Toxinology

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Toxins and Drug Discovery

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Toxinology

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 microorganisms 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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Toxins and Drug Discovery

With 70 Figures and 44 Tables

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ISBN 978-94-007-6451-4 ISBN 978-94-007-6452-1(eBook)
ISBN 978-94-007-6453-8 (print and electronic bundle)
DOI 10.1007/978-94-007-6452-1

Library of Congress Control Number: 2017933846

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer Science+Business Media B.V.
The registered company address is: Van Godewijckstraat 30, 3311 GX Dordrecht, The Netherlands

Preface

The term TOXIN is derived from the Greek word *Toeikov* 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.

This volume deals mainly with the specialized aspect of toxins and drug discovery. Man has been using natural resources especially plants to treat diseases from very early times in history of mankind. Although many drugs derived from plants and microbes have been discovered and being used in clinical practice, not many drugs have been developed from venoms and toxins although many laboratories

worldwide are actively working on it. This volume describe some of the recent developments in this research area, such as snake venoms, conotoxins, bioinformatics in drug discovery from peptide toxins.

Singapore

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Acknowledgments

I would like to sincerely thank the section editors of this volume, Lourdes J. Cruz and Sulan Luo 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.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Sarah Mathews, Sunali Mull, Meghna Singh, Mokshika Gaur, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

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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. Prof. Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

Editors

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Dr. Lourdes J. Cruz is professor emeritus of the University of the Philippines Diliman (UPD) and a national scientist. Dr. Cruz obtained her B.S. Chemistry degree from UPD in 1962 and her M.S. and Ph.D. in Biochemistry from the University of Iowa in 1966 and 1968. She worked briefly at IRRI before joining the Department of Biochemistry in UP Manila in 1970 where she served as chair of the Department of Biochemistry and Molecular Biology from 1980 to 1986, then transferred to the Marine Science Institute of UPD in 1989. From 1976 to 2006, she spent 3–6 months every year at the University of Utah collaborating with Professor Baldomero M. Olivera on the elucidation of the biochemical and molecular nature of toxins from the venomous *Conus* marine snail, which are now used as pharmacological tools in the study of brain function and in drug development. Her research activities include marine toxins involved in harmful algal blooms. Dr. Cruz served for 12 years as the delegate of the Philippine Society for Biochemistry and Molecular Biology (PSBMB) to the federation FAOBMB and for 9 years as delegate to the international union, IUBMB. Her international awards include the Sven Brohult Award of the International Foundation for Science (IFS) in 1993, ASEAN Outstanding Scientist and Technologist in 2001, the L'Oreal-UNESCO Award for Women in Science in 2010, and the Outstanding Alumnus Award of the University of Iowa's Carver College of Medicine in 2011. Professor Cruz is a member of the National Academy of Science and Technology, Philippines, and a fellow of The World Academy of Sciences. As the current chair of the Regional Committee for Asia and the Pacific of the International Council for Science (ICSU), she is very actively involved in the newly established program on the "Sustainability Initiative for the Marginal Seas of South and East Asia."

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Professor Sulan Luo, Ph.D., is distinguished professor of Chang Jiang Scholars Program and the director of Key Lab for Tropical Biological Resources, Ministry of Education at Hainan University. She is the director of Key Lab for Marine Drug of Haikou at Hainan University, as well. Professor Luo is the team leader of "Program for Changjiang Scholars and Innovative Research Team in University" of Ministry of Education. She is Science and Technology Innovation Leading Talent of "National High-level Personnel of Special Support Program of China" ("Ten Thousand Talents Program"). Presently, Professor Luo acts as secretary of Asia-Pacific section of The International Society on Toxinology and president of 19th World Congress of the International Society on Toxinology (IST 2017 Haikou).

Her research interests focus on structure and function of conopeptides native to Hainan and their molecular receptors (ion channels), molecular biology, *Xenopus* oocytes electrophysiology, biotechnology and marine medicine, and neuropeptides in marine organisms, among others. She established a new center for experimental biotechnology and department of pharmacy in Hainan University.

Professor Luo has been the core leader of Major International Joint Research Project of National Natural Science Foundation of China, the State High-Tech Research and Development Project (863) of the Ministry of Science and Technology of China Grant, Program for International Science & Technology Cooperation Program of China Grant, and National Natural Science Foundation of China Grant, among others. She is the author of 145 scientific publications of various journals, including *Proceedings of the National Academy of Sciences*, *The Journal of Biological Chemistry*, *The FASEB Journal*, *Journal of Medicinal Chemistry*, *Scientific Reports*, etc. She is also the inventor of 15 patents.

Professor Luo discovered six new Hainan conotoxins that target nicotinic acetylcholine receptor (nAChR) subtypes, including atypical α-conotoxin LtIA that targets a novel microsite of the α3β2 nAChR, α-conotoxin TxIB that selectively targets α6/α3β2β3 nAChR, α4/7-conotoxin LvIA that selectively blocks α3β2 vs. α6/α3β2β3, α-conotoxin TxID that potently blocks rat α3β4 nAChR, a novel inhibitor of α9α10 nAChR that delineates a new conotoxin superfamily, and an αO-conotoxin GeXIVA which is a potent α9α10 nAChR antagonist with antihypersensitive effect of neuropathic pain. Structure and function characterization of more Hainan conopeptides is ongoing continuously. The nAChRs are potential targets for the treatment of various neuropsychiatric diseases, including pain, cancer, addiction, epilepsy, Alzheimer's disease, Parkinson's disease, schizophrenia, etc. These Hainan conotoxins show great potential in medical applications.

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Part I

Snake Venoms

Crotoxin from *Crotalus durissus terrificus* and Crotoxin-Related Proteins: Structure and Function Relationship

1

Grazyna Faure, Dorota Porowinska, and Frederick Saul

Abstract

Snake venom presynaptic phospholipases A_2 (PLA_2s) are β-neurotoxins present in monomeric form or as multimeric complexes with various quaternary structures. Three classes of β-neurotoxins from snake venom have been described. Here in the heterodimeric CACB crotoxin complex, a potent β-neurotoxin from *Crotalus durissus terrificus* venom, and its natural isoforms are presented. Crotoxin and crotoxin-related proteins possess PLA_2 activity and display diverse pharmacological properties. Many of these properties are conferred by regions of the structure not involved in catalysis but directly implicated in protein-protein interactions (PPI) with PLA_2-receptor targets. Mono- and multimeric PLA_2s are involved in various biological functions and can modulate specific disease processes. Numerous attempts have been made to correlate PLA_2 structures with these pharmacological properties and to identify PPI sites. These sites represent potential lead structures for the development of new compounds for modulation of specific disease processes. However, PPI sites are difficult to discover and design in the absence of 3D structural studies (co-crystallization with protein targets), and few structures of PLA_2-receptor

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_7

complexes have been reported. The acidic CA subunit of crotoxin may be considered as a natural target of the basic PLA_2 CB subunit. The 3D structure of the crotoxin CACB complex provides a detailed structural model of the interaction between the CA and CB subunits. Identification of the molecular interface between the two subunits of crotoxin is essential to predict other biologically relevant PPI sites. This chapter is focussed on the structure-function relationship of crotoxin and crotoxin-related proteins and recent investigations to identify new biological targets of crotoxin.

Keywords
Crotoxin • Isoform • Neurotoxic phospholipase A_2 • Snake venom • PLA_2 targets

Contents

Introduction

Snake venom is a complex mixture of components that exhibit numerous pathophysiological effects. The venom of the South American rattlesnake *Crotalus durissus terrificus* contains four proteic components: gyroxin, crotoxin, convulxin, and crotamine, of which crotoxin represents the principal toxic component. Crotoxin is a potent β-neurotoxin that acts primarily at the presynaptic level of the neuromuscular junction and inhibits acetylcholine release; blockage of neuromuscular transmission leads to muscle paralysis and death by asphyxia (Brazil and Excell 1971; Hawgood and Smith 1977). Crotoxin can also act at the postsynaptic level, stabilizing a desensitized form of the acetylcholine receptor (AChR) (Bon et al. 1979; Brazil et al. 2000).

In addition to neurotoxicity, crotoxin exhibits other significant pharmacological properties and biological effects such as myotoxic (Gutiérrez et al. 2008), cardiotoxic (potentiation of L-type Ca^{2+} channel) (Zhang et al. 2010), cytotoxic (chronic seizure effect by release of glutamate from cerebrocortical synaptosomes via N and P/Q Ca^{2+} channel in the central nervous system) (Lomeo Rda et al. 2014), bactericidal (Oliveira et al. 2002; Toyama et al. 2003; Perumal Samy et al. 2006), anti-inflammatory (Cardoso et al. 2001; Sampaio et al. 2005; Zambelli et al. 2008; Nunes et al. 2010), antitumoral (Rudd et al. 1994; Costa et al. 1998; Cura et al. 2002;

Yan et al. 2007), and analgesic (Zhang et al. 2006; Nogueira-Neto Fde et al. 2008) effects and antiviral activity (Muller et al. 2012; Muller et al. 2014).

Crotoxin from *Crotalus durissus terrificus* venom was the first animal neurotoxin isolated and characterized (Slotta and Fraenkel-Conrat 1938). Other crotoxin-like proteins commonly occur in the venoms of *Crotalus* species, e.g., *Crotalus durissus cascavella* (Beghini et al. 2000; Beghini et al. 2004; Rangel-Santos et al. 2004; Fonseca et al. 2006), *Crotalus durissus collilineatus* (Ponce-Soto et al. 2002; Rangel-Santos et al. 2004; Toyama et al. 2005; Ponce-Soto et al. 2007; Salvador et al. 2009), *Crotalus durissus cumanensis* (Pereañez et al. 2009; Cavalcante et al. 2015), *Crotalus durissus ruruima* (Dos-Santos et al. 2005), *Crotalus scutulatus* (Mojave toxin) (Bieber et al. 1975; Gopalakrishnakone et al. 1980), and *Crotalus simus* (Calvete et al. 2010; Castro et al. 2013; Durban et al. 2013), and *Erotalus vegronolis* (Viala et al. 2015). Heterodimeric complexes of crotoxin-like proteins have also been identified in the venom of non-rattlesnake species belonging to Crotalinae subfamily: pit vipers *Gloydius intermedius* (Gintexin) (Yang et al. 2015a, b) and *Bothriechis nigroviridis* (Nigroviriditoxin) (Fernández et al. 2010; Lomonte et al. 2015).

A comparision of the structure, biological properties, and toxicity of crotoxin from *Crotalus durissus terrificus* and other crotoxin-like proteins is presented here. Other biological targets of crotoxin and its basic PLA_2 CB subunit are also described.

Crotoxin

Crotoxin is a heterodimeric protein complex formed by the non-covalent association of an acidic, nontoxic, nonenzymatic CA subunit with a basic, weakly toxic CB subunit with phospholipase A_2 activity (group IIA $sPLA_2$) (Hendon and Fraenkel-Conrat 1971). Individual snake venom contains up to 16 natural crotoxin complexes having different biochemical and biological properties (Faure and Bon 1988). These complexes result from the association of different isoforms of the CA and CB subunits (Faure et al. 1991, 1993). Moreover, the profiles of crotoxin isoforms vary between individual snakes of the same species (Faure and Bon 1987).

CA Subunit

The CA subunit of crotoxin (crotapotin, component A, or crotoxin A) is an acidic, nontoxic protein comprising three disulfide-linked polypeptide chains α, β, and γ. The CA subunit does not possess catalytic activity and in snake venom is present only in complex with the basic CB subunit, enhancing its lethal potency. CA prevents the formation of oligomers between PLA_2 subunits and targets CB to reach the specific crotoxin receptor. CA also inhibits the PLA_2 and anticoagulant activities of CB (Faure et al. 1991, 1993, 2007).

Table 1 Molecular masses of CA subunit isoforms of crotoxin and crotoxin-like proteins

	Molecular mass [Da]	References
Crotalus durissus terrificus		
CA_1	9597	This review
CA_2	9429.9 (±1.4)	This review
CA_3	9645 (±0.2)	This review
CA_4	9687	This review
Gloydius intermedius		
Gintexin-A	9569	Yang et al. (2015a)
	9487	Yang et al. (2015a)
	9947	Yang et al. (2015a)
	9670	Yang et al. (2015a)
Bothriechis nigroviridis		
Nigroviriditoxin A_1	9605.6	Lomonte et al. (2015)
Nigroviriditoxin A_2	9421.5	Lomonte et al. (2015)

Molecular masses were determined by mass spectrometry

Four CA isoforms (CA_{1-4}) have been identified in *Crotalus durissus terrificus* venom. Briefly, crotoxin was isolated from crude venom by gel filtration chromatography, and the CA and CB subunits were separated by ion-exchange chromatography in the presence of 6 M urea. Four CA isoforms were purified from a mixture of isoforms on Mono Q column by an anion-exchange chromatography. The three chains of each purified CA isoforms from *Crotalus durissus terrificus* were separated by reverse-phase (RP)-HPLC (Vydac C18 column) and the sequences determined by Edman degradation (Faure et al. 1991). Several isoforms of an acidic CA-like protein were found in the non-rattlesnake species *Gloydius intermedius* (Yang et al. 2015a) and *Bothriechis nigroviridis* (Lomonte et al. 2015) and were isolated from the crude venoms using directly RP-HPLC in acetonitrile gradient (Yang et al. 2015a; Lomonte et al. 2015). Gel filtration chromatography was performed in the case of Gintexin-A (Yang et al. 2015a). The molecular masses of CA subunit isoforms isolated from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis* are shown in Table 1.

All CA subunit isoforms of crotoxin derive from a pro-CA PLA_2-like precursor protein homologous to nontoxic acidic group IIA $sPLA_2$ and result from post-translational modifications by removal of three peptide segments (Faure et al. 2011) (the enzymes responsible for this process have not yet been identified). During protein maturation a conversion of N-terminal glutamine residues to pyrrolidone carboxylic acid occurs in the β and γ chains. This modification increases protection of the protein from proteolytic degradation. The mature CA protein is composed of three polypeptide chains (α, β, γ) linked by two intra- and five interchain disulfide bonds (Bouchier et al. 1991; Faure et al. 1991; Faure et al. 2011). Differences between CA isoforms of crotoxin and crotoxin-like proteins result from slightly different lengths and amino acid sequences of the

Fig. 1 Multiple sequence alignment of precursors and mature proteins of CA from *Crotalus durissus terrificus* (Faure et al. 1991) and Gintexin-A from *Gloydius intermedius* (Yang et al. 2015a). *pro-CA* precursor of the acidic subunit of crotoxin from *Crotalus durissus terrificus*, *pro-Gintexin-A* precursor of the acidic subunit of the crotoxin-like protein from *Gloydius intermedius*. Amino acid numbering is according to Renetseder et al. 1985. In *blue*, point mutations between sequences; *black boxes*, chain α, β, and γ in mature proteins; *red boxes*, pyrrolidone carboxylyl residues, converted from glutamine by posttranslational modification; underlined, residues determined only by amino acid composition (not sequenced). Sequence alignment was performed with CLC Sequence Viewer 6

individual polypeptide chains (Fig. 1) (Faure et al. 1991). The post-translational maturation of pro-CA is a prerequisite for an appropriate assembly of the three independent CA chains with the basic CB subunit to form the crotoxin complex (Faure et al. 1991).

The crystal structure of only one CA isoform has been solved, the isoform CA_2 in complex with isoform CBb in the 3D structure of crotoxin (PDB 3R0L) (Faure et al. 2011). This structure revealed that the two long α-helices C and D in the positions expected for group IIA $sPLA_2$ are preserved in the CA subunit, but the long α-helix A and the short α-helix B are absent (as expected from posttranslational cleavage of pro-CA) (Faure et al. 2011). Despite the preservation of the amino acid sequence in the predicted Ca^{2+}-binding loop, an extended conformation unlike the canonical Ca^{2+}-binding loop of group IIA PLA_2S structures was revealed, explaining in part why the CA subunit does not perform a catalytic function. The amino acid sequence of the precursor of the acidic subunit from *Gloydius intermedius* (Gintexin-A) displays 76% identity with pro-CA from *Crotalus durissus terrificus* (Fig. 1). Similar to the CA subunit of crotoxin, mature Gintexin-A consists of three polypeptide chains resulting from post-translational modifications of a precursor protein, and the structure is stabilized by seven disulfide bonds (Yang et al. 2015a). The α, β, and γ chains of this protein show high amino acid sequence identity (87% and 69% with $CA_{1,2,4}$ for chain α and 85% and 66% with CA_3 for chain β of crotoxin and 78% for chain γ of CA).

CB Subunit

The CB subunit of crotoxin (component B or crotoxin B) is a basic, weakly neurotoxic protein that possesses PLA_2 activity and catalyzes the hydrolysis of the *sn*-2 ester bond of phospholipids, producing free fatty acids and lysophospholipids (Hendon and Fraenkel-Conrat 1971; Faure and Bon 1988). In snake venom, isoforms of the CB subunit of crotoxin occur in complex with CA. The isolated CB subunit also acts at the presynaptic level of the neuromuscular junction and blocks neuromuscular transmission, but tenfold higher doses of this protein are needed compared to the CACB complex.

Four isoforms of the CB subunits (CBa_2/CB2, CBb, CBc/CB1, and CBd) in *Crotalus durissus terrificus* venom have been identified. These isoforms were purified from crude venom using a four-step chromatographic procedure: (i) gel filtration (isolation of crotoxin), (ii) ion-exchange chromatography in the presence of 6 M urea (separation of the CA and CB subunits each containing a mixture of isoforms), (iii) cation-exchange column using double gradient (0–1 M NaCl and 0–3 M urea, purification of CB isoforms), and (iv) RP-HPLC, Vydac C4 column (additional purification step) (Faure and Bon 1988). The CB isoforms are products of different mRNAs (Bouchier et al. 1991; Faure et al. 2011). However, in *Crotalus durissus terrificus* venom, several additional CB isoforms (CB-like proteins: F15 (Toyama et al. 2003), F16 (Hernandez-Oliveira et al. 2005), F17 (Oliveira et al. 2002), and Intercro (Vieira et al. 2013)) have been identified. Proteins F15, F16, and F17 were isolated from the crotoxin fraction by RP-HPLC. Intercro was eluted from the column as a fraction located between crotamine and crotoxin during gel filtration chromatography. In the case of non-rattlesnake species, the presence of basic CB-like isoforms has also been observed. Two isoforms of Nigroviriditoxin B, a CB-like basic protein, were purified by RP-HPLC from the crude venom of *Bothriechis nigroviridis* (Lomonte et al. 2015). A single isoform of Gintexin-B was isolated from crude venom of *Gloydius intermedius* using gel filtration and RP-HPLC (Yang et al. 2015a). Both rattlesnake and non-rattlesnake CB and CB-like proteins contain 122 amino acid residues with a molecular weight of approximately 14 kDa and pI values around 9 (Table 2).

All CB isoforms from *Crotalus durissus terrificus* are secreted group IIA phospholipases structurally homologous to the inflammatory, non-pancreatic human group IIA $sPLA_2$ (EC 3.1.1.4). The crystal structures of isoforms CBa_2, CBb, and CBc show the presence of highly conserved canonical structural features of group IIA $sPLA_2$ stabilized by seven disulfide bonds (Marchi-Salvador et al. 2008; Faure et al. 2011). The 3D structures of CB-like PLA_2s have not yet been determined experimentally, but as shown in Fig. 2, they share a high degree of sequence homology with CB. CB isoforms and other CB-like proteins isolated from *Crotalus durissus terrificus* venom (F15, F16, F17, and Intercro) show sequence identities of more than 90% (except CBc/F17) (Table 3). The high sequence identity between CBa_2 and Intercro (97.5%) is surprising since the latter is not part of the crotoxin fraction (Vieira et al. 2013). These proteins differ by only three residues (positions CBa_2/Intercro: W70/F, Y117/L, and Y120/F) (Fig. 2), resulting in lower PLA_2

Table 2 Biochemical properties of CB and CB-like proteins from *Crotalus durissus terrificus*, *Bothriechis nigroviridis*, and *Gloydius intermedius*

	Molecular mass [Da]	pI	References
Crotalus durissus terrificus			
CBa_2	4245 (±1)^	8.74	Faure et al. (1994)
CBb	4152 (±1)^	8.74	Faure et al. (1994)
CBc	4186 (±0.9)^	8.74	Faure et al. (1994)
CBd	4234 (±1)^	8.74	Faure et al. (1994)
F17	4664.14^	8.74	Oliveira et al. (2002)
F16	4860^	9.01	Hernandez-Oliveira et al. (2005)
F15	14479.7*	9.16	Toyama et al. (2003)
Intercro	4188^ 4282^	8.75	Vieira et al. (2013)
Gloydius intermedius			
Gintexin-B	14,177^	8.73	Yang et al. (2015a)
Bothriechis nigroviridis			
Nigroviriditoxin B	14,083^ 14,113 (±2)^	8.5	Lomonte et al. (2015)

^ Molecular masses determined by ES mass spectrometry, * based on the protein sequence with the program ProtParam
pI values were determined based on the protein sequence with the use of ProtParam program

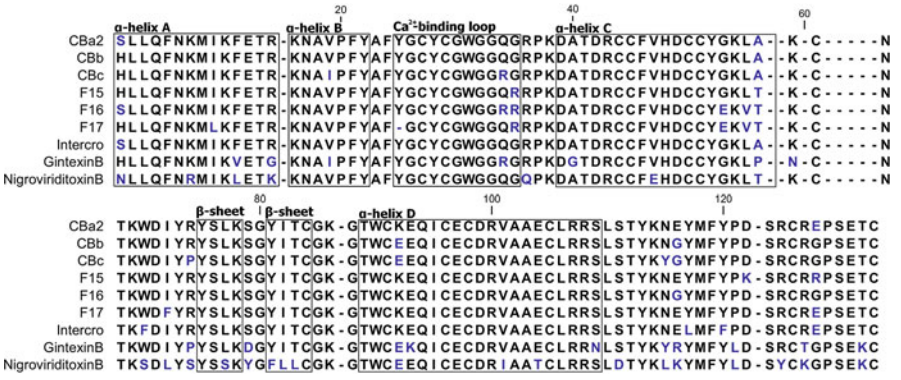

Fig. 2 Multiple sequence alignment of CB isoforms and CB-like proteins F17, F16, F15, and Intercro from *Crotalus durissus terrificus* and non-rattlesnake basic subunits Gintexin-B from *Bothriechis nigroviridis* and Nigroviriditoxin B from *Gloydius intermedius*. Amino acid numbering is according to Renetseder et al. 1985. In *blue*, point mutations occurring in sequences of analyzed proteins; *black boxes*, highly conserved regions of $SPLA_2$-IIA: α-helix A, helix B, Ca^{2+}-binding loop, α-helix C, the β-wing, and α-helix D.

activity and toxicity of Intercro and preventing complex formation with CA (Table 4). The amino acid sequences of CB-like subunits isolated from non-rattlesnake species Gintexin-B and Nigroviriditoxin B reveal the identities up to 90% and 80%, respectively, to all PLA_2s isolated from *Crotalus durissus terrificus*

Table 3 Sequence identities (%) of $sPLA_2$ isoforms from *Crotalus durissus terrificus*, *Bothriechis nigroviridis*, and *Gloydius intermedius* venom

	CBa_2	CBb	CBc	F17	F16	F15	Intercro	Gintexin-B
CBb	96.72	–	–	–	–	–	–	–
CBc	93.44	96.72	–	–	–	–	–	–
F17	94.21	92.56	89.26	–	–	–	–	–
F16	94.26	94.26	92.62	95.04	–	–	–	–
F15	95.9	95.08	91.8	95.04	94.26	–	–	–
Intercro	97.54	94.26	90.98	91.74	91.8	93.44	–	–
Gintexin-B	84.43	86.89	90.16	80.99	83.61	83.61	81.97	–
Nigroviriditoxin B	78.69	80.33	78.69	76.03	77.05	77.87	77.05	77.05

(Table 3). Several sequence differences occur in the C-terminal region often considered to be responsible for the neurotoxicity of the venom PLA_2s. The phylogenetic relationship of the CB and CB-like proteins analyzed here is shown in Fig. 3.

CB isoforms and CB-like proteins in the presence of acidic subunit CA form different classes of crotoxin complexes (see below), confirming that natural mutations in CB isoforms determine different properties of crotoxin complexes (Faure and Bon 1988). The phospholipase A_2 activities of all CB isoforms are quite similar (Table 4) (Faure et al. 1993). However, significant differences appear in the presence of the CA subunit (except for isoform CBa_2 where binding of CA does not influence PLA_2 activity). The crystal structure of the crotoxin CA_2CB_6 complex (Faure et al. 2011) revealed that the natural point mutation at position 1 (Ser1 in CBa_2, His1 in CBb,c) is crucial for the functional differences between crotoxin isoforms. The presence of Ser at position 1 in CBa_2 associated with a displacement of Trp70 and Trp31 prevents their interaction with Asp89 and Asp99 of the CA subunit (chain β), leading to greater substrate access to the catalytic site of CB (His48 and Asp99) (Faure et al. 2011). These structural differences explain the higher PLA_2 activity of the CA_2CBa_2 complex.

For the CBb, CBc, and CBd isoforms, a two- to fourfold decrease in enzymatic activity is observed after CA binding, depending on the CB isoform (Table 4) (Faure et al. 1993). Once again, these differences result from point mutations in the CB subunit (Fig. 2). For isoforms F15, F16, and F17, very weak inhibition of enzymatic activity is observed in complexes with the CA subunit (Table 4). Intercro does not form complexes with CA (Vieira et al. 2013). In agreement with previous results (Faure et al. 2011), the mutation at position 70 (Phe70 in Intercro, Trp70 in CBa_2) explains the absence of binding of Intercro with CA. Nigroviriditoxin B alone shows very similar PLA_2 activity to CB (Lomonte et al. 2015).

The enzymatic activity of all PLA_2s is strictly dependent on the presence of Ca^{2+} ions (Mayer and Marshall 1993; Murakami and Kudo 2002). The calcium binding sites (Cys29, Gly30, Gly32), as well as Trp31 which affects stability in this region, are conserved in all CB isoforms and other crotoxin-like proteins analyzed here

Table 4 Enzymatic activity of CB isoforms and CB-like PLA_2s from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis*

	PLA_2 activity	Influence of CA on PLA_2 activity
Crotalus durissus terrificus		
CBa_2	V_{max} = 24 (±2) μmol/min/mg K_m = 0.06 (±0.02) μM[a*]	For CA_2 V_{max} = 25 (±2) μmol/min/mg K_m = 0.05 (±0.03) μM[a*] For CA_3 V_{max} = 24 (±1) μmol/min/mg K_m = 0.05 (±0.02) μM[a*]
CBb	V_{max} = 22 (±2) μmol/min/mg K_m = 0.07 (±0.02) μM[a*]	For CA_2 V_{max} = 10 (±2) μmol/min/mg K_m = 0.3 (±0.1) μM[a*] For CA_3 V_{max} = 9 (±2) μmol/min/mg K_m = 0.2 (±0.05) μM[a*]
CBc	V_{max} = 18 (±2) μmol/min/mg K_m = 0.07 (±0.02) μM[a*]	For CA_2 V_{max} = 7 (±1) μmol/min/mg K_m = 0.22 (±0.05) μM[a*] For CA_3 V_{max} = 6.6 (±1) μmol/min/mg K_m = 0.2 (±0.05) μM[a*]
CBd	V_{max} = 19.7 (±2) μmol/min/mg K_m = 0.06 (±0.03) μM[a*]	For CA_2 V_{max} = 4.6 (±1) μmol/min/mg K_m = 0.35 (±0.05) μM[a*] For CA_3 V_{max} = 4 (±1) μmol/min/mg K_m = 0.3 (±0.1) μM[a*]
F17	V_{max} = 0.0082 μmol/min/mg K_m = 31.2 mM[b] ^	Slight inhibition of PLA_2 activity[b]
F16	V_{max} = 0.0107 μmol/min/mg K_m = 27.3 mM[c] ^	V_{max} = 0.0098 μmol/min/mg K_m = 53.4 mM[c] ^
F15	V_{max} = 0.0085 μmol/min/mg K_m = 38 mM[d] ^	V_{max} = 0.0082 μmol/min/mg K_m = 58.4 mM[d] ^
Intercro	Activity represent ~40% of CB[e x #]	No complex formation with CA[e]
Gloydius intermedius		
Gintexin-B	nd	nd
Bothriechis nigroviridis		
Nigroviriditoxin B	Activity represent ~80% of CB[f ^ #]	Inhibition of PLA_2 activity: 2x[f]

Substrates: *2 μM 1-palmitoyl-2-(l0-pyrenyldecanoyl)-sn-glycero-3-monomethyl phosphatidic acid, ^ 0.32 mM synthetic 4-nitro-3-octanoylbenzoic acid, [x]1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine, *nd* not determined, # activity given as a fluorescence intensity

[a]Faure et al. (1993)
[b]Oliveira et al. (2002)
[c]Hernandez-Oliveira et al. (2005)
[d]Toyama et al. (2003)
[e]Vieira et al. (2013)
[f]Lomonte et al. (2015)

Fig. 3 Phylogenetic tree of CB and CB-like PLA_2 isoforms F17, F16, F15, and Intercro from *Crotalus durissus terrificus* and non-rattlesnake basic subunits Gintexin-B from *Gloydius intermedius* and Nigroviriditoxin B from *Bothriechis nigroviridis*. The phylogenetic tree was calculated with the UPGMA algorithm.

(Fig. 2). Moreover it has been shown that Mg^{2+} stimulates PLA_2 activity of Intercro (Vieira et al. 2013).

All CB isoforms display neurotoxic properties, CBa_2 being the least toxic (Table 5). Binding of the acidic CA subunit increases the lethal potency of the CB subunit, and this toxic effect is correlated with the stability of CACB complexes (Faure et al. 1993, 2011). CBa_2 in complex with CA forms less stable and less neurotoxic complexes, with affinity to CA sixfold lower compared to the other CB isoforms (Table 6). CBb, CBc, and CBd have comparable affinity to CA (K_D = 3.5–5 nM), and these complexes are at least four times more toxic. However, the stability of these complexes is different. The half-life of CACBd is two times greater than that observed for CACBb (Table 6) (Faure et al. 1993). Slight variations in the neurotoxic activities of these isoforms are shown in Table 5. Differences in the stability and neurotoxicity of crotoxin complexes result from the point mutations in CB isoforms (see below). F15, F16, and F17 PLA_2s in complex with CA reveal much lower toxicity (Oliveira et al. 2002; Toyama et al. 2003; Hernandez-Oliveira et al. 2005), whereas Intercro does not display neurotoxic properties (Vieira et al. 2013). Surprisingly, the toxicity of the Gintexin complex is similar to that observed for crotoxin (Yang et al. 2015a). Uncomplexed Nigroviriditoxin B revealed almost sixfold lower potency in comparison with CB isoforms. The toxicity of the complex (Nigroviriditoxin B with Nigroviriditoxin A) increases only slightly and is 20-fold lower than that determined for crotoxin (Table 5) (Lomonte et al. 2015).

Based on their enzymatic and neurotoxic properties, crotoxin isoforms can be divided into two classes (Faure and Bon 1988). Class I includes complexes formed by CBb, CBc, CBd, and CA isoforms, forming stable complexes with high toxicity and low phospholipase A_2 activity. CBa_2, which belongs to crotoxin complexes of class II, forms less toxic and less stable complexes with higher enzymatic activity.

The F15, F16, F17, and Intercro proteins show very weak or no toxicity and low phospholipase A_2 activity. However, binding of the CA subunit has little effect on their enzymatic activity. Based on these data, complexes of F15, F16, and F17 with CA can be classified as class II crotoxin-like complexes similar to the CA_2CBa_2 crotoxin complex.

Table 5 Neurotoxic properties of CB isoforms and CB-like PLA_2s from *Crotalus durissus terrificus*, *Gloydius intermedius*, and *Bothriechis nigroviridis*

	Neurotoxic activity		
	Chick biventer cervicis preparation	Mouse phrenic nerve-diaphragm preparation	LD_{50} intravenous injection [μg/kg tissue]
Crotalus durissus terrificus			
CBa_2	With CA_2: 50% blockage after 155 (±10) min (dose 35 nM)[a] With CA_3: 50% blockage after 230 (±8) min (dose 35 nM)[a]	Alone: ~30% blockage after 90 min With CA: 50% blockage after 30 min (dose 10 μg/ml)[b]	Alone: 700 (±100)[a] With CA_2: 420 (±70)[a] With CA_3: 450 (±60)[a]
CBb	With CA_2: 50% blockage after 44 (±6) min (dose 35 nM)[a] With CA_3: 50% blockage after 60 (±5) min (dose 35 nM)[a]		Alone: 500 (±100)[a] With CA_2: 110 (±20)[a] With CA_3: 95 (±20)[a]
CBc	With CA_2: 50% blockage after 87 (±11) min (dose 35 nM)[a] With CA_3: 50% blockage after 77 (±5) min (dose 35 nM)[a]		Alone: 480 (±100)[a] With CA_2: 80 (±20)[a] With CA_3: 110 (±45)[a]
CBd	With CA_2: 50% blockage after 67 (±7 min) (dose 35 nM)[a] With CA_3: 50% blockage after 69 (±3) min (dose 35 nM)[a]		Alone: 480 (±120)[a] With CA_2: 70 (±10)[a] With CA_3: 90 (±20)[a]
F17	nd	No blockage (alone or with CA) (dose 10 μg/ml)[c]	nd
F16	Alone – 50% blockage after 26 (±2) min (dose 20 μg/ml)[c] With CA: 50% blockage after 20 (±2) min (dose 20 μg/ml)[c]	Alone – no blockage[c] With CA: 50% blockage after 50.9 (±6.2) min (dose 10 μg/ml)[c]	nd
F15	Alone: 50% blockage after 28.4 (±4.3) min (dose 10 μg/ml)[d]	Alone: 23 (±3.5) % blockage after 120 min (dose 20 μg/ml)[d]	nd
Intercro	nd	No blockage after 90 min (dose 10 μg/ml)[b]	nd
Gloydius intermedius			
Gintexin-B	nd	nd	With Gintexin-A: 40–120[e]
Bothriechis nigroviridis			
Nigroviriditoxin B	nd	nd	Alone: 2900[f] With Nigroviriditoxin A: 2200[f]

nd not determined
[a]Faure et al. (1993)
[b]Vieira et al. (2013)
[c]Hernandez-Oliveira et al. (2005)
[d]Toyama et al. (2003)
[e]Yang et al. (2015a)
[f]Lomonte et al. (2015)

Complexes of CB with Different Protein Targets

The CA and CB subunits of crotoxin are present in snake venom as non-covalent, high (nanomolar) affinity complexes. However, CB isoforms obtained *in vitro* after separation of the two subunits from a mixture of crotoxin isoforms can also

Table 6 Stability of CACB complexes

	Affinity of CB to CA K_D [nM]	Half-life of dissociation of complex with CA_2 [min]
CBa_2	With CA_2: 28 (±3) With CA_3: 22 (±2)	1.4 (±0.4)
CBb	With CA_2: 5 (±2) With CA_3: n.d.	10.5 (±1)
CBc	With CA_2: 5 (±1.5) With CA_3: 3.5 (±1)	Nd
CBd	With CA_2: 5 (±2) With CA_3: 4 (±2)	22 (±3)

nd not determined
Faure et al. (1993)

interact with other protein targets such as human factor Xa (hFXa), CICS (a natural crotoxin inhibitor from snake blood), GLIC pentameric receptor, and CFTR chloride channel, affecting their pharmacological properties and biological functions. The crystal structure of crotoxin (pdb 3R0L) provides a detailed model (Fig. 4) to explore possible modes of binding of CB to different protein targets.

CB Interaction with Human Coagulation Factor Xa

It has been shown that CB can form complexes with human coagulation factor Xa (hFXa) (Faure et al. 2007). This interaction influences the coagulation pathway by inhibiting the prothrombinase complex formation and retarding the formation of thrombin. Surface Plasmon Resonance (SPR) analysis showed that binding affinity depends on the CB isoform. CBc interacts with hFXa with very high affinity ($K_D = 0.6 \pm 0.3$ nM) compared to CBa_2 ($K_D = 52 \pm 4$ nM), which is correlated with anticoagulant activity (stronger inhibition of prothrombinase complex formation) (Faure et al. 2007).

The anticoagulant binding site of CB was identified by mutagenesis, affinity-binding studies, functional assays, and molecular docking calculations (Faure et al. 2007; Faure and Saul 2011). Two adjacent regions were identified. The first region is composed of residues located in helices A and B of the CB subunit and a loop between helix C and the β-wing. The second region includes part of the Ca^{2+} binding loop and the C-terminal region of the protein. Seven regions in hFXa (five in the heavy chain and two in the light chain) are involved in binding with CB. The catalytic sites of both proteins are not involved in these interactions (Faure and Saul 2011). Moreover, analysis of crystallographic structures showed that His1, Arg34, and Gly128 may be essential in the binding of hFXa by isoform CBc. Mutations of these residues in isoform CBa_2 (Ser1, Gln34, and Glu128) lead to conformational changes in adjacent residues and result in lower affinity for hFXa (Faure et al. 2007; Faure and Saul 2011).

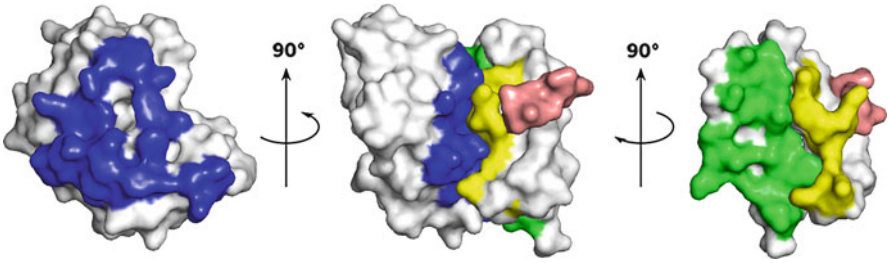

Fig. 4 Molecular binding interface of the CA and CB subunits of crotoxin. The crystal structure of crotoxin (pdb 3R0L) provides a detailed model to explore possible modes of binding of CB to other protein targets. *Left*: surface representation of the CB subunit (*in blue*, amino acid residues in contact with the CA subunit). *Center*: the assembled CACB complex. *Right*: surface representation of the CA subunit (*in yellow*, α-chain residues in contact with CB; *in green*, β-chain residues in contact with CB; *in pink*, the γ-chain of the CA subunit makes no contact with CB)

CB Interaction with CICS

Several natural PLA_2 inhibitors (PLI) have been identified in the blood of venomous snakes (Faure 2000). They can be divided into three types (PLI-α, PLI-β, and PLI-γ) according to their structure and properties. The crotoxin inhibitor from *Crotalus durissus terrificus* serum (CICS) belongs to PLI-γ type inhibitors, homologous to proteins of the Ly-6 superfamily. CICS is a 130 kDa acidic glycoprotein formed by the non-covalent association of six 23–25 kDa subunits (Perales et al. 1995; Faure et al. 2000). SPR analysis revealed a very high affinity of crotoxin and its CB subunit to CICS (nM affinity) (Faure et al. 2000). CICS neutralizes the toxic effect and inhibits the PLA_2 activity of crotoxin by interacting with the CB subunit. Binding of CICS with crotoxin leads to dissociation of the crotoxin complex and the release of CA (Perales et al. 1995).

Using peptide arrays Fortes-Dias and co-workers (Fortes-Dias et al. 2009) identified three possible interaction regions involved in CICS/CNF binding. According to their studies, the CA-CB and CICS-CB interfaces partially overlap.

CB Interaction with Prokaryotic Receptor GLIC

Crotoxin is primarily a presynaptic neurotoxin. However, some studies have shown that it also displays postsynaptic action. Recently, the proton-gated ion channel GLIC from *Gloeobacter violaceus*, a bacterial homolog of pentameric ligand-gated ion channel receptors, was identified as a new protein target of the CB subunit of crotoxin, and a novel function of CB as an inhibitor of proton-gated ion channel activity was reported (Ostrowski et al. 2016). Using SPR studies, it was shown that the interaction PLA_2-GLIC involves the extracellular domain of GLIC and that the enzymatic activity of PLA_2 is enhanced during interaction with GLIC (Ostrowski et al. 2016). Formation of the CB-GLIC complex leads to conformational changes in

the secondary structure of the proteins, and it was proposed that PLA_2 would be a new negative allosteric modulator of GLIC (Ostrowski et al. 2016).

CB Interaction with CFTR

Numerous studies have shown that neurotoxic PLA_2 can enter cells and interact with various intracellular protein targets, resulting in a wide range of pharmacological effects. PLA_2s as multifunctional proteins could potentially interfere as binders with ion channels. It has recently been shown that crotoxin and its CB subunit interact with the nucleotide-binding domain (NBD1) of CFTR (cystic fibrosis transmembrane regulator, a cyclic AMP-regulated chloride channel) and increase its chloride channel currents (Faure et al. 2016). Interestingly, the CB subunit possesses high affinity for both wild type and ΔF508-CFTR (the most frequent mutation associated with cystic fibrosis). CB behaves as a dual modulator of CFTR activity, as a potentiator and as a corrector (Faure et al. 2016).

Conclusion

Knowledge at the molecular level of the crotoxin CACB-binding interface and the 3D structures of natural isoforms of CB and CB-like proteins may help to identify other biologically relevant PPI sites. Two examples cited here (the CB-FXa and CB-CFTR/ΔF508CFTR complexes) open interesting perspectives for the future development of noncompetitive inhibitors of human FXa as anticoagulant agents and new correctors and potentiators for the treatment of cystic fibrosis.

