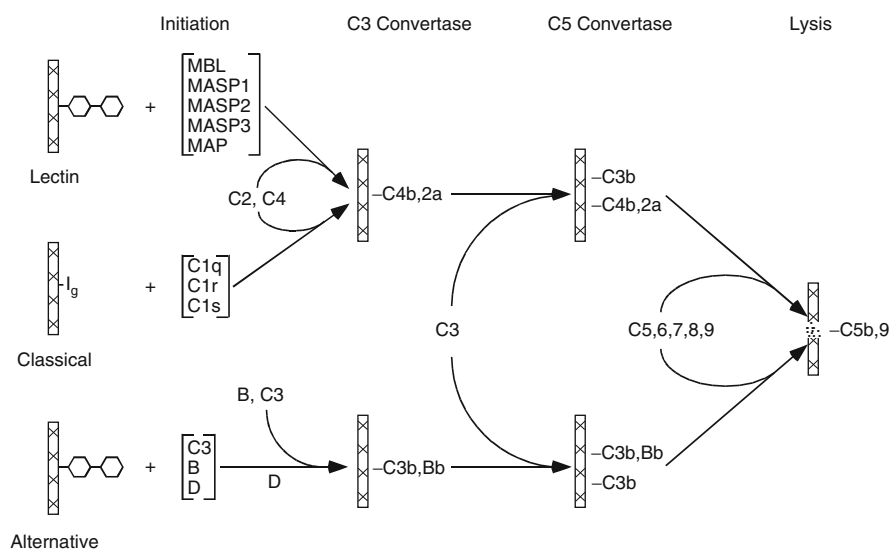


Fig. 1 Schematic representation of the three pathways of complement activation (lectin, classical, alternative), converging into the final pathway of membrane attack

complement system, it was stated that “undoubtedly, without CVF, the field of complement would not be as advanced as it is, and the role of complement in numerous diseases may never have been recognized and investigated” (Tambourgi and Van den Berg 2014).

The toxins found in venoms are highly potent pharmacological agents and represent potential lead substances for drug development. Although the enormous pool of natural toxins for drug development is largely untapped, several toxin-derived drugs are in clinical use (McCleary and Kini 2013). Here too, CVF has more recently served as a lead venom component for the development of an experimental therapeutic agent for complement depletion in diseases with complement pathology (humanized CVF), as described further below (Vogel et al. 2014; Vogel and Fritzing 2007).

The focus of this review will be on the biochemistry of CVF and its molecular interaction with the complement system and its structural and functional homology to the complement component C3. The survival benefit for the cobra of having CVF in its venom will be discussed, as well as the use of CVF as an experimental tool to deplete laboratory animals of complement or to target complement activation. Lastly, an overview of the efficacy of humanized CVF for therapeutic complement depletion in preclinical animal models of human diseases with complement pathology will be provided.

It should be noted that snake venoms, as well as the venoms of other poisonous animals, contain other venom components that interact with complement (Tambourgi and Van den Berg 2014). Two anticomplementary factors, including a high molecular weight inhibitor from *N. naja* (Ballow and Cochrane 1969) and a low molecular weight basic peptide from *N. haje* (von Zabern et al. 1981), have never been investigated beyond their original description, and attempts to confirm the presence of the former have failed (Vogel 1991).

Of significantly greater importance are venom proteases and in particular snake venom metalloproteases that act on complement components. Whereas proteases are relatively rare in elapid venoms (Iwanaga and Suzuki 1979; Markland and Swenson 2013), they are dominant components of viperid and crotalid venoms and known to be responsible for many toxic activities including hemorrhage, bleeding, intravascular clotting, edema, inflammation, and necrosis. Venom metalloproteases have been shown to cleave many serum proteins including fibrinogen, fibrin, and von Willebrand factor, as well as complement proteins such as C3, C4, and C5 (Tambourgi and Van den Berg 2014; Man and Minta 1977; Eggertsen et al. 1980; Tambourgi et al. 1994; Farsky et al. 2000; Pidde-Queiroz et al. 2010, 2013; Tanaka et al. 2012). The cleavage of complement components C3 and C5 by venom metalloproteases can generate the pro-inflammatory C3a and C5a anaphylatoxins, and metalloproteases are sometimes referred to as “complement activating” although the release of anaphylatoxins does not constitute true activation of the complement system. They are also referred to as complement inhibitors as the reduced concentration of activatable complement components interrupts the activation pathway in the case of subsequent complement activation. Whereas the release of anaphylatoxins is helpful to the venomous animal (see below), inhibition of complement, by metalloproteases or CVF, does not contribute to the toxic effects of the venom and likely constitutes an epiphenomenon. A detailed

review of the snake venom metalloproteases cleaving serum complement proteins is beyond the scope of this review.

Occurrence of CVF in Different Snake Species

CVF has so far been isolated only from a small number of species of the elapid family. Most authors purified CVF from the Indian cobra *Naja naja* or the monocellate or monocled cobra (*N. kaouthia*, formerly considered a subspecies (*N. n. kaouthia*) of the Indian cobra, just like all other Asiatic cobras; see Appendix for a discussion on the taxonomy changes) (Ballou and Cochrane 1969; Müller-Eberhard and Fjellström 1971; Vogel and Müller-Eberhard 1984; Eggertsen et al. 1981), the Chinese cobra (*N. atra*) (Takahashi and Hayashi 1982), the Egyptian cobra (*N. haje*) (von Zabern et al. 1980, 1982; Malih et al. 2014), the white-lipped or forest cobra (*N. melanoleuca*) (Osipov et al. 2005), the king cobra (*Ophiophagus hannah*) (Zeng et al. 2012), and the lowland copperhead (*Austrelaps superbus*) (Rehana and Kini 2007). Based on immunological cross-reactivity, pattern of complement consumption, or proteomic analysis, CVF is also present in the Cape or yellow cobra (*N. nivea*), the black-necked or spitting cobra (*N. nigricollis*), the equatorial spitting cobra (*N. sumatrana*), and the ringhals cobra (*Hemachatus haemachatus*) (Birdsey et al. 1971; Warrell et al. 1976; Eggertsen et al. 1981; Yap et al. 2014). It is probably safe to assume that CVF is present in the venom of all *Naja* species. CVF is not present in other elapid venoms (coral snakes (*Micrurus* sp.), kraits (*Bungarus* sp.), and mambas (*Dendroaspis* sp.)) or in the venoms of the viperid and crotalid families (Eggertsen et al. 1980; Tanaka et al. 2012; Tambourgi and Van den Berg 2014).

The CVF proteins isolated from the different species exhibit very similar structures and activities, although there are some differences in glycosylation and C5-cleaving activity (see below). Complete sequence information is available for *N. kaouthia* (Fritzinger et al. 1994), *A. superbus* (Rehana and Kini 2007), and *O. hannah* (Zeng et al. 2012).

In several species, CVF isoforms have been found, including *N. kaouthia* (Fritzinger et al. 1991, 1992a; Bammert 2002; Bammert et al. 2002a), *A. superbus* (Rehana and Kini 2007), *N. melanoleuca* (Osipov et al. 2005), and *O. hannah* (Vonk et al. 2013).

Most *Naja* species are protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (www.cites.org).

Structure and Function of CVF

Protein Structure of CVF

As outlined above, CVF has now been isolated from a number of elapid snakes. The overall structural characteristics of CVF from different elapid species are highly similar. The vast majority of biochemical work has been reported for CVF from the

Table 1 Physicochemical parameters of CVF^a

Molecular mass	
(Svedberg equation)	135,000 Da
(Equilibrium sedimentation)	136,000 Da
(Protein sequence, sum of three chains)	141,464 Da
(SDS-PAGE, sum of three chains)	149,000 Da
(Gel filtration, undenatured)	160,000 Da
Sedimentation coefficient	
(Analytical ultracentrifugation)	6.8 S
(Sucrose density gradient centrifugation)	6.7 S
Diffusion coefficient	
(Gel filtration)	$4.56 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$
(Stokes-Einstein law)	$4.51 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$
Stokes' radius	
(Gel filtration)	47.3 Å
(Stokes' law)	47.5 Å
Stokes' volume	$4.43 \times 10^{-19} \text{ cm}^3$
Partial specific volume	$0.73 \text{ cm}^3 \text{ g}^{-1}$
Frictional coefficient	$8.97 \times 10^{-8} \text{ g s}^{-1}$
Frictional ratio	1.4
Dimensions	
(Electron microscopy)	$137 \text{ Å} \times 82 \text{ Å}$
(Crystallography)	$150 \text{ Å} \times 76 \text{ Å} \times 66 \text{ Å}$
Molar extinction coefficient	$1.35 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$
Isoelectric point	pH 5.5 – pH 6.5
Electrophoretic mobility	$2.82 \times 10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$

^aData are from Vogel and Müller-Eberhard (1984), Vogel et al. (1996), and Krishnan et al. (2009)

Indian cobra *N. naja* or the closely related species *N. kaouthia* (Vogel and Müller-Eberhard 1984; Eggertsen et al. 1981; Fritzinger et al. 1994). CVF is a three-chain protein with a molecular mass of approximately 150,000 Da (Table 1), consisting of an α -chain (68,500 Da), a β -chain (48,500 Da), and a γ -chain (32,000 Da) (Figs. 2 and 3, Table 2). Multiple authors reported size heterogeneity of the γ -chain (Vogel and Müller-Eberhard 1984; Pepys et al. 1979; Eggertsen et al. 1981; Osipov et al. 2005; von Zabern et al. 1982; Rehana and Kini 2007). As the N-terminal amino acid sequence of the CVF γ -chain was found to be homologous to the N-terminus of the C3 α' -chain (Figs. 2 and 3) (Eggertsen et al. 1981), the size heterogeneity of the CVF γ -chain must be due to differential proteolytic processing at its C-terminus. The size heterogeneity of the γ -chain is likely the cause for the presence of multiple bands on isoelectrofocusing. Table 1 summarizes the physicochemical parameters of CVF.

CVF is a somewhat elongated molecule with a frictional ratio of 1.4, consistent with a somewhat irregularly ellipsoidal ultrastructure as shown by high-resolution transmission electron microscopy (Fig. 4) (Smith et al. 1982; Vogel et al. 1984;

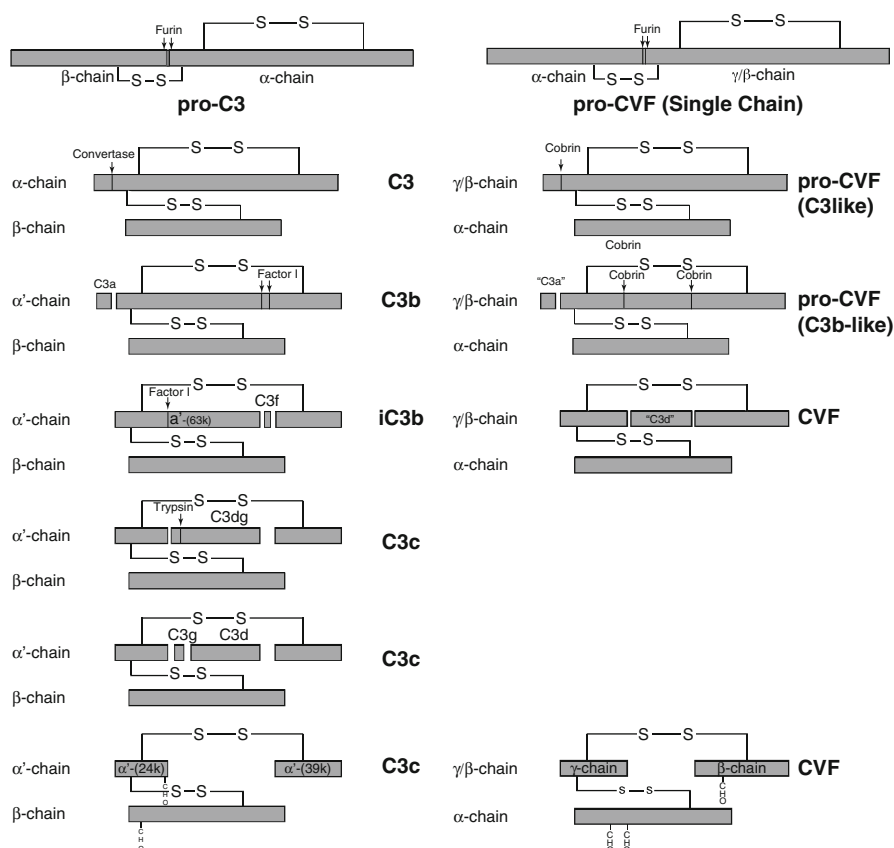


Fig. 2 Schematic representation of the chain structures of complement component C3 and its physiological degradation products (*left panel*) and CVF (*right panel*). Homologous forms of C3 and CVF are juxtaposed. The proteolytic cleavage sites and the two interchain disulfide bonds are shown. The locations of the two glycosylation sites of human C3 and the three glycosylation sites of CVF are also shown (*bottom drawings only*)

Janssen et al. 2009). More recently, the three-dimensional structure of CVF, both free and in complex with factor B, was solved by x-ray crystallography (Fig. 5) (Janssen et al. 2009; Krishnan et al. 2009). Mature three-chain CVF consists of eleven domains: eight macroglobulin domains, a linker domain, the CUB domain which is functionally important for convertase formation (Janssen et al. 2009; Vogel and Fritzinger 2010), and the C345C domain at the C-terminus of the CVF β-chain which binds to the Bb fragment and is important for convertase stability (Janssen et al. 2009; Hew et al. 2012; Fritzinger et al. 2009). Figure 3 shows the location of the domain borders within the amino acid sequence of CVF and, by extension, of pro-CVF. Table 3 summarizes the domain data.

Molecular cloning of CVF revealed that it is synthesized as a single-chain preproprotein. The CVF mRNA is greater than 5,950 nucleotides in length

C3 β-chain, MG1			
hC3	1	SPMYSIIITPNILRLESEETMVLEAHDAQGDVPVTVTVHDFPGKKLVLSSE	50
CVF	1	ALYTLITPAVLRTDTEEQILVEAHGDSTPKQLDIFVHDFPRKQKTLFQT	49
CVF α-chain, MG1			
hC3	51	KTVLTPATNHMGNTVFTIPANREFKSEKGRNKFVTVQATFGTQVVEKVVL	100
CVF	50	RVDMNPAGGMLVTPTIEIPA-KEVSTDSRQNYVVVQVTGPQVRLEKVVL	98
MG2			
hC3	101	VSLOSGLFIQTDKTIYTPGSTVLYRIFTVNHKLLPVGRVTMVNIENPEG	150
CVF	99	LSYQSSFLFIQTDKGIYTPGSPVLYRVFSMDHNTSKMNTKTVIVEFQTPEG	148
MG2			
hC3	151	IPVKQDSLSSQNQLGVLPPLSWDIPELVNMGQWKIRAYYENSPQQVFSTEF	200
CVF	149	ILVSSNSVDLN-----FFWPYNLPDLVSLGTWRIVAKYEHSPENYATAYF	192
MG3			
hC3	201	EVKEYVLPSEFEVIVEPETEKFYYIYNEKLEVTITARFLYGGKVEGTAFVI	250
CVF	193	DVRKYVLPSEFEVRLQPSKFFYIDGNENFHVSIITARYLYGEEVEGVAFVL	242
MG3			
hC3	251	FGIQDGEQRISLPESLKRIPIEDGSGEVVLSRKVLLDGVNLRADLVGK	300
CVF	243	FGVKIDDAKKSIPDSLTRIPIIDGDGKATLKRDTFRSRFPNLN--ELVGH	290
MG4			
hC3	301	SLYVSATVILHSGSDMVQAERSGIPIVTSPYQIHFTKTPKYFKPGMPFDL	350
CVF	291	TLYASVTVMTESGSDMVVTEQSGIHIVASPYQIHFTKTPKYFKPGMPYEL	340
MG4			
hC3	351	MVFVTNPDGSPAYRVPVAVQGEDTVQSLTQGDGVAKLSINTHPSQKPLSI	400
CVF	341	TVYVTNPDGSPAAPHVPVSEAFHSMG-TTSLSDGTAKLILNIPLNAQSLPI	389
MG5			
hC3	401	TVRTKKQELSEAEQATRTMQALPYSTVGNSNNYLHLSVLRTELRPGETLN	450
CVF	390	TVRTNHGDLPRERQATKSMTAIAYQTQGGSGNYLHVAITSTEIKPGDNLP	439
MG5			
hC3	451	VNFLLRMDRAHEAKIRYYTYLIMNKGRLKAGRQVREPQDGLVVLPLSIT	500
CVF	440	VNFNVKGNANSLKQIKYFTYLI LNKGKIFKVGRQPRRDGQNLVTMNLHIT	489
MG6^β			
hC3	501	TDFIPSFRLVAYYTLIGASGQREVVADSVWVDVFDSCVGLSVVKSGQSED	550
CVF	490	PDLIPSFRLFVAYY-----QVGNNEIVADSVWVDVKDTCMGTLVVKG---DN	532
MG6^β			
LNK			
hC3	551	RQPVPGQQMTLKIEGDHGARVVVLVAVDKGVFVLNKKNKLTQSKIWDVVEK	600
CVF	533	LIQMPGAAMKIKLEGDPGARVGLVAVDKAVYVVLNDKYKISQAKIWDTIEK	582
LNK			

Fig. 3 (continued)

hC3	601	ADIGCTPGSGKDYAGVFS DAGLTFTSSSGQQT AQRAELQCPQPAARRRR	649
CVF	583	SDFGCTAGSGQNNLGVFEDAGLALTTSTNLNTKQRSAAKCPQPANRRRRS	632
		4 Arg	
		4 Arg	
hC3	650	SVQLTEKRMDKVGKYP-KELRKCCEDGMRENPMRFSCQRRTRFISLGEAC	698
CVF	633	SVLLLDSTASKAAEFQDQDLRKCCEDVMHENPMGYTCEKRAKYIQEGDAC	682
		"C3a", ANA	
hC3	699	KKVFLDCCNYITELRRQHARASHLGLAF SNLDEDIIEENIVSRSEFPES	748
CVF	683	KAAFLECCRYIKGVRDENQRESEFLAFDDNEDGFIADSDIISRSDFPKS	732
		α' -NT, C3 α' -chain	
		α' -NT, CVF γ -chain	
hC3	749	WLWNVEDLKEPPKN-GISTKLMNIFLKDSITWEILAVSMSDKKGICVAD	797
CVF	733	WLWLTKDLTEEPNSQGISSKTMSFYLRDSITTWVVLAVSFTPTKGICVAE	782
		MG6 α	
hC3	798	PFEVTVMQFFIDLRLPYSVVRNEQVEIRAVLYNYRQNELKVRVELLHN	847
CVF	783	PYEIRVMKVFIDLQMPYSVVKNEQVEIRAILHNY-VNEDIYVRVELLYN	831
		MG7	
hC3	848	PAFCSLATTKRRHQQTVTIPPKSSLSVPYVIVPLKTGLQEVEVKAAYVHH	897
CVF	832	PAFCSASTKGQRYRQQFPIKALSSRAVPFVIVPLEQGLHDVEIKASVQEA	881
hC3	898	FISDGVRKSLKVVPEGIRMNKTVAVRTLDPERLGRGEGVOKEDIPPADLSD	947
CVF	882	LWSDGVRKSLKVVPEGVOK-SIVTIVKLDPRAGVGGTQLEVIKARKLDD	930
		CUB β	
		C3g	
hC3	948	QVPDTESETRILLOQTPVAQMTEDAVDAERLKLHLIVTPSGCGEQNMIGMT	997
CVF	931	RVPDTEIETKIIIQGDPVAQIIENSIDGSKLNHLIITPSGCGEQNMIRMA	980
		TED	
		C3d	
		* *	
		"C3d"	
hC3	998	PTVIADVHYLDETEQWEKFGLEKRQGALELIKGYTQQLAFROPSSAFAAF	1047
CVF	981	APVIATYYLDTTEQWETLGINRRTEAVNQIVTGAAQQMVKADHSYAAF	1030
hC3	1048	VKRAPSTWLTAYVVKVFS LAVNLIA-IDSQVLCGAVKWLILEKQKPDGVF	1096
CVF	1031	TNRASSSWLTAYVVKVFAMAAMVAGISHEIICGGVRWLILNRQQPDGAF	1080
hC3	1097	QEDAPVIHQEMIGGLRNNNEKDMALTAFVLISLQEAKDICEEQVNSLPGS	1146
CVF	1081	KENAPVLSGTMQGGIQG-AEEFVYLTAFILVALLESKTI CNDYVNSLDSS	1129
hC3	1147	ITKAGDFLEANYMNLQRSYTVAIAGYALAQMGRLKGPLLNKFLT TAKDKN	1196
CVF	1130	IKKATNYLLKKYEKLQRPYTTALTAYALAAADQLND--DRVLMMASTGRD	1177

Fig. 3 (continued)

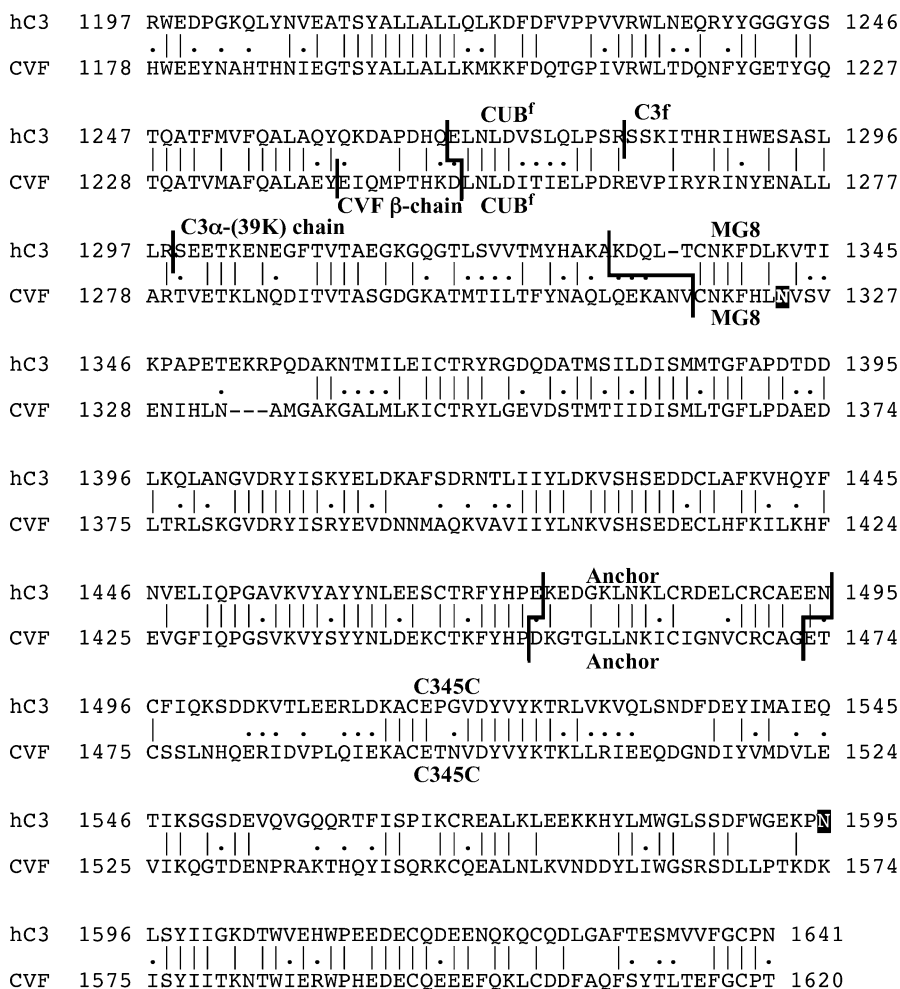


Fig. 3 Protein sequences of CVF and human C3. Shown is a sequence comparison of human pro-C3 and pro-CVF. Identical amino acid residues (I) and conservative replacements (*) are indicated; nonconservative changes have no symbol. The amino acid residue numbering is according to the sequences of the two pro-proteins (without signal sequence) (Fritzinger et al. 1994, 1995; de Bruijn and Fey 1985). The locations of the N-termini of all chains and chain fragments as well as of all domains as determined by x-ray crystallography (Janssen et al. 2005, 2006, 2009; Krishnan et al. 2009) are shown. All asparagine residues at potential glycosylation sites are highlighted. The cysteine and glutamine residues involved in the formation of the intramolecular thioester are indicated (*)

(Fig. 6, Table 4) (Fritzinger et al. 1994, 1995). It has an open reading frame that encodes for 1,642 amino acid residues of pre-pro-CVF, consisting of a 22-residue signal sequence, the 627-residue α -chain, four arginine residues, and the 989-residue γ/β -precursor chain from which the mature γ -chain (252 residues) and β -chain (379 residues) of CVF are derived (Table 2) (Fig. 3). The γ/β -precursor chain also

Table 2 CVF chains and fragments

CVF chains	Amino acid residues	No. of amino acid residues	Protein molecular mass ^a
Pre-pro-CVF	–22 to –1 and 1–1,620	1,642	184,516 Da
Pro-CVF (single chain)	1–1,620	1,620	182,200 Da
CVF α -chain	1–627	627	69,506 Da
CVF γ/β -precursor chain	632–1,620	989	112,087 Da
CVF β -chain	1,242–1,620	379	43,573 Da
CVF γ -chain	711–962 ^b	252	28,385 Da
“C3a” ^c	632–710	79	9,070 Da
“C3d” ^c	963–1,241	279	31,112 Da

^aCalculated from the amino acid sequence, without taking glycosylation into consideration

^bThe CVF γ -chain exhibits size heterogeneity at its C-terminus

^cThe “C3a” and “C3d” domains are only part of the pro-CVF molecule. They are not present in the mature three-chain CVF protein

contains the C3a-like region (79 residues) and C3d-like region (279 residues) which are not present in mature CVF. The CVF mRNA has a 5'-untranslated region of at least three nucleotides, a 3'-untranslated region of 1,001 nucleotides, and a poly-A tale of at least 20 nucleotides (Fritzinger et al. 1994, 1995; Vogel et al. 1996; Bammert 2002).

The posttranslational processing of the pre-pro-CVF protein in the venom gland involves the removal of the signal sequence and the four arginine residues between the α -chain and the γ/β -precursor chain, presumably by a furin-type protease. The mature γ - and β -chains are derived from the γ/β -precursor chain by the removal of the C3a-like domain and a domain roughly corresponding to the C3d domain of C3 (Figs. 2 and 3). The C3a-like domain is entirely homologous to the C3a anaphylatoxin of C3. The C3d-like domain of pro-CVF is 29 amino acids (2,675 Da) shorter than the C3d fragment of C3 (Figs. 2 and 3). The CVF γ -chain includes the homologous C3g fragment of C3 at its C-terminus, and the N-terminus of the CVF C3d-like domain corresponds to the N-terminus of the C3d fragment of C3 (Fig. 3). However, the abovementioned size heterogeneity of the CVF γ -chain implies that in some instances the N-terminus of the removed C3d-like domain of CVF may either lie within the C-terminal portion of C3g or the N-terminal portion of C3d. The N-terminus of the CVF β -chain includes the C-terminal portion of the C3d fragment and the entire C3f fragment (Fig. 3).

The proteases involved in the processing of the γ/β -precursor chain of pro-CVF in the venom glands are not known. However, in 1984, a metalloprotease, named cobrin, was identified, purified, and subsequently cloned from cobra venom that was able to cleave human C3, generating a novel cleavage product, named C3o (Table 4) (O'Keefe et al. 1984, 1988; Petrella et al. 1991; Bambai 1998; Bambai et al. 1998). Cobrin cleaves the human C3 α -chain between E736/E737,

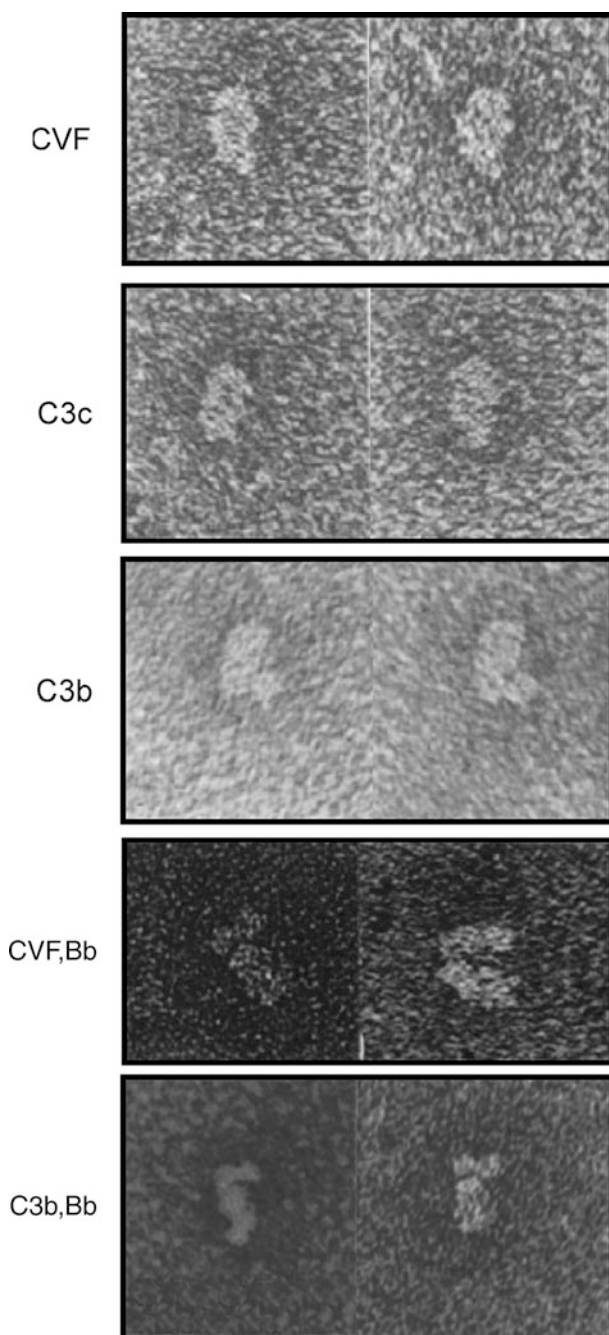


Fig. 4 Electron microscopic images of CVF, C3c, and C3b and of the CVF,Bb and C3b,Bb bimolecular enzymes. Please note the presence of the TED domain causing the embryo-like structure of C3b. Please also note the two globular domains (SP and vWA domains) of Bb attached to C3b or CVF (at the C345c domain) opposite from the TED domain (Smith et al. 1982, 1984; Vogel et al. 1984)

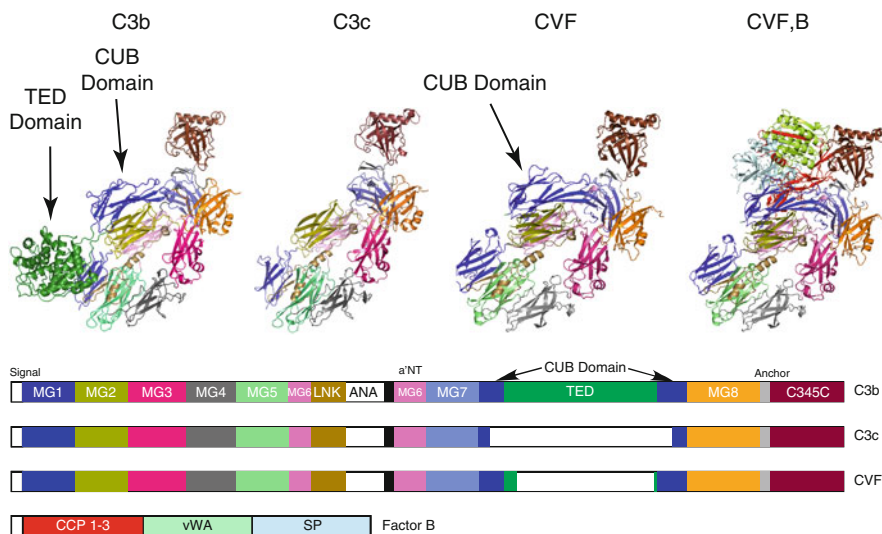


Fig. 5 Structures of C3b, C3c, CVF, and CVF,B. The *upper panel* shows the three-dimensional domain structures of CVF (2.2 Å), C3b (4.0 Å), C3c (2.4 Å), and CVF,B (2.2 Å) as determined by x-ray crystallography (Janssen et al. 2005, 2006, 2009). The *lower panel* shows the schematic domain structures of CVF and C3. Please note the absence of a complete CUB domain in C3c compared to CVF and C3b. Only C3b has the TED domain. The three domains of factor B are the complement control protein domains (CCP1-3), the von Willebrand domain (vWA), and the serine protease domain (SP) (Milder et al. 2007; Ponnuraj et al. 2004). For the description of the domains of CVF and C3, please refer to Table 3. Please note that the glycosylation sites of CVF (in the MG2 and MG8 domains) and human C3 (in the MG1 domain and near the N-terminal end of the CUB^g domain) are distant from Factor B

Q967/M968, and K1331/D1354 (O’Keefe et al. 1988). Accordingly, C3o has a three-chain structure resembling both CVF and C3c. In contrast to C3c, C3o exhibits the CVF functional properties of being able to support factor B activation and convertase formation (O’Keefe et al. 1987, 1988). This functional property is consistent with the fact that C3o contains the entire functionally important CUB domain, whereas the C3c α' -(24 K) chain lacks over half of the CUB^g domain at its C-terminus (Fig. 3, Tables 3 and 6). Cobrin exhibits a high specificity for C3 as it cleaves C3 even in whole human or guinea pig serum, causing complement-dependent hemolysis (O’Keefe et al. 1989). Recombinantly expressed cobrin cleaves both human C3 and pro-CVF (Bambai 1998). It has been suggested that cobrin may be the enzyme in the venom gland responsible for the posttranslational processing of the γ/β -precursor chain of pro-CVF, generating the γ - and β -chains of the mature three-chain CVF (O’Keefe et al. 1988; Vogel and Fritzinger 2010).

Table 5 shows the amino acid compositions of pre-pro-CVF, pro-CVF, mature CVF, and the three chains of mature CVF as derived from the cDNA which correlate well with the amino acid compositions derived by earlier biochemical analysis (Vogel and Müller-Eberhard 1984; Eggertsen et al. 1981). The GC composition of the open reading frame for pre-pro-CVF is 43.5 % (Vogel et al. 1996). This is very

Table 3 Domain structures of human C3 and CVF

Domain ^a	Human C3 residues ^b	No. of AA res.	CVF residues ^c	No. of AA res.
Signal sequence	–22 to –1	22	–22 to –1	22
MG1	1–104	104	1–101	101
MG2	105–209	105	102–198	97
MG3	210–328	119	199–318	120
MG4	329–426	98	319–417	99
MG5	427–534	108	418–521	104
MG6 ^β	535–577	43	522–560	39
LNK	578–645	68	561–627 ^d	67
4 Arg	646–649 ^e	4	628–631 ^f	4
ANA	650–726	77	632–710 ^f	79
α'NT	727–745	19	711–728 ^g	18
MG6 ^α	746–806	61	729–790	62
MG7	807–911	105	791–896	106
CUB ^g	912–962	51	897–946	50
TED	963–1,268	306	947–1,250 ^h	304
CUB ^f	1,269–1,330	62	1,251–1,317	67
MG8	1,331–1,474	144	1,318–1,452	135
Anchor	1,475–1,495	21	1,453–1,472	20
C345C	1,496–1,641	146	1,473–1,620 ⁱ	148
Total residues (with signal sequence)		1,663		1,642
Total residues (without signal sequence)		1,641		1,620

^aNomenclature of domains. MG, macroglobulin domain (analogous to immunoglobulin domain) (Janssen et al. 2005); LNK, linker domain (Janssen et al. 2005); ANA, anaphylatoxin domain (Janssen et al. 2005); α'NT, C3 α'-chain N-terminal domain (Janssen et al. 2005); CUB, complement C1r/C1s, Uegf, Bmp1 domain (Janssen et al. 2005, 2009; Feinberg et al. 2003; Bork and Beckmann 1993); TED, thioester-containing domain (Janssen et al. 2005); C345C, C-terminal domain of C3, C4, and C5 (Janssen et al. 2005; Bramham et al. 2005). MG6^β and MG6^α refer to the two noncontiguous portions of the MG6 domain in the C3 β- and α-chains, respectively (Janssen et al. 2005). CUB^g and CUB^f refer to the noncontiguous portions of the CUB domain in the C3 α-chain, with the CUB^g including 64 % of C3g at its C-terminus (residues 933–962) and CUB^f including all of C3f (residues 1,282–1,298) (Janssen et al. 2005).