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Dexamethasone Antagonizes Myotoxic and Inflammatory Effects Induced by *Bothrops* Snake Venoms

2

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Abstract

Treatment of snakebite around the world is a challenge because of the diversity of snake species and the complexity of their venom components. Most snakebites induce intense local inflammatory response that leads to extensive tissue damage which can result in late important disabilities. Among the pit vipers in the American continent, the snakes of genus *Bothrops* are very important, due to the great number of snakebites and mainly because their venom induces prompt local injury characterized by hemorrhage, edema, and myonecrosis. The main protocol for the treatment is based on the administration of animal-derived antivenom. However, despite reducing some systemic effects, this therapy is not able to completely neutralize all of the local venom effects. The problem is bigger when the therapy is delayed because of geographical problems or lack of accessibility to health support. The use of the glucocorticoid is proposed to decrease the acute inflammatory response to snakebites, and recent data indicate that dexamethasone is effective in reducing the local inflammation response and myonecrosis caused by *Bothrops* envenomation. This chapter shows a general view of venom injury caused by snakebites and revises the experimental use of dexamethasone as adjuvant treatment for these accidents, while detailed descriptions of specific venom components and its effects are present in other sections of this book.

Keywords

Snakebites • Snake venoms • Myonecrosis • Inflammation • Dexamethasone

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_9

Contents

Introduction

Snake venoms are a complex mixture of enzymes and nonenzymatic proteins used for both the immobilization and digestion of snake prey, while humans can be accidentally caught in the middle of this natural circle of life. Venomous snakebites are a problem globally neglected, and although they have been present since the human being started reporting the history, it was not until recently that they have been considered a public health issue (Scheske et al. 2015 and references therein). With a global estimated incidence of 2,500,000 cases and mortality in circa of 100,000 deaths per year, its importance is due especially to the morbidity caused by these accidents (Chippaux and Goyffon 1998; Kasturiratne et al. 2008). The envenoming involves a large range of biologically active substances present in the venoms, such as metalloproteases, acetylcholinesterases, phospholipases, cationic peptides, and hemorrhage factors (Kang et al. 2011; Lomonte and Rangel 2012; Markland and Swenson 2013). The tissue responses to the presence of crude snake venom and its purified constituents have been investigated under a variety of in vitro and in vivo approaches in order to understand their local cytotoxicity and systemic effects. Among the surprising variety of pathophysiological manifestations induced by different snake venoms and their toxins are locally intense erythema, edema, hemorrhage, blistering, skin and muscle necrosis, and systemic effects like coagulation deficiencies, nervous system disturbances, and heart and kidney failure (Sanchez et al. 1992; Gutierrez et al. 1986; Melo and Ownby 1999; Teixeira et al. 2009). However, several evidences indicate that besides direct toxin effects, such as membrane disruption by phospholipases, the inflammatory response induced by snake venoms importantly contributes to further development of damage (Gutierrez et al. 1986; Farsky et al. 1997; Milani et al. 1997; Zamuner et al. 2001; Costa et al. 2002; Olivo et al. 2007; Carneiro et al. 2008).

Inflammation is the reaction of all tissues to injury or presence of offending agents. It is an extensive protective response which tends to block and remove the aggressor organism and sets the stage for healing and reconstitution of normal function in the damaged tissue. The vascular and cellular responses of both acute and chronic inflammation are mediated by endogenous chemical factors derived from plasma or cells and triggered by the inflammatory stimulus. These different factors play key roles, not only initiating but also regulating the host responses. The

process involves functional alterations of local microvessels, leading initially to accumulation of fluid and leukocytes into extravascular space of the involved tissues. Presence and activation of phagocytic leukocytes is a key process in the innate immune response to invading pathogens. Activation of these cells results in the assembly of a nicotinamide adenine dinucleotide phosphate (NADP) oxidase-2 enzyme complex at the plasma membrane and a subsequent "respiratory burst," in which O_2 is reduced, at the expense of reduced NADP, to superoxide radicals (O_2^-). This radical undergoes rapid spontaneous or catalyzed (by superoxide dismutase) dismutation to yield molecular oxygen and hydrogen peroxide (H_2O_2). High concentrations of H_2O_2 can be cytotoxic or bactericidal. These properties of activated leukocytes have been attributed to the actions of myeloperoxidase (MPO), a heme enzyme released by activated neutrophils. This green enzyme is the most abundant protein in neutrophils, accounting for up to 5% of their dry mass; the measurement of its activity is an indirect indicator of local activated neutrophil presence and consequently inflammatory response. Hence, if persistent and uncontrolled, leukocyte infiltrate itself becomes the offender, just like in the several chronic inflammatory diseases in which leukocytes are overactivated (Klebanoff 2005; Davies 2011 and references therein). Several conditions can exemplify the inflammatory apparatus as either the main actor or at least a leading support in disastrous tissue damage. For example, in the acute infarcted myocardium, necrotic cardiomyocytes release danger signals, activating an intense inflammatory response, whose pathways play a crucial role in regulation of a wide range of cellular processes involved in injury, repair, and remodeling of the infarcted heart (Saxena et al. 2015 in press). Moreover, asthma, in pathological terms, is characterized by airway inflammation and by structural changes in airway tissues, collectively referred to as airway remodeling. Since an inhaled-allergen challenge in atopic asthma induces eosinophilic inflammation of the airway and changes in the extracellular matrix, and since a reduction in airway eosinophils has been reported to reduce certain markers of airway remodeling, such structural changes in the tissues have been considered a consequence of eosinophilic airway inflammation (Grainge et al. 2011). A last example is severe sepsis, in which the specific response in any patient depends on the causative pathogen (load and virulence) and the host (genetic characteristics and coexisting illnesses), with differential responses at local, regional, and systemic levels. The composition and direction of the host response probably change over time in parallel with the clinical course. In general, acute pro-inflammatory reactions (directed at eliminating invading pathogens) are thought to be responsible for collateral tissue damage in severe sepsis, whereas anti-inflammatory responses (important for limiting local and systemic tissue injury) are implicated in the enhanced susceptibility to secondary infections (Angus and van der Poll 2013).

Treatment of most of human-occurring snakebites is largely life supportive, which includes analgesia, sedation, and occasionally fluid resuscitation. The main recommended specific therapy to snakebite envenomation is still based on the intravenous administration of animal-derived antivenom. Currently, antivenoms are either whole immunoglobulin G molecules or divalent or monovalent

immunoglobulin fragments purified from the plasma of animals, mostly horses or sheep, immunized with venoms. Although it is clear that in life-threatening situations the antivenom should be used, its use in moderate or light envenomation is not so clear. That can be explained by the long-known capacity of the antivenom to neutralize the systemically circulating toxins that are mainly responsible for the snakebite mortality. However, the local damage induced by snake venoms is described as being only partially neutralized by either the specific or the polyvalent antivenom even if it is locally injected (Gutierrez et al. 2007; da Silva et al. 2007). The problem is bigger when the therapy is delayed, either by geographical problems or lack of accessibility to health support. Even when available, the use of antivenom can still elicit allergic reactions, such as an anaphylactic shock, once they are animal-derived products (Chippaux and Goyffon 1998; Pardal et al. 2004; Gutierrez et al. 2007; Scheske et al. 2015 and references therein). It has been difficult to develop new drugs for snakebite envenoming treatment, either from plants or from new planed synthetic molecules, because they are not attractive to developed countries nor to big companies once they will hardly return the investment and the endeavor (Gutierrez et al. 2007; Lomonte et al. 2009). Although many studies have shown new substances, mainly from plants, that are able to decrease the powerful venom effects, it remains a challenge (Olivo et al. 2007; Gutierrez et al. 2007; Melo et al. 2010; Patrão-Neto et al. 2013).

Snake Toxins and Muscle Damage

Many previous clinical and experimental observations had described that the local and systemic skeletal muscle degeneration is a common consequence of envenomation due to snakebites. Within snake venoms, a complex presence of components which are both nonenzymatic or active catalytic enzymes that act in combination has been described, resulting in local and systemic effects. They are grouped and named by their described catalytic properties such as acetylcholinesterases, L-amino acid oxidases, serine proteinases, metalloproteinases, and phospholipases A_2 (PLA2s) (Kang et al. 2011; Markland and Swenson 2013 and references therein). Gutierrez et al. (2005) described that the pathogenesis of venom-induced hemorrhage involves the direct damage to microvessels, performed by hemorrhagic toxins, combined with a wide variety of effects that viperid venoms exert in hemostasis. Hemorrhage induced by viperid snake venoms can be the result of direct angiorrhexis or many combined activities involving endothelial cell damage and a prothrombin activator (Gutierrez et al. 2005; Markland and Swenson 2013). Although the direct vascular damage can compromise the muscle fiber nutrition and metabolic supply, which could per se induce the myonecrosis, it is well established that snake venoms contain many myotoxic components that can alone cause damage to muscle cells (Melo and Ownby 1999; Fuly et al. 2003; Melo et al. 2004). These so-called myotoxins induce direct cytotoxicity on skeletal muscle cells, and they have been investigated under many different protocols, evaluating the muscle damage and the changes that they can induce in the local

environment, such as the edema as part of an intense inflammatory response (Melo and Ownby 1999; Teixeira et al. 2009; Lomonte and Rangel 2012 and references therein). The isolation and characterization of myotoxins have shown that they are abundant in many venoms and some are enzymatically active such as PLA2. Further studies on PLA2 myotoxins revealed that those which contain arginine residues are enzymatically active and the other with lysine in position 49 (Lys-49) are devoid of this action, but these are strongly cationic molecules (Murakami et al. 2008 and references therein; Lomonte and Rangel 2012). These toxins are able to interact with the sarcolemma and disrupt it, inducing many individual morphological appearance that were described in different ways under light or electron microscopy investigations (Calil-Elias et al. 2002 and references therein). One important observation was that the amount of cationic charge affects the myotoxic effect of the toxins isolated from *Bothrops* venoms. Investigations with *B*. asper myotoxin II and *B*. *jararacussu* botrhopstoxin I showed that a polysulfated polyanion named suramin was able to neutralize the surface positive charges of these myotoxins and decreased the sarcolemmal damage (Murakami et al. 2008 and references therein). They proposed that the interfacial surface charge and free accessibility to the PLA2-active site-like region are essential requirements for the activity of Lys49 PLA2 homologues.

Besides direct cytotoxicity against skeletal muscle cells, other actions of venoms and their toxins can take place in myonecrosis, such as the altered blood supply commented above, and the devastating inflammatory response elicited just after the accidents and reproduced in in vivo experiments.

Inflammatory Response to Snake Venoms

Local edema, erythema, and heat are observed immediately after the snakebite, and the patients report intense pain, all signs and symptoms of a typical acute inflammatory reaction. Local myonecrosis and inflammatory response are critical to the commonly observed residual damage which could lately be responsible for loss of limb function or even amputation (Milani et al. 1997; Gutierrez et al. 2006; Teixeira et al. 2009). Studies of the inflammatory response elicited by different snake venoms show local edema and the presence of inflammatory white blood cells on the injection site (Fig. 1) and correlate it with tissue damage and mediators present in the bloodstream (Gutierrez et al. 1986; Fuly et al. 2003; Teixeira et al. 2009). It is known that *Bothrops* venoms are able to activate leukocyte oxidative stress due to the presence of MPO enzymes in these cells (Zamuner et al. 2001; Elifio-Esposito et al. 2011; Patrão-Neto et al. 2013), but exactly how the venom elicits this inflammatory response is still to be answered. The local presence of leukocytes after viperid venom injections has been investigated under several experimental conditions with various snake species, such as *Bothrops asper*, *Bothrops lanceolatus*, *Bothrops jararaca*, and *Crotalus durissus terrificus*, in different inoculation sites like the peritoneum, skin, and skeletal muscles (Gutierrez et al. 1986; Farsky et al. 1997; Zamuner et al. 2001; Costa et al. 2002). Among the possible

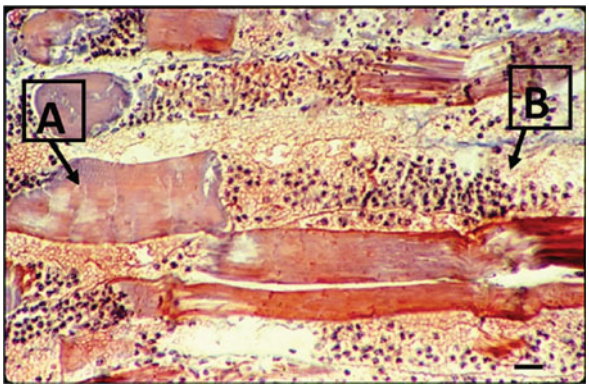

Fig. 1 Light microscopy of mouse *extensor digitorum longus* muscle 24 h after perimuscular injection of 1.0 mg/kg *Bothrops jararacussu* venom. Longitudinal section stained with hematoxylin and eosin. Note necrotic myofibers in different stages of degeneration, as shown in (**a**), and the intense acute inflammatory infiltrate in (**b**) (bar = 10 μm) (Original in Saturnino-Oliveira et al. (2012))

mechanisms involved in cell migration to the inoculation site is the ability of a lectin from *B. jararacussu* venom to directly activate human neutrophil migration (Elifio-Esposito et al. 2011). Nevertheless, according to Farsky et al. (1997), the local leukocyte increase induced by injection of *B. jararaca* venom is dependent on activation or secretion of endogenous compounds such as cytokines. In agreement, Moreira et al. (2007) showed the *B. jararaca* venom ability of inducing cyclooxygenases expression as part of the pathophysiological process of inflammation and hyperalgesia caused by this venom. Interestingly, in the same study, this response was not observed with *C. d. terrificus* venom, indicating that various mechanisms may be present among different species. Finally there is the ability of metalloproteinases, present in viperid snake venom, to disrupt the vascular membrane (Costa et al. 2002). These enzymes are capable of degrading some basement membrane and associated extracellular matrix proteins facilitating the local cell and fluid leakage (Escalante et al. 2011). Basal membrane damage combined with the venom ability to induce distinct classes of inflammatory mediators may also be responsible for the capillary leakage of cells and for the local edema observed after venom inoculation (Moreira et al. 2012 and references therein). Additionally, chronification of this venom-induced inflammatory response has been reported as responsible for inducing skin squamous cell carcinoma (Mello et al. 2000).

Glucocorticoid Mechanisms

Glucocorticoids (GC) are multitasking agents with complex and varied actions that are beyond the scope of this review and can be found elsewhere (Buckingham 2006). Particularly important is their well-known anti-inflammatory action with

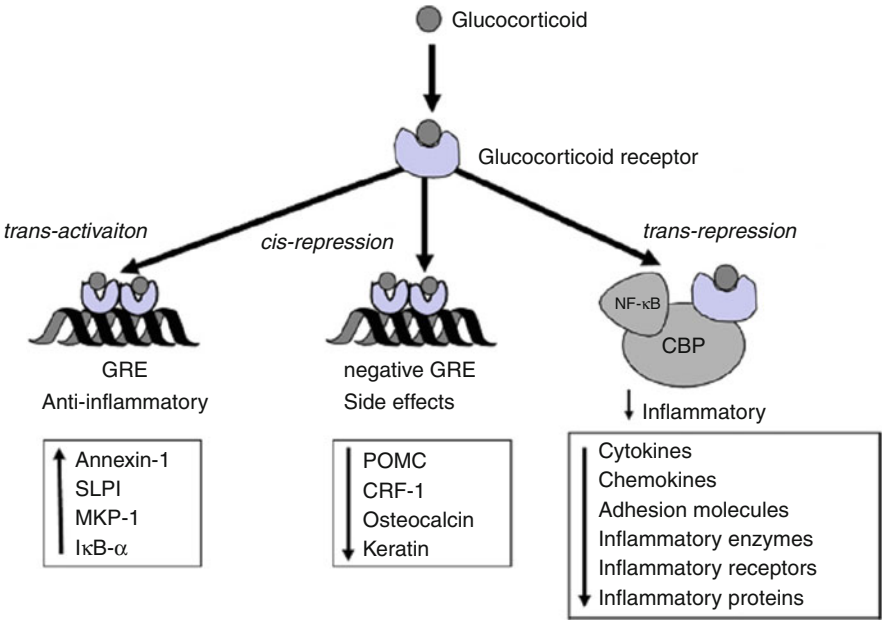

Fig. 2 The glucocorticoid regulation of gene expression in several ways. *GRE* glucocorticoid-response elements, *NF-κB* nuclear factor κB, *CBP* CREB-binding protein (*CBP*), *SLPI* secretory leukoprotease inhibitor, *MKP-1* mitogen-activated kinase phosphatase-1, *IκB-α* inhibitor of NF-κB, *GILZ* glucocorticoid-induced leucine zipper protein, *POMC* proopiomelanocortin, *CRH* corticotrophin releasing factor (Reprinted from The Journal of Steroid Biochemistry and Molecular Biology, 120, Peter J. Barnes, Mechanisms and resistance in glucocorticoid control of inflammation, 76–85, Copyright (2010), with permission from Elsevier)

established effectiveness as therapy for immune and chronic inflammatory diseases such as asthma. Briefly, GC may modulate the inflammatory reaction in several ways as proposed by Barnes (2010 and references therein) and shown in Fig. 2. Their major action is to switch off the genes activated by the inflammatory stimuli (Clark 2007). To achieve that effect, GC diffuse rapidly across cell membranes and bind to glucocorticoid receptors (GR) in the cytoplasm, which together translocate to the nucleus where they can directly or indirectly regulate the gene transcription. The GC-GR then dimerizes and interacts with glucocorticoid-response elements (GRE) leading to increase in anti-inflammatory gene transcription of proteins such as annexin-1 or inhibitor of NF-κB (IκB-α), in a mechanism known as transactivation. This gene activation is associated with a selective acetylation of lysine residues in histones, resulting in increased gene transcription (Ito et al. 2000). Negative GRE have also been described, and their interaction with the GC-GR dimer may account for the GR systemic side effects caused by endocrine hormone gene-related suppression (cis repression). The GC-GR homodimer complex may also act alone interacting with other activated transcription factors by protein-protein binding. This transrepression mechanism seems to be particularly important

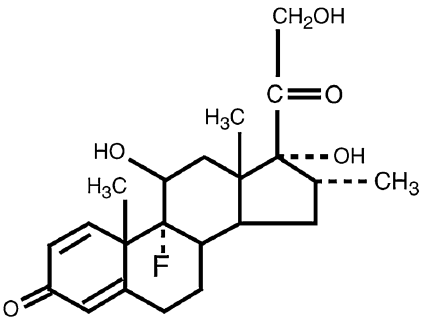

Fig. 3 Chemical structure of anti-inflammatory synthetic steroid dexamethasone

in cells with overexpressed genes and attenuates the inflammatory response already elicited by pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB) (Barnes 2010). Dexamethasone (Fig. 3) is a GC widely used in medical practice that has recently been investigated regarding its ability to preserve muscle tissue exposed to *Bothrops* venoms in mice (Patrão-Neto et al. 2013).

Dexamethasone and Snake Venom

One of the first writings describing the use of dexamethasone (DEXA) against snake venom local effects was published by Rosenfeld et al. in 1969. The authors reported a reduction of cutaneous necrosis in mice treated with DEXA after *B. jararaca* crude venom injection. Since then DEXA has been sporadically investigated under different protocols, mostly as second comparative option, but has never been meticulously explored as an antivenom substance. Crosland (1991) described that DEXA increased the survival rate on lethality test induced by the administration of *Bungarus multicinctus* and *Oxyuranus scutellatus* crude venoms or the isolated toxins β-bungarotoxin, crotoxin, or taipoxin. Kato et al. (1985) suggested a possible direct interaction between DEXA and venom constituents. Other investigators did not find any in vitro effect of DEXA against snake venoms in isolated skeletal muscle, neuromuscular junction, or perfused kidney preparations (Havt et al. 2001; Patrão-Neto et al. 2013). Failure to protect isolated organs contrasts with the in vivo observations. Indeed when kidneys were selectively perfused in vivo with venom via mesenteric artery, DEXA was able to prevent renal damage (Martins et al. 1998). On the other hand, when applied topically on the eyes as treatment of *Naja nigricollis* and *Naja sumatrana* venom-induced ophthalmia, DEXA failed to protect animals experimentally (Chu et al. 2010). These results show the importance of the *systemic* response in vivo and indicate that DEXA may act in modulating the inflammatory response.

Recent observations have shown that parenteral administration of DEXA was effective against snake venom-induced local muscle damage since it was able to prevent later manifestation of myotoxicity. This effect was demonstrated in the study, in vivo, as preserved muscle CK content (Fig. 4) and reduced myofiber

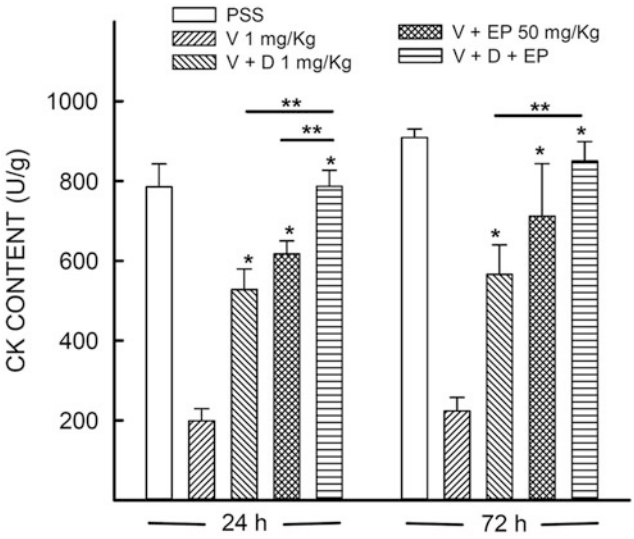

Fig. 4 Effect of dexamethasone (*D*) and *Eclipta prostrata* (*EP*) treatments in muscle CK content following *B. jararacussu* venom injection. Muscles were dissected 24 and 72 h after venom injection, and homogenized, and their creatine kinase (*CK*) content was determined and expressed as U/g. Other abbreviations: *PSS* physiological saline solution, *V* venom (Reprinted from Toxicon, 69, Patrão-Neto, Fernando Chagas; et al, Dexamethasone antagonizes the in vivo myotoxic and inflammatory effects of Bothrops venoms, 55., Copyright (2013), with permission from Elsevier)

degeneration observed on light microscopy, after intramuscular *Bothrops* sp. venom injection (Patrão-Neto et al. 2013). The authors showed that a single dose of DEXA alone preserved as much as 67% of mice muscle tissue up to 72 h after the *B. jararacussu* injection.

It is a tempting conclusion that most likely DEXA would be effective in snakebite envenoming therapy basically for the reduction of inflammation, but to assume that, one must accept the importance of inflammatory response in the myonecrosis induced by snake venoms. Indeed DEXA reduces local edema and cellular infiltrate induced by *Bothrops* venom injection in mice (Wagner and Fessler 1986; Melo et al. 2010; Patrão-Neto et al. 2013). Moreover, treatment with DEXA showed leukocyte count and MPO activity reduction in mouse skeletal muscle (Fig. 5), and similar DEXA effect has been reported with *B. jararacussu* inoculated in the peritoneum (Pereira et al. 2009). Perretti and Flower (1993), although not using venoms in their investigations, described an antimigratory effect of DEXA on mouse leukocytes and correlated this effect with annexin-1 production. Mancuso et al. (1995) showed that DEXA did not alter the rolling and adherence of leukocytes to the vascular wall, but increases their detaching rate from the endothelium, making the white cells return to the bloodstream. They also showed an increase in the leukocyte diapedesis duration and the disappearance of this effect with annexin-1 antagonists. DEXA could mediate the leukocyte migration activated directly by snake venom components by endogenous mediators,

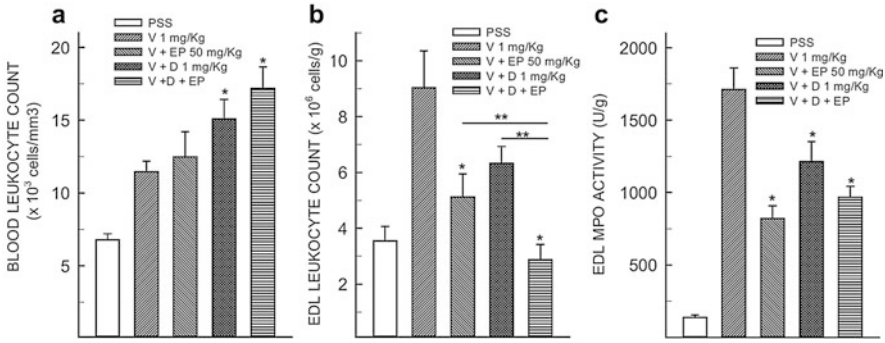

Fig. 5 Effect of dexamethasone (*D*) and *Eclipta prostrata* (*EP*) in local inflammatory parameters induced by *B. jararacussu* venom. Data on panel (**a**) show blood leukocyte count. On panel (**b**) data show leukocyte count in EDL muscle, and panel (**c**) presents the myeloperoxidase (*MPO*) enzyme activity on the muscular tissue. Other abbreviations: *PSS* physiological saline solution, *V* venom (Reprinted from Toxicon, 69, Patrão-Neto, Fernando Chagas; et al, Dexamethasone antagonizes the in vivo myotoxic and inflammatory effects of Bothrops venoms, 55., Copyright (2013), with permission from Elsevier)

decreasing systemic responses produced by cell damage (Fig. 5), keeping the cells on the bloodstream without stopping their recruitment from the bone marrow as reported by Patrão-Neto et al. (2013). Additionally, treatment with DEXA reduced the MPO activity in mouse muscle tissue corroborating the reduction found in the inflammatory cell count in the same muscle. Unlike DEXA, which seems to show only in vivo antivenoms effect, the authors suggest that antiophydic agents, such as natural compounds present in plant crude extracts, such as *Eclipta prostrata*, antagonize directly the venom effects, while DEXA decreases the endogenous inflammatory response elicited by venom injection (Wagner and Fessler 1986; Melo et al. 2010; Patrão-Neto et al. 2013). DEXA has a known anti-inflammatory property ascribed to the ability of inhibiting the expression of pro-inflammatory factors, consequently reducing the inflammatory reaction and the cell damage (Euzger et al. 1999; Clark 2007).

Conclusion and Future Directions

In conclusion, just like clearly accepted as of pivotal importance in many severe pathological conditions, there is absolutely no reason to ignore, despite the direct actions of toxins on target structures, the role of the inflammatory process in the local muscle damage induced by snake venoms, once the use of DEXA protected the muscle tissue from the venom myonecrotic effect. The idea of the presence of large amounts of inflammatory cells on the damaged fibers being responsible, at least in part, by the myonecrosis induced by snake venoms is strongly attractive and represents a new approach to the problem. Understanding and modulating the immune system response to the snake venom injury may be the first step in studying

a common way of treating different toxins from a variety of poisonous animals. Researchers should focus on the investigation of the role of inflammation on snakebites, and efforts should be made to find new anti-inflammatory agents, natural or synthetic, that can aid on venom neutralization and prevent the late disabilities observed after these accidents. Meanwhile, since DEXA is a well-known, safe, and largely available worldwide medication, controlled clinical trials using DEXA should be designed and performed in order to ascertain whether in human cases there is a reproduction of its protective effect.

Cross-References

- ▶ Antiproliferative Effects of Snake Venom Phospholipases A_2 and Their Perspectives for Cancer Treatment
- ▶ Inflammatory Action of Secretory Phospholipases A_2 from Snake Venoms
- ▶ Myotoxin Inhibitors
- ▶ Natural Inhibitors of Snake Venom Metallopeptidases

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Inflammatory Action of Secretory Phospholipases A_2 from Snake Venoms

3

Soraia K. P. Costa, Enilton A. Camargo, and Edson Antunes

Abstract

Secretory phospholipases A2 (sPLA2s) play an important role in the generation of inflammatory events in several human diseases. Secretory PLA2s obtained from snake venoms have been widely used as pharmacological tools to elucidate the physiopathology of inflammatory conditions, as well as to reproduce the clinical manifestations of human envenomation by snake venoms. In venoms from *Bothrops* genus, two main types of sPLA2s have been characterized, namely, the catalytically active Asp-49 enzymes and the catalytically inactive variants Lys-49, that present Lys instead of Asp at position 49. Injections of Asp-49 and Lys-49 sPLA2 variants into experimental animals evoke classical inflammatory events such as local edema formation and leukocyte recruitment. Additionally, Lys-49 and the Asp-49 sPLA2s induce mechanical hyperalgesia and allodynia in rodent models. An array of endogenous agents such as histamine, 5-hydroxytryptamine (5-HT), bradykinin, tachykinins, arachidonic acid metabolites, pro-inflammatory cytokines, and nitric oxide (NO) have been implicated in sPLA2-induced responses. The catalytic activity and/or the

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_10

cationic content of sPLA2 has been proposed to be of great importance in triggering its edematogenic, cell infiltrating, and pain responses. Venom Subscript>/Subscript> has also been used to mimic the clinical findings of acute pancreatitis associated with abdominal hyperalgesia. Bradykinin, substance P, and nitric oxide (NO) play important roles in pancreatic and remote lung inflammation during sPLA2-induced pancreatitis. This chapter summarizes the current knowledge on the local inflammatory mechanisms of venom sPLA2 in rodent models. The ability of venom sPLA2s to induce acute pancreatitis and underlying mechanisms will also be presented here.

Keywords

Catalytic activity • Cationic charge • Leukocyte • Pancreatitis • Pain

Contents

Introduction to Phospholipases A_2 (PLA2s)

Biochemistry, Division (sPLA2, cPLA2, iPLA2, lp-PLA2), Mechanism, Regulation, Lys-49, and Asp-49 Variants

Phospholipases A2 (PLA2s) are esterolytic hydrolases which catalyze the hydrolysis of 3-sn-phosphoglycerides at the sn-2 position producing lysophosphatidic and free fatty acid. Hydrolysis of membrane phospholipids by PLA2s generates a large number of pro-inflammatory lipid mediators, including the arachidonic acid-derived metabolites (prostaglandins, prostacyclin, thromboxane A2, and leukotrienes) and platelet-activating factor (PAF). Accordingly, studies have shown increased PLA2 activity in different types of human inflammatory conditions such as rheumatoid arthritis, psoriasis, gram-negative septic shock, allergic rhinitis, acute pancreatitis, atherosclerosis, ocular inflammation, and osteoarthritic cartilage, among others.

Phospholipases A2 are widely distributed in mammalian cells and pancreatic juice, as well as in snake and bee venoms. These enzymes have been divided in five

groups based on molecular weight, amino acid sequence, and number of disulfide bridges and requirement of calcium for its enzymatic activity. They comprise the so-called secretory (sPLA2), cytosolic (cPLA2s), calcium-independent (iPLA2), PAF acetylhydrolases (PAF-AH), and the lysosomal PLA2s. A calcium-dependent PLA2 (AdPLA or group XVI PLA2) expressed in adipose tissue and induced in obesity conditions has been described (Duncan et al. 2008). Assignment of the enzymes to a particular group relies mainly on the catalytic mechanism (His/Asp, Ser/Asp, or Ser/ His/Asp hydrolase), as well as on functional and structural features. For review see Schaloske and Dennis (2006), Murakami et al. (2011), and Gutiérrez and Lomonte (2013).

The cPLA2s are divided into six subgroups (GIVA to GIVF) and found in murine and human cells. These enzymes are large cytosolic proteins with molecular sizes varying from 61 to 114 kDa and use a catalytic serine. They require Ca^{2+} for translocation from the cytosol to their site of action on membrane phospholipids upon binding to a C2 domain, despite they do not require calcium for catalytic activity per se. All contain a conserved Ser/Asp catalytic dyad and Arg that are required for the catalytic activity. Cytosolic PLA2 shows preferential specificity for arachidonic acid in the *sn*-2 position.

The iPLA2s are found in human/murine cells and comprise the subgroups GVIA-1, GVIA-2, GVIB, GVIC, GVID, GVIE, and GVIF. They are localized intracellularly and utilize a serine for catalysis, but do not require Ca^{2+} for membrane association or for catalytic activity.

The sPLA2s are subdivided into several groups, as follows: GIA, GIB, GIIA, GIIB, GIIC, GIID, GIIE, GIIF, GIII, GV, GIX, GX, GXIA, GXIB, GXII, GXIII, and GXIV. These sPLA2s are small secreted proteins (14–18 kDa) usually containing 6–8 disulfide bonds and require micromolar levels of calcium for catalysis. An exception is the mammalian/murine subgroup III that possess a high molecular mass (approximately 55 kDa). These enzymes use an active site histidine in close proximity to a conserved aspartate and generally show a high activity with anionic phospholipids. The sPLA2s represent the most abundant components in Viperidae snake venoms. Two main types of sPLA2s have been characterized in bothropic venoms: (i) catalytically active PLA2s, which have the conserved residues at the catalytic network and at the calcium binding loop including Asp-49, and (ii) catalytically inactive variants having Lys, Ser, Asn, or Arg instead of Asp at position 49. Generally, the non-Asp-49 PLA2 enzymes exhibit strong myotoxic activity. In this latter group, the most studied toxins are the basic and homodimeric Lys-49 PLA2s that induce marked local myonecrosis through calcium-independent mechanism. Table 1 summarizes the different types of snake venom sPLA2s.

Secretory PLA2s from snake venoms have been largely studied due to its ability to induce acute muscle damage and to mimic local inflammatory reactions (edema formation, leukocyte influx into tissues, and pain) that may reproduce the clinical manifestations in human envenomation by snake venoms (Teixeira et al. 2003; Gutiérrez and Lomonte 2013). Additionally, studies on venom

Table 1 Secretory phospholipases A2s (sPLA2s) isolated from snake venoms

Asp-49 PLA2 (D49 PLA2)	**Snake venoms**
BthTX-II (bothropstoxin-II)	*Bothrops jararacussu*
PrTX-II (piratoxin-II)	*Bothrops pirajai*
LmTX-I	*Lachesis muta muta*
Bl-PLA2	*Bothrops leucurus*
BaltTX-II	*Bothrops alternatus*
CVV myotoxin	*Crotalus viridis viridis*
Myotoxin I/III	*Bothrops asper*
EcTx-I	*Echis carinatus*
Cdc PLA2	*Crotalus durissus collilineatus*
VL-PLA2	*Macrovipera lebetina obtusa*
PhTX-II	*Porthidium hyoprora*
Lys-49 PLA2 (K49 PLA2)	
BthTX-I (bothropstoxin-I)	*Bothrops jararacussu*
PrTX-I (piratoxin-I)	*Bothrops pirajai*
BaltTX-I	*Bothrops alternatus*
ACL myotoxin	*Agkistrodon contortrix laticinctus*
Basp-II (myotoxins II and IV)	*Bothrops asper*
Myotoxin II	*Atropoides nummifer*
Myotoxin II	*Cerrophidion godmani*
MjTX-I/MhTX-II	*Bothrops moojeni*
BnSP-6 (and BnSP-7)	
Acla, AppK	*Agkistrodon p. piscivorus*
Ser-49 PLA2 (S49 PLA2)	
Ecarpholin S	*Echis carinatus sochureki*
Ammodytin L	*Vipera ammodytes ammodytes*
Vur-S49	*Vipera ursinii renardi*
Asn-49 PLA2 (N49 PLA2)	
TsR6	*Trimeresurus stejnegeri*
CTs-R6	*Trimeresurus stejnegeri*
TM-N49	*Protobothrops mucrosquamatus*
Arg-49 PLA2 (R49 PLA2)	
Promutoxin	*Protobothrops mucrosquamatus*
Zhaoermiatoxin	*Zhaoermia mangshanensis*
PeBP(R)-I/PeBP(R)-II	*Protobothrops elegans*

sPLA2 may also shed light into the mechanisms triggered by mammalian sPLA2 in the diverse human inflammatory conditions. This chapter summarizes the current knowledge on the local inflammatory mechanisms of venom sPLA2 in rodent models, focusing the importance of its catalytic activity and cationic content in triggering its effects. Studies addressed to investigate the effects of venom sPLA2s in experimental pain and pancreatitis have also been reviewed in the present chapter.

Role of Catalytic Activity in the Inflammatory Actions of Venom sPLA2s

Early studies showed the snake venom sPLA2s are able to induce in vivo rat mast cell degranulation and paw edema (Damerau et al. 1975; Brain et al. 1977). Other sPLA2s isolated from *Naja mocambique mocambique* and *Vipera russelli* snake venoms as well as from *Apis mellifera* bee venom were later confirmed to possess edematogenic activity (Cirino et al. 1989). Around the same time, two distinct and independent sites of action responsible for the catalytic and pharmacological actions of venom sPLA2 were described (Vishwanath et al. 1987). It was also suggested that PLA2 presents affinity toward a specific protein rather than to lipid domains (Kini and Evans 1989). The catalytic activity and the cationic content of sPLA2 were proposed to be of great importance for its physiopathological actions (Wang and Teng 1990). Secretory PLA2 homologues presenting little or no enzyme activity (Asp-49 and Lys-49 sPLA2 homologues, as detailed above) were used to further examine the importance of catalytic activity in triggering the inflammatory responses. These homologues include, for instance, bothropstoxin-I (Lys-49 PLA2), bothropstoxin-II (Asp-49 PLA2), and piratoxin-I (Lys-49 PLA2), among several others (see Table 1). In addition, *p*-bromophenacyl bromide (BPB) has widely been employed to evaluate the involvement of the catalytic activity of sPLA2 since it alkylates the histidine-48 residue located in the active site of the enzyme resulting in an inhibition of the enzymatic activity (Díaz-Oreiro and Gutiérrez 1997). Bothropstoxin-I, bothropstoxin-II, piratoxin-I, and LmTX-I (Asp-49 homologue purified from *Lachesis muta muta* venom) were shown to increase the microvascular permeability in rat paw and skin by mechanisms dependent on in vivo mast cell activation (Landucci et al. 1998), although mast cell-independent mechanisms have also been reported in the rabbit skin (Landucci et al. 2000a). A novel enzymatically inactive Arg-49 PLA2 purified from *Protobothrops mucrosquamatus* venom (promutoxin) also increases the microvascular permeability in rat skin associated with mast cell degranulation (Wei et al. 2010). Mast cells are resident in tissues throughout the body and play an important role in the pathogenesis of several inflammatory disorders due to its ability to produce granule-associated mediators, lipid-derived substances, cytokines, and chemokines. These cells also express and release type II PLA2, which is thought to modulate mast cell degranulation. Interestingly, BPB inhibited both edema formation and in vitro mast cell degranulation induced by bothropstoxin-I, bothropstoxin-II, and piratoxin-I, suggesting that besides inhibiting enzymatic activity, BPB may have additional effects on the PLA2 structure which result in conformational changes of the molecule (Landucci et al. 1998, 2000a). Alkylation of a Lys-49 PLA2 purified from *Bothrops moojeni* venom (MjTX-II) with BPB also reduced the mice edema-induced activity, confirming a dissociation (or partial overlapping) of the pharmacological and toxic effects (Stábeli et al. 2006). Since the cytotoxic and bactericidal activities by MjTX-II were not affected by BPB, it was hypothesized that the functional site for these effects is the C-terminal domain rather than the catalytic site. Moreover, structural studies and biological activities

have shown that the myotoxic activity of Lys-49 sPLA2 is independent of the catalytic activity site (Montecucco et al. 2008). A previous study reported the use of mutants of *Oxyuranus scutellatus scutellatus* toxin 1 (OS1) and toxin 2 (OS2) at the catalytic site (Rouault et al. 2006). The enzymatic activity of the active site mutant (H48Q) was found to be 500-fold lower than that of the wild-type protein, while neurotoxicity produced by intracerebroventricular injections was only 16-fold lower, which is indicative that the catalytic activity is a minor factor that determines toxicity at the central nervous system. In addition, the C-terminal region of OS2 (residues 102–119) was found to be critical for enzymatic activity, but not for central neurotoxicity, again dissociating both of these phenomena (Rouault et al. 2006).

It is well established that hydrolysis of membrane phospholipids by PLA2s generates a large number of pro-inflammatory lipid mediators, some of which are potent chemoattractants for polymorphonuclear cells, including leukotriene B4 (LTB_4). Incubation of polymorphonuclear leucocytes with different PLA2s results in significant LTB_4 generation (Shimizu et al. 1994). Honey bee venom sPLA2 also activates human basophils in vitro to produce LTB_4 (Mustafa et al. 2008). Interestingly, human recombinant PLA2 promotes cell infiltration in the rat air pouch model by mechanisms unrelated to generation of arachidonic acid metabolites (Cirino et al. 1994), which seems to be consistent with studies showing that PLA2 homologues bothropstoxin-I, bothropstoxin-II, and piratoxin-I recruit leukocyte into the pleural cavity by mechanisms unrelated to enzymatic activity (De Castro et al. 2000). In addition, group IA (*Naja mocambique mocambique* venom) and IIA (recombinant human synovial) sPLA2s are able to activate macrophage by a mechanism independent of their enzymatic activities, which is probably related to the activation of the mannose receptor or sPLA2-specific receptors (Trigianni et al. 2000). Venom sPLA2s are also potent stimuli for cytokine production in human lung macrophages, blood monocytes, and eosinophils (Triggiani et al. 2002). Secretory PLA2 from *Naja mocambique mocambique* venom induces the production of cytokines and chemokines in human macrophages by a nonenzymatic mechanism that involves the PI3K/Akt, MAPK p38, ERK1/2, and NF-kappaB signaling (Granata et al. 2006). An Asp-49 PLA2 homologue (*Bl*-PLA2) purified from *Bothrops leucurus* venom promotes secretion of the pro-inflammatory cytokines IL-12p40, TNF-α, IL-1β, and IL-6 from human monocytes (Nunes et al. 2011). The cytokine production by venom PLA2 may not be secondary to enzymatic activity since it can be obtained with the enzymatically inactive Lys-49 family of sPLA2. Two basic Subscript>/Subscript>s purified from *Bothrops asper* venom, namely, MT-II (catalytically inactive) and MT-III (catalytically active), increased the IL-1 and IL-6 levels in peritoneal fluid (Zuliani et al. 2005). Interestingly, BaltTX-I (a catalytically inactive Lys-49 PLA2 isolated from *Bothrops alternatus* venom) stimulated complement receptor-mediated phagocytosis at noncytotoxic concentrations in macrophages, whereas BaltTX-II (catalytically active Asp-49 PLA2 isolated from the same venom) failed to produce this response (Setúbal et al. 2013). Recently, a Lys-49 PLA2 homologue devoid of catalytic activity from *Bothrops asper* venom (homologue MT-II) was shown to

directly activate murine macrophages to form lipid droplets by a mechanism related to perturbation of the membrane phospholipid bilayer independently on its enzymatic activity (Giannotti et al. 2013).