^bDomain assignment for human C3 is according to Janssen et al. (2005)

^cDomain assignment for CVF is according to Krishnan et al. (2009)

^dIncludes residues 625–627 not assigned to the CVF LNK domain by Krishnan et al. (2009)

^eThe four arginine residues (646–649) are not present in mature two-chain C3

^fThe four arginine residues (628–631) and the ANA domain (632–710) are not present in mature three-chain CVF

^gIncludes residues 711–716 not assigned to the CVF α'NT domain by Krishnan et al. (2009)

^hMature three-chain CVF lacks the TED domain. Only TED domain residues 947–962 (representing the C-terminus of the γ-chain) and residues 1,242–1,250 (representing the N-terminus of the β-chain) are present in mature CVF

ⁱIncludes residues 1,619–1,620 not assigned to the CVF C345C domain by Krishnan et al. (2009)

ACCATGGAGAGGATGGCTCTCTATCTGGTGGCTGCTCTATTGATTGGTTTTCCAGGGTCT	60
M E R M A L Y L V A A L L I G F P G S	-4
5'-UTR Begin signal sequence	
Exon 1 Exon 2	
TCTCATGGGGCTCTCTACACCCTCATCACCCCTGCTGTTTTGCGAACAGACACAGAAGAG	120
S H G A I Y T L I T P A V L R T D T E E	17
End signal sequence Begin α-chain	
CAAATTTTGGTGAGGCCCATGGAGACAGTACTCCAAAACAGCTTGACATCTTTGTTTCAT	180
Q I L V E A H G D S T P K Q L D I F V H	37
GATTTTCCACGGAAGCAGAAAACCTTGTTCCAAACCAGAGTAGATATGAATCCAGCAGGA	240
D F P R K Q K T L F Q T R V D M N P A G	57
Exon 3	
GGCATGCTTGTCACTCCAACATATAGAGATTCCAGCAAAAGAAGTGAGTACGGACTCCAGG	300
G M L V T P T I E I P A K E V S T D S R	77
CAAATCAATATGTGGTTGTGCAAGTAACTGGTCTCAAGTGAGATTGGAAAAGGTGGTT	360
Q N Q Y V V V Q V T G P Q V R L E K V V	97
CTCCTTTCTTACCAGAGTAGCTTTCTGTTTATCCAGACAGATAAAGGCATCTATACACCA	420
L L S Y Q S S F L F I Q T D K G I Y T P	117
Exon 4	
GGGTCTCCAGTACTCTATCGTGTTTTTCTATGGATCACAACACAAGCAAGATGAACAAA	480
G S P V L Y R V F S M D H N T S K M N K	137
Exon 5	
ACTGTGATTGTTGAGTTTCAGTCTCCAGAAGGCATTCTTGTCACTTCTAATTCAGTTGAC	540
T V I V E F Q T P E G I L V S S N S V D	157
Exon 6	
CTAACTTCTTCTGGCCTTACAATTTACCAGACCTTGTCACTTTGGGGACTTGGAGGATT	600
L N F F W P Y N L P D L V S L G T W R I	177
GTGGCCAAATATGAACATTCCTCCAGAGAATTATACTGCATATTTTGATGTCAGGAATAT	660
V A K Y E H S P E N Y T A Y F D V R K Y	197
Exon 7	
GTGTTGCCAAGCTTTGAAGTCCGTCTGCAACCATCAGAGAAGTTTTTTTACATTGACGGC	720
V L P S F E V R L Q P S E K F F Y I D G	217
Exon 8	
AATGAAAATTTCCACGTGTCTATCACTGCAAGGTACTTGTATGGAGAGGAAGTGGAAGGT	780
N E N F H V S I T A R Y L Y G E E V E G	237
GTGGCCTTTGTCTCTTTGGAGTGAAAATAGATGATGCTAAAAAGAGTATTCAGACTCA	840
V A F V L F G V K I D D A K K S I P D S	257
Exon 9	
CTCACGAGAATTCCGATTATTGATGGAGATGGGAAAGCAACACTAAAAAGAGATACATTC	900
L T R I P I I D G D G K A T L K R D T F	277
CGTTCTCGATTTCCAAATCTCAATGAGCTTGTTGGGCATACTCTGTATGCATCTGTAACA	960
R S R F P N L N E L V G H T L Y A S V T	297
Exon 10	
GTCATGACAGAATCAGGCAGTGATATGGTAGTGACTGAGCAAAGCGGCATTTCATATTGTG	1020
V M T E S G S D M V V T E Q S G I H I V	317
GCATCTCCCTATCAGATCCACTTCACAAAAACCCCAATATTTCAAGCCAGGAATGCCA	1080
A S P Y Q I H F T K T P K Y F K P G M P	337

Fig. 6 (continued)

Exon 11

TATGAACTGACGGTGTATGTTACCAACCCTGATGGCTCACCAGCTGCCCATGTGCCAGTG 1140
 Y E L T V Y V T N P D G S P A A H V P V 357

GTATCAGAGGCCTTTTCATCTATGGGAACCACCTTGAGTGATGGGACTGCTAAGCTCATC 1200
 V S E A F H S M G T T L S D G T A K L I 377

Exon 12

CTGAACATACCATTGAATGCTCAAAGCCTACCAATCACTGTTAGAACTAACCATGGAGAC 1260
 L N I P L N A Q S L P I T V R T N H G D 397

CTCCCAAGAGAACGCCAGGCAACAAAGTCCATGACAGCCATAGCCTACCAAACCCAGGGA 1320
 L P R E R Q A T K S M T A I A Y Q T Q G 417

GGATCTGGAACTATCTTCATGTAGCCATTACATCTACAGAGATTAAGCCCGGAGATAAC 1380
 G S G N Y L H V A I T S T E I K P G D N 437

TTACCTGTCAATTTCAATGTGAAGGGCAATGCAAATTCCTGAAGCAGATCAAATATTTTC 1440
 L P V N F N V K G N A N S L K Q I K Y F 457

Exon 13

ACATACCTCATATTGAATAAAGGGAAGATTTTCAAGGTTGGCAGGCAACCCAGGAGAGAT 1500
 T Y L I L N K G K I F K V G R Q P R R D 477

GGGCAGAATCTGGTGACCATGAATCTGCATATCACTCCAGATCTCATCCCTTCCTTCCGG 1560
 G Q N L V T M N L H I T P D L I P S F R 497

TTTGTGGCTTACTACCAAGTGGGAACAACGAAATTGTGGCTGATTCTGTCTGGGTGGAT 1620
 F V A Y Y Q V G N N E I V A D S V W V D 517

Exon 14

GTGAAGGATACCTGCATGGGAACCTTGTTGTGAAAGGAGACAATCTAATACAAATGCCA 1680
 V K D T C M G T L V V K G D N L I Q M P 537

GGAGCTGCAATGAAAATCAAATTGGAAGGGGATCCAGGTGCTCGGGTGGTCTTGTGGCT 1740
 G A A M K I K L E G D P G A R V G L V A 557

Exon 15

GTGGACAAAGCAGTATATGTTCTCAATGATAAATATAAGATTAGCCAAGCTAAGATATGG 1800
 V D K A V Y V L N D K Y K I S Q A K I W 577

GACACAATAGAAAAGAGTGACTTTGGCTGTACAGCTGGCAGTGGCCAGAATAATCTGGGT 1860
 D T I E K S D F G C T A G S G Q N N L G 597

GTGTTTGAAGATGCTGGACTGGCTCTGACAACCAGCACTAATCTCAACACCAAACAGAGA 1920
 V F E D A G L A L T T S T N L N T K Q R 617

Exon 16

TCAGCTGCAAAGTGTCCTCAGCCTGCAAAATCGGAGGCGTCGCAAGTTCTGTTTTGCTGCTT 1980
 S A A K C P Q P A N R R R R S S V L L L 637

End α -chain
Exon 17
Begin "C3a"

GACAGCAACGCAAGCAAACCGGCAGAATTTTCAGGATCAAGACCTGCGTAAATGCTGTGAA 2040
 D S N A S K A A E F Q D Q D L R K C C E 657

GATGTCATGCATGAGAACCCCATGGGGTACACTTGTGAAAAGCGTGCAAAATACATCCAG 2100
 D V M H E N P M G Y T C E K R A K Y I Q 677

Fig. 6 (continued)

GAGGGAGATGCTTGTAAGGCTGCCTTCCTTGAATGCTGCTCGCTACATCAAGGGGGTCCGA 2160
 E G D A C K A A F L E C C R Y I K G V R 697
 GATGAAAACCAACGGGAGAGCGAGTTGTTTCTGGCAAGAGATGATAATGAAGATGGTTTC 2220
 D E N Q R E S E L F L A R D D N E D G F 717
 End "C3a" | Begin γ -chain
 ATAGCAGATAGTGATATCATCTCAAGGTCTGATTTCCCCAAGAGTTGGTGTGGCTAACAA 2280
 I A D S D I I S R S D F P K S W L W L T 737
 AAGGACTTGACCGAGGAGCCTAACAGTCAAGGATTTCAAGCAAGACAATGTCTTTTTTAT 2340
 K D L T E E P N S Q G I S S K T M S F Y 757
 CTGAGGGATTCCATCACAACTGGGTGGTGTGGCTGTAAGCTTTACACCCACCAAGGG 2400
 L R D S I T T W V V L A V S F T P T K G 777
 ATCTGTGTGGCTGAACCTTATGAAATAAGAGTCATGAAAGTCTTCTTCATTGATCTTCAA 2460
 I C V A E P Y E I R V M K V F F I D L Q 797
 ATGCCATATTCAGTAGTGAAGAATGAGCAGGTGGAGATTCGAGCTATTCTGCACAACCTAC 2520
 M P Y S V V K N E Q V E I R A I L H N Y 817
 GTTAACGAGGATATTTATGTGCGAGTGGAAGTGTATACAACCCAGCCTTCTGCAGTGCT 2580
 V N E D I Y V R V E L L Y N P A F C S A 837
 TCCACAAAAGGACAAAGATACCGACAGCAGTTCCTCAATTAAGCCCTGTCTCCAGAGCA 2640
 S T K G Q R Y R Q Q F P I K A L S S R A 857
 GTACCGTTTGTGATAGTCCCATTAGAGCAAGGATTGCATGATGTTGAGATTAAAGCAAGT 2700
 V P F V I V P L E Q G L H D V E I K A S 877
 GTCCAGGAAGCGTTGTGGTCAGACGGTGTGAGGAAGAACTGAAAGTTGTACCTGAAGGG 2760
 V Q E A L W S D G V R K K L K V V P E G 897
 GTACAGAAATCCATTGTGACTATGTAAACTGGACCCAAGGGCAAAGGAGTTGGTGGA 2820
 V Q K S I V T I V K L D P R A K G V G G 917
 ACACAGCTAGAAGTGATCAAAGCCCGCAAATTAGATGACAGAGTGCCTGACACAGAAATT 2880
 T Q L E V I K A R K L D D R V P D T E I 937
 GAAACCAAGATTATCATCCAAGGTGACCTGTGGCTCAGATTATTGAAACTCAATTGAT 2940
 E T K I I I Q G D P V A Q I I E N S I D 957
 GGAAGTAAACTCAACATCTCATTATCACTCCTTCTGGCTGTGGGGAGCAAATATGATC 3000
 G S K L N H L I I T P S G C G E Q N M I 977
 End γ -chain | Begin "C3d"
 CGCATGGCCGCACCAAGTATTGCCACCTACTACCTGGACACCACAGAGCAGTGGGAGACT 3060
 R M A A P V I A T Y Y L D T T E Q W E T 997
 CTCGGCATAAATCGCAGGACTGAAGCTGTCAATCAGATCGTGACTGTTATGCCAGCAG 3120
 L G I N R R T E A V N Q I V T G Y A Q Q 1017
 ATGGTGTAAGAAAGCAGATCATTCCTATGCAGCATTTACAAACCGTGCATCTAGTTCT 3180
 M V Y K K A D H S Y A A F T N R A S S S 1037

Fig. 6 (continued)

Exon 26
 TGGCTAACAGCATATGTCGTAAGTCTTTGCCATGGCTGCCAAAATGGTAGCAGGCATT 3240
 W L T A Y V V K V F A M A A K M V A G I 1057

AGTCATGAAATCATTGTGGAGGTGTGAGGTGGCTGATTCTGAACAGGCAACAACAGAT 3300
 S H E I I C G G V R W L I L N R Q Q P D 1077

Exon 27
 GGAGCGTTCAAAGAAAATGCCCTGTACTTTCTGGAACAATGCAGGAGGAATTCAAGGT 3360
 G A F K E N A P V L S G T M Q G G I Q G 1097

GCTGAAGAAGAAGTATATTTAACAGCTTTCATTCTGGTTGCGTTGTTGGAATCCAAAACA 3420
 A E E E V Y L T A F I L V A L L E S K T 1117

Exon 28
 ATCTGCAATGACTATGTCAATAGTCTAGACAGCAGCATCAAGAAGGCCACAAATTATTTA 3480
 I C N D Y V N S L D S S I K K A T N Y L 1137

CTCAAAAAGTATGAGAACTGCAAAGGCCTTACACTACAGCCCTCACAGCCTATGCTTTG 3540
 L K K Y E K L Q R P Y T T A L T A Y A L 1157

Exon 29
 GCTGCTGCAGACCAACTCAATGATGACAGGGTACTCATGGCAGCATCAACAGGAAGGGAT 3600
 A A A D Q L N D D R V L M A A S T G R D 1177

CATTGGGAAGAATACAATGCTCACACCCACAACATTGAAGGCACCTTCCTATGCCITGTTG 3660
 H W E E Y N A H T H N I E G T S Y A L L 1197

GCCCTGCTGAAAATGAAGAAATTTGATCAAAGTGGTCCCATAGTCAGATGGCTGACAGAT 3720
 A L L K M K K F D Q T G P I V R W L T D 1217

Exon 30
 CAGAATTTTATGGGGAAACATATGGACAAACCCAAAGCAACAGTTATGGCATTTCAGCT 3780
 Q N F Y G E T Y G Q T Q A T V M A F Q A 1237

CTTGCTGAATATGAGATTCAGATGCCTACCCATAAGGACTTAACTTAGATATTACTATT 3840
 L A E Y E I Q M P T H K D L N L D I T I 1257

End "C3d" Begin β -chain

GAACTGCCAGATCGAGAAAGTACCTATAAGGTACAGAATTAATTATGAAAATGCTCTCCTG 3900
 E L P D R E V P I R Y R I N Y E N A L L 1277

Exon 31
 GCTCGACAGTAGAGACCAAACTCAACCAAGACATCACTGTGACAGCATCAGGTGATGGA 3960
 A R T V E T K L N Q D I T V T A S G D G 1297

[Exon 32 of human C3]
 AAAGCAACAATGACCAATTTTGACATTCTATAACGCACAGTTGCAGGAGAAGGCAAATGTT 4020
 K A T M T I L T F Y N A Q L Q E K A N V 1317

Exon 32
 TGCAATAAATTTTCATCTTAATGTTTCTGTTGAAAACATCCACTGAAATGCAATGGGAGCC 4080
 C N K F H L N V S V E N I H L N A M G A 1337

Exon 33
 AAGGGAGCCCTCATGCTCAAGATCTGCACAAGGTATCTGGGAGAAGTTGATTCTACAATG 4140
 K G A L M L K I C T R Y L G E V D S T M 1357

Exon 34
 ACAATAATTGATATTTCTATGCTGACTGGTTTTCTCCCTGATGCTGAAGACCTTACAAGG 4200
 T I I D I S M L T G F L P D A E D L T R 1377

CTTTCTAAAGGAGTGGACAGATACATCTCCAGATATGAAGTTGACAATAATATGGCTCAG 4260
 L S K G V D R Y I S R Y E V D N N M A Q 1397

Fig. 6 (continued)

		Exon 35	
AAAGTAGCTGTATCATTACTTAAACAAGGTCTCCCACTCTGAAGATGAATGCCTGCAC	4320		
K V A V I I Y L N K V S H S E D E C L H	1417		
TTTAAGATTCTCAAGCATTTTGAAGTTGGCTTCATTCAGCCAGGATCAGTCAAGGTGTAC	4380		
F K I L K H F E V G F I Q P G S V K V Y	1437		
		Exon 36	
AGCTACTACAATCTAGATGAAAAATGTACCAAGTTCTACCATCCAGATAAAGGAACAGGC	4440		
S Y Y N L D E K C T K F Y H P D K G T G	1457		
		Exon 37	
CTTCTCAATAAGATATGTATTGGTAACGTTTGCCGATGTGCAGGAGAAACCTGTTTCCTCG	4500		
L L N K I C I G N V C R C A G E T C S S	1477		
CTCAACCATCAGGAAAGGATTGATGTTCCATTACAAATTGAAAAAGCCTGCGAGACGAAT	4560		
L N H Q E R I D V P L Q I E K A C E T N	1497		
		Exon 38	
GTGGATTATGCTACAAAACCAAGCTGCTTCGAATAGAAGAACAAGATGGTAATGATATC	4620		
V D Y V Y K T K L L R I E E Q D G N D I	1517		
		Exon 39	
TATGTCATGGATGTTTTAGAAGTTATTAACAAGGTACTGACGAAAAATCCACGAGCAAAG	4680		
Y V M D V L E V I K Q G T D E N P R A K	1537		
ACCCACAGTACATAAGTCAAAGGAAATGCCAGGAGGCTCTGAATCTGAAGTGAAATGAT	4740		
T H Q Y I S Q R K C Q E A L N L K V N D	1557		
		Exon 40	
GATTATCTGATCTGGGGTTCCAGGAGTGACCTGTTGCCCACGAAAGATAAATTTCTCTAC	4800		
D Y L I W G S R S D L L P T K D K I S Y	1577		
ATCATTACAAAGAACACATGGATTGAGAGATGGCCACATGAAGACGAATGTCAGGAAGAA	4860		
I I T K N T W I E R W P H E D E C Q E E	1597		
GAATTCAAAAAGTTGTGTGATGACTTTTGCTCAGTTTAGCTACACATTGACTGAGTTTGGC	4920		
E F Q K L C D D F A Q F S Y T L T E F G	1617		
TGCCCTACTTAAAGTTCAGAAGAATCAATGATAGGAAGGAAATTCAGAAAGACAGATT	4980		
C P T ! *	1620		
End β-chain ! 3' UTR			
TTTGAGCCAATGCATATATGTTACTTTTGCTCTTGATCTTTTAGTTTTATGTCAATTTGC	5040		
TCTGTTATTTTCCCTTAAATTGTTTATACATAAAATAAATAATCGATTTCCTACTTTGAT	5100		
ATGTTCTTGATTTTAAATAACAATGGTGATTCATGATTATTTTTTCTTCTCTGATCC	5160		
GTCCAATATTTGAAGTGCTCTGAACAGAGCACTTATGGAGTAATGTTTTAGTGATGGATG	5220		
AATAAGTTGGTGAGTCAATATTATCAGGCCCTATATACTCTTATGGAAGATCGATTTGTA	5280		
CCCAAGAAACATAGATTGAAATGTGTTACTTTGAAAACAGAGGTTTCAGTTGTATATGT	5340		
TTACACTTGGATACAATCTTAACCTCTTAATAAACACTGATCTCAGAACATTTAACAGCTG	5400		
CTATTTAATAATGACAAAATATCTTTGACTGCACCCACAGAAAACATTGCATTACATTA	5460		
GAAATGGGTTTTATCAGATGACTAAGCTGCTAGACTTGCCATCTGTCAAAATGTGCCTCT	5520		
TCCCCAGTCAAGGATTGGTTGAGGGGTTATGCCAATGTGATTGAAGGGTTTCACCCATG	5580		
AGGTGTGGTAGCCACTGAGTTTCCCTGGATGACACTAGAAGCTGGCAGCACACTGCAGCC	5640		
TGGTGGAGGGGGCCTCTTTTGCTATCCCATGAGCTTCTATTCATCCTCTTATCTGTTGGG	5700		
ATGGGGATGGGACGCTCTGATTTTCCAGGTATACAGGTGATCTCATTACTAATCATCAC	5760		
CACTAAGTTCAGGATTGGTTGAGGGGTTATGCCAATGTGATTGAAGGGTTTCACCCATG	5820		
TGAATCTATTCTCCAATCCCAATGCTGTATCTATGCTGCTCATTTCTGCTTGTAATAATG	5880		
GTATAAAAAGAATAAACACTGCCAGGCAGTCAGACATCTTTGGACACTG	5930		

Fig. 6 cDNA sequence and derived amino acid sequence of CVF from *N. kaouthia*. **Bold broken lines** indicate the beginning and end of the CVF chains. **Solid lines** indicate the position of exon borders. A **thin broken line** indicates the position between nucleotides 3,975 and 3,976 in exon 31 where an additional intron is present in the otherwise highly conserved human C3 gene, causing the human C3 gene to have 41 exons. The **asterisk** marks the TAA stop codon

Table 4 Accession numbers for *Naja kaouthia*, *Ophiophagus hannah*, and *Austrelaps superbis* CVF, C3, and cobrin proteins

Protein	Species	Protein sequence	cDNA sequence
CVF	<i>N. kaouthia</i>	Q91132.1	U09969.2
OVF	<i>O. hannah</i>	I2C090.1	EF080836.1
AVF1	<i>A. superbis</i>	Q0ZZJ6.1	DQ110889.1
AVF2	<i>A. superbis</i>	A0RZC6.1	DQ110890.1
C3	<i>N. kaouthia</i>	Q01833.1	L02365
C3-1	<i>A. superbis</i>	DQ149984.1	DQ149984.1
C3-2	<i>A. superbis</i>	ABA28746.1	AY902391.1
Cobrin	<i>N. kaouthia</i>	AAF00693.1	AF063190

Table 5 Amino acid composition of CVF^a

Residue	α -Chain	β -Chain	γ -Chain	Mature CVF	Pro-CVF	Pre-pro-CVF
Ala	37	17	13	67	110	113
Arg	23	15	12	50	70	71
Asn	34	23	8	65	81	81
Asp	35	27	18	80	97	97
Cys	3	13	2	18	27	27
Gln	27	16	12	55	75	75
Glu	27	29	17	73	98	99
Gly	42	17	12	71	91	94
His	13	10	2	25	32	33
Ile	37	30	24	91	109	110
Leu	50	35	18	103	135	139
Lys	39	29	19	87	107	107
Met	13	8	3	24	35	37
Phe	28	11	9	48	58	59
Pro	37	11	13	61	68	69
Ser	41	17	20	78	96	98
Thr	51	27	12	90	115	115
Trp	4	3	4	11	16	16
Tyr	26	18	7	51	71	72
Val	60	23	27	110	129	130
Total	627	379	252	1,258	1,620	1,642

^aData derived from Fritzing et al. (1994, 1995) and Vogel et al. (1996)

similar to cobra C3 (43 %) (Fritzing et al. 1992b) and other cobra proteins (43.6 %, cobra nerve growth factor (Selby et al. 1987); 44.2 %, cobra acetylcholine receptor (Neumann et al. 1989)) but more than 10 % lower than that found for human and murine C3 (de Bruijn and Fey 1985; Lundwall et al. 1984; Wetsel et al. 1984). The low GC composition is reflected in the codon usage of CVF where G- and C-rich codons are underrepresented compared to mammalian C3s (Table 6), likely representing a codon preference for the *Naja* species. The unusual codon frequency

Table 6 Codon frequency of CVF and human C3^a

Amino acid	Codon	CVF	Human C3	Amino acid	Codon	CVF	Human C3
Gly	GGG	0.18	0.27	Trp	UGG	1.00	1.00
Gly	GCA	0.40	0.17	End	UGA	0.00	1.00
Gly	GGU	0.21	0.12	Cys	UGU	0.56	0.31
Gly	GGC	0.20	0.44	Cys	UGC	0.44	0.69
Glu	GAG	0.34	0.69	End	UAG	0.00	0.00
Glu	GAA	0.66	0.31	End	UAA	1.00	0.00
Asp	GAU	0.64	0.28	Tyr	UAU	0.57	0.18
Asp	GAC	0.36	0.72	Tyr	UAC	0.43	0.82
Val	GUG	0.39	0.50	Leu	UUG	0.22	0.10
Val	GUA	0.16	0.05	Leu	UUA	0.09	0.01
Val	GUU	0.26	0.12	Phe	UUU	0.59	0.22
Val	GUC	0.18	0.33	Phe	UUC	0.41	0.78
Ala	GCG	0.04	0.08	Ser	UCG	0.01	0.10
Ala	GCA	0.35	0.14	Ser	UCA	0.16	0.08
Ala	GCU	0.38	0.19	Ser	UCU	0.27	0.17
Ala	GCC	0.23	0.59	Ser	UCC	0.17	0.29
Arg	AGG	0.34	0.20	Arg	CGG	0.08	0.24
Arg	AGA	0.25	0.10	Arg	CGA	0.14	0.15
Ser	AGU	0.22	0.10	Arg	CGU	0.10	0.10
Ser	AGC	0.16	0.27	Arg	CGC	0.08	0.22
Lys	AAG	0.44	0.73	Gln	CAG	0.51	0.80
Lys	AAA	0.56	0.27	Gln	CAA	0.49	0.20
Asn	AAU	0.62	0.21	His	CAU	0.70	0.22
Asn	AAC	0.38	0.79	His	CAC	0.30	0.78
Met	AUG	1.00	1.00	Leu	CUG	0.29	0.49
Ile	AUA	0.16	0.10	Leu	CUA	0.07	0.08
Ile	AUU	0.52	0.15	Leu	CUU	0.13	0.05
Ile	AUC	0.32	0.75	Leu	CUC	0.21	0.27
Thr	ACG	0.05	0.15	Pro	CCG	0.03	0.14
Thr	ACA	0.43	0.15	Pro	CCA	0.55	0.22
Thr	ACU	0.28	0.12	Pro	CCU	0.29	0.19
Thr	ACC	0.23	0.58	Pro	CCC	0.13	0.45

^aData derived from Fritzing et al. (1994, 1995), Vogel et al. (1996), and De Bruijn and Fey (1985)

of CVF affected the production of recombinant CVF in *E. coli* in which the most frequently used codons in CVF for arginine (AGG and AGA; 59 %) represent only 7 % of arginine codons used by *E. coli*. Accordingly, recombinant expression of CVF in *E. coli* was toxic to the bacteria because of arginine tRNA depletion (Kock 1996; Vogel et al. 2004). This toxic effect could be overcome by co-expression of the rare tRNA_{Arg4}, resulting in normal cell growth kinetics and viability as well as CVF synthesis (Kock 1996; Vogel et al. 2004).

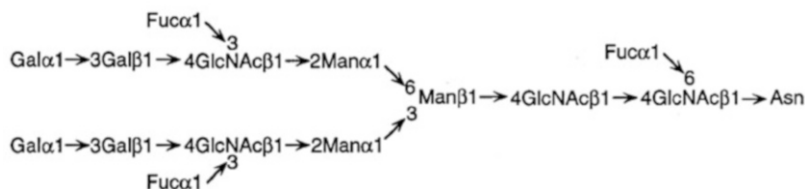


Fig. 7 Structure of the major oligosaccharide chain of the CVF. The structure is a symmetric fucosylated biantennary complex-type N-linked chain with an α -galactosylated Le^x structure at the nonreducing end

CVF Glycosylation

The glycoprotein nature of CVF was described by multiple investigators (Müller-Eberhard and Fjellström 1971; Eggertsen et al. 1981; Vogel and Müller-Eberhard 1984). The reported carbohydrate content of CVF varied between 5 % and 11 % (w/w). Carbohydrate staining of CVF after SDS-PAGE showed glycosylation of the α - and β -chains only (Vogel and Müller-Eberhard 1984; Grier et al. 1987) although some investigators also reported carbohydrate staining of the γ -chain (von Zabern et al. 1980; Eggertsen et al. 1981; Takahashi and Hayashi 1982). Carbohydrate composition analyses consistently found *N*-acetylglucosamine, suggesting that N-linked oligosaccharides represent the predominant (if not exclusive) form of CVF glycosylation, although some differences exist among investigators about the carbohydrate composition (Vogel and Müller-Eberhard 1984; Müller-Eberhard and Fjellström 1971; Johnson and Kucich 1977), with some investigators also reporting the presence of *N*-acetylgalactosamine (Eggertsen et al. 1981; Takahashi and Hayashi 1982). A comprehensive study of the oligosaccharide chain structures of CVF from *N. kaouthia* found only N-linked oligosaccharides, with the vast majority being of the complex type (Gowda et al. 2001). The major CVF oligosaccharide is a symmetric fucosylated biantennary complex-type chain with an unusual α -galactosylated Le^x structure at its nonreducing end (Fig. 7) (Gowda et al. 1992, 2001).

Humans, like apes and old-world monkeys, lack the α 1-3 galactosyltransferase (Galili et al. 1988). As α -galactosyl residues at the nonreducing end of saccharide structures are very common in nature, up to 1 % of natural human antibodies are anti- α -Gal antibodies. Human anti- α -Gal binds to CVF but it does not affect its function as CVF activates complement in human and cynomolgus monkey serum, both in vitro and in vivo (see Figs. 14 and 15 below) (Gowda et al. 1994; Gowda 1998; Vogel and Fritzing 2010; Warrell et al. 1976). However, the exposure of the Le^x antigen by removal of the terminal α -galactose leads to faster clearance from the circulation by the liver (Fu et al. 1997), indicating that the terminal α -galactose residues prevent recognition of the Le^x structure by the liver. Consistent with this observation is the fact that both chemical modification of the terminal galactose residues and complete deglycosylation do not affect the plasma clearance of CVF (Fu et al. 1997).

Glycosylation of CVF is not required for its functional activity. Removal of the terminal α -galactose residues or their oxidation with subsequent hydrazide

derivatization, complete deglycosylation, or even the introduction of charged sialic acid residues has no effect on the activity of CVF (Gowda et al. 1994; Gowda 1998), collectively confirming that the CVF oligosaccharides are not important for CVF activity, a conclusion that is corroborated by the identical functional activity of recombinant CVF with its insect-type glycosylation (see below) (Kock et al. 2004; Vogel et al. 2004). Moreover, the locations of the CVF glycosylation sites (in the MG2 and MG8 domains, see below) are sterically removed from the functionally important CUB and C345C domains (Figs. 2 and 5), both of which interact with factor B. In the presence of tunicamycin, secretion of recombinant CVF from insect cells into the supernatant was inhibited, pointing at a role of the CVF oligosaccharides chains for intracellular trafficking and secretion (Kock et al. 2004).

The molecular cloning of CVF from *N. kaouthia* revealed that both the CVF α - and β -chains are glycosylated, confirming the biochemical data of no glycosylation of the CVF γ -chain (Fritzinger et al. 1994). There are three potential *N*-glycosylation sites in the CVF α -chain (at positions 131, 136, and 187 in the MG2 domain) and one potential glycosylation site in the CVF β -chain (at position 1,324 in the MG8 domain) (Figs. 2 and 3). Because of the proximity of positions 131 and 136, it is likely that only one is glycosylated. A total of three CVF glycosylation sites is also consistent with the carbohydrate composition of CVF (Gowda et al. 1992, 1994, 2001). In addition, there is another glycosylation site in the C3a-like domain of pro-CVF at residue 640 which is not present in the mature CVF protein. CVF from *O. hannah* has four glycosylation sites of which only two are present in the mature three-chain protein, both in the α -chain (Zeng et al. 2012). One isoform of CVF from *A. superbus* (AVF-2) has three glycosylation sites, one in the α -chain and two in the β -chain, whereas the other isoform (AVF-1) is not glycosylated (Rehana and Kini 2007).

The CVF Gene

The CVF gene is larger than 89 kb. It consists of 40 exons. Exon sizes vary from 46 bp to 213 bp, and introns vary from 100 bp to 9,000 bp (Bammert et al. 2002b; Bammert 2002). Figure 6 shows the locations of the exon borders. Please note that the exon borders neither correlate with the CVF chains nor the CVF domains. A more recent analysis of the *O. hannah* genome showed that its CVF gene is somewhat smaller (71 kb) but also consists of 40 exons (Vonk et al. 2013).

CVF Activity: Molecular Interaction with the Complement System

CVF activates complement and leads to complement consumption when it comes into contact with human or mammalian serum either by being added in vitro, by being injected into a laboratory animal, or by envenomation after a cobra bites a prey or human victim. The molecular basis for that activity involves the initial binding of CVF (through its CUB domain) to complement factor B (through its complement control protein domains (CCP1–3)) of the alternative pathway (Fig. 5) (Götze and

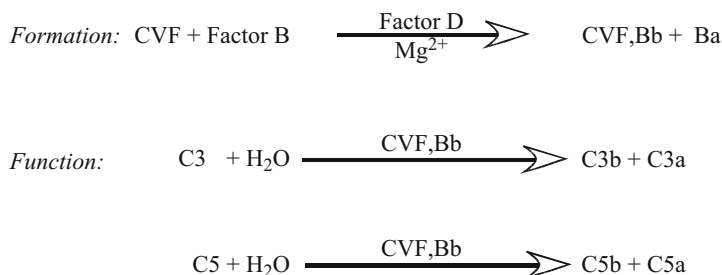


Fig. 8 Formation and enzymatic functions of the CVF,Bb C3/C5 convertase

Müller-Eberhard 1971; Hensley et al. 1986). Factor B, when in complex with CVF, is cleaved by complement factor D, resulting in the release of the Ba activation fragment (CCP 1–3 domains) and the formation of a bimolecular complex consisting of the Bb fragment of factor B and CVF (Janssen et al. 2009; Vogt et al. 1974; Lesavre et al. 1979). The Bb fragment consists of two globular domains, the von Willebrand domain (vWA) and the serine protease domain (SP) (Milder et al. 2007; Ponnuraj et al. 2004). The bimolecular complex CVF,Bb is an enzyme, referred to as C3 convertase as it cleaves complement component C3 (Cooper 1973; Bitter-Suermann et al. 1972; Vogel and Müller-Eberhard 1982), with the active site being in the Bb subunit. In addition to cleaving C3, CVF,Bb can also cleave C5 (at least with CVF from certain species, including *N. kaouthia*), and the enzyme is therefore usually referred to as C3/C5 convertase (von Zabern et al. 1980; DiScipio et al. 1983; Petrella et al. 1987). Consistently, a single EC number has been issued for the C3/C5 convertase (EC 3.4.21.47). Figure 8 summarizes the formation and activity of the CVF-dependent C3/C5 convertase.

The CVF,Bb enzyme shows spontaneous decay-dissociation into its two subunits which inactivates the C3- and C5-cleaving activities. However, the decay-dissociation is relatively slow ($t_{1/2}$ is 7 h at 37 °C) (Vogel and Müller-Eberhard 1982; Pangburn and Müller-Eberhard 1986; Fritzing et al. 2009), and the CVF,Bb enzyme is resistant to inactivation by the complement regulatory enzymes factors H and I (Lachmann and Halbwachs 1975; Alper and Balavitch 1976; Nagaki et al. 1978). As a consequence, CVF,Bb continuously cleaves C3 and C5. The C5 activation product C5b will in turn activate the terminal complement components C6, C7, C8, and C9. The action of CVF will therefore consume factor B, C3, C5, C6, C7, C8, and C9, leading to depletion of the serum (or plasma) complement activity both in vitro and in vivo. However, it has been shown that the depletion of the complement activity is primarily a function of C3 consumption (Van den Berg et al. 1991). Figure 9 shows the depletion of complement in mice after a single i.p. injection with CVF. Decomplementation is rapid, occurring within minutes, and complement activity returns to normal levels after several days through resynthesis of complement components in the liver. Figure 9 also shows the serum complement

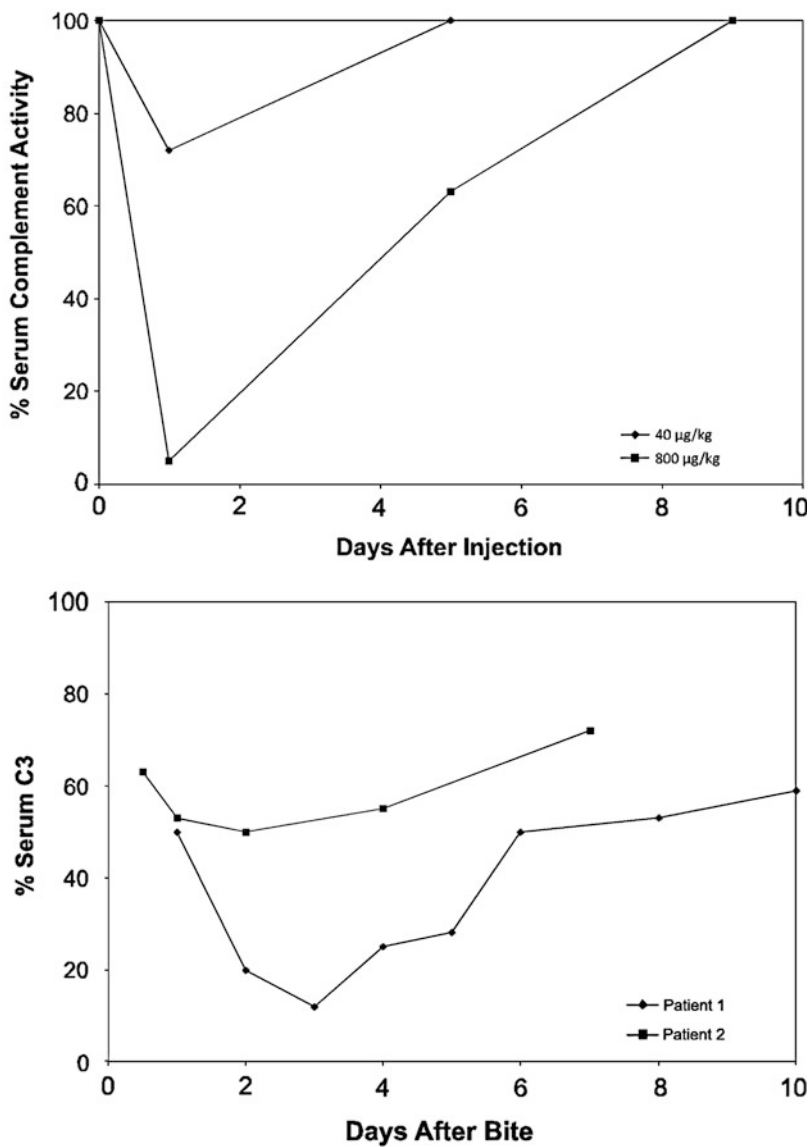


Fig. 9 In vivo complement depletion by CVD. The *upper panel* shows a time course of the serum complement activity in mice after a single i.p. injection of CVD at doses as indicated. The *lower panel* shows a time course of the percent of serum C3 in two patients after being bitten by *N. nigracollis* (Modified from Warrell et al. 1976) [The authors would like to thank Dr. David Warrell, University of Oxford, England, for his generous permission to reproduce his results of complement depletion in *N. nigracollis* snakebite victims]

C3 levels of two patients after being bitten by *N. nigricollis* in the elbow or ankle, respectively (Warrell et al. 1976). C3 depletion and return to normal levels in the patients mirrors the findings from laboratory animals. These results demonstrate that, after envenomation, CVF reaches the bloodstream and leads to complement depletion, however to a varying extent, likely a consequence of the amount of venom injected and the local physical environment at the envenomation site. Consistent with the well-documented lack of toxicity of complement depletion by CVF in laboratory animals (see below), the systemic complement depletion in the patients does not correlate with either the serious local pathology observed on the affected limb or any of the systemic toxic effects of the venom (Warrell et al. 1976). In contrast to the varying degree of systemic C3 activation, complete activation of C3 was observed in the wound and blister fluids of patients (Warrell et al. 1976). Taken together, these results indicate that massive complement activation by CVF occurs at the site of envenomation and that systemic complement activation appears to be an epiphenomenon without toxic consequences for the bite victim.

CVF has been shown to be able to deplete complement in the serum of all vertebrates tested except cobras (Dias da Silva et al. 1984; Vogel and Müller-Eberhard 1985a; Vogel and Fritzinger 2010). Although cobras have a complement system like all other vertebrates (Vogel and Müller-Eberhard 1985a, b), CVF does not affect the complement hemolytic activity of cobra plasma, indicating that cobras have developed a protective mechanism against complement depletion by its own CVF. CVF when added to cobra plasma changes its electrophoretic mobility, suggesting its binding to a plasma protein which inhibits formation of a convertase (Vogel and Fritzinger 2010). Apparently, cobra factor B serves as CVF inhibitor by binding to it but preventing convertase formation and complement depletion (Grunwald et al. 1996).

CVF: Why Is It in Cobra Venom?

Taken together, these results indicate that CVF is not a toxic component of cobra venom. Rather, it causes massive complement activation at the site of envenomation by cleaving C3 and C5 and thereby locally releasing the highly active C3a and C5a anaphylatoxins. C3a and C5a cause increased blood flow and increased vascular permeability at the site of envenomation. As a consequence, the toxic venom components will enter into the bloodstream faster and reach their intended targets for toxic action (e.g., the neuromuscular end plates) more quickly, resulting in a prey animal being paralyzed or killed faster. This reduces the struggle with the prey or its chance to escape, representing a definitive survival advantage for the cobra. Accordingly, CVF acts as a toxin facilitator or toxin enhancer, representing a different category of a venom component. Consistent with its apparent importance for the cobra, CVF is present in significant quantities in elapid venoms, representing 2 % of dry venom weight in *N. kaouthia* (Vogel and Müller-Eberhard 1984) and 5.5 % of the venom proteome in *O. hannah* (Vonk et al. 2013). CVF is an excellent example of how evolution has modified an existing gene (complement component C3) into a

powerful venom component (CVF), harnessing the prey's complement system for the benefit of the venomous animal.

Complement Component C3: Structural and Functional Homology to CVF

Structure of C3

Complement component C3 is a core component of the complement system. It is involved in the activation of the complement system by all three pathways (classical, alternative, and lectin). It is present in the plasma of all vertebrates, representing the fourth most abundant plasma protein (after albumin, immunoglobulin, and fibrinogen) and the third most abundant protein in serum (approximately 1.3 g/l or 6 μ M in human serum).

Like CVF, C3 is synthesized as a single-chain preproprotein. The human C3 mRNA is greater than 5,067 nucleotides long (de Bruijn and Fey 1985). It has an open reading frame that encodes for 1,663 amino acid residues of pre-pro-C3, consisting of a 22-residue signal sequence, the 645-residue β -chain, four arginine residues, and the 992-residue α -chain (Table 7). Posttranslational processing in the liver removes the four arginine residues by a furin protease, resulting in the mature two-chain C3 protein. The C3 mRNA has a 5'-untranslated region of at least

Table 7 Human C3 chains and fragments

Human C3 chains	Amino acid residues	No. of amino acid residues	Protein molecular mass ^a
Pre-pro-C3	–22 to –1 and 1–1,641	1,663	187,163 Da
Pro-C3	1–1,641	1,641	184,966 Da
C3 α -chain	650–1,641	992	113,027 Da
C3 β -chain	1–645	645	71,332 Da
C3a	650–726	77	9,095 Da
C3b α' -chain	727–1,641	915	103,951 Da
iC3b α' -(63 K) chain	727–1,281	555	62,477 Da
C3c α' -(24 K) chain	727–933	206	23,719 Da
C3c α' -(39 K) chain	1,299–1,641	343	39,488 Da
C3f	1,282–1,298	17	2,021 Da
C3dg	933–1,281	349	38,905 Da
C3g	933–979	47	5,137 Da
C3d	980–1,281	302	33,787 Da

^aCalculated from the amino acid sequence, without taking glycosylation into consideration

60 nucleotides and a 3'-untranslated region of at least 15 nucleotides (de Bruijn and Fey 1985).