Role of Cationic Content in the Inflammatory Actions of Venom sPLA2s

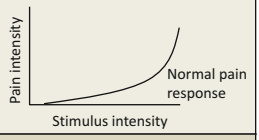
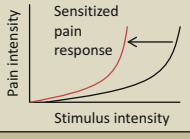
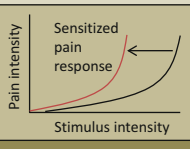
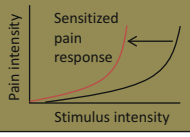
The positive charge content of the various sPLA2s has been postulated to play an important role in triggering its physiopathological activities (Kini and Evans 1989; Melo and Ownby 1999). The human group IIA protein is highly basic, and the positive charge is globally distributed over the protein surface. This feature results in the ability of the protein to form supramolecular aggregates in the presence of anionic phospholipid vesicles (Bezzine et al. 2002). The presence of numerous arginine and lysine residues on the surface of proteins is consistent with the ability of these sPLA2s to bind to the polyanions heparin and heparan sulfate proteoglycans (Birts et al. 2010). This is in agreement with findings showing that basic sPLA2s are usually more potent than the acidic ones and that heparin prevents sPLA2-mediated responses in different biological models (Wang and Teng 1990). Additionally, heparin and/or dermatan sulfate efficiently prevented edema formation in both rat and rabbit and inhibited bothropstoxin-I-, bothropstoxin-II-, and piratoxin-I-induced mast cell degranulation, all suggesting that the cationic charge of sPLA2 plays a major role in such inflammatory responses (Landucci et al. 1998, 2000a, b; De Castro et al. 2000). Additionally, the Lys-49 (bothropstoxin-I and piratoxin-I) and Asp-49 homologues (bothropstoxin-II) as well as the sPLA2 from *N. m. mocambique* and *A. mellifera* venoms caused LTB4- and PAF-neutrophil chemotaxis via signaling pathways involving activation of protein kinase C (PKC) and endogenous iPLA2/cPLA2. These chemotactic responses were inhibited by heparin, reinforcing a major role for cell surface heparin/heparan binding sites (Gambero et al. 2002, 2004). Heparin and other proteoglycans are able to inhibit toxic effects triggered by sPLA2s such as bothropstoxin-I, ACL myotoxin (*Agkistrodon contortrix laticinctus* myotoxin), Basp-II (*Bothrops asper* myotoxin II), BnSP-6 and BnSP-7 (*Bothrops neuwiedi pauloensis* isoforms), *Crotalus durissus collilineatus* venom, and *Trimeresurus mucrosquamatus* venom (Wang and Teng 1990; Melo and Ownby 1999; Soares et al. 2000; Chacur et al. 2003; Toyama et al. 2005; Stábeli et al. 2006). It is likely that heparin binds at the hydrophobic interfacial surface of sPLA2 (Murakami et al. 2007) attenuating their toxic actions. Recently, a basic PLA2 isolated from *Echis carinatus* snake venom (EcTx-I) was shown to display a heparin-binding C-terminal region responsible for its cytotoxic and bactericidal effects (Perumal et al. 2010). In addition, the myotoxic effect of a Lys-49 from *Bothrops neuwiedi* venom reflects the presence of hydrophobic amino acid residues near the C-terminus and the number of basic amino acids present in this region, which allows an electrostatic binding interaction and penetration in the lipid bilayer (Delatorre et al. 2011). The main structural determinants of toxic effects of Lys-49 PLA2 have been experimentally mapped to

a region near their C-terminus, which combines cationic and hydrophobic/aromatic amino acid residues and present heparin-binding properties (Gutiérrez and Lomonte 2013).

Pain-Related Process and sPLA2

Pain can be defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage (International Association for the Study of Pain, IASP, 2011). There are multiple sources (e.g., thermal, chemical, and mechanical stimuli) of pain and at least three basic kinds of pain (see Farquhar-Smith 2007; Table 2). Upon stimuli, painful transmission occurs through nociceptors distributed throughout the body, in particular in the skin, providing the nociception (Abrahamsen et al. 2008). Based on the degree of axon myelination and size of the fibers, the conduction velocities range from 1 m/s to 25 m/s. The myelinated Aδ and unmyelinated C-type fibers transmit the first and second pain sensation, respectively, and most nociceptors are C fibers (See reviews: Farquhar-Smith 2007; Petho and Reeh 2012). Transduction is the first nociceptor event responsible for detecting and converting this aversive stimulus into electrical activity via cell membrane proteins represented mainly by ion channels, G protein-coupled receptors (GPCR), and cytokine receptors. Signals and conduction pathways for pain modulation from nociceptors may together

Table 2 A simplified diagram showing the different types of pain and its cause

TYPES	CAUSES	OUTCOME
I) Nociceptive pain	triggered by the activation of the peripheral nerve sensory fibres (nociceptors).	usually timelimited and susceptible to non opioid analgesics 
II) Inflammatory pain	associated with inflammatory process in response to noxious stimuli	usually timelimited usually respond to non opioid analgesics /stops when inflammation is solved 
III) Neuropathic pain	triggered by primary lesion, disease or dysfunction of the somatosensory system	usually timeunlimited and resistent or poorly sensistive to non opioid analgesics 
IV) Mixed	triggered by a combination of primary lesion and secondary effects	

undergo temporary changes, and thus the pain intensity (Table 2). Sensitization can be defined as the increase in magnitude of sensory nerve fibers' response to stimuli, leading to a reduction in the associated threshold activation of sensory fibers (Table 2), which may lead to a phenomenon known as allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (represents increased pain on suprathreshold stimulation) (Gold and Gebhart 2010).

Inflammatory process is usually associated with nociceptive process (e.g., pain or hyperalgesia or allodynia) (Table 2). Thus, one might expect that inflammation seen in accidents by both the viperine and crotaline snake species would trigger related pain or hypernociception in both human and experimental models (Teixeira et al. 2009). The use of animal models has provided much evidence to support this hypothesis in addition to the fact that antivenom therapy does not always fully reverse the local manifestations of envenomation by these species. Besides a precise role for the stored and newly generated inflammatory mediators in mediating the inflammation caused by the viperine and crotaline venom (Farsky et al. 2005; Teixeira et al. 2009), the role of venom components in nociceptive mechanisms is more limited. Nonetheless, there has been some evidence to implicate toxins and PLA2s in the pathogenesis of pain and hyperalgesia regardless of their catalytic activities (Picolo et al. 2002; Chacur et al. 2003, 2004a, b). Toxins isolated from animal venoms such as spider, bee, or wasp may activate or inhibit ion channels (e.g., transient receptor potential cation channel subfamily V member 1 [TRPV1]) expressed in primary sensory neurons, to mediate pain or the nociceptive behavior in their predators (Cromer and McIntyre 2008). They also can mimic algesic effects of endogenous peptide (e.g., kinins) and non-peptide (e.g., glutamate) substances via interaction with their receptors or indirectly via activation of sensory nerve terminals (Costa et al. 2002; Adams 2004; Yshii et al. 2009). Interestingly, venom peptide toxins can be potent PLA2 activator in addition to their potential to inhibit PKC and several other protein kinases (Cromer and McIntyre 2008). Furthermore, venom-induced pain seems to be highly dependent on bradykinin B2 receptors and leukotriene-mediated mechanisms (Chacur et al. 2003, 2004a, b). PLA2s comprise approximately 30% of *Bothrops asper* venom; thus, it should be expected that they are likely to play an important role in the venom-mediated inflammatory and nociceptive responses. A limited number of PLA2s molecules exerting a modulatory function in the transmission of nociceptive information and pain perception is summarized in Table 3.

Increased sPLA2 mRNA, protein expression, and enzyme activity in the spinal cord have been associated with nociceptive behavior in experimental models of inflammation and peripheral tissue injury, as the effect can be inhibited by a selective sPLA2 inhibitor (Svensson et al. 2005). Spinal cyclooxygenase (COX) products such as PGE2 may be involved in the facilitatory processes leading to inflammatory hyperalgesia. Both group IVA cPLA2 and group VI iPLA2 are the predominant PLA2 messages in the rat spinal cord, and peripheral inflammation does not change spinal expression of PLA2; however, intrathecal (i.t.) administration of both group IVA and group VI iPLA2 inhibitors, but not group VI iPLA2 inhibitors, prevented carrageenan-induced thermal hyperalgesia as well as formalin-induced flinching in the rat hind paw (Lucas et al. 2005).

Table 3 Endogenous mediators/mechanisms relevant to pain evoked by PLA2 obtained from snake venoms

PLA2s	Obtained from	Response	Pain mediators/ mechanisms	References
Lys-49 PLA2	*Bothrops asper*	Mechanical hyperalgesia in rodent paw	Influenced by histamine H1 and 5-HT receptors or COX blockade	Chacur et al. 2003
Asp-49 PLA2	*Bothrops asper*	Mechanical hyperalgesia in rodent paw Mechanical allodynia	Influenced by histamine H1 and 5-HT receptors Influenced by generation of nitric oxide, IL-1 and IL-6	Chacur et al. 2003 Chacur et al. 2004a, b
sPLA2	*Crotalus durissus terrificus*	Abdominal hyperalgesia	Regulated by NK1 receptors iNOS	Camargo et al. 2011 Camargo et al. 2013

In addition, VI iPLA2 inhibition decreased the release of NMDA-induced prostaglandin E2 (PGE2) into the spinal cord, thus reinforcing a role for constitutive group IVA cPLA2 in spinal nociceptive processing mechanisms (Lucas et al. 2005). Inhibition of spinal cPLA2 expression by an antisense oligonucleotide attenuated tissue injury-induced hyperalgesia (Kim et al. 2008), thus reinforcing the suggestion that group IVA cPLA2 specifically play a role in spinal nociceptive processing mechanisms.

Secretory Phospholipases A_2 and Acute Pancreatitis

The Concept of Acute Pancreatitis and the Role of Endogenous sPLA2

Acute pancreatitis causes morbidity and mortality and has an increasing incidence worldwide. This disease is mainly characterized by increased serum pancreatic enzyme levels, pancreatic inflammation and necrosis, as well as abdominal pain and secondary damage to other organs, such as the lung and kidney (Cruciani and Jain 2008; Harper and Cheslyn-Curtis 2011). Despite a large number of experimental studies have been undertaken to investigate the pathophysiology and improvement of pharmacological treatment of acute pancreatitis, the clinical management of pancreatitis is still a challenge to physicians and the treatment is rather supportive.

In spite of the complex pathophysiology of acute pancreatitis, a well-established early event in this disease is the activation of zymogen granules in the pancreatic acinar cells. Although trypsin is one of the most important pancreatic enzymes to start damaging the pancreatic tissue and its levels are linked to the severity of pancreatitis, an important role for sPLA2 has been suggested. Actually, some studies have shown that this enzyme may be used as an additional diagnostic tool for acute pancreatitis (Lempinen et al. 2005).

Schmidt and Creutzfeldt (1969) proposed that PLA2 could play a role in acute pancreatitis. Latter studies reinforced this hypothesis and indicated that the serum levels of PLA2 are correlated with the severity of this disease (Schröder et al. 1980; Büchler et al. 1989). Among all the PLA2 isoforms known at that time, it was shown that levels of both group I (pancreatic) and group II (non-pancreatic) isoforms (both secretory enzymes) were augmented in serum of patients with acute pancreatitis. However, the serum concentration of sPLA2 belonging to group II was more related to the systemic complications of severe acute pancreatitis than any other isoform (Hietaranta et al. 1999).

The Use of sPLA2 Inhibitors in Experimental Acute Pancreatitis

In accordance with the importance of sPLA2 in acute pancreatitis, over the last 20 years, many sPLA2 inhibitors were developed and tested in experimental models as potential therapeutic agents for the treatment of this disease. One of these compounds, named LY311727, which is a potent inhibitor of sPLA2 ($IC_{50} = 23$ nM for human synovial fluid isoform), dose-dependently inhibited the elevated levels of human sPLA2 in transgenic mice that expressed high levels of this enzyme (Schevitz et al. 1995). It was also demonstrated that another inhibitor of sPLA2, BN162056, reduced the serum catalytic activity of type II sPLA2 and concomitantly diminished the pancreatic injury in a rat model of taurocholate-induced severe pancreatitis (Uhl et al. 1998). Likewise, an indole derivative with inhibitory activity on the group II sPLA2, S5920/LY3159820Na, increased the survival rate of rats with severe acute pancreatitis induced by the infusion of trypsin plus sodium taurocholate into the common bile duct. In addition, animals treated with this drug presented lower serum amylase, lipase, and sPLA2 levels than untreated animals (Yoshikawa et al. 1999). Another study tested the effects of S5920/LY3159820Na and showed that the pretreatment with this drug reduces the sPLA2 activity in plasma and ascites, as well as serum pancreatic enzymes levels and hemorrhage in the pancreas of rats injected with a mixture of sodium taurocholate and porcine pancreatic sPLA2 (Tomita et al. 2004). These studies indicated that sPLA2 inhibitors possess a potential to treat pancreatitis; however, the great variety of sPLA2 isoforms and their widespread distribution and role in the physiopathology of living cells complicate the therapeutic use of these substances due to potential side effects.

Pancreatitis Induced by sPLA2 Obtained from Snake Venoms

Considering that sPLA2s are involved in the pathophysiology of acute pancreatitis, Camargo et al (2005) proposed that the injection of a sPLA2 isolated from snake venoms into the common bile duct of rats could induce acute pancreatitis in rodents. Piratoxin-I increased pancreatic plasma protein extravasation and myeloperoxidase activity in the pancreas and lung, along with the concentrations of serum amylase. These alterations were more evident at 4 or 8 h, indicating that this sPLA2

induced acute alterations in the pancreas and lung of rats, which mimic those observed in patients with acute pancreatitis. A comparison between sPLA2 with different catalytic activity (piratoxin-I, bothropstoxin-II, and the sPLA2 from *Naja mocambique mocambique* venom) demonstrated that the pancreatic inflammatory responses (plasma protein extravasation and increased myeloperoxidase activity) are not related to the catalytic activity. In contrast, both neutrophil infiltration into the lung tissue and the serum amylase levels are higher after injection of bothropstoxin-II or sPLA2 from *Naja mocambique mocambique* venom in comparison with piratoxin-I. This raised the suggestion that remote pulmonary inflammation depends, at least partly, on the sPLA2 catalytic activity.

Besides these effects, the induction of acute pancreatitis by sPLA2 from *Naja mocambique mocambique* venom also caused impairment of the endothelium-dependent relaxation response in isolated mesenteric artery rings of rats (Camargo et al. 2008a), which is associated with high plasma levels of NO metabolites produced by the acute inflammatory response found in animals with sPLA2-induced acute pancreatitis. Moreover, it is also possible that the higher concentrations of TNF-α found in serum of animals submitted to sPLA2-induced acute pancreatitis contribute to the impaired relaxation responses (Camargo et al. 2008b). Of interest, a previous study showed that the rat mesenteric artery relaxation responses to acetylcholine and bradykinin are reduced in vitro by the addition of TNF-α (Wimalasundera et al. 2003).

Many mediators are involved in the mechanisms underlying the pathophysiology of acute pancreatitis. The importance of cytokines, neuropeptides, bradykinin, NO, reactive oxygen species, and lipid mediators has been recognized in the last few years. In order to establish some of the mechanisms underlying the acute pancreatitis induced by sPLA2, Camargo et al. (2008b) investigated the contribution of bradykinin and substance P to the pancreatic inflammatory effects provoked by sPLA2 from *Naja mocambique mocambique* venom in rats. Specifically, they determined the involvement of activation of B_2 and tachykinin neurokinin-1 (NK-1) receptors, respectively. These authors prophylactically treated rats with the NK-1 antagonist SR140333 and the bradykinin B2 receptor antagonist icatibant (also known as HOE-140) and showed that the blockade of both B_2 and NK-1 receptors inhibited the pancreatic inflammation and attenuated the serum concentration of TNF-α. However, only B_2 receptor blockade reduced the lung neutrophil influx and serum amylase concentration seen in sPLA2-induced acute pancreatitis. These findings indicated that while bradykinin mediates both pancreatic and lung inflammation, the action of substance P is limited to the pancreas inducing plasma protein extravasation and neutrophil infiltration.

Acute pancreatitis induced by sPLA2 from *Crotalus durissus terrificus* venom in rats is similar to that observed by using the sPLA2 from *Naja mocambique mocambique* venom (Camargo et al. 2011). Pancreatitis induced by *Crotalus durissus terrificus* venom is also accompanied by abdominal hyperalgesia (Camargo et al. 2011), a characteristic that resembles the pain observed in patients with acute pancreatitis and is an important clinical symptom of this disease. The nociceptive response in rats was described as an early response that is maximal at 4 h after

induction of sPLA2-induced pancreatitis. Hyperalgesia was reduced at 8 and 16 h and returned to basal levels after 24 h. Additionally, antagonism of NK-1 receptor by SR140333 reduced the abdominal hyperalgesia, although the pancreatic inflammatory effect of venom sPLA2 was not affected by the treatment with the NK-1 antagonist.

Interestingly, sPLA2s from *Naja mocambique mocambique* or *Naja naja* venoms are not able to depolarize the nociceptive fibers in the rat-isolated vagus nerve preparation in a grease-gap system (Camargo et al. 2011). The rat vagus nerve contains approximately 75% of capsaicin-sensitive C fibers and is a useful preparation for testing the ability of substances to cause depolarization. It was suggested that the inability of sPLA2 to directly depolarize sensory fibers in vitro indicates that the sensitization must involve cellular or humoral components, such as bradykinin or substance P, which were previously shown to mediate pancreatic inflammation during sPLA2-induced acute pancreatitis (Camargo et al. 2008b).

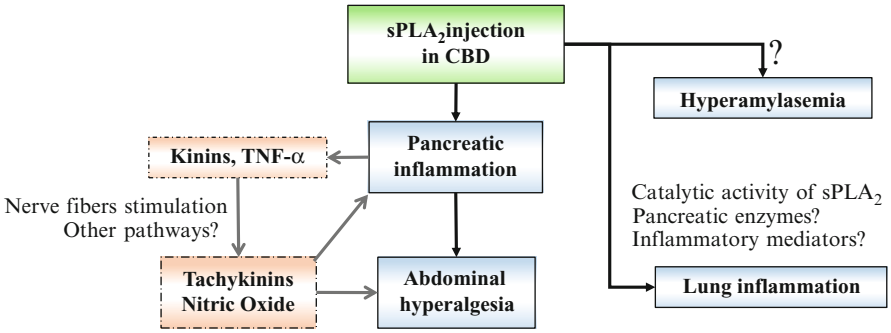
A recent study investigated the role of NO on the rat acute pancreatitis induced by sPLA2 from *Crotalus durissus terrificus* venom, by using prophylactic or therapeutic treatment with the following NO synthase (NOS) inhibitors: N^{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME, a nonselective inhibitor of NOS), aminoguanidine (a preferential inhibitor of inducible NOS), and 7-nitroindazole (7-NI, a selective inhibitor of neuronal NOS) (Camargo et al. 2014). The therapeutic blockade of iNOS reduced both hyperamylasemia and leukocyte influx to the pancreas, indicating that iNOS is the main NOS involved in the inflammatory effects during sPLA2-induced acute pancreatitis. More importantly, either prophylactic or therapeutic treatment with L-NAME, aminoguanidine, or 7-NI reduced the abdominal hyperalgesia observed in this model. Considering that bradykinin is involved in the rat pancreatic inflammation and hyperamylasemia induced by sPLA2 (Camargo et al. 2008b), it is then reasonable to suggest that common bile duct injection of sPLA2 induces the formation of kinins, well known to stimulate C fibers and neuropeptide release (i.e., substance P), in addition to NO from peripheral nociceptive pathways. Table 4 summarizes the proposed mechanisms described for acute pancreatitis induced by sPLA2.

Altogether, the studies involving the role of sPLA2 in acute pancreatitis indicate not only that the endogenous enzymes are involved in the pathophysiology of this disease, but also sPLA2 isolated from snake venoms can be used to mimic the main clinical findings of acute pancreatitis in humans, which is of great importance to understanding the mechanism underlying the development of pancreatitis.

Conclusion and Future Directions

The present chapter has summarized the current knowledge on the inflammatory and nociceptive actions of sPLA2s from snake venoms in different experimental models. Snake venoms constitute rich sources of sPLA2s, and those enzymes purified from Viperidae (mainly from *Bothrops* genus) have been widely used in experimental research. These include the Asp-49 sPLA2 (that contain little catalytic activity) and the Lys-49 sPLA2 (that are devoid of catalytic activity) isolated from

Table 4 Mechanism(s) of acute pancreatitis and the role of sPLA2



B. jararacussu, *B. asper*, and *B. neuwiedi* venoms, among several other venoms. Generally, injections of these Asp-49 and Lys-49 sPLA2 evoke local inflammatory reactions characterized by increased microvascular permeability and edema formation, leukocyte recruitment, and pain. It has been a matter of debate in literature to what extent the catalytic activity contributes to the venom sPLA2-induced inflammation. The positive charge content of various venom sPLA2 is globally distributed over the protein surface and that polyanions like heparin attenuate sPLA2-mediated inflammatory responses in different biological models. Therefore, it is also believed that cationic charge of sPLA2 via electrostatic interactions with anionic sites in cell membranes plays a major role in physiopathology of venom sPLA2s. Over 40 years it has been suggested that PLA2 play a role in inflammatory diseases such as acute pancreatitis and remote organ failure (mainly pulmonary insufficiency and renal failure) that correlates with increased catalytic phospholipasic activity in serum. Therefore, this chapter also attempted to detail the use of snake venom sPLA2 to reproduce acute pancreatitis and remote lung inflammation associated with abdominal hyperalgesia. Injection of venom sPLA2s into the common bile duct of rats induces acute pancreatitis characterized by edema and neutrophil infiltration in the pancreas and lungs, as well as increased serum amylase levels and abdominal allodynia. The local pancreatic inflammatory alterations (plasma protein extravasation and increased myeloperoxidase activity) in response to venom sPLA2 are not related to the catalytic activity of tested sPLA2, while remote inflammation (lung neutrophil infiltration) and higher serum amylase levels may depend, at least partly, on its catalytic activity. Bradykinin, tachykinins, and nitric oxide (NO) have been postulated to play important roles in sPLA2-induced pancreatitis. Additionally, iNOS-derived NO and neuronal NO as well as neuronal substance P and its NK_1 receptor may be highly relevant to the pathophysiology of abdominal pain in acute pancreatitis associated with sPLA2. Therefore, discovery of new sPLA2 inhibitors appears to be an innovative therapy for treatment of pancreatitis associated with abdominal pain and other chronic inflammatory conditions.

Cross-References

► Bee Venom and Pain
► Synthetic Peptides and Drug Discovery

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Natural Inhibitors of Snake Venom Metallopeptidases

4

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Abstract

Protein inhibitors of peptidases are widely distributed among animals, plants, and microorganisms. They are important regulatory molecules that function to avoid proteolysis in various biological systems. From the plasma of animals that are naturally resistant to snake envenomation, a special class of peptidase inhibitors has been identified. These inhibitors have antihemorrhagic properties and form inactive noncovalent complexes with snake venom metallopeptidases. In nonresistant animals, these toxic metallopeptidases induce microvascular damage and are responsible for systemic and local hemorrhage, key events in the pathogenesis of viperid envenomation. The inhibitors isolated from mammalian plasma are grouped into the MEROPS I43 family of immunoglobulin-related proteins; those from reptiles show a typical cystatin-like fold and belong to the MEROPS I25C subfamily. The inhibitors show a reversible tight-binding reaction mechanism of inhibition, although due to a lack of three-dimensional information for the enzyme-inhibitor complexes, the structural features that govern the interaction are largely unknown. This review is intended to highlight the latest advances in the field, analyzing future perspectives in the area of natural immunity against

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_19

snake venom. The discussion will focus on how endogenous protein inhibitors can be used as structural templates, providing valuable insights into the molecular determinants of selective metallopeptidase inhibition. The wide range of natural toxin inhibitors may constitute a rich source of information leading to new possibilities in intervention not only against snake venom metallopeptidases but also against other metzincins, such as mammalian MMPs and ADAMs.

Keywords

Antihemorrhagic activity • BJ46a • Habu serum factor (HSF) • Cystatin • DM43 • Immunoglobulin • Jararhagin • Mammalian matrix metallopeptidases • MEROPS • Natural immunity • Peptidase inhibitors • Phospholipase A2 (PLA2) • Snake venom metallopeptidases (SVMPs) • Venom-resistant animals

Contents

Introduction

Since 1936, when Kunitz's work pioneered the field of protein inhibitors of peptidases, several naturally occurring inhibitors have been described in viruses, Archaea, *Bacteria*, and Eukarya. According to the MEROPS database statistics (http://merops.sanger.ac.uk/inhibitors), as of August 2013 (release 9.9), there were 542 protein species (containing $\geq$ 14 amino acid residues) of "experimentally characterized and sequenced" inhibitors of peptidases distributed among 76 families and 39 clans (Rawlings et al. 2014). In this hierarchical scheme, homologues (sequence-similar inhibitors) are grouped in the same family, and families are organized in different clans whenever they share a common evolutionary origin, mainly indicated by the conservation of tertiary structure. Additionally, 49 protein species were classified as compound inhibitors, i.e., peptidase inhibitors presenting multiple reactive sites.

Although multiheaded inhibitors may be generated by the (non)covalent association of several peptide chains, most often they derive from gene elongation through duplication/multiplication (Laskowski and Kato 1980). In a few cases, they show inhibitory activity against peptidases from different mechanistic classes (Bode and Huber 2000). Therefore, MEROPS classification system calculates structural similarities based on the sequence of inhibitory domains or "inhibitory units" instead of considering the entire protein sequence (Rawlings et al. 2004).

It now seems clear that protein peptidase inhibitors "have arisen on many different occasions during the evolution of living organisms" (Rawlings et al. 2004). Broadly speaking, their key function is the regulation of potentially hazardous proteolysis, although the physiological target enzyme is often ignored (Laskowski and Kato 1980). With the exception of the serpins (family I4), macroglobulins (I39), and the baculovirus p35 caspase inhibitor (I50), which follow an irreversible "trapping" mechanism of action (suicide inhibitors), most peptidase inhibitors interact with their target enzymes through a reversible tight-binding reaction (Rawlings et al. 2004). Well-known examples include mammalian blood plasma proteins involved in the regulation of the coagulation cascade (Laskowski and Kato 1980), plant tissue proteins implicated in defense roles against insects and pests (Gomes et al. 2011), and anticoagulant proteins found in the saliva of hematophagous animals (Huntington 2014). Within the last few years, there has been much interest in exploiting these endogenous molecules in biotechnology and agriculture, as well as using them as novel therapeutic agents in medicine. This review chapter will discuss another fascinating group of natural peptidase inhibitors, represented by the antiophidic proteins isolated from the blood plasma of venom-resistant reptiles and mammals.

Natural Resistance to Snake Envenomation

The existence of venom-resistant animals was first reported by the Italian scientist Felice Fontana in 1781 [apud (Phisalix 1922)]. Current scientific evidence convincingly shows that several reptiles and mammals have evolved efficient molecular strategies to resist snake envenomation (for a historical overview, see Domont et al. 1991; Thwin and Gopalakrishnakone 1998). Well-characterized examples include snake species from the Viperidae, Elapidae, and Colubridae families as well as several mammals from the families Didelphidae (opossums), Erinaceidae (hedgehog), and Herpestidae (mongooses) (Faure 2000; Lizano et al. 2003; Perales et al. 2005). From an evolutionary perspective, natural selection from snake predation seems to represent an important driving force underlying venom proteome variability. As discussed previously (Casewell et al. 2013), "the evolving venom resistance in prey and the evolution of novel venom composition exert reciprocal selective pressure on each other."

As shown in Table 1, at least two molecular mechanisms may explain the biological phenomenon of natural resistance to snake venom. First, protective

Table 1 Natural resistance to snake envenomation

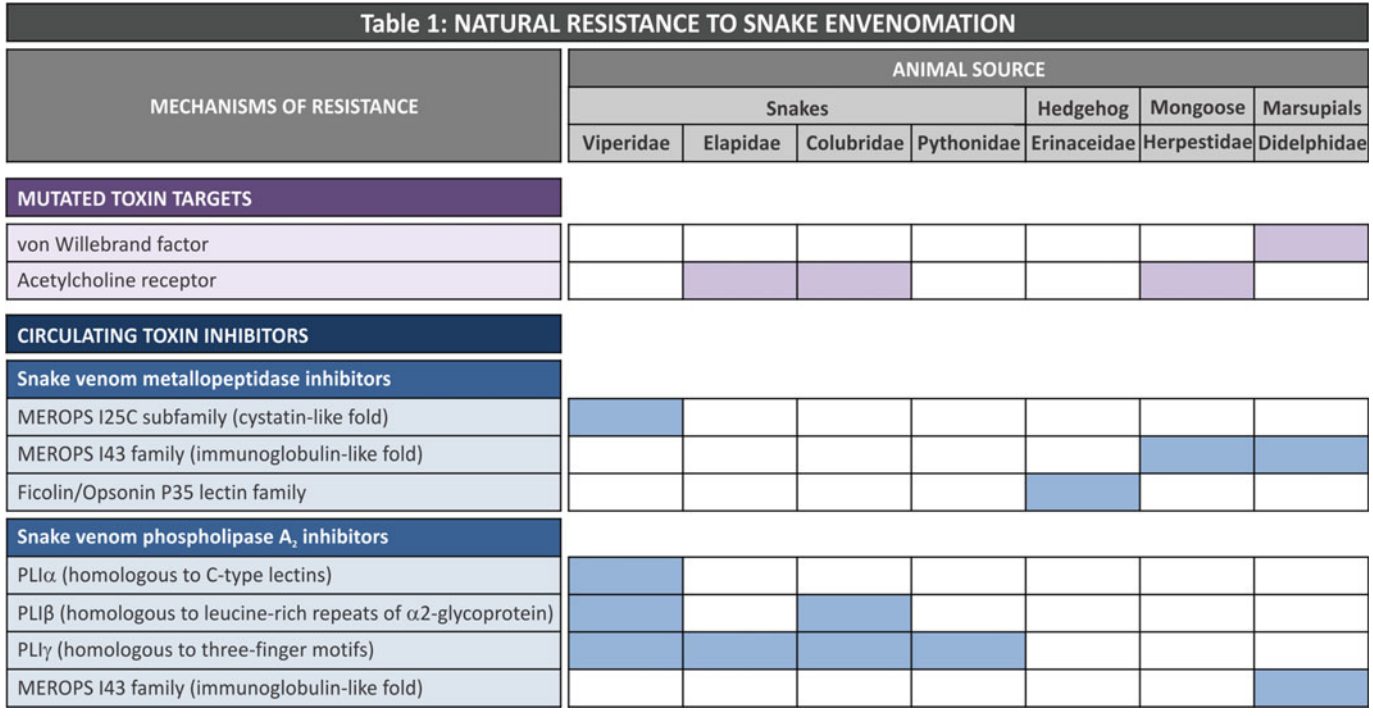

Table 1: NATURAL RESISTANCE TO SNAKE ENVENOMATION								
MECHANISMS OF RESISTANCE	ANIMAL SOURCE							
	Snakes				Hedgehog	Mongoose	Marsupials	
	Viperidae	Elapidae	Colubridae	Pythonidae	Erinaceidae	Herpestidae	Didelphidae	
MUTATED TOXIN TARGETS								
von Willebrand factor								
Acetylcholine receptor								
CIRCULATING TOXIN INHIBITORS								
Snake venom metallopeptidase inhibitors								
MEROPS I25C subfamily (cystatin-like fold)								
MEROPS I43 family (immunoglobulin-like fold)								
Ficolin/Opsonin P35 lectin family								
Snake venom phospholipase A_2 inhibitors								
PLIα (homologous to C-type lectins)								
PLIβ (homologous to leucine-rich repeats of α2-glycoprotein)								
PLIγ (homologous to three-finger motifs)								
MEROPS I43 family (immunoglobulin-like fold)								

amino acid substitutions in venom-targeted substrates/ligands would render them insensitive to the deleterious effect of venom toxins. Classical examples are the nicotinic acetylcholine receptors of elapid and *Natrix tessellata* snakes (Neumann et al. 1989) and of the mongoose *Herpestes ichneumon* (Barchan et al. 1992), whose mutations impair the binding of α-bungarotoxin without interfering with the physiological ligand. Recently, another interesting example of such adaptive evolution was suggested, based on the comparative analysis of partial vWF (von Willebrand Factor) gene sequences in several didelphids (Jansa and Voss 2011). The authors observed that some vWF sequence substitutions were associated with venom-resistant ophiophagous marsupials. They hypothesized that the corresponding amino acid replacements (leading to changes in protein net charge and hydrophobicity) would give rise to a less suitable binding target for botrocetin, a C-type lectin-like venom protein that promotes dysfunctional platelet aggregation. Therefore, given the accelerated evolution observed in the blood protein of venom-resistant animals that feed on snakes, the existence of a biochemical "arms race" scenario where snakes would play a role not only as predators but also as preys has been suggested.

Venom resistance can also be explained by preexisting circulating proteins (different from antibodies) with antiophidic properties that effectively bind, neutralize, and trigger the removal of the toxic component from the host. Two main classes of inhibitors have been isolated from the blood plasma/serum of venom-resistant animals: (a) SVMPI (snake venom metallopeptidase inhibitors), responsible for antihemorrhagic activity, and (b) PLI (snake venom phospholipase A_2 inhibitor), which may present antimyotoxic and/or antineurotoxic properties (Neves-Ferreira et al. 2010).

Endogenous protein inhibitors isolated from venom-resistant animals are congenitally inherited factors (Perales et al. 1986). As discussed later in this chapter, they show sequence homology to proteins commonly associated with roles in the immune system, such as the killer cell immunoglobulin-like receptor (Neves-Ferreira et al. 2002), the immunomodulatory cystatins (Ochieng and Chaudhuri 2010), and the lectins of the ficolin/opsonin family (Omori-Satoh et al. 2000). It has been suggested that, as a ready-made line of defense against harmful toxins, these circulating proteins form part of these animals' innate immune system (Perales and Domont 2002). The following selected examples seem to support this hypothesis.

Virginia opossums (*Didelphis virginiana*) injected several times with 40 mg of crude *Crotalus atrox* venom (~15 mg/kg body weight) did not mount any detectable serum antibody response against the venom, as determined by ring precipitation, double gel diffusion (Ouchterlony assay), and ELISA (enzyme-linked immunosorbent assay). On the other hand, control marsupials responded to the same amount of bovine serum albumin as did control rabbits, which mounted a strong response against far lower doses of venom (McKeller and Perez 2002). Interestingly though, there was an effective increase in the opossum's antihemorrhagic response, which was triggered by the venom. Although the clearance mechanism of the toxin-

antitoxin complexes remains largely unknown, this important self-protective process seems to be fast enough to impair the adaptive immune response.

The second example is illustrated by the venomous snake *Gloydius brevicaudus* (renamed from *Agkistrodon blomhoffii siniticus*), which has three phospholipase inhibitors in its serum: PLI-α (homologous to C-type lectins), PLI-β (to leucine-rich repeats of α_2-glycoprotein), and PLI-γ (to three-finger motifs). Within a few hours after intramuscularly injected with a single dose of the autologous crude venom, there was an upregulation (quantified by real-time qRT-PCR, quantitative reverse transcription polymerase chain reaction) of the expression of all PLI genes in the snake liver tissue (Kinkawa et al. 2010). The results were confirmed by the concurrent immunological detection of increased amounts of the inhibitors in the blood plasma of the snake. When its own acidic PLA_2 (phospholipase A_2) was used as the injection challenge, only PLI-α and PLI-β were upregulated (although different expression time courses were observed), irrespective of its catalytic activity. Interestingly, the same authors previously showed that PLI-β is an ortholog of mammalian LRG (leucine-rich α_2-glycoprotein), a component of the acute-phase response and an important player in the innate immune system (Shirai et al. 2009).

Continuing the analogy with innate immunity, the endogenous antivenom proteins could be compared to soluble PRM (pattern recognition molecules), albeit far more specific than the canonical components of this system's humoral arm (e.g., collectins, ficolins, and pentraxins) (Bottazzi et al. 2010). Several proteomic studies have shown that snake venoms are complex mixtures of proteoforms belonging to a small ($4 < n < 20$) number of protein families [reviewed by (Calvete 2013; Valente et al. 2014)]. In other words, snake venoms constitute an ensemble of rather homologous toxins, some of which may be recognized by a limited number of plasma inhibitors. According to this view, the innate surveillance system of venom-resistant animals may fit the criteria of (narrow) pattern recognition capacity. Therefore, given that PRM are considered functional ancestors of antibodies (Bottazzi et al. 2010), in terms of specificity, endogenous antitoxins would be situated somewhere between these two extremes. Similarly to immunoglobulins, metallopeptidase inhibitors from venom-resistant opossums have been found in female milk and serum (Jurgilas et al. 1999).

From the examples above, it seems clear that the antitoxic proteins may readily increase their constitutive expression level, effectively neutralizing and triggering the immediate elimination of different toxin threats. This specialized effector response is able to restore the animal's homeostasis, regardless of any antibody response. Unfortunately, the signaling mechanisms mediating this positive "acute-phase-like" protein response are unknown.

The aforementioned amino acid substitutions in venom-targeted substrates/ligands and the antimyotoxic/antineurotoxic PLI are beyond the scope of this chapter and will not be discussed. Instead, this review will focus on the "transferrable" resistance to snake venom hemorrhagic toxins. This is not intended to be an exhaustive inventory of all snake venom metallopeptidase inhibitors known, as several authors have already reviewed this topic (Neves-Ferreira et al. 2010; Voss and Jansa 2012). Rather, an effort has been made to further discuss recent advances

in the molecular understanding of three thoroughly studied examples, taken as paradigms of this class of inhibitors: DM43 isolated from *Didelphis aurita* opossum, BJ46a isolated from *Bothrops jararaca* snake, and HSF isolated from *Protobothrops flavoviridis* snake (renamed from *Trimeresurus flavoviridis*). A concise discussion on the research perspectives in this field will also follow.

Snake Venom Metallopeptidases

SVMPs (snake venom metallopeptidases) are abundant components of most viperid and colubrid venoms. They are key players in the pathogenesis of snake envenomation and are directly involved in disruption of capillary vessel structure and hemostasis impairment (Gutiérrez et al. 2010). These toxins have been associated with local and systemic hemorrhage, fibrinolysis and fibrinogenolysis, activation of prothrombin and factor X, pro-apoptotic and pro-inflammatory activities, and/or inhibition of platelet aggregation (Escalante et al. 2011; Markland and Swenson 2013). From a structural point of view, SVMPs belong to the MEROPS peptidase family M12, subfamily M12B, together with mammalian ADAM (a disintegrin and metallopeptidase) and ADAMTS (ADAM with a thrombospondin type 1 motif) proteins. Phylogenetic analyses indicate that SVMPs evolved from ancestral forms of ADAM proteins (most likely ADAM 7, ADAM 28, and ADAM DEC1) recruited into the venom before the radiation of caenophidian snakes (Casewell 2012). These zinc-dependent multidomain proteins are also known as reprolysins. Proteolytically processed SVMPs may comprise the catalytic domain alone (P-I class); alternatively, this domain can be C-terminally bound to a canonical disintegrin domain (P-II class) or to a disintegrin-like/cysteine-rich domain. Subclass P-IIId is also characterized by two C-type lectin-like domains linked by disulfide bonds to the rest of the molecule. SVMPs belonging to the P-II and P-III classes are further divided into subclasses, depending on whether they undergo proteolytic processing and/or dimerization. Both phenomena seem to be modulated by the presence of a variable number of cysteinyl residues forming different disulfide bond arrangements among different SVMPs (Fox and Serrano 2005; Fox and Serrano 2008).

Regulation of Snake Venom Metallopeptidase Activity

SVMPs are synthesized as pre-proenzymes in the cytoplasm of venom gland secretory epithelial cells. The signal sequence of the nascent polypeptides directs them to the endoplasmic reticulum, where they undergo folding, disulfide bond formation, glycosylation, and/or multimerization. After moving to the Golgi apparatus, proenzymes are packed into secretory vesicles before being released into the venom gland lumen (Fox and Serrano 2008).

In latent SVMP, a conserved cysteinyl residue located in a consensus sequence of the prodomain binds to the zinc ion of the catalytic domain, thus avoiding the occupation of the fourth coordination site by a water molecule; three other zinc

coordination sites are occupied by histidines in the consensus sequence **H**EXX**H**XXGXX**H**, which is followed by a Met turn. Peptide sequences structurally similar to the prodomain consensus site have already been shown to inhibit SVMP, albeit with very low efficiency (Fox and Bjarnason 1998). Differing from MMPs (matrix metallopeptidases) (Tallant et al. 2010), SVMPs are not susceptible to activation by organomercurials. Their "cysteine-switch"-like mechanism of activation is triggered by the proteolytic removal of the prodomain by other metallopeptidases in the crude venom (Shimokawa et al. 1996).

Using antibodies produced against the recombinant prodomain of jararhagin (P-IIIb class from *Bothrops jararaca* venom) (Paine et al. 1992), it was recently shown that SVMPs are released from the venom secretory cells primarily as zymogens (Portes-Junior et al. 2014). Unlike what was previously thought (Fox and Serrano 2008), it seems that most SVMP processing occurs within the lumen of venom glands, immediately following enzyme secretion (i.e., after the trans-Golgi network). The processed prodomain undergoes further proteolytic degradation and is rarely detected in the venom sample (Valente et al. 2009a). Recently, uncommon large numbers of prodomain-derived peptides have been identified in *Bothrops jararaca* main venom gland extracts (Luna et al. 2013). This may further indicate that SVMPs remain within the secretory cells mainly as proenzymes.

As far as is known, the integrity of the venom gland environment (including epithelial cells, the extracellular matrix, and other venom proteins) is maintained by a rather low pH in the lumen of both the venom glands and the secretory vesicles. Apparently, this acidification is ensured by mitochondrion-rich proton-secreting cells in the epithelium of the venom gland (Weinstein et al. 2010). Additionally, the presence of millimolar concentrations of short pyro-glutamyl peptides in the venom may also contribute to the temporary modulation of the activity of SVMPs. Pyro-glutamyl tripeptides have been shown to inhibit SVMPs with Ki values in the low micromolar range. Following appropriate dilution, their inhibitory effect would be readily reversed (Fox and Bjarnason 1998). Citrate has also been identified as a very abundant component of several venoms (Odell et al. 1998). However, the role of citrate in maintaining the latency of SVMP is less clear; it has been demonstrated that the removal of zinc ions from atrolysin E, in the absence of immediate replacement with cobalt(II), induces the irreversible denaturation of SVMP (Bjarnason and Fox 1983). Therefore, a possible biological function of citrate as a chelating agent of divalent metal ions regulating SVMP activity seems unlikely (Fox and Bjarnason 1998).

A few proteomic studies have detected small amounts of SVMPI and PLI in snake venom/venom gland extracts (Luna et al. 2013; Terra et al. 2009). Assuming this is not due to venom sample contamination with blood proteins during sampling procedures, a possible role of toxin inhibitors as additional modulators of venom activation/integrity could be envisaged. However, unless lower-affinity inhibitor proteoforms are present in the venom, a regulatory mechanism based on virtually irreversible binding does not seem biologically plausible. Alternatively, similar to the Kunitz-type serine peptidase inhibitors frequently found in snake venoms (Mukherjee et al. 2014), SVMPI and PLI variants found in the venom could be

more specifically tailored to interact with prey targets, resulting in a variety of biological functions outside of the inhibition of venom-protein targets. Finally, it has been suggested that endogenous inhibitors could be constitutively synthesized by several reptile tissues (including venom glands), without necessarily implying any biological significance (Luna et al. 2013).

Upon injection into prey tissues, SVMPs are expected to be fully active. Typically, SVMPs belonging to the P-III class are the most potent hemorrhagic toxins, inducing both local and systemic bleeding (Bjarnason and Fox 1994; Gutiérrez et al. 2005). This enhanced in vivo activity appears to be modulated by the presence of the cysteine-rich domain, which effectively targets these toxins to relevant substrates/sites (e.g., the vWF A domain of extracellular proteins) where the proteolytic activity would be more effective (Fox and Serrano 2010). Given the conformational characteristics of the integrin-binding loop of P-III SVMPs, which is inaccessible to protein-protein interactions (Takeda et al. 2006), the disintegrin-like domain is likely related to a structural role rather than a protein binding role (Gutiérrez et al. 2010).

Another important issue affecting hemorrhagic potency of SVMPs is their partial to complete resistance to human α2-macroglobulin (Baramova et al. 1990; Gutiérrez et al. 2005), a 720 kDa tetrameric plasma glycoprotein inhibitor with broad specificity against endopeptidases (Sottrup-Jensen et al. 1984). Although most P-I SVMPs are usually inhibited by these high molecular mass molecules, the same is not true for P-III members. This is most likely a consequence of the steric hindrance caused by the presence of additional disintegrin-like/cysteine-rich domains in this latter toxin group. Apparently, P-III SVMPs are too large to access the inhibitor's bait region, which cleavage triggers a conformational change that leads to peptidase entrapment within the macroglobulin molecule.

A Brief Overview of Protein Inhibitors of Snake Venom Metallopeptidases

Most SVMPIs isolated from venom-resistant animals are acidic plasma glycoproteins in the 40–95 kDa mass range that show marked pH and thermal stability. Following protein-protein interaction, no proteolysis of either the inhibitor or the peptidase is observed. Instead, thermodynamically stable tight complexes are formed through noncovalent interactions, thus inactivating the toxin (Neves-Ferreira et al. 2010; Perales et al. 2005).

Protein inhibitors of snake venom metallopeptidases isolated from mammalian plasma belong to family I43, the only member of clan JM. Although it is considered one of the largest families, with more than 5,000 homologous sequences reported, only three biochemically characterized homologues have been assigned MEROPS identifiers: oprin (I43.001), alpha1B-glycoprotein (I43.950), and immunoglobulin-alpha Fc receptor (I43.951). All members of family I43 show a characteristic Ig (immunoglobulin)-related fold, although only those assigned to MEROPS identifier I43.001 have been characterized as functional inhibitors (Rawlings 2010).

The holotype (well-studied inhibitor or type example) of family I43 is DM43 (UniProtKB P82957), a SVMPI isolated from the plasma of the South American opossum *Didelphis aurita* (renamed from *Didelphis marsupialis*) (Neves-Ferreira et al. 2000). In the MEROPS database, it is referred to as "oprin" (opossum proteinase inhibitor), a name originally coined to describe the ortholog isolated from the serum of the North American opossum *Didelphis virginiana* (Catanese and Kress 1992). Oprin was the first SVMPI bearing an Ig-like fold to be characterized (albeit not completely sequenced). Other partially sequenced homologues have been described in the plasma of the marsupials *Didelphis aurita* (DM40) (Neves-Ferreira et al. 2000), *Didelphis albiventris* (DA2-II) (Farah et al. 1996), and *Philander frenatus* (PO41, originally published as *Philander opossum*) (Jurgilas et al. 2003) and of the mongoose *Herpestes edwardsii* (AHF1, AHF2, and AHF3) (Qi et al. 1994).

SVMPIs with atypical high molecular masses have been described in the European hedgehog (*Erinaceus europaeus*). From skeletal muscle extracts, a 1,000 kDa antihemorrhagic protein that does not inhibit serine peptidases has been purified. The multimeric protein erinacin was shown to be composed of α (37 kDa) and β (35 kDa) subunits forming an $\alpha_{10}.2\beta_{10}$ structure. Although not fully sequenced, it was classified as a member of the ficolin/opsonin P35 lectin family (Omori-Satoh et al. 2000). A 700 kDa β-macroglobulin, made of 34 kDa and 39 kDa subunits, was also isolated from its plasma, although no structural information is available (de Wit and Westrom 1987). In addition to the antihemorrhagic activity, the inhibitor was also effective against trypsin, chymotrypsin, plasmin, and papain.

On the other hand, protein inhibitors of snake venom metallopeptidases isolated from reptile plasma belong to clan IH, inhibitor family I25. Interestingly, this family includes inhibitors of peptidases of different catalytic types: subfamilies A and B contain typical cysteine peptidase inhibitors (type-1 and type-2 cystatins, respectively). Subfamily C (cystatin-like proteins) includes snake venom metallopeptidase inhibitors, although most members lack peptidase inhibitory properties (Rawlings et al. 2004). The holotype of subfamily I25C is BJ46a (UniProtKB Q9DGI0), a SVMPI isolated from *Bothrops jararaca* plasma (Valente et al. 2001). Both BJ46a and the ortholog HSF (Habu serum factor) isolated from *Protobothrops flavoviridis* (Yamakawa and Omori-Satoh 1992) have been assigned the MEROPS identifier I25.026.

SVMPI from Mammals: DM43 as the Holotype Inhibitor of Family I43

Biochemical Characterization

Homogeneous DM43 was purified from the serum of the opossum *Didelphis aurita* following two sequential chromatographic steps: anion exchange on a DEAE-Sephacel column and hydrophobic interaction on a Phenyl-Sepharose CL-4B column. Protein analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) under (non)reducing conditions showed a single polypeptide chain of ~48 kDa. This value was later shown to be slightly overestimated, as a

consequence of the glycoprotein nature of the inhibitor. Under native conditions, molecular mass estimates by SEC (size exclusion chromatography) and DLLS (dynamic laser light scattering) of 73 kDa and 78 kDa, respectively, indicate a homodimeric structure (Neves-Ferreira et al. 2000; Neves-Ferreira et al. 2002).

Edman degradation sequencing was used to determine the complete primary structure of DM43 (Neves-Ferreira et al. 2002). The inhibitor is homologous to oprin (86% identity over 237 residues) (Catanese and Kress 1992) and to human alpha1B-G (alpha1B-glycoprotein, 37% identity over 271 residues) (Ishioka et al. 1986), members of the immunoglobulin superfamily. Human alpha1B-G is a plasma glycoprotein of 63 kDa, consisting of five Ig-like domains. It binds human CRISP-3 (cysteine-rich secretory protein 3) with high affinity, forming a 1:1 stoichiometric complex (Udby et al. 2004). It has been suggested that alpha1B-G would protect humans against the potential deleterious consequences of free circulating CRISP-3. Although little is known about the biological function of this 28 kDa protein, mammalian CRISPs show a wide distribution: in addition to their occurrence in plasma, they have been described in the reproductive tract, in neutrophils, and in exocrine glands (salivary glands, pancreas, and prostate) (Udby et al. 2010).

DM43 monomers are composed of 291 amino acid residues, including four experimentally confirmed glycosylation sites (Asn^{23}, Asn^{156}, Asn^{160}, and Asn^{175}) in the consensus sequon Asn-Xaa-Ser/Thr (Xaa $\neq$ Pro). The glycan moiety of DM43 (9,039 Da) is characterized by complex-type oligosaccharides (containing N-acetylglucosamine, mannose, galactose, and sialic acid), most likely forming biantennary N-linked chains. The glycan moiety corresponds to 21% of the average molecular mass of the monomer (42,691 Da), as determined by MALDI-TOF MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry) (León et al. 2012; Neves-Ferreira et al. 2002).

Recently, DM43 was analyzed on a high-resolution ESI-Q-TOF MS (electrospray ionization quadrupole time-of-flight mass spectrometer) (Brand et al. 2012). The deconvoluted spectrum showed four main peaks, with molecular masses ranging from 43,123 Da to 44,362 Da. The mass difference observed between the first two most abundant proteoforms of DM43 was 291 Da, which matches a sialic acid residue (N-acetyl-neuraminic acid); the same mass difference was observed between the other two most abundant DM43 peaks. The mass heterogeneity was completely abolished following the enzymatic treatment of DM43 with PNGase F, indicating the existence of different glycoforms of DM43. The glycoforms could result from microheterogeneity (different carbohydrate structure) and/or macroheterogeneity (different site occupancy). The presence of sialic acid residues may account for the very acidic pI (isoelectric point) of DM43 (<3.5), which is lower than expected from its amino acid sequence (4.8, calculated using ExPASy – Compute pI/Mw tool).

Biological Activity Characterization

When tested in vivo, DM43 partially inhibited the hemorrhage induced by *Bothrops jararaca* venom (maximum 70% inhibition). It also showed antilethal,

antihyperalgesic, and antiedematogenic activity against crude venom (Neves-Ferreira et al. 2000). However, the hemorrhagic effect was completely inhibited by crude opossum serum or by a partially purified DM43 fraction (ABC/ABF, standing for antibothropic complex/fraction) (Neves-Ferreira et al. 1997). These results indicate that DM43 (irrespective of the glycoforms referred to above) is not the only endogenous antihemorrhagin present in venom-resistant *Didelphis aurita* opossums.

Indeed, at least two homologues have already been described in this marsupial. In addition to DM40, a far less abundant inhibitor that was co-purified along with DM43, the complete cDNA coding for DM43b (UniProtKB Q8HYX5) was cloned, although the protein has never been isolated. Amino acid sequences of DM43 and DM43b share 81% identity and 88% similarity. A third homologous inhibitor of 64 kDa has also been purified from opossum serum (Rocha et al. 2002). Surprisingly, instead of inhibiting metallopeptidases, DM64 neutralizes myotoxicity induced by venom proteins bearing PLA_2 structure. Therefore, it is likely that inhibitors with complementary specificities function in a concerted manner to protect the resistant animal against a wide repertoire of toxins.

In vitro, DM43 effectively inhibited the proteolytic activity of *B. jararaca* venom on casein, fibrinogen, and fibronectin. A similar inhibition profile was attained with EDTA, a metal chelating agent. Additionally, DM43 completely inhibited the hemorrhagic activity of jararhagin, as well as its hydrolytic activity on fibrinogen or on the fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba. The inhibition was accompanied by a stable inactive complex formed through noncovalent interactions between DM43 and jararhagin (Neves-Ferreira et al. 2000; Neves-Ferreira et al. 2002). On the other hand, venom trypsin-like serine peptidase activity on specific synthetic substrates was not inhibited by the antibothropic complex, nor was trypsin, chymotrypsin, or bacterial collagenase (Neves-Ferreira et al. 1997). Based on these results, DM43 was characterized as a snake venom metallopeptidase inhibitor.

After treatment with neuraminidase and β-galactosidase, DM43 was still able to form a complex with jararhagin (León et al. 2012). Both native and exoglycosidase-treated DM43 appeared to be equally efficient in terms of inhibiting the proteolytic activity of jararhagin on casein or fibrinogen. Therefore, the presence of sialic acid and galactose does not seem to affect the inhibitory properties of DM43 against SVMPs, although the possible impact on the protein half-life and solubility remains unknown. On the other hand, the inhibitory activity of DM43 was reduced by 50% following partial deglycosylation by PNGase F under nondenaturing conditions. Although N-linked carbohydrates could not be completely removed, this result suggests that the glycan portion of DM43 somehow influences the inhibitory activity of DM43. It should be determined whether glycans directly interact with the toxin and/or contribute to the overall structural stability of the inhibitor.

The specificity of DM43 against different venom targets has been further explored. The inhibitor was immobilized in a HiTrap NHS-activated column, through which several crotalid venoms were chromatographed (Rocha et al. 2009). Bound and unbound fractions were analyzed by 2D-PAGE (two-dimensional polyacrylamide gel electrophoresis) and MALDI-TOF/TOF MS. The results showed that DM43 can bind a wide spectrum of metallopeptidases from the venoms of *Bothrops*

atrox, *Bothrops jararaca*, *Bothrops insularis*, and *Crotalus atrox* snakes. Numerous proteoforms of P-III and P-I SVMPs have been "fished" by the inhibitor, calling attention to the hypervariability of the SVMP subproteome. It is well known that such molecular diversity may give rise to different functional outcomes, ultimately improving venom toxicity (Fry et al. 2009).

It has been previously shown that DM43 does not interact with jararhagin-C, a processed form of jararhagin consisting of DC domains (disintegrin-like and cysteine-rich domains) only (Neves-Ferreira et al. 2002). This result indicates that the metallopeptidase domain is necessarily involved in the binding of jararhagin to DM43. Therefore, several DC fragments observed in the DM43-bound fraction were most likely artifactually generated following toxin binding to the inhibitor through its catalytic domain. Venom chromatographic fractions that did not interact with DM43 were markedly enriched in serine peptidases, C-type lectins, C-type lectin-like proteins, and L-amino acid oxidase.

SVMP spots observed by 2D-PAGE in the unbound fractions had their tryptic peptides analyzed by MS/MS: those showing molecular masses of ~30 kDa most likely constitute preprocessed DC domains that do not interact with DM43, as mentioned above; other unbound SVMP spots showed higher molecular masses (>67 kDa) and may represent highly glycosylated SVMPs. Accordingly, DM43 does not inhibit atrolysin A (Neves-Ferreira et al. 2002) or HF3 (Asega et al. 2014), two highly glycosylated P-IIIa SVMPs (~70 kDa) isolated from *Crotalus atrox* and *Bothrops jararaca* venoms, respectively. A hypothesis is that this may be due to the steric hindrance caused by toxin glycan moiety, thus precluding the interaction with the inhibitor. Five putative glycosylation sites have been described in HF3, three of which are located within the metallopeptidase domain, whereas jararhagin has only one. Under native conditions, partial deglycosylation of HF3 could be achieved, but is not sufficient to render the SVMP susceptible to inhibition by DM43 (Asega et al. 2014). Similar to DM43, its homologue oprin also lacked inhibitory activity against atrolysin A; the presence of a second inhibitor fully active against this SVMP has been reported in the plasma of a North American opossum (Catanese and Kress 1992).

From the results discussed above, it can be concluded that DM43 is a broad-spectrum (although not universal) SVMP inhibitor. This observation is corroborated by several additional experiments in which DM43 was tested against isolated SVMPs. Bothropasin (Assakura et al. 2003) is a P-IIIb class SVMP sharing 95.5% identity with jararhagin. As expected, its proteolytic and hemorrhagic activities were effectively neutralized by DM43 (Asega et al. 2014). Moreover, DM43 inhibited the proteolytic activity of the following P-I class SVMPs: bothrolysin (UniProtKB P20416) (Neves-Ferreira et al. 2002), BJ-PI (*Bothrops jararaca*) (Asega et al. 2014), BaP1 (P83512, *Bothrops asper*), leucurolysin-a (P84907, *Bothrops leucurus*), and atroxlysin (*Bothrops atrox*, Sánchez et al. 2010) (manuscript in preparation). Hemorrhagic activity of BaP1 was neutralized by DM43 as well. Using surface plasmon resonance, the interaction between DM43 immobilized on the sensor chip with bothropasin and BJ-PI has been unequivocally shown (Asega et al. 2014). Previously, only a poor inhibition of atrolysin C from *Crotalus atrox*

venom has been observed when an excess molar concentration of DM43 was used (Neves-Ferreira et al. 2002).

DM43 has also been used as a bait protein in proteomic experiments aimed to exploit the metallopeptidase content of the rear-fanged snake *Thamnodynastes strigatus* (Ching et al. 2011). In addition to binding canonical SVMPs, DM43 effectively interacted with svMMPs (snake venom matrix metallopeptidases). Differing from SVMPs, svMMPs seem to have evolved from a MMP-9 ancestor, although some features (e.g., hemopexin and fibronectin domains) have been lost. Enzymatically active svMMPs have been characterized as the main venom component of this colubrid species. However, their biological contribution to venom toxicity remains unknown.

It has previously been shown that oprin is not effective against mammalian MMP-1–3 (Catanese and Kress 1992). Contrary to this result, immobilized DM43 has been successfully used for capturing peptidases from MMP-enriched samples, such as osteoarthritis synovial liquid and supernatants of breast (MCF-7 cell line) and prostate (MDA cell line) adenocarcinomas (Jurgilas et al. 2005). DM43-bound fractions showed proteolytic activity on gelatin zymograms. When the same fractions were analyzed by Western blot, a few bands were recognized by monoclonal antibodies raised against MMP-2, MMP-3, and/or MMP-9. However, before drawing any definitive conclusions about the specificity of DM43 against mammalian MMPs, it will be important to identify the bound fraction components by MS/MS and also to test the inhibitor against isolated MMPs.

Structural Characterization

Unfortunately, attempts to crystallize DM43 for X-ray studies have been unsuccessful, most likely due to heterogeneities present in its N-glycan moiety, as previously discussed (Brand et al. 2012). Therefore, a predicted structure for DM43 was determined by homology modeling, based on the crystal structure of the extracellular region of the KIR2DL1, a natural killer cell inhibitory receptor (Neves-Ferreira et al. 2002). The parent structure has two Ig-like domains and shows a rather low (~28%) sequence identity with DM43; it was used as a template to model the second and third domains of DM43 (D1 and D2). The first domain of DM43 (D0) was built on the second domain of the killer cell receptor due to its higher sequence identity.

All members of the immunoglobulin superfamily are composed of homologous domains of approximately 100 residues, forming a sandwich of two β-sheets linked by a conserved disulfide bond. Depending on the number of antiparallel strands in each β-sheet, they may present four different structural folds: V (variable), C1 (constant-1), C2 (constant-2), and I (intermediate). Molecules bearing Ig-related domains are largely involved in immune response and cell surface recognition (Smith and Xue 1997; Williams and Barclay 1988). The modeled structure of DM43 was found to consist of three Ig-like domains showing the characteristic I-type fold. A pair of cysteines linked by a disulfide bond was assigned to each

domain. Due to the lack of a structural template bearing three Ig-like domains, relative orientation between the D0 and D1/D2 domains could not be envisaged.

By analogy with homologous receptor-ligand complexes, six loops connecting DM43 strands in domains D1/D2 have been predicted to encompass the metallopeptidase binding site (Neves-Ferreira et al. 2002). Both the hypothetical SVMP binding region on DM43 (especially the presumably most solvent-exposed segment linking D1 and D2, corresponding to residues 188–193) and the entrance to the active-site cleft of atrolysin C show an overall negative charge, which may preclude the interaction between these proteins. On the other hand, the corresponding regions of the crystal structures of bothropasin and BaP1 are rather neutral. As expected, these last two SVMPs are effectively neutralized by DM43.

Ig-related molecules are prone to dimerization (Williams and Barclay 1988), and as previously mentioned, DM43 is no exception. In fact, DM43 monomers have never been observed under native conditions. Therefore, a DM43 model allowed for the prediction of a dimerization interface characterized by a cluster of hydrophobic residues on domain D2 (Neves-Ferreira et al. 2002). The association would involve the same surface on both monomers, related by a twofold symmetry axis. Accordingly, studies on DM43 denaturation induced by chemical (guanidine hydrochloride) or physical (high pressure) agents have detected a significant increase in extrinsic (bis-ANS) fluorescence intensity during the transition from native dimer to partially folded monomer (Chapeaurouge et al. 2009). This observation is likely explained by the exposure of previously hidden hydrophobic areas on DM43 monomers.

Although a DM43 homodimer is readily observed in solution, it was not detected by mass spectrometry under nondenaturing conditions (i.e., 600 mM ammonium acetate) (Brand et al. 2012). This suggests that the interaction of DM43 monomers is mainly guided by hydrophobic interactions, which are substantially weaker/nonexistent in the gas phase. It has been previously observed that hydrophobic residues are more frequently found at intermolecular interfaces of homodimers, which are seldom observed as monomers. On the contrary, because heterodimers usually involve proteins that are expected to exist independently, the presence of exposed hydrophobic patches on the protein surface does not seem to be a thermodynamically favorable characteristic (Jones and Thornton 1996).

Mechanism of Inhibition

An effective protein inhibitor of a peptidase will ultimately preclude substrate binding to the enzyme's active site due to steric hindrance. Three general mechanisms of action have already been described: (a) blocking enzyme's active site in a substrate-like manner, (b) docking to adjacent sites (exosite-binding inhibitors), and (c) binding to distant allosteric sites (Bode and Huber 2000). With the exception of substrate-like inhibitors of serine peptidase (serpins), which follow a "standard" or "Laskowski" mechanism, most inhibitors target areas flanking the peptidase catalytic site rather than the active site itself (Rawlings 2010; Rawlings et al. 2004). Remarkably, reactive-site residues in inhibitors are much less conserved than active-

site residues in peptidases, and this hypervariability often precludes the prediction of inhibitory activity (Laskowski and Kato 1980; Rawlings et al. 2004).

Specifically for metallopeptidases, at least three different mechanisms of inhibition have been detailed in the literature: (a) interaction with the active-site cleft of the catalytic domain (e.g., TIMP-1 from inhibitor family I35), (b) displacement of activated water by providing an additional metal ligand (e.g., *Pseudomonas* inhibitor from family I38), and (c) an induced mix of disulfide bonds masking the peptidase active site (e.g., pro-eosinophil major basic protein from family I63) (Rawlings 2010). Regarding mammalian and reptile SVMPIs, the structural features that govern the interaction are largely unknown. Although structural data for almost 20 SVMPs have been deposited in the Protein Data Bank (Cerda-Costa and Gomis-Ruth 2014), no three-dimensional structures are known for SVMPIs or their noncovalent complexes.

The stoichiometry of the interaction between DM43 and jararhagin has been analyzed by size exclusion chromatography (Neves-Ferreira et al. 2000) and confirmed by mass spectrometry under nondenaturing conditions (Brand et al. 2012). Although DM43 shows a dimeric quaternary structure, the inhibitor interacts with the toxin as a monomer. At equimolar toxin-to-inhibitor ratios (considering the molecular mass of the monomer), a saturating amount of complex was formed, and no chromatographic peaks corresponding to free ligands were detected by SEC. The molecular mass of the heterocomplex DM43-jararhagin was 94 kDa by ESI-Q-TOF, corroborating a 1:1 stoichiometry. In contrast to human serpins, such as α_1-proteinase inhibitor, antithrombin III, C1-esterase inhibitor, and α_2-antiplasmin, which are readily inactivated by catalytic amounts of SVMPs (Kress 1988), DM43 was not hydrolyzed by jararhagin, bothrolysin (Neves-Ferreira et al. 2002), HF3, BJ-PI, or bothropasin (Asega et al. 2014).

The interaction between DM43 and jararhagin was analyzed by surface plasmon resonance on a Biacore 3000 instrument (Brand et al. 2012). To avoid enzyme autolysis (thereby ensuring a uniform binding surface), jararhagin was captured on a CM5 chip to which anti-jararhagin monoclonal antibodies (MAJar 2) (Tanjoni et al. 2003) had been immobilized. Therefore, a fresh jararhagin-coated surface could be prepared just before each experimental cycle. The antibody binds to the disintegrin-like domain of the SVMP and does not interfere with substrate/inhibitor accessibility to the catalytic domain. DM43 and jararhagin form a high-affinity complex, with an estimated equilibrium dissociation constant (K_D) of 0.33 ± 0.06 nM, calculated as the ratio of the dissociation rate constant ($k_d = 1.16 \pm 0.07 \times 10^{-5}$ s^{-1}) by the association ($k_a = 3.54 \pm 0.03 \times 10^4$ M^{-1} s^{-1}).

Reversible tightly binding inhibitors typically show low rate constants of complex dissociation (k_d) and low equilibrium dissociation constants of the enzyme-inhibitor complex (K_i or K_D), indicating high affinities (Bieth 1995). Therapeutically efficacious antibodies, for example, are expected to show K_D values within the range of 1 pM to 1 nM (Drake et al. 2004). Therefore, for a significant inhibition, there is no need for a large molar excess of inhibitor, a very convenient defense strategy against snake envenomation. Unfortunately, very few protein inhibitors of SVMP have been characterized from a thermodynamic point of view. Rather than estimating the kinetic parameters of the peptidase-inhibitor interaction, most studies are restricted to the description of "percent inhibition" values. This is most likely a

consequence of the high sample amounts needed for the progress curve experiments in the presence or absence of the inhibitor.

SVMPI from Reptiles: BJ46a and HSF as Holotype Inhibitors of Subfamily I25C

Biochemical Characterization

BJ46a was isolated from *Bothrops jararaca* serum following four purification steps: ammonium sulfate precipitation (40–60% saturation fraction), hydrophobic interaction on Phenyl-Sepharose, and two chromatographic runs on a C4-reversed phase column. The homogeneous single-chain inhibitor has an acidic character (pI 4.55) and a molecular mass of 46,101 Da by MALDI-TOF MS. Size exclusion chromatography, performed under nondenaturing conditions, gave a molecular mass of 79 kDa. A similar molecular mass was obtained by DLLS, indicating that the native structure of BJ46a is a dimer (Valente et al. 2001).

The cDNA corresponding to BJ46a was the first full-length cDNA to be sequenced for an endogenous SVMP inhibitor (Valente et al. 2001). It included the 5′- and 3′-noncoding regions, a typical secretory signal sequence, and a poly(A) tail. The remaining cDNA sequence encoded a protein of 322 amino acids, including 12 cysteine residues and four putative glycosylation sites (Ans^{76}, Asn^{185}, Asn^{263}, and Asn^{274}) on asparagine-linked consensus motifs. Following complete chemical deglycosylation with trifluoromethanesulfonic acid, the glycan moiety of BJ46a was estimated to account for 17% of the molecular mass of the inhibitor.

The primary structure for BJ46a predicted from cDNA sequencing was extensively confirmed (81% sequence coverage) by Edman degradation. BJ46a shares 85% sequence identity with HSF, the first inhibitor of snake venom metallopeptidase with cystatin-like structure to be isolated (Yamakawa and Omori-Satoh 1992). The Habu serum factor is a glycoprotein of 47 kDa, bearing residues of glucosamine, mannose, and galactose as major constituents. All 323 amino acid residues of HSF were sequenced by Edman degradation. In addition to three putative N-glycosylation sites at positions 123, 185, and 263, 13 cysteine residues forming six disulfide bridges were characterized. Because no free SH group was detected, the authors speculated on the possible interaction between the remaining cysteine residue and other available thiol groups, such as in glutathione. From the sera of *Gloydius blomhoffii* snakes, two additional cystatin-like SVMPIs with antihemorrhagic activity have been isolated and completely sequenced: cMSF (Chinese mamushi serum factor) and jMSF (Japanese mamushi serum factor) (Aoki et al. 2008).

Biological Activity Characterization

BJ46a inhibited the enzymatic activities of atrolysin C and jararhagin on the fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nbz. It also showed antihemorrhagic

activity against *Bothrops jararaca* venom (Valente et al. 2001). BJ46a did not interact with processed DC domains of jararhagin, indicating that the catalytic domain of the SVMP is a key target for the inhibitor; additionally, neither atrolysin C nor jararhagin were able to hydrolyze BJ46a. Although evolutionally related to cysteine peptidase inhibitors, snake venom cystatin-like proteins do not inhibit cysteine peptidases, such as papain and cathepsin B. The inhibitory specificity shift may be explained by the absence of a conserved motif (QXVXG) in cystatin-like domains, which is predicted to form part of a wedge-shaped structure that interacts with the active site of cysteine peptidases (Yamakawa and Omori-Satoh 1992). Interestingly, there are a few reports in the literature indicating that some cystatins may bind MMPs and therefore avoid their autolytic inactivation, without inhibiting the proteolytic activity of the metallopeptidases on endogenous substrates (Ochieng and Chaudhuri 2010).

The biological activity of BJ46a homologue HSF against several metallopeptidases isolated from the venoms of *Protobothrops flavoviridis* (HR1A, HR1B, HR2a, HR2b, and H2) and *Gloydius halys brevicaudus* (brevilysins H2, H3, H4, H6, L4) has been extensively studied (Deshimaru et al. 2005). Of all the mentioned SVMPs, only brevilysin H2 was not inhibited by HSF. Therefore, similar to DM43, SVMPI bearing cystatin-like structures also show a broad inhibitory specificity against different enzymes, irrespective of their biological activity (hemorrhagic or not) and domain composition (P-I or P-III).

Recently, a second biological function has been attributed to HSF in Habu snake serum (Shioi et al. 2011). It has been shown that HSF binds Habu SSPs, small serum proteins (6.5–10 kDa) whose physiological function is largely unknown. As a carrier protein, HSF binds all five SSPs with high affinity. It has been shown that SSP-1 can bind HV1, an apoptosis-inducing SVMP, although the activity of the enzyme was not affected. On the other hand, HSF does not bind/inhibit HV1. Nevertheless, the complex made of HSF and SSP-1 was able to bind HV1. Within the ternary complex made with SSP-1 and HSF, the SVMP was rendered catalytically inactive (Shioi et al. 2013).

A recombinant form of BJ46a (rBJ46a) has been produced in the methylotrophic yeast *Pichia pastoris* (Ji et al. 2013). The glycosylated rBJ46a showed a molecular mass of ~58 kDa, which was reduced to 38.7 kDa after the enzymatic treatment with endoglycosidase H. rBJ46a was reported active against "MMPs," although, based on the available information, it is not clear which peptidases were assayed. When tested in vitro using a trans-well migration assay, rBj46a reduced the invasion of B16F10 melanoma cells and MHCC97H hepatocellular carcinoma cells into Matrigel. In vivo, the recombinant protein partially inhibited lung colonization in C57BL/6 mice inoculated with B16F10 cells. It also decreased the number of spontaneous lung metastases in BALB/c nude mice injected with MHCC97H cells. The antitumoral activity of rBJ46a was attributed to the inhibition of MMP-2 and MMP-9, although no direct evidence was presented.

Structural Characterization

Both BJ46a and HSF, classified in subfamily I25C of the cystatin family, have a three-domain structure typical of fetuins: two contiguous cystatin-like domains (double headed) in the N-terminal region, followed by a His-rich non-cystatin C-terminal domain. The cystatin fold is characterized by an alpha helix lying on top of an antiparallel beta strand (Rawlings 2010). Fetuin domains are stabilized by conserved disulfide bonds between 12 cysteine residues. Based on the structure of the human fetuin α-2-HS-glycoprotein (Kellermann et al. 1989), the following distribution of disulfide bridges has been hypothesized in BJ46a and HSF: two disulfide bridges (A1 and B1 loops) in the N-terminal cystatin domain and three others (A2, B2, and the smaller C loop) distributed along the second cystatin domain. A pair of cysteines at both ends of the molecule would form an additional S-S bridge connecting the N- and C-termini of the protein (D loop).

Limited information is available in the literature regarding the structural determinants of SVMP inhibition by BJ46a or HSF. Lysine and tryptophan residues on HSF have been submitted to trinitrophenylation (TNP, 2,4,6-trinitrobenzene sulfonic acid) or oxidation (N-bromosuccinimide), respectively (Deshimaru et al. 2005). After chemical modification, the inhibitory activity against brevilysin H6 was strongly affected; on the other hand, chemical modifications on arginine, tyrosine, and histidine residues had no effect. Different SVMPs were complexed with HSF before treatment with TNP, followed by acid-induced dissociation. Complexes with HR1A or HR1B yielded full recovery of inhibitory activity, whereas HSF that had interacted with H6 was partially active. This result indicated that Lys residues on/near the reactive site of HSF are mostly buried upon binding with the SVMPs. Because modified residues are resistant to Lys-C hydrolysis, the unmodified residues (Lys^{15}, Lys^{41}, Lys^{103}) were easily identified following HSF digestion with Lys-C peptidase and Edman sequencing (Aoki et al. 2007).

To further evaluate the structure-function relationship of HSF, the inhibitor was digested with papain. The generated fragment B (~14 kDa) was cleaved with cyanogen bromide, producing the highly antihemorrhagic non-glycosylated fragment C (10.2 kDa), consisting of two polypeptide chains: residues 5–89 in the first cystatin-like domain were disulfide-linked to residues 312–317 in the C-terminal His-rich domain. Based on the crystal structure of human cystatin C, a molecular model for the two cysteine-like domains of HSF was proposed. In addition to confirming the typical cystatin fold, the model indicates that Trp^{17}, Trp^{48}, Lys^{15}, and Lys^{41} form an exposed cluster on domain 1, all facing the same molecular plane as Lys^{103}. Therefore, this specific region of the first cystatin-like domain of HSF appears to be more associated with the inhibitory activity of HSF (Aoki et al. 2007). The molecular model of BJ46a shows that, in addition to the above-referred residues, the unique Trp^{52} and Lys^{58} could form a second cluster on the first domain of the *Bothrops jararaca* inhibitor (Valente et al. 2009b). It remains to be verified whether these residues are responsible for binding a second SVMP molecule, as predicted

from the stoichiometry data, or whether the second cystatin-like domain of BJ46a is also involved in the interaction.

Interestingly, three HSF-like proteins (HLP) showing neither antihemorrhagic nor antiproteolytic activities against SVMPs have been reported in the sera of two snakes (HLP-A and HLP-B, from *Gloydius blomhoffii brevicaudus*, and HLP, from *Protobothrops flavoviridis*) (Aoki et al. 2009). Despite their homology, only HLP-B was characterized as a true snake fetuin, i.e., a serum protein capable of binding minerals, thus preventing systemic calcification. The amino acid sequences of the antihemorrhagic HSF (and its active ortholog BJ46a, cMSF, and jMSF) and the inactive HSF-like proteins (HLP, HLP-A, and HPL-B) have been compared. The following results further suggest that the amino-terminal cystatin-like domain accounts for the anti-SVMP activity: (a) both active and inactive proteins may present a deletion of 17 residues in the C-terminal domain, and (b) the second cystatin-like domain is highly conserved among all proteins (84–94% identity), whereas a lower identity (~60%) was observed in the first domain, particularly involving residues 1–16, 42–58, and 96–105.

Mechanism of Inhibition

The stoichiometry of the interaction between BJ46a and atrolysin C or jararhagin was determined by a titration assay. Toxin-antitoxin were mixed at different molar ratios and submitted to analysis by SEC and SDS-PAGE under nonreducing conditions (Valente et al. 2001). At a 2:1 SVMP/BJ46a molar ratio (considering the molecular mass of monomeric BJ46a), a saturable amount of complex was formed, and no remaining free ligands were observed. Therefore, the BJ46a monomer binds noncovalently to two molecules of peptidase. A different stoichiometric ratio has been determined for HSF, which appears to bind only one SVMP molecule as a monomer (Deshimaru et al. 2005; Yamakawa and Omori-Satoh 1992). This controversy is worthy of further investigation, preferentially using new technologies, such as mass spectrometry.

To measure the affinity between BJ46a and jararhagin, real-time interaction experiments were performed on a Biacore instrument, as previously described for DM43. The preliminary binding data (sensograms) were best fit to a simple bimolecular interaction model. The measured kinetic parameters were $k_a = 5.76 \pm 0.12 \times 10^3\ M^{-1}\ s^{-1}$ and $k_d = 7.68 \pm 0.11 \times 10^{-5}\ s^{-1}$. The K_D, determined from the k_a and k_d values, was 13.8 nM, indicating a high-affinity interaction (unpublished results).

Conclusions and Future Directions

Natural resistance to snake venom is a complex biological phenomenon that deserves integrated research strategies. As previously discussed (Voss and Jansa 2012), combined efforts by biochemists and evolutionary biologists will be instrumental in achieving a better understanding of this important trophic adaptation,

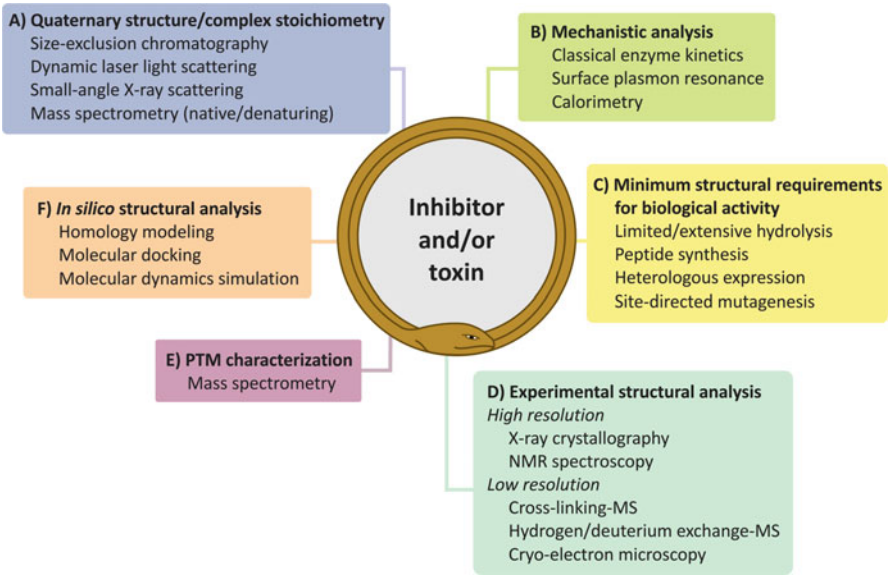

Fig. 1 Methodological perspectives in the molecular study of protein inhibitors of snake venom toxins. Several complementary approaches may be applied to better address the structure-function relationship of antiophidic proteins and their target toxins, adding information on (**a**) the subunit composition of the inhibitor and the stoichiometry of the toxin-antitoxin complex, (**b**) the type of inhibition and the binding affinity of the interaction, (**c**) the minimum required primary structure of the inhibitor needed for both interaction and biological activity, (**d**) the interacting regions/residues/atoms on the toxin-antitoxin complex, (**e**) the presence of posttranslational modifications in the inhibitor and their biological significance, and (**f**) the use of experimental and theoretical data for structural predictions/validations. *MS* mass spectrometry, *NMR* nuclear magnetic resonance, *PTM* posttranslational modification

including its possible impact on the evolution of snake venom toxins. Specifically regarding the resistance to SVMPs, two aspects should be further explored: from a physiological point of view, emphasis should be placed on understanding the mechanisms underlying this intriguing innate response, including the characterization of stimulus-response specificity/kinetics and the identification of cellular targets and regulatory circuits; from a biomedical perspective, structural biology methods (e.g., MS combined with cross-linking and hydrogen/deuterium exchange, X-ray crystallography, and NMR spectroscopy) (Alber et al. 2008) should be a strong focus in the field moving forward. This will enable the high-resolution characterization of interaction sites of enzymes and inhibitors, providing valuable insights into the three-dimensional determinants of selective inhibition. A summary of the methodological perspectives in the molecular study of protein inhibitors of snake venom toxins is depicted in Fig. 1.

Many important pathophysiological effects of SVMPs, such as hemorrhage, coagulopathy, and pro-inflammatory activity, are directly dependent on their catalytic activity. Poor muscle regeneration related to microvasculature impairment is

also closely associated with SVMP toxicity (Gutiérrez et al. 2010). Additionally, local tissue damage induced by metallopeptidases (and myotoxic PLA_2s) is only partially neutralized by venom immunotherapy. Therefore, developing effective inhibitors of this class of toxins may be an interesting research strategy leading to improved snakebite treatment and a successful regenerative process. Future trends in antivenom therapy already point to the coadministration of SVMPI inhibitors (in situ) and hyperimmune serum (intravenously) as an emerging alternative (Gutiérrez et al. 2007).

Most peptidase inhibitors used clinically are low molecular mass synthetic peptidomimetic compounds, which traditionally show lower susceptibility to proteolysis and improved bioavailability. ACE (angiotensin-converting enzyme) inhibitors for treatment of hypertension and HIV (human immunodeficiency virus) aspartyl protease inhibitors to prevent development of AIDS (acquired immunodeficiency syndrome) are good examples of similar successful therapeutic agents (Scott and Taggart 2010). In the case of mammalian matrix metallopeptidases, small-molecule active-site-directed inhibitors were not selective enough in clinical trials, showing important unwanted off-target effects (Brew and Nagase 2010). Synthetic peptide inhibitors of MMPs (batimastat, marimastat, CGS-270 23A, AG-3340, and Bay-12 9566) have been tested against SVMPs, showing a few encouraging results (Escalante et al. 2000; Howes et al. 2007). Novel SVMP inhibitors are also being pursued by a combination of chemical library screening and rational peptide design (Villalta-Romero et al. 2012).