Mature human C3 has a molecular mass of approximately 190,000 Da. It circulates in plasma as a two-chain protein, consisting of an α -chain (110,000 Da) and a β -chain (75,000 Da) (Figs. 2 and 3, Table 7). C3 has a unique molecular structure in its α -chain, an intramolecular thioester between a cysteine residue (position 988) and a glutamic acid residue (encoded as glutamine) (position 991) (Fig. 3) which is the molecular structure responsible for the covalent binding to cell surfaces during complement activation (Tack et al. 1980).

Complement activation through either pathway will lead to the formation of a C3 convertase (Fig. 1) which will cleave human C3 at position 726, thereby removing the C3a anaphylatoxin and generating nascent C3b which will covalently bind through its now highly reactive thioester in its α' -chain to cell surfaces. In the presence of factor H, factor I will cleave the α' -chain twice (at positions 1,281 and 1,298), generating cell-bound iC3b and releasing the 17-residue-long C3f fragment (Figs. 2 and 3, Table 7). With complement receptor CR1 as cofactor (instead of factor H), factor I will cleave the α' -(63 K) chain of iC3b at position 932, releasing C3c and generating the cell-bound C3dg fragment which, in turn, is cleaved by a trypsin-like enzyme into C3g and cell-bound C3d (Figs. 2 and 3, Table 7).

The molecular cloning of CVF and C3 from various species revealed that both proteins are synthesized as a single-chain preproprotein that is subsequently processed into the mature two-chain C3 protein and three-chain CVF protein, respectively (Figs. 3 and 6). There is extensive sequence homology between CVF and C3 from different species at both the protein and DNA level (Table 8). CVF shows a protein sequence identity of about 50 % with various vertebrate C3 molecules and a protein similarity of about 70 % (Table 8). The protein sequence identity between CVF and cobra C3 is 85 %, with a similarity of 92 % and a DNA sequence identity of 93 %. The molecular cloning of C3 and CVF also demonstrated the homology of the CVF α -chain to the C3 β -chain and the CVF γ/β -precursor chain to the C3 α -chain (Figs. 2 and 3) (Fritzinger et al. 1994; de Bruijn and Fey 1985; Vogel et al. 1996). Polyclonal antisera to CVF have been shown to cross-react with CVF in the venoms of other elapid snakes, with cobra C3 and with human C3 (Alper and Balavitch 1976; Eggertsen et al. 1981; Vogel et al. 1984; Dias da Silva et al. 1984; Grier et al. 1987; Vogel and Fritzinger 2010), with chain-specific antisera confirming the abovementioned chain relationships between CVF and C3 (Eggertsen et al. 1983).

X-ray crystallography of CVF and human C3 and its derivatives revealed that both proteins exhibit an identical domain structure (Fig. 5, Table 3) (Janssen et al. 2005, 2006, 2009; Krishnan et al. 2009). Both CVF and C3 have a somewhat elongated structure, with C3b exhibiting a prominent protrusion at one end which gives it a somewhat embryo-like structure on electron microscopy (Fig. 4) (Vogel et al. 1984). The crystal structures of C3 and C3b revealed this protrusion to be the C3d region (essentially corresponding to the TED domain) which is removed by conversion of C3b to C3c (Fig. 5, Table 3) (Janssen et al. 2005, 2006; Wiesmann et al. 2006). These studies also confirmed that the three-chain structure of CVF

Table 8 Homology of *N. kaouthia* CVF to CVF and C3 from other species^a

	% Protein identity	% Protein similarity	% DNA identity
<i>CVF species</i>			
<i>O. hannah</i>	88	92	93
<i>A. superbis</i> (AVF-1)	83	90	90
<i>A. superbis</i> (AVF-2)	82	89	90
<i>C3 species</i>			
<i>N. kaouthia</i>	85	92	93
<i>A. superbis</i> (C3-1)	83	90	90
<i>A. superbis</i> (C3-2)	82	89	90
Chicken	55	72	57
Human	50	69	56
Mouse	52	70	57
Rat	51	70	58
Rabbit	49	68	57
Guinea pig	53	70	56
<i>Xenopus</i>	51	67	55
Carp (C3-H1)	52	62	51

^aData either taken from or derived from (using the MacVector 11.1.2. program) Fritzinger et al. (1992b, 1994, 1995), Zeng et al. (2012), Rehana and Kini (2007, 2008), De Bruijn and Fey (1985), Lundwall et al. (1984), Wetsel et al. (1984), Misumi et al. (1990), Auerbach et al. (1990), Kusano et al. (1986), Nakao et al. (2000), Lambris et al. (1995), and Mavroidis et al. (1995)

resembles that of the C3 degradation product C3c (Figs. 2 and 3) (Vogel et al. 1984). However, in contrast to C3c, CVF has an intact CUB domain which has been shown to be important for its ability to form a convertase with factor B (Fig. 5) (O'Keefe et al. 1987; Janssen et al. 2006, 2009; Vogel and Fritzinger 2010).

The crystal structures of the CVF,B and C3b,Bb complexes demonstrated that factor B is bound to CVF or C3b at the C345C domain (Fig. 5) (Janssen et al. 2009; Rooijakkers et al. 2009; Forneris et al. 2010), confirming the earlier electron microscopic images of the C3 convertases (Fig. 4) (Smith et al. 1982, 1984; Torreira et al. 2009a, b).

The structural homology of CVF and C3 as revealed by molecular cloning and x-ray crystallography correlates with high-resolution transmission electron microscopy images (Fig. 4) (Smith et al. 1982; Vogel et al. 1984; Janssen et al. 2009) and confirms the earlier prediction of structural homology between the two proteins from circular dichroism spectroscopy and N-terminal protein sequencing (Vogel et al. 1984; Eggertsen et al. 1981).

Glycosylation of C3

Like CVF, C3 is glycosylated. The carbohydrate content of human C3 is with 1.7 % (w/w) significantly lower than that of CVF (Tomana et al. 1985). Human C3 has three potential N-linked glycosylation sites (Fig. 3), of which only two, at positions

63 and 917, are glycosylated (Hirani et al. 1986; de Bruijn and Fey 1985). The human C3 oligosaccharides have been identified as high-mannose type (Hase et al. 1985; Hirani et al. 1986). The extent, location, and type of glycosylation vary for C3 from different species (Miki et al. 1986), suggesting that glycosylation is, like for CVF, of no functional relevance. Interestingly, cobra C3 is not glycosylated (Table 4) (Fritzing et al. 1992; Alsenz et al. 1992).

Cysteine Residues, Disulfide Bonds, and Thioester Formation in CVF and C3

All CVF and C3 proteins purified from different species share 26 highly conserved cysteine residues that form 13 disulfide bonds (Dolmer and Sottrup-Jensen 1993; Huber et al. 1980; Janssen et al. 2009; Krishnan et al. 2009; Vogel et al. 1996). There is only one disulfide bond each connecting the CVF α -chain to the CVF γ -chain (Cys⁵²²–Cys⁷⁷⁹) and the CVF γ -chain to the CVF β -chain (Cys⁸³⁵–Cys¹⁴⁷⁰). The corresponding interchain disulfide bonds in human C3 connect the C3 β -chain to the C3 α -chain (Cys⁵³⁷–Cys⁷⁹⁴) and the C3c α' -(24 K) chain to the C3c α' -(39 K) chain (Cys⁸⁵¹–Cys¹⁴⁹¹) (Figs. 2, 3, and 10). All other disulfide bonds are intra-chain bonds and mostly clustered within the CVF β -chain (and especially within the functionally important C-terminal half (anchor and C345C domains) of the CVF β -chain (Grunwald et al. 1993; Vogel and Fritzing 2010)) and the anaphylatoxin domain (Huber et al. 1980; Janssen et al. 2009). Figure 10 shows the location of the 13 disulfide bonds in pro-CVF.

In addition to the 26 cysteine residues involved in disulfide bonds, there is a 27th highly conserved cysteine present in all C3 proteins (at position 988 in human C3). This cysteine residue is involved in C3 proteins in the formation of an intramolecular thioester with a glutamine residue (at position 991 in human C3) (Fig. 3), forming a 15-atom ring structure in the C3d region. The intramolecular thioester is an essential structure for the covalent attachment to surfaces of C3b

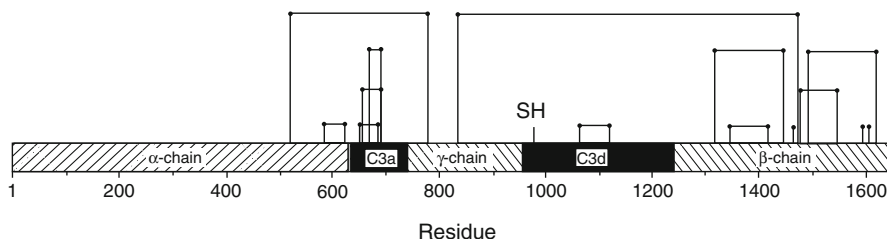


Fig. 10 Location of disulfide bonds. Shown is a schematic drawing of pro-CVF indicating the location of the highly conserved 13 disulfide bonds. Please note the presence of only two interchain disulfide bonds, connecting the α -chain and γ -chain and the γ -chain and β -chain, respectively. Please also note the clustering of disulfide bonds in the C3a portion and the functionally important C-terminal half of the β -chain. The free thiol group in the C3d portion (TED domain) involved in the intramolecular thioester in C3 is shown

during complement activation (Tack et al. 1980; Pangburn and Müller-Eberhard 1980). The formation of the thioester in C3 is a nonenzymatic process (Pangburn 1992a, b). There are several highly conserved amino acid residues in the vicinity of the thioester that have been shown to play an important role in the thioester formation. These include two proline residues flanking the thioester (at positions 985 and 998 in human C3) as well as a glutamic acid residue at position 990 (Fig. 3) (Isaac and Isenman 1992). Two more residues important in the formation of the thioester are further C-terminal from the thioester site in C3d, a histidine and a glutamic acid (at positions 1,104 and 1,106 in human C3) (Fig. 3) (Nagar et al. 1998).

Interestingly, the cysteine and glutamine residues forming the thioester in C3 are also present in CVF proteins from *N. kaouthia* (residues 971 and 974) (Fig. 3), *O. hannah*, and *A. superbis* (AVF-1 isoform). The conservation of these two residues may appear surprising because the thioester site is in the C3d region (TED domain) which is not present in the mature three-chain CVF proteins. Moreover, the biological function of CVF is to form a fluid-phase enzyme, not a surface-bound enzyme like C3b, and a functional thioester is therefore neither required nor wanted. However, the presence of these residues in CVF proteins indicates that the gene duplication resulting in separate genes for C3 and CVF occurred rather recently. This is consistent with the fact that the cysteine in AVF-2 from *A. superbis* is replaced by a serine, caused by a single base mutation (Rehana and Kini 2007).

All conserved residues in the vicinity of the thioester in C3 important for thioester formation are also present in all CVF proteins (proline at positions 968 and 982, glutamic acid at position 973, *N. kaouthia* numbering). Interestingly, the conserved proline residue in CVF from *N. kaouthia* (position 982) and *O. hannah* is one residue further C-terminal than in CVF from *A. superbis* (both isoforms) and in C3 proteins, including cobra C3 (Fig. 3). However, the two functionally important residues further C-terminal from the thioester site (histidine, glutamic acid) are only conserved in the AVF-1 isoform from *A. superbis* (Rehana and Kini 2007); in CVF from *N. kaouthia* and *O. hannah*, they are replaced by a serine and threonine (positions 1,088 and 1,090 in *N. kaouthia*) (Fig. 3) (Fritzinger et al. 1994; Zeng et al. 2012). In the AVF-2 isoform of CVF from *A. superbis*, they are replaced by a leucine and glycine (Rehana and Kini 2007). Collectively, these data allow the assumption that the CVF proteins from *N. kaouthia*, *O. hannah*, and *A. superbis* (AVF-2 isoform) never formed a thioester. As the AVF-1 isoform of CVF from *A. superbis* has all known conserved C3 amino acid residues, it is conceivable that a thioester might form during its biosynthesis. However, as AVF-1 purified from venom exhibits the typical three-chain structure of mature CVF proteins and forms a C3 convertase (Rehana and Kini 2007), it must have a C3b-like conformation, consistent with the absence of a thioester.

Lastly, CVF from *O. hannah* contains, in addition to the 26 cysteine residues involved in disulfide bonds and the 27th cysteine at the thioester site, a 28th cysteine residue in its γ -chain, caused by a single base mutation (Zeng et al. 2012).

The Human C3 Gene

The gene for human C3 is located on the short arm of chromosome 19 (19p13.3-p13.2) (Schneider and Rittner 1997). It is 42 kb in length (Vik et al. 1991; Fong et al. 1990) and therefore significantly smaller than the genes for CVF in *N. kaouthia* (Bammert et al. 2002b; Bammert 2002) and *O. hannah* (Vonk et al. 2013). However, the intron/exon structure is highly conserved between the human C3 gene and the CVF gene. All splice donor/acceptor sites are conserved, with the exception that the human C3 gene has a total of 41 exons. The additional exon is created by an additional intron which separates the corresponding exon 31 of CVF into two separate exons (Fig. 6). This additional intron is also missing in CVF from *O. hannah* (Vonk et al. 2013) as well as in the gene for cobra C3 (Bammert et al. 2004; Bammert 2002).

Functional Homology of CVF and C3

Given the enormous structural homology between CVF and C3, it is not surprising that the two proteins also share functional homology. Although the three-chain structure of CVF resembles the C3 breakdown product C3c (Figs. 2 and 3), CVF functionally resembles the two-chain C3 activation product C3b. Like CVF, C3b contains the CUB domain required for convertase formation (Fig. 5). C3b, like CVF, binds factor B in the presence of Mg^{2+} ions. The C3b,B complex, like the CVF,B complex, is called the proconvertase, and it is the substrate of factor D which cleaves factor B within the complex, resulting in the release of the Ba activation peptide and the generation of a bimolecular complex C3b,Bb (Forneris et al. 2010; Lesavre et al. 1979). C3b,Bb is the C3 convertase of the alternative complement pathway, cleaving C3 and, as outlined below, also C5. Accordingly, both the formation of the C3b,Bb enzyme and its function mirror those of the CVF,Bb enzyme (compare Fig. 8).

Functional Differences Between Convertases Formed with CVF and C3b

The two enzymes CVF,Bb and C3b,Bb share the molecular architecture of consisting of a structural subunit (CVF or C3b) and the identical active site-bearing subunit Bb. Accordingly, they are identified by the same EC number (EC 3.4.21.47). The two enzymes also share the substrate specificity for C3 and C5. However, the two enzymes exhibit considerable functional differences:

1. Both bimolecular enzymes exhibit spontaneous decay-dissociation into the two respective subunits which abolishes the enzymatic activity. The decay-dissociation of the C3b,Bb enzyme is rapid, exhibiting a half-life of 1.5 min at 37 °C (Pangburn and Müller-Eberhard 1986; Medicus et al. 1976). In contrast, the

CVF,Bb enzyme is significantly more stable with a half-life of approximately 7 h at 37 °C (Vogel and Müller-Eberhard 1982; Pangburn and Müller-Eberhard 1986; Miyama et al. 1975).

2. The C3b,Bb enzyme and C3b are subject to rapid and efficient inactivation by the complement regulatory proteins factors H and I. Factor H dissociates C3b,Bb (Pangburn et al. 1977) and serves as cofactor for the proteolytic inactivation of C3b by factor I (Whaley and Ruddy 1976), generating the cleavage products of iC3b and C3f (Figs. 2 and 3). In contrast, both the CVF,Bb enzyme and CVF are completely resistant to the regulatory actions of factors H and I (Lachmann and Halbwachs 1975; Alper and Balavitch 1976; Nagaki et al. 1978). As CVF is not cleaved by factor I, it can reform a convertase with factor B, which is in contrast to both iC3b and C3c.
3. An important difference between C3b,Bb and CVF,Bb is the site of enzyme formation of the two enzymes. Complement activation occurs on the surface of a target cell or particle to which nascent C3b binds through its intramolecular thioester. Accordingly, the C3b,Bb enzyme is surface bound. In contrast, the CVF,Bb enzyme is a fluid-phase enzyme.
4. The two enzymes exhibit substantial differences in the kinetics of C5 hydrolysis. Whereas soluble fluid-phase C3b,Bb can cleave C5, the K_m of the monomeric C3b,Bb enzyme (24 μM) is almost two orders of magnitude above the physiological C5 concentration on plasma (0.37 μM) which implies that C3b,Bb does not exhibit C5-cleaving activity under physiological conditions (Rawal and Pangburn 2001). On the surface of a target cell, however, additional C3b molecules will attach in close proximity to the C3b,Bb convertase. The additional C3b molecules can bind the substrate C5 which results in a gradual increase in the affinity of the C3b,Bb convertase for C5 and a concomitant reduction of the K_m for C5 to as low as 0.016 μM , more than an order of magnitude below the normal plasma concentration of C5, essentially switching the C3-cleaving activity to the C5-cleaving activity (Rawal and Pangburn 1998, 2000, 2001; Vogt and Schmidt 1978; Vogt et al. 1978). In contrast, the CVF,Bb enzyme exhibits fluid-phase C5-cleaving activity, consistent with a K_m of 0.036 μM , well below the normal plasma concentration of C5 (Rawal and Pangburn 2000).

Whereas CVF from *N. kaouthia* exhibits C5-cleaving activity (Fig. 12) (von Zabern et al. 1980; DiScipio et al. 1983; Petrella et al. 1987; Vogel and Fritzinger 2010), the CVF proteins from *N. haje* and *A. superbis* lack C5-cleaving activity (von Zabern et al. 1980; Rehana and Kini 2007). For CVF from *N. haje*, it has been shown that it lacks the ability to bind C5 (von Zabern et al. 1980) which is in contrast to CVF from *N. kaouthia* which exhibits C5-binding activity (von Zabern et al. 1980; Fritzinger et al. 2009). It has been suggested that C5 binds to the same CVF molecule that is part of the CVF,Bb convertase (Vogt et al. 1977). Oxidation of three to four methionine residues in CVF from *N. kaouthia* reduces the C5-cleaving activity by approximately 50 % (Fu et al. 1998). Functional analysis of chimeric proteins

derived from CVF and cobra C3 (see below) suggested that the region from the C-terminal end of the CVF α -chain through most of the CVF γ -chain harbors crucial structures for the C5-cleaving activity (Hew et al. 2012). The crystal structure of a CVF/C5 complex showed an interaction with C5 in this particular region (MG6 and MG7 domains) (Laursen et al. 2011), although a much larger interface between C5 and CVF is in the CVF MG4 and MG5 domains which are entirely part of the CVF α -chain and appear to be of no functional relevance for C5 cleavage (Hew et al. 2012). Like CVF, recombinant CVF exhibits C5-cleaving activity (see below) (Fig. 12). In contrast, recombinant CVF modified with a histidine tag at its C-terminus is devoid of C5-cleaving activity (Kock 1996; Vogel and Fritzing 2010), and humanized CVF (see below) similarly lacks C5-cleaving activity (Fig. 12) (Fritzing et al. 2009). In conclusion, the molecular basis for the fluid-phase C5-cleaving activity of CVF,Bb remains elusive.

Purification of CVF

The purification of CVF from cobra venom has been reported by multiple investigators and involves sequential column chromatography (Ballow and Cochrane 1969; Müller-Eberhard and Fjellström 1971; Vogel and Müller-Eberhard 1984; Sharma et al. 2001; Kölln et al. 2007). Although these procedures yield CVF with good yields and purity, it has been noted that CVF preparations were often contaminated with phospholipase A₂ and, more importantly, that effects observed after treatment of laboratory animals with CVF were caused by the contaminating phospholipase A₂ and were not a consequence of complement activation by CVF (Ballow et al. 1973; Waldmann and Lachmann 1975; Shaw et al. 1978). In at least two instances, it was shown that an effect originally thought to be a consequence of CVF treatment (Azar et al. 1968; Day et al. 1970) was actually caused by the contaminating phospholipase A₂ (Hall et al. 1972; Morrison et al. 1976). Multiple procedures to reduce the phospholipase A₂ contamination have been described. An additional chromatographic step on hydroxyl apatite (Waldmann and Lachmann 1975; Pepys et al. 1979) and preparative polyacrylamide electrophoresis (Ulevitch and Cochrane 1977) or isoelectric focusing (Lachmann et al. 1976) were only partially effective or rather laborious. Better success was achieved with an additional chromatographic step using dye agarose (Vogel and Müller-Eberhard 1984), which had been shown to bind cobra venom phospholipase A₂ (Darke et al. 1980) or with chemical inactivation of phospholipase A₂ by treatment of CVF preparations with *p*-bromophenacyl bromide (Shaw et al. 1978). Another group of investigators reported a purification procedure involving a single HPLC chromatographic step resulting in CVF virtually free of phospholipase A₂ (Beukelman et al. 1987). With the ability to recombinantly produce functionally active CVF (Kock et al. 2004; Vogel et al. 2004) (see below), contamination with phospholipase A₂ is no longer a concern.

Assays for CVF

Because of its immunogenicity, polyclonal antisera to CVF can easily be raised in laboratory animals, including goats (Vogel et al. 1984), rabbits (Vogel et al. 1984; Eggertsen et al. 1981), and mice (Grier et al. 1987). Using polyclonal anti-CVF antiserum, CVF can be detected and quantified in immunological assays including immunoelectrophoresis (Vogel and Fritzinger 2010), radial immunodiffusion (Vogel and Müller-Eberhard 1984), and ELISA (Grier et al. 1987). The determination of CVF as CVF antigen by immunological methods allows the quantitation of CVF in crude mixtures, including whole venom (Vogel and Müller-Eberhard 1984).

The concentration of purified CVF can be determined by spectrophotometry at 280 nm using $E_{1\text{cm}}^{0.1\%} = 0.99$ (Vogel and Müller-Eberhard 1984). To determine CVF activity, two hemolytic assays are frequently employed. One assay is based on the anticomplementary activity of CVF. The CVF-containing sample is incubated with normal human or guinea pig serum. Subsequently, the remaining serum complement activity is determined in a hemolytic assay using sensitized sheep erythrocytes as targets (Ballow and Cochrane 1969; Cochrane et al. 1970). A second assay for CVF activity is based on the fluid-phase C5 cleavage and ensuing bystander lysis of non-sensitized erythrocytes (Pickering et al. 1969; Ballow and Cochrane 1969; Brai and Osler 1972; Miyama et al. 1976). The CVF-containing sample is incubated in the presence of normal guinea pig serum and guinea pig erythrocytes. Subsequently, unlysed erythrocytes are sedimented by centrifugation, and the released hemoglobin is determined spectrophotometrically in the supernatant (Vogel and Müller-Eberhard 1984). Neither functional assay, although routinely employed, is specific for CVF activity, especially in the presence of other venom components because cobra venom contains both other anticomplementary as well as hemolytic components as mentioned above (Vogel 1991).

The determination of the C3-cleaving and C5-cleaving activities of the CVF, Bb enzyme requires the formation of the enzyme from purified components factors B and D and use of purified C3 or C5 as substrate (Vogel and Müller-Eberhard 1982; von Zabern et al. 1980; DiScipio et al. 1983; Petrella et al. 1987).

CVF: An Experimental Tool to Study Complement Function

Ever since it was demonstrated that CVF can be safely administered to laboratory animals for temporary depletion of complement activity (Cochrane et al. 1970; Maillard and Zarco 1968; Nelson 1966), CVF has become widely used as an experimental tool to study the biological functions of complement as well as its involvement in the pathogenesis of diseases by comparing normal (complement-sufficient) with complement-depleted animals (Vogel 1991). It is beyond the scope of this chapter to review the large number of studies where complement depletion with CVF led to the identification of the role of complement in the pathogenesis of

diseases. CVF is immunogenic which limits its use essentially to a single application (Cochrane et al. 1970; Pryjma and Humphrey 1975). There is some evidence that the unusual carbohydrate structures of CVF may significantly contribute to its immunogenicity (Vogel et al. 2014). As outlined further below, humanized CVF is apparently devoid of immunogenicity, allowing multiple sequential applications for complement depletion (Rayes et al. 2014b).

The only side effect from massive intravascular fluid-phase activation of complement *in vivo* by CVF has been an acute but fleeting inflammatory lung injury (Till et al. 1982, 1987; Mulligan et al. 1996). The lung inflammation is mediated by the C5a anaphylatoxin (as well its C5a-des-Arg derivative after carboxypeptidase N has removed the terminal arginine residue). Both C5a and C5a-des-Arg activate neutrophils and lead to their subsequent sequestration to the lungs. In the presence of an inhibitor of carboxypeptidase N, massive fluid-phase activation of complement by CVF can be lethal (Huey et al. 1983). However, even under these conditions of carboxypeptidase N inhibition, lower doses of CVF, though still causing lung damage, are no longer lethal, and animals recover fully (Huey et al. 1983). Collectively, these data indicate that decompensation by CVF is safe, entirely corroborated by decades of use of CVF for decompensation of laboratory animals, from mice to primates.

In addition to any potential complications from massive fluid-phase activation of complement by CVF, it is reasonable to expect that a prolonged state of being depleted of circulating complement may cause infectious complications. However, transgenic mice constitutively expressing CVF and living with low C3 and serum complement levels (between 30 % and below 10 %) do not exhibit any increased susceptibility to infections or any other pathology (Andrä et al. 2002; Fritzing et al. 2010). This is in contrast to C3 knockout mice which have been shown to have a tendency to develop infections (Circolo et al. 1999; Sylvestre et al. 1996). Moreover, patients with homozygous C3 deficiency develop recurrent gram-positive infections (Botto et al. 2009). In the case of CVF transgenic mice, as well as animals depleted of complement with CVF, it is important to recognize that C3 depletion will never be complete: as the C3 concentration in serum decreases, so will the turnover of the remaining C3 by the CVF, Bb enzyme, a consequence of Michaelis-Menten enzyme kinetics (Vogel and Müller-Eberhard 1982). This fact will always ensure the presence of residual C3 and complement activity. Moreover, the ability to generate C3 locally by immune cells in response to infectious agents or other inflammatory stimuli is not impaired.

Antibody Conjugates with CVF: Tools for Targeted Complement Activation

The property of CVF to form a stable C3/C5 convertase and continuously activating the alternative pathway of complement has been exploited for the targeting of CVF and, therefore, complement activation by coupling CVF to monoclonal antibodies. Such antibody-CVF conjugates have been created as an experimental therapeutic

concept, primarily for complement-mediated killing of tumor cells. Antibody-CVF have been shown to induce specific killing of target cells, including human melanoma cells (Vogel and Müller-Eberhard 1981; Vogel et al. 1985), human lymphocytes and leukemia cells (Müller et al. 1986; Müller and Müller-Ruchholtz 1986, 1987), and human neuroblastoma cells (Juhl et al. 1990, 1997). Conjugates with CVF have also been made for other purposes such as lysing erythrocytes (Ganu et al. 1984; Parker et al. 1986), targeting complement activation to endothelial cells (Marks et al. 1989), and increasing tumor uptake of therapeutic antibodies (Juhl et al. 1995). It is important to note that conjugates with CVF do not exhibit intrinsic toxicity; all biological activities depend on the presence of complement and are mediated by the binding of C3b to target cells, the release of anaphylatoxins, or the cytotoxic activity of the membrane attack complex (MAC) of complement.

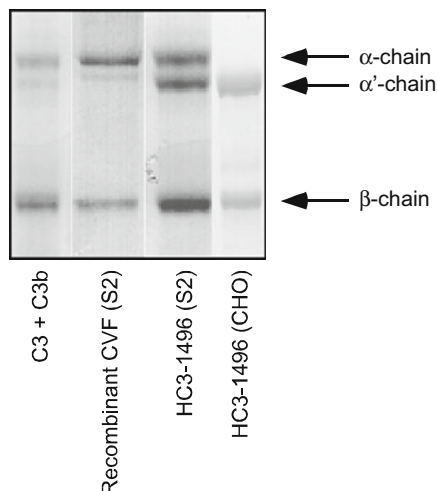
Most conjugates used monoclonal IgG antibodies as the targeting moiety. However, monoclonal IgM antibodies (Zara et al. 1995), F(ab')₂ fragments (Juhl et al. 1990), or a lectin (Marks et al. 1989) have also been employed.

All conjugates were created by chemical cross-linking of CVF to the targeting moiety protein using heterobifunctional cross-linking reagents (Vogel 1987, 1988). Heterobifunctional cross-linking reagents result in the formation of only heteroconjugates that contain at least one or a few of each protein to be coupled. Most cross-linking reagents derivatize amino groups exposed on the surface of a protein. As the derivatization of amino groups is random, functional or steric inactivation of coupled proteins occurs (Petrella et al. 1987; Vogel 1988). Moreover, the chemical structure of the linker portion can also affect the plasma half-life and biodistribution (Vogel 1988). To avoid inactivation of the antibody binding site, antibody-CVF conjugates have also been made with carbohydrate-directed cross-linking reagents which link CVF to the antibody carbohydrate chains in the hinge region (Zara et al. 1991, 1995; Vogel 2004). Conversely, as the CVF carbohydrate chains are also located away from functionally important sites in the CVF molecule, CVF has also been linked to antibodies through its carbohydrate chains (Fu and Gowda 2001; Gowda 1998).

Recombinant CVF

CVF has been recombinantly produced using eukaryotic expression systems (Kock et al. 2004; Vogel et al. 2004). In baculovirus-infected Sf9 insect cells, recombinant CVF (rCVF) is predominately expressed as a single-chain pro-CVF protein of 185,000 Da. In stably transfected *Drosophila* S2 cells, single-chain pro-CVF is converted into a two-chain C3-like form of pro-CVF by removal of the four arginine residues between the CVF α -chain and the CVF γ/β -precursor chain (Fig. 3). The C3-like form of pro-CVF can be processed further into a C3b-like form of pro-CVF by removal of the C3a anaphylatoxin-like domain (Fig. 3), and production of recombinant pro-CVF in S2 cells usually results in a mixture of both the C3-like and C3b-like forms of pro-CVF (Fig. 11) (Kock et al. 2004; Vogel et al. 2004). Continued incubation of Sf9 cells also leads to partial conversion of single-chain

Fig. 11 Chain structures of rCVF and hCVF. Shown are Coomassie-stained polyacrylamide gels of rCVF and hCVF protein HC3-1496 produced in *Drosophila* S2 cells and of hCVF protein HC3-1496 produced in CHO cells. A mixture of purified human C3 and C3b serves as control. Please note that rCVF and hCVF produced in S2 cells are a mixture of a C3-like and C3b-like form. hCVF produced in CHO cells is in the C3b-like form only



pro-CVF to the C3- and C3b-like forms (Kock et al. 2004). There is no further processing of pro-CVF in insect cells into the mature three-chain CVF as it occurs in cobra venom.

rCVF produced in insect cells is glycosylated, exhibiting insect-type glycosylation including N-linked oligosaccharides of the high-mannose type and O-linked oligosaccharides of the simple galactose- β (1-3)-*N*-acetylgalactosamine type. No complex N- or O-linked glycosylation was detected (Kock et al. 2004; Vogel et al. 2004). In the presence of tunicamycin, the secretion of recombinant CVF from Sf9 cells was inhibited, causing intracellular accumulation and degradation, indicating a functional role of the oligosaccharide chains for intracellular sorting and secretion.

rCVF of *N. kaouthia* does not form an intramolecular thioester although it contains the C3d-like portion (TED domain) (Figs. 2 and 3) with the thioester site. rCVF displays a single free thiol group (cysteine at position 971) (Fig. 10), and the amino acid residue at position 974 was identified as glutamine (Kock et al. 2004), indicating that an intramolecular thioester had never formed – as thioester hydrolysis would result in a glutamic acid residue at position 974. These results are consistent with the abovementioned amino acid replacements of two important residues for thioester formation in the C3d-like region (residues 1,088 and 1,090). As rCVF forms a fluid-phase convertase just like natural CVF (see below), the lack of a thioester is of no functional relevance.

Surprisingly, all three forms of recombinantly produced pro-CVF (rCVF) exhibit functional activity indistinguishable from mature three-chain CVF from cobra venom. All three forms of rCVF support the activation of human factor B in the presence of factor D and Mg^{2+} ions, forming a stable bimolecular convertase rCVF,Bb that exhibits enzymatic activity for both C3 and C5 (Fig. 12) and depletes serum complement activity (Kock et al. 2004; Vogel et al. 2004). The ability to deplete serum complement activity demonstrates that rCVF,Bb not only forms a

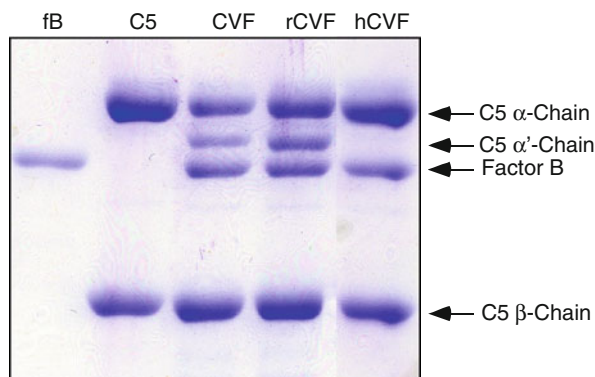


Fig. 12 C5-cleaving activity of convertases formed with human factor B. Preformed convertases with CVF, rCVF, and hCVF were incubated with purified human C5. C5-cleaving activity is indicated by the conversion the C5 α -chain into the C5 α' -prime chain upon polyacrylamide gel electrophoresis. Note that both natural CVF and rCVF exhibit C5-cleaving activity. In contrast, hCVF (HC3-1496) lacks C5-cleaving activity. Purified human C5 and factor B serve as control

stable convertase like CVF, Bb but also exhibits resistance to degradation by factors H and I (Fritzing, unpublished).

The surprising observation that recombinant CVF exhibits identical functional activity of native CVF poses the question of the biological significance of the processing of pro-CVF in the venom gland into the mature three-chain protein. As mentioned above, cobrin is a metalloprotease with high specificity for C3, removing a C3d-like portion from the human C3 α -chain, thereby generating the three-chain C3 derivative termed C3o (O'Keefe et al. 1988; Bambai et al. 1998). As C3o resembles CVF both structurally (three-chain, complete CUB domain) and functionally (supporting factor B activation and forming a convertase) (O'Keefe et al. 1988), it was suggested that cobrin is the protease responsible for the processing of pro-CVF into its mature three-chain form in the venom gland. That suggestion was further supported by the knowledge, at the time, that elapid venoms had only weak peptidase activity and were devoid of proteolytic activity (Iwanaga and Suzuki 1979). In contrast, as mentioned above, viperid and crotalid venoms are rich in metalloproteases and have been shown to cleave many plasma proteins, including complement proteins such as C3, C4, and C5 (Tambourgi and Van den Berg 2014; Man and Minta 1977; Eggertsen et al. 1980; Tambourgi et al. 1994; Pidde-Queiroz et al. 2010, 2013). More recently, however, metalloproteases have also been found in elapid venoms (Tanaka et al. 2012), including in *Naja* and *Ophiophagus* species which also have CVF (Takeda et al. 2012; Vonk et al. 2013; Ali et al. 2013; Kulkeaw et al. 2007; Malih et al. 2014; Yap et al. 2014)). These findings suggest that collectively metalloproteases in elapid venoms may have a somewhat broader specificity for plasma proteins beyond the C3-cleaving activity of cobrin. It is therefore conceivable that the processing of pro-CVF in the venom gland (by cobrin or related metalloproteases) is entirely incidental and not required to generate active CVF. There are some additional observations that would support this

conclusion. Cobrin cleaves the C3 α -chain at multiple sites, at least four, when generating C3o (O'Keefe et al. 1988), and at least two different cobrin enzymes were found (Petrella et al. 1991). Similarly, the observed size heterogeneity at the C-terminus of the CVF γ -chain indicates that the pro-CVF processing enzyme(s) in the venom gland can cleave the CVF γ/β -precursor chain at multiple cleavage sites, implying lower substrate specificity than would be expected for a dedicated pro-CVF processing protease.

The three chains of CVF have also been recombinantly expressed individually in *E. coli*. However, the individual chains or a mixture of all three chains did not exhibit CVF activity (Ziegelmüller et al. 1999), an observation that is not surprising in light of the current knowledge of the importance of both the CUB and C345C domains.

Chimeric Proteins of CVF and C3

The high degree of structural homology between C3 and CVF allowed the recombinant production of chimeric proteins derived from the two parent proteins C3 and CVF (Fritzing et al. 2009; Kölln et al. 2004; Vogel and Fritzing 2007; Vogel and Fritzing 2010; Vogel et al. 2014; Hew et al. 2004). There was a twofold purpose for creating such chimeric (or hybrid) proteins. For one, the approach of creating chimeric proteins might allow the identification of functionally important regions of the two molecules and, in particular, to identify the molecular structures responsible for the functional differences between C3 and CVF, such as being able to form a physicochemically stable convertase or to be resistant to inactivation by factors H and I. In addition to being research tools, chimeric proteins of C3 and CVF might also allow the generation of human C3 derivatives with CVF-like function such as being able to deplete complement in clinical situations where complement activation is part of the disease process. As it turns out, chimeric proteins were successfully created for both purposes: the generation of chimeric proteins did indeed allow the identification of functionally important regions or amino acid residues, and human C3 derivatives with CVF-like function (referred to as humanized CVF (hCVF)) have indeed become a novel therapeutic concept for complement inhibition in complement-mediated diseases (Vogel et al. 2014).

A first approach was to generate loss-of-function chimeric proteins by exchanging amino acid sequences in CVF with homologous sequences from cobra C3 (Wehrhahn et al. 2000; Hew et al. 2012). Using this approach, it was found that the C-terminus of pro-CVF (corresponding to the CVF β -chain) is responsible for forming a stable convertase and being able to deplete serum complement. The replacement of only the 113 C-terminal residues of CVF, representing approximately three quarters of the C345C domain, with the corresponding cobra C3 residues resulted in the loss of this CVF function (Hew et al. 2012). The use of loss-of-function chimera also identified the functional importance for C5-cleaving activity to the region spanning from the C-terminal end of the CVF α -chain through most of the CVF γ -chain as outlined above (Hew et al. 2012). Moreover, substitutions of only a very small number of amino acid residues resulted in functional changes, allowing

the identification of functionally important amino acid residues (Fritzinger et al. 2004; Hew et al. 2012).

The second experimental approach was to generate gain-of-function hybrids by creating chimeric proteins where portions of human C3, or only a few amino acid residues, were replaced with the corresponding sequences or amino acid residues from CVF. Whereas this approach also allowed the identification of functionally important amino acid residues (Fritzinger et al. 2007a, b; Hew 2010; Hew et al. 2008, 2010), replacement of the C-terminal portion of human C3 with corresponding sequences from CVF resulted in human C3 derivatives with CVF-like function, collectively referred to as humanized CVF (hCVF) (Fritzinger et al. 2009; Vogel and Fritzinger 2007; Vogel et al. 2014).