MMPs are the main molecules responsible for controlling extracellular matrix catabolism. They are involved in several human pathologies, including cancer, cardiovascular disease, and arthritis; therefore they should be strictly regulated (Tallant et al. 2010). The search for potent synthetic inhibitors with target selectivity is hampered by similar catalytic site architectures of MMPs, ADAMs, and ADAMTSs (Gomis-Rüth 2009). Alternatively, a strategy for developing new metallopeptidase inhibitors aims to systematically uncover and exploit the molecular diversity of TIMPs (tissue inhibitors of metalloproteinases) (Brew and Nagase 2010). These broad-spectrum inhibitors of MMPs are currently being engineered to generate variants with altered inhibitory specificity. To be viable as therapeutic agents, protein inhibitor molecules should be reduced to the smallest portion that still retains the inhibitory activity and specificity; ideally, the lower molecular mass peptide should be easily produced by chemical synthesis or heterologous expression systems.

Similarly, natural inhibitors from venom-resistant animals could be used as structural templates to further the search for more efficient antivenom therapies. As previously discussed, SVMPIs with complementary specificities are frequently observed in venom-resistant animals. Therefore, the wide repertoire of natural toxin inhibitors may constitute a rich source of information that could reveal new possibilities of intervention not only against SVMPs but also against ADAMs and MMPs. These metallopeptidases are classified as metzincins (Gomis-Rüth 2009) and show a high degree of tertiary structure conservation and overlapping substrate specificity. Therefore, it is expected that SVMPIs will ultimately lead to scaffolds that target

peptidases (and other toxins) with more specificity than that which can be achieved with small-molecule inhibitors (Scott and Taggart 2010). Additionally, natural inhibitors can contribute to a better understanding of the structure-function relationship of reprolysins, especially regarding the characteristics of their extended substrate binding sites (Fox and Bjarnason 1998).

Acknowledgment We are grateful to Heloisa M. N. Diniz (Image Production and Treatment Service – IOC/Fiocruz) for producing the artwork for the table and figure.

Cross-References

► Myotoxin Inhibitors
► Random Peptide Library for Ligand and Drug Discovery
► Synthetic Peptides and Drug Discovery

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Production and Utilization of Snake Antivenoms in South America

5

Jorge Kalil and Hui Wen Fan

Abstract

Venomous snakebites represent an important public health issue in many parts of the world, including South America, causing high morbidity and mortality. In this continent, antivenoms are currently manufactured in Argentina, Bolivia, Brazil, Colombia, Peru, and Venezuela, and by more than one laboratory in some of these countries. Eight of them are public laboratories (three in Brazil) that produce specific mono- and polyvalent antivenoms, derived from the antibodies produced mostly by horses immunized with either one or a pool of venoms from snakes, spiders, scorpions, and caterpillars of medical importance. $F(ab')_2$ fragments or whole IgG are purified from plasmas of immunized horses. Studies on the cross-neutralization between antivenoms and venoms from different species countries, as well as clinical trials confirming the efficacy and safety of antivenoms may improve the antivenom availability at a regional level.

Keywords

Antivenom Production • Snake • South America

Contents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_26

Introduction

Snake antivenom has been first produced in South America in 1901, when the Serum Therapy Institute of São Paulo State, Brazil (the current Instituto Butantan) was founded. Dr. Vital Brazil Mineiro da Campanha was designated as the Institute's first director, with the immediate responsibility of producing a serum to be used against the epidemics of bubonic plague afflicting the country at that time. A few months after the delivery of the first vials of anti-bubonic-plague serum, two types of snake antivenom also entered production. Vital Brazil studied snake venoms and human envenomation. He observed differences in clinical symptomatology provoked by the Brazilian viper *Bothrops jararaca* and the South American rattlesnake *Crotalus durissus terrificus*. Immunizing horses with *C.d. terrificus* venom and with a mixture of *Bothrops* species venoms, Vital Brazil obtained specific antivenoms. He demonstrated that the antivenom produced with the venom from one of the species could not neutralize the effects of the other species' venom (Brazil 1903). The principle of specificity in antivenom therapy was then established. Soon after distribution throughout the country, the efficacy of antivenom was proved as snakebite case-fatalities rates started falling.

The success of Instituto Butantan stimulated other Brazilian state governments to create other institutes to produce immunobiologicals, such as Fundação Ezequiel Dias, in 1907, and Instituto Vital Brazil, in 1919. In parallel, in Argentina, Bernard Houssay started to investigate snake venoms and to produce sera against venomous snakes at the Institute of Bacteriology of the National Department of Hygiene, currently National Administration of Laboratories and Institutes of Health Dr. Carlos G. Malbrán (Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos G. Malbrán" – ANLIS) (Argentina. Ministerio de Salud 2011). Later on, the National Institute of Health (Instituto Nacional de Salud) in Colombia (Toro et al. 1998) and Peru, and the National Institute of Health Laboratories (Instituto Nacional de Laboratorios de Salud) Bolivia, also started antivenom production. Uruguay and Ecuador no longer produce antivenom. From the 45 public and private laboratories that manufacture antivenoms in the world (Gutierrez 2012), eight are located in South America. Most laboratories belong to public institutions, though private manufacturers also produce antivenoms. Technological development and compliance with good manufacturing practices are highly variable, contributing to the heterogeneity of laboratories, some with production deficiencies, and unable to supply their country's demand for antivenom (WHO 2007). Instituto Butantan became the main immunobiologicals manufacturer in the continent, producing about 500,000 vials of antivenoms, antitoxins, antirabies immunoglobulins, which amounts to 50% of the nationally produced immunoglobulins that are distributed without cost to the entire population of the country by the Brazilian Health Ministry (De Franco and Kalil 2014). Recently the plasma purification plant was renewed and Butantan obtained the Good Manufacture Practice (GMP) certificate from the Brazilian national regulatory agency.

Antivenom Production

Antivenom production and control involves five basic stages: (1) selection of medically important snake venoms, (2) immunization of animals with relevant snake venoms, (3) bleeding and fractionation of animal blood to obtain the active substance, (4) formulation and dispensing in the final containers, and (5) quality control of the final product. The manufacturing process developed and adopted at Instituto Butantan will be described here as an example of antivenom production.

1. Selection of medically important snake venoms
   Most South American countries have a surveillance system with compulsory report of snakebites. Data from this system provide information on the venomous snakes responsible for envenomation and subsides to establish a list of medically important species. A list of the species of venomous snakes was summarized by WHO (2010) in categories for South America:
   - Venomous snakes of highest medical importance: Viperidae species: *Bothrops alternatus*, *B. asper*, *B. atrox*, *B. bilineatus*, *B. brazili*, *B. diporus*, *B. jararaca*, *B. jararacussu*, *B. leucurus*, *B. mattogrossensis*, *B. moojeni*, *B. pictus*, *B. venezuelensis*; *Crotalus durissus*; *Lachesis muta*;
   - Minor venomous snake species: some species of *Micrurus* coral snake

   For each country, Table 1 shows the medically important viperid snakes. From the 12 countries in South America, only Chile has no medically important venomous snakes.

   The geographical distribution of venomous snakes may lead to variation in venom composition (Nunez et al. 2009; Calvete et al. 2011; Furtado et al. 2010). Specificity and efficacy of antivenom may differ greatly from place to place. Other factors can influence venom composition, such as age (Saldarriaga et al. 2003; Zelanis et al. 2012, 2010), season of the year, and gender (Menezes et al. 2006), apart from the genetic diversity. Therefore, venoms to be used in the immunization process should be ideally pooled from snakes of different ages, genders, and geographical origins.

   Usually manufacturers maintain a snake farm and handle different species for antivenom production. At Instituto Butantan, animals collected or brought by the population are selected to be quarantined for two months and examined by a veterinarian for parasites and infections. The snakes are then transferred to production rooms, where they are kept in individual cages. Rooms are left undisturbed, at constant temperature and humidity. Animals are fed with living mice or other prey, according to the species, once a month, usually soon after being milked. Under these conditions, reproduction in captivity has been successful for *Bothrops* and *Crotalus* species and adult snakes have been living in captivity for over 10 years.

   Snakes are regularly milked according to a defined schedule, usually once a month, depending on the species. Animals are removed from their cage and

Table 1 Medically important viperid snakes in South America, according to categories of threat to public health (Modified from WHO 2010)

Snake	Species	Argentina	Bolivia	Brasil	Colombia	Ecuador	French Guyana	Guyana	Paraguay	Peru	Suriname	Uruguay	Venezuela
Bothrops	*alternatus*	1		2					1			1	
	asper				1	1				2			2
	atrox		1	1	1	1	1	1		1	1		1
	bilineatus		2	2	1	1	1	1		1	1		2
	brazili		2	2	2	2	1	1		2	1		2
	diporus	1		2					2				
	jararaca	2		1					2				
	jararacussu	2	2	1					2				
	leucurus			1									
	mattogrosensis	2	1	2					2	2			
	moojeni		2	1					2				
	neuweidi	2		2					2				
	pictus									1			
	pubescens	2		2								2	
	santaecrucis		2										
	taeniatus		2	2	2	2	2	2		2	2		
	venezuelensis												1

Bothriechis	*schlegelii*				2	2				2			2
Bothrocophias	*hyoprora*				2	2				2			
	microphtalmus				2	2				2			
Porthidium	*nasatum*				2								
	lansbergii				2								2
Crotalus	*durissus*	1	1	1	1		1	1	1	1	1	1	1
Lachesis	*muta*		2	1		1	2	2		1	2		2
Micrurus	*circinalis*												2
	corallinus	2		2					2			2	
	lemniscatus	2		2	2	2	2	2	2	2	2		2
	mipartitus				2	2				2			2
	nigrocintus				2								2
	spixii			2	2	2			2	2			2
	surinamensis			2	2	2	2	2		2	2		

Category 1: Highest medical importance – highly venomous snakes that are common or widespread and cause numerous snakebites, resulting in high levels of morbidity, disability, or mortality

Category 2: Secondary medical importance – highly venomous snakes capable of causing morbidity, disability, or death, for which exact epidemiological or clinical data may be lacking, and/or which are less frequently implicated (due to their activity cycles, behavior, habitat preferences, or occurrence in areas remote to large human populations)

anesthetized with carbon dioxide for approximately 10–15 min, to reduce the risk of accident to the snake-handler. Milking is performed by grasping the head of snake just behind the angle of the jaw, which opens under gentle pressure. The fangs are exposed and pushed through a flexible paraffin film stretched over the lip of a glass vessel, partially immersed in ice, and venom is squeezed out. For coral snakes, capillary tubes applied to the front fangs may be necessary to collect venom. Several specimens of the same species are milked. The fresh venom is then centrifuged to remove cellular debris, and stored at – 20 °C. Once a sufficient amount of venom from snakes of different origins, genders, and ages has been collected, venom is pooled, freeze-dried, and stored in the dark at −20 °C. For each batch produced, quality control tests are performed, including protein concentration, SDS-Page eletrophoresis, and LD50.

National reference venoms from *Bothrops* and *Crotalus* snakes are produced by Instituto Butantan. Only wild snakes, collected from different regions, are milked and venom is prepared according to the same procedures described above. About 2,000–2,500 snakes are necessary to obtain a batch of a national reference venom, which is delivered to the National Institute of Quality Control in Health, an organ of the Brazilian Ministry of Health, responsible for performing potency assay and testing for lots of antivenom prior to batch release (Araujo et al. 2000).

2. Immunization

The selection of venoms to be used in immunization is based on epidemiological and clinical information, as well as on the immunological cross-reactivity between the venoms of different species. For different manufacturers and products, venoms selected come from the snakes of the highest medical importance. For the Brazilian *Bothrops* AV, the capacity to induce antibody production and mutual cross-reaction were assayed for the venoms of ten different *Bothrops* species, of which five constituted the mixture that showed to be the best to be used in horse immunization for antivenoms (Dias da Silva et al. 1989). As there are more than 30 *Bothrops* species in South America, the widespread occurrence of cross-reactivity between venoms of this genus facilitates the design of immunizing mixtures composed of venoms from few species, as established by the manufacturers (Table 2).

Micrurus antivenom is usually monovalent, except in Brazil, which is bivalent. *Micrurus* AV is only produced by two Brazilian laboratories. Antigenic cross-reactivity among components of Brazilian *Micrurus* snake venoms indicated that *M. corallinus* and *M. frontalis* are good immunogens that contain many cross-reactive molecules and that their toxic components are neutralized by the bivalent antivenom (Higashi et al. 1995). However, recent cross-reactivity tests of specific antivenom with *Micrurus* venoms showed that it does not correlate well with the expected neutralizing capacity (Tanaka et al. 2016), suggesting that the mixture of venoms used should be reviewed to attain a broader neutralizing activity.

The horses remain the animal of choice for antivenom production for South American manufacturers, except in Bolivia where donkeys are currently used (Fernandez et al. 2010). Immunization schedules consist of repeated

Table 2 List of public snake antivenom manufacturers in South America and pool of venoms used in the immunization process

Country	Manufacturer	Product	Pool of venoms	Active substance – precipitated by	Neutralizing potency (mg venom/mL AV)
Argentina (Instituto Nacional de Productos Biológicos, Argentina 2016)	Instituto Nacional de Producción de Biológicos, Administración Nacional de Laboratorios y Institutos de Salud "Carlos Malbrán" – ANLIS	Bivalent *Bothrops* AV	*B. alternatus*, *B. diporus*	$F(ab')_2$ – ammonium sulfate	3.37 mg/mL v *B. neuwiedi*
		Tetravalent *Bothrops* AV	*B. alternatus*, *B. diporus*, *B. jararaca*, *B. jararacussu*		
		Crotalus AV	*C.d. terrificus*		
Argentina (Instituto Biológico 2016)	Instituto Biológico Dr. Tomás Perón – Ministerio de Salud de la Provincia de Buenos Aires	*Bothrops* AV	*B. alternatus*, *B. neuwiedii*	$F(ab')_2$ – ammonium sulfate	2.5 mg/mL v *B. alternatus* 1.5 mg/mL v *B. neuwiedii*
Bolivia (Fernandez et al. 2010)	Instituto Nacional de Laboratorios de Salud - INLASA	*Bothrops-Crotalus* AV	*B. mattogrossensis*; *C.d.terrificus*	IgG – caprylic acid	1.5 mg/mL v *B. mattogrossensis*
		Bothrops-Lachesis AV	*B. mattogrossensis*; *Lachesis muta*		
Brazil (Raw et al. 1991)	Instituto Butantan Fundação Ezequiel Dias Instituto Vital Brazil	Pentavalent *Bothrops* AV	*B. jararaca*, *B. alternatus*, *B. jararacuçu*, *B. moojeni*, *B. neuweidi*	$F(ab')_2$ – ammonium sulfate	5.0 mg/mL v *B. jararaca*
	Instituto Butantan Fundação Ezequiel Dias Instituto Vital Brazil	*Bothrops-Lachesis* AV	*B. jararaca*, *B. alternatus*, *B. jararacuçu*, *B. moojeni*, *B. neuweidi*; *Lachesis muta*		5.0 mg/mL v *B. jararaca* 3.0 mg/mL v *Lachesis muta*
	Instituto Butantan Fundação Ezequiel Dias Instituto Vital Brazil	*Bothrops-Crotalus* AV	*B. jararaca*, *B. alternatus*, *B. jararacuçu*, *B. moojeni*, *B. neuweidi*, *C.d. terrificus*, *C.d. collilineatus*		5.0 mg/mL v *B. jararaca* 1.5 mg/mL v *Crotalus durissus*
	Instituto Butantan Fundação Ezequiel Dias Instituto Vital Brazil	*Crotalus* AV	*C.d. terrificus*, *C.d. collilineatus*		1.5 mg/mL v *Crotalus durissus*

(*continued*)

Table 2 (continued)

Country	Manufacturer	Product	Pool of venoms	Active substance – precipitated by	Neutralizing potency (mg venom/mL AV)
	Instituto Butantan Fundação Ezequiel Dias	*Micrurus* AV	*M. corallinus*, *M. frontalis*		1.5 mg/mL v *M. frontalis*
Colombia (Instituto Nacional de Salud, Colombia 2016)	Instituto Nacional de Salud	Polyvalent *Bothrops-Crotalus* AV	*B. atrox*, *B. asper*; *C.d. cumanensis*	IgG – ammonium sulfate	7.0 mg/mL v *B. atrox* 1.0 mg/mL v *C.d. cumanensis*
Peru (Instituto Nacional de Salud, Peru. Suero antibotrópico polivalente 2016; Instituto Nacional de Salud, Peru. Suero antilachésico monovalente 2016; Instituto Nacional de Salud, Peru. Suero crotálico monovalente 2016)	Instituto Nacional de Salud	Polyvalent *Bothrops* AV	*B. atrox*, *B. barnetti*, *B. brazili*, *B. pictus*, *B. hyoprora*	IgG – caprylic acid	2.5 mg/mL v *B. atrox*
		Lachesis AV	*Lachesis muta*		2.5 mg/mL v *L. muta*
		Crotalus AV	*C.d. terrificus*		1.5 mg/mL v *C.d. terrificus*
Venezuela (Centro de Biotecnologia de la Universidad Central de Venezuela 2016)	Universidad Central de Venezuela	Polyvalent snake AV	*B. atrox*, *B. colombiensis*, *B. venezuelensis*; *P.l. hurmanni*; *C.d. cumanensis*, *C.d. ruruima*	$F(ab')_2$ – ammonium sulfate	2.0 mg/mL v *B. colombiensis*, 1.5 mg/mL v *C.d. cumanensis*

subcutaneous injections of low doses of venom dissolved in distilled water or buffer, and added to adjuvant.

Immunization with venoms should aim at obtaining a high neutralizing antibody titre without significant harm to the immunized animals, which is usually achieved through repeated injections of low doses of venom. In general, the optimum immunization dose is obtained by trial and error.

Although some manufacturers inactivate venoms in order to reduce toxicity, this strategy may affect the structure of relevant epitopes, thus compromising antibody response; therefore, it is preferred to use native venoms for immunization. Suppressive components may affect the response of horses to venoms. For instance, a chromatographic fraction of the venom of *Lachesis muta* snake, containing a main component of 27 kDa, was found to suppress antibody production. Removal of this fraction from the whole venom resulted in a highly significant improvement in the response of the horses, thus improving the efficiency in the *Lachesis* antivenom production (Stephano et al. 2005).

For most South American manufacturers immunization protocols are generally based on the use of Freund's complete and incomplete adjuvants, for the first immunizations, followed by subsequent injections of venom with other adjuvants. However, sterile abscess and granuloma at the sites of venom injection is frequent with the use of Complete Freund Adjuvant. Butantan has been using a mixture of Montanide adjuvants containing highly purified oil and injectable emulsifying agents in a multisite, low dose, and low volume primary immunization protocol (Waghmare et al. 2009; Waghmare et al. 2014). This protocol utilizes minimum amount of venoms, increasing the effectiveness of plasma production, with a clear benefit for the health of the horses.

Moreover, the immunization schedule using lower venom concentrations and extending the dose intervals generate higher antibody levels, since these conditions seem to guarantee an efficient memory induction and avoidance of clonal deletion or anergy to relevant epitopes.

3. Bleeding and plasma collection

The antibody titres of the immunized animals are verified throughout the immunization process by enzyme immunoassay. Bleeding is performed when an immunized animal develops the antibody titre that reaches the specification, usually after a 3–4 dose reimmunization schedule. The area surrounding the venipuncture site is shaved, cleaned, and disinfected, and then the animal is bled. Horses with 370–400 kg are bled up to three times with intervals of 48 h. Each bleeding volume corresponds to about 2% of the animal's weight. The blood is collected in disposable double plastic bags containing citrate anticoagulant. During the session, which takes about 1 h, blood is gently and continuously agitated to ensure a homogenous distribution of the anticoagulant and avoid formation of clots.

Next, red blood cells are separated by precipitation for 24 h at 2–8 °C. The content of the bag, containing only red blood cells, is suspended with apyrogenic saline solution and reinfused into the donor horse.

In theory, automatic plasma collection has the advantage of reducing errors, particularly in the reinfusion of blood cells to the donor. Plasma is filtrated, thus resulting in higher yields, and tends to contain fewer contaminating blood cells. However, the collection process is longer, lasting up to 4 h, which can stress the animal kept in the bleeding room for such a long time. In practice, none of the South American manufacturers collects plasma by using plasmapheresis machines. Bags are stored in a refrigerated condition in the dark until the fractionation step.

4. Purification

There is a great variety of technologies used in the purification process. Currently, some of the South American antivenoms consist of an ammonium sulfate-precipitated, pepsin-digested, concentrated $F(ab')_2$ fraction of the IgG molecule. Other manufacturers produce antivenoms by adding caprilic acid precipitation, to obtain whole IgG (Gutierrez et al. 2007). Both types of antivenoms show a similar profile of effectiveness concerning the neutralization of toxins with local and systemic effects (Leon et al. 2013). None of South American laboratories produce F(ab) fragments (Table 2).

In the 1980s, a Brazilian national program for self-sufficient production of immunobiological products led to the first modernization process in the biological products plants of public laboratories (Ponte 2003). Large-scale production was introduced, and a semi-enclosed industrial plant was constructed at Instituto Butantan (Raw et al. 1991). In 2016, a completely enclosed and automated plant replaced the previous plant (Fig. 1). Following GMP rules, this renewed plant facilitates the transport of raw materials and intermediate products, and reduces the risks of contamination, since there is no exposure or manipulation during the production process. Traceability is assured, as well as the timing control for each step of the process.

At Instituto Butantan, plasma from individual animals is submitted to sterility test, by bioburden assay, before fractionation. Upon approval, they are checked for macroscopic clots and precipitates. Plasma is pooled in a 300 L stainless steel tank. Samples are collected for protein concentration, electrophoresis, and potency assay. The starting plasma pool is submitted to fractionation by ammonium sulfate with stirring for 1 h, and then the precipitate is centrifuged. The liquid fraction is discarded and the precipitate is solubilized. The pH is adjusted for enzymatic digestion by pepsin, which degrades the non-IgG proteins, and cleaves the molecule into $F(ab')_2$ and Fc fragments. A second precipitation of IgG is performed by adding ammonium sulfate and pH is adjusted to caprylic acid addition, and the solution is stirred. Next, thermocoagulation at 56 °C separates unspecific thermolabile proteins. Ammonium sulfate is again added to the solution, which is stirred. The precipitate is discarded and the supernatant is submitted to ultrafiltration, to eliminate ammonium sulfate and other low-molecular-mass contaminants, and to ion-exchange chromatography as an additional step of purification (Fig. 2). In this process, antibodies that bind to venom components are retained in the column, while other proteins flow through. The column is treated to release the antibodies, which are collected and concentrated. Phenol is

Fig. 1 Enclosed and automated purification plant of Instituto Butantan, showing the fractionation tanks and the ultracentrifuge used in the process of antivenom production

Fig. 2 Ion-exchange chromatography in the antivenom production at Instituto Butantan

added as preservative. The concentrated $F(ab')_2$ solution is submitted to clarifying and sterile filtration, the resulting solution is maintained in disposable plastic bags. At this step quality control tests are performed, including pyrogen, sterility, sodium chloride, pH, protein concentration, phenol level, and potency. Once

approved, the concentrate is formulated to adjust osmolarity and pH, and then filtrated through 0.22 micra filters. The bulk product is packaged in plastic bags. Quality control tests at this step include sterility, pyrogen, pH, chloride, ammonium sulfate, total and non protein nitrogen, protein concentration, and potency assay.

Since antivenom is obtained from purified hyperimmunized plasma of horses, these animals should be ideally free of viral pathogens. However, the lack of diagnosis and vaccines for all equine viruses require strict control of infectious risks. Therefore, viral inactivation becomes essential in the downstream process of equine plasma. Phenol has been proved to be an effective virucidal preservative in the process of antivenom production at Instituto Butantan (Caricati et al. 2013). This purification method demonstrated to be cost-effective and eliminates viruses to the levels required by WHO for a safe product (WHO 2010).

5. Dispensing and labeling
The product is bottled in glass vials (10 mL) and submitted again to sterility test and pyrogen. The approved antivenom is labeled, identified, according to rules established by the national regulatory authorities. The final product is stored under 2–8 °C.

Although stability studies are scarce, liquid antivenoms are relatively stable, suffering few different rates of activity loss, depending on the temperature applied. At ambient temperature, most liquid antivenoms maintain its clarity and neutralizing potency for years after expiring period (Gutierrez et al. 2009).

6. Quality control tests
Quality control tests are essential to assure the quality of the intermediates and final product in the antivenom production process. The results meet the specification approved by the National Pharmacopoeia of each country. In Brazil, since 1959 and until the last revision in 2010, the Brazilian Pharmacopoeia determines the analytical methodologies for batch release of antivenoms (Brazil. Agência Nacional de Vigilância Sanitária 2010). The lethality neutralization assay performed in mice estimates the antivenom potency for *Bothrops* and *Crotalus* antivenoms. For in vivo tests, male and female Swiss mice weighing 18–22 g are used after approval of the Ethics Committee on the Use of Laboratory Animals. The median lethal dose is assessed by intraperitoneal injection of 0.5 mL of five dilutions in groups of ten mice. Deaths are recorded after 48 h and LD50 is calculated by probit analysis. To determine the potency and median effective dose (ED50), a constant amount of venom corresponding to 5 LD_{50}/animal is incubated for 45 min at 37 °C with serial dilution of antivenoms. After incubation, 0.5 mL aliquots of the mixture are inoculated by intraperitoneal route in four or five groups of eight mice each and the number of survivors is recorded after 48 h. The ED50 is calculated by probit analysis, and the potency of the antivenom is expressed as the amount of venom (in mg) that is neutralized by 1.0 mL of antivenom.

A National Control Laboratory of the Ministry of Health of Brazil was created in 1987, to test lots of antivenoms prior to batch release, including phenol, sodium chloride, protein, protein nitrogen, ammonium sulfate, pH, volume, sterility, and

pyrogen. The potency test is performed for *Bothrops* and *Crotalus* antivenoms, for which national reference venoms are provided by Instituto Butantan. Between years 2000 and 2006, only 1.3% of the Brazilian 619 lots of antivenoms were rejected (Araujo et al. 2000).

7. Availability

If compared to other continents, South America is not in a bad condition in terms of availability of antivenoms. However, there are still some deficiencies as some countries of the region do not produce their own antivenoms (Ecuador, Guiana, French Guiana, Paraguay, Suriname, and Uruguay). Additionally, local production of antivenoms may be insufficient to cover the national demands (Gutierrez et al. 2007). In some cases, antivenoms are purchased from other countries, ideally from those with overlap in the geographical distribution of species and studies of cross-neutralization of antivenoms produced in different countries. This assures effectiveness of the products distributed to a given country, in terms of neutralization capacity of the venoms of medical importance.

In the 1990s Brazil became self-sufficient, supplying the total amount of antivenom required nationally, as a consequence of a program for self-sufficiency in antivenom production and distribution. Conducted by the Ministry of Health, in partnership with three official manufacturers, the success of this program was based on (1) modernization of industrial plants, (2) standardization of venoms used in the immunization process, (3) standardization of criteria for the treatment of envenomation, and (4) implementation of a surveillance system. According to this program, all antivenoms produced by official manufacturers are delivered to the Ministry of Health. Distribution is decentralized at the regional level and antivenoms are allocated to reference health centers. Antivenoms in Brazil are therefore not commercialized. However, limitations in the cold chain and insufficiency of health professionals in remote regions lead to serious deficiencies in the accessibility to antivenoms. Large distances from rural areas to hospitals where antivenoms are available have been contributing to the high morbidity and mortality of snakebites. In other South American countries, antivenom distribution follows national health policies. Mostly, governments purchase antivenoms from public or private manufacturers. Antivenom therapy is free of charge for the patient, and restricted to hospitals and clinics. Eventually, antivenoms may be commercialized in pharmacies, as in Colombia, representing a small quantity of the antivenoms available.

With the increasing requirements for good manufacturing practices, as in Colombia, legal constraints for the collection of wild animals, and the lack of financial and political support, some public manufacturers have been limiting the production and distribution. Therefore, in some South American countries, local production of antivenoms is becoming insufficient to cover national demands, and products have to be purchased from other countries. Moreover, those that do not have a local antivenom manufacturer depend on the imported antivenom. Preclinical studies on the neutralization profile of antivenoms manufactured in different countries have been conducted to give subsidies to health authorities and national regulation agencies to make the decision to import a product. The

capacity of antivenoms produced in Argentina, Bolivia, Brazil, Colombia, Costa Rica, and Peru to neutralize toxic activities of *Bothrops* venoms has been demonstrated experimentally (de Roodt et al. 1998; Laing et al. 2004; Segura et al. 2010) and clinically confirmed in trials performed in Brazil, Colombia, and Ecuador (Cardoso et al. 1993; Otero et al. 2006; Otero-Patino et al. 2012; Pardal et al. 2004; Smalligan et al. 2004).

Antivenom Therapy

Antivenom administration follows national guidelines, with very similar recommendations (Argentina. Ministerio de Salud 2007; Brazil. Ministerio da Saude 2001; Colombia. Ministerio de la Protección Social 2008; Ecuador. Ministerio de Salud Pública 2008; Paraguay. Ministerio de Salud Publica y Bienestar Social 2008; Peru. Ministerio de Salud 2004): antivenom should be administered by intravenous route, diluted in isotonic fluid, and infused over approximately 60 min. Preferably, patients should be hospitalized for antivenom therapy and be monitored in the first 24 h for early anaphylactic reactions. In most countries, the number of vials or ampoules to be administered is judged empirically, according to severity, in equal doses for children and adults.

Efficacy

Antivenom therapy should start as soon as indications are fulfilled. Delay in antivenom therapy is the main determinant for poor prognosis, as observed in *Bothrops* envenomation, in which case failure of antivenom in reversing clinical and laboratory effects is more likely to occur in patients admitted more than 6 h after bite (Bucaretchi et al. 2001; Feitosa et al. 2015). However, even a long delay between bite and admission to hospital should not exclude the indication of antivenom therapy if symptoms and signs of systemic envenomation are still evident.

Polyspecific antivenom is able to neutralize the most relevant toxic components of venoms to which the antivenom is designed. In particular, clinical studies have shown that, when administered early after bite, antivenoms are highly effective in reversing hematological disturbances, in stopping local and systemic bleeding caused by viperid snake venoms (Bucaretchi et al. 2002; Jorge and Ribeiro 1997; Otero-Patino 2009; Sano-Martins et al. 1997, 2001; Santoro et al. 2008) and in preventing progress of neurotoxic *Micrurus* venom effects. On the other hand, the efficacy of antivenom in reducing local tissue damage is limited, unless antivenom is given within a few hours of the bite.

Usually, coagulation disturbances are reversed in the first 24 h of antivenom therapy and no relapse occurs if the recommended dose is given. Clinical trials performed in Brazil confirmed the efficacy of the three *Bothrops* $F(ab')_2$ antivenoms to clear venom from the circulation and to restore coagulation parameters in

B. jararaca (Cardoso et al. 1993) and *B. atrox* envenomation (Pardal et al. 2004). In parallel, enzyme immunoassays to compare the effect and assess the dosage regimens of Brazilian *Bothrops* antivenoms suggest that patients have been treated with large amounts of antivenom (Jorge et al. 1995), with a high persistence of antivenom in the circulation for approximately 10 days, and still detectable for 30–50 days after administration (Theakston et al. 1992). In Uruguay, pattern of the evolution of venom antigen concentration and antivenom levels were similar, with a complete neutralization of the venom and its effects in almost all cases (Morais et al. 2012).

Antivenoms composed of either whole IgG or $F(ab')_2$ fragments, manufactured in Brazil, Colombia, Costa Rica, and Mexico, have been tested in Colombia for *B. asper* and *B. atrox* envenomation (Otero et al. 1996, 1999, 2006; Otero-Patino et al. 1998, 2012). Trials showed that antivenoms were equally efficient for the neutralization of both local and systemic envenomation with no necessity of additional doses after the initial treatment.

Safety

Adverse effects of antivenoms, whose frequency and severity considerably vary, can be related to several factors: contamination with endotoxins during the manufacturing process, lack of purity and presence of protein aggregates, and anticomplementary activity and immunogenicity of antivenoms (Squaiella-Baptistão and Marcelino 2014).

1. Pyrogenic reactions
   Antivenom contaminated with pyrogenic substances can elicit pyrogenic reactions, characterized by fever, rigors, chills, sweating, vasodilation, and hypotension, which appear during antivenom infusion. Pyrogenic reactions can be caused by endotoxins generated by the formation of lipopolysaccharides (LPS) of Gram-negative bacteria.
2. Acute reactions
   The nature of acute reactions to antivenom is not clear and can involve different pathogenic mechanisms:
   - Type I hypersensitivity seems not to be responsible in most cases, since acute reactions often occur in patients with no history of previous exposure to equine proteins. However, in-process quality control of antivenom should investigate traces of antibiotics used to treat horses that could cause IgE-mediated reaction.
   - Anticomplementary activity could be involved (Pidde-Queiroz et al. 2010; Squaiella-Baptistão and Marcelino 2014; Tanaka et al. 2012); however, complement activation has not been demonstrated in clinical studies.
   - The presence of human heterophilic antibodies towards equine immunoglobulins has also been suggested, although the presence of these antibodies in patients treated with *Bothrops* antivenom in Brazil did not correlate with the frequency of early reactions (Leon et al. 2008).

Independent of the mechanisms involved, clinical symptoms of early reaction are indistinguishable and have been reported in variable frequencies, ranging from 11% (Otero-Patino et al. 1998) to 25% (Fan et al. 1999), and 73% (Smalligan et al. 2004), with no apparent differences between antivenom consisting of whole IgG or $F(ab')_2$ fragment (Table 3). Mostly acute reactions are mild, including pruritus, urticaria, nausea, vomiting, and abdominal pain. Dyspnea and hypotension indicate severe reaction and demand urgent intervention.

Risk of early reaction depends on the dose, speed of administration, and the quality of purification of the antivenom. A widespread practice in the antivenom therapy is to recommend the slow infusion of antivenom, often achieved by diluting the antivenom in isotonic fluid, although clinical studies have not provided full support that the speed of antivenom infusion correlates with the frequency of acute reaction.

Intradermal/conjunctival hypersensitivity tests do not predict antivenoms reactions and are no longer indicated.

There is no consensus regarding pretreatment. The administration of antihistamines and/or corticosteroids 15–20 min prior to antivenom therapy does not prevent early reactions (Bucaretchi et al. 1994; Fan et al. 1999). The use of subcutaneous adrenaline (epinephrine) has been advocated to reduce the risk of severe early reactions.

Table 3 Frequency of early reactions caused by antivenom

Manufacturer	Active substance	Frequency of early reaction	Country of study
Instituto Butantan, Brazil	$F(ab')_2$	37%	Colombia (Leon et al. 2001)
Instituto Butantan, Brazil	IgG (caprylic)	11%	
Instituto Nacional de Salud, Colombia	IgG (caprylic)	82%	
Instituto Butantan, Brazil	$F(ab')_2$	18%	Brazil (Otero-Patino 2009)
Fundação Ezequiel Dias, Brazil	$F(ab')_2$	19%	
Instituto Clodomiro Picado, Costa Rica	IgG (sulfate)	52%	Colombia (Leon et al. 2008)
Instituto Clodomiro Picado, Costa Rica	IgG (caprylic)	25%	
Instituto Nacional de Higiene y Medicina Tropical "Leopoldo Izquieta Perez," Ecuador	$F(ab')_2$	17%	Ecuador (Ponte 2003)
Instituto Butantan, Brazil	$F(ab')_2$	53%	
Instituto Nacional de Salud, Colombia	IgG (sulfate)	73%	
Instituto Clodomiro Picado, Costa Rica	$F(ab')_2$ (caprylic)	29%	Colombia (Morais et al. 2012)
Instituto Clodomiro Picado, Costa Rica	IgG (caprylic)	21%	

3. Late reactions
   Late reaction or serum sickness is a type III or immune-complex mediated hypersensitivity disorder. It has an onset of 5–21 days after antivenom therapy, and symptoms are self-limited in most cases, although symptoms can eventually persist for days. Clinically late reactions manifest with mild self-limited symptomatology, such as pruritic probit analysis rash, low fever, arthralgia, and headache.

Conclusion and Future Directions

South American antivenom manufacturers have been producing different types and quantities of snake antivenom. Most are public institutions, some founded in the beginning of the 20th century, being the Brazilian scientist Vital Brazil Mineiro da Campanha a pioneer in the research on snake venom and envenomation, as in the antivenom production. Currently, laboratories present variable degrees of technological development and compliance with good manufacturing practices. Comparing processes among the South American laboratories, some steps of antivenom production present clearly differences in terms of the venoms used in the immunization schedules, which reflects the variation in the geoghrapical distribution of the snakes of medical importance. The final products are derived from purification process that results on of F(ab)'2 fragments or whole IgG immunoglobulins, both showing equal efficacy and safety. Brazil has achieved self-sufficiency in supplying the total amount of antivenom required, in consequence of a program conducted by the Ministry of Health, based on the modernization of industrial plants of local public manufacturers, standardization of venoms used in the immunization process, guidelines for the treatment of snake envenomation, and epidemiological surveillance. There is a need to provide antivenom for all South American continent, especially considering that there are countries that do not produce their own antivenoms, and others where local production do not sufficient to cover the national demands. Thus, it is necessary to implement a strategy for increasing the production of antivenoms, involving standardization of antivenom production and quality control processes, based on the World Health Organization *Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins*, implementation of knowledge and technology transfer between well-developed manufacturing laboratories and less-develop production facilities. At the same time, technological innovation in antivenom production should be encouraged, as the studies of stability of liquid antivenoms versus freeze-dried antivenoms.

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Snake Venom Components as Basis for Biologically Active Synthetic Peptides

6

Igor E. Kasheverov and Victor I. Tsetlin

Abstract

In this chapter, an attempt is made to collect data on synthetic peptides derived from peptide and protein components from snake venoms. The focus is on shorter fragments of natural-occurring compounds and to a lesser extent their mutant analogs. In general, these synthetic variants were prepared either to identify the "active sites" of the natural molecules, as well as the structural and functional features of their targets, or to design new compounds with potential for practical use. Taking into account the huge diversity of peptide-protein components in the snake venoms (and limited volume of this Chapter), the selection of components for the review was limited to only the most important classes of compounds. Among these are myotoxins, so-called three-finger toxins, phospholipases, metalloproteinases, and disintegrins, as well as several groups of small peptides with different structure and properties which we called "disulfide poor" compounds (containing two or less disulfides).

Keywords

Synthetic peptides • Snake venom components • Myotoxins • Three-finger toxins • Disintegrins • Sarafotoxins • Waglerins • Azemiopsin • Bradykinin-potentiating peptides

Contents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_23

Introduction

Snake venoms are a naturally occurring source of a huge number of biologically active compounds, considerable portion of which are peptides and proteins. For many of these compounds, the functional roles have been elucidated and the molecular targets identified. Snake venom components have become the "classical tools" for research on the respective biological targets. In recent times, both the snake venoms as such and the individual components have found practical application for diverse medical purposes. This trend is reflected in a large number of reviews on description and promising applications of the respective compounds from snake venoms, in particular in the present handbook series on "Toxinology."

Parallel to the discovery and characterization of novel snake venom components, efforts have been focused on the creation of synthetic products based on known structures. In such a "competition with Nature," the main purpose in most cases is to pinpoint the "active sites" of the peptide or protein components and open the way to active but shorter analogs, to create mutant forms to shed light on the mechanisms of action, and finally and ideally, to design drugs starting from the natural product leading to more stable, effective, and selective analogs. Research of this sort has been carried out on snake venom compounds of various classes. This chapter provides a brief overview of relevant information encompassing the most important proteins and peptides from snake venoms, namely three-finger neurotoxins, cytotoxins, phospholipases A2, disintegrins, sarafotoxins, myotoxins, waglerins, and some linear peptides.

Synthetic Peptides Derived from Myotoxins (Small Myotoxins, PLA2, Cardiotoxins)

The term "myotoxins" embraces various compounds which induce necrosis of the muscle tissue. Apparently, the first compound with the mentioned activity isolated from snake venom was crotamine discovered over 50 years ago in the South American rattlesnake *Crotalus durissus terrificus.* Due to the endeavors of Anthony Tu, in the 1970s, a myotoxin from the rattlesnake *Crotalus viridis viridis* was

discovered and analyzed. The primary and three-dimensional structures were established for the two above-mentioned homologous polypeptides (42 amino acid residues, 3 disulfides). Conventionally classified as a family of Small Basic Polypeptide Myotoxins, these were later joined by several other members, mainly from the venom of *Crotalus* snakes. They were characterized as Na^+ channel affecting toxins (crotamine), as inhibitors of Ca^{2+} -ATPase of skeletal muscle sarcoplasmic reticulum, and as potent voltage-gated potassium channel inhibitors (myotoxin a). The data in literature on analgesic, antimicrobial, antifungal, and antitumor activities explain the term "exceptional biological versatility" applied to these compounds. At present one of the most promising developments is on their cell-penetrating ability, which allows the toxins to cross cell membranes and to accumulate in the nucleus. This opens the perspectives of their use for intracellular vesicle tracking and as a cell cycle marker, as well as for delivering DNA into replicating mammalian cells (see reviews (Oguiura et al. 2005; Rádis-Baptista and Kerkis 2011)).