Humanized CVF: Structure and Activity

Figure 13 shows the schematic chain structure of two hCVF proteins. hCVF can be produced in S2 cells, resulting in a mixture of a C3-like and C3b-like form, just like rCVF (Fig. 11) (Fritzinger et al. 2009). Production in stably transfected Chinese hamster ovary (CHO) cells resulted in high yields of the C3b-like form only (Fig. 11) (Vogel et al. 2014). hCVF proteins form convertases as stable or even more stable than CVF and are able to deplete complement in serum both in vitro (Fig. 14) and in vivo (Fig. 15) indicating that they also exhibit significant resistance to factors H and I (Fritzinger et al. 2009; Vogel et al. 2014). The depletion is very rapid, within minutes, but does not last as long as depletion with natural CVF (Fig. 15), in all likelihood because hCVF remains partially susceptible to inactivation by factors H and I (Fritzinger et al. 2009; Vogel et al. 2014). Like recombinant CVF, hCVF does not have an intramolecular thioester (Fritzinger et al. 2009). This fact is unexplained because hCVF contains the C3d portion of human C3 (TED domain) and exhibits sequence identity of approximately 95 % with human C3, and all important amino acid residues known to be required for thioester formation in human C3 (see above)

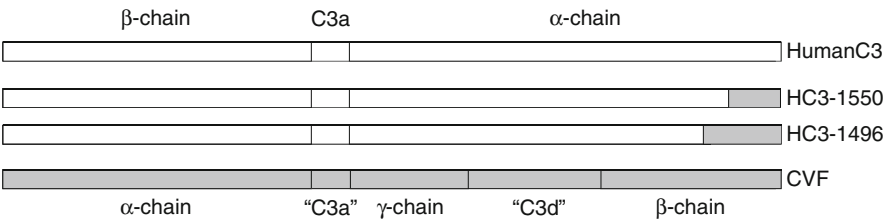


Fig. 13 Schematic representation of the chain structures of hCVF proteins. The chain structures of two hCVF proteins (HC3-1550 and HC3-1496) are shown. The number given in the name of an hCVF protein (e.g., HC3-1496) is the first amino acid residue replaced by CVF sequence, using human pre-pro-C3 numbering. The N-terminus is to the left. The chain homologies with human C3 and CVF are indicated. Please note that even in the CVF portion of the hCVF proteins, approximately 45 % of the amino acid residues are identical to human C3

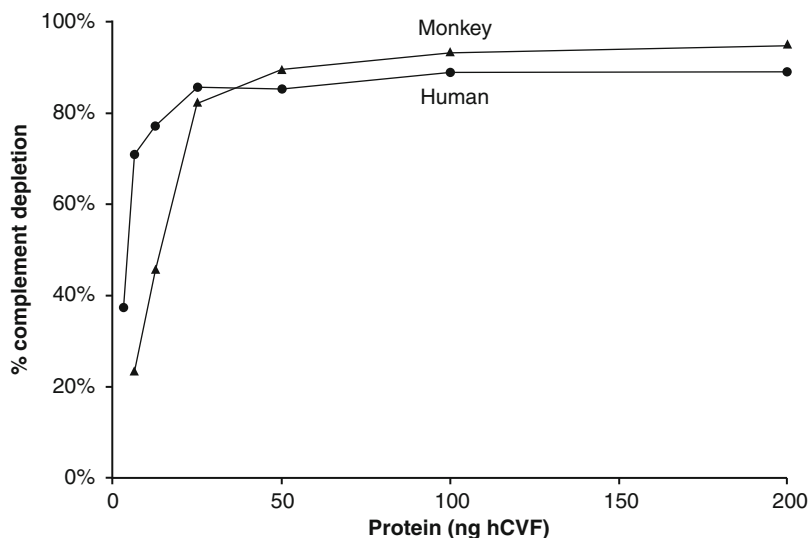


Fig. 14 In vitro complement depletion by hCVF. Shown are dose response curves for in vitro complement depletion of human and cynomolgus monkey serum by hCVF protein HC3-1496

are present in hCVF. Convertases formed with hCVF exhibit C3-cleaving activity just like C5b,6 and rC5b,6. In contrast, hC5b,6 does not exhibit C5-cleaving activity for purified human C5 (Fig. 12). The lack of C5-cleaving activity of the hC5b,6 convertase is corroborated by a lack of bystander lysis activity and the absence of C5a generation in monkey serum, both in vitro and in vivo (Fritzinger et al. 2009; Vogel and Fritzinger 2010; Vogel et al. 2014). The lack of C5-cleaving activity is a fortuitous property of hCVF in light of potential human application (see below).

Humanized CVF: A Novel Experimental Therapeutic Agent for Complement Depletion

The complement system is an integral component of both innate and adaptive immunity. However, complement is also involved in the pathogenesis of many diseases, and significant efforts are being made to develop drugs and biologicals for therapeutic complement inhibition. Most approaches aim to inhibit complement by either preventing the activation of a complement component or blocking the activity of an activated complement component. Examples include low molecular weight inhibitors of complement enzymes, receptor antagonists for the C3a and C5a anaphylatoxin receptors, and a cyclic peptide (compstatin) binding to C3 and inhibiting convertase formation, and inhibiting the C3 convertase by a recombinant fluid-phase form of complement receptor CR1 or by a fusion protein of factor H with complement receptor CR2 (Lachmann and Smith 2009; Ricklin and Lambris 2013a, b). The use of a monoclonal antibody to C5, inhibiting its activation, has been in clinical use for a

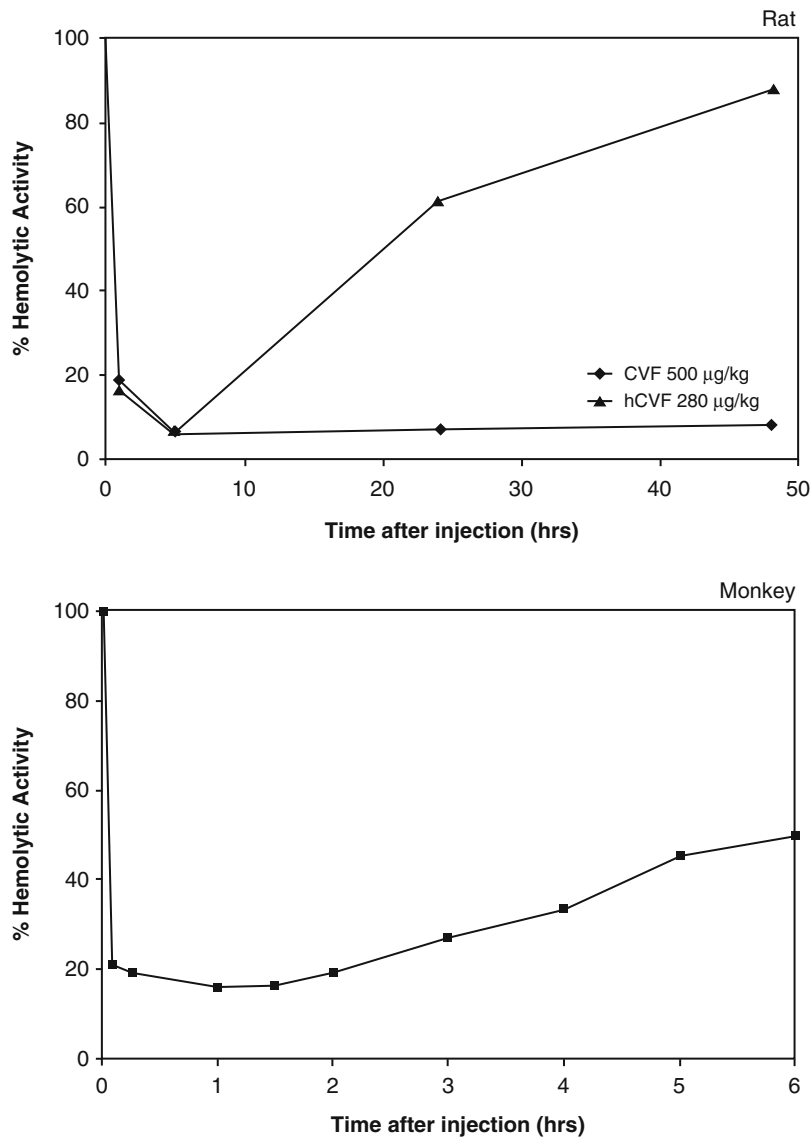


Fig. 15 In vivo complement depletion by hCVF. Shown is a time course of complement depletion in rat after i.p. injection of hCVF protein HC3-1496 at 280 µg/kg (*upper panel*) and in cynomolgus monkey after intra-arterial injection of hCVF protein HC3-1496 at 1,000 µg/kg (*lower panel*). Please note the rapid depletion of complement within minutes of injection. Please also note the longer period of complement depletion achieved by natural CVF

number of years in patients with paroxysmal nocturnal hemoglobinuria (PNH) (Hillmen et al. 2004; Roth et al. 2011).

hCVF represents a distinctly different approach to pharmacological intervention of complement which is neither based on inhibition of a complement component nor its activated fragment but on depletion of complement. The therapeutic efficacy of hCVF has been studied in a number of preclinical disease models (Vogel and Fritzing 2010; Vogel et al. 2014). In all studies, hCVF protein HC3-1496 was used which is a human C3 derivative in which the C-terminal 168 amino acid residues of the C3 α -chain have been replaced with the corresponding 168 amino acid residues from the C-terminus of the CVF β -chain (Fig. 13) (Vogel and Fritzing 2010). It is important to note that even in this stretch of 168 amino acids from CVF, 73 amino acids (43.5 %) are identical to human C3, 34 amino acids (20.2 %) represent conservative replacements, and only 61 amino acids (36.3 %) represent CVF residues. Accordingly, only 3.7 % (exclusive of conservative replacements) or 5.7 % (inclusive of conservative replacements) of the total number of amino acid residues in HC3-1496 of the 1,641 amino acids of human C3 are different (Fig. 3). The convertase formed with HC3-1496 is more stable than CVF_{Bb} and exhibits a higher C3-cleaving activity (Vogel et al. 2014; Vogel and Fritzing 2010). It causes rapid complement depletion, within minutes (Fig. 15), and, importantly, is devoid of C5-cleaving activity like all other hCVF proteins tested so far (Fig. 12) (Vogel et al. 2014; Vogel and Fritzing 2010). Because of these very favorable properties, HC3-1496 has been the primary candidate of hCVF in preclinical studies. It has been produced in S2 cells and in CHO cells, resulting in a mixture of C3-like and C3b-like form like rCVF (in S2 cells) or solely as a C3b-like form (in CHO cells) (Fig. 11) (Vogel et al. 2014).

Therapeutic Efficacy of hCVF in Preclinical Models of Human Disease

hCVF protein HC3-1496 was used for complement depletion in eight murine models of human disease as well as one in vitro model of a human disease. The hCVF dose employed in the in vivo models was between 250 μ g/kg and 500 μ g/kg. Figure 16 shows the beneficial therapeutic effect of hCVF in preventing myocardial ischemia-reperfusion injury. In this model, myocardial ischemia was induced by ligation of the left anterior descending coronary artery for 30 min. Subsequently, the myocardium was reperfused for 4 h. Depletion with hCVF resulted in a significant reduction of the reperfusion injury as demonstrated immunohistochemically by reduced deposition of C3b (Fig. 16, upper panel), morphologically by a smaller infarct size (Fig. 16, lower left panel), and functionally by a larger ejection fraction (Fig. 16, lower right panel) (Gorsuch et al. 2009). Similarly, complement depletion with hCVF was also protective in a murine model of gastrointestinal ischemia-reperfusion injury (Vogel et al. 2015).

Other murine models of human disease where complement depletion by hCVF has been shown to result in significant therapeutic benefit include age-related macular degeneration (AMD) (Fritzing et al. 2010), ventilator-induced lung injury (Takahashi et al. 2011), collagen-induced arthritis (Fritzing et al. 2008b), and myasthenia gravis (Huda et al. 2011; Vogel et al. 2014). In a murine model of

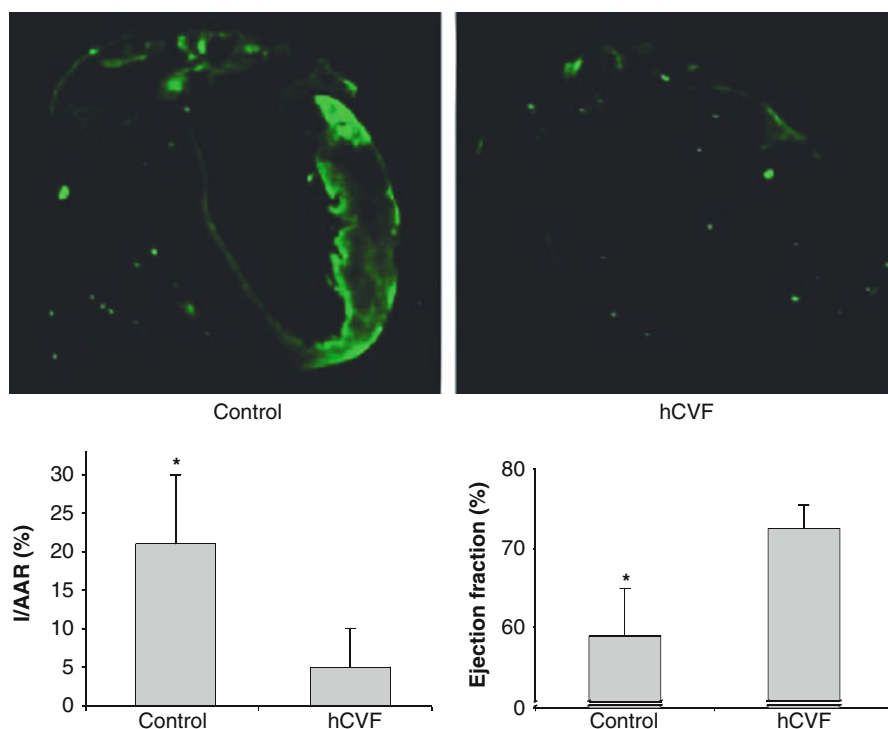


Fig. 16 Effect of complement depletion with hCVF protein HC3-1496 in a murine model of myocardial ischemia-reperfusion injury. The *upper panel* shows microscopic images after immunohistochemical staining for C3b deposition. The *lower left panel* shows the size of the infarcted area as a percentage of the area of risk. The *lower right panel* shows the ejection fraction as a measure of left ventricular function (* $p < 0.002$ for infarct size, * $p < 0.05$ for ejection fraction)

hemophilia A, complement depletion with hCVF prior to weekly injections of recombinant factor VIII prevented the generation of a functionally relevant antibody response to factor VIII, a complication observed in up to 30 % of hemophilia A patients (Rayes et al. 2014a).

In a murine model of lymphoma therapy with a monoclonal antibody (38C13 lymphoma cells; MF11G6 anti-lymphoma monoclonal antibody) it was found that complement depletion with hCVF 4 h prior to antibody injection, followed by a second dose of hCVF 2 days later, resulted in survival of 80 % of the mice (Fig. 17). In contrast, all mice died in the three control groups without treatment or treated with the antibody or hCVF alone (Fig. 17). This result suggests that complement depletion with hCVF prevents the binding of C3b to the lymphoma cells, thereby facilitating NK cell-mediated lysis (Wang et al. 2009).

In an *in vitro* model of human paroxysmal nocturnal hemoglobinuria (PNH), it was found that complement depletion with hCVF protected human PNH erythrocytes from complement-dependent lysis (Fritzinger et al. 2008a; Vogel et al. 2014).

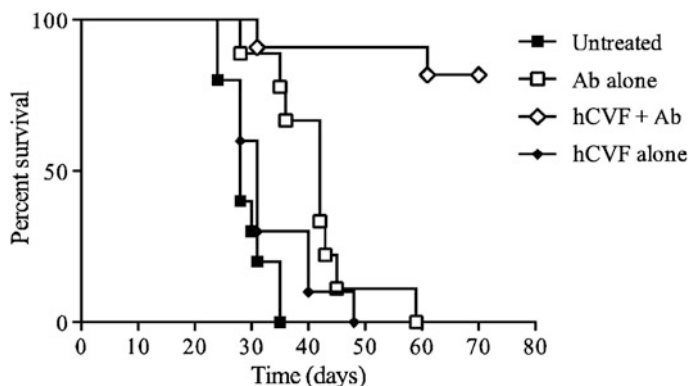


Fig. 17 Effect of complement depletion with hCVF protein HC3-1496 on the therapeutic efficacy of monoclonal antibody therapy in a syngeneic mouse model of lymphoma. Mice were depleted of complement with an initial dose of 400 $\mu\text{g/kg}$ of hCVF protein HC3-1496 injected i.p. 3 days after the inoculation of 38C13 lymphoma cells and 4 h prior to the injection of the MF11G6 anti-lymphoma monoclonal antibody and with a second dose of hCVF 2 days later. Please note that 80 % of the complement-depleted mice treated with antibody survived

Collectively, these results from multiple preclinical studies demonstrate the therapeutic efficacy of complement depletion by hCVF (Vogel et al. 2014, 2015; Vogel and Fritzinger 2010).

Lack of Toxicity of hCVF

No side effects of complement depletion by hCVF were observed in any of the abovementioned animal models of disease. As mentioned further above, the only side effect of massive fluid-phase complement activation by natural CVF is a consequence of the anaphylatoxins C3a and C5a derived from the complement components C3 and C5, respectively. The C3a anaphylatoxin is readily inactivated by carboxypeptidase N which removes the C-terminal arginine. Whereas carboxypeptidase N also removes the C-terminal arginine residue from the C5a anaphylatoxin, the resulting C5a-des-Arg retains its ability to bind to and activate neutrophils, resulting in sequestration to the lungs and causing a fleeting inflammatory lung injury (Till et al. 1982, 1987; Mulligan et al. 1996). As mentioned above, hCVF lacks C5-cleaving activity and, therefore, does not generate C5a (Fig. 12) (Fritzinger et al. 2009; Vogel et al. 2014; Vogel and Fritzinger 2010). Accordingly, no lung damage would be expected from complement depletion with hCVF. To assess any potential side effect of complement depletion with hCVF, cynomolgus monkeys were depleted of their complement by intra-arterial administration of hCVF into the pulmonary artery. As shown in Fig. 18, injection of hCVF into the pulmonary artery had no effect on pulmonary or cardiac function (Fritzinger et al. 2008c; Vogel et al. 2014; Vogel and Fritzinger 2010). No other acute side effects were observed in the monkeys or in any of the murine models mentioned above, consistent with the experience of well over 40 years of complement depletion with natural CVF in laboratory animals.

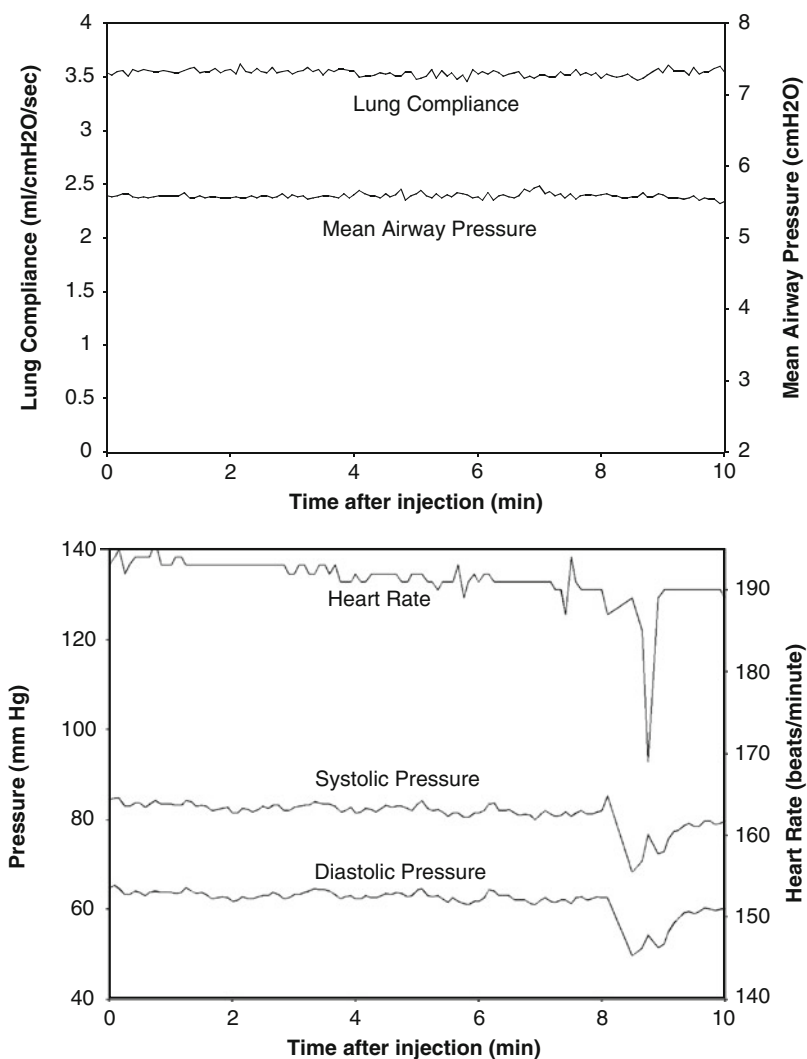


Fig. 18 Effect of complement depletion with hCVF on pulmonary and cardiac function in cynomolgus monkey. Complement depletion was achieved by intra-arterial injection into the pulmonary artery of hCVF protein HC3-1496 at 250 $\mu\text{g/kg}$. Mean airway pressure and lung compliance (*upper panel*) as well as heart rate and systolic and diastolic blood pressure (*lower panel*) were continuously monitored. Please note that complement depletion by hCVF had no measurable effect on pulmonary or cardiac functions

Another potential adverse effect from complement depletion by hCVF could be a consequence of being in a state of prolonged complement depletion. So far, mice have been depleted by hCVF for up to 30 days without any signs of infectious complications (Fritzinger et al. 2008b, 2010; Huda et al. 2011; Vogel et al. 2014), a period significantly longer than can be achieved with natural CVF. As mentioned

above, complement depletion by CVF or hCVF always results in the presence of residual C3 in serum and in residual serum complement activity, and transgenic mice constitutively expressing CVF have not shown any tendency to develop infections and exhibit a normal lifespan (Andrä et al. 2002; Fritzinger et al. 2010).

Potential Immunogenicity of hCVF

As mentioned above, CVF is immunogenic, limiting its use for complement depletion to a single application (Cochrane et al. 1970; Pryjma and Humphrey 1975). The immunogenicity of CVF is most likely due to both differences in the amino acid sequence and the presence of its unusual oligosaccharide chains. hCVF was designed to minimize its immunogenicity for human applications. As outlined above, hCVF proteins are human C3 derivatives with an overall sequence homology of approximately 95 % to human C3 (Fritzinger et al. 2009; Vogel et al. 2014), with amino acid differences being limited to the very C-terminal region of the α -chain (Fig. 13). Moreover, the three-dimensional structure in this region is highly conserved between CVF and C3 (Fig. 5), with essentially identical three-dimensional structures of human C3 and hCVF predicted by *in silico* modeling (Fritzinger et al. 2008b; Vogel et al. 2014). In addition, hCVF proteins are produced in eukaryotic insect or hamster cells, resulting in glycosylation that is far more similar to human (Kock et al. 2004). Importantly, recent experimental results confirmed the prediction of very low or even absent immunogenicity of hCVF. In the abovementioned mouse model of hemophilia A, repeated injection with hCVF in weekly intervals resulted in efficient depletion of C3 and the prevention of the generation of anti-factor VIII antibodies, indicating the absence of a functionally relevant immune response to hCVF (Rayes et al. 2014b). This was in stark contrast to natural CVF whose effectiveness for complement depletion was essentially limited to a single application which failed to prevent the generation of anti-factor VIII antibodies (Rayes et al. 2014b).

Ultimately, the immunogenicity of hCVF in humans cannot be predicted. However, as repeated injections of hCVF did not elicit a measurable immune response in mice, it is reasonable to expect that hCVF would be even less immunogenic in humans as 95 % of the amino acid sequence of hCVF is identical to human C3 which is likely to be immunogenic in mice.

Collectively, these results indicate that hCVF is a conceptually different therapeutic approach for complement depletion in diseases with complement pathology. The apparent lack of toxicity and immunogenicity as observed in laboratory animals, from mice to primates, makes it reasonable to predict that hCVF will become an effective clinical tool for complement depletion.

Conclusion

CVF has been the object of investigation for well over a century. It is a highly unusual venom component. While not exerting toxic activity, it has evolved to exploit the prey's complement system to accelerate the toxic actions of other

venom components by facilitating their faster uptake into the bloodstream. CVF was also an important tool in deciphering the molecular reaction sequence of the alternative pathway of complement activation. After its safe use for complement depletion of laboratory animals had been shown, CVF has served for well over four decades and continues to serve as a valuable research tool to understand the biological functions of the complement system and its role in the pathogenesis of disease. More lately, CVF has also served as the lead substance to design a novel experimental biological agent (humanized CVF) with potential therapeutic application in many human diseases.

Appendix

The taxonomy of the Asiatic cobras is complex and has undergone changes. There currently are eleven recognized species within the Asiatic cobra complex (Wüster 1996), six of which occur on the Asian mainland (*N. naja* (Indian or spectacled cobra), *N. kaouthia* (monocellate or monocled cobra), *N. atra* (Chinese cobra), *N. oxiana* (Central Asian cobra), *N. siamensis* (Indochinese spitting cobra), *N. mandalayensis* (Burmese spitting cobra)), including two species that were only rather recently described or redescribed (*N. siamensis*, *N. mandalayensis*) (Slowinski and Wüster 2000; Wüster et al. 1997), and five that occur only on Southeast Asian archipelagos of Indonesia, the Philippines, and the Andaman Islands (*N. sputatrix* (Southern Indonesian or Javan spitting cobra), *N. philippinensis* (northern Philippine cobra), *N. sagittifera* (Andaman cobra), *N. samarensis* (southern Philippine cobra), *N. sumatrana* (Equatorial spitting cobra)). Formerly, there was only one recognized Asiatic cobra species (*N. naja*) with multiple subspecies (e.g., *N. n. naja*, *N. n. kaouthia*, etc.) (Mehrtens 1987; Phelps 1981; Wüster 1996). Accordingly, many earlier reports in the toxinological literature, including reports on CVF, identify *N. naja* as the venom source (Cochrane et al. 1970; Müller-Eberhard and Fjellström 1971). Many studies denote the origin of the venom from *N. n. siamensis* (Eggertsen et al. 1981; Vogel and Müller-Eberhard 1984). However, *N. n. siamensis* was not a recognized species or subspecies until recently, and the older designation by venom suppliers of *N. n. siamensis* refers to the fact that the venom originated from Thailand. Most likely, the venom was derived from *N. kaouthia*, although the precise origin will remain obscure, and it may even represent a mixture of more than one species (Vogel 1991; Wüster and Harvey 1996; Wüster et al. 1997).

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Non-Cobra Venom Factor Venom Components Acting on Complement Proteins

16

Denise V. Tambourgi and Carmen W. van den Berg

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Abstract

The complement system, one of the first lines of defense of the innate immunity, is an important mechanism by which the body recognizes foreign substances and pathogens. This system consists of over 35 proteins, among them regulators and membrane-bound receptors and plasma proteins that interact with multiple cells and mediators of the immune system. These interactions vary according to the pathophysiologic context and occur at different stages of the immune reaction. Snake venoms contain potent cocktails of proteolytic enzymes, and the possibilities of these enzymes to interact with the complement system by either activating

D.V. Tambourgi (✉)

Immunochemistry Laboratory, Research Division, Butantan Institute, São Paulo, SP, Brazil

e-mail: denise.tambourgi@butantan.gov.br

C.W. van den Berg

Institute of Molecular and Experimental Medicine, School of Medicine, Cardiff University, Cardiff, UK

e-mail: vandenbergcw@cardiff.ac.uk

it or inhibiting have been investigated in many studies. The activation of the complement system by snake venoms, by generation of the potent anaphylatoxin C5a, could help the spread of the other toxic components and thereby enhancing the immobilization of the prey. Discovery of snake components that interact with the complement system may elucidate more about how the venom of snakes is spread in their prey, as well as the role of this interaction on the pathogenesis of the envenomations. It may also lead to discovery of components that aids the studies of the roles of complement in health and disease and also may yield novel anticomplement therapeutic agents. This review summarizes the key contributions regarding the action of snake venom toxins on the complement system.

Keywords

Complement system • Snake venoms • Toxins • Inflammation

Introduction

Snake venoms contain a plethora of toxic molecules including many proteolytic enzymes. These proteolytic enzymes have a range of functions in the pathology caused by the envenomation, and some may aid to immobilize and/or kill the prey. The complement system (C) is an important target of the venom of the cobra, as cobras contain an analogue of the third component of complement C3. This analogue, named Cobra venom factor (CVF) (also subject of another chapter in this book), causes activation of the complement system and the generation of large amounts of C5a. C5a is a potent anaphylatoxin and its generation leads to vasodilation, which may facilitate the spread of other venom components and leads to hypotension, which can lead to loss/reduced consciousness, while C5a-induced bronchoconstriction could lead to asphyxiation. C5a can also contribute to edema. In this chapter, it is explored the mechanisms other than CVF whereby snake venoms induce complement activation, which may aid the immobilization and killing of the prey and may contribute to human pathology after envenomation.

The Complement System

The complement system, one of the first lines of defense of the innate immunity, is a major mechanism by which the body recognizes foreign substances and pathogens. This system consists of over 35 proteins, among them regulators, membrane-bound receptors, and plasma proteins that interact with multiple cells and mediators of the immune system. These interactions vary according to the pathophysiologic context and occur at different stages of the immune reaction.

The main function of the complement has been linked to the recognition and elimination of pathogens by opsonization by C3b and/or direct lysis and stimulation of phagocytosis and removal of immune complexes. Furthermore, generation of C5a

leads to inflammation by induction of vasodilation (anaphylaxis), chemotaxis, and general cell activation. However, in recent decades, it has been proven that this can also play important immunoregulatory functions. These include enhancing of the humoral immune response, through the interaction of the fragments C3d with CD21 (CR2: C3d Receptor) or CD35 (CR1: C3b/C4b Receptor) present in the follicular dendritic and B cells; assisting in maintenance of antibody secreting cells and memory B cells; reducing the activation threshold for specific T cells; modeling the development of the repertoire of natural antibodies; and regulating the tolerance to own nuclear antigens, such as DNA and chromatin. Another biological function of the complement system is the removal of undesirable debris, such as apoptotic and necrotic cells. In addition to its immunoregulatory functions, many studies have been conducted on the pathogenic role of complement in inflammatory, autoimmune, and ischemic diseases (reviewed by Bajic et al. 2015).

C-Activation

The complement system is activated by three mechanisms (Fig. 1), which allow the body to respond to inflammatory, infectious, ischemic, or necrotic events, as well as self and foreign antigens. Thus, the Classical Pathway (CP) is activated by binding of C1q, a pentameric molecule, which in the presence of Ca^{2+} forms a complex with serine proteases C1r₂ and C1s₂, to the Fc regions of IgM antibodies and certain IgG isotypes complexed to antigens. The interaction of C1q to the antibody allows activation of C1r, and this activated cleaves C1s, which in turn acts on the C4 and C2 components forming the complex C3 convertase (C4b2a) and acting subsequently on C3 and forming the C5 convertase C4b2a3b (reviewed by Merle et al. 2015).

The Alternative Pathway (AP), phylogenetically the oldest pathway, can be activated on the surface of pathogens or other targets by a process called “tick over” auto activation mechanism (Müller-Eberhard 1988). Serum C3 undergoes spontaneously and in solution conformational change generating a form called C3 (H₂O) (“tick over” rate of 1 % per hour), which permits factor B to interact in the presence of Mg^{2+} . Subsequently, factor B (FB) can be cleaved by factor D (FD) into to Ba and Bb, thereby producing a C3 convertase, C3(H₂O)Bb, which cleaves additional C3 molecules, resulting in the generation of C3b that covalently binds the target surface by means of a thioester bond (reviewed by Merle et al. 2015).

In addition, the alternative pathway can be an “amplification loop” of the classical and lectin pathways. C3b, generated by through these pathways, when fixed onto a target it binds factor B, resulting again, after the conformational change of FB, in cleavage by FD, similarly to the process of “tick over” (Müller-Eberhard 1988).

The last identified pathway of complement activation, the lectin pathway (LP), can be activated by the interaction of mannose-binding lectin (MBL) or ficolins to repetitive mannose residues and *N*-acetyl glucosamine, respectively, present on the surface of microorganisms. Similar to C1, MBL and ficolins are multimeric proteins and are complexed with the serine proteases MASPs (mannan-binding lectin associated serine proteases). The MASPs have identical organization of domains, which

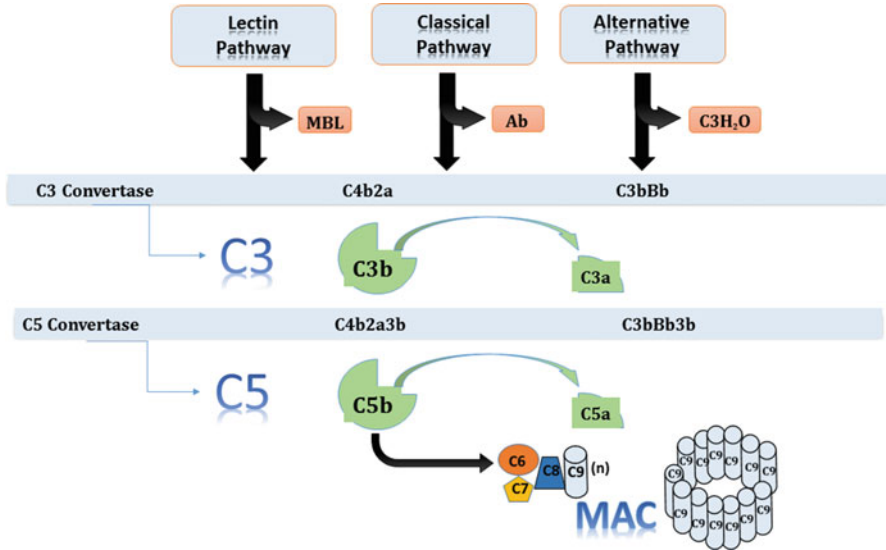


Fig. 1 Complement activation pathways. Classical, lectin, and alternative complement pathways activation leads to the formation of C3 convertase complexes: C4b2a for classical and lectin pathways and C3bBb for the alternative pathway. C3 convertases are able to cleave C3 in C3a and C3b. Additional interaction of C3b molecules to the C3-convertases results in the formation of C5 convertases: C3bBbC3b for the alternative pathway and C4b2a3b in the classical and lectin pathways. C5 convertases are able to cleave C5 initiating the lytic terminal pathway of the complement system. This process results in the generation of the anaphylatoxin C5a and the fragment C5b. C5b constitutes the first component of the terminal pathway and the subsequent binding of C6, C7, C8, and multiple C9 molecules and forms the membrane attack complex (MAC/C5b-9)

are similar to the serine proteases from the classical pathway, C1r and C1s. When MBL/ficolin is bound to the target, the MASPs are activated in a manner similar to C1r and C1s, resulting in the cleavage of C4 and C2 to form the C3 convertase C4b2a, a common product to the activation of the classical and lectin pathways (reviewed by Merle et al. 2015).

Activation of the initiation pathways of complement, by any of the three mechanisms, results in the formation of C5-convertase, which is formed by binding of an additional C3b molecule to the C3-convertases, resulting in C3bBbC3b for the alternative pathway, and C4b2a3b in the classical and lectin pathways; both cleave C5 initiating the lytic terminal pathway of complement. This process results in the generation of the anaphylatoxin C5a and the fragment C5b; the latter constitutes the first component of the terminal pathway and subsequent binding of C6, C7, C8, and multiple C9 molecules and forms the membrane attack complex (MAC/C5b-9). Activation of the terminal pathway is responsible for many inflammatory reactions particularly through interaction of C5a with its receptors (C5aR), but also through membrane attack complex-induced cell activation (reviewed by Merle et al. 2015).

Anaphylatoxins

The anaphylatoxins generated by activation of the complement system, C3a and C5a, play important roles in the inflammatory process. The anaphylatoxins are potent inflammatory mediators that act on both immune and nonimmune cells. Especially C5a is a potent molecule, and it can directly or indirectly (e.g., via mast cell degranulation) induce vasodilation, increase vascular permeability, and induce bronchoconstriction. In immune cell, C5a can induce the respiratory burst and enhance phagocytosis. The anaphylatoxins can also enhance adhesion of immune cells to endothelial cells, and C5a is a potent chemotactic agent. In addition, the anaphylatoxins are also known to influence the generation of adaptive immune responses. They are small polypeptides consisting of 74–77 amino acids, with 38 % amino acid identity, sharing therefore a high degree of structural homology. C5a in general is a more potent proinflammatory molecule than C3a, which can also be anti-inflammatory. A conserved five amino acid sequence in the C-terminal portion of C3a and C5a is responsible for the binding to and activation of their respective receptors C3aR and C5aR, both of which are seven transmembrane G-protein-coupled receptors (reviewed by Klos et al. 2009).

C-Regulation

As unregulated C-activation can lead to fast amounts of pro-inflammatory anaphylatoxin C5a generation, activation pathways are tightly regulated (Fig. 2).

Regulation of the CP and LP is accomplished by C1-Inhibitor (C1-INH), a serpin, which in addition to the complement components C1r, C1s, and the MASPs, also inhibits several coagulation proteins including kallikrein, FXIa, and FXIIa. Deficiency in C1-INH can cause hereditary angioedema (HAE), which is a life-threatening condition and can lead to death by asphyxiation. The edema caused by C1-INH deficiency is not so much caused by C-activation, but rather by the uncontrolled activity of kallikrein, leading to excess bradykinin production, a potent vasodilator. Nevertheless, C1-INH deficiency will also lead to generation of C5a, as spontaneous activation of the CP and LP may occur.

Other important regulatory steps take place at the level of the C3-convertase by several structurally homologous proteins, which can either dissociate the C3-convertase, for example, Decay accelerating Factor (DAF), C4 Binding Protein (C4BP), Factor H (FH), and Complement Receptor 1 (CR1), or act as a co-factor (MCP: Membrane Cofactor Protein; C4 Binding Protein: C4BP; FH and CR1) for enzymatic degradation by Factor I (FI). Deficiency of some of these regulators has been shown to result in, for example, kidney failure due to atypical Hemolytic Uremic Syndrome (aHUS).

In addition to the regulation of the activation of the complement system, it is also important to regulate the activity of the anaphylatoxin C5a, which is accomplished by enzymatic cleavage of the C-terminal arginine by carboxypeptidases present in the plasma. No deficiencies in regulation of the C5a activity have been reported, but

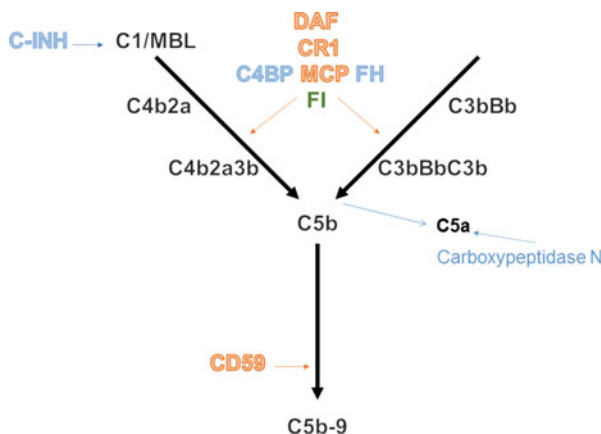


Fig. 2 Regulation of the Complement system activation. Regulation of the CP and LP is accomplished by C1-Inhibitor (C1-INH), which in addition to inhibit C1 and the MASPs, it also acts on coagulation proteins, such as kallikrein, FXIa, FXIIa. Important regulatory steps also take place at the level of the C3-convertase by proteins, which can either dissociate the C3-convertase (DAF, C4BP, FH, and CR1) or act as a co-factor (MCP, C4BP, FH, and CR1) for enzymatic degradation by FI. The activity of the anaphylatoxin C5a is also regulated by enzymatic cleavage of the C-terminal arginine by carboxypeptidases present in the plasma. The main regulator of the membrane attack complex is CD59, a protein that prevents the unfolding of C9 and thus the lytic pore formation

as several different carboxypeptidases can accomplish the inactivation of C5a, multiple deficiencies would be required to result in potential symptoms. Inhibition of C5a generation and C5a-mediated activation of the C5aR is a major target for pharmaceutical drug development (reviewed by de Cordoba et al. 2012). The main regulator of the membrane attack complex is CD59, a glycosylphosphatidylinositol anchored protein that prevents the unfolding of C9 and thus prevents lytic pore formation.

Snake Venoms that Contain Substances that Interact with the Complement System

Cobra venom has been known for a long time to contain a component named Cobra venom factor (CVF), which is a powerful activator of the complement system. It is an analogue of C3 and forms a very stable complex with Bb, and by activating the terminal pathway, it is able to generate a vast amount of C5a. CVF has been instrumental in the elucidation of the complement cascade, and in our knowledge and understanding of the role of complement in health and disease. Applications of CVF have been discussed in recent reviews (Vogel and Fritzinger 2010; Vogel et al. 2014) and will be presented in another chapter of this book.

While cobra venom contains CVF, an analog of the human complement component C3, many snake venoms contain potent cocktails of proteolytic enzymes, and

the possibilities of these enzymes to interact with the complement system, by either activating or inhibiting it, have been investigated in many studies. In this chapter, it will be reviewed how snake venoms with their potent enzymatic activities interact with the complement system, and if any snake venoms, other than cobra venom and CVF, have been identified that display such an activity. Complement activation by snake venoms, resulting in the generation of the potent anaphylatoxin C5a, could help spreading the other toxic components and thereby enhancing the immobilization of the prey, although this has not even been investigated for the well-described complement activator CVF. Discovery of snake components that interact with the complement system may elucidate more about how the venom of snakes is spread in their prey. It may also lead to discovery of components that can be used in investigating the role of complement in health and disease, and in addition, it could potentially lead to the development of new therapeutic agents to modulate complement activity in disease.

After a thorough search of the literature, it was identified the following snakes that have substances in their venom that can interact with the complement system and can contribute potentially to the pathology observed. This will be reviewed here by genus.

Elapidae Family

Genus *Naja* (Cobra)

In addition to the C-activating CVF, the venoms of the genus *Naja* have also been shown to contain other components that can interfere with complement. While these components were only partially purified, a high molecular weight component (h-CoF) was suggested to interfere with the activation pathways (Ballow and Cochrane 1969). Furthermore, a 26-kDa molecule in the venom of *Naja haje*, which had the ability to bind native and activated C3 and C4, inhibited the CP and AP (von Zabern et al. 1981). Shoibonov et al. (2005) described oxiagin, a 50 kDa glycoprotein from *N. oxiana*, which prevented IgG binding and interaction of C4b and C2. This molecule is a member of the metalloprotease proteins of repolysins; however, no cleavage of C-components could be detected. It was described the proteolytic degradation of pure C3 by venoms *N. n. siamensis* (O'Keefe et al. 1988), *N. nigricollis*, and *N. naja* (Tambourgi et al. 1994), resulting in C3c- and C3d-like fragments. A metalloprotease, named atarase, that cleaved the terminal components C6, C7, and C8 and the AP component FB, resulting in inhibition of hemolytic activity, was described and isolated from the venom of *N. atra* (Sun and Bao 2010).

CVF Like Molecules in Genera *Austrelaps* and *Ophiophagus*

The cobra belongs to the family Elapidae, and CVF has been identified in many cobra species venoms. Two snakes in this Elapidae family, but belonging to different genera, *Austrelaps superbis* (lowland copperhead) and *Ophiophagus hannah* (king cobra), have also been shown to contain CVF-like activities in their venoms.

Their proteins obtained after cloning and expressing confirm this. *A. superbis* components AVF1 and AVF2 showed 83 % identity with CVF (Rehana and Manjunatha Kini 2007), while *O. hannah* component OVF shared 87.5 % identity with CVF from *N. kaouthia* (Zeng et al. 2012). Thus far, no other snakes venoms have been reported to contain a CVF-like molecule.

Genus *Micrurus* (Coral Snake)

It was recently showed that several snake species from the genus *Micrurus* contained metalloproteinases that directly cleaved purified C3 and C1-INH, while in whole serum their activity resulted in the activation the CP and/or LP (possibly by resulting from auto activation as a consequence of the C1-INH inactivation) and generation of significant amounts of C3a, C4a, and C5a in complete serum (Tanaka et al. 2012). It was not observed any reactivity with anti-CVF in the *Micrurus* venoms.

Viperidae Family

Genus *Crotalus*

Minta and Man isolated four proteins from *C. atrox* (rattlesnake) venom that inhibited the hemolytic activity of human serum (Minta et al. 1977). Heat labile metalloprotease proteins with Mr of 19.2 and 55 kDa consumed C1, C4, C2, C3, C5, and factor B in a whole serum assay, while a 14.5 kDa protein partially destroyed C2, C3, and C4 activities and a 12.6 kDa protein inactivated factor B, and to a lesser degree C2, C3, and C5. A C3-cleaving component was partially purified from *C. scutulatus scutulatus* venom (Rael and Jones 1983).

Genus *Bothrops* (Pitviper)

Farsky and collaborators showed that *B. jararaca* venom generated chemotactic activity for leukocytes in human sera (Farsky et al. 1997). From *B. asper* venom, it was identified a metalloproteinase, BAP-1, responsible for the induction of chemotactic activity (Farsky et al. 2000). It was showed that this chemotactic activity was blocked by sCR1 and by anti-C5aR antibodies, suggesting the generation and involvement of C5a. BAP-1 did not directly generate C5a, but activated the complement system (Farsky et al. 2000). A 30-kDa protein isolated from *B. atrox* venom, induced C-activation in serum, and also cleaved purified C3. Intramuscular injection of this protein in mice caused edema, neutrophil influx, and hemorrhage. C5-deficiency or C5-depletion reduced the neutrophil influx but not the hemorrhage (Rodrigues et al. 2004).

It was also demonstrated that venoms of several *Bothrops* species activated all three-complement pathways. A metalloprotease (C-SVMP) isolated from *B. pirajai* venom induced the complement cleavage fragments C3a, C4a, and C5a, in serum, as well as using purified complement components (Pidde-Queiroz et al. 2010, 2013). In addition, C1-INH was cleaved by C-SVMP resulting in an 83 kDa fragment (Pidde-Queiroz et al. 2013). C-SVMP treatment of mice resulted in partial complement, albeit it was not as efficient as CVF. Different complement dysregulation

mechanisms of C-SVMP and CVF may account for this, while C-SVMP acts by direct enzymatic cleavage of C-components and C-inhibitors, in mice CVF forms a very stable C3/C5 convertase with factor B, which has an in vivo half-life of 11.5 h (van den Berg et al. 1991), resulting in long-lasting activation and depletion. *B. pirajai* snake venom contains the serine proteases, BpirSP27 and BpirSV41, which inhibited the three complement activation pathways (Menaldo et al. 2013). Ayres et al. (2015) showed recently that venoms from *B. jararacussu* and *B. pirajai* and two isolated components, the serine protease (BjussuSP-I) and L-amino acid oxidase (BpirLAAO-I), inhibited the CP and AP likely as a consequence of complement consumption and both venoms induced chemotactic activity.

Genus *Trimeresurus*

Flavoxobin, a serine protease, isolated from the venom of *Trimeresurus flavoviridis* snake (Habu, pit viper), activated the AP by cleaving the C3 α -chain into two fragments. Edman degradation assay revealed that the flavoxobin induced cleavage in C3 took place at the same site as the cleavage induced by C3-convertases (Yamamoto et al. 2002). On human erythrocytes, *Trimeresurus flavoviridis* venom also slightly reduced CD55/DAF and CD59 expression, but the component responsible for this has not been identified (Yamamoto et al. 2001).

Genus *Agkistrodon*

A recombinantly expressed fibrinogenase (rFII) from *Agkistrodon acutus* (sharp-nosed viper) inhibited C-activity and membrane attack complex (MAC formation) on human umbilical vein endothelial cells (HUVECs), by cleaving and inactivating human C5, C6, and C9. In rats, rFII reduced C1q, C3, and C4. In an in vivo guinea-pig-to-rat heart transplant model, rFII showed to protect against hyper acute complement mediated rejection, which led the authors to suggest it as a potential therapeutic for xenotransplantation (Lin et al. 2013).

Genus *Daboia*

The genus *Daboia* has only one member *D. russelii* (Russell's viper), and human envenomation was found to be associated with increased anaphylatoxins C3a and C5a in the patients sera (Stone et al. 2013).

Conclusion and Future Directions

In conclusion, many snake venoms contain components that interfere with the complement system. Many of these components are proteases and can directly cause generation of anaphylatoxins such as C5a by their direct action on the C-components. This generation of C5a can cause direct and indirect (via mast cells) vasodilation and inflammation. The C-activation by these venoms/components can thus contribute to both the spread of the venom and the inflammatory response. Some venom components also cause C-activation by inactivation of C1-INH. While this also leads to the generation of C5a, a much more potent effect is likely induced by the

dysregulation of bradykinin generation as the enzyme responsible for this, kallikrein, is also inhibited by C-INH. C1-INH deficiency is a well-known phenomenon, resulting in extensive edema formation in hereditary angioneurotic edema, which is largely thought to be caused by excess bradykinin production. The components in snake venoms that can inactivate C1-INH likely contribute to the edema formation observed in many envenomations. Many of the above-described studies did not clearly identify the mechanism of activation or study the active component in isolation. Further studies are required and isolation and characterization of the active components may not just result in a better understanding of the complexity of how snake venoms work, but may also yield useful research tools and potentially novel therapeutic agents.

Cross-References

- [Cobra Venom Factor: The Unique Component of Cobra Venom That Activates the Complement System](#)

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Snake Venom Components Affecting the Coagulation System

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Miryam Paola Alvarez-Flores, Fernanda Faria, Sonia Aparecida de Andrade, and Ana Marisa Chudzinski-Tavassi

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Abstract

Snake venoms are rich sources of proteases of medical importance that affect hemostasis. In this chapter, the factors affecting blood coagulation are classified into two separate groups: the procoagulant and the anticoagulant proteins. The procoagulant proteins are subclassified as clotting factor activators and thrombin-like enzymes. The anticoagulant proteins are able to prevent blood clotting and maintain blood incoagulability and can include phospholipases A2, fibrin(ogen)olytics, protein C activator, and L-amino acid oxidase (enzymatic anticoagulants)

M.P. Alvarez-Flores (✉) • F. Faria (✉) • S.A. de Andrade (✉) • A.M. Chudzinski-Tavassi (✉)
Biochemistry Laboratory, Butantan Institute, São Paulo, Brazil
e-mail: miryam.flores@butantan.gov.br; fernanda.faria@butantan.gov.br; sonia.andrade@butantan.gov.br; ana.chudzinski@butantan.gov.br

or C-type lectin-like proteins, three-finger toxins (TFTs), and Kunitz-type proteinase inhibitors (nonenzymatic anticoagulants). The study of toxins from snake affecting blood coagulation contributed to the current understanding of the mechanism of activation and inhibition of clotting factors. Moreover, they have been useful in the therapeutic area, for example, as defibrinogenating agents (thrombin-like enzymes) and as antithrombotic agents (fibrinolytic enzymes). Here, the recent findings will be introduced.

Keywords

Snake venom • Procoagulant • Anticoagulant • Toxin • Coagulation

Introduction

Snake venoms are complex mixtures of toxins used as offensive weapons for immobilizing, killing, and digesting their preys turning their toxins important object of study for understanding the pathophysiology of envenoming. Moreover, snake venoms are rich sources of new molecules for drug discovery and potential tools for applications in health and biotechnology. A great diversity of snake venom toxins has been purified and well characterized, and new toxins are constantly being described. New approaches such as proteomic, transcriptomic, and genomic have enabled to acquire more knowledge about the great diversity of proteins and peptides in snake venoms. In addition, these strategies have allowed obtaining information about the similarity and variability of snake venoms in several aspects such as geographic, ontogenic, taxonomic, and even sex-based variations (Lomonte et al. 2014; Serrano et al. 2005; Zelanis et al. 2016).

A great percentage of toxins from snake venoms, especially from the Viperidae and Crotalidae families, affect different targets in the hemostatic system inducing coagulopathy and hemorrhage due to the action of toxins such as clotting factor activators and inhibitors, toxins affecting fibrinolysis and platelet, and hemorrhagins that disrupt the endothelium. Components acting on hemostasis are also found in Elapidae, but they are not common. Coagulopathy is one of the main causes of mortality. Traditional treatments for coagulopathy such as replacement therapy and heparin cannot be applied in snakebite coagulopathy due to the presence of thrombin-like enzymes which, unlike human thrombin, are not inhibited by heparin. This feature makes thrombin-like enzymes useful for assaying antithrombin III and other hemostatic parameters in heparin-containing samples. In general, the most effective treatment for envenoming is the antivenom, although its effectiveness in all cases of consumption coagulopathy is controversial (Maduwage et al. 2015).

Some toxins are responsible for coagulation disturbances because they can activate the coagulation factors X and II, degrade fibrinogen, and inhibit platelet aggregation. The presence or absence of a certain class of toxins, their abundance, and structural differences can reveal differences in the toxicological profile. For example, the hemorrhagic effects induced by viperids are well known to depend

upon the presence of snake venom metalloproteinases (SVMPs). Besides inducing hemorrhagic effects, SVMPs exhibit other biological effects such as coagulant, anticoagulant, and antiplatelet effects (Fox and Serrano 2008). On the other hand, the absence of SVMPs could be correlated with the lack of hemorrhagic effect as it was observed in the proteome of *Bothriechis nigroviridis* venom (Lomonte et al. 2014). Similarly, the procoagulant effect could be associated with the presence of snake venoms serine proteinases (SVSPs). A good example of this was shown in previous studies in Costa Rica, where specimens of *Bothrops asper* from the Pacific versant are more proteolytic, while specimens from the Caribbean versant are more procoagulant, hemorrhagic, and myotoxic. Both venoms showed differences in the toxin content: *B. asper* from the Pacific versant has more phospholipase A₂ (PLA₂), while specimens from the Caribbean versant have more SVSPs and disintegrins (Lomonte et al. 2014).

The study of toxins from snake affecting coagulation contributed to the current understanding of the mechanism of activation of coagulation factors. Moreover, they have been useful in the therapeutic area, for example, as defibrinogenating agent (thrombin-like enzymes) and as antithrombotic agents (fibrinolytic enzymes). Here, the recent findings in this area were described.

Toxins Affecting Blood Coagulation

In general, toxins interfering with coagulation can be procoagulant (prothrombin activator, thrombin-like enzymes, and factor X and factor V activators) or anticoagulant. These toxins could have structures with enzymatic and nonenzymatic activity on coagulation. Based on sequence and structure, they can be classified as metalloproteinases and serine proteinases (including FX, FV activators, prothrombin activator, and fibrin(ogen)olytic enzyme), Kunitz-type proteinase inhibitors, phospholipases A₂, L-amino acid oxidases, C-type lectin-like proteins, protein C activators, or three-finger toxins. Members of a particular group often exhibit marked selectivity on different targets (clotting factors, platelets, or endothelium). Most of the toxins affecting blood coagulation are metalloproteinases or serine proteinases (for details, see Fox and Serrano 2008; Lu et al. 2005; Sajevec et al. 2011; Serrano 2013).

Snake Venom Serine Proteinases (SVSPs)

SVSPs represent between 4 % and 26 % of the whole venom in viperids and affect mainly the hemostatic system. They are also present in venoms of Elapidae and Colubridae snakes. They show a high degree of sequence identity and a highly conserved catalytic triad (His57, Asp102, and Ser195) but different specificity toward macromolecular substrates. Besides acting in the digestion of the prey, SVSPs are able

to affect various physiological functions, as platelet aggregation, blood coagulation, fibrinolysis, the complement system, blood pressure, and the nervous system. They are inhibited by serine-modifying reagents such as phenylmethylsulfonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP), and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF). Most of them are monomers, but a few factor X activators and prothrombin activators have multiple subunits. Several studies have shown the high similarity in structure and function of prothrombin activators from snake venom and mammalian clotting factors (Serrano 2013).

Snake Venom Metalloproteinases (SVMPs)

SVMPs are the most abundant toxins affecting blood coagulation, particularly in snake venoms belonging to the Viperidae and Crotalidae families. SVMPs are present in all viperids, except in *B. nigroviridis* venom, in a range between 6 % and 72 % (Lomonte et al. 2014). SVMPs are critical to induce coagulopathy and hemorrhage or even to increase tissue factor levels in plasma than could be contributing to the intravascular coagulation syndrome during envenomation (Markland and Swenson 2013).

Hemorrhagic SVMPs destroy the basement membrane through degradation of the extracellular matrix proteins degradation and endothelium disruptions. SVMPs affect hemostasis by exerting effects in fibrinogenolysis, platelet aggregation, and blood coagulation as prothrombin activators. SVMPs are endoproteolytic enzymes that exhibit a characteristic zinc-binding active site motif HEXXHXXGXXH. Indeed, they are completely inactivated by ethylenediaminetetraacetic acid (EDTA) through chelation of divalent cations.

SVMPs are categorized into four classes according to their sizes and domains (for details, see Bjarnason and Fox 1994; Fox and Serrano 2008; Markland and Swenson 2013; Sajevic et al. 2011).

P-I class are the small SVMPs, having molecular masses between 20 and 30 kDa. They contain only a pro domain and the proteinase domain; examples of this class are hemorrhagic toxins (HT-b, c, d) isolated from *C. atrox* venom with molecular masses of 24 kDa.

P-II class are the medium-size SVMPs and molecular masses between 30 and 60 kDa. They contain a pro domain, a proteinase domain, and a disintegrin domain. P-II class can be exemplified by bilitoxin-1, a hemorrhagic glycoprotein of 32 kDa from *Agkistrodon bilineatus*. The disintegrin domain contains a Met-Gly-Asp (MGD) sequence instead of Arg-Gly-Asp (RGD) sequence found in many other disintegrins and does not have platelet aggregation inhibitory activity.

P-III class are the large SVMPs, having molecular masses between 60 and 100 kDa. They contain a structure formed by a pro domain, a proteinase domain, a disintegrin-like domain, and a cysteine-rich domain. The hemorrhagic toxin-a (HT-a, 68 kDa) from *C. atrox* venom and Jarharagin from *B. jararaca* are examples of this class. Note that P-III are generally more hemorrhagic than those of P-I class,

suggesting that the disintegrin-like domains play a key role in facilitating the action of these metalloproteinases by affecting platelet aggregation.

P-IV class are the proteinases that contained the P-III domain structure plus lectin-like domains connected by disulphide bonds. Nowadays this class has been phased out. In the current classification of SVMPs the P-IV class was included into the P-III class because there are no reports of a P-IV mRNA transcript and that the P-IV structure may just be another posttranslational modification of the P-III structure. Accordingly, in the most recent classification scheme, the P-IV class was embedded within the P-III class (Fox and Serrano 2008). This class can be exemplified by the factor X activator from *V. lebetina* (VLFXA) venom with a molecular mass of 85–91 kDa.

A large number of SVMPs has been sequenced over the years, and also the crystal structures of SVMPs are available, but, mostly are from the P-I class, still, some SVMPs of other classes also have been crystallized.

Procoagulant Molecules from Snake Venoms

In the cascade model of coagulation (Fig. 1), blood coagulation can initiate with the formation of the extrinsic tenase complex formed by factor VIIa and tissue factor (FVIIa/TF complex) or by the intrinsic pathway complex formed by factor XII (FXIIa), prekallikrein (PK), and high molecular weight kininogen (HMWK). Both complexes activate the zymogen factor X (FX). In both pathways, intrinsic or extrinsic, calcium ions and phospholipids are crucial for activation. FXa is the most important physiological component of the prothrombinase complex, which also comprises FV, calcium ions, and negatively charged phospholipids. In this complex, FXa converts prothrombin to thrombin (factor IIa). Thrombin is a key factor of blood coagulation because it catalyzes the final stage of the cascade by cleaving fibrinogen to fibrin. Moreover, thrombin contributes to the generation of a platelet plug by activating factors V, VIII, and XIII.

The cell-based model proposed by Hoffman and Monroe (2007) explains very well that, under physiological conditions, the extrinsic and intrinsic pathways do not operate in vivo as independent processes like a “cascade.” Instead of that, both processes take place in a cellular environment where complex interactions between blood factors, endogenous inhibitors, and cofactors occur for balancing the whole process via feedback mechanisms.

Many procoagulant proteins from snake venoms have been described, metalloproteinases being the majority. Procoagulant toxins promote blood coagulation by acting at different points of the coagulation “cascade” leading to coagulation disturbances such as disseminated intravascular coagulation. Their specific mechanism of action toward zymogens of blood coagulation makes them good tools for diagnostic uses for studying the binding sites and understanding the recognition mechanism of zymogens and activators.

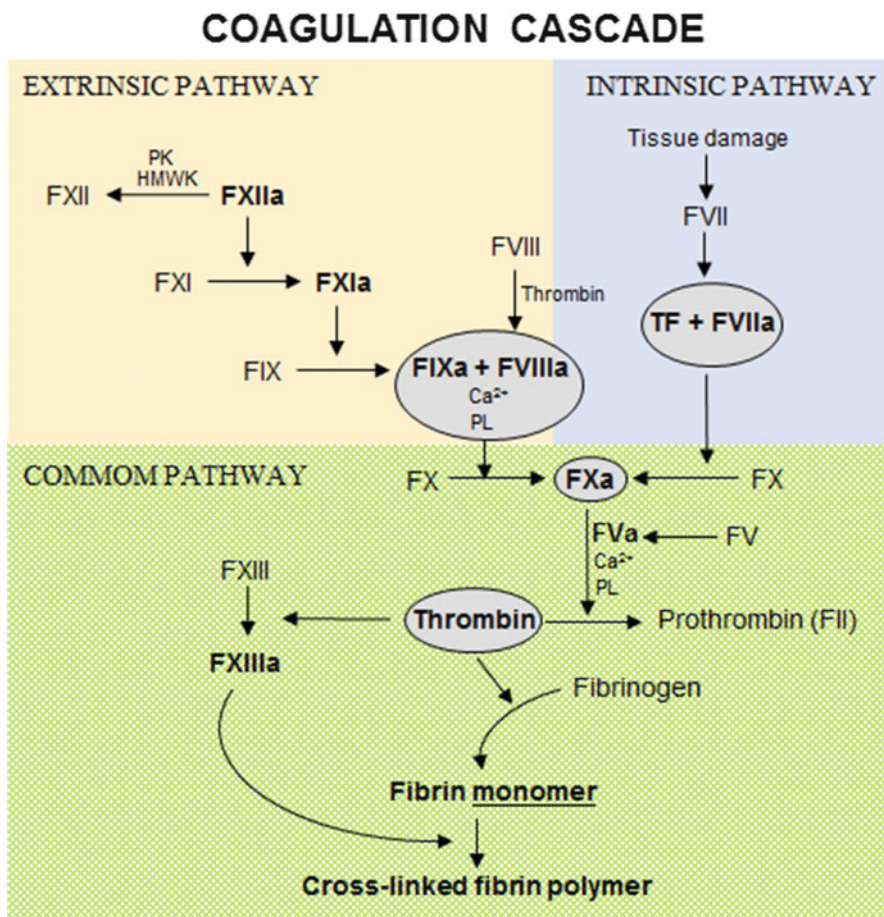


Fig. 1 Summary of the coagulation cascade. *TF* tissue factor, *FVIIa* activated factor VII, *FXIIa* activated factor XII, *FXIa* activated factor XI, *FIXa* activated factor IX, *FVIIIa* activated factor XVIII, *FXa* activated factor X, *FVa* activated factor V, *FII* prothrombin, *FIIa* thrombin, *FXIIIa* activated factor XIII, *PK* prekallikrein, *HMWK* high molecular weight kininogen, Ca^{2+} calcium ions, *PL* phospholipids

Factor V Activators

Factor V (FV) is a critical component in blood coagulation acting in both procoagulant and anticoagulant pathways. It circulates in plasma as an inactive form consisting of six domains with 330 kDa. It can be activated by thrombin or active factor X (FXa) after three specific proteolytic cleavages at 709, 1018, and 1545 resulting in the release of the highly glycosylated B domain and the exposition of the FV-binding site. As procoagulant, the active form of FV (FVa) expresses its function as a nonenzymatic cofactor of FXa in the activation of prothrombin to thrombin. FVa also enhances FXa proteolytic activity. As anticoagulant, FV is a

cofactor to activated protein C (APC) for the inactivation of FVIIIa and FVa (Cramer and Gale 2012). Moreover, FVa inhibits thrombin generation and factor X activation through inhibition of the TF/FVIIa complex (Al Dieri et al. 2013).

In snake venoms, FV activators present serine proteinase-like activity and can be found in the venoms of the families Crotalidae (*Bothrops atrox*), Elapidae (*Naja naja oxiana*, *Naja nigricollis nigricollis*), and Viperidae (*Daboia russelli*, *Vipera lebetina*, *Vipera ursinii*). RVV-V is a FV-activating SVSP purified from Russell's viper venom (*D. russelli*). Unlike thrombin, RVV-V cleaves one of the specific thrombin-susceptible bonds, between Arg1545 and Ser1546, without releasing the B domain of FV (Segers et al. 2006). In spite of that, FV generated through RVV-V action can bind to FXa and shows the same procoagulant activity that FV generated by thrombin. Although RVV-V is a thrombin-like enzyme, it has no multiple substrates as thrombin has. Structural analysis of the complex RVV-V and a fragment of FV have allowed to define the binding recognition regions and to explain the specificity of RVV-V toward FV (Nakayama et al. 2011). FV is until now the only substrate for RVV-V, which makes RVV-V a useful tool for laboratory studies and diagnostic uses.

Factor X Activators

FX is a vitamin K-dependent serine proteinase composed of two-chain polypeptide containing light (17 kDa) and heavy (45 kDa) chains connected by a disulfide bond (Hoffman and Monroe 2007). The amino-terminal region of the light chain contains the Gla domain composed of 11 γ -carboxyglutamic acid residues necessary for Ca^{2+} -dependent binding of FX to negatively charged phospholipids. The Gla domain is followed by two epidermal growth factor (EGF)-like domains. The heavy chain contains the catalytic (serine proteinase) domain covered by the activation peptide formed by 52 amino-terminal residues. Upon activation, FX is cleaved at Arg194-Ile195, the activation peptide is released, and the active site becomes exposed.

In snake venoms, the appearance of FX activators is widespread among the families Viperidae and Crotalidae, but also in Elapidae. A limited number have been isolated and characterized. They can be grouped according to their structure, number of subunits, molecular mass, and susceptibility to protease inhibitors. The majority of FX activators belong to the group of SVMs, but a few SVSPs were also described in Elapidae venom (Lu et al. 2005; Sajevec et al. 2011).

FX activators of the SVSPs group are single-chain proteins with low molecular mass, which are strongly dependent on calcium ions. Until now, the primary structure of this group of FX activator is unknown. FX activators of the SVMs group are glycosylated and are made up of three subunits (one heavy chain and two C-type lectin light chains) held together by disulfide bonds. They also require calcium ions for their activity. Mature forms of FX activators metalloproteinases have a heavy chain composed of a metalloproteinase, a disintegrin-like domain, and a cysteine-rich domain.

RVV-X (Russell's viper venom factor X activator) from *Daboia russelli* and VLFXA (*Vipera lebetina* factor X activator) from *Macrovipera lebetina* are the

two better characterized FX activators whose three subunits were cloned and fully sequenced (Takeya et al. 1992; Siigur and Siigur 2006). All the chains of both activators are synthesized from different genes. FX activators of the SVSPs group are structurally and functionally similar to RVV-X. Functionally, RVV-X and VLFXA critically depend upon the presence of Ca^{2+} to activate FX by hydrolysis at the same cleavage site of factors IXa and VIIa. RVV-X and VLFXA are also capable to activate the human factor IX (FIX) and protein C through a similar catalytic mechanism, but in a weaker form.

Crystallographic studies of the complex RVV-X/FX support the hypothesis that RVV-X primarily recognizes the Gla domain in FX. Calcium ions act inducing a conformational change in the Gla domain enhancing its binding to the two C-type lectin-like protein light chains of RVV-X that probably form an exosite. Following the first recognition, the conformational change facilitates the cleavage of FX by the metalloproteinase domain of RVV-X (Takeya et al. 1992; Takeda et al. 2007).

Prothrombin Activators

One of the central reactions of blood coagulation is the activation of prothrombin in thrombin by the prothrombinase complex (Hoffman and Monroe 2007). Prothrombin is a single-chain glycoprotein (72 kDa) which belongs to the vitamin K-dependent clotting factors. It must have a functional Gla domain including 8–10 γ -carboxyglutamic acid residues to be activated. FXa is the enzyme responsible for in vivo activation of prothrombin and requires the presence of calcium ions, a negatively charged membrane, and the cofactor FVa for activation to occur efficiently.

In the absence of prothrombinase complex, the mechanism of cleavage of prothrombin by FXa proceeds through prethrombin-2 rather than through meizothrombin. FXa cleaves two peptide bonds during the conversion of prothrombin into thrombin. First, the cleavage between Arg271 and Thr272 generates the intermediate product called fragment 1.2. Then, thrombin is formed from the remaining intermediate called prethrombin-2 by cleavage between Arg320 and Ile321. In the prothrombinase complex, FXa first cleaves prothrombin at Arg320-Ile321 producing meizothrombin (containing the fragment 1.2) which is later cleaved between Arg320 and Ile321 producing fragment 1.2 and active thrombin. Thrombin is a two-chain enzyme with a heavy chain containing the active site (32.5 kDa) and a light chain (4.6 kDa).

A large number of snake venoms contain prothrombin activators, which hydrolyzes prothrombin into meizothrombin or thrombin. Based on their structures, the specific cleavage site on prothrombin, the products formed (meizothrombin or α -thrombin), the susceptibility to protease inhibitors, and the cofactor requirements for its activities (calcium ions, phospholipids, and FVa), they are classified into four groups (Kini 2005b).

Group A activators are metalloproteinases, which convert prothrombin into meizothrombin (cleavage at Arg320) without requiring cofactors. The best example

is ecarin (55 kDa) from *Echis carinatus* venom. It is a single-chain protease composed of a metalloproteinase catalytic domain, a disintegrin-like domain, and a cysteine-rich domain.

Group B activators are metalloproteinases, which convert prothrombin into meizothrombin, but require Ca^{2+} for activity. They contain two subunits linked noncovalently: a heavy chain (homologous to ecarin) and a C-type lectin-like dimer linked via disulfide bridges. The presence of C-type lectin chains makes these activators more similar to RVV-X than ecarin. An example is Carinactivase-1 (CA-1) from *Echis carinatus leucogaster* venom, which has a similar structure to RVV-X. Like RVV-X, which uses their C-type lectin domains for recognition of the Gla domain, prothrombin activators belonging to the group B are unable to activate undercarboxylated forms of prothrombin. This property makes this kind of activators useful to monitoring plasma of patients under anticoagulant therapy and for quantification assays for the binding of prothrombin to procoagulant membranes.

Group C activators are serine proteinases exclusively found in some Australian Elapidae venoms, which are able to generate active thrombin similar to factor Xa by cleavage of Arg271-Thr272 and Arg320-Ile321 bonds of prothrombin. They require Ca^{2+} and negatively charged phospholipids, but not FV for maximal activity. This kind of activators are large multi-subunit proteases of approximately 300 kDa composed of enzymatic (FXa-like subunit) and nonenzymatic (FV-like subunit) subunits that resemble the factor Xa-Va complex (prothrombinase). The two chains of the FXa-like catalytic subunit (60 kDa) are disulfide-linked, and as do FXa, this subunit has a weak catalytic activity alone, whereas activity is greatly stimulated by the presence of the factor Va-like subunit. The two chains of the factor Va-like subunit (200 kDa) are held together by noncovalent interactions. Oscutarin C from *Oxyuranus scutellatus* (Taipan snake) venom and pseutarin C from *Pseudonaja textilis* venom are the most extensively studied members of this group. Oscutarin C was also capable of cleaving zymogen factor VII (FVII) and producing active FVII (FVIIa). This activity was potentiated in the presence of calcium ions and phospholipids. Despite this finding, specific FVII activators are not known. Oscutarin C was produced recently in its recombinant form (Lövgren 2013).

Group D activators are FXa-like serine proteinases (45–47 kDa) from Australian Elapidae venoms which are strongly dependent on Ca^{2+} , negatively charged phospholipids, and FVa to generate thrombin. They share many properties with FXa, including high sequence identity. They have two chains held together by disulfide bonds and cleave prothrombin at the same sites than FXa (Arg271 and Arg320). The serine proteinase active site is located in the heavy chain. Their light chains consist of an N-terminal Gla domain, followed by two epidermal growth factor-like domains, EGF-I and EGF-II. Hopsarin D from *Hoplocephalus stephensii* and trocarin D from *Tropidechis carinatus* are two examples of this group. Trocarin D (47 kDa) is a highly expressed toxin in the venom of the snake *Tropidechis carinatus*. This protein is structurally similar to mammalian FX. Its complete amino acid sequence was described and the complete molecule produced in its recombinant form (Lövgren 2013).

Thrombin-Like Enzymes

During envenoming, thrombin-like enzymes play a pivotal role in the coagulation disorders. Thrombin is a multifunctional enzyme that plays a role in thrombus formation, platelet activation, cell proliferation, and inflammation. The key role of thrombin in coagulation makes the study of those toxins essential for understanding all the disturbances caused for the venom (Hoffman and Monroe 2007).

Thrombin-like enzymes (TLEs) are single-chains serine proteinases (one exception is the TLE from *Cerastes cerastes* with two identical disulfide-linked chains) whose structure and procoagulant activity are similar to human thrombin. They are present in Viperidae and Colubridae snake venoms showing high sequence identity among themselves (about 67 %) but less than 40 % similarity with human thrombin. They have a molecular mass of 26–33 kDa and some may be glycosylated (Lu et al. 2005; Sajevec et al. 2011).

TLEs display procoagulant activity by converting fibrinogen to fibrin, but in vivo, they can have an anticoagulant effect by causing fibrinogen depletion. In contrast to fibrinogenolytic enzymes that usually cleave fibrinogen on the C-terminal portion hindering clot formation, TLEs hydrolyze fibrinogen at the N-terminal end of the A α and/or B β chain, releasing relatively small portions of the fibrinogen molecule, called fibrinopeptides A and B, respectively. This cleavage in the fibrinogen molecule allows its polymerization, with the formation of the fibrin clot. However, in contrast to thrombin, the majority of the thrombin-like enzymes from snake venoms preferentially release fibrinopeptide A and are not able to activate FXIII, necessary to form insoluble and cross-linked fibrin clots. Consequently, these enzymes form an instable fibrin clot, which is easily removed by the fibrinolytic system.

TLEs are not inhibited by heparin: thus they can be used to test plasma samples containing this anticoagulant or to remove fibrinogen from samples containing heparin. Two well-characterized thrombin-like enzymes, ancrod and batroxobin, are currently being used therapeutically as defibrinogenating agents, which will be discussed later (Marsh and Williams 2005).

Anticoagulant Molecules from Snake Venoms

Snake venom toxins that prolong blood coagulation are, as a rule, protein molecules and sometimes glycoproteins possessing molecular masses ranging from 6 to 350 kDa, which inhibit blood coagulation by different mechanisms (Kini 2006). Focusing on anticoagulant molecules, many studies have greatly contributed to clarify various molecular mechanisms involved in the physiological processes. It is noteworthy that these molecules can also often contribute to the development of new therapeutic agents for the treatment of hematological and cardiovascular disorders.

Some of these molecules have enzymatic activities, but only a few molecules have their well-defined mechanism, for example, PLA₂ (phospholipase A₂) and proteinases, while others do not exhibit this kind of activity, such as the C-type lectins. For example, thrombin-like enzymes (TLEs), a class of procoagulant toxin

described above, is also another member of the serine proteinases class that could be considered as an anticoagulant because of their capacity to make the plasma incoagulable by depletion of fibrinogen. TLEs have a wide distribution between the snakes' genera *Agkistrodon*, *Bothrops*, *Crotalus*, *Lachesis*, *Trimeresurus*, *Bitis*, and *Cerastes*, including the colubrid, *Dispholidus typus*. Recently, it was described as a new peptidic inhibitor (443.6 kDa) of both thrombin and factor Xa called Ruviprase from *Daboia russelli russelli*. This peptide has no similarity with any existing thrombin or FXa inhibitor and inhibits both factors by binding to their active sites via a nonenzymatic mechanism (Thakur et al. 2015).

Molecules with Enzymatic Activity

Phospholipases A₂ (PLA₂) Enzymes

Snake venoms are rich sources of PLA₂ enzymes that induce a wide variety of pharmacological activities, among them cardiotoxic, neurotoxic, myotoxic, antiplatelet, anticoagulant, and hemorrhagic effects. These enzymes have been purified and characterized. In general, they are proteins rarely glycosylated with about 13 kDa, 116–124 amino acid residues, and six or seven disulphide bonds. So far, three-dimensional structures have been determined for some PLA₂ enzymes. These analyses indicate that snake venom PLA₂ enzymes share strong structural similarity to mammalian pancreatic enzymes, as well as secretory PLA₂ enzymes (Kini 2006). Moreover, the catalytic function is also similar to mammalian enzymes; both are able to hydrolyze phospholipids at the sn – 2 position. Among them only snake venom PLA₂ enzymes are toxic and induce a wide spectrum of pharmacological effects (Kini 2003; Kini 2005a; Lu et al. 2005; Sajevic et al. 2011).

The inhibition of the blood coagulation is one of the actions that snake venom PLA₂ enzymes can promote. However, the properties of PLA₂ enzymes have been classified into different levels: strongly anticoagulant (when acting at concentrations below 2 µg/mL), weakly anticoagulant (shows effects between 3 and 10 µg/mL), or PLA₂ enzymes (do not prolong the clotting times significantly even at 15 µg/mL) (Kini 2006). Sometimes, in the venom of the same snake species, the PLA₂ enzymes can be classified into different levels, for example, CM-I, CM-II, and CM-IV from *Naja nigricollis* venom. They were classified as strongly (CM-IV) or weakly (CM-I, CM-II) anticoagulant PLA₂ enzymes. Besides, CM-IV shows at least 100-fold more potent anticoagulant activity than others (Kini 2005a).

It is worth emphasizing that phospholipids play a crucial role in the formation of several coagulation complexes, and the destruction of the phospholipid surface might be the first target of the PLA₂ enzymes. However, strongly anticoagulant PLA₂ enzymes also affect blood coagulation by other mechanisms, independent of phospholipid hydrolysis; see below.

The PLA₂ from *N. nigricollis* venom were used in different clotting time assays (prothrombin time, Russell viper venom time, and thrombin time) in order to identify the stage in the extrinsic coagulation cascade they act (for details, see Kini 2005a; Kini and Banerjee 2005; Stefansson et al. 1989). Thus, it was shown that the extrinsic tenase

complex (tissue factor/factor VIIa: TF/FVIIa) is inhibited by all three anticoagulant PLA₂ enzymes from *N. nigricollis* venom (CM-I, CM-II, and CM-IV), whereas the prothrombinase complex is only inhibited by CM-IV. That is, CM-IV (the strongly anticoagulant enzyme) inhibits both the extrinsic tenase and prothrombinase complexes, acting two steps of the coagulation cascade, while CM-I and CM-II (the weakly anticoagulant enzymes) only inhibit the coagulation cascade in one step (TF/FVIIa).

The inhibition of the extrinsic tenase complex occurs by all three PLA₂ from *N. nigricollis*. While CM-IV inhibits the tenase by both enzymatic and nonenzymatic mechanisms, the weakly anticoagulants (CM-I and CM-II) inhibit the complex mostly by their enzymatic activity (Kini and Evans 1995).