It was supposed that the above-mentioned properties are due to the presence in the primary structure of two nuclear localization signal motifs (Rádis-Baptista and Kerkis 2011). On the basis of one of the motifs (crotamine fragment 27–39), a series of 38 analogs has been synthesized by Jha et al. (2011), shortened at the *N*-terminus or bearing various substitutions, with the purpose of establishing the optimal length for the cell-penetrating properties. The most active compounds are presented in Table 1A and the crotamine fragment CRWRWKCCKK today is one of the best cell-penetrating peptides. Another crotamine feature, namely, the capacity to target nucleolus, was utilized by Rádis-Baptista et al. (2008) to synthesize its fragments as the nucleolar-targeting peptides, of which the most efficient is the peptide YKQCHKKGGKKGSG shown in Table 1A or NrTP1.

The main purpose of preparing synthetic fragments of myotoxin a was to map its binding surface involved in the recognition of the Ca^{2+}-ATPase of the skeletal muscle sarcoplasmic reticulum (see Table 1A). From researches of the 1990s, it was surmised that the *N*- and *C*-terminal fragments rather than the central part of the molecule are important for binding (Baker et al. 1992). It was also assumed that the binding site on Ca^{2+}-ATPase is situated in the vicinity of Cys-344 in the phosphorylation domain and Lys-515 resides in the nucleotide binding domain (Baker et al. 1995).

Another class of proteins with potent myotoxic activity was revealed in the middle 1980s in crotalid snake venoms (mainly from the *Bothrops* genus). These are nonneurotoxic phospholipases A2 (PLA2), quite often existing in the same venom as two subtypes, "Asp49" and "Lys49" PLA2 myotoxins. The latter subtype does not have a catalytic activity, thus suggesting the operation of two different mechanisms in their myotoxicity. A large number of myotoxic phospholipases of both types are known at present, for which the X-ray structures have been determined and the structural determinants of their myotoxic action established (see a recent review (Fernandes et al. 2014)).

A search for determinants of toxic activity by Lomonte et al. (1994) resulted in synthesis of fragments of myotoxin II (PLA2 myotoxin from *Bothrops asper* snake venom), which correspond to the amino acid residues 1–27, 69–80, and 115–129.

Table 1 Synthetic peptides based on snake venom components

Family	Name of origin (species)	Sequence of fragments (a.a. number in origin or analog' given name)	Purpose/result of synthesis	References
A. Synthetic peptides derived from myotoxins				
Small Basic Poly-peptide Myotoxins	crotamine (*Crotalus durissus terrificus*)	KMDCRWRWKCCKK (27-39) MDCRWRWKCCKK (28-39) CRWRWKCCKK (30-39) KMDCRWRWKCKK (27-36,38-39)	Design of new effective group of cell-penetrating peptides	Jha et al. (2011)
		YKQCHKKGGKKGSG (1-9,38-42)	Design and synthesis of the nucleolar-targeting peptides	Rádis - Baptista et al. (2008)
	myotoxin a (*Crotalus viridis viridis*)	YKQCHKKGGHCFPKEK (1-16) KGGHCFPKEKICIPPS(7-22) PKEKICIPPSSDLGKM(13-28) IPPSSDLGKMDCRWKW(19-34) LGKMDCRWKWKCCKKGSG(25-42) DCRQKWKCCKKGSG (29-42)	Characterization of binding site(s) to Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum	Baker et al. (1992, 1995)
Non-neurotoxic Lys49-PLA2	myotoxin II (*Bothrops asper*)	LFELGKMILQETGKNPAKSYGAYG (1-27) KKDRYSYSWKDK (69-80) KKYRYYLKPLCKK (115-129)	Identification of toxic activities site(s)	Lomonte et al. (1994)
		KKWRWWLKPLCKK (p115-W3)	Design of potent myotoxic analog	Lomonte et al. (1999)
		KKWRWWLKALAKK (pEM-2)	Design of antimicrobial peptide analog	Santamaría et al. (2005)
	(*Agkistrodon piscivorus piscivorus*)	KKYKAYFKLKCKK (115-129)	Identification of myotoxic site(s)	Núñez et al. (2001)

(*continued*)

Table 1 (continued)

B. Synthetic peptides derived from "three-finger" toxins				
Cytotoxins	cardiotoxin IV (*Naja mossambica mossambica*)	LIPPFWK (6-12)	Identification of binding site to lipids	Dufourcq et al. (1982)
		LIPPFWK-NH_2 RLIPPFWK-NH_2	Identification of binding site to lipids and lethality in mouse	Dufourcq et al. (1982), Marchot et al. (1988)
		RNRLIPPFWKTR-NH_2 (R^3(4-13)R^{14})	Lethality in mouse	Marchot et al. (1988)
	cardiotoxin III (*Naja naja atra*)	LKCNKLVPLFYKTC (1-14) (L1AD3)	Producing a compound with selective toxicity towards leukemic T-cells	Smith and Hinman (2004)
α-Neuro-toxins	short neurotoxin 1 (*Naja philippinensis*)	TCSGETNC^{Ec}YKKWWSDHRGTIIERGCGC^{Ec}PKVK PG (16-48)	Verification of the existence of a lethal "active center"	Juillerat et al. (1982)
	lapemistoxin (*Lapemis hardwickii*)	CCNQQSSQPKTTTNC (3-17) CYKKTWSDHRGTRIERGC (22-39) YKKTWSDHRGTRIERG CPQVKPGIKLEC (41-52)	Studies on structure-function relationships of neurotoxin loops' interactions with the acetylcholine receptor	Miller and Tu (1991)
	α-cobratoxin (*Naja kaouthia)*	WCDAFCSIRGKR (25-36)	Studies on structure-function relationships of neurotoxins loop 2 interactions with GABA-A receptor	Kudryavtsev et al. (2015)
	long neurotoxin b (*Ophiophagus hannah*)	YTKTWCDGFCSSRGKRIDLG (25-44) YTKTWCDGFCSSRGKR TWCDGFCSSRGKR DGFCSSRGKR	Studies on structure-function relationships of neurotoxins loop 2 interactions with the acetylcholine receptor	Lentz (1991)
	α-bungarotoxin (*Bungarus multicinctus*)	MWCDAFCSSRGKV (28-40) MWCAAFCSSRGKV MWCDATCSSRGKV MWCDAFCSSDGKV MWCDAFCSSRWKV DAFCSSRGKV		

(*continued*)

Table 1 (continued)

	long neurotoxin b (*Ophiophagus hannah*)	YTKTWCDGFCSSRGKRIDLG (25-44)	Studies on affinity for neuronal nAChRs and the brain targeting efficiency in vitro and in vivo	Zhan et al. (2010)
Fasciculins	Fasciculin-1 (*Dendroaspis angusticeps*)	Ac-CYRKSRRHPPKMVLCG ((22-35)$C^{36}G^{37}$) C^{25}SRRHPPKC^{33} (C^{25}(26-32)C^{33}) GDDNLEVKCPGCYRKCRRHPPKMVLCRG-NH_2 ((44-52)PG(22-25)C^{26}(27-35)C^{36}(37-38))	Studies on structure-function relationships of fasciculin loops' interactions with the acetylcholine esterase	Falkenstein et al. (2004)
C. Synthetic peptides derived from disintegrins and domains of metalloproteinases				
Disintegrin-like domains	Jararhagin (*Bothrops jararaca*)	ASMSECDPAEH (423-433) SECDPA SECD, SECA, SEAD, SACD ECDP ECD SEC MSE, MSA, MAE	Identification of jararhagin fragment responsible for inhibition of aggregation of platelets	Kamiguti et al. (1997)
	Atrolysin A (*Crotalus atrox*)	Ac-(Acm)CRPARSECDIAESC-NH_2 (268-281) Ac-CRPARSECDIAESC(Acm)-NH_2 Ac-(Acm)CRPARSECDIAESC(Acm)-NH_2 (Acm) Ac-(Acm)CRPARSESDIAESC(Acm)-NH_2 Ac-CRPARSACDIAESC(Acm)-NH_2 Ac-CRPARSECAIAESC(Acm)-NH_2 Ac-CRPARSACAIAESC(Acm)-NH_2 Ac-(Acm)CRPARGDADIAESC(Acm)-NH_2	Investigation of atrolysin A fragments' cyclization on inhibiting ability of platelets' aggregation	Jia et al. (1997)

(*continued*)

Table 1 (continued)

	Jararhagin (*Bothrops jararaca*)	CWSNGDKITC (212-219) CEQQRYDPYKC (151-159) CKLPDSEAHAC (103-111) CHYSPDGREIC (46-54) CPADVFHKNC (441-449) CTRKKHDNAQC (241-249) CTRKKHDNAQC CTRKKHDC CTRKKHDNAC CYSNDDEHKGC (537-545) CRASMSECDPAEHC(Acm) (421-434)	Identification of jararhagin fragment responsible for binding to integrin α_2I domain and impact on this activity of fragment cyclization	Ivaska et al. (1999)
	Kaouthiagin(*Naja kaouthia*)	RAAKHDCDLPELC (258-270) RAAKHDCDLPELC	Identification of fragments responsible for disintegrin-like activity in kaouthiagin	Ito et al. (2001)
Cys-rich domains	Kaouthiagin(*Naja kaouthia*)	CFDLNMRGDDGSFC (323-336) CFDLNMRGEDGSFC		

(*continued*)

Table 1 (continued)

	Jararhagin (*Bothrops jararaca*)	YCYNGNCP (315-322) NCPIMYHQCY (320-329) QCYALFGADVYEAEDSCF (327-344) SCFKDNQKGNYYGYCR (342-344) YCRKENGKKIPCA (355-357) PCAPEDVKCG (365-374) KCGRLYCK (372-379) YCKDNSPGQNNPCK (377-390) PCKMFYSNDDEHKGMVLPGTKCA (388-410) KCADGKVCS (408-416) VCSNGHCV (414-421)	Identification of jararhagin regions which could bind to von Willebrand factor	Pinto et al. (2007)
Disintegrins	Obtustatin(*Vipera lebetina obtusa*)	CWKTSLTSHYC (19-29) CAKTSLTSHYC CWATSLTSHYC CWKASLTSHYC CWKTALTSHYC CWKTSATSHYC CWKTSLASHYC CWKTSLTAHYC CWKTSLTSAYC CWKTSLTSHAC	Identification of the integrin-binding sequence motif in obtustatin	Moreno-Murciano et al. (2003)
	VLO5 (*Vipera lebetina obtusa*)	CKRTMLDGLNDYC (39-51)	Identification of the causes of specificity to certain types of integrins	Bazan-Socha et al. (2004)
	EC3 (*Echis carinatus sochureki*)	CKRAMLDGLNDYC (38-50)		

(*continued*)

Table 1 (continued)

D. Synthetic peptides derived from "disulfide poor" components				
Sarafotoxins	Sarafotoxin b (*Atractaspis engaddensis*)	HQDVIW (16-21) HQDIIW	Structure-function studies on biological activities of sarafotoxins and endothelins	Rovero et al. (1990)
		CSCKDMTDKECLYFCHQD (1-18) CSCKDMTDKECLYFCVQD CSCADMTDKECLYFCHQD CSCSDMTDKECLYFCHQD CSCKDMTDKECLYFCMSEMS CSCSDMTDKECLYFCMSEMS	Design of metalloproteinase inhibitors on the base of sarafotoxin b	Lauer-Fields et al. (2007)
Waglerins	Waglerin-I (*Trimeresurus wagleri*)	LRPCHPPCHYIPRPKPR (6-22)	Structure-function studies on lethality	Schmidt and Weinstein (1995)
		PCHPPCHYIPR-NH_2 (8-18) GGKPDLRPCHP-NH_2 (1-11) PCHYIPRPKPR-NH_2 (12-22)	Establishment of the impact of two adjacent residues Cys and His on Ni^{2+} ion binding	Kulon et al. (2008)
Azemiopsin	Azemiopsin (*Azemiops feae*)	WYPKP-NH_2 (3-7)	Design of shorten active fragments for practical use	Kasheverov et al. (2015)
Bradykinin-potentiating peptides	SQ 20881 (*Bothrops jararaca*)	(2S)-1-[(2S)-2-methyl-3-sulfanylpropanoyl]-P (captopril)	Design of specific inhibitors of angiotensin-converting enzyme	Ondetti et al. (1977)

The last peptide resembles myotoxin II in its capacity to bind heparin – a known blocker of the toxic action of phospholipases (Table 1A). The same publication reported a cytolytic activity of the 115–129 fragment, which was later shown to be responsible for the bactericidal activity of myotoxin II. Substitution of three tyrosine residues for tryptophans in this peptide (Table 1A) generated an analog (peptide p115-W3), which retained the cytolytic activity, had enhanced bactericidal properties, and acquired the mitotic activity (Lomonte et al. 1999). This work confirmed a hypothesis on the role of hydrophobic amino acid residues in the mechanism of toxicity. Later the peptide p115-W3 was used by Santamaria et al. (2005) as a starting point for a series of 10 analogs, one of which (peptide pEM-2) (Table 1A) revealed a high bactericidal activity and reduced toxicity, presenting it as a promising candidate for further evaluation of its antimicrobial potential *in vivo*. This analog was found to have potent fungicidal activity against a variety of clinically relevant *Candida* species.

The dominant contribution of the C-terminal fragment to toxicity was elucidated by Nuñez et al. (2001) for many other myotoxic Lys49-PLA2 as shown with the corresponding 115–129 fragment of the protein from the *Agkistrodon piscivorus piscivorus* venom (see Table 1A). This peptide had potent myotoxic and cytotoxic activities (with the antitumor effects reported later), contrary to the analogous C-terminal fragment of Asp49-phospholipase A2 from the same venom. These findings suggest that Lys49 and Asp49 PLA2s might exert their myotoxic actions through different molecular mechanisms.

Quite often amid myotoxins under consideration are some representatives of neurotoxic phospholipases A2, whose toxicity is due to the presynaptic action. Among the best studied is notexin from *Notechis scutatus scutatus*, which induces impressive skeletal muscle necrosis at very low doses via binding to the sarcolemma, as well as crotoxin from *Crotalus durissus terrificus*, consisting of two (basic and acidic) subunits. In the last decade, in addition to neurotoxicity and myotoxicity, crotoxin has been found to have many other activities such as immunomodulatory, anti-inflammatory, antimicrobial, antitumor, and analgesic actions (see, for example, review (Sampaio et al. 2010)). Moreover, there were attempts to use crotoxin as such (named NSC-624244) or in equimolar association with cardiotoxin from *Naja Naja atra* venom (VRCTC-310-ONCO, formerly VRCTC-310) as an antitumor agent in patients with refractory cancer.

Literature gives several publications where different reagents were employed to modify some amino acid residues in notexin and crotoxin to elucidate their role in toxicity and in catalytic or other activities. Development of methods for producing, purification, and refolding of the heterologously expressed proteins provided a number of mutants both of notexin and of a close homolog of the basic crotoxin subunit – trimucrotoxin from *Trimeresurus mucrosquamatus* venom. In particular, the notexin analogs with substitutions of the *N*-terminal amino acid residue or with additional residues at the *N*-terminus were prepared, as well as a [Asp49Lys]-mutant deprived of catalytic activity but possessing a residual toxicity (Simonato et al. 2014). Analysis of expressed 37 mutants (with correct refolding and processing) of trimucrotoxin identified a series of amino acid residues

(Asn1, Asn6, Lys7, Ile11, Met12, Gly53, Thr79, His108, and Met118) important to its neurotoxicity (Tsai et al. 2011).

With the use of peptide arrays approach, Fortes-Dias et al. (2009) synthesized a huge set of overlapping dodecapeptides, with one residue frame shift, spanning the whole amino acid sequences of two main isoforms of the basic crotoxin subunit to map possible interfacial interaction sites in its complex with the *Crotalus* neutralizing factor (CNF), an acidic protein. The latter is a member of the major class of endogenous PLA2 inhibitors identified in the circulating blood of snakes, which is capable of replacing the acidic subunit CA in the CA-CB crotoxin complex thus deleting its toxicity. From the results obtained, it was presumed that CB the basic crotoxin subunit binds CA through two main sites located at the amino-terminus and the beta-wing regions. Peptide segments at the carboxyl-terminus of the basic crotoxin subunit were also suggested to play a secondary role in the binding of both the acidic subunit and CNF.

In some instances, "myotoxins" were described as cardiotoxins because of their capacity to depolarize cardiac myocytes, thus revealing their cytotoxicity (myotoxicity). However, snake venom cardiotoxins, based on their structure and three-dimensional organization (so-called three-finger folding) belong to a large group of cytotoxins and will be considered in the respective chapter.

Synthetic Peptides Derived from the Three-Finger Toxins (Cytotoxins, α-Neurotoxins, Fasciculins)

The proteins described by the terms "three-finger proteins," "three-finger toxins," and by abbreviations "3F" or "TFP" encompass diverse components of snake venoms differing in biological activity but having in general the same type of three-dimensional structure characterized by three disulfide-confined loops protruding from the central core formed by 4–5 disulfide bridges. The best known among those 3F proteins are cytotoxins, α- and k-neurotoxins, muscarinic toxins, and "nonconventional" toxins (the first found toxins from the latter group were virtually nontoxic and got the name of "weak toxins"). Among the most recently discovered 3F toxins should be mentioned the mambalgins that target the acid-sensitive ion channels (ASICs).

Cytotoxins, some of which are also referred to as cardiotoxins, are relatively small proteins containing the polypeptide chain of 60–65 amino acid residues fixed by four disulfides; these proteins interact with the lipid bilayers resulting in the disruption of cell membranes leading to cell death. Information about the structural features of different cytotoxins can be found elsewhere (see, for example, recent review (Dubovskii and Utkin 2014)). Only a small number of publications have dealt with the preparation and research on the cytotoxin fragments. One of the first investigations focusing on the cytotoxin site implicated in the interaction with a lipid bilayer, compared proteolysis of free and membrane-bound cytotoxins and suggested the involvement of the cytotoxin loop I (residues Leu1-Thr13). This hypothesis was confirmed by Dufourcq et al. (1982) who showed the interaction

of lipids with the tryptic and synthetic fragments of loop I (see Table 1B) and demonstrated the role of Arg5 in ensuring a high affinity of toxin for a bilayer. Later two fragments were shown by Marchot et al. (1988) to be toxic to mice with symptoms resembling that of the whole cytotoxin. Investigation of the role of all three loops in the cytolytic activity of cardiotoxin from the *Naja sputatrix* venom done by Ma et al. (2002) indicated loops I and II to be mainly responsible for activity. This conclusion was drawn employing recombinant chimeric toxins built from various combinations of three loops borrowed from a cardiotoxin and from a short neurotoxin from the same venom.

Notably, L1AD3, a cyclic analog of loop I from the *Naja naja atra* cardiotoxin III (Table 1B), was found by Smith and Hinman (2004) to be selectively cytotoxic towards proliferating human leukemic T-cells. Micromolar concentrations of L1AD3 almost totally inhibited thymidine incorporation. In contrast to the whole cardiotoxin molecule, this cyclic peptide does not make cell membranes permeable to vital dyes. Further, L1AD3-induced apoptosis seems to occur only in human leukemic T-cells, but not in their healthy white blood cell counterparts. Thus, this compound has potential as a useful adjunct for cancer chemotherapy (Smith and Hinman 2004).

The group of α-neurotoxins comprises those venom proteins which block the neuro-muscular transmission due to direct interaction with the nicotinic acetylcholine receptors (nAChRs). α-Neurotoxins are subdivided into two groups (short-chain and long-chain ones) depending on their structure. Short-chain α-neurotoxins are built of 60–62 amino acid residues, contain four disulfides, and block only muscle nAChRs and the muscle-type nAChRs from the electric organs of eels and rays. Long-chain α-neurotoxins (up to 74 amino acid residues) have an extended *C*-terminus and an additional 5th disulfide in the central loop II. These toxins not only block muscle-type nAChRs, but also have a high affinity for the homooligomeric nAChRs composed of α7 or α9/α10 subunits.

The effects of chemical modification or mutagenesis of some residues in the central loop on the biological activity of α-neurotoxins stimulated solid-phase synthesis of the peptide corresponding to the whole loop II (fragment 16–48) of the major toxin of *Naja naja philippinensis* (short-chain α-neurotoxin, 61 amino acid residues with 4 disulfides). When Juillerat et al. (1982) tested it (Table 1B) on the isolated *Torpedo* nAChR, the peptide showed a relatively high affinity (K_d 220 nM), but still much lower than the affinity of the whole native toxin. Among the three synthesized loops of another short-chain toxin, lapemis toxin from the sea snake *Lapemis hardwickii* (see Table 1B), Miller and Tu (1991) found that only the central loop II (residues 22–39) could bind to *Torpedo californica* nAChR (K_d 40 nM as compared to 2 nM for the native neurotoxin). However, the toxicity on mice even for loop II was over 100-fold lower than for the whole-size toxin. Thus, although the other loops are probably necessary for overall toxicity, the above-mentioned publications disclosed a principal role of the central loop in α-neurotoxins for their interaction with nAChRs.

Finding the capacity of some α-neurotoxins to interact quite efficiently not only with nAChRs but also with ionotropic GABA-A receptors stimulated research on

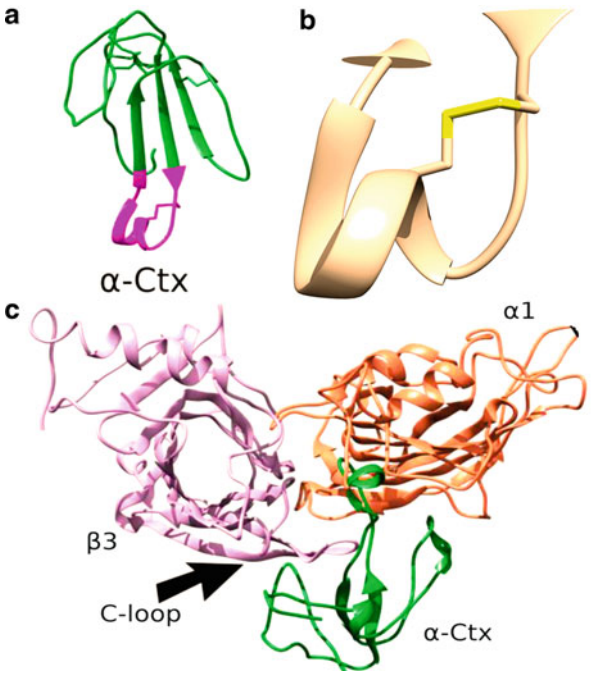

Fig. 1 (**a**) Structure of α-cobratoxin (α-Ctx) with the tip of the loop II colored in *magenta*. (**b**) Chemically synthesized WCDAFCSIRGKR peptide that mimics tip of α-Ctx loop II at 100 μM inhibited GABA-evoked current. (**c**) *Top view* of the molecular model of complex formed by α-Ctx with the orthosteric site of the GABA-A receptor. Toxin (shown *green*) resides under the loop C of the β3 (shown *plum*) subunit. GABA-A receptor α1 subunit is colored *coral* (Taken from Kudryavtsev et al. (2015))

which toxin loop is involved in binding. Kudryavstev et al. (2015) synthesized the tip of the central loop II (fragment 25–36) of the α-cobratoxin (long-chain α-neurotoxin) from the *Naja kaouthia* venom (see Table 1B and Fig. 1a, b) and found that at 100 μM, fragment 25–36 inhibited (by 28%) the agonist-induced current in the α1β3γ2 GABA-A receptor. Taking into account these data, a computer model for the receptor complex with α-cobratoxin was proposed (Fig. 1c).

A certain homology detected between the central loop fragments of some α-neurotoxins and rabies virus glycoprotein prompted the synthesis of fragments of the proteins of both types and their testing for interactions with the nAChRs by radioligand analysis on *T. californica* nAChR and through inhibition of the agonist-induced $[^{22}Na]^+$ influx on the BC3H-1 cells. The relevant results were presented in a series of publications from the Lentz laboratory (see, for example, (Lentz 1991)).

The central loop fragments of toxin b from the king cobra *Ophiophagus hannah* and α-bungarotoxin from the venom of multibanded krait *Bungarus multicinctus* (see Table 1B) had a micromolar affinity for nAChR (IC_{50} from 1 to 200 μM, depending on the peptide and the method of measurement). The analysis done by Lentz (1991), based on the activities of fragments differing in their length and on the affinity changes induced by mutations, provided the evidence for the importance of Lys27, Trp29, Phe33, Arg37, and Gly38 residues for the α-neurotoxin interaction with the nAChR. The central loop II of toxin b from the king cobra venom, with the closed disulfide bridge (Table 1B), was shown by Zhan et al. (2010) to have high affinity for the neuronal nAChRs from the rat brain (IC_{50} 32.5 nM) via competition

with the radioactive α-bungarotoxin. Moreover, this publication also revealed that the loop could be endocytosized after binding with nAChRs and thus might enhance intracranial drug delivery for the treatment of central nervous system diseases.

Synthesis of α-neurotoxin loops was also carried out for preparing the antisera and for checking the efficiency of the latter against poisoning by the whole-size toxins. The relevant examples are the fragments of toxin α from *Naja nigricollis* cobra venom, the most effective being antiserum against fragment 24–41 of the loop II (Léonetti et al. 1990). In the case of α-bungarotoxin, antisera against fragments (3–16, 26–41 и 66–74) gave positive effects (Dolimbek and Atassi 1996).

Structure-function studies on three-finger toxins of other classes, such as nonconventional toxins, muscarinic toxins, kappa-neurotoxins or mambalgins, were conducted mostly by preparing their point mutants, while data on the synthetic fragments of these proteins are lacking in the literature. The only exception is fasciculins, inhibitors of the acetylcholinesterases. Two such proteins are fasciculins-1 and -2 isolated from the venom of green mamba *Dendroaspis angusticeps*. They contain 61 amino acid residues with four disulfides and were characterized most comprehensively, including high-resolution X-ray structures of the respective complexes with acetylcholinesterases. Site-directed mutagenesis of fasiculins revealed the importance of the central loop II for the interaction with the enzyme. In view of these results, synthetic fragments of fasciculin-1 loops I and II (with the termini of each peptide connected by disulfide) were prepared by Falkenstein et al. (2004), as well as a chimeric peptide embracing the loops II and III fixed by two disulfides (Falkenstein et al. 2004) (Table 1B). These peptides confirmed the importance of the fasciculin loop II in acetylcholinesterase recognition, but their affinity (in micromolar range) was several orders of magnitude lower than that of the naturally occurring toxin. On the other hand, Kafurke et al. (2015) starting from structure-based design followed by computational optimization recently prepared synthetic peptides mimicking the binding site of human acetylcholinesterase for fasciculin-2, which bind to toxin at micromolar concentrations.

Synthetic Peptides Derived from Disintegrins and Disintegrin-Like Domain of Metalloproteinases

Disintegrins are generated in the *Viperidae* and *Crotalinae* snake venoms as a result of proteolytic processing of some snake venom metalloproteinase (SVMP) precursors which contain an appropriate disintegrin-like domain. However, it has also been shown that disintegrins may originate due to the short-coding mRNAs present in the cDNA library of venom glands without a metalloproteinase domain. In all cases, disintegrins are small (40–100 amino acid residues) Cys-rich polypeptides. Biochemical, structure-function, and other aspects of disintegrin research, as well as perspectives for their medical applications, are covered in numerous reviews (including a recent one by Calvete (2013)), while an impressive list of disintegrins known at present can be found in the review by Arruda Macedo et al. (2015). In 1987, Huang et al. (1987) found the first disintegrin, trigramin in *Trimeresurus gramineus* snake

venom, which inhibited fibrinogen-induced aggregation of platelets stimulated by ADP and aggregation of chymotrypsin-treated platelets. Synthetic tetrapeptide RGDS efficiently blocked both ^{125}I-fibrinogen binding and ^{125}I -trigramin binding to platelets. Such a characteristic activity of disintegrins was ascribed to the triplet fragment RGD of the above tetrapeptide. RGD, representing the putative platelet binding sites in fibrinogen, was found in many other disintegrins. However, as found with the synthetic fragments, various disintegrins contained some other triplets responsible for the activity, namely, MLD, KTS, KGD, VGD, and some others. Most often the fibrinogen-binding sites of disintegrin-like domains of metalloproteinases are the triplets ECD, MVD, DCD, MSE, and a few others. These active triplets were distinguished with the aid of linear synthetic peptides. However, many publications reported that the activity of disintegrin fragments (in particular, inhibitory effects on the collagen- or ADP-dependent platelet aggregation) is enhanced upon cyclization of the fragments. Literature covering various fragments of disintegrins and disintegrin-like domains comprising an "active triplet" is very abundant, but only some publications will be discussed in this review.

A series of short linear peptides was synthesized by Kamiguti et al. (1997) based on the disintegrin-like domain of jararhagin, a metalloproteinase from *Bothrops jararaca* venom, overlapping with the RGD sequence of venom disintegrins (Table 1C). Only those peptides containing the triplet ECD could effectively inhibit platelet aggregation in response to collagen, thrombin, or ADP. In addition, jararhagin and its fragments were shown for the first time to inhibit the release of 5-hydroxytryptamine (5-HT) from platelets preloaded with [^{14}C]5-HT and stimulated with collagen. The importance of cyclization of the ECD-containing synthetic peptides in the inhibition of platelet aggregation was demonstrated by Jia et al. (1997) for the disintegrin-like/cysteine-rich domains of the *Crotalus atrox* hemorrhagic metalloproteinase atrolysin A expressed in insect cells. Linear synthetic fragments of this domain, contrary to their cyclized derivatives, showed lack of inhibition of collagen-stimulated platelet aggregation (see Table 1C). The role of cyclization in the activity was confirmed by Ivaska et al. (1999) with synthetic fragments of the above-mentioned disintegrin-like domain of jararhagin. In this study, a series of short cyclic peptides corresponding to regions along the whole protein (see Table 1C) was prepared and tested, in parallel with jararhagin itself, for the capacity to directly interact with recombinant I domain (which binds such ligands as collagen, laminin, and others) in the integrin α_2 subunit, as well as for the ability to inhibit collagen binding. Only cyclic fragment (241–249) was active, while its linear form had no activity. Alanine-scanning mutagenesis revealed the importance of triplet RKK for its activity. Interestingly, fragment (421–434), an enlarged cyclic form of the peptide (423–433) (see (Kamiguti et al. 1997) above and Table 1C), did not show a marked capacity to bind integrin α_2I domain or to compete with collagen.

Synthetic fragments of metalloproteinase were prepared on the basis of the so-called Cys-rich domain as well. For example, in the unique structure of kaouthiagin, a metalloproteinase from the venom of cobra *Naja kaouthia*, the RGD sequence is located in the Cys-rich domain and the respective triplet in the disintegrin-like domain is presented by the DCD sequence. Thus Ito et al. (2001)

prepared synthetic cyclized peptides from the disintegrin-like domain and from the Cys-rich domain (see Table 1C) and examined the peptide's inhibitory effect on collagen-induced platelet aggregation. The peptides from the Cys-rich domain, encompassing the RGD sequence, were 20-fold more active, while their linear forms were not active. A series of 11 synthetic cyclic fragments of the Cys-rich domain of jararhagin (see Table 1C) was synthesized by Pinto et al. (2007) in an attempt to identify regions which could bind to von Willebrand factor (vWF), a known binding partner for jararhagin. Two fragments (365–374) and (372–379) could support binding to vWF, as well as block the recombinant cysteine-rich domain of jararhagin binding to vWF. These findings were the start for modeling the structure of jararhagin and for docking the modeled cysteine-rich structure of that protein to the A1 domain of vWF.

Application of synthetic fragments of disintegrins can be illustrated by the following two examples. Obtustatin, one of the shortest disintegrins built of 41 amino acid residues, was isolated by Moreno-Murciano et al. (2003) from the venom of the *Vipera lebetina obtusa* viper. It was characterized as the first potent and selective inhibitor of integrin $\alpha_1\beta_1$ binding to collagen IV. In contrast to known short disintegrins, the integrin-binding loop of obtustatin (amino acid residues 19–29) is two residues shorter and does not contain the classical RGD sequence. Alanine-scanning mutagenesis of this integrin-binding loop afforded 10 synthetic peptides (see Table 1C), which were tested for the inhibitory activity on the adhesion of cells transfected with $\alpha_1\beta_1$ integrin to collagen IV. The result was identification of the KTS motif as the integrin-binding sequence. A three-dimensional model of obtustatin strongly indicated that the KTS motif resides at the tip of a flexible integrin-binding loop. Bazan-Socha et al. (2004) reported a structure-functional study of three disintegrins, namely, VLO5, EO5, and EC3, belonging to heterodimeric family of snake venom-derived disintegrins, which were isolated from the *Vipera lebetina obtusa, Echis ocellatus*, and *Echis carinatus sochureki,* respectively. These are potent inhibitors of certain leukocyte integrins that act via the MLD motif present in one of their subunits and displaying specificity for different kinds of integrins, namely, α4β1, α4β7, and α9β1. Application of two synthetic fragments of VLO5 and EC3 (see Table 1C) unraveled a link between the above-mentioned specificity and the presence of a particular residue (T or A) preceding the MLD motif.

Synthetic Peptides Derived from "Disulfide Poor" Compounds (Containing Two or Less Disulfides – Sarafotoxins, Waglerins, Azemiopsin, Bradykinin-Potentiating Peptides)

Sarafotoxins

The endothelin-like peptides, sarafotoxins a–d, were first isolated by Hayashi et al. (2004) from the venom of one species of stiletto snake, *Atractaspis engaddensis*. These are highly homologous peptides that contain 21 amino acid residues and two disulfides in positions 1–15 and 3–11 (Fig. 2). A similar peptide bibrotoxin was

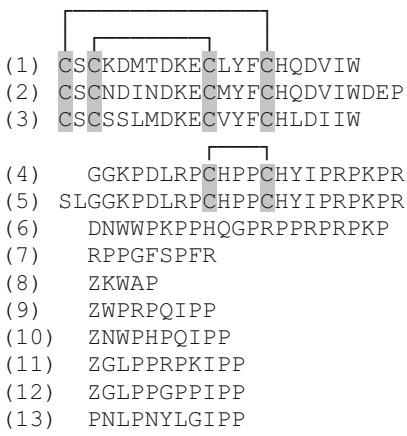

Fig. 2 Selected representatives of the "Disulfide poor" compounds: (1) sarafotoxin b, (2) sarafotoxin m, (3) endothelin-1, (4) waglerin I, (5) waglerin SL-I, (6) azemiopsin, (7) bradykinin, and bradykinin-potentiating peptides from venoms of *Bothrops jararaca* (8), (9), (10), *Agkistrodon halys blomhoffii* (11), (12), *Crotalus durissus cascavella* (13). Z – pyrrolidonecarboxylic acid

isolated later from the venom of *Atractaspis bibroni*. Several years after, the venom of *Atractaspis microlepidota* provided a number of highly homologous long sarafotoxins possessing three additional amino acid residues at the *C*-terminus (see Fig. 2 and (Hayashi et al. 2004)).

The main structural feature of the first "classical" sarafotoxins is their high (over 60%) homology with the endogenous endothelins, having the same chain length and identical disposition of the disulfide bridges (Fig. 2). Endothelins were first isolated from endothelial cells and shown to be powerful vasoconstrictor peptides. However, in the sequences of endothelins, there is a neutral protease cleavage site (involved in their inactivation), which is lacking in sarafotoxins, rendering safarotoxins resistant to hydrolysis of this type. This is why sarafotoxins exert a strong vasoconstriction of coronary blood vessels, which ultimately leads to cardiac arrest. Biological effects of endothelins and sarafotoxins are realized through the interactions with the two main types of endothelin receptors located in the cardiovascular system as well as in other tissues. The K_d of sarafotoxins differ depending on their nature and tissue localization of the receptors, but in general the affinity is high (K_d values start from 100 pM in radioligand tests) and the slow dissociation rates of receptor-ligand complexes eventually result in the increase in intracellular Ca^{2+} and in the induction of smooth muscle contraction.

In the light of high homology between endothelins and sarafotoxins, most of the publications devoted to preparation of analogs, isomers, and fragments focused on endothelins. The investigations clearly demonstrated the importance of both disulfides with the correct pairing (1–15 and 3–11) for vasoconstrictor action and for tight binding to the appropriate endothelin receptors. The presence of the C-terminal tryptophan residue and of the Asp8, Glu10, and Phe14 is also essential. Similar conclusions can be drawn from a limited number of publications dealing with the sarafotoxin analogs and fragments. In particular, in radioligand tests, a considerable decrease in the binding potency was registered, and marked reduction of biological activity (lethal, vasocontractile activities, cardiovascular effects) up to

complete loss was observed by Hirata et al. (1989) for the sarafotoxin b isomer having incorrect disulfide bridges (1–11 and 3–15). The C-terminal fragment (16–21) of sarafotoxin b and its analog (see Table 1D) did not show any contractile activity on the guinea pig bronchus. Interestingly, the same fragment of endothelins was found by Rovero et al. (1990) to produce a concentration-dependent contraction. Moreover, Heyl et al. (1993) used a series of synthetic fragments on the basis of long C-terminal linear [Cys11Ala, Cys15Ala] fragment 8–21 with the substitutions in the positions Glu/Lys9 and/or Asn/Tyr13, which are present in sarafotoxin-c and endothelin-3, respectively. Some of these analogs could interact with the endothelin receptor B, rather than A, whereas their monocyclic 11–15 disulfide-containing variants failed to bind to either endothelin ET_A or ET_B receptors.

A considerable drop in biological activity may originate not only from the shortening of the sarafotoxin molecule, but also from its extension, as demonstrated by Aumelas et al. (1999). The authors found that grafting two additional amino acid residues Lys-Arg at the N-terminus generates a salt-bridge between Arg(-1) and Asp8 in sarafotoxin b. As a result, its contractile activity decreased markedly. On the other hand, a loss of biological activity is not always undesirable. For instance, basing on the well-known fact that cutting off the C-terminal residues from sarafotoxin b is accompanied by loss of both the toxicity and vasocontractile activity (Schneider et al. 2007), its shorter nontoxic and inactive fragment (1–18) was employed as a starting point for developing metalloproteinase inhibitors (see Table 1D and (Lauer-Fields et al. 2007)).

Waglerins

These are 22–24-membered peptides bearing one disulfide in the central part of the molecule (Fig. 2) found in the *Trimeresurus (Tropidolaemus) wagleri* snake venom. Total number of discovered waglerins is four (I, II, SL-I, SL-II). Their lethality arises from the efficient interactions with the muscle nAChR, although there are publications indicating that waglerin I may affect the GABA currents in some neurons. A functional characteristic of waglerins is their considerably higher affinity for the α/ε-binding site of the nAChR than for the respective α/γ site, the amino acid residues of α and ε subunits responsible for this property being identified. Another feature of waglerins is a marked species specificity of their action: a remarkable difference in waglerin I toxicity between mice and rats was observed.

The structural elements responsible for waglerin activity were identified by Schmidt and Weinstein (1995) using chemical modifications and point mutations. In particular, the role of a single disulfide for toxicity manifestation was demonstrated. Alanine-scanning mutagenesis of waglerin I revealed the role of amino acid residues near the disulfide, particularly His10. The residues close to the N-terminus are also important as found from the low toxicity of waglerin I fragment (6–22) (see Table 1D) shortened at the N-terminal part. Three waglerin I fragments (N-, C-terminal, and the central one) (see Table 1D) were obtained by Kulon et al. (2008) to establish the influence of two adjacent residues Cys and His

on Ni^{2+} ion binding. As a result, the Cys–His motif was found to be crucial for the coordination of Ni^{2+} ions.

Azemiopsin

Based on its biological activity, this 21-amino acid peptide (Fig. 2) isolated by Utkin et al. (2012) from the venom of *Azemiops feae* viper resembles waglerins. However, azemiopsin is the first peptide from animal venoms which has no disulfides but still can efficiently block muscle nAChRs. Similarly to waglerins, azemiopsin distinguishes, although with less efficiency, two types of binding sites on the muscle nAChR and has a comparable toxicity. Alanine-scanning mutagenesis along the whole azemiopsin sequence identified the residues governing the affinity for muscle nAChR, namely, residues 4–6, 8–11 and 13 (see Fig. 3). Taking into account these data, a series of 48 pentapeptide including fragment (4–6) were synthesized by Kasheverov et al. (2015). The most active of them (see Table 1D) showed moderate inhibiting potency toward muscle-type nAChR (IC_{50} 23 μM) with low toxicity and some practical potential in cosmetology (reduction of facial wrinkles).

Bradykinin-Potentiating Peptides

The history of bradykinin-potentiating peptides (abbreviated as BPPs) seems to be quite long: the first mention of BPPs found in the venom of *Bothrops jararaca* appeared in the mid of 1960s followed by their isolation from *B. jararaca* venom (Ferreira et al. 1970; Ondetti et al. 1971) as well as from *Agkistrodon halys blomhoffii* venom (Kato and Suzuki 1971) (see Fig. 2) in the early 1970s. A few years later on the basis of BPP, Ondetti et al. (1977) designed an amino acid derivative Captopril (see Table 1D), which at present is still used as efficient drug for lowering blood pressure. Then followed discoveries of novel BPPs from different venoms (not only of snakes but also of scorpions, frogs) and their structure-functional studies using substitutions of different residues of the naturally occurring peptides or even by reversing the peptide chain direction and configuration of amino acid residues (retro-inverso analogs) (Bonelli et al. 1984). The number of BPPs has been increasing each year through isolation from different snakes, followed by cloning and sequence analysis of a *Bothrops jararaca* cDNA that encodes a precursor of seven bradykinin-potentiating peptides (Murayama et al. 1997). For different BPPs, the two activities usually are observed which are potentiation of bradykinin and inhibition of angiotensin I converting enzyme (ACE).

At present, more detailed information is available about the interactions between BPPs and ACE. Somatic angiotensin ACE contains two functional active sites and some BPPs were found to behave as selective inhibitors of the C-domain of ACE using new fluorogenic synthetic substrate of ACE. The effects exerted by BPPs as well as captopril involve interactions with ACE; therefore, human somatic ACE is an important therapeutic target for the treatment of hypertension and cardiovascular

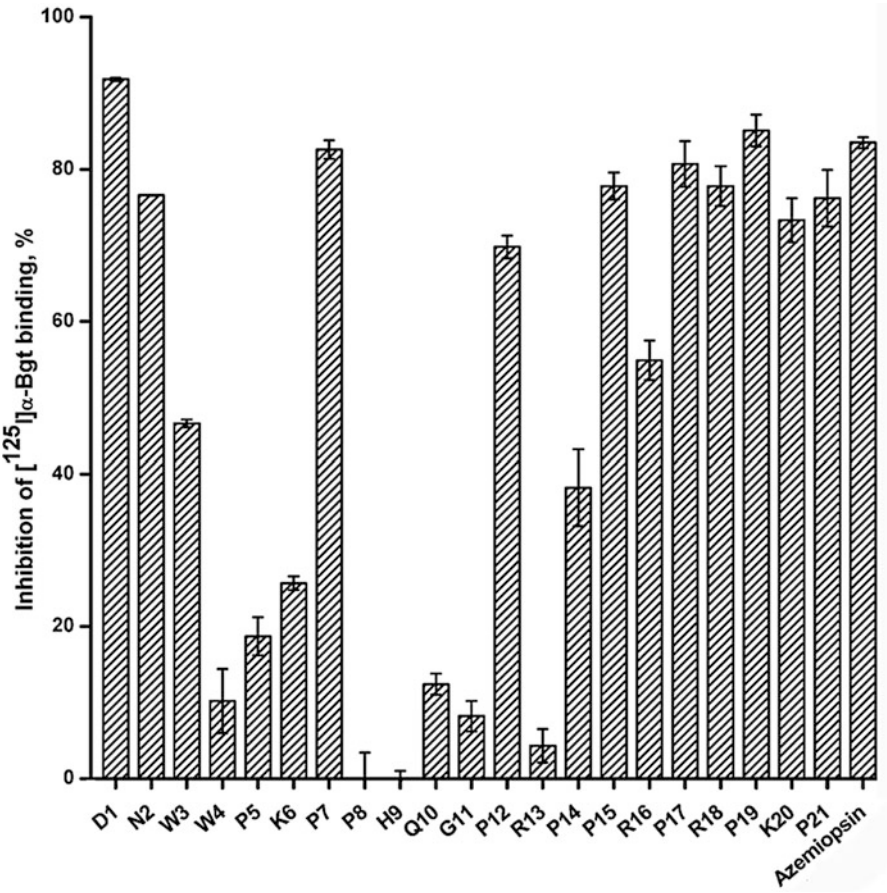

Fig. 3 Biological activity of Ala analogs of azemiopsin according to Utkin et al. (2012). *Abscissa* indicates azemiopsin amino acid residues replaced by alanine. ^{125}I-labeled-α-bungarotoxin binding in the absence of peptide was taken as control (no inhibition) (Taken from Utkin et al. (2012)

disorders. This zinc-dependent exopeptidase catalyzes the conversion of decapeptide angiotensin I to the octapeptide angiotensin II by removing a C-terminal dipeptide. At present the three-dimensional information on this enzyme and its complexes is available from the X-ray analysis of an ACE homologue from *Drosophila melanogaster* (designated as AnCE) bound to mammalian peptides (bradykinin, angiotensin I, and BPP). The results obtained show that both angiotensin II (the cleaved product of angiotensin I) and BPP bind in an analogous manner at the active site of AnCE, but also exhibit significant differences (Akif et al. 2012). Another recent X-ray analysis shows the crystal structures of ACE from human testis (designated as tACE) and of AnCE in complex with the promising selenium analog of captopril at 2.4 Å resolution. The inhibitor binds at the active site of tACE

and AnCE in a similar way as found for captopril and provides the first examples of a protein–selenolate interaction (Akif et al. 2011).

There are some indications that not all pharmacological activity of BPPs could be explained by inhibition of the activity of somatic ACE. In particular, nAChRs expressed in blood vessels may be also involved in blood pressure regulation. Although decapeptide BPP-10c from *Bothrops jararaca* venom (compound (10) in Fig. 2) did not induce nAChR-mediated ion flux, it alleviated MK-801-induced inhibition of nAChR activity, broadening possible mechanisms of the BPPs effects (Nery et al. 2008).

With respect to the diversity of BPPs, it is appropriate to mention the proteomic analysis of the *Bothrops jararaca* venom focusing on possible sex-based differences in BPPs among the males and females of the same siblings. Four new peptides were detected by Pimenta et al. (2007) only in venoms of females and identified by *de novo* sequencing as cleaved BPPs lacking the C-terminal Q-I-P-P sequence. The synthesized cleaved BPPs were less potent than the full-length BPP-10c in potentiating the bradykinin hypotensive effect, suggesting that the C-terminus is critical for the interaction of the BPPs with their mammalian molecular targets.

Nowadays a search for novel BPPs is going on. In a recent publication of Kodama et al. (2015), the peptide fractions from four snake venoms (*Bitis gabonica gabonica, Bitis nasicornis, Bitis gabonica rhinoceros*, and *Bitis arietans*), which showed inhibitory activity against ACE, were analyzed by mass spectrometry, then eight proline-rich peptides were synthesized and their biological activity analyzed *in vitro* and *in vivo*. For some peptides, inhibition assays showed inhibitory potentials of angiotensin I cleavage ten times greater as compared to bradykinin. A new structurally atypical BPP, termed BPP-Cdc (compound (13) in Fig. 2), has been recently isolated by Lopes et al. (2014) from the venom of South American rattlesnake *Crotalus durissus cascavella*. Containing the classical IPP sequence at the C-terminus, it has a completely atypical N-terminal sequence of very low homology with all other BPPs. The pharmacological effects of BPP-Cdc in general were similar to those of BPP-9a from *Bothrops jararaca* (compound (9) in Fig. 2) and captopril, but differed in duration, suggesting more selective action on arterial blood system than seen with BPP-9a.

Interestingly, most BPPs have earlier been isolated from viperids, but analysis of two elapid snake venoms identified them in those of genera *Naja* and *Notechis* (*N. m. mossambica* and the Peninsula tiger snake, *N. scutatus*). A high degree of similarity between BPPs from elapid and viperid snake venoms was observed by Munawar et al. (2014), suggesting that these molecules play a key role in snake venoms.

Conclusion and Future Directions

The subtitles in this review and especially the Table show how versatile are the protein and peptide components of snake venoms and how much efforts was spent to synthesize new peptides on the basis of these naturally occurring proteins and peptides. The major aim of that research was, and still is, to have more active and more specific peptides representing in fact the appropriate “active centers” or “active sites” of those biologically active compounds. In earlier studies, the main empirical

approaches were amino acid substitutions to first detect those active centers and then passing to shorter peptides, more or less retaining the original activity. In many cases, indeed, the fragments essential for the structural stability and for the activity of the respective protein or peptide were identified, thus giving initial information about the mechanisms of action of the original peptides and proteins. One of those still rare examples of a fast successful transfer from the snake venom component to a drug is bradykinin-potentiating peptides and the resulting drug Captopril. Only much later, the binding mode of the bradykinin-potentiating peptides to their biological target, angiotensin-converting enzyme (ACE), was elucidated due to high-resolution X-ray structure of ACE complex with the selenium analog of captopril. At present the most accurate information is provided by the X-ray analysis of the snake venom proteins and peptides (including the synthetic fragments and variants of both of them) with the respective biological targets. Here should be mentioned the X-ray structures of fasciculins (three-finger neurotoxins) in complexes with acetylcholinesterases, as well as structures of α-bungarotoxin (also a three-finger neurotoxin, but of another class) bound to a high affinity peptide (HAP), modeling a very short fragment of the nicotinic acetylcholine receptor, and the X-ray structure of α-cobratoxin in complex with the acetylcholine-binding protein, a much better naturally occurring model of the whole ligand-binding domain of nicotinic receptors. Similar structure-function information is now available for a number of snake venom proteins and peptides. However, in spite of already compiled experimental data for a huge amount of synthetic peptides designed on the basis of many snake proteins differing in structure and function, as well as on the peptides, short and long, with or without disulfide bridges and appearing to be the shortest way to a desired drug, getting such a drug is still a challenging and very difficult task. Apparently, application of new methods is required, including novel ways of computer modeling and other approaches.

Cross-References

- ▶ Crotoxin from *Crotalus durissus terrificus* and Crotoxin-Related Proteins: Structure and Function Relationship
- ▶ Myotoxin Inhibitors
- ▶ Synthetic Peptides and Drug Discovery

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Antiproliferative Effects of Snake Venom Phospholipases A_2 and Their Perspectives for Cancer Treatment

7

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Abstract

Secretory phospholipases A2 (PLA2s) are ubiquitous enzymes taking part in different biological pathways including cell growth and differentiation. Mammalian PLA2s mostly enhance tumor cell proliferation, while snake venom PLA2s (svPLA2s) are capable to inhibit it. This effect of svPLA2s was discovered almost two decades ago; however, so far its biochemical basis is not completely clear. As PLA2s are the most abundant proteins in snake venoms, their numerous biological effects including antiproliferative ones are well studied. This chapter is the first attempt to systemize the data about the biochemical mechanism of the antiproliferative effect of svPLA2s. The antiproliferative effect shows no direct correlation with svPLA2 cytotoxicity and enzymatic activity. While the products of phospholipid hydrolysis by PLA2s and their metabolites influence several cellular processes including proliferation, svPLA2 antiproliferative effects may be accomplished by other mechanisms in which cell surface membrane proteins (e.g., PLA2 receptors) and intracellular signal transduction pathways take part. As nerve growth factor (NGF), another snake venom component, also induces differentiation in neuronal cell lines, respective biochemical pathways are compared basing mostly on the data obtained for endogenous mammalian PLA2s and NGFs. The comparison uncovered the partial overlap of two pathways. Antiproliferative svPLA2s may realize their effects also through interaction with growth factor receptors and integrins. As carcinogenesis and hypercoagulation promote each other, svPLA2s possessing both antiproliferative and anticoagulant properties would be promising candidates in cancer research. svPLA2 application for the study of antiproliferative effects may boost the discovery of a new biochemical mechanism for inhibition of tumor cell growth.

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_13

Keywords
Phospholipase A2 • Anti-proliferative effect • Biochemical mechanism • Snake venom • Cancer

Contents

Introduction

Despite the impressive achievements of modern medicine, oncological diseases rank second worldwide after cardiovascular diseases as the cause of human death and thus represent a serious social problem. Acuteness of the problem lies in the inability in some cases to achieve complete recovery. In this regard, an urgent need is to find new approaches for anticancer therapy based on a more selective suppression of the tumor cell growth, and the recent works show definite progress in this direction.

In general, polypeptide substances possess a higher selectivity toward their biological targets as compared to their low molecular weight counterparts. If such a polypeptide or protein can suppress the development of tumor cells, its use in research may open a new approach to cancer therapy. At the present time, they can be utilized mainly for the characterization of the targets, responsible for the antitumor effect.

Snake venoms are complex mixtures of highly active peptides and proteins. The low amount of venom proteins, which can be injected in the victim at a single bite, suggests their high biological activity and a targeted effect. Usually, the single venom contains more than 100 of individual highly active compounds that differ in biological effects both in principle and in details. Extension of biological activities of snake venom proteins belonging to a certain structural type is realized by a mechanism of accelerated exon evolution of surface residues involved in the interaction with tissue targets. This phenomenon results in rapid production by snakes of proteins with diverse biological activities including antiproliferative ones. Snake venom proteins which have shown an antitumor effect become objects for scrupulous consideration. Thus, the antitumor effects were revealed for disintegrins, α-neurotoxins, nerve growth factor (NGF), and several other proteins. The studies of these proteins are expanding rapidly. There is one more class of proteins derived

from snake venom that affect cell proliferation. These are phospholipases A2 (PLA2s), the antiproliferative effects of which have not received so far an appropriate attention. To date, numerous evidences about their effect on tumor cells have been accumulated (see, e.g., a review on the medical and biological aspects of the problem Rodrigues et al. 2009). Despite the fact that the activation of mammalian PLA2 is associated mainly with the proliferative effect and the development of cancer (for instance, see Cummings 2007; Scott et al. 2010), PLA2s from snake venoms (svPLA2s) have been quite effective in withholding the development of tumor cells of different origins. The effects are so pronounced that svPLA2s could be considered seriously as anticancer agents (Rodrigues et al. 2009). However, the svPLA2 as a protein is a xenobiotic, a priori possessing immunogenicity that may hinder substantially its practical use. In our opinion, instead of direct attempts to apply svPLA2s for cancer treatment in vivo, it may be more reasonable to utilize them for exploration of new biochemical mechanisms involved in carcinogenesis which can open a new way for antitumor therapy. In this chapter, the biochemical aspects of the problem will be discussed. To this end, information on the structural determinants and mechanism of antitumor PLA2 action will be considered. The data published so far are fragmentary and sometimes contradictory and therefore require systematization and understanding for further targeted work.

Snake Venom PLA2s: An Overview

Phospholipases A2 (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) are widely spread in nature. They hydrolyze phospholipids and are usually most efficient on lipids with polyunsaturated fatty acids in the sn-2 position. A lot of PLA2 effects are caused by the released arachidonic acid (AA) and its metabolites as well as by lysophospholipids, another important hydrolysis product.

All PLA2s can be classified into secretory PLA2s, cytosolic PLA2s, calcium-independent PLA2s as well as the platelet-activating factor acetylhydrolases, the lysosomal PLA2s, and adipose-specific PLA2s. In total, there are 15 main groups of secretory PLA2s (designated by a roman numeral) with further division into subgroups (Burke and Dennis 2009; Schaloske and Dennis 2006). For example, in mammals at the gene level, secretory PLA2s of group II are presented by IIA, IIC, IID, IIE, and IIF subgroups. PLA2s from snake venoms belong to groups IA, IB, IIA, and IIB of secretory PLA2s. SvPLA2s of groups IA and IB are monomers with molecular mass of 12–15 kDa; "neurotoxic" venoms of Elapidae snake family contain only svPLA2s of these two groups. "Hemotoxic" snake venoms of the Viperidae family contain only svPLA2s of groups IIA and IIB. Sometimes, the svPLA2s of group II exist as dimers or higher oligomers composed of up to five subunits. SvPLA2s are major components of snake venom and are represented by a series of homologues often differing greatly by their toxicity and spectrum of biological activity. Some svPLA2s manifest such prominent properties as neurotoxicity (Rouault et al. 2006) and anticoagulant effect

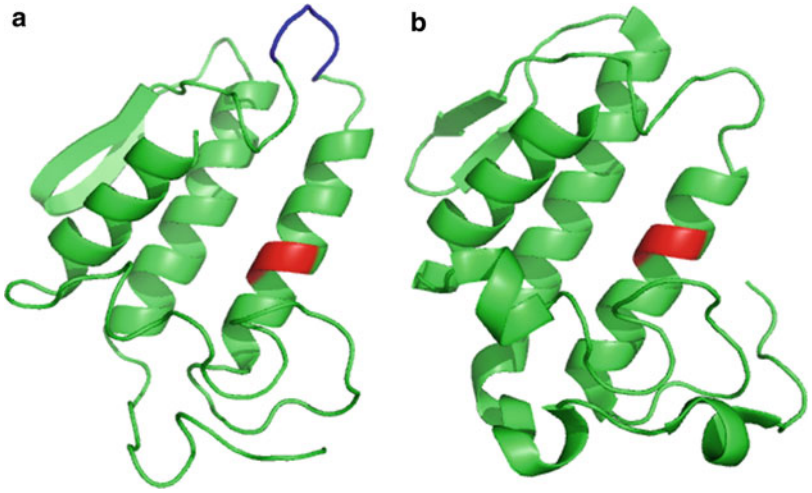

Fig. 1 Crystal structures of PLA2s. (**a**) Group IB PLA2 Mipla3 from *Micropechis ikaheka* (PDB ID: 1OZY); (**b**) group IIA ammodytoxin A from *Vipera ammodytes ammodytes* (PDB ID: 3G8G). Active site His48 and Asp49 are shown in *red*, pancreatic loop in *blue*

(Kini 2005), and their phospholipase activity is not always required for these manifestations. In some cases, it is necessary, while in others it is not (Kini 2005; Rouault et al. 2006).

Enzymatic activity of PLA2s from groups I and II is calcium dependent. Catalytic site is formed by four amino acid residues His48, Asp49, Tyr52, and Asp99 with conserved water molecule bound to His48 and Asp49 by hydrogen bonds (amino acid residue numeration as in Kang et al. 2011; Fig. 1). Depending on the residue present at position 49, the snake venom enzymes are further subdivided into Asp49 or Lys49 PLA2s; Ser49 homologues of svPLA2s from group IA are also known. Lys49 and Ser49 svPLA2s are unable to bind Ca^{2+} and have been shown to be catalytically inactive on artificial substrates. At the same time, they possess general toxicity, are very active in inducing myonecrosis in vivo, and sometimes are called as myotoxins. They often manifest a potent Ca^{2+}-independent membrane-damaging activity in vitro (Rodrigues et al. 2009).

Numerous literature data (Kini 2005; Kang et al. 2011) indicate that, despite the high degree of homology among individual proteins, different subgroups and clans within the subgroups of svPLA2s have certain structural features, which entail a clear distinction in their biological activity. There is a wealth of published data on structure and biochemical properties of svPLA2s demonstrating the molecular mechanisms of their action (de Paula et al. 2009). For example, N-terminal region is considered important for neurotoxicity (Rouault et al. 2006), while C-terminus determines the anticoagulant effect (Prijatelj et al. 2006). However, it should be noted that at the present moment the structural determinants for nonenzymatic activity of snake PLA2s still are not completely clear.

Hydrolysis of Phospholipids by PLA2 and Cell Proliferation

A number of studies (e.g., Cummings 2007; Hammamieh et al. 2007; Cai et al. 2012) have shown involvement of endogenous secretory PLA2s and the products of phospholipid hydrolysis in cellular processes related to cell proliferation. Other data indicate on their differentiating activity and cytotoxic properties. These data are briefly summarized in Hammamieh et al. (2007).

It was shown that lysophosphatidylcholine (LPC) but not other lysophospholipids or AA induced cell differentiation in rat pheochromocytoma cell line PC12 (Nakashima et al. 2003). It was established further that in PC12 LPC acted through G2A, a G-protein-coupled receptor involved in LPC signaling (Ikeno et al. 2005). Overproduction or suppression of G2A resulted in the enhancement or reduction, respectively, of neurite outgrowth induced by PLA2 treatment. Another way for LPC signaling is an activation of cytosolic phospholipase D2. Phospholipase D2 hydrolyzes the lysophospholipids with the formation of lysophosphatidic acid, which enhances cell proliferation (Moolenaar et al. 2004).

AA is cytotoxic itself both as the detergent (Farooqui et al. 2004) and as an inductor of mitochondrial permeability and swelling as well as an uncoupling of oxidative phosphorylation (Cummings 2007). However, once released, AA is rapidly oxidized by cyclooxygenases (COX) or lipoxygenases (LOX) and cytochrome P-450 to eicosanoids such as prostaglandins, prostacyclin, thromboxane, and leukotrienes. These eicosanoids modulate the cell function via binding to G-protein-coupled receptors and are able to promote cell proliferation. Both prostaglandins and leukotrienes directly stimulate the growth of malignant cells. Expression of COX and multiple LOX isoforms is increased in various cancers, and levels of their products correlate to cancer cell growth, proliferation, and invasion (reviewed briefly by Cummings 2007). For example, the presence of 12-LOX potentiates prostate cancer progression, while this enzyme is not detected in normal prostate tissue samples (Yin et al. 2011). Thus, prostaglandin E2 (PGE2), a downstream product in the AA metabolic pathway, is an angiogenic and tumor-promoting factor; genetic deletion of microsomal PGE2 synthase results in marked and persistent suppression of intestinal cancer and large adenomas (Nakanishi et al. 2008). Interleukin-1-β and leukotriene LTB4 increase basal NGF secretion (the relationship between NGF and cell proliferation will be discussed below) from rat neonatal cortical astrocytes in primary culture; treatment of cells with the PLA2 inhibitor mepacrine inhibited basal and IL-1-stimulated NGF secretion by 50% and 80%, respectively (Carman-Krzan and Wise 1993). Interestingly, different blockers of AA metabolism inhibit to varying degrees proliferation of tumor cells and have very small effect on the growth of colonies of stem cells and hematopoietic cells. The inhibitors of LOX pathways have been found to be most effective in blocking breast cancer proliferation (Hammamieh et al. 2007).

The lipoxygenase, cyclooxygenase, leukotrienes, and prostaglandins discussed above are enzymes and products in the eicosanoid pathway which includes PLA2 also. Although there is no direct relation between these molecules and svPLA2s, this paragraph has been included because all of them are downstream AA, the main

product of lipid hydrolysis by svPLA2s. In summary, the data discussed above show that, in overall, the products of phospholipid hydrolysis and their metabolites most probably increase proliferation.

Cytotoxicity Versus Differentiation

At the present time, there is no generally accepted view which of the PLA2 activities contributes mostly to its antiproliferative effect. Some results point to the cytotoxic properties, while others to differentiating ones. Moreover, there are very conflicting data about the relation between these effects of svPLA2s and their enzymatic activity. And so far it is not clear how these activities (differentiating, cytotoxic, enzymatic, and strictly speaking antiproliferative) are related with each other.

As an example, the enzymatically inactive svPLA2 MT-II from *Bothrops asper* venom can be considered. At low concentrations (0.5 μg/ml), it enhances cell proliferation in lymphoblastoid cell line CRL-8062. At medium concentrations (up to 25 μg/ml), it induces apoptosis and at high (above 25 μg/ml) necrosis (Mora et al. 2005). This example clearly shows that the cytotoxicity and the effect on proliferation can depend on the concentration of svPLA2, but not on enzymatic activity.

The secretory mammalian PLA2s are known to possess a very broad spectrum of biological activities and among other functions participate in the regulation of various cellular processes, including stimulation of neurite outgrowth in cells of neuronal origin. In 2003, it was found that PLA2s from bacteria, fungi, and bee venom induced neurite outgrowth in PC12 cells (Nakashima et al. 2003) that halted the cell proliferation and could be considered as an indicator of cell differentiation. Later, PLA2s of the groups IA and II from the venom of cobra (Elapidae) and viper (Viperidae), respectively, have been shown also to evoke the differentiation of PC12 cell line (Makarova et al. 2006).

To address the problem of cytotoxicity, it should be noted that high concentrations of cytotoxic svPLA2s induce necrosis, and this phenomenon is not specific for tumor cells only. However, some svPLA2s can provoke apoptosis in tumor cells, such as lymphoblastoid line CRL-8062 (Mora et al. 2005) or human neuroblastoma SK-N-SH; key role in the latter case belongs to the activation of Ca^{2+}- and ROS-evoked p38 mitogen-activated protein kinase (MAPK) (Chen et al. 2009).

It has been shown that the enzymatic activity of svPLA2s is not absolutely necessary for the cytotoxicity. Thus, the data about influence of svPLA2s on PC12 cells (Makarova et al. 2006) allow suggesting that antiproliferative activity and cytotoxicity of svPLA2s are not directly related. Discussing the influence of phospholipid hydrolysis on neuritogenesis, Nakashima et al. (2004) claim that "the enzymatic activity rather than the binding activity to the putative N-type receptor for neurotoxic sPLA2s is the critical determinant for the neuritogenic response." In other papers, this group (Ikeno et al. 2005) suggests that the ability of different PLA2s to stimulate growth of neurites is also in direct relation to their ability to release LPC from the cell membrane. However, this statement is also questionable.

Certainly, it is known that the addition of LPC results in the differentiation of PC12 cells (Nakashima et al. 2003) indeed, but the antitumor effect of PLA2s is not entirely determined by the ability to produce LPC.

To get an answer to this question, another groups of researchers performed an alkylation of His48 residue in the active center of Lys49 svPLA2, which itself is considered to be enzymatically inactive (Gebrim et al. 2009; Samel et al. 2013). Nevertheless, after modification, the svPLA2 cytotoxicity to different cells decreased significantly, but antitumor activity was preserved for leukemic cell line K-562 in vitro (Samel et al. 2013) and sarcoma S180 cell in vivo (Gebrim et al. 2009). Moreover, a synthetic peptide corresponding to the C-terminal fragment 115–129 of the svPLA2 amino acid sequence also showed antitumor activity and even was toxic, although a priori it was enzymatically inactive (Gebrim et al. 2009). This means that the enzymatic svPLA2 activity only promotes but does not define the anticancer properties. It is quite possible that the natural svPLA2s with low cytotoxicity and high antitumor activity may exist. SvPLA2 TI-Nh from *Naja haje* cobra venom can be mentioned as an example. This svPLA2 possesses very low enzymatic activity and is practically devoid of cytotoxicity (Osipov et al. 2010). Nevertheless, in PC12 cell line, it manifests differentiating activity that is stronger than those of toxic and enzymatically highly active svPLA2s (Osipov et al. 2010). It should be noted that the TI-Nh belongs to the group IB of secretory PLA2s and contains a fragment of amino acid sequence called "pancreatic loop" (Fig. 1) which is responsible for a significant decrease in enzymatic activity.

As to structural features determining snake PLA2 activity, at least three specific sites that affect its toxicity can be mentioned: active center, pancreatic loop, and, possibly, the C-terminal region, while antitumor activity was shown only for the C-terminal region (Gebrim et al. 2009). Thus, synthetic peptide corresponding to the C-terminal fragment 115–129 of bothropstoxin-I from *Bothrops jararacussu* venom produced the lytic action on different tumor cell lines (Gebrim et al. 2009). Injection of the peptide in mice, 5 days after transplantation of S180 tumor cells, reduced 36% of the tumor size on day 14th and 79% on day 60th. However, it should be noted that this fragment in snake venom svPLA2 is highly variable.

Thus, the above considerations suggest that the antitumor effect of svPLA2s may be expressed through the necrosis, apoptosis, and differentiation.

PLA2s and Growth Factors

As it was noted above, the induction of neurite outgrowth in neuronal tumor cell lines can be regarded as an indicator of cell differentiation. However, along with svPLA2s, snake venoms contain NGF that is also capable to induce neurite outgrowth and is effective at nanomolar concentrations, in contrast to svPLA2s, which in most cases display differentiating activity in micromolar range. NGF is a protein with the molecular mass close to that of monomer svPLA2 (~13 kDa). NGF from snake venom, being homologous to mammalian counterparts, is a member of the family of neurotrophic factors (neurotrophins) that regulates the development and

maintenance of the nervous system. To perform this function, they bind to specific receptors. There are two types of neurotrophin receptors for NGF: high-affinity TrkA and low-affinity p75. NGF activates TrkA on axon terminals and triggers activation of kinases PI3K/Akt, MEK/ERK (extracellular signal-regulated kinase), and phospholipase C signaling pathways. The signal then travels retrograde along the axon to the cell body to promote neuronal survival. Receptor p75 mediates a fundamentally different signaling pathway leading to apoptotic cell death.

These considerations pose several questions about the possible relationship or similarity in mechanisms of PLA2 and NGF activities. Do PLA2 and NGF act synergistically? Do they act competitively at the same process? Are the effects of one protein (e.g., PLA2) not caused by admixture of the other (in particular, NGF) because of not very adequate purification? These questions can be answered using the data obtained for endogenous mammalian NGFs and PLA2s capable to arrest the cell proliferation.

Biochemical pathways for PLA2 and NGF have many track crossing, but they are not identical. For examples, NGF treatment of breast cancer cells activates NF-κB resulting in the inhibition of ceramide-induced apoptosis. Inhibitors of both NF-κB and NGF receptors (both p75 and TrkA) manifest antiproliferative and pro-apoptotic activities in breast cancer cells (Naderi and Hughes-Davies 2009), while inhibition of group IIa sPLA2 attenuates NF-κB activity and also reduces proliferation but increases apoptosis in lung cancer cells (Yu et al. 2012a).

Indeed, the mechanisms of PC12 differentiation induced by PLA2 or NGF have very much in common. For example, it was found that phospholipases A2 and C probably stimulated choline acetyltransferase activity and neurite outgrowth in PC12 cells through their ability to increase cyclic AMP levels. A great deal of compounds including PLA2, phospholipase C, and NGF, which stimulate neurite outgrowth, increase intracellular cAMP level as well. All compounds which elevate cAMP level stimulate the metabolism of phosphatidylinositol and phosphatidic acid (the so-called PI effect) (Traynor 1984). Thus, a common way for PLA2 and NGF action is outlined: both of them increase the concentration of cAMP, which is followed by a "PI effect" and then by a growth of neurites.

NGF-induced neurite outgrowth can be suppressed by pretreatment of cells with selective inhibitors of tyrosine kinase (ST638 and genistein), phospholipases C (neomycin B) and A2 (p-bromophenacyl bromide and indomethacin), as well as the inhibitor of intracellular calcium mobilization, TMB-8. AA release, induced by NGF, from the cell lipids is reduced following previous inhibition of abovementioned enzymes. It is suggested that NGF requires tyrosine kinase at the early phase of action (within 60 min) in PC12h cells; later on, phospholipases C and A2 are necessary to release Ca^{2+} from intracellular stores and AA from cellular phospholipids (Tsukada et al. 1994).

The following data support synergism in PLA2 and NGF action (Masuda et al. 2008). Adenoviral expression of human PLA2 of group III (PLA2-III) in PC12 cells or dorsal root ganglion explants facilitated neurite outgrowth, whereas expression of a catalytically inactive PLA2-III mutant or use of PLA2-III-directed small interfering RNA (siRNA) reduced NGF-induced neuritogenesis. PLA2-III also suppressed

neuronal death induced by NGF deprivation. The authors assume that the differentiating activity of PLA2-III is related to LPC production, while NGF does not hydrolyze lipids. These observations suggest the potential contribution of PLA2-III to neuronal differentiation (Masuda et al. 2008). In principle, this work demonstrates that the effects of NGF and PLA2 are different; however, they complement each other: on the one hand, the overexpression of PLA2 replaces NGF; on the other hand, if PLA2 is switched off, NGF is not effective.

The augmentation in the activity of intracellular PLA2s, which belong to other main type, by NGF was demonstrated in Akiyama et al. (2004). The authors investigated the effect of NGF on the cytosolic phospholipase A2 (cPLA2) expression and its signaling pathways in PC12 cells and found that the treatment with NGF increased cPLA2 mRNA level after 4 h and its protein level 24 h after NGF addition. The NGF-induced increase in cPLA2 mRNA was inhibited by actinomycin D (inhibitor of RNA transcription). NGF caused phosphorylation of MAPKs, sustained phosphorylation of ERK1/2, and transient phosphorylation of p38 MAPK. NGF responses (cPLA2 mRNA and its protein) were inhibited by selective inhibitors for the ERK1/2 pathway, p38 MAPK, and c-Jun NH(2)-terminal kinase. Epidermal growth factor, which transiently activates ERK1/2, did not modify cPLA2 expression. Although phorbol 12-myristate 13-acetate, an activator of protein kinase C (PKC), alone showed no effect, NGF-induced cPLA2 mRNA expression decreased due to the inhibition of PKC. These findings suggest that NGF-induced cPLA2 expression is regulated by gene transcription via the ERK1/ERK2, p38 MAPK, and PKC pathways in PC12 cells (Akiyama et al. 2004). Although this study involved cPLA2s which structurally are quite different from svPLA2s, it indicated that NGF can control PLA2 expression. However, in other cancer types, specific inhibition of group IIa secretory PLA2 directly reduces viability and proliferation of cells by attenuating ERK1/2 activation (Sadaria et al. 2013).

It is interesting to note that in PC12 cells NGF stimulates inclusion of unsaturated fatty acids in diacylglycerol and then further into phospholipids (Li and Wurtman 1999). This process is opposite to that which NGF triggers through PLA2 enzymatic activity (Tsukada et al. 1994).

Thus, NGF binds to its receptors on the cell surface, and as a result a biochemical cascade (involving ERK1/2, p38 MAPK, and PKC pathways) activates intracellular PLA2s. One of the subsequent effects of this activation is an increase in the metabolism of polyunsaturated fatty acids, which being coupled with the raise of cAMP concentration finally leads to the growth of neurites. One can suggest that the exogenous svPLA2s which are always active can be transported inside the cell, for example, by a PLA2 receptor (transporter) discussed below, and then there they activate a cascade alternative to that of NGF. This scheme may explain partly why an excess of PLA2 can substitute for NGF at its deprivation.

Since the minimal active concentration of NGF required for the induction of cell differentiation is usually lower than that of PLA2 by about two orders of magnitude, the questions arise: is not an admixture of NGF (at the level of about 1%) present in the svPLA2 sample under study, and is not the observed effect due to this admixture? The answers to these questions can be found in the study of heterodimeric svPLA2s

from the venom of viper *Vipera nikolskii*. As mentioned above, these svPLA2s induce neurite outgrowth in PC12 cells. Both svPLA2s consist of two different subunits which can be separated by reversed-phase chromatography. The isolated subunits lose the capacity to induce differentiation in PC12; however, this capacity completely restores after dimer renaturation from separated subunits (Makarova et al. 2006). Such recovery would be hardly possible if neurite outgrowth was caused by an admixture of NGF. Another example was given in the work (Ikeno et al. 2005) where the important biochemical evidence for nonidentical effect of NGF and PLA2 was provided. Unlike NGF effect, neuritogenesis induced in PC12 cells by PLA2s was insensitive to tyrosine kinase inhibitor K-252a. Otherwise, inhibition of L-type Ca^{2+} channel or depletion of extracellular Ca^{2+}, which was ineffective in blocking NGF-induced neuritogenesis, inhibited neurite outgrowth induced by PLA2.

PLA2 binding to the receptors of other growth factors also may be considered as a possible way leading to cell differentiation. For example, RVV-7 from *Vipera ammodytes ammodytes* venom is an enzymatically inactive Lys49 svPLA2 with strong myoxicity. Similarly to vascular endothelial growth factor (VEGF-A165), RVV-7 binds to the extracellular domain of the kinase domain containing the receptor with subnanomolar affinity and shares the binding epitope on this receptor with VEGF-A165 (Yamazaki et al. 2005). This binding inhibits the proliferative effect of VEGF in the endothelium. The pretreatment of B16F10 melanoma cells with RVV-7 at sublethal dose significantly decreased growth of tumor in C57BL/6 mice (Yamazaki et al. 2005).

Antitumor effect has been shown for crotoxin, a svPLA2 derived from the venom of *Crotalus durissus terrificus* (Cura et al. 2002). Cytotoxicity of crotoxin has been shown on a variety of tumor cells both in vitro and in vivo; this property is now associated with type II apoptosis (via autophagy) (Yan et al. 2007). Crotoxin was most effective in suppressing growth of cells expressing high level of receptor for epidermal growth factor (EGF). No evidence for direct binding of crotoxin to this receptor was found, although pretreatment with EGF resulted in partial suppression of the antiproliferative activity of crotoxin. However, in intact A431 cells, tyrosine phosphorylation of EGF receptor was stimulated by crotoxin in a concentration-dependent manner (Donato et al. 1996). In this chapter, a direct relation of the PLA2 enzymatic activity with the antiproliferative effect was distinctly shown. Recently, it has been shown that secreted PLA2 induces proliferation in astrocytoma through the EGF receptor, which is frequently overexpressed in tumors (Hernández et al. 2010).

PLA2s and Cell Adhesion

Tumor invasion can be facilitated by expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), which is sometimes considered as a signal molecule. Intracellular secretory PLA2 activity effects ICAM-1 expression, and pharmacological inhibition of group IIa secretory PLA2 decreases ICAM-1 expression and invasion in lung cancer cells (Yu et al. 2012b).

Recently, group IA svPLA2s from the venoms of Tunisian vipers *Cerastes cerastes* and *Macrovipera lebetina transmediterranea* were described. The distinctive property of these svPLA2s was that they completely abolished adhesion and migration of various human tumor cells. The authors demonstrated that their antitumor effects were mediated by integrins αv and α5β1. The amino acid sequences of these svPLA2s do contain neither classical RGD triad nor SECD sequence, which are necessary for interactions with integrins. So other structural element(s) should be involved in these effects (Zouari-Kessentini et al. 2013). There are indications that the mammalian secretory PLA2 influences the proliferation and/or survival of limb mesenchymal cells. Treatment of these cells with exogenous PLA2 promotes cell death by activating matrix metalloproteinase-9 and increasing type I collagen degradation (Choi et al. 2009). Another svPLA2, from *Daboia russelii* venom, reduces the migration of SK-MEL-28 cells with an IC_{50} of 0.35 μg/ml, which is about one order of magnitude lower than that of crude venom (Khunsap et al. 2011).

The svPLA2s discussed above were isolated from venoms of snakes from the Viperidae family. The venoms of snakes from this family are known to contain disintegrins, proteins inhibiting integrin receptors. Without compromising by no means the purity of the abovementioned svPLA2 preparations, it should be noted that svPLA2s of groups IA and IB from cobra venoms containing no disintegrins do not disturb the adhesion of tumor cells (as, e.g., found by Osipov et al. 2010), but act by some other way, which needs to be established.

PLA2s from *C. cerastes* venom are not cytotoxic to tumor cells, and blocking of His47 in the active site leads to only a small decrease of antitumor activity. These data again evidence in favor of insignificance of phospholipid hydrolysis products and PLA2 cytotoxicity in antitumor effect.

Cancerogenesis and Hypercoagulation

Many svPLA2s can affect hemostasis. For example, the abovementioned RVV-7 (Yamazaki et al. 2005) antagonizes platelet aggregation which itself suppresses both solid tumor growth and metastasis. However, in different svPLA2s, these effects are expressed at varying degrees; moreover, they may be mediated both by enzymatic (hydrolysis of phospholipids in the complexes of the blood-clotting cascade) and nonenzymatic mechanisms (Kini 2005). With this in mind, one cannot exclude the possibility that the selective PLA2 anticoagulant with low toxicity may exist. It is well known that the processes of oncogenesis and hypercoagulation promote each other; thrombin, a central enzyme in blood coagulation cascade, is a strong mitogen. From a practical point of view, the most interesting would be noncytotoxic PLA2s possessing simultaneously anticoagulant and antiproliferative properties. TI-Nh, PLA2 from *Naja haje* venom, might be mentioned as an example. This is the only thrombin inhibitor from the venom of snakes from the Elapidae family and the only thrombin inhibitor, possessing intrinsic antiproliferative activity (Osipov et al. 2010). It is reasonable to expect that other anticoagulant PLA2s can manifest the antitumor effect as well. Indeed, the abovementioned novel tumor-suppressing

PLA2s from Tunisian viper venoms are anticoagulants and are not cytotoxic themselves (Zouari-Kessentini et al. 2013). Another thing is that their anticoagulant effect is usually pronounced enough, and therefore they may be highly toxic and even lethal in vivo because of the possible massive uncontrolled hemorrhage. It is worth to note that thrombin inhibitors of other types were found in venoms of snakes from the Viperidae family. These are C-type lectin-like proteins; however, nothing is known about their antiproliferative activity.

What Are Possible Mechanisms?

The effect of products generated by PLA2 during phospholipid hydrolysis on cell proliferation has been considered in detail above.

The available data suggest that the expression of some mammalian group IIA PLA2s correlates with either tumor promotion (very often) or inhibition (seldom), depending on the organ affected and the biochemical microenvironment of tumors (Scott et al. 2010). While mammalian PLA2s more often contribute to the tumor development, the most of svPLA2s can exert only tumor inhibition.

The data discussed above suggest that in tissues PLA2s recognize not only phospholipids but also some proteins which play a role of the receptors (Kini 2005); this means that the existence of PLA2s selectively toxic to the cells of one particular type of tumor is fairly possible. For example, taipoxin, svPLA2 from the venom of Australian elapids, is quite toxic to small-cell lung carcinoma and nontoxic to many "normal" cells (Poulsen et al. 2005). Earlier, the ability of taipoxin to bind a specific protein of pentraxin family on the cell membrane has been shown (Schlimgen et al. 1995).

No single svPLA2 exhibits all variety of biological properties pertinent to this protein family. To explain the svPLA2 functional specificity, Kini and Evans (1989) have proposed the so-called target model. According to this model, surface of a target cell or tissue has a "target site" which is recognized by a complementary "pharmacological site" in the svPLA2 molecule. This "target site" may be represented by a protein that is called a "target protein."

The affinity between svPLA2s and target proteins lies in the nanomolar range, whereas the affinity between svPLA2s and phospholipids covers the micromolar range. Such a difference may explain why the interaction of svPLA2 with target protein governs the pharmacological specificity (Kini 2005). The "pharmacological site" is independent of, but sometimes overlapping with, the active enzymatic site. Naturally, neighboring phospholipids may also contribute to the interaction.

Some types of cells have on their surface a special receptor for secretory PLA2s (basically, it binds effectively PLA2s of group IB; with lower affinity, those of groups IA, IIA, and X). This is a type I transmembrane glycoprotein related to the C-type animal lectin family. It regulates a variety of biological responses elicited by PLA2 (Hanasaki 2004). Interestingly, PLA2 receptor has endocytic properties and rapidly internalizes PLA2 ligands, which can thus penetrate into the cell. Entered into the cell, then PLA2 can exert its effect.