Interestingly, CM-IV inhibits the prothrombinase complex via a nonenzymatic mechanism and more strongly in the absence of phospholipids than in the presence. In contrast, despite the complete hydrolysis of phospholipids by CM-I and CM-II, thrombin formation is not significantly reduced; they fail to inhibit the prothrombinase complex. In other words, the inhibition of the prothrombinase complex is independent of phospholipid hydrolysis (Stefansson et al. 1990).

Fibrin(ogen)olytic SVMPs

Some of the SVMPs inhibit blood coagulation – most are fibrinogenases – and they directly split off fragments from the C-terminal of α , β , and γ fibrinogens, rendering it incoagulable by thrombin. They are classified, on the basis of their specificity for the A α or B β chain of fibrinogen, into α - and β -fibrinogenases (Swenson and Markland 2005). As an example, recently, a fibrinogenolytic SVMP from *Bothrops moojeni* venom, named moojenin, was purified and sequenced. Moojenin consists of a single polypeptide chain and has a molecular mass of about 45 kDa. It cleaves the A α -chain of fibrinogen first, followed by the B β -chain, and shows no effects on the γ -chain. Moojenin showed a coagulant activity on bovine plasma about 3.1-fold lower than crude venom. It rendered the blood incoagulable when it was intraperitoneally administered into mice. This SVMP may be of medical interest because of its anticoagulant activity (De Moraes et al. 2012).

Considering that, the subtle physical destruction leads to the anticoagulant action of metalloproteinases. The relation structure versus function of the SVMPs with respect to their anticoagulant effects needs to be clarified.

Protein C Activators

The protein C activators are the only ones presenting a direct anticoagulant function. The protein C, activated by thrombin, degrades FV/FVa and FVIII/FVIIIa and releases a tissue-type plasminogen activator. It also stimulates fibrinolysis through its interaction with plasminogen activator inhibitor (Fay and Owen 1989; Sakamoto et al. 2003).

Protein C activators are mainly present in the venom from *Agkistrodon* snakes genus (*A. contortrix contortrix*, *A. contortrix mokasen*, *A. contortrix pictigaster*, *A. piscivorus piscivorus*, *A. piscivorus leucostoma*, *A. bilineatus*, *A. blomhoffii ussuriensis*, *A. halys halys*) but have been also described in the venom from other snakes such as *Bothrops moojeni*, *Bothrops pradoi*, *Vipera lebetina*, *Daboia russelli*,

Cerastes cerastes, and *Trimeresurus mucrosquamatus* (Gempeler-Messina et al. 2001). These protein C activators are glycoproteins with a molecular mass of about 36–40 kDa, which activate the protein C at low salt concentrations in the absence of calcium ions (Kini 2006). First amino acid sequence of the protein C activator has been determined from *A. c. contortrix* venom.

L-Amino Acid Oxidases

L-amino acid oxidases catalyze the oxidative deamination of a number of L-amino acids, generating hydrogen peroxide (H_2O_2). They are enzymes found in several organisms, including venoms of snakes, where they contribute to the toxicity of ophidian envenomation. Their toxicity is primarily due to enzymatic activity, but other mechanisms have been recently proposed which require further investigation. Among the L-amino acid oxidase effects are the actions on platelet aggregation and the induction of apoptosis, hemorrhage, and cytotoxicity. Sakurai and colleagues showed that L-amino acid oxidase purified from *Agkistrodon halys blomhoffii* exhibits anticoagulant activity. This enzyme affects only the intrinsic pathway, having little effect on the extrinsic pathway (Sakurai et al. 2003). In general, these molecules present a high biotechnological potential for the development of antimicrobial, antitumor, and antiprotazoan agents. For details about L-amino acid oxidase biochemical properties, pharmacological effects, and their structure/activity relationship, see Izidoro et al. 2014.

Nonenzymatic Molecules

C-type Lectin-Like Proteins (Snaclecs or SVCLPs)

Snaclecs were among the first nonenzymatic proteins characterized from snake venoms. Snaclec anticoagulants can be IX/X-bp (factor IX/X binding protein), IX-bp (factor IX binding protein), or X-bp (factor X binding protein), and they are able to inhibit coagulation factors IX and X. The anticoagulant activities of these molecules are conferred by their calcium-dependent binding to Gla-containing domains of coagulation factors IX and X. The anticoagulants IX/X-bp, IX-bp, and X-bp are heterodimeric proteins that consist of two homologous C-type lectin-like chains of 14 and 15 kDa. The binding of IX/X-bp to factor IX and factor X in the presence of Ca^{++} has high affinity (nanomolar) (Morita 2004).

Bothrojaracin is a C-type lectin-like described from the venom of the Brazilian snake jararaca (*Bothrops jararaca*). Structurally, it is a heterodimeric protein held together by an interchain disulphide bond. Both chains are similar to C-type lectin-like proteins. Bothrojaracin is an example of snaclec anticoagulant that interacts specifically with thrombin/prothrombin. This molecule is a unique thrombin inhibitor binding to both exosite-I and exosite-II with high affinity (Kd 0.6 nM) but not to the active site. Bothrojaracin inhibits FV activation by thrombin. It is known to bind to partially exposed pro-exosite-I in prothrombin (Kd 175 nM), thus inhibiting its activation to thrombin (for details, see Kini 2006). Bothrojaracin was also described as a potent antithrombotic agent tested

in *in vivo* assays, with hope that could further help the development of new prothrombin-directed drugs (Zingali et al. 2005).

Another snakec was obtained from *Agkistrodon halys brevicaudus* venom. Like bothrojaracin, it has fibrinogen clotting inhibitory activity, and because of this, it increases fibrinogen clotting time (induced by thrombin). Even so, its mechanism of action is a little different from that bothrojaracin. Salmorin forms a complex with prothrombin, but it also inhibits prothrombin activation independently of the presence of FV (Koh et al. 2000).

Others similar to bothrojaracin molecules have been described in *Bothrops* species (*B. alternatus*, *B. atrox*, *B. cotiara*, *B. jararacussu*, *B. moojeni*, *B. neuwiedi*, and *B. insularis*) and in small amounts in *Lachesis muta* venom, but not as well characterized yet (Kini 2006; Oliveira-Carvalho et al. 2008).

Three-Finger Toxins (TFTs)

Three-finger toxins are nonenzymatic proteins with 60–74 amino acid residues firstly found in elapid venoms and, recently, demonstrated in colubrid and viperid venoms as well (for details, see Kini 2006; Sajevic et al. 2011). They are characterized by specific folding of three β -sheet loops, named “fingers,” extending from the central core and by four conserved disulphide bridges. These molecules have mainly cardio and neurotoxic actions; however, despite of their structural similarity, some of them are also acting on the hemostatic system.

Hemextin AB complex is the first synergistic anticoagulant complex isolated from snake venom. It has two TFTs, hemextin A and hemextin B, as subunits, and it was obtained from *Hemachatus haemachatus*. While hemextin A shows only mild anticoagulant activity, hemextin B is inactive per se. However, the tetrameric complex formation is fundamental for the FVIIa potential inhibition. This complex formation is an enthalpically driven process. The hemextin AB complex is a highly specific natural inhibitor of the initiation of blood coagulation, inhibiting the TF-FVIIa complex noncompetitively, with a K_i of 50 nM.

Very recently, a TFT from *Naja kaouthia* cobra (Nk-3FTx) was biochemically and biologically characterized; this study revealed that it exhibits mild anticoagulant activity when tested on citrated human plasma (Das et al. 2016).

Kunitz-Type Proteinase Inhibitors

Several snake venoms contain a number of small molecules with inhibitory activity toward proteinases involved in hemostatic processes. Some toxins demonstrate structural and functional identity to the Kunitz class of serine proteinase inhibitors. Kunitz-type inhibitors have been isolated from elapid and viperid venoms. They contain 57–60 amino acid residues and three disulphide bridges and belong to the Kunitz pancreatic trypsin-inhibitor family. Basically, they are similar in their three-dimensional structures. Considering that all the proteinases in blood coagulation and fibrinolysis are serine proteinases, this group of molecules was thought to be potential anticoagulants (Kini 2006).

Snake Venom Components and Their Applications in Clinical and Biotechnology

Currently, exogenous factors are used as (a) reagents in diagnostic kits to detect hemostatic disturbances and deficiencies of a clotting factor, (b) components for kits to monitor hemostatic parameters, and (c) therapeutic agents for disturbances that involve unbalanced hemostasis, such as deep vein thrombosis, myocardial infarction, stroke, priapism, and sickle cell crisis (Liu et al. 2003).

In general, the exogenous factors can be divided into two separated groups: procoagulants and anticoagulants. Exogenous inhibitors usually display anticoagulant activity by specifically inhibiting the activity of a clotting factor (e.g., toxins of the serpin family). However, proteolytic enzymes that act on fibrinolytic system have also been described, e.g., plasminogen activators, which have anticoagulant activity, and the fibrinolytic enzymes, which are in general antithrombotic, because they can act on cross-linked fibrin (Swenson and Markland 2005). The protein C activator isolated from *A. c. contortrix* has been produced in a commercial preparation named Protac[®], and it has considerably simplified the determination of protein C. Protac[®] converts the protein C (zymogen) very fast, independently of thrombomodulin, into a serine proteinase, which exerts the enzymatic and anticoagulant effects of protein C. Besides, differently from thrombin, the activation product of Protac[®] is not influenced by the inhibitory effect of protein C inhibitor (Gempeler-Messina et al. 2001).

Numerous procoagulant proteins have been isolated in their native form from snake venoms. They have been sequenced and cloned, but the majority has not been functionally expressed as recombinant molecules. Many efforts have been currently made in this sense, but the potential risks associated with toxicity and immunogenicity of the clinical use of some proteins in recombinant form as therapeutic agents and the need for several regulatory procedures and approvals for its development and production turned the research and development involving the use of procoagulant proteins still incipient in comparison to the use of exogenous inhibitors in the therapeutic field.

Several toxins have been proved to be useful as reagents in laboratory tests for diagnosis. The diagnostic uses of snake venom toxins have been reviewed by different authors (Schöni 2005; Moore 2014). Among snake venom FV activators, commercially available RVV-V – a toxin from Russell's viper venom – is one of the most used and is the key reagent of different diagnostic kits (Schöni 2005). One of them is a clotting test based on the prothrombinase complex and is used to monitor patients undergoing anticoagulant therapy, except for vitamin K antagonists. In the presence of RVV-V, the prothrombinase complex is formed very quickly, generating active thrombin that cleaves fibrinogen to fibrin.

Another kit using RVV-V is applied to detect factor V Leiden mutation genotype. This test consists of two steps, the first one involving the activation of FV by RVV-V followed by inactivation of FVa by the addition of APC. In the second step, the

group D prothrombin activator nescarin is added to generate active thrombin and finally the fibrin clot. Since nescarin activity is dependent on FVa as a cofactor, patients with factor V Leiden mutation have short clotting times in comparison to normal individuals (Schöni 2005). Another toxin from Russell's viper venom is RVV-X, a FX activator also used for diagnostic tests and for detection of factor X deficiency (Bezeaud 1995) and lupus anticoagulant (Triplett 2000), which is also an important risk factor for thrombophilia (Marsh 2001; Marsh and Williams 2005). The assay to detect lupus anticoagulant is the dilute Russell's viper venom time dRVVT, which is based on the activity of RVV-X. There are various commercially available kits to assay dRVVT, which contain purified RVV-X or the whole venom from Russell's viper.

In the therapeutic field, currently there are two basic potential applications for exogenous procoagulant agents. One is as defibrinogenating agents to prevent thrombosis and decrease blood viscosity. The other may be as a hemostatic agent to arrest bleeding, for example, during surgical procedures (Andrade 2012).

Among the procoagulant proteins from snake venoms currently in use as therapeutic tools, the most relevant are the thrombin-like enzymes. Thrombin-like enzymes from snake venoms are not inhibited by heparin, a glycosaminoglycan, clinically used in the prevention of both arterial and venous thromboses (Eley et al. 2013), and they are used for detecting dysfibrinogenemias and to remove fibrinogen for different assays (Marsh and Williams 2005). Unlike thrombin, which cleaves both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from fibrinogen, many thrombin-like enzymes usually only cleave FPA and do not activate FXIII. This aspect makes the thrombin-like enzymes interesting tools to remove fibrinogen from plasma (defibrinogenation) without the risk of thrombosis, because the fibrin clot formed is very unstable in contrast to cross-linked fibrin (Koh et al. 2006). Therefore, the fibrin is rapidly removed by the fibrinolytic system. Otherwise, administration of a thrombin-like enzyme as a defibrinogenating agent has a low bleeding risk in comparison to other anticoagulants currently in use (Crowther and Warkentin 2008).

Ancrod and batroxobin, which are also commercially named Arvin and Defibrase, respectively, are indicated as defibrinogenating drugs to patients victimized by stroke, deep vein thrombosis, myocardial infarction, peripheral arterial thrombosis, priapism, and sickle cell crisis (Qin et al. 2013). Ancrod is a serine proteinase toxin from the Malayan pit viper snake *Agkistrodon rhodostoma* (*Calloselasma rhodostoma*) that causes reduction in plasma fibrinogen concentration in vivo by formation of soluble fibrin complexes, which are degraded by plasmin. In addition, ancrod induces plasminogen activations and leads to a fibrinolytic response (Dempfle et al. 2001). Batroxobin is a thrombin-like enzyme present on venom from *B. atrox moojeni* (Qin et al. 2013). This serine proteinase only releases fibrinopeptide A by specific cleavage of the Arg16-Gly17 bond in the A α -chain of fibrinogen, and it is not inhibited by antithrombin or heparin cofactor II (Serrano 2013).

The thrombin-like enzymes can be also used as procoagulants for hemorrhage management. A mixture of two enzymes from the venom of *B. atrox*, a thrombin-like

enzyme and a thromboplastin-like enzyme, forms a clot-promoting product called Hemocoagulase[®]. These enzymes cooperate, targeting different points at the coagulation cascade to form blood clots. The thrombin-like enzyme directly cleaves fibrinogen into fibrin monomers, and the thromboplastin-like enzyme activates FX, which in turn converts prothrombin into thrombin (McCleary and Kini 2013).

The diversity of known exogenous factors and the many recently identified ones also suggest that there are many other molecules to be discovered, which could belong to the established classes or even present new biochemical properties and concomitantly bring new perspectives for the use of these proteins in the study and treatment of a wide range of clinical dysfunctions.

Conclusion and Future Directions

Many toxins acting as activators or inhibitors of blood coagulation have been described. Some procoagulant toxins from snake venoms, especially from viperids, have been used as diagnostic tools or by their application in medicine. For example, batroxobin (TLE from *Bothrops atrox*) is useful for measuring fibrinogen levels in an assay known as Reptilase[™] time, and RVV-X (a FX activator from *D. russelli*) is useful for measuring lupus anticoagulant (Marsh and Williams 2005). Nevertheless, little is known about the possible roles of new discovered toxins, especially blood coagulation inhibitors, at the physiological level. Additional effects of those new toxins, or currently known, could open new perspectives for the application of these enzymes as tools in the therapeutic and biotechnological area. In addition, the great diversity of proteins and peptides in snake venoms of unknown factors recently identified by new approaches such as proteomic, transcriptomic, and genomic have allowed the realization that there are many toxins and functions to be discovered. Further studies should be performed to better understand the great versatility of toxins affecting blood coagulation, which can trigger various biological activities at the same time of the organism.

Cross-References

- ▶ [Endothelial Cell Toxicity of Vascular Apoptosis-Inducing Proteins from Hemorrhagic Snake Venom](#)
- ▶ [Snake Venom Protease Inhibitors: Enhanced Identification, Expanding Biological Function, and Promising Future](#)

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Abstract

Disintegrins are generally involved in cell-cell and cell-matrix adhesion. Although disintegrins were first identified in viper venoms, their presence in many other organs, tissues, and secretions was reported later. In snake venoms, these small cysteine-rich peptides strongly inhibit platelet aggregation and thereby antagonize clotting of blood in bite victims. The integrin binding is generally dependent upon a conserved arginine-glycine-aspartic acid (RGD) motif. However, many disintegrins lacking this RGD motif can also bind and block integrins. Disintegrins from hematophagous animals show considerable variation in the signature tripeptide disintegrin motif. However, in most cases the aspartic acid residue (D) is present. Disintegrins found in snake venoms and mammalian ADAM (disintegrin and metalloproteinase) are believed to share a common membrane-bound protein ancestor, which underwent modifications and acquired novel functions through millions of years. Recently, disintegrins have attracted researchers for their ability to strongly intervene in tumor growth and

D. Chakrabarty (✉) • C. Chanda

Department of Biological Sciences, Birla Institute of Technology and Science, Pilani, Zuarinagar, Goa, India

e-mail: diba27@yahoo.com; dibakarchakrabarty@goa.bits-pilani.ac.in;
chandrasekharchanda02@gmail.com

angiogenesis. Some drugs have already been designed in mimicking the molecular structures of disintegrins. Currently, many laboratories are engaged in isolating and characterizing disintegrins with a focus on developing novel anticancer and antithrombotic drugs.

Keywords

Snake venom • Disintegrin • Metalloproteinase • ADAMs • GP IIb/IIIa

Introduction

Disintegrins

Pathetic and amazing consequences of snake envenomation have revealed many wonderful molecules of Nature. One such unfortunate consequence is dramatic hemorrhage caused by viper envenomation. Many (Gould et al. 1990) hemorrhagins have been isolated and studied to reveal their modes of action in recent past. Some thought that hemorrhage is caused by extensive proteolysis. However, some of the pure hemorrhagins were not able to digest common protein substrates, like bovine serum albumin or casein in vitro. Some investigators cinematographically recorded the hemorrhagic event after application of *Trimeresurus flavoviridis* venom or hemorrhagins HR1 and HR2 purified from the same venom (Ohsaka et al. 1971; Tsuchiya et al. 1974). They noted extravasation of erythrocytes through pinpoint holes developed due to disruption of junctions between endothelial cells and the basement membrane. Few years later, specific proteins capable of disrupting cell-cell adhesion and cell-basal lamina adhesion were found in viperine venoms. Those proteins were found to block specific integrins present on many cell surfaces for adhesion purpose. These proteins were called disintegrins.

The term disintegrin was coined in 1990 by Gould et al. (Gould et al. 1990) to describe low molecular weight, cysteine-rich peptides from viper venoms having Arg-Gly-Asp (RGD) motif(s). However, disintegrins were later found in many different types of tissues in mammals also. Several disintegrins are also reported from hematophagous (blood-consuming) animals, e.g., leeches, lice, bedbugs, etc. Most of the disintegrins identified till date are potent platelet aggregation inhibitors which block fibrinogen binding to $\alpha_{IIb}\beta_3$ integrin (GP IIb/IIIa receptor) on ADP, collagen- and thrombin-stimulated platelets. They have also been found to bind $\alpha_v\beta_3$, $\alpha_4\beta_1$, or $\alpha_5\beta_1$ integrins expressed on endothelial cells, fibroblasts, phagocytes, and tumor cells, impairing integrin-mediated cell adhesion onto selective extracellular matrix (ECM) proteins. Although RGD tripeptide motifs are more prevalent in snake venom disintegrins, blood-sucking invertebrate disintegrins may contain KGD, VGD, MLD, KTS, RTS, WGD, or RED in addition to RGD (Assumpcao et al. 2012). Snake venom disintegrins are mostly found in the venoms of Viperinae and Crotalinae subfamilies of Viperidae. It is believed that snake venom disintegrins are derived by proteolytic processing of multi-domain zinc-dependent proteinases

known as snake venom metalloproteinases (SVMPs). SVMPs can be classified into four types according to their domain compositions:

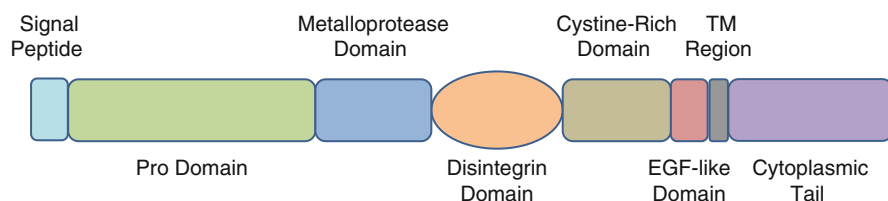
1. **P-I** SVMPs contain a peptide signal, a prodomain, and a metalloproteinase domain.
2. **P-II** class has a metalloproteinase and a disintegrin domain.
3. **P-III** class contains a cysteine-rich domain in addition to the metalloproteinase and the disintegrin-like domain.
4. **P-IV** proteinases contain lectin-like domains in addition to P-III domain structure.

Binding of cells to adhesive glycoprotein is primarily dependent on a family of special receptors. These receptors bind large glycoproteins on the extracellular space and also bind to the cytoskeleton on the intracellular space, thereby, integrating both intracellular and extracellular scaffolds to work together. This large family of receptors was named **integrins** (Horwitz 1986). The integrins are known to play important roles in a wide variety of physiological processes from fertilization to embryonic development. In adult animals they constitute important steps in inflammatory reactions, tissue remodeling, cell adhesion, migration, angiogenesis, and other biological processes (Hynes 1987, 1992), including platelet aggregation for hemostasis. Cells must adhere to the basal lamina to develop into tissues and organs. It is known that binding of cells to the basal lamina is essential for viability of many cell types. Integrins are therefore extremely important components for development of tumors (Anna and Alan 2011). Ability of disintegrins to interfere in the cell-cell adhesion and cell-matrix adhesion by blocking integrins can be exploited to stop tumor development. One of the most important steps in tumor development is establishing blood supply by angiogenesis. Disintegrins also inhibit angiogenesis by interfering with integrin activity in endothelial cells. The integrin-inhibitory activity of disintegrins depends on an active tripeptide sequence and appropriate pairing of cysteine residues, which determine the conformation of the inhibitory loop. A number of protein ligands, such as fibronectin, vitronectin, osteopontin, collagens, thrombospondin, laminin, fibrinogen, and von Willebrand factor (vWF), contain the tripeptide sequence arginine-glycine-aspartic acid (RGD) representing a common integrin recognition site (Ruoslahti and Pierschbacher 1987).

Disintegrins constitute the nonenzymatic domain of the snake venom metalloproteinases which bind with high-affinity to extracellular β -subunit of integrin receptors present on platelets and other endothelial cells. Taiwanese investigator Tur-Fu Huang and colleagues isolated the first disintegrin from *Trimeresurus gramineus* venom, which inhibited platelet aggregation by competitively blocking fibrinogen binding to GP IIb/IIIa receptors of ADP-stimulated platelets (Huang et al. 1987, 1989).

ADAM constitutes a family of membrane-bound proteins containing a disintegrin and a metalloproteinase domain. These molecules are also known as the **ADAMalysin** family or MDC (metalloproteinase-like, disintegrin-like, and

ADAM Structure



P III SVMP Structure

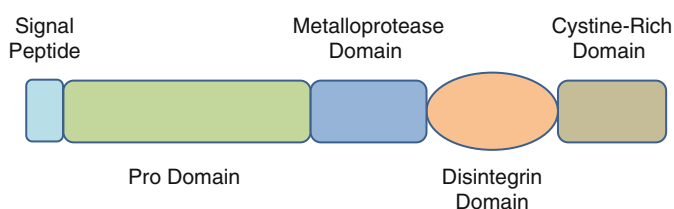


Fig. 1 P-III SVMP in comparison with ADAM structure

cysteine-rich) family. ADAM proteins by having zinc-dependent metalloproteinase domain were found to shed the extracellular domain of the membrane-bound growth factors, cytokines, and their receptors and playing a vital role in cellular signaling (White 2003; Schlondorff and Blobel 1999; Moss and Lambert 2002). ADAM proteins have been reported in many mammalian tissues and other lower eukaryotes. Members of this family were found having 700–800 amino acids with a prodomain, metalloproteinase domain, disintegrin domain, cysteine-rich domain, EGF-like domain, and cytoplasmic tail (Fig. 1). These domains of the ADAM family of proteins were having no similarity with other membrane-anchored adhesion molecules; instead they were found to be similar to the domain of snake venom metalloproteinases (SVMPs) (Kamiguti et al. 1998; Huang 1998).

Snake Venom Disintegrins

Snake venom is a rich source of many pharmacologically active molecules, proteins and peptides (Markland 1998; Fry 1999; Warrell 1989). Venoms of Elapidae and Viperidae family snakes contain many hemorrhagic components which interfere at different stages of the coagulation cascade. Hemorrhagic effects observed are mainly due to the presence of zinc-dependent metalloproteinase, serine proteinases, and disintegrins (Than et al. 1987; Kini 2005, 2006; Ouyang et al. 1979). Several disintegrins have been purified till date from snake venoms after the first report of Trigramin (Huang et al. 1987). These snake venom disintegrins were found to bind with a wide variety of heterodimeric integrins present on blood platelets and other

cells. Some of the reported disintegrins, their characteristics, and their mechanisms of action are discussed below.

Trigramin a low molecular weight disintegrin was from Taiwan viper, *Trimeresurus gramineus*, and is a highly specific inhibitor of fibrinogen as well as vWF binding to platelet receptors GP IIb/IIIa. Trigramin prolonged the bleeding time in hamsters by inhibiting the formation of platelet plug. It also inhibited binding of melanoma cells to fibronectin and fibrinogen (Cook et al. 1989; Knudsen et al. 1988).

Contortrostatin purified from *Agkistrodon contortrix contortrix* binds to $\alpha_2\beta_3$, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$ integrins present on the blood platelets and other endothelial cells (Zhou et al. 2000). Contortrostatin was found to inhibit platelet aggregation in human, canine, and rabbit platelet-rich plasma and also found to inhibit tumor cell invasion in human breast and ovarian cancer cells in immunodeficient mice and rodent glioma models (Pyrko et al. 2005; Swenson et al. 2005; Ifigeneia et al. 2013; Trikha et al. 1994). Contortrostatin in combination with docetaxel acted as a potent inhibitor of prostate cancer both in vitro and in vivo studies (Lin et al. 2010).

Rhodostomin a disintegrin purified from the venom of *Calloselasma rhodostoma* inhibited basic fibroblast growth factor (bFGF)-induced human umbilical vein endothelial cell (HUVEC) proliferation by blocking $\alpha_v\beta_3$ integrins and inhibited HUVEC migration and invasion in Matrigel system (Yeh et al. 2001). Yet another function of disintegrin has been reported recently. Tur-Fu Huang's team at the National Taiwan University reported that rhodostomin inhibited neutrophil binding to immobilized fibrinogen and also attenuated superoxide production suggesting that it might have anti-inflammatory properties. However, the identity of the entity mediating disintegrin binding to neutrophils remains to be studied (Tseng et al. 2004). The same team made another interesting observation that rhodostomin inhibited adhesion, migration, cytokine production, and mitogen-activated protein kinase (MAPK) activation of lipopolysaccharide-induced macrophages. Further they also reported that rhodostomin significantly decreased production of tumor necrosis factor α (TNF- α) and several interleukins apart from attenuation of blood pressure, heartbeat, and thrombocytopenia in LPS-induced endotoxemic mice. Their results indicate toward the potential therapeutic use of disintegrin in endotoxemia (Hsu et al. 2010).

Echistatin purified from *Echis carinatus* is a potent blocker of $\alpha_{IIb}\beta_3$ integrin and thereby inhibits platelet aggregation. It was also found to inhibit $\alpha_v\beta_3$ integrins expressed on osteoclasts and preventing their binding with bone extracellular matrix thus inhibiting bone resorption (Kumar et al. 1997; Nakamura et al. 1998).

Triflavin (*Trimeresurus flavoviridis*), **albolabrin** (*Trimeresurus albolabris*), **applagin** (*Agkistrodon piscivorus*), **barbourin** (*Sistrurus miliaris barbouri*), **batroxostatin** (*Bothrops atrox*), **bitistatin** (*Bitis arietans*), **halysin** (*Agkistrodon halys*), and **kistrin** (*Agkistrodon rhodostoma*) are potent inhibitors of platelet aggregation by blocking GP IIb/IIIa (Scarborough et al. 1991; Williams et al. 1990; Calvete et al. 1991; Rucinski et al. 1990; Shebuski et al. 1989; Huang et al. 1991a; Yasuda et al. 1991; Huang et al. 1991b).

Salmosin an RGD-containing disintegrin purified from *Agkistrodon halys brevicaudus* was found to bind with $\alpha_v\beta_3$ integrin and inhibited proliferation of basic bFGF-induced bovine capillary endothelial (BCE) cells. It is also found to inhibit platelet aggregation by binding to GP IIb/IIIa (Kang et al. 1998; Chung et al. 2003).

Obtustatin a KTS disintegrin purified from *Vipera lebetina obtuse* is a potent selective inhibitor of $\alpha_1\beta_1$ and found to show antiangiogenic properties in chicken chorioallantoic membrane assay and in the Lewis lung syngeneic mouse model. It also inhibited human melanoma growth in nude mice models (Marcinkiewicz et al. 2003; Brown et al. 2008).

Eristostatin, an RGD-containing disintegrin purified from *Eristicophis macmahoni*, was observed to inhibit melanoma cell motility by interfering with $\alpha_4\beta_1$ integrin (Tian et al. 2007; Danen et al. 1998).

Altrnagin-C a non-RGD disintegrin-like protein purified from *Bothrops alternatus* was found to modulate $\alpha_2\beta_1$ integrin signaling and induce neutrophil migration. It was also found to induce endothelial cell proliferation in vitro (Mariano-Oliveira et al. 2003; Cominetti et al. 2004).

Colombistatin is an RGD-containing disintegrin purified from the venom of *Bothrops colombiensis* found to inhibit ADP-induced platelet aggregation by blocking GP IIb/IIIa receptors. It also inhibited human urinary and skin melanoma cancer cell adhesion to fibronectin (Sanchez et al. 2009).

Accutin purified from the venom of *Agkistrodon acutus* inhibited thrombin-, ADP-, and collagen-induced platelet aggregation by blocking GP IIb/IIIa receptors. Accutin was also found to block $\alpha_v\beta_3$ integrin and prevent the adhesion of HUVEC to fibrinogen, fibronectin, and vitronectin. It also showed angiogenic properties in CAM models (Yeh et al. 1998). Another disintegrin, acurhagin-C, recently purified from *Agkistrodon acutus* was found inducing apoptosis via caspase-3 activation and inhibiting $\alpha_v\beta_3$ -mediated human endothelial cell functions (Wang 2010).

Saxatilin an RGD-containing disintegrin purified from *Gloydius saxatilis* suppressed ovarian cancer cell invasion induced by TNF- α and suppressed tumor-induced human lung cancer angiogenesis by regulating VEGF expression. It also inhibited ADP-induced platelet aggregation by blocking GP IIb/IIIa receptors and inhibited the binding of HUVEC and smooth muscle cells to vitronectin by blocking the $\alpha_v\beta_3$ integrin (Kim et al. 2007; Jang et al. 2007; Hong et al. 2002).

Classification

Disintegrins can be divided into four subfamilies according to their number of amino acid residues and the number of disulfide bonds (Calvete et al. 2003).

Group I includes disintegrins with 84-residue polypeptides cross-linked by seven disulfide bonds.

Group II is formed by disintegrins with about 70 residues and 6 disulfide bonds.

Group III is composed of homo- and heterodimers. Dimeric disintegrins contain subunits of about 67 residues with 10 cysteines involved in the formation of four intra-chain disulfide bonds and two interchain cysteine linkages (Bilgrami et al. 2004, 2005; Calvete et al. 2000).

Group IV comprises the short disintegrins composed of 41–51 residues and four disulfide bonds. They possess a transmembrane domain and cytoplasmic tail. These peptides are involved in cell-cell fusion events including sperm-egg binding and fusion, myoblast fusion, and other cell-cell and cell-matrix interactions.

Evolution, Structure, and Function

The high degree of homology among molecular structures of different functional regions of disintegrin, SVMP, and ADAM points at their evolutionary relationship. These proteins are believed to have diverged from membrane-bound types at the base of the so-called Colubroidea radiation that happened around 60–80 Ma ago and recruited as important soluble components of venoms (Moura-da-Silva et al. 1996). Disintegrins in the venoms of Viperidae family evolved by gene duplication of disintegrin-like domains present in P-III class of metalloproteinase hemorrhagins (SVMPs). SVMPs are known to be closely related to ADAMs and mammalian metalloproteinases involved in matrix degradation. It is believed that ADAMs and SVMPs evolved from a common ancestral gene through gene duplication and divergence (Glassey and Calvete 2004). These genes were then included in the venom proteome. This new soluble status was achieved by minimization of both genomic and protein structures by gradual loss of introns as well as cysteine pairs. Products of these modified genes underwent positive Darwinian selection of different combinations of integrin recognition sites and the C-terminal ends that gave rise to their present-day divergence (Calvete 2010). This divergent family of proteins proved to be efficient toxic components in different types of venoms. The primary reason for their successful recruitment in venoms is probably their ability to inhibit platelet aggregation. This function is particularly important to support “bleeding to death” action of the hemorrhagins.

Viper venom disintegrins are generated by either proteolysis of Serpentes-specific P-II-SVMP precursor proteins (Kini and Evans 1992) or direct synthesis from mRNAs lacking the metalloproteinase-coding region (Okuda et al. 2002). Snake venom disintegrins formed by proteolysis of P-II SVMPs are 50–80 amino acid long ligands of integrin (Juárez et al. 2008). These disintegrins interact with integrins with a 13-amino acid motif with RGD or any other tripeptide sequence capable of binding to integrin β -subunit at the tip (Niewiarowski et al. 1994). The RGD motif present in most disintegrins represents a sequence capable of recognizing the integrin motifs that emerged from P-III SVMPs housing an RDCED sequence.

Mammalian ADAM group of proteins were first identified in rodent sperm protein fertilin β containing a disintegrin and metalloproteinase domain (Wolfsberg et al. 1993). However, ADAMs generally do not contain an RGD motif. Human

ADAM15 is the only member of the ADAM family having RGD motif in its disintegrin-like domain. The primary sequences of disintegrin-like domains in the ADAM family are homologous to snake venom disintegrins. They constitute one subfamily of the so-called ADAMalysins, belonging to met-zinc superfamily of metalloproteinases. Members of this subfamily of proteins are found in several different types of animals (Dong et al. 2010).

The disintegrin domain of ADAM proteins contains about 90 amino acid residues. Disintegrin domains of SVMs are similar to the ligand binding site of matrix proteins (fibronectin). Many have an RGD consensus sequence housed in a 13-amino acid zone projecting from the surface of the protein. This region is called the **disintegrin loop**. This disintegrin loop allows binding to $\alpha_{\text{IIb}}\beta_3$ and $\alpha_v\beta_3$ integrin receptors (Blobel et al. 1992). A conserved RGD sequence in the disintegrin domain of human ADAM15 binds with $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Although most ADAMs do not contain an RGD sequence, they are still capable of binding with integrin receptors. Like the SVMs, regulation of metalloproteinase activity of ADAM may also occur by a “cysteine-switch” mechanism. This involves maintaining a prodomain cysteine in inactive state by blocking the active site Zn^{+2} (Fox and Bjarnason 1996). However, all ADAMs are not known to be proteolytic.

Future Directions and Conclusions

Disintegrins with $\alpha_{\text{IIb}}\beta_3$ integrin-inhibiting activity are potent antiplatelet agents and act as desirable candidates in treating pathological conditions like acute coronary ischemia and thrombosis. The specific integrin blocking ability and the therapeutic importance of disintegrins may lead researchers to design RGD mimetics which acts as antithrombotic agents. Tirofiban (Aggrastat) and Eptifibatide (Integrilin) have been designed based on snake venom disintegrins and are available in the market as antiplatelet agents (Koh and Kini 2012). Tirofiban is a synthetic, non-peptide inhibitor acting at glycoprotein GPIIb/IIIa receptors.

Several snake venom disintegrins can block the integrin molecule $\alpha_v\beta_3$ involved in multiple steps of malignant cancer development, including tumor angiogenesis and metastasis. Most of the antiangiogenic agents employed in current research are monoclonal antibodies (mAb) which act on specific pathways and inhibit specific signaling cascade to prevent the tumor growth. It is possible for tumor cells to develop resistance against these mAb therapies by choosing an alternative pathway for signaling. Snake venom disintegrins block integrins in a more generalized way. These disintegrins inhibit most of the major integrins on the tumor cells which are involved in the tumor signaling, thus producing more systemic effects and preventing the tumor at different stages. Disintegrin treatment may simply disrupt the cell-basal lamina adhesion or cell-cell adhesion to stop tumor growth. Interfering with endothelial cell adhesion system also prevents angiogenesis. Therefore, proper utilization of these extremely useful molecules provided by Nature will empower us to fight many life-threatening diseases. Apart from their possible use against cancer and thrombosis, the disintegrins probably can act against some viral infections

(Adam et al. 2010) which have shown that cytomegalovirus uses a glycoprotein disintegrin-like domain on its envelope to bind with integrin receptors to enter fibroblasts and endothelial cells. In the future, snake venom disintegrins may be used to challenge such virus-cell binding to prevent viral infections. One should also note that use of disintegrins as attack weapons has started quite early in the history of life!

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Part VI

Snake Envenomation and Snakebite Management

Role of Lymphatic System on Snake Venom Absorption

19

Dayanira Paniagua, Irene Vergara, Leslie Boyer, and
Alejandro Alagón

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Abstract

For several decades, advances have been made in venom characterization, mechanism of toxicity, and antivenom therapy. Much of this research has been based on models of the blood vascular system, to analyze the pharmacokinetics of venoms

D. Paniagua (✉) • I. Vergara • A. Alagón

Departamento de Medicina Molecular y Bioprocesos, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México

e-mail: dashpame@ibt.unam.mx; dashpame@gmail.com; irene@ibt.unam.mx; alagon@ibt.unam.mx

L. Boyer

Venom Immunochemistry, Pharmacology, and Emergency Response (VIPER) Institute, University of Arizona, Tucson, AZ, USA

e-mail: boyer@viper.arizona.edu

and antivenoms. However, in clinical envenomations, venom is injected into the interstitial space and an absorption process is necessary before it reaches the bloodstream. Absorption may occur by way of the blood or lymphatic capillaries, depending on the physicochemical properties of the molecules involved. Until recently, the role of the lymphatics in envenomation remained essentially unexplored, although several reports have demonstrated the fundamental role of the lymphatic system in the absorption of therapeutic proteins, administered subcutaneously. This review describes the absorption process, from the interstitial space and extracellular matrix through the entry into the blood capillaries and early lymphatics. Venom toxins interact with hyaluronic acid in the extracellular matrix, facilitating interstitial spread before entry into the vessels, and they induce local damage to the vascular endothelium, resulting in local hemorrhage and edema and altering the absorption characteristics of damaged vessels. Large molecules are absorbed primarily via the lymphatics, providing them a fundamentally different toxicokinetic profile from that of smaller toxins for which direct access to the blood capillaries is possible. Improved knowledge of the mechanism and factors influencing the subcutaneous venom absorption can improve the understanding of the role of edema, patterns of local injury, the toxicokinetics of envenomation, the effect of pressure immobilization, the pharmacodynamics and dosing of antivenom, and the phenomenon of recurrent venom effect.

Keywords

Venom absorption • Pharmacokinetics • Lymphatic system • Antivenom • Recurrence

Introduction

Snakebites represent a significant public health problem and clinical challenges in individual cases, especially in subtropical countries where they are responsible for significant morbidity and mortality. The World Health Organization reports that at least 421,000 envenomations and 20,000 deaths occur worldwide from snakebite each year and that these figures may be as high as 1,841,000 envenomations and 94,000 deaths. The highest burden of snakebites is in South Asia, Southeast Asia, and sub-Saharan Africa (Kasturiratne et al. 2008; WHO 2013).

Venomous snakes are found in the families Colubridae, Elapidae, Viperidae, and Atractaspididae (Mebs 2002). Venoms are a complex mixture of substances, mainly proteins, with different biological activities. Symptoms of envenomation differ in each case, but the principal toxicities include neurotoxic, hemorrhagic, cytotoxic, myotoxic, and systemic effects (Chippaux and Goyffon 1998). The factors that influence the severity of envenomation include the victim's age, size, and health and the venom composition, quantity, and delivery (Gold et al. 2004).

The usual treatment of envenomation involves the intravenous administration of specific neutralizing antibodies (IgG) or antibody fragments (Fab or F(ab')₂). The pharmacokinetics in the blood of each of these has been studied using different

animal models or healthy human volunteers (Ismail et al. 1998; Krifi et al. 2004; Morais et al. 1994; Pepin et al. 1995; Rivière et al. 1997; Vazquez et al. 2005, 2010a, b). Antivenom dosing takes into consideration the circumstances of the bite, local and systemic effects, and local medical capabilities (Chippaux and Goyffon 1998). In some cases, after treatment with antivenoms, local and systemic recurrences (worsening after initial improvement) have been observed. Local recurrence may result in greater tissue injury, and hemotoxic recurrence may result in the risk of hemorrhage. The latter is of particular concern because hemotoxic recurrence usually occurs after the patient is discharged from the hospital. Recurrence has been observed with ovine and digoxin-specific Fab, as well as with Fab, F(ab')₂, and IgG antivenoms from a variety of source animals (Seifert and Boyer 2001; Gutiérrez et al. 2003; Bush et al. 2013; Boyer et al. 2013). The recurrence of venom effects in Fab-treated patients appears to be the result of a pharmacokinetic and pharmacodynamic mismatch between the antivenom and target venom components. That is, tissue penetration and venom neutralization may be incomplete, and clearance of unbound antivenom (antivenom that has not bound its venom target) is significantly faster than the clearance of some venom components, allowing the signs and symptoms of envenomation to recur (Seifert and Boyer 2001). Recurrence depends also in the further absorption of venom from a depot area at the bite site as reviewed by Theakston (1997). The pharmacokinetic parameters from venom blood levels in experimental animals are known for some snake venoms (Audebert et al. 1994; Ismail et al. 1998); however, very little is known about the mechanisms of venom recurrence in the blood.

Rattlesnake venom absorption to the blood circulation, via the lymphatic system, was suggested as early as 1940 by Fidler et al. In 1941, Barnes and Trueta demonstrated that snake venoms containing components of high molecular weight are not absorbed from the limbs when lymphatics are obstructed or from an extremity that is completely immobilized. On the other hand, cobra venom possessing smaller toxic molecules is absorbed into the blood rapidly from obstructed or immobilized limbs.

This review describes the absorption process through the lymphatic pathway, with particular emphasis on venoms, and the impact of some toxins, specifically from snake venoms. Knowledge of the mechanism and factors influencing the subcutaneous venom absorption can help to better understand the role of edema in pathogenesis, patterns of local injury, the toxicokinetics of envenomation, the pharmacodynamics and dosing of antivenom, and the phenomenon of recurrent venom effect following initial therapy.

The Lymphatic System

The lymphatic system is a network of vessels distributed in almost all tissues of the body except the central nervous system, eyes, and bones. At intervals along the collecting vessels of the lymphatic system, there are encapsulated aggregations of lymphocytes and other accessory cells of the immune system, called lymph nodes.

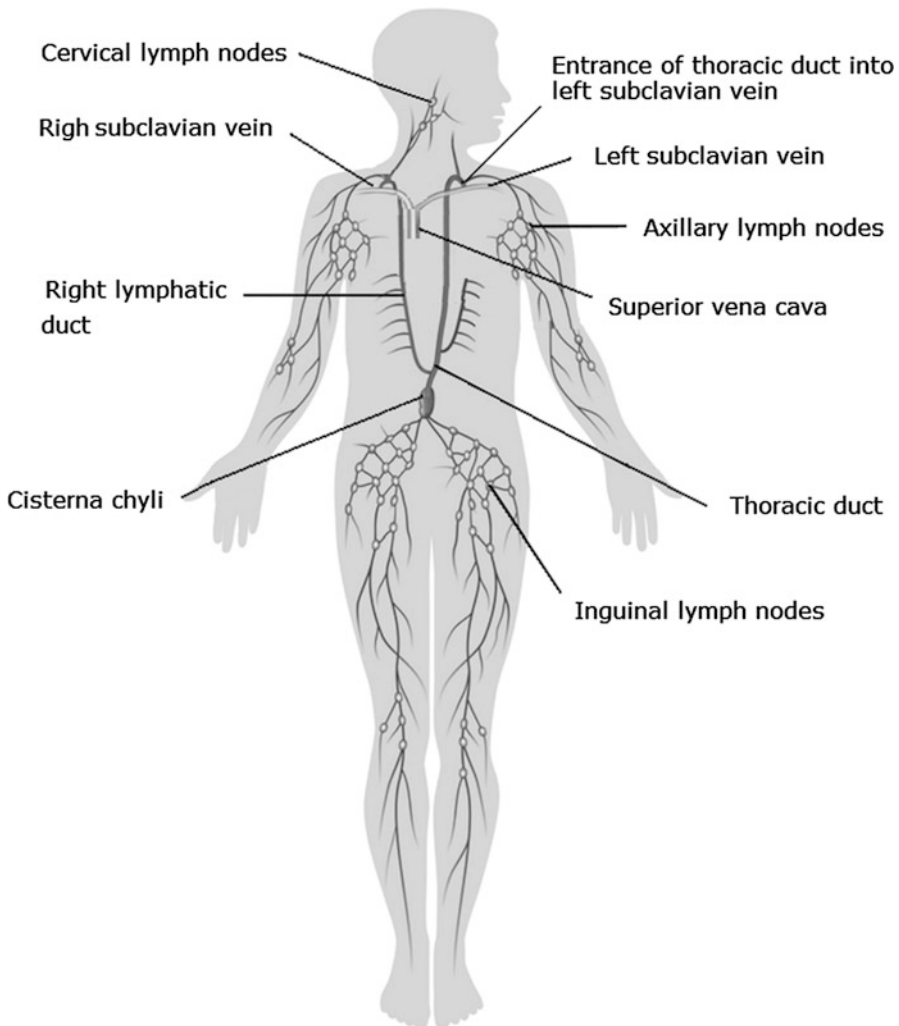


Fig. 1 Lymphatic system. Anatomy of the lymphatic network, lymph nodes, and ducts

These increase the contact between the lymph and the blood circulation, enabling fluid exchange and interactions between the cells of the immune system. The prenodal, or *afferent*, lymphatics deliver peripheral lymph from the tissues to the lymph nodes, while *efferent* or central lymph channels transport lymph to nodes further along the lymphatic chain in a unidirectional way, until they reach one large vessel, the thoracic duct, and one smaller vessel, the right lymphatic duct, which drain directly into the great veins in the root of the neck (Swartz 2001; Fig. 1).

The lymphatic system is a secondary vascular system with significant physiological function including the maintenance of fluid homeostasis, returning excess fluid,

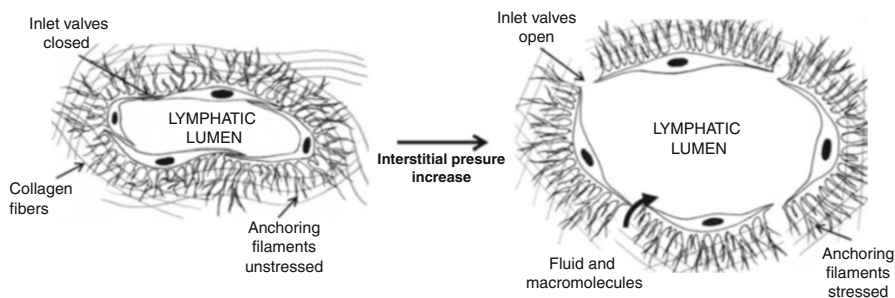


Fig. 2 Initial lymphatics. Conformational changes of the initial lymphatics when the interstitial pressure increases due to extrinsic lymphatic pump mechanism

proteins, and waste products from the interstitial space into the bloodstream. In humans, 3–4 L per day of lymph is returned to the blood. The lymphatic system transports lipophilic compounds including long-chain fatty acids, triglycerides, cholesterol esters, and lipid-soluble vitamins (Guyton and Hall 2011a). The removal of large macromolecules and particulate matter from the interstitial space is a unique attribute and a critical function of the lymphatic system, given that the blood vessels are not significantly permeable to macromolecules (Guyton and Hall 2011b). The lymphatic system is also an essential component of the immune system.

Lymph formation starts when fluid and macromolecules from the interstitium diffuse across the interstitial space into the initial lymphatics. The initial lymphatics are endothelialized structures conformed by cells that are attached to collagen fibers by anchoring filaments. These filaments are attached only to the center of the endothelial cells, leaving the unattached edges of the cell to function as inlet valves to the lymphatic lumen. When the surrounding tissue expands, the valves open, allowing unrestricted movement of fluid, macromolecules, and cells into the initial lymphatic. When the tissue is compressed, these valves close, sealing the endothelial barrier and preventing lymph from leaking back into the interstitial space (Fig. 2). This dependence on extrinsic tissue deformation is referred to as the *extrinsic lymphatic pump*, which affects the flow of lymph into the initial lymphatics. Mechanisms contributing to the extrinsic pump include vasomotion and pulse pressure changes in neighboring arterioles, skeletal muscle contraction, respiration, walking, skin tension, and external tissue compression.

Several initial lymphatics join to form a collecting duct, which is a segmented structure. Each segment, called lymphangion, is delimited by a bicuspid valve that prevents the retrograde flow of lymph and an intrinsic smooth muscle that compresses the lymphatic lumen. The rhythmic contraction of lymphatic smooth muscle is referred to as the *intrinsic lymphatic pump*, which propels lymph downstream from the initial lymphatics (Fig. 3).

The predominant mechanism driving lymph formation appears to be the development of transient fluid pressure gradients between the interstitium, the initial lymphatic, and the downstream collecting lymphatic vessels.

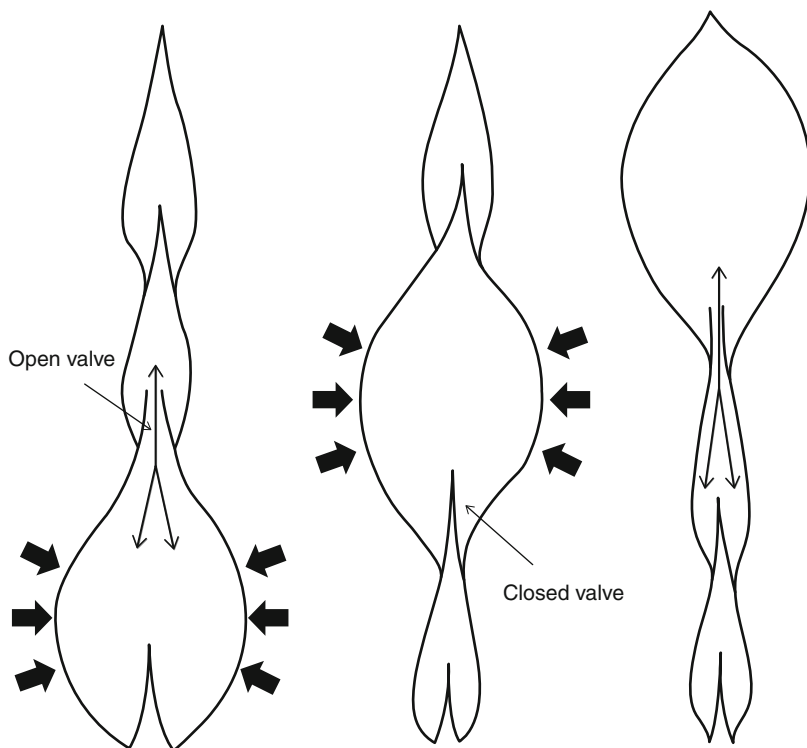


Fig. 3 Lymphangion. Structure of the lymphatic duct and the intrinsic lymphatic pump mechanism

The interstitial fluid volume, and therefore the interstitial fluid pressure, is kept within narrow limits because even a small increase in interstitial fluid volume leads to an increase in interstitial fluid pressure, which leads to greatly increased lymph flow rate. Lymph flow can increase 20–25-fold, resulting in a fall in interstitial fluid protein concentration, thereby lowering the tissue colloid osmotic pressure and reducing the net filtration of fluid from the capillaries and in turn helping to prevent an increase in interstitial fluid volume. This “washdown” of protein in the interstitial fluid is an important edema-preventing mechanism (Wiig and Swartz 2012).

Interstitial Space

The interstitial space consists of the connective and supporting tissues and parenchymal cells, and this space is vascularized by abundant blood and lymphatic vessel networks (Bosman and Stamenkovic 2003). It is composed of two phases: the structural molecules called the extracellular matrix (ECM) that function as an exclusion gel matrix and the interstitial fluid (IF), in which the diffusive and convective transports of water and molecules occur.

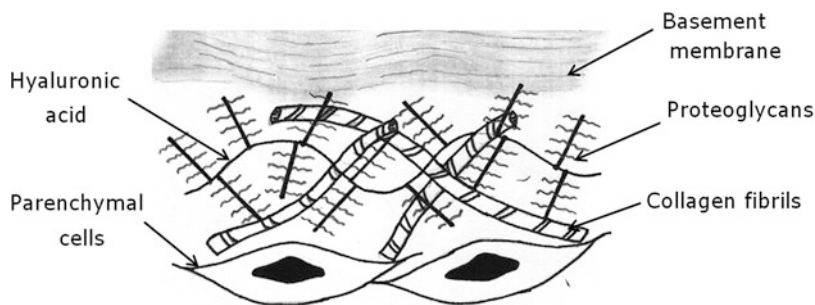


Fig. 4 Extracellular matrix. Macromolecules that conform to the extracellular matrix

Extracellular Matrix (ECM)

The ECM provides support and architecture for the extracellular space. It not only functions as a scaffold, supporting the tissues and preserving their physical integrity, but it also plays an important role in the development, migration, proliferation, shape, and function of cells in contact with it.

The ECM is comprised of four kinds of macromolecules: collagens, elastins, proteoglycans, and glycoproteins. The collagen and elastin system forms the architecture of extracellular matrix (Fig. 4). Collagens are ubiquitous proteins that form supra-macromolecular structures of three associated polypeptide chains, called fibrils. Collagens are responsible for maintaining the structural integrity of vertebrates and many other organisms. To date, 27 distinct collagens with different functions have been identified. They are classified according to the way they assemble as fibrillar collagens (types I, II, III, IV, and XI) and non-fibrillar collagens (VI, VII, VIII, and X). Elastin is the protein that enables stretching or contracting by many tissues in the body. It is an insoluble polymer, constituted from soluble tropoelastin molecules and a glycoprotein called fibrillin. Elastin forms different amounts of fibers depending on the tissue. Other glycoproteins, such as laminin and tenascin, adhere to the scaffold and interact with the cells adjacent to the matrix. Glycosaminoglycans (GAGs) are among the most complex polysaccharide chains that are either covalently linked to protein cores (to form proteoglycans, PGs) or free as unsulfated hyaluronan. The main GAGs are hyaluronan (or hyaluronic acid or hyaluronate or HA), dermatan, keratan, chondroitin sulfate, and heparan sulfate (Bosman and Stamenkovic 2003; Tanzer 2006).

The PGs participate in the adhesion of cells and other matrix components. GAGs and PG have an important role in the maintenance of pH and of hydric equilibrium due to the presence of sulfate and carboxylate groups that confers a high density of negative charges. Structural interstitial macromolecules, particularly GAGs and collagen, restrict the space available to macromolecules in the interstitial space simply because the molecules cannot occupy the same space. This phenomenon is called geometrical, or steric, interstitial exclusion.

The degradation of extracellular matrix components is tightly controlled, and their regulated turnover is critical to a variety of important processes. In all tissues,

there is a continuous turnover of the extracellular matrix as a result of degradation and resynthesis.

Interstitial Fluid (IF)

The IF consists of interstitial water and a variety of solutes. This fluid transports nutrients and waste products between cells and blood capillaries, signaling molecules between cells and antigens, and cytokines to local draining lymph nodes for immune regulation. The IF volume is kept fairly constant at 20 % of body weight, under normal conditions, involving structural changes, adjustment of forces acting across the capillary wall, and lymph flow.

IF forms when fluid and proteins extravasate from the plasma to the interstitium through the blood capillary wall. The extravasation process is governed by the imbalance of the hydrostatic and oncotic pressures between the interstitial fluid and plasma. These forces are known as Starling forces. When equilibrium is reached, some fluid remains in the interstitial space and that is when lymph formation starts (Aukland and Reed [1993](#)).

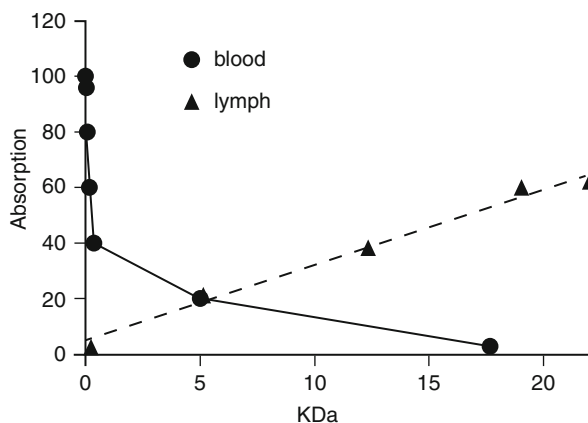
Blood Capillary Vascular Endothelium

The structure of the blood capillary wall is complex and varies greatly in different organs and tissues. It generally comprises four layers, namely, the plasma-endothelial interface, the endothelium, the basal lamina, and the adventitia. Based on the morphology and the continuity of the endothelial layer and the basal membrane, the blood capillary endothelium can be divided into three general categories: continuous, fenestrated, and discontinuous. Continuous capillaries are found in the skeletal, cardiac, and smooth muscles, as well as in the lungs, skin, and subcutaneous and mucous membranes. The capillary endothelial cells join by tight junctions and an uninterrupted subendothelial basement membrane (BM) (Takakura et al. [1998](#)). The BM is a specialized structure of the ECM; in general BMs separate the endothelium from the stroma of any given tissue. BM is always in contact with cells providing structural support, dividing tissues into compartments, accumulating growth factors, and participating in the communication between intracellular and extracellular environments. The main components of BMs include type IV collagen, laminin, heparan sulfate proteoglycans, and nidogen/entactin (Kalluri [2003](#)). In tissue remodeling and inflammatory processes, BM components are degraded by endogenous metalloproteinases (Shapiro and Senior [1999](#); Page-McCaw et al. [2007](#)).

Differential Absorption to Systemic Blood Circulation of Small and Large Proteins

Small proteins in the interstitial space are preferentially absorbed by the blood capillaries due to their largely unrestricted permeability and the high rate of filtration and reabsorption of fluid across the vascular capillaries (in the range of 20–40 L/day,

Fig. 5 Permeability. Correlation between molecular weight and absorption in the blood and lymph (Modified from Guyton and Hall 12th ed, and Supersaxo et al. 1990)



in comparison to approximately 2–4 L/day of fluid drained by the lymph). In contrast, permeability to large macromolecules is restricted across the vascular endothelium, where tight endothelial junctions make it difficult for macromolecules to pass through. The lymphatic system provides macromolecules an alternative absorption pathway from the interstitial space to the blood because lymphatic capillaries have incomplete basal lamina, which enables almost unrestricted drainage of macromolecules from the interstitial space (Porter and Charman 2000). In Fig. 5, the correlation of permeability and molecular size is shown for the vascular and lymphatic endothelia. As molecular size increases, the permeability of blood vascular endothelium decreases, and the permeability of lymphatics increases.

Subcutaneous (SC) injections are widely used as a delivery route for compounds with limited oral bioavailability, and the pharmacokinetics of several therapeutic proteins administered SC have been analyzed. Absorption of these molecules is not always complete, with absorption fraction values ranging from 20 % to 100 % of the administered dose and with a relatively slow rate of absorption as evidenced by a prolonged terminal half-life in comparison to that observed after intravenous administration. Maximum plasma concentrations after SC injection occur from 2 to 20 h post-dosing (reviewed by Porter and Charman 2000). The rate of absorption and total bioavailability after SC administration both in humans and animals depend on many physiological factors (e.g., age, body mass index, site of injection and the application of heat and massage, and restriction of active movement), as well on the physicochemical properties (e.g., size, shape, charge, and hydrophobicity) of the injected molecules (Kota et. al. 2007; Porter and Charman 2000; Reddy et al. 2006).

Hyaluronidases as Spreading Factors

Enzymes that degrade hyaluronic acid (HA) are called hyaluronidases. These enzymes are classified, based on the products of degradation and mechanism of action, into three groups: (1) hyaluronate 4-glycanohydrolase/hyaluronoglucosaminidase (EC 3.2.1.35),

e.g., testicular hyaluronidases and snake venom hyaluronidases; (2) hyaluronate 3-glycanohydrolase/hyaloglucuronidase (EC 3.2.1.36), e.g., salivary leech hyaluronidase; and (3) bacterial hyaluronidases that cleaves HA at $\beta(1-4)$ glycosidic linkages (EC 4.2.99.1) through an eliminase/lyase mechanism, while the first two classes are hydrolases (Girish and Kemparaju 2007; Kemparaju et al. 2010).

Hyaluronidase is perhaps the most ubiquitous enzyme present in venoms. The great majority of venoms of scorpions, snakes, bees, wasp, spiders, caterpillars, fishes, and lizards contain hyaluronidase (Girish et al. 2002; Tanaka et al. 2010). In venom, hyaluronidase plays an important role because it acts as a spreading factor promoting venom absorption (Girish and Kemparaju 2007).

HA is the simplest of the GAGs because it is not bound to a polypeptide chain. It is present in the ECM of all vertebrates. HA is a linear, polysaccharide, unsulfated acid of high molecular weight of up to 10^7 Da, formed by repeated sequences of D-glucuronic acid-*N*-acetylglucosamine (Fraser et al. 1997). It is produced in large quantities during wound repair and it is an important constituent of joint fluid, where it serves as a lubricant. It has been isolated from the vitreous humor, the umbilical cord, the mucoid of group A hemolytic streptococci, synovial fluid, and the pleural fluid of a patient with a mesothelioma.

By depolymerizing hyaluronan, hyaluronidase rapidly reduces the viscosity of the ECM, changing the interstitial glycosaminoglycans from a gel-like phase to a fluid-liquid phase, thereby making increased bulk fluid flow possible through the collagen fibers.

Duran-Reynals, in 1939, reported for the first time the presence of a “spreading factor” and suggested that this factor was independent of the toxic factor in snake venoms. In 1940, Chain and Duthie related the “spreading factor” to hyaluronidase activity. “Spreading agents” derived from animal testicular extracts containing interstitial matrix-degrading enzymes have been used clinically for over 50 years to facilitate the dispersion and absorption of other drugs. Bookbinder et al. (2006) provided evidence that highly purified recombinant human hyaluronidase (rHuPH20) enhanced the infusion rates and penetration of molecules up to 200 nm in diameter up to 20-fold. In addition to increasing infusion rates and dispersion across a broad range of molecular weights, rHuPH20 was found to enhance the systemic bioavailability of locally injected biotherapeutics with an absolute bioavailability comparable to the levels obtained by intravenous delivery. The effect of rHuPH20 has been demonstrated on the pharmacokinetics and bioavailability of a 31 kDa pegylated cytokine, peginterferon alfa-2b (60 kDa), and a 149 kDa monoclonal antibody, infliximab. Local co-injection with rHuPH20 increased the absolute bioavailability of the cytokine from 61 % to 108 %, and the maximal plasma concentration increased twofold. These results indicate that rHuPH20 markedly improves the pharmacokinetic profiles of large molecule biopharmaceuticals administered via the interstitial route and drives the pharmacokinetic profile toward an intravenous administration.

Not surprisingly, the hyaluronidase activity in venoms has a role in the diffusion and distribution of toxins during envenomation (Yingprasertchai et al. 2003). Girish et al. (2002) showed that hyaluronidase treatment of various tissues resulted in the

major loss of ECM structure integrity and hence facilitated the diffusion of toxins. Hyaluronidase promotes the spread of hemorrhage in mice when injected with hemorrhagic toxin from *Trimeresurus flavoviridis* (Tu and Hendon 1983). Hyaluronidase from *Naja naja*, coadministered with myotoxin VRV-PL-VIII and hemorrhagic complex I from *Daboia russelii*, potentiated their myotoxicity and hemorrhagic activities. In vivo experiments, with a rabbit dermonecrosis model, clearly showed that *Loxosceles* venom hyaluronidase increased the dermonecrotic effect of a sphingomyelinase D from the same spider venom (Ferrer et al. 2013). Thus, hyaluronidase activity in venoms affects not only local damage but also the magnitude of the systemic effects of envenomation (Girish et al. 2004).

Effect of Venom Molecules on Lymphatic System Components

Venoms that induce local damage contain components that affect the blood vascular and lymphatic endothelial cells and their underlying BM. Those alterations result in changes in their permeability to blood cells, proteins, and fluids. Prominent edema and extensive local hemorrhage are some of the resulting clinical findings. Two classes of venom components are clearly involved in the genesis of these phenomena, metalloproteinases and PLA₂ myotoxins.

In the Viperidae family, snake venoms are a rich source of metalloproteases (SVMs), which comprise a subfamily of zinc-dependent enzymes that differ in their domain structure. These fall into four groups: P-I, containing only the metalloproteinase domain; P-II, having a metalloproteinase domain and a disintegrin-like domain; P-III, comprising metalloproteinase, disintegrin-like, and cysteine-rich domains; and P-IV, having, additionally, a lectin-like domain (Bjarnason and Fox 1994; Paine et al. 1992).

One of the most serious effects induced by SVMs is local hemorrhage; but other alterations of high pathophysiological impact are also promoted by these enzymes, including myonecrosis, blistering, coagulopathy, platelet effects, and proinflammatory activity (Bjarnason and Fox 1994; Hati et al. 1999; Kamiguti et al. 1998). A microscopic observation of affected tissues reveals abundant extravasated erythrocytes associated with prominent damage in the microvasculature.

In 1995, Gutiérrez et al. showed that a metalloproteinase (P-I) isolated from *Bothrops asper* venom (BaP1) induced paw edema in mice after intramuscular injection, with blister formation and infiltration of leukocytes into the dermis. Edema was associated with degranulation of mast cells and enlarged macrophages (Rucavado et al. 1998, 1999). The cytokines could mediate the local inflammatory events induced by BaP1, since IL-1 and IL-6 were released in the muscle tissue (Rucavado et al. 2002); however, mechanisms involved in the proinflammatory action of mammalian metalloproteinases are only partially explained. Other pathophysiological effects include the degradation of BM proteins, but their cleavage per se does not result in hemorrhage, since BM is a structural scaffold that provides mechanical support to endothelial cells. The disruption of the endothelial cell integrity, with consequent extravasation, involves a second step, the thinning of

endothelial cells caused by the hemodynamic forces that tend to distend the capillary wall (Gutiérrez et al. 2005). Microvessel disruption by metalloproteinases also damages the skeletal muscle, contributing to permanent tissue loss after snakebites (Bjarnason and Fox 1994).

In an elegant paper, Mora et al. 2008 found that *Bothrops asper* venom applied to the mouse mesentery induced contraction of lymphatic vessels with reduction of their lumen and eventual interruption of lymph flow. The effect was reproduced by pure myotoxin II, a phospholipase A₂ homologue. The hemorrhagic metalloproteinase BaPI and a thrombin-like enzyme isolated from the same venom were inactive in the mesenteric preparation. The venom and myotoxin II induced edema in the mouse footpad model and caused cytotoxicity in cultured smooth muscle cells. The authors conclude that the alterations observed in the collecting lymphatic vessels are due to the effect of myotoxin II on smooth muscle cells of the lymphatic vessel wall.

On the other hand, non-myotoxic, catalytically active, Asp 49 phospholipases A₂ from several snake venoms induce edema due to arachidonic acid release from the sn-2 position of phospholipids. Arachidonic acid is the major precursor of prostanoids, such as prostaglandins and leukotrienes. These inflammation mediators cause alteration in blood microvessels, leading to increased permeability and edema formation at the site of snakebite (Doley et al. 2010; Teixeira et al. 2003).

Role of the Lymphatic System on Snake Venom Absorption

The envenomation process begins with venom delivery and the absorption of toxins to systemic circulation, until they reach their target. Depending on the snake's anatomic structure and fang size, venom delivery occurs subcutaneously, or occasionally intramuscularly, after which toxins are released into the interstitial space. From the interstitium, toxins must diffuse through the extracellular matrix until they reach a vessel with permeable endothelium, where they can be absorbed. The permeability of the blood vascular endothelium decreases as molecular size increases; in contrast, the physiology of the lymphatic system allows the entrance of large molecules that cannot be absorbed by the blood vessels. The principal factors governing these processes are the physicochemical properties of the toxins (size, charge, hydrophobicity) and microenvironmental conditions (integrity of the extracellular space, blood and lymphatic capillaries vascularity near the inoculation site) (Fidler et al. 1940; Kota et al. 2007; McLennan et al. 2005; Mora et al. 2008; Porter and Charman 2000; Reddy et al. 2006).

Some venoms, such as those of scorpions and elapid snakes, are rich in low-molecular-weight neurotoxins of high diffusivity and large volume of distribution that reach their tissue targets rapidly after injection (Calderón-Aranda et al. 1999; Hammoudi-Triki et al. 2007; Krifi et al. 2001, 2004). In contrast, venoms rich in high-molecular-weight toxins, such as those of viperid snakes, have a pharmacokinetic profile characterized by a rapid initial absorption phase followed by a complex and slow absorption process from the site of venom injection. Venom antigens

appear in the blood circulation 10 min after the IM injection of *Vipera aspis* venom and reach maximal concentration after 1.5–5 h, with high concentrations being maintained for more than 3 days; at the same time, the apparent terminal half-life was threefold higher than that measured after IV injection of venom (Audebert et al. 1994). This pharmacokinetic behavior suggests the participation of lymphatic circulation. As lymph has relatively slow flow and low volume, absorption by this route should affect the residence time in the body as well as the absorption rate into the blood vascular circulation. However, the absorption of venom via the lymphatic route has been poorly explored (Paniagua et al. 2012).

Past models of venom pharmacokinetics have relied primarily on the measurement of blood venom levels, occasionally supplemented by urine and/or selected organ venom levels. Paniagua et al. (2012) demonstrated that lymphatic absorption at the envenomation site plays a major role in the availability and kinetics of subcutaneously injected *Micrurus fulvius* venom, whose principal toxins are around 13,000 Da. They found that after 6 h, the observation period, only 69 % of the initial dose had been absorbed, of which 25 % was via the lymphatic system. The highest concentration of venom found in lymph was more than 25-fold higher than the venom concentration reached in the blood. This could be explained considering the differences between lymph and blood flow rates. The kinetic analysis of lymphatic venom absorption also enabled, for the first time, an explanation of sustained venom levels in the blood following envenomation. The analysis of both lymph and blood venom levels suggested that the lymph pool provides a sustained inoculum of venom and associated high-molecular-weight products that are carried into the bloodstream via the lymphatics.

The measurement of venom in lymph enhances the understanding of the dynamic process of how venom passes from the site of injection into the systemic circulation. Analysis of the injection site, 6 h following injection, confirmed the presence of 22 mcg of venom per g of skin. This suggests the retention of the macromolecules administered SC in the inoculation site, functioning like a depot as suggested by Seifert et al. (1997) and Theakston et al. (1992). Further experiments are needed to analyze the venom depot and its absorption process. Interestingly, Wu et al. (2012) investigated the influence of the molecular weight of proteins on the rate of loss from the SC injection site and found a direct correlation between the residence time of protein in the inoculation site and its molecular weight.

Recurrence of Envenomation and Lymphatic Transport of Venom from the Inoculation Site

Recurrence of venom in the blood circulatory system occurs when there is an unfavorable pharmacokinetic match between antivenom and venom. Reappearance of venom in the blood, followed by repeated worsening of laboratory test values and clinical symptoms after initial improvement following antivenom administration, is a well-known phenomenon in snake envenomations, especially by viperids (Bogdan et al. 2000; Boyer et al. 1999, 2001; Warrell, et al. 1986). The cause of this

recurrence phenomenon has been suggested to involve a delayed absorption of venom from the site of injection, either subcutaneous or muscular tissues (Dart et al. 2001; Ho et al. 1990; Meyer et al. 1997).

The findings of Paniagua et al. (2012) on the role of the lymphatic system in the absorption of venom from the inoculation site and in the maintenance of significant amounts of venom in the blood circulation can help to explain, to a great extent, the coagulopathic recurrence of envenomation. Now it is known that the venom lymph pool, together with the venom pool in the depot, represents a long-term source of venom that can last several days (Paniagua et al. 2012).

Particularly in the case of Fab antivenom therapy for rattlesnake envenomation, coagulopathic recurrence appears to reflect an approximately 2-week subacute phase of envenomation, which is unmasked when antivenom is cleared within this time (Boyer et al. 2013). Fab fragments have short elimination half-life and, consequently, treatment consensus favors repeated dosing of Fab following the initial control of envenomation (Lavonas et al. 2011). Alternatively, it has been suggested that Fab be administered as a continuous IV infusion, after initial control has been achieved, for ongoing neutralization of venom components that reach circulation from tissue stores and the lymphatics at later times (Bush et al. 2013). Much less frequently, recurrence of envenomation has also been described for IgG and F(ab')₂ antivenoms, which have pharmacokinetic profiles more compatible with those of venoms (Otero-Patiño et al. 1998; Warrell et al. 1986; Bogdan et al. 2000). It could be hypothesized that recurrence in the case of IgG or F(ab')₂ antivenoms occurs when the initial dose of antivenom falls short of what would be required to neutralize the total amount of venom inoculated that later reaches the circulation.

Use of the Pressure Immobilization Technique to Retard Systemic Venom Absorption

Several techniques have been recommended to retard the flow of venom through the lymphatic system to slow the systemic spread of venom (Anker et al. 1983). These techniques are not tourniquets and are designed to preserve deep venous and arterial flows. One of them, the pressure immobilization technique (PIT), developed in the 1970s by Struan Sutherland (Sutherland et al. 1979), has two components: pressure to prevent lymphatic drainage and immobilization of the bitten extremity to prevent the pumping action of the skeletal muscles. PIT is recommended as a field first-aid technique for elapid bites in Australia because it delays systemic toxicity (Pear et al. 1981). Most elapid venoms are predominantly neurotoxic and cause limited local damage. PIT could prove to be a reasonable intervention for coral snake bites since there is a major transport of coral venom from the injection site to the regional lymph nodes and the thoracic duct and, from there, into the general blood circulation (Paniagua et al. 2012). Moreover, in a porcine model, PIT delayed toxicity in coral snake envenomation (German et al. 2005). However, real-life conditions to apply

PIT may be such that instead of buying time for antivenom administration, there may be loss of precious time before the use of the specific treatment.

In addition to the potential time delay to definitive treatment, the pressures achieved must be within certain ranges, which differ from the upper to lower extremities and which are likely not directly determinable in the field. The proper technique may not be applied or retained even by trained individuals and result in either too low a pressure being achieved, which will be ineffective, or too high a pressure, which will result in a tourniquet effect (Norris et al. 2005; Simpson et al. 2008; Canale et al. 2009).

Lymph flow-retarding procedures are problematic, however, for snakebites that cause substantial local damage, as is often the case with viperid bites. The application of PIT in viperid bites can lead to increased and/or permanent limb injury while saving virtually no lives (Seifert et al. 2011). The use of PIT for the prehospital treatment of North American Crotalinae envenomation is not recommended (American College of Medical Toxicology et al. 2011), and that position could be generalized for other snakebites associated with drastic local tissue pathology, including prominent edema, hemorrhage, blistering, dermonecrosis, and/or myonecrosis.

Conclusion and Future Directions

The lymphatic system has significant physiological functions, the impact of which on snakebite envenomation has been largely neglected. In the past years, substantial scientific effort has gone into an improved understanding of the role of the lymphatic system in cancer, edema, absorption of therapeutic proteins, and the immune response. In particular, research on the absorption, distribution, and bioavailability of parenterally administered biopharmaceuticals has shed light on the importance of the lymphatics in those processes. The main toxic components of snake venoms are proteins of varying molecular weight, and they are generally injected subcutaneously or intramuscularly; so regardless of the paucity of past research, lymph physiology must play a very important role in the envenomation process.

Using a sheep lymphatic cannulation model, Paniagua et al. (2012) showed that the absorption of *Micrurus fulvius* venom via the lymphatics is very important following SC administration and that lymph pool provides a sustained inoculum of venom destined for entry into the bloodstream. These findings help to explain that systemic signs and symptoms of paralysis may be delayed for many hours, for as long as 12 h (Kitchens and Van Mierop 1987). Since the venom of *Micrurus fulvius*, as that of many elapids, lacks significant proteolytic activity and its local tissue effects are minimal to none, its direct pathological actions on lymph and blood capillaries around the injection site could be regarded as minimal.

On the other hand, one of the local signs in viperid snakebite envenomations is rapid development of edema. This excess fluid accumulation in the extracellular space could result from an abnormal leakage of fluid from the plasma to the

interstitial space across the capillaries, failure of the lymphatics to return the fluid from the interstitium into the blood (lymphedema), or both. Inflammatory mediators that cause increased permeability of the capillaries are present in viperid venoms (Doley et al. 2010; Rucavado et al. 1998, 1999, 2002; Teixeira et al. 2003). Another cause of increased permeability is degradation of the basement membrane proteins of the blood capillaries by metalloproteinases (Bjarnason and Fox 1994; Gutiérrez et al. 2005; Paine et al. 1992). Factors that increase the interstitial fluid pressure, such as increased permeability of blood capillaries, also increase lymph flow if the lymph vessels are functioning normally. Under this view, massive edema ought to increase the speed of venom uptake considerably. This would be an evolutionary reason for the edema-inducing components of venom to allow for the distribution of the other toxins. However, the findings of Mora et al. (2008) indicate that *Bothrops asper* venom damages the smooth muscle of lymphatic collecting vessels, inducing their contraction and halting of lymph flow. In addition, hypovolemic shock following rapid edema formation (third spacing) occurs with some viperid envenomations and may have been evolutionarily favored as a mechanism of prey immobilization. These findings suggest that lymphedema could play an important role in the local edema of viperid snakebite envenomations and, thus, in the distribution and availability of many venom components.

The use of combined blood and lymphatic sampling in large animal models should provide insights into the absorption and distribution of venoms, including selected individual venom toxins, as well as the interactions of venoms and antivenoms. Future studies of the pharmacokinetics of antivenoms in the blood must be enriched by their simultaneous evaluation in lymph. Characterization of the effect of antivenoms on the blood and lymph venom levels should help to better understand the neutralization capabilities of IgG-, Fab- and F(ab')₂-based antivenoms, their dosage, and the influence of administration route, and also provide guidance in designing better therapeutic strategies.

Better understanding of the dynamics of venom clearance from local tissues would improve the safety and effectiveness of early treatment measures, such as the use of compression wraps or other local first-aid measures. Improved knowledge of the relative role of capillary leak and lymphedema, coupled with better data on the role of hyaluronidase and other toxins, could inform the early dosing of antivenom, in the phase of disease when edema continues to spread despite initial antivenom use. A clearer understanding of the role of the intrinsic inflammatory response could lead to the more rational use of adjunctive therapies in critically ill patients. And subacute toxicodynamic studies could lead to a better balance of wound management and coagulopathy prevention during the subacute phase of disease, when ongoing envenomation and early tissue healing may take place simultaneously (Boyer et al. 2013).

Mora et al. (2008) considered the effects of viper snake venom on lymphatic vessels as the hidden aspect of envenomation. Because of this, the incipient field of lymphotoxinology should help to gain a better and deeper understanding of the many pathophysiological effects of venoms, to improve knowledge of the mechanisms and factors that affect parenteral absorption of venoms, to better understand their pharmacokinetics, and to improve antivenom therapy (Witte 2012).