There are some evidences on the presence of the PLA2 receptor in tumors. Thus, the expression of the M-type PLA2 receptor was demonstrated in human ovarian carcinoma effusions (Gorovetz et al. 2006). However, in general, the data about the structure and function of PLA2 receptors (Hanasaki 2004) in tumor cells are not numerous. In particular, downregulation of PLA2 receptor 1 favors an escape from oncogenic stress-induced senescence in human epithelial cells in vitro or in mouse epidermis in vivo. The knockdown of PLA2 receptor 1 increases the transformation potential of cancer cells, whereas its ectopic expression inhibits cancer cell transformation. Further, PLA2 receptor 1 mRNA levels generally are decreased in cancers. Together, these data support a tumor-suppressive role of PLA2 receptor 1 (Vindrieux et al. 2013). However, it is not established if the binding of svPLA2s to the receptors results in antiproliferative effect. Nevertheless, it is known that the specific binding of mammalian PLA2s induces non-tumor cell proliferation (Arita et al. 1991). Indeed, proliferative effects of some endogenous secretory PLA2s were shown in several works (for instances, see Cummings 2007; Martín et al. 2009; Scott et al. 2010; Cai et al. 2012; and others). It is possible that svPLA2s, being similar to endogenous ones in general, also bind this receptor, but due to some differences in mechanism, this binding results in the opposite effect. On the other hand, several types of PLA2 receptors may exist, and some types are simply not identified yet. It is also possible that PLA2 receptors may act as carriers delivering ligands into the cell (similar to the glucocorticoid or insulin receptors), and there exogenous PLA2s incorrectly affect the intracellular components, which usually interact with endogenous PLA2s. The mentioned "target model" suggests that svPLA2s may recognize some phospholipids together with proteins within a "target site" on the cell surface. In any case, hydrolysis of phospholipids, being fairly a straightforward process, even with the release of polyunsaturated fatty acids, cannot itself mediate two opposite effects without some outside guiding influence. These effects are even difficult to explain by hydrolysis of phospholipids only. It should be added that, explaining the mechanism of PLA2 activity, Chen et al. (2009) notice that the cytotoxicity of AA and LPC is not associated with upregulation of Fas and FasL; this process, along with the activation of p38 MAPK, is induced by reactive oxygen species (ROS). Interestingly, a proliferative human group IIA PLA2 also induces ROS, activates MAPK cascade, and evokes phosphorylation of kinases Act and ERK in human astrocytoma cells (Martín et al. 2009).

With the exception of AA and LPC, what are the other cell components which may mediate the antiproliferative effect of svPLA2? Toxic svPLA2s at doses lower than those inducing PC12 cell death evoked cessation of their proliferation and then differentiation. Since Ca^{2+}- and ROS-evoked p38 MAPK was shown to be involved in the death of the cells (Chen et al. 2009) and p38 MAPK is also implicated in the induction of cell proliferation (through ADAM17), one can cautiously suggest that the antiproliferative effect of svPLA2s may arise from the binding to PLA2 receptor or to some intracellular regulatory molecule. This binding may result then in changes of kinase activity.

The blockers of endogenous secretory PLA2 (mainly for the subgroup IIA) were proposed for therapeutic use (Cummings 2007). However, how can one achieve the

blocker selectivity with regard to this subgroup? From the standpoint of antitumor drug design of the future generation, it is worth considering the possibility of modulating the receptors of endogenous PLA2s by their counterparts from the venoms.

A number of papers (Nakashima et al. 2003, 2004; Ikeno et al. 2005) argue that antiproliferative activity of PLA2s is directly related to enzymatic activity. The binding of bee venom PLA2 to the putative neuronal N-type receptor was suggested unlikely to be involved in PLA2-induced neurite outgrowth in PC12 cells (Nakashima et al. 2004). However, the data shown on Fig. 6 in Nakashima et al. (2003) are contradictory to these conclusions: catalytically inactive mutants of PLA2s from groups V and X in which the active site His residue has been replaced by Ala residue exhibit in PC12 cells neuritogenic activity that is only two times smaller than the native forms. Therefore, the His residue in the active site only promotes but does not define the neuritogenic activity of PLA2s in PC12 cells.

To attack the prey organism, the components of snake venom often use a strategy of "bad ligand," i.e., they affect the same targets as endogenous counterparts but with a perverse effect. For example, thrombin-like enzymes from venoms hydrolyze fibrinogen similarly to thrombin and induce local hypercoagulation, but in a manner that ultimately perturbs blood clotting. Cobra venom factor is similar to the C3b component of the complement system, but it activates the system in such a way that depletes it completely. α-Neurotoxins are, similar to lynx1, an endogenous modulator of nicotinic acetylcholine receptors in the mammalian brain, but instead of "modulation" they produce a complete block of the receptor. It is possible that the same strategy is utilized by svPLA2s. As a case in point, endogenous PLA2s affect certain cell structures, which, as a rule, lead to the proliferation; svPLA2s may affect the same structure(s), but, as components of snake venoms, with the perverse effect, i.e., they inhibit proliferation.

Conclusion and Future Directions

As it can be concluded from the above considerations, the problem is interesting, and the solution is very promising, but fairly complicated and poorly understood. The mechanism of the antiproliferative effect of svPLA2 is not well defined at the moment, but further study on the nonenzymatic function of this protein may give some clue on how it exerts the antiproliferative function in tumor cells. Of course, there is no certainty whether it could be the competitive binding of this enzyme with the endogenous mammalian PLA2 on the receptors that gives rise to a distorted signaling. On the other hand, the difference in the amino acid sequences between snake and mammal PLA2s could result in activation of different downstream molecular targets. Nevertheless, two conclusions can be drawn with certainty: (1) the PLA2 cytotoxicity correlates to some extent with enzymatic activity; (2) anticancer PLA2 properties generally are not directly related to the enzymatic activity. Sometimes, enzymatic activity enhances antitumor ones; in other cases,

these properties are not related at all. In general, the considerations given above suggest that svPLA2s interact with certain molecules on the cell surface or inside the cell (but not only with phospholipid substrates), and this interaction leads eventually to the cessation of the tumor development. However, it should be kept in mind that cytotoxicity, myotoxicity, and neurotoxicity are characteristic to most of svPLA2s, and these activities obviously may affect normal tissues, not only tumors.

Although the data about antiproliferative effects of svPLA2s in vitro are encouraging, the information about suppression of tumor growth in vivo (in a living organism) is very limited. Only few examples of svPLA2 antitumor in vivo activity can be mentioned: crotoxin (Cura et al. 2002), BPB-modified PLA2 BthTX-I from *Bothrops jararacussu* and its cationic synthetic peptide derived from the C-terminal region 115–129 (Chen et al. 2009), as well as two svPLA2s from *Daboia russelii* venom (Maity et al. 2007; Khunsap et al. 2011). In the two last cases, however, the cytotoxic svPLA2s had been incubated with B16F10 murine skin melanoma cells before they were injected into mice but not administered when the tumor had been already developed in animals.

Thus, to the present moment for the svPLA2s, three main modes for the inhibition of tumor growth may be proposed. These are cytotoxicity, impaired adhesion, and differentiating activity. Apparently, they are independent from each other, and in terms of use, all of them pose logical questions about the specificity of the effects. Further investigations are necessary to resolve the following problems:

1. PLA2 cytotoxicity against tumor cells is not always expressed specifically, and the cells of normal tissues may be destroyed at the same time. As a rule, cytotoxic PLA2s have a high overall toxicity in vivo.
2. It should be clarified if svPLA2s arrest the proliferation of normally dividing cells such as stem and epithelial cells and gametes, along with tumor cells.
3. The anti-adhesive PLA2 properties may affect the integrity of the endothelium of the blood vessels. It should be kept in mind that snake venom disintegrins can foster hemorrhage.
4. Finally, the structural features of svPLA2s should be determined that result in the effects opposite to the mainly proliferative action of mammalian PLA2s.

To ensure further progress in this field, answers to these practical questions should be given. But in any case, the introduction of svPLA2s as tools for the study of molecular mechanisms of carcinogenesis at the level of basic research seems quite appropriate and timely.

Cross-References

► Inflammatory Action of Secretory Phospholipases A_2 from Snake Venoms

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Part II

Conotoxins

Conotoxins and Drug Discovery with Special Reference to Hainan Species

8

Sulan Luo, Xiaopeng Zhu, Yong Wu, and Dongting Zhangsun

Abstract

Cone snails native to tropical marine waters are unique as they have ability to use various small disulfide-bridged peptides (conotoxins or conopeptides, CTxs) for prey capture. So far conotoxins have attracted much interest as neuropharmacological tools, as potential leads in drug development, or indeed as drugs themselves due to their high potency and specificity for a range of important physiological targets. Given that up to 700 species of cone snails exist in the world and over >1000 peptides are present in each species, it is estimated that more than 500,000 conotoxins may await discovery. This natural library of bioactive peptides thus provides a rich source of potential drug leads or neurophysiological probes. Less than 0.1% of this diverse library of compounds has been examined. Cone snails native to Hainan are unique biological resources of marine drugs in China. Conotoxins adopt a variety of 3D structures and can be regarded as "mini-proteins." They range from simple helical structures for non-disulfide-bonded conopeptides to tightly folded structures with cross-bracing disulfide bonds for the cystine-rich conotoxins. In general, this type of structural motif provides conotoxins with high stability compared with non-disulfide-bonded peptides. The structure and biological function of most conotoxins has not yet been defined, especially for Hainan *Conus* species. This chapter discusses Hainan conotoxins and drug discovery including their diversity, synthesis, structure-activity relationships, pharmacology, and therapeutic potential.

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_21

Keywords
Hainan cone snails • Conotoxins • nAChRs • Ion channels • Molecular target • Peptide synthesis • Recombinant expression • Structure • Function • Analgesic agents • Anti-addiction • Moleculer probes • Drug leads

Contents

Introduction

Predatory cone snails (genus *Conus*) from tropical marine waters produce a rich array of venoms and have evolved >500,000 different disulfide-rich peptides (i.e., conopeptides, or conotoxins, CTxs) (Davis et al. 2009; Espiritu et al. 2001). An estimated 500–700 different cone snail species in the world prey on fish, worms, or other snails, in which at least 1/10, >50 cone snail species native to Hainan, China (South China Sea). Every *Conus* species appears to have a repertoire of >1000 different venom components, most of which are the disulfide-rich conopeptides (Davis et al. 2009). Each *Conus* venom, moreover, has its own distinct complement of peptides, with essentially no molecular overlap between the venoms of different species (Olivera and Teichert 2007). There are more than 50,000 distinct Hainan conopeptide libraries waiting to be tapped. Over the last few decades, the conopeptides have revealed a remarkable diversity of pharmacological function and utility (Olivera et al. 1990; Myers et al. 1993). To date, only a small fraction of these conotoxins have been identified, and <0.1% have been functionally characterized. Therefore, despite longer than three decades of intensive conotoxin research, this field can be regarded as still at an early stage. Conotoxins are a real pharmaceutical cornucopia waiting to be excavated (Mayer et al. 2010; Norton and Olivera 2006).

The lethal toxins produced by cone snails are in hot demand for neuroscience research and are being developed as potent drugs. Today, the venom's painkilling properties are just one facet of a burgeoning field of research and drug development. Unlike most venomous animals, which produce one or a few poisons, a single snail can produce >1000 individual toxins (Davis et al. 2009). The researchers have demonstrated that the different venom components had specific targets in the nervous system – in particular they block different ion channels in cell membranes, which control the transmission of impulses by nerves, or specific receptors for the

neurotransmitter chemicals that transmit signals from cell to cell. One venom component discovered at the University of Utah called ω-conotoxin MVIIA causes tremors in mice (Olivera et al. 1985). Further work found that it blocks the N-type voltage-sensitive calcium channel (McCleskey et al 1987) that has been implicated in chronic neuropathic pain, caused by damage to the nervous system. The toxin has since been developed as a drug called ziconotide (the commercial product, Prialt) which was approved by the U.S. FDA in December 2004 and is now in wide clinical use for patients with cancer and AIDS who are suffering from chronic neuropathic pain that cannot be relieved by opiates (Sanford 2013). Several conopeptides are being explored as different antinociceptive agents, cardiac reperfusion, cardiovascular/BPH, epilepsy, mood, etc. for clinical development (Lewis et al. 2012).

Different groups of conotoxins whose pharmacology has been designated by various letters of the Greek alphabet target other ion channels, such as those for calcium, sodium, and potassium, or receptors and transporters for neurotransmitters such as acetylcholine, glutamate, serotonin, neurotensin, and noradrenaline (Olivera et al. 1990; Terlau and Olivera 2004). In addition to their value as drugs, neuroscientists have used conotoxins as ultraprecise tools to selectively disable particular ion channels, or to block the action of individual neurotransmitters (Janes 2005). In addition, researchers have used fluorescently labeled toxins to find the pattern of distribution of ion channels in cell membranes. Pursuing this research potential depends on the steady discovery and characterization of molecular targets of a great number of novel conopeptides. The complexity is possibly even greater as the molecular profiles of toxins produced by an individual snail are not uniform over time. Instead, the snails produce different toxins at different times possibly influenced by external conditions.

Recently Hainan conotoxins, also referred to as conopeptides discovered from cone snail species native to Hainan, China, have been undergoing accelerated research. There are around 1000 cDNA or genes found from Hainan cone snail species (Kaas et al. 2012). Some Hainan conotoxins that have been functionally characterized show great drug developing prospect. This chapter focuses on Hainan conotoxins and drug discovery including their diversity, synthesis, structure-activity relationships, pharmacology, and therapeutic potential.

Various Conotoxins from Hainan Conus Species

Three criteria, i.e., gene superfamilies, Cys frameworks, and pharmacological families, are used to classify various conotoxins (Terlau et al. 2004; Kaas et al. 2010, 2012). Diverse conotoxins could be grouped into a small number of gene superfamilies according to similarities in the endoplasmic reticulum signal sequences of the conotoxin precursors. Conotoxins identified to date may be divided into 27 gene superfamilies, including A, B1, B2, B3, C, D, E, F, G, H, I1, I2, I3, J, K, L, M, N, O1, O2, O3, P, Q, S, T, V, and Y superfamilies. More than 1000 cDNA or genes have been found from Hainan cone snail species, which

Table 1 Selected protein precursor sequences of conotoxin gene superfamilies from species of Hainan, China.

Superfamily	Peptide	Cysteine Framework & Pattern	Connectivity	Precursor Sequence (ER signal peptide, *N-terminal pro-regions*, ↓Mature peptide and ↓*C-terminal pro-regions*)	Target Receptors	References
A	LtIA	I, CC-C-C	1-3, 2-4	**MGMRMMFIMFMLVVLATTVVTFTS**↓ *DRALDAMNAAASNKASRLIALAVR* ↓GCCARAACAGIHQELC↓GGGR	α nAChRs	Luo et al. (2010), Pi et al. (2006a)
A	LvIA	I, CC-C-C	1-3, 2-4	*FRGRDAAAKASGLVGLTDRR*↓GCCSHPACNVDHPEIC↓G	α nAChRs	Luo et al. (2014)
A	SII	II, CCC- C-C- C		**MGMRMMFTVFLLVVLATTVVS**↓*FPSDRASDGRDDEAK* *DERSDMHESDRNGR*↓GCCCNPACGPNYGCGTSCS↓RTL	α nAChRs	Ramilo et al. (1992), Wang et al. (2003)
B3	αB-VxXXIVA	XXIV, C-CC-C		**METLTLLWRASSSCLLVVLSHSLLRLLG** ↓VR**C**LEKSGAQPNKLFRPP**CC**QKGPSFARHSR**C**VYYTQSRE	α nAChRs	Luo et al. (2013a)
C	contulakin-Lt1	C-C		**MRTAYWVMVMMMVGITAPLSEG** ↓*RKLNDAIRGLVADYLTPQLLQSLVSAPYPEFQLDDPNLE* ↓IPVCIWKVCPPIPW↓RRRDLKKRNK		Pi et al. (2006a)
D	Im20.2	XX, C-CC-C-CC-C-C-C-C		**MPKLEMMLLVLLILPLCYIDAGLL** ↓*EPWVLEEKEIIQRWIDR*↓NCDSPNFQAW AMCCLESRCGTGGCCSQEVCDCNGPQSKECNCPP		Liu et al. (2012)
I1	Tx11.3	XI, C-C-CC-CC-C-C		MKLCVTFLLVLVILPSVTGVKSSERTLSGAALRGDR ↓GTCSGRGQECKHDSDCCGHLCCAGITCQFTYIPCK		Wu et al. (2013)
I2	BtX	XI, C-C-CC-CC-C-C		**MMFRVTSVGCLLLVIVFLNLVVPTSA** ↓CRAEGTYCENDSQCCLNECCWGGCGHPCRHP ↓GKRSKLQEFFRQR	κ, K(+) channels	Fan et al. (2003)
I3	Lt6.1	VI/VII, C-C-CC-C-C		**MKLVLAIVLILMFLSLSAGA** ↓*ETSDNGVSRGGHRPQYWPVTPPSIV* ↓CLRSGEDCENNTPCCPGLSCRVSADLATLKLSLACD		Yuan et al. (2009)
J	Eu14.1	XIV, C-C-C-C	1-3, 2-4	**MTSVQSVTCCCLLWLMLSLTLVTP** ↓GSPGPAQLPGHRAARVPAEPILEEICP DMCNSGEGEIFCTCGSRQFVVTLPVIE↓RKRSMAV		Liu et al. (2012)
L	LtXIVA	XIV, C-C-C-C	1-3, 2-4	**MKLSVMFIVFLMLTMPMTCA**↓*GISRSATNGGEAD* *VRAHDKAANLMALLQER*↓MCPPLCKPSCTNCG		Pi et al. (2006a)

M	Vt3.1	C-C-	disulfide-crosslinked dimers	**MLKMGVVLFIFLVLFPLATLQLNA**↓*DQPVERNAENIQDL NPDKRVIKIPVP*RRR↓GPYRRYGNCYCPIG	BK channels	Li et al. (2014), Wu et al. (2010)
M	Vt3.2	C-C-	disulfide-crosslinked dimers	**LLFPLATLQLNA**↓*DQPVERNAENIQDLN PDKRFIFMPVPRRRGPYGSVHRRR*↓GPYRRHGNCFCPS↓G		Wu et al. (2010)
M	MrIIIE	III, CC-C-C-CC	1-5, 2–4, 3-6	**MLKMGVVLFIVLVLFPLATLQLDA**↓DQPVERYAENKRL LNPDERRGIILHALGQR↓VCCPFGGCHELCYCCD↓G		Han et al. (2006), Wang et al. (2008b)
M	Lt3a	III, CC-C-C-CC	1-5, 2–4, 3-6	**MLKMGVLLFTFLVLFPLTTLELD**↓*TDRPVERHAAIKQ DLKPQERRGIRLHAPR*↓DECCEPQWCDGACDCCS	ι (iota) (TTX-S) Na channel	(Wang et al. 2009)
M	VxII	II, CCC-C-C-C		**MMSKLGVLVTICLLLFPLTA**↓*LPLDGDQPADHPAK RTQDHNLASPISA*↓WIDPSHYCCCGGGCTDDCVNC		Jiang et al. (2006a)
M	Ac3.1		No cys	**LGVLVTIFLVLFPMATL**↓*QLDGDQTADRHAGERDQD PLEQYRNLKHVLRR*↓TRNYYLYPARPENSWWT		Wu et al. (2010)
M	Conomarphin-Im1		No cys	**MMSKLGVLLCIFLVLFPMATL**↓*QLDGDQTADRHA DQRGQDLTEQQRNSKRVLKKR*↓DWEYHAHPKPNSFWT↓LV		Wu et al. (2010)
M	conomarphin-Mr1		No cys	**MMSKLGVLLCIFLVLFPMATL**↓*QLDGDQTADRHAD QRGQDLTEQQRNSKRVLKKR*↓DWEYHAHPKPNSFWTLV		Han et al. (2008)
M	Lt0.3	C		**MSKLGVVLFIFLVLFPMATLQLD** ↓*GDEKDLTQQYLNLRRVLQR*↓GLVCAHASPYHNAVWS		Pi et al. (2006a)
M	Eu5	C-CC-C-C-C		**MSKLGVVLLIFVVLLALTS**↓*PHHYGNRFAGYQARQ MGVQQRKNALANAVRRS*↓GCGYLGEPCCIS PKRAYCHGDLECNNVAMCVN		Liu et al. (2012)
M	LtXVIA	XVI, C-C-CC		**MPKLGVSLFIFLVLFPLATLQLD**↓*GDQSAGRHAQ ERGEDLFKMYQYLRRALERR*RTGEDFLEECMGG CAFDFCCK↓RSLRDTTSD		Pi et al. (2006a)
N	Qc6.3	VI/VII, C-C-CC-C-C		**MHTLEMMLLLLLLLPLAPGEG** ↓*DGQAVAGDRNPSEARSTYKRFLQRPARRIDRR* ↓ECTPCGPNLCCQPGKTCGTSTHHDHYGEPACV		Lu et al. (2014)

(*continued*)

Table 1 (continued)

Superfamily	Peptide	Cysteine Framework & Pattern	Connectivity	Precursor Sequence (ER signal peptide, *N-terminal pro-regions*, ↓Mature peptide and ↓*C-terminal pro-regions*)	Target Receptors	References
O1	Lt0.1			**MKLTCMMIVAVLFLTAWTFVTA**↓*DDPRDGLEDR* ↓GGWGQAGGWGKLFS↓KARNEMKNPKASKLDNTERRI		Pi et al. (2006a)
O1	MiK41	VI/VII, C-C-CC-C-C	1-4, 2–5, 3-6	**MKLTCVLIITVLFLTACQLTTA**↓*VTYSRGEHKHR* *ALMSTGTNYRLPK*↓TCRSSGRYCRSPYDCCRRYCRRITDACV		Luo et al. (2007)
O1	MiEr92	VI/VII, C-C-CC-C-C		**MKLTCVLIVIMLFLTVCPLITA**↓*DHSRDKQEHP* *AMRLKDRIRYLRRGKLTR* ↓DCKHQNDSCAEEGEECCSDLRCMTSGAGAICVT		Luo et al. (2007)
O2	BeB54	VI/VII, C-C-CC-C-C	1-4, 2–5, 3-6	**MEKLTILLLVAAVLMSTQALI** ↓*QSDGEKRQQAKINFLS.R* ↓KSTAESWWEGECKGWSVYCSWDWECCSGECTRYYCELW		Zhangsun et al. (2006)
O2	Contryphan-S	C-C-		**MEKLTILVLVAAVLLSTQVMVQG** ↓*DADQPADRDAVPRDDNAGGTDGKFMNVQRRS* ↓GCPWEPWC↓G		Pi et al. (2006b)
O2	Contryphan-Vi	C-C		**MGKLTILVLVAAVLLSTQVMVQG** ↓*DGDQPADRNAVRRDDNPGGLSGKF* *MNVLRRS*↓GCPWHPWC↓G		Liu et al. (2012)
O2	Bt15a	XV, C-C-CC-C-C-C-C		**MEKLTILVLVATVLLAIQVLVQS** ↓*DGEKPLKRRVKQYAAKRLSALMRGPR* ↓QCTPRNQRCEGDAECCPNLVCKCFTRPDCQSGYKCDTS		Jiang et al. (2006b)
O2	LtXVA	XV, C-C-CC-C-C-C-C		**MEKLTILILVATVLLAIQVLV** ↓*QSDGENPVKGRVKHYAAKRFSALFRGPR* ↓ECTTKHRRCEKDEECCPNLECKCLTSPDCQSGYKCKP		(Pi et al. 2006b)
O3	LiCr173	VI/VII, C-C-CC-C-C	1-4, 2–5, 3-6	**MSGLGTMVLTLLLLVFMVTSHQDGG** ↓*KKQATQRNAVNIRRRKSITQR* ↓TTDEKCNEYCEERDRNCCGKANGEPRCARMCF↓G		Zhangsun et al. (2006)
P	Im9.12	IX, C-C-C-C-C-C-C	1-4, 2–5, 3-6	**MHLSLASSAVLMLLLLFALGNFVGVQP** ↓*GQITRDADNLRNLRSQWKKRGLFKSLDKR* ↓TNCEAHSCSPSCPDECYCDTNEDTCHPERRGH		Liu et al. (2012)
P	Lt9a	IX, C-C-C-C-C-C-C		**MTLTKSAVLILVLLLAFDNFA** ↓*DVQPGLITMGGGRLSNLLSKR* ↓VRIWFCASRTCSAPADCNPCTCESGVCVDWL		Pi et al. (2006a)

Q	Fla16.1	XVI, C-C-CC		**MHTLEMLLLLLLLSPLALG** ↓*EGDGQAVAGDRNPSEARSTHEHFLQKLLGRIDGR* ↓DCQPCGHDVCCPP		Lu et al. (2014)
S	Ac8.1	VIII, C-C-C-C-C-C-C-C-C-C		**LKMGAMFVLLLLFTLASS** ↓*QQEGDVQARKTSLKSDFYRALRQYDR* ↓QCTLVNNCDRNGERACNGDCSCEGQ ICKCGYRVSPGKSGCACTCRNAK		Liu et al. (2008)
S	Tx8.1	VIII, C-C-C-C-C-C-C-C-C-C		**LKMGAMFVLLLLFTLASS** ↓*HREGDIQARKTHLKSDFYRTLPRFAR* ↓GCTISCGYEDNRCQGECHCPGKTNCYCTSGHHNKGCGCAC		Liu et al. (2008)
T	BeB34	V, CC-CC	1-3, 2-4	**MRGLPVFVILLLLIASEPSVDA** ↓*RPKTKADVPLTSLNDNAKRTLQILRNKR*↓ACCPYEPSCCI		Luo et al. (2006)
T	Lt5d	V, CC-CC-	1-3, 2-4	**MRCLPVFIILLLLIPSAPSVDA***QPTTK* *DDVPLASLHDNAKRALQMFWNKR* ↓DCCPAKLLCCNP	(TTX-S) Na channels	Pi et al. (2006a)
T	Pu5.1	V, CC-CC-	1-3, 2-4	MRCVPVFVILLLLIASTPSVDA ↓*RPNPKDDVPLASFHEDANGILQMLWKKGR* ↓SCCPSPTSCCPW↓GKRK		Peng et al. (2007)
T	TxVC	V, CC-CC	1-3, 2-4	KPCCSIHDNSCCGL-NH2	α-nAChRs	Liu et al. (2008)
T	CMrX	X, CC-C.[PO]C	1-4, 2-3	**MRCLPVLIILLLLTASAP**↓*GVDVLPKTEDDV* *PLSSVYGNGKSILRGILRK*↓GICCGVSFCYPC		Liu et al. (2012)
V	ViXVA	XV, C-C-CC-C-C-C-C		**MMPVILLLLLSLAIRCADG**↓*KAVQGDSDPSASL* *LTGDKNHDLPVKR* ↓DCTTCAGEECCGRCTCPWGDNCSCIEW↓GK		Peng et al. (2008)
V	Vt15.1	XV, C-C-CC-C-C-C-C		**MMPVILPLLLSLAIRGGDG** ↓*QAIQGDRDLIAKLFKRYQEHGLSVKR* ↓ACHTCDDGTECCDSRCSCPWNTCTCIPW↓GK		Peng et al. (2008)
Y	CaXVIIA	XVII, C-C-CC-C-CC-C		**MQKATVLLLALLLLLPLSTA** ↓*QDAEGSQEDAAQREVDIATR* ↓CGGTGDSCNEPAGELCCRRLKCVNSRCCPTTDGC		Yuan et al. (2008)

belong to 21 different gene superfamilies. Selected protein precursor sequences of conotoxin gene superfamilies from Hainan species are shown in Table 1. None of the Hainan conopeptides discovered to date could be assigned to B1, B2, E, F, H, or K gene superfamily. Initially conotoxins are synthesized as precursor proteins that are processed subsequently into the mature toxin (Woodward et al. 1990). Cysteine frameworks are the arrangement of cysteines along the mature peptide region, which influence the final three-dimensional structure of conotoxins. According to cysteine frameworks, conotoxins are grouped into 26 cysteine patterns, such as pattern I, CC-C-C; II, CCC-C-C-C; III, CC-C-C-CC, etc. (Ye et al. 2012). Cysteine number of Hainan conotoxins is from none (e.g., Lt0.1) to 10 (e.g., Im20.2) (Table 1). The conotoxins are also classified into pharmacological families according to the specific receptor or ion channel target. Hypervariation exists in non-Cys residues within a given family of conotoxins, which is believed to enable selective action on a given target subtype (Olivera 2006). Most Hainan conotoxins are not functionally characterized to date.

New native conopeptides have been obtained by venom purification historically (Mayer et al. 2010). With advances in molecular biology, more and more novel conotoxins are identified by nucleic acid analysis recently (Kaas et al. 2010). Conotoxins are translated as prepropeptide precursors initially, which are cleaved by proteolysis and yield the final product(s) (Woodward et al. 1990; Kaas et al. 2012). Conopeptides within the same superfamily have highly conserved prepropeptide precursor sequences, which allows the search for and direct identification of new peptides belonging to a particular superfamily by cDNA sequencing (Santos et al. 2004; Zhangsun et al. 2006). Most Hainan conotoxins have been identified by gene cloning or cDNA library sequencing (Table 1).

Hainan Conotoxins Targeting nAChRs

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated cationic channels, which are broadly distributed throughout the central and peripheral nervous systems of animals (Gotti and Clementi 2004). The nAChRs are very important in the nervous system because their structures are highly conserved over a wide species. The seven families of conotoxins (α*-CTxs) that target nAChRs include family α-(Myers et al. 1991), αA- (Jacobsen et al. 1997), αC- (Jimenez et al. 2007), αD- (Loughnan et al. 2006), αS- (Teichert et al. 2005), αB- (Luo et al. 2013a), and αO-CTxs (Luo et al. 2015). Overall, ten Hainan α*-CTxs are known to target nAChRs (Table 2). The α-conotoxins are among the smallest conopeptides with 12–20 amino acid residues, and selective antagonists of various nAChR subtypes (Lewis et al. 2012). Seven α-conotoxins from Hainan cone snails α-CTx LtIA, LvIA, TxIB, TxID, Qc1.2, Ac1.1a, and Ac1.1b were discovered to block nAChRs (Table 2). The other Hainan α*-CTxs are αB-VxXXIVA, αO-GeXIVA, Pu14a, and Lt14a (Table 2). Generally the globular form of α-conotoxins is active isomer with two disulfide bonds, which has a disulfide connectivity of Cys I–II, III–IV (Fig. 1a).

Table 2 Sequence, nAChR subtype selectivity preference and IC50 values etc. of Hainan α*-conotoxins

Peptide	Species	Diet	Sequence	Residue number	Mass (mono)	nAChR select	IC_{50} [nM]	References
α-LtIA	*C. litteratus*	V	GCCARAACAGIHQELC*	16	1599.68	α3β2 α6/α3β2β3	9.79 84.4	Luo et al. (2010)
α-LvIA	*C. lividus*	V	GCCSHPACNVDHPEIC*	16	1678.64	α3β2 α6/α3β2β3 α6/α3β4	8.7 108 121	Luo et al. (2014)
α-TxIB	*C. textile*	M	GCCSDPPCRNKHPDLC*	16	1738.68	α6/α3β2β3	28.4	Luo et al. (2013b)
α-TxID	*C. textile*	M	GCCSHPVCSAMSPIC*	15	1488.58	α3β4 α6/α3β4	12.5 94	Luo et al. (2013c)
αB-VxXXIVA	*C. vexillum*	V	VRCLEKSGAQPNKLFRPPCCQ KGPSFARHSRCVYYTQSRE^	40	4622.27	α9α10	1200	Luo et al. (2013a)
αO-GeXIVA	*C. generalis*	V	TCRSSGRYCRSPYDRRRR YCRRITDACV^	28	3450.66	α9α10 nAChR	3.8	Luo et al. (2015)
α-Ac1.1a	*C. achatinus*	P	NGRCCHPACGKHFNC*	15	1640.63	α1β1γδ	35.9	Liu et al. (2007b)
α-Ac1.1b	*C. achatinus*	P	NGRCCHPACGKHFSC*	15	1613.62	α1β1γδ	25.8	Liu et al. (2007b)
Qc1.2	*C. quercinus*	V	ZCCANPPCKHVNCR^	13	1410.54	α3β2 α3β4	N.D.	Peng et al. (2009)
Pu14a	*C. pulicarius*	V	DCPPHPVPGMHKCVCLKTC^	19	2059.92	α1β1γδ, α6/α3β2 α3β2	N.D.	Peng et al. (2010)
Lt14a	*C. litteratus*	V	MCPPLCKPSCTNC*	13	1390.53	nAChR	N.D.	Peng et al. (2006), Sun et al. (2011)

Notes: *P* fish, *M* molluscs, *V* worms, * C-terminal amidation, *N.D.* not determined, *Z* pyroglutamic acid, ^ C-terminal. All the Hainan α*-conotoxins in the table contains 4 cysteine residues and two disulfide bonds.

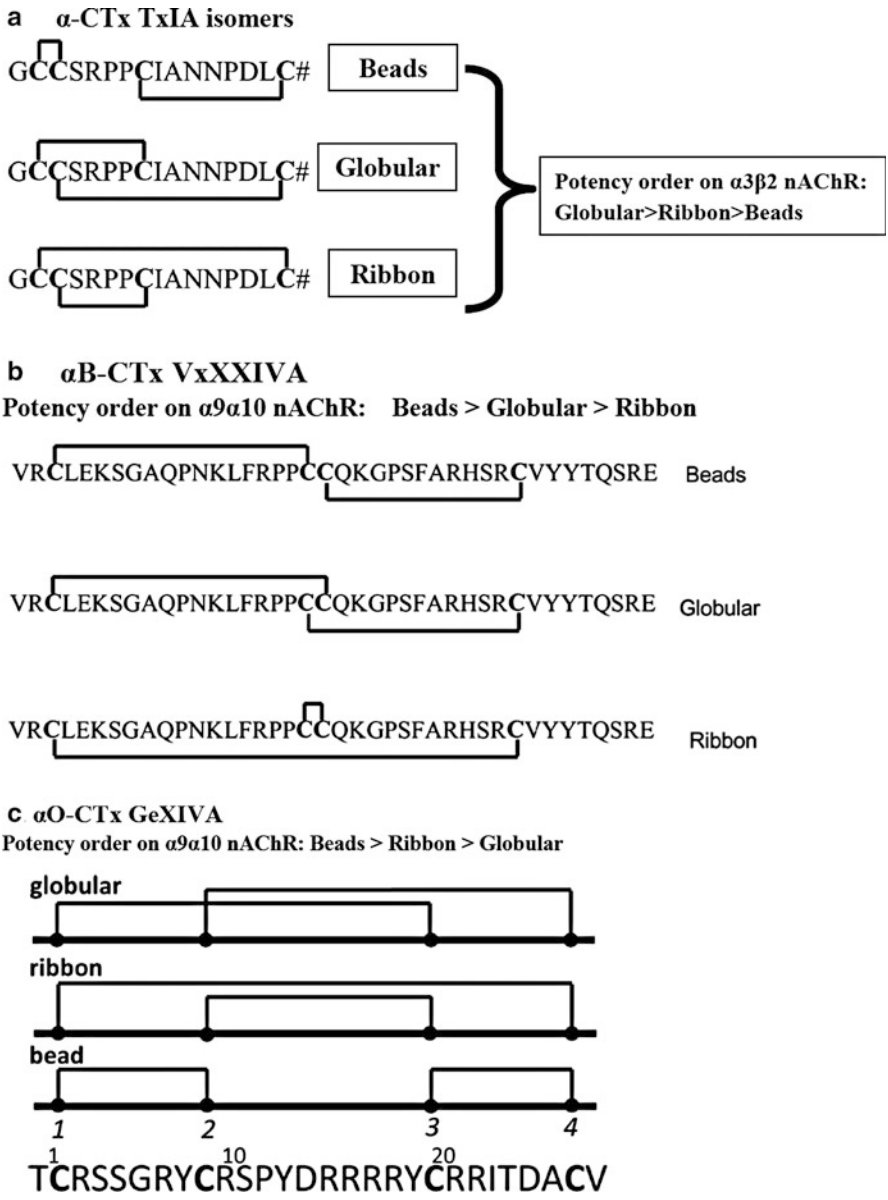

Fig. 1 Disulfide bond isomers and their activities of representative Hainan α*-conotoxins (Luo et al. 2013a, 2015; Wu et al. 2014)

α-CTx LtIA (α-LtIA, LtIA) containing 16 amino acids is from *Conus litteratus* with an atypical sequence, which targets a novel microsite of the rat α3β2 nAChR subtype with a 9.8 nM IC_{50}) (Luo et al. 2010). α-CTx LtIA lacks highly conserved motif of Ser-Xaa-Pro. This conserved motif is crucial for potent nAChR interaction

with the other known α-conotoxins. 1 μM LtIA could block ACh-evoked currents on α3β2 nAChRs completely, while 10 μM LtIA showed little or no block of rat α4β2, α3β4,α2β2, α2β4, α4β4, α7, α9α10 and mouse α1β1δε nAChRs. LtIA is also active on α6/α3β2β3 nAChRs with an IC50 of 84.4 nM, and much less potent on α6/α3β4 nAChRs with an IC50 of 5990 nM. When a β4 rather than β2 nAChR subunit was coexpressed with the α3 subunit, the IC_{50} for LtIA on α3β4 was more than 1000-fold higher than IC_{50} of α3β2. This indicates that the amino acid residue differences between the homologous β subunits (β4 and β2) influence LtIA potency significantly. The off-rate of LtIA was rapid compared with α-conotoxin MII with Ser-Xaa-Pro motif, an α3β2 nAChR antagonist. The binding sites of LtIA and MII to α3β2 nAChRs apparently overlap because preblock of α3β2 nAChRs with LtIA prevented the slowly reversible block by MII. Mutations of α3β2 nAChRs and molecular docking simulations proved that LtIA had a surprisingly shallow binding site on the α3β2 nAChR that includes β2 F119 and K79. LtIA had a >900-fold increased IC_{50} of 9190 nM on α3β2(F119Q) than IC_{50} of 9.79 nM on wild type nAChRs, which was not like MII. LtIA binding was disrupted by the K79A mutant, but without effect on an LtIA analog (LtIA[A4S;A6P]) with the Ser-Xaa-Pro motif.

α-CTx LvIA (α-LvIA, LvIA) from *Conus lividus* blocks rat and human α3β2 versus α6/α3β2β3 nAChRs selectively (Luo et al. 2014). It is a 16-amino-acid peptide with two disulfide bonds and C-terminal amidation. LvIA is the first α-Ctx reported to show high selectivity for α3β2 versus α6β2 nAChRs, which has the highest affinity for α3β2 nAChRs with an IC_{50} of 8.7 nM. 100 nM LvIA could block >90% of α3β2 nAChR ACh-evoked currents. The block was fast reversible and required 2 min of toxin washout only. The IC_{50}s of LvIA on α6/α3β2β3, α6/α3β4, and α3β4 nAChRs were >100 nM. The next highest affinities for LvIA binding were found at α6/α3β2β3 and α6/α3β4 nAChRs with an IC_{50} of 108 nM and 121 nM respectively. The IC_{50}s of LvIA on all other subtypes were ≥3 μM, including α2β2, α2β4, α4β2, α4β4, α7, α9α10, and α1β1δε nAChRs. Even 10 μM LvIA showed little or no block of α2β2 or adult muscle nAChRs. LvIA selectivity at human α3β2 versus α6β2 subtype showed 300-fold greater preference for α3β2 over α6β2 nAChRs. Previously characterized α-conotoxins, e.g., PeIA, MII, LtIA, BuIA, etc. lack selectivity between α3β2 and α6/α3β2β3 nAChR subtypes.

α-CTx LvIA can differentiate the α3β2 nAChR subtype from the α6β2* and α3β4 nAChR subtypes. The key residues in the nAChR β2 subunit contribute to LvIA binding determined by receptor mutations (Zhangsun et al. 2015). Residues of the β2 subunit versus those of the β4 subunit on the binding of LvIA on rat α3β2 nAChR were mutated to assess their influence at LvIA potency. Seven α3β2 nAChRs mutants on the binding of α-CTx LvIA were investigated, in which the four mutants, α3β2[F119Q], α3β2[T59K], α3β2[T59L], and α3β2[V111I] affected LvIA binding significantly. Three mutants, α3β2[Q34A], α3β2[K79A], and α3β2[T59I] had little or no detectable effect on LvIA activity. The α3β2 receptor mutants influenced LvIA recovery significantly too. Recovery of wild-type α3β2 and α3β4 nAChRs were 2 min and 20–26 min after toxin washout respectively. The mutant α3β2[T59K] showed the slowest recovery time, with less than 3% recovery 20 min after washout. The β2 mutation α3β2[V111I] substantially reduced LvIA potency.

Residue V111 is critical and facilitates LvIA binding, although this residue has not previously been identified as important to binding of other α-conotoxins. The two β2 mutations α3β2[F119Q] and α3β2[T59K] strongly increased the affinity of LvIA indicating interference of LvIA binding by Thr59 and Phe119 of the β2 subunit. Therefore β2 subunit contributes to LvIA binding and selectivity. The three positions on the receptor β2 subunit, T59, V111, and F119 are key to LvIA binding.

α-CTx TxIB (α-TxIB, TxIB) from *Conus textile* that targets rat α6/α3β2β3 selectively showed little or no block of other tested subtypes at concentrations up to 10 μM (Luo et al. 2013b). TxIB is a novel 16-amino-acid C-terminally amidated peptide with two disulfide bonds. TxIB blocked rat α6/α3β2β3 nAChRs potently with an IC_{50} of 28 nM, which is the most selective ligand of this subtype. The block of α6/α3β2β3 nAChR by TxIB was rapidly reversible. Substantial block of ACh-evoked currents by TxIB with 1 μM toxin on α6/α3β2β3 nAChR was observed, but 10 μM TxIB had little or no block of rat α6/α3β4, α3β2, α3β4, α2β2, α2β4, α4β2, α4β4, α7, α9α10 and Mouse α1β1δε nAChRs. Both amino acid residue differences between the highly homologous α6 and α3 subunits and between the homologous β2 and β4 subunits influence TxIB potencies, as supported by the >400-fold higher IC_{50} of TxIB on α6/α3β2β3 than α3β2 and α6/α3β4 nAChRs.

α-CTx TxID (α-TxID, TxID) is an α-4/6-conotoxin from *C. textile*, which potently blocks rat α3β4 nAChRs with a 12.5 nM IC_{50}. TxID consists of 15 amino acid residues with two disulfide bridges, which is most selective and potent α3β4 nAChR antagonist. TxID also blocked rat α6/α3β4 nAChRs with an IC_{50} of 94 nM, but TxID showed little or no block on the other rat nAChR subtypes, including