Cross-References

- ▶ Anticoagulant and Membrane Damaging Properties of Snake Venom Phospholipase A₂ Enzymes
- ▶ Antivenom Safety and Tolerance for the Strategy of Snake Envenomation Management
- ▶ Cellular Mechanisms of Action of Snake Phospholipase A₂ Toxins
- ▶ Snake Venom Disintegrins

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Antivenom Safety and Tolerance for the Strategy of Snake Envenomation Management

20

J. P. Chippaux, R. P. Stock, and A. Massougbodji

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J.P. Chippaux (✉)

Institute of Research for Development (IRD – UMR 216 “Mother and Child Facing Tropical Diseases”), Cotonou, Bénin

Faculté de Pharmacie, University Paris Descartes, Sorbonne Paris Cité, Cotonou, France

Center for the Study and Research of Malaria Associated with Pregnancy and Childhood (Cerpape), Cotonou, Benin

e-mail: jean-philippe.chippaux@ird.fr

R.P. Stock

Institute of Biotechnology, National Autonomous University of Mexico (IBT-UNAM), Cuernavaca, Mexico

e-mail: rstock@ibt.unam.mx

A. Massougbodji

Center for the Study and Research of Malaria Associated with Pregnancy and Childhood (Cerpape), Cotonou, Benin

e-mail: massougbodjiachille@yahoo.fr

Abstract

Discovered 120 years ago, passive immunotherapy remains the sole etiological treatment of envenomations, particularly those caused by snakes and scorpions. Antivenoms are composed of immunoglobulins produced by animals immunized against certain venoms. Improvement of antivenoms, most notably by advances in the methods used for immunoglobulin purification, enzymatic digestion, and fractionation, has allowed a considerable reduction of adverse effects while maintaining antivenom efficacy. This has expanded antivenom indication, particularly for children and pregnant women to limit the risks of complications due to venom action.

However, antivenom accessibility remains the main obstacle to widespread use; antivenoms are still very expensive and very poorly distributed in high-risk areas. A special effort is required of health authorities, producers, and distributors of antivenoms as well as treating physicians and even patients to alleviate the absence of antivenoms where they are most useful – rural health centers – to increase their use and reduce the morbidity and mortality caused by envenomations.

The authors describe the desired properties of antivenoms as tools of public health (efficacy, tolerability, stability, and accessibility, particularly economic), explore the difficulties that still plague their use, and suggest some steps to improve accessibility.

Keywords

Envenomation • Antivenom • Adverse reactions • Treatment • Immunoglobulin snake • Scorpion

Introduction

Invented in the end of the nineteenth century, antivenoms remain the only etiological treatment of envenomations. Antivenoms are antibodies – mostly immunoglobulins G (IgG) – produced by animals upon immunization with antigens from one or several venoms (Chippaux and Goyffon 1998). Immunization is conducted by repeated injection of low doses of venom, sometimes detoxified and/or associated to adjuvants. In cases of envenomation, antivenom is administered to the patients to neutralize and eliminate venom. While numerous early clinical and experimental observations established their general utility, antivenoms were not systematically studied in clinical trials or pharmacological research until after 1950 (Chippaux et al. 2010). For a long time, their therapeutic value was accepted despite their occasionally severe (and sometimes lethal) adverse side effects, until developments in emergency medicine led to a decline in use (Chippaux and Goyffon 1998).

Antivenoms are made of heterologous proteins capable of provoking undesirable reactions in patients. Caution is required in terms of the pertinence of administration, adequate surveillance of patients, and appropriate measures in case of intolerance.

Table 1 Milestones in the evolution of antivenom manufacture

Date	Procedure	Product	Contribution
1894	Decantation/centrifugation	Antiserum (first generation)	Etiological treatment of envenomations
1924	Formaldehyde detoxification of venoms	Antiserum	Improvement in immunization
1930	Precipitation with ammonium sulfate	Purified IgG (second generation)	Concentration of active principle
1936	Enzymatic digestion	IgG fragments (third generation)	Improvement in safety (tolerability)
1970	Ultrafiltration – dialysis	Highly purified IgG fragments	Improvement in safety (tolerability)
1990	Lyophilization	–	Improvement in stability
2000	Immunization with enriched toxic fractions or recombinant toxins	–	Improvement in immunization

Early in the twentieth century, the original Calmette protocol of antivenom preparation was rapidly implemented throughout the world. Later on, the protocol was improved in order to increase antivenom efficacy and reduce, as much as possible, the risk of adverse effects (Table 1). Since then, progressively more complex and costly manufacturing processes have resulted in antivenoms that are not available in many countries of the Global South, where envenomations are most common (Chippaux 1998, 2002). Recent epidemiological information, however, confirms the importance of improving antivenom distribution in those regions where envenomations are frequent and, often, therapeutic resources very limited.

This chapter attempts to summarize the requirements modern antivenoms must meet to be useful public health tools. First and foremost, they must be efficacious and available in rural health centers, close to the places where envenomations by snakebite and scorpion sting occur. This is essential, as early antivenom administration is far more effective in preventing and arresting complications. In tropical countries, rural health centers often do not have even the barest essentials and, furthermore, healthcare professionals are simultaneously few and poorly trained. Therefore, the real-world utility of antivenoms will be essentially determined by four properties – efficacy, safety (tolerability), stability, and accessibility – such that they will benefit patients even when administered by poorly qualified personnel (Chippaux 2002; WHO 2010).

Mode of Action of Antivenoms

The action of passive immunotherapy depends on the binding of antibodies (antivenom) administered to the antigens (venom) present in the organism. This evidently requires that the antivenom antibodies and venom antigens find

themselves in the same compartment at the same time. It is also necessary for venom components to be neutralized upon antibody binding and that antigen-antibody complexes be eliminated.

After inoculation, venom components diffuse rapidly in the organism via lymphatic and blood circulation (Rivière et al. 1998; Krifi et al. 2005; Sevcik et al. 2004; Paniagua et al. 2012), and venom concentration tends to equilibrate between different compartments, i.e., blood and deep organs, a process influenced by the route of inoculation. Venom injected intravenously is eliminated in 2–3 days (Rivière et al. 1997, 1998), mostly through renal route, although some components can take more than a week to disappear from the organism. During the natural course of an envenomation, venom originally inoculated intramuscularly or subcutaneously can persist for much longer times; a recirculation from the site of the bite or another reservoir compartment, particularly the lymphatic system, is possible (Paniagua et al. 2012).

The rate of diffusion and distribution of antibodies depends on their nature and the route of administration (Rivière et al. 1997, 1998; Krifi et al. 2005; Sevcik et al. 2004; Vázquez et al. 2010). The apparent volume of distribution of IgG antibodies is superimposable to the vascular compartment, which translates into their persistence in this compartment for several days (Table 2). Maximal IgG concentration is reached in about 6 h in superficial tissues and in about 30 h in deeper ones. Antibody fragments ($F(ab')_2$) have an apparent volume of distribution about double that of plasmatic volume, suggesting a greater distribution into tissues than whole IgG. Furthermore, these fragments diffuse more rapidly: 1 h for superficial tissues and 6 h for deeper ones. The affinity of monovalent Fab fragments for deep tissues is five times greater than that of whole IgG and twice that of $F(ab')_2$. However, the rate of diffusion is not markedly different than that of $F(ab')_2$ (Rivière et al. 1998).

The presence of antibodies in the vascular compartment results in a transfer of venom components from peripheral tissues to the blood, where they form venom-antibody complexes (Rivière et al. 1997, 1998; Sevcik et al. 2004). When antibodies are administered in low quantity, venom concentration in blood drops temporarily before returning to the original levels as antibodies is exhausted. When administered

Table 2 Comparative pharmacokinetics of IgG and its fragments

Properties	IgG	$F(ab')_2$	Fab
Production	Precipitation	Pepsin + precipitation	Papain + precipitation
Distribution	>6 h	3 h	1 h
Elimination (half-life)	>100 h	60 h	10 h
Affinity for tissues	1	2	5
Complement fixation	Yes	No ^a	No
Antibody valence	Bivalent	Bivalent	Monovalent
Excretion	Immunocompetent cells	Immunocompetent cells	Renal

^aComplement activation by the alternate pathway

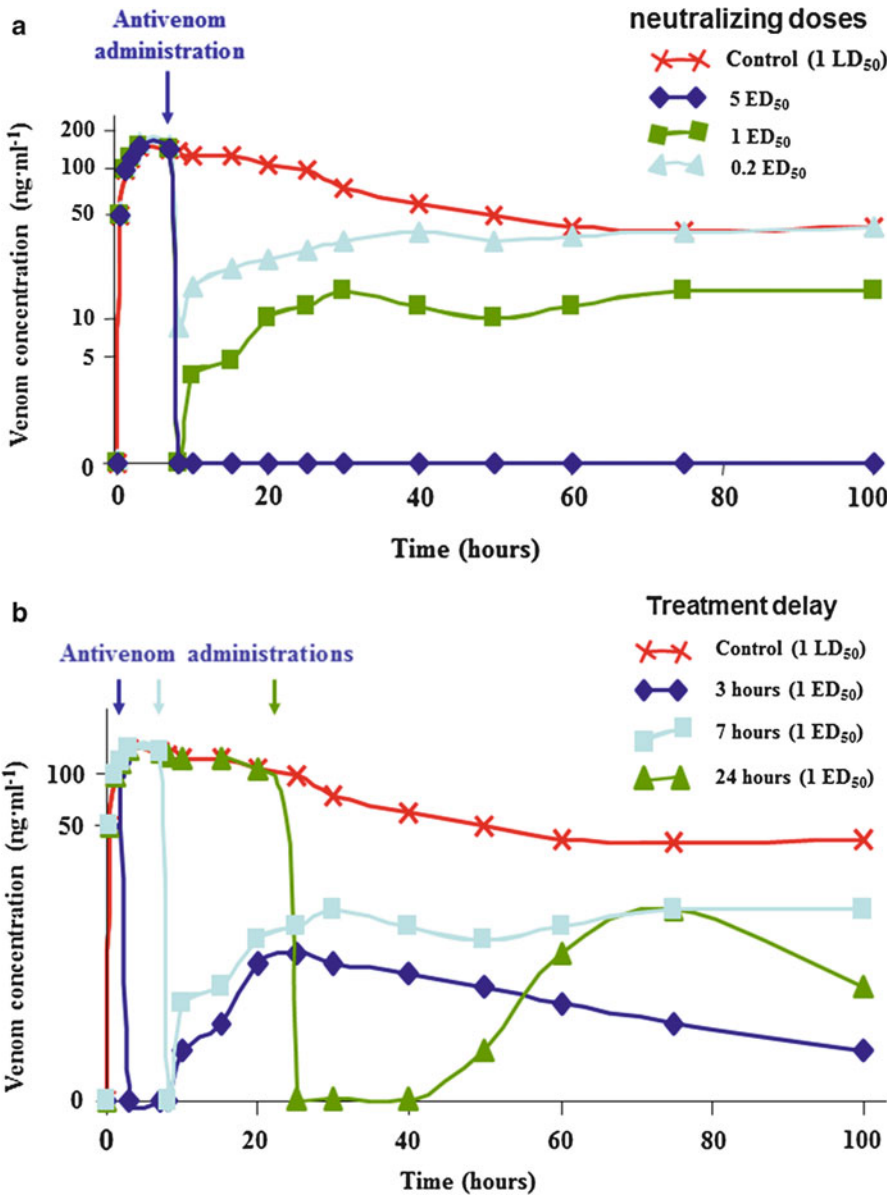


Fig. 1 Pharmacokinetics of F(ab')₂ according to the dosage (a) and treatment delay (b) (After Rivière et al. 1997)

in excess, the antibodies remain in the vascular compartment and continuously complex with venom, effectively removing it from circulation permanently (Fig. 1a). Extending the delay between venom and antivenom inoculation does not seem to alter this process (Fig. 1b).

Complexes composed of IgG or F(ab')₂ and venom antigens are destroyed by the immune system. However, the fate of Fab fragment complexes remains controversial. They do not seem to be phagocytized by immunocompetent cells by virtue of their solubility. Fab fragments are normally eliminated via the kidneys except when they are complexed to venom, and the combined molecular weight exceeds the glomerular filtration limit (Rivière et al. 1998). However, renal insufficiency was noted by the authors without identifying the causes.

In practical terms, three types of antivenom antibodies are commercialized: (i) whole IgG, mostly purified by caprylic acid precipitation, (ii) F(ab')₂, and (iii) Fab (WHO 2010). The main advantage of Fab-based antivenoms relative to F(ab')₂- and whole IgG-based ones is their rapid availability within deeper tissues, which in principle should allow it to form complexes with venom already there (Rivière et al. 1998). However, their rapid elimination requires that they be frequently readministered, which simultaneously increases both the quantity of heterologous protein injected to patients and the cost of treatment (Rivière et al. 1998; Boyer et al. 2001). Furthermore, it is unknown how Fab-antigen complexes are eliminated, which can entrain residual toxicity or undesired effects if neutralization is incomplete (Boyer et al. 2013b).

From the clinical perspective, however, the three types of antibody preparations have been shown to be of therapeutic benefit. Efficacy seems to depend mostly on the quality of immunization of production animals and the procedures used for antibody purification. The latter seem to determine how well they will be generally tolerated by patients.

Safety of Antivenoms: Causes of Adverse Effects and Their Prevention

Antivenom administration has been historically associated with adverse effects of variable frequency and intensity. This constitutes one of the main drawbacks for their use by health personnel with limited training and lacking the therapeutic resources necessary to manage potentially severe iatrogenic reactions. Assuring the safety and tolerability of antivenoms has constituted a major challenge to producers and distributors because they are the essential preconditions for effective care of envenomations in real-world rural health centers.

The causes of adverse effects are multifactorial and can be generally attributed to (a) nonspecific and adaptive immune responses to the heterologous immunoglobulins, (b) non-immunoglobulin contaminants present in plasma used for antivenom production or introduced during manufacture (Burnouf et al. 2004; WHO 2010), and (c) the venom itself, which is a mixture of heterologous proteins sometimes very allergenic by themselves (Morais et al. 2012). It is very important to avoid confusion between the adverse effects attributable to the antivenom itself and those resulting from the toxic action of venom during treatment. Criteria of imputability of adverse effects to antivenom are summarized in Table 3.

Table 3 Criteria of imputability of adverse effects to antivenom treatment

Criteria based on circumstances of onset	
1. Absence of symptom before the start of antivenom treatment	
2. Appearance in the hour after antivenom injection	
3. Disappearance upon interruption of administration	
4. No reoccurrence after renewal of treatment	
Criteria based on etiologic considerations	
1. Very suggestive symptoms: abrupt decrease in arterial pressure or shock in the minutes that follow antivenom injection, pruritus, cutaneous eruption, or glottal edema	
2. Suggestive symptoms that could nonetheless be related to the envenomation itself: digestive trouble (nausea, vomiting), respiratory problems (laryngeal irritation, cough, dyspnea), inflammatory syndrome (fever), neurovegetative manifestations (vertigo)	
3. Absence of other reasonable etiology	

Strong criteria are in **boldface**

Table 4 Classification and characteristics of different types of hypersensitivity

Type	I	II	III	IV
Promoters	Proteins: venoms, antivenoms	IgG, IgM complement	Immune complexes	Variable
Sensibilization	Yes	No	No	Yes
Effectors	IgE	IgG/IgM	IgG/IgM	Cells
Delay	Immediate/intermediate	Intermediate	Intermediate	Late
Cells	Mast cells, basophiles	Phagocytes	Phagocytes	Lymphocytes, macrophages
Mediators	Histamine, leukotrienes	Complement	Complement	Cytokines
Urgent treatment	Adrenalin, antihistamines		Anti-inflammatories	Corticosteroids

Action of Heterologous Proteins

The injection of heterologous proteins – whether originating in antivenom or venom – can produce two types of responses causing adverse effects, immediate or delayed, whose severity can vary from a simple local eruption to fatal anaphylactic shock (Table 4).

Immediate (<1 h) and Intermediate (<12 h) Reactions

Administration of antivenom – IgG or its fragments – can trigger hypersensitivity reactions of Type I in sensitized individuals. These reactions are caused by the action of IgE antibodies produced by a previous contact with the same antigen. Specific IgE antibodies bind mast cells which, in turn, release chemical mediators such as biogenic amines (histamine, serotonin, dopamine, heparin) and enzymes (notably

tryptase, carboxypeptidase), responsible for clinical manifestations appearing during the hour following introduction of the antigen (cutaneous eruption, angioedema, hypotension, dyspnea, anaphylactic shock). These molecules stimulate the release of other mediators (prostaglandins, leukotrienes, cytokines) with effects which can become manifest at later times (5–12 h after contact). Sensitization is generally the consequence of a previous immunotherapy with IgG of the same origin (anti-tetanus antiserum or antivenom). It is also possible that sensitization is the result of contact with animals of the same species as those used for antivenom production (Herrera et al. 2005) or from their consumption as food (Sevcik et al. 2008).

The incidence and severity of immediate reactions depends on the nature of the antigens promoting hypersensitivity, particularly their molecular mass, and a variety of amplifying factors, such as complement activation. The latter is naturally activated by the circulation of whole IgG, immune complexes, certain mediators during the immune response, and some constituents of venoms (Pugh and Theakston 1987).

An anaphylactic reaction appears in a patient sensitized to the trigger antigen, whereas an an IgE-mediated anaphylactic (= anaphylactoid) reaction occurs in the absence of sensitization, although some academic debate still exists regarding the application of these terms (Brown 2004; León et al. 2013; Pugh and Theakston 1987; Reid 1980). The severity of anaphylaxis can be expressed in degrees (Table 5). The incidence of IgE-mediated anaphylactic reactions is proportional to the quantity of administered proteins, which is not the case in non-IgE-mediated anaphylactic reactions (Reid 1980; León et al. 2013), underscoring the importance of adequate, and if possible minimal, dosage. In communities where heavy helminthic infections are widespread, as is often the case in sub-Saharan Africa, mast cell saturation by IgE would reduce the incidence and severity of anaphylactic reactions (Pugh and Theakston 1987).

Reports of the frequency of immediate reactions vary substantially (Boels et al. 2012; Boyer et al. 2013a; Schaeffer et al. 2012). Certain antivenoms of low purity can result in up to 80 % of adverse effects, although most are benign. Strong anaphylactic shock, potentially endangering the life of the patient, seems infrequent, a fortiori in the case of highly purified immunoglobulins or their fragments. Besides the difference in the type and purity of antivenoms commercialized and the doses administered, variations in the incidence and severity of adverse reactions can also reflect how they are perceived by the physicians and the patients and also by how case information is collected. In this respect, accurate and consensual criteria of imputability of adverse events to treatment are essential to consistently assess the safety of antivenoms (Table 3).

Delayed Hypersensitivity Reactions

Type III hypersensitivity reactions are associated to circulating immune complexes before their elimination. As they circulate, they may activate platelets, inducing their aggregation and release of vasoactive amines that can cause capillary thrombi and complement activation. The kidneys are particularly affected by virtue of their filtration function.

Table 5 Severity scale of anaphylactic shock (After Brown 2004)

Degree	Definition	Cutaneous troubles	Digestive troubles	Cardiac troubles	Respiratory troubles	Vital prognosis
I	Benign: cutaneous or subcutaneous tissue	Localized or generalized	None	None	None	Good
II	Moderate: same + general signs	Present	Dysphagia, nausea, vomiting, diarrhea	Arterial pressure drop, tachycardia	Cough, dyspnea	Good
III	Severe: multiple organs affected	Facultative and delayed	Facultative and delayed	Collapse, arrhythmias	Dyspnea	Compromised
IV	Vital risk	None	None	Circulatory arrest	Respiratory failure, acute pulmonary edema	Very compromised

Type III events have two main manifestations. The Arthus phenomenon appears 6–8 h after antivenom administration and persists 2–4 days, which consists of an edematized erythema next to the site of antivenom inoculation with acute vascularization. The Arthus reaction is rare and discussed by some authors. Serum sickness can appear later on – between 1 and 3 weeks after treatment – and results from the synthesis of antibodies against the immunoglobulins or other components (notably traces of serum albumin) in the antivenom. The immune complexes precipitate in the wall of small vessels and provoke a variety of symptoms, generally moderate and transient: fever, urticaria, adenopathy, arthralgias, and nephropathy with proteinuria (LoVecchio et al. 2003). Severe forms with glomerulopathy and neuropathy are very rare. Evolution is generally benign, with spontaneous healing in 2–4 days. The end of clinical troubles is marked by the appearance of free antibodies against antivenom components – and sometimes against venom, which can also elicit serum sickness – around 15 days after treatment (Morais et al. 2012).

Treatment and Prevention of Adverse Effects

Adverse effects resulting from antivenom administration require treatment with antihistamines, corticosteroids, or adrenaline depending on the severity of the symptoms. Antihistamines are simple to use and are indicated for light anaphylactic or non-IgE-mediated anaphylactic reactions. Treatment of type III hypersensitivity reactions is symptomatic: if symptoms are severe, antihistamines and corticosteroids are a recourse. Finally, the rare instances of anaphylactic or non-IgE-mediated anaphylactic shock require treatment with adrenaline. Hydrocortisone acts slowly and in a sustained manner, whereas the effects of adrenaline are immediate and of short duration. It may therefore be useful to combine them.

The prophylactic administration of corticosteroids or adrenaline remains controversial (Nuchpraryoon and Garner 2000). Most studies conclude that antihistamines (Fan et al. 1999), corticosteroids (Gawarammana et al. 2004), adrenaline (Premawardhena et al. 1999), or a combination of these (Bucaretschi et al. 1994) are of no prophylactic benefit. Nevertheless, some studies have shown that adrenaline administration significantly reduced adverse events (Gawarammana et al. 2004; de Silva et al. 2011) and that a combination of antihistamines and corticosteroids was beneficial especially when antivenom was administered by perfusion (Caron et al. 2009).

Some authors maintain that the rate of antivenom administration is an important factor in the manifestation of adverse events (Reid 1980). According to this view, slow administration by perfusion is preferable to direct intravenous push. Contrary to this, in several studies, a slow direct intravenous injection (5–10 min) did not cause significantly higher rates of adverse effects than perfusion. It has the advantage that it is more rapid and less costly, particularly in poorly equipped health centers (Malasit et al. 1986; Pugh and Theakston 1987; Chippaux et al. 2007). In any case, procedures of antivenom administration must be supervised such that it can be stopped if onset of adverse effects requiring immediate attention is ascertained.

Improvement of antivenom quality in each stage of production remains the best solution to control the risk of adverse effects. It is especially important so antivenoms can be used safely in peripheral health facilities that are poorly equipped, as is often the case in developing nations, and where the staff often lacks the training necessary to adequately handle severe adverse effects.

Proteins from animals used for antivenom production are more capable of eliciting adverse effects the more exposed humans are to them. In fact, the more frequent contacts between humans and animals are, the more likely it is for people to become sensitized to the animal proteins (Herrera et al. 2005; Sevcik et al. 2008). However, the choice of animals for antivenom production is generally determined by logistic imperatives such as cost, availability, yield, and husbandry requirements (WHO 2010).

Adverse effects attributable to sensitization of patients to horse proteins, as well as cultural or economic considerations, have led some producers to propose the use of animal species such as sheep, goats, rabbits, dogs, hens or ostriches (eggs), dromedaries, and llamas. At present, the horse and to a lesser extent sheep remain the main species used for antivenom production. The former combines a stable immune response, an excellent yield of antibodies and relatively simple husbandry requirements.

Animals must be in perfect health to avoid the risk of transmission of infectious agents to patients treated with products derived from their plasma. Since they are often kept in herds of tens and even hundreds of individuals, surveillance must be rigorous to prevent epizootic outbreaks conceivably transmissible to humans (Burnouf et al. 2004; WHO 2010) and possibly curtailing production.

Precipitation of immunoglobulins with ammonium sulfate, their enzymatic digestion with pepsin or papain to prepare F(ab')₂ or Fab fragments, respectively, are accompanied by the formation of protein aggregates that must be eliminated by centrifugation and filtration. Further reduction of contaminants may be attained by applying chromatography (Morais and Massaldi 2009; WHO 2010).

A procedure for purification of IgG currently in use involves the precipitation of unwanted serum proteins with caprylic acid. With this method, IgG is not precipitated, and the formation of aggregates is negligible (Rojas et al. 1994). The subsequent steps of purification are then simplified. However, the addition of an enzyme digestion with caprylic acid precipitation allows to join the streamlined production of IgG fragments with the safety of antivenoms (WHO 2010).

Administration of IgG fragments is associated to a significant reduction of incidence and severity of adverse effects when compared with whole IgG (Chippaux et al. 2007; Otero-Patiño et al. 2012; Smalligan et al. 2004), provided that they are adequately purified and not aggregated. Whole IgG antivenoms prepared by caprylic acid precipitation are also of low reactogenicity (Otero et al. 2006, 2012). In any case, the high incidence of adverse events is associated to high protein doses, incomplete antibody purification (Visser et al. 2008), and protein aggregation (Morais and Massaldi 2009). Although removal of the Fc portion of IgG does not completely eliminate reactogenicity, it probably reduces the incidence – and severity – of adverse effects.

Parasites, bacteria, and virus (or their components) may be present in production animals or accidentally introduced during antivenom manufacture. Careful screening, selection and husbandry of animals, immunization procedures, handling of plasma, and, finally, antivenom production must conform to accepted norms, now coded in World Health Organization guidelines. Several procedures aimed at inactivation (acid or thermal, preservatives) or elimination (chromatography, ultrafiltration) of potential pathogens are currently available to manufacturers (Burnouf et al. 2004).

Pyrogenic substances present in antivenoms can result from protein fractions not adequately removed during purification or from microbial growth during different phases of manufacture (WHO 2010). These contaminants may cause systemic inflammatory reactions characterized by high fevers. Microbiological control of antivenoms may require addition of preservatives, generally phenol, thimerosal, or cresol. However, some of these may accelerate protein degradation and aggregation of low molecular weight material that can cause intolerance in some patients (García et al. 2002). In addition, some preservatives such as phenol are irritants in concentrations close to those used for preservation (García et al. 2002; WHO 2010).

Several analytical laboratory methods, such as gas or liquid chromatography and protein electrophoresis, can be conducted to rule out the presence of unwanted proteins, aggregates, or low molecular weight substances that can cause adverse reactions (for a discussion of analysis of an antivenom, see Ramos-Cerrillo et al. 2008). Injection of small volumes of antivenom to guinea pigs or rabbits is a standard and effective procedure to establish the presence of pyrogens.

Clinical evaluation of an antivenom is indispensable before its commercialization. Clinical studies must seek to measure the incidence of adverse effects. It is not necessary to use a randomized clinical trial, unless clinical criteria are very precise, and imputability to the antivenom is of high probability (Chippaux et al. 2007). It is of particular importance to avoid confusion with symptoms derived from the envenomation itself and to the frequent and legitimate anguish of the patient.

Cutaneous or conjunctival tests have been proposed to recognize subjects likely to develop intolerance to antivenom. In general, their predictive value is considered insufficient (Malasit et al. 1986). In any case, a positive test should not dispense the patient of antivenom treatment if the clinical picture requires it; consequently, the trend has been to abandon these procedures.

Stability of Antivenoms Under Current Conditions of Use

The limited stability of antivenoms is a recurrent problem. It limits distribution and compromises storage, particularly in tropical countries where the climate is hot and humid and infrastructure, particularly the cold chain, is very deficient and unreliable. Antivenoms are often formulated in liquid form – usually requiring storage at +2 °C to +8 °C – or lyophilized, which allows storage at room temperature in the dark.

Studies have shown that efficacy outlives expiration dates, sometimes by a wide margin, and that liquid antivenoms left at room temperature for several days, and

sometimes even weeks, can retain their neutralizing properties (Al-Abdulla et al. 2013; Segura et al. 2009). However, a discrete amount of protein degradation, even if it does not significantly affect neutralization as measured in animals, can reduce safety and pose a risk to patients in terms of adverse reactions. Physicochemical changes in proteins in solution can, if fact, result in aggregation and/or the appearance of pyrogenic low molecular weight species that can cause adverse effects, sometimes severe, in patients. This is often manifested by the appearance of turbidity in the antivenom, and even flocculent precipitates, which may make injection risky (García et al. 2002). The organization and maintenance of a cold chain remain a challenge in most of the territory of developing nations, particularly rural areas where antivenom is most needed. Consequently, lyophilized antivenoms are preferable particularly in developing countries where cold chain is difficult to maintain. Interestingly, most envenomations occur in these regions.

The stability – in terms of preservation of venom neutralization – of lyophilized antivenoms has not been contested. However, lyophilization of whole IgG or its fragments is delicate, and the results can be less than satisfactory if it is not properly optimized and conducted. Adequately lyophilized antivenoms will dissolve rapidly and completely. Many antivenoms, however, will take long times to dissolve, and this may render them unsuitable for urgent treatments. Certain antivenoms require more than 45 min to dissolve in the injection solution (Hill et al. 2001), and some do not actually yield transparent homogeneous solutions even after several hours (Alejandro Alagón, personal communication). Before complete dissolution, it is risky to inject antivenoms as suspended material can be highly reactogenic.

Indications and Use of Antivenoms

The efficacy of quality antivenoms is now widely accepted, in spite of some controversy surrounding the utility of antivenom in envenomations by scorpions (Abroug et al. 2011). In addition, when highly purified antivenoms are used, the incidence and severity of adverse effects have significantly decreased, i.e., life-threatening severe ones have become exceedingly rare. An analysis of the use of passive immunotherapy over the last 30 years, however, reveals the conceptual and practical, particularly logistical, difficulties encountered in tropical countries.

As quality antivenoms have become safer, indications of use have been extended to include children and pregnant women, to whom they must be administered even in cases of apparently benign envenomations to prevent the possible rapid onset of severe complications. Although there is no time limit to the potential benefit of antivenom use, the swiftness of treatment will largely determine the rapidity of healing and the prevention of complications (Boels et al. 2012).

The failures of antiscorpion immunotherapy – and there are many reports – can almost always be attributed to, on one hand, delays in antivenom administration – antivenom administered within the hour following the sting is significantly more effective (Krifi et al. 2005) – and, on the other hand, a low neutralizing potency or inappropriate procedures of antivenom administration (low dose,

wrong route of injection). This is the case of certain antivenoms against Old World scorpions, of low neutralizing potency due to poor manufacturing methods. This explains why some studies have evidenced clear therapeutic failures, whereas others have shown equally clear successes (Abroug et al. 2011; Boyer et al. 2009).

For a long time, monovalent antivenoms were considered more efficacious and better tolerated because it was thought that lower doses were required. Very little hard evidence for such beliefs is available. It has been shown, however, that polyvalent antivenoms are essentially equivalent – and sometimes superior – due to paraspecific and potentiating effects during immunization of production animals (Dos-Santos et al. 2011). Furthermore, since identification of offending animals is difficult, polyvalent antivenoms are preferable for early and opportune treatment of envenomations caused by unknown species.

After decades of supposition, and trial and error, the intravenous route of administration is the only one recommended at present. Subcutaneous or intramuscular injections have been shown, experimentally and clinically, to result in lower efficacy (Krifi et al. 2005; Rivière et al. 1997; Vázquez et al. 2010). Immunotherapy can be administered by perfusion or by slow direct intravenous injection; however, the latter is effective more rapidly and less costly and can be also stopped upon onset of immediate adverse reactions (Malasit et al. 1986; Pugh and Theakston 1987).

The dose of antivenom is based on the initial symptoms, the delay in initiation of treatment, clinical evolution, the neutralizing potency of the antivenom, and the medical environment, particularly in terms of ancillary treatments. This is without doubt one of the principal questions that require study and documentation, particularly since antivenom accessibility is severely compromised. Several authors have shown that high doses, *a priori* legitimate to quickly neutralize the venom, are not necessarily more effective than lower ones (Agarwal et al. 2005; Tiarang et al. 1999). Lower doses offer the advantage of lower cost and a reduced risk of adverse effects.

Accessibility: The Crisis of Antivenom Immunotherapy

An analysis of antivenom sales and availability has shown an increasing scarcity, denounced already 15 years ago (Chippaux 1998, 2002). This scarcity has opened the way to dangerous developments, such as the distribution of poorly purified antivenoms and even gross falsifications completely devoid of immunoglobulins (Warrell 2008). In numerous African countries, antivenoms manufactured in India using venoms from Asian snakes are available. Although some species may belong to genera common to Africa and Asia, the venoms of Asian species are usually very different. These antivenoms are not suitable for the treatment of envenomations in Africa (Visser et al. 2008; Warrell 2008).

The reasons for the antivenom deficit, particularly in Africa, are many and involve all relevant actors: manufacturers, distributors (gross and retail), prescriptors, and patients. Health authorities bear the responsibility of validation and registration of

products to be placed on the market and of assuring their accessibility. The combination of factors has been described as a vicious cycle condemning antivenom immunotherapy to extinction where it is needed most (Chippaux 2002).

Antivenom manufacturers, much to the contrary of what is observed with most medicines, are excessively prudent regarding the distribution of their products. In Europe, for example, accessibility of antivenom is severely restricted (exclusively in hospitals) despite the absence of counterindications, the ease of use, and the low risk of adverse effects. In developing countries, manufacturers have emphasized the elevated costs of production of highly purified immunoglobulins or fragments and the uncertainties of limited and/or insolvent markets. The difficulties in ascertaining the extent of these markets have led most producers to abandon production of antivenoms or to limit production considerably to adapt to persistently receding and very unstable demand.

Distributors insist on the complexity of maintaining stocks without being able to anticipate the fluxes. Relatively rapid expiration of the products and the organization of cold chains all along the circuit of distribution, particularly in countries where climate is extreme, are certainly major handicaps.

Health authorities lack necessary epidemiological information, although in some tropical countries, the problem of envenomation is by now reasonably well defined. However, in many places, the incidence of envenomation is still poorly known and frequently underestimated, and the location of accidents remains imprecise. It is therefore difficult to guide manufacturers and distributors on the quantities of necessary antivenoms and the sites where they should be stockpiled.

The problem for physicians treating snakebites is double: passive immunotherapy is generally not accessible due to defective stocks and high cost, and, lacking adequate training, they do not master antivenom use in terms of indications, route of administration, dosages, therapeutic surveillance, and criteria of evolution.

Patients are very aware of the shortcomings of the system, starting by the absence of antivenom at the local health center and the unencouraging reception by health personnel, often anguished by their own impotence when facing the sometimes dramatic effects of envenomations. If antivenom is available, and even if it is effective, the high cost is very dissuasive; its acquisition often represents several months of income, and the form of the savings of rural populations (domestic fowl, farm animals, stored grains) is not suited to the context of urgency in which victims of envenomation find themselves in.

Consequently, different actors find alternatives reflecting their needs: an increase in price in the case of manufacturers and distributors (usually compounded), symptomatic treatments – usually not better mastered but more accessible – for treating physicians, and traditional medicine for many patients, who do not request or expect healing but rather some relief or comforting practice often involving supernatural aspects of the condition.

Reverting the process to favor a virtuous cycle requires simultaneous engagement of all actors (Stock et al. 2007). Health authorities must realize that envenomations do not constitute a fatality and that they must identify the therapeutic needs by way

Table 6 Essential information about *candidate antivenoms (supplied by manufacturers and independently validated by health authorities)*

Compositional information (in vitro analyses)
Protein content (by Bradford, BCA, or equivalent assays)
Type of antivenom (whole IgG, F(ab') ₂ , Fab)
State of aggregation <i>in solution</i> (by size exclusion chromatography)
Purity (by SDS-PAGE)
Neutralization potency (in vivo testing)
At least neutralization of lethality of the most important species (ED ₅₀)
Stability
In terms of integrity and state of aggregation after storage (safety)
In terms of neutralization potency (efficacy)

of epidemiological surveys measuring the incidence of bites, the severity of ensuing envenomations, and their geographic distribution. Health authorities should create the conditions for a reasonable and optimal choice of the best antivenom regarding the country. They have to organize quality control or ensure that it is conducted by independent laboratories to guarantee the properties and relevance of the antivenom (Table 6). They must also train health personnel in the use of antivenoms. As for many other public health problems, they must devote reasonable resources to address envenomations productively.

Manufacturers and distributors must agree on the regularity of production and distribution based on information provided by health authorities. They can also participate in the training of health professionals by providing accurate information on their products. The WHO guidelines are an excellent resource to standardize and improve manufacture of antivenoms. In addition, the use of lyophilized antivenoms can simplify their transport and storage and prolong their therapeutic usefulness.

Treating physicians must improve care by following rigorous treatment protocols that are now known to allow for significant reductions in dosage without compromising treatment efficacy. Moreover, the careful association of ancillary treatments enhancing passive immunotherapy would constitute a major improvement.

Finally, patients who arrive quickly at health centers will receive earlier treatment with antivenom, which in turn increases its efficacy, particularly in the case of scorpion envenomations – and maybe elapid bites too – and allows dose reduction.

It is reasonable to expect that these combined actions will improve the care of envenomation victims and adjust the consumption of antivenoms to real needs, which in turn will – almost mechanically – lead to a reduction in price. It will be no less important to develop economic devices that will distribute more equitably the cost of treatment: inclusion of antivenoms in lists of essential medicines, attribution of orphan drug status, financial participation by the state, and creation of insurance and mutual health structures and, in general, mechanisms that will compensate for

the failure of market-based strategies when the populations at need are mostly rural, poor, and distant from urban centers.

Conclusion and Future Direction

Improvements of antivenoms since the beginning of their clinical use relate mostly to their safety, which today is accepted to be very high, especially when antivenoms are manufactured according to the general guidelines now codified by the World Health Organization (WHO 2010). Indications of use have been extended, and the modality of administration is now more precise; injected intravenously, immunotherapy benefits symptomatic treatments, particularly reanimation procedures.

It is clear that the first criterion to consider for an antivenom is its efficacy, which will depend on (a) the valence of the antivenom, that is, the choice of venoms used for immunization of production animals, (b) the immunization protocol used, and (c) the quality and yield of the antivenom production process. Efficacy is evaluated by appropriate experimental tests now well standardized (WHO 2010). However, clinical confirmation, just as necessary in principle, is more difficult to standardize (Chippaux et al. 2010).

The safety and tolerability of the antivenoms are also of utmost importance insofar as they will make them useful in rural health centers, close to where envenomations occur. Safety is assured by adequate purification of the antibodies and their fragmentation or preparation with caprylic acid, in short, by assuring that no contaminants and protein aggregates are present in the final preparations. This in turn requires careful quality control during manufacture.

An adequate response to the problem of snake and scorpion envenomations is not only technological, even if some advances can be expected on this front. At present, the two major problems are (i) the unavailability of quality antivenoms and (ii) the lack of rationalization of therapeutic protocols. The unavailability of antivenoms results from a complex process based both on the scarcity of the product and care access, described elsewhere and for which solutions are currently being studied (Chippaux 2002). The responsibility for antivenom accessibility is shared between manufacturers, distributors, prescriptors, and victims. An improved knowledge of the needs, estimated by adequate epidemiological surveys, is an indispensable requisite that must be supplemented with a more equitable division of the cost of antivenom treatment, which remains a powerful constraint. The goal is to assure the presence – and use – of antivenoms at peripheral health centers where they are most needed. Simultaneously with standardization of the therapeutic protocols and training of medical personnel in the proper use of antivenoms, these measures should abate morbidity and mortality considerably.

In addition, it is essential to ensure the adaptation of antivenoms to the local fauna. On this matter, the World Health Organization (WHO 2010) has published recommendations for the production, quality control, clinical validation, and

registration of antivenoms directed at manufacturers and health authorities in charge of drug registration.

Cross-References

- ▶ [Role of Lymphatic System on Snake Venom Absorption](#)
- ▶ [Snake Venom Toxins Targeted at the Nervous System](#)

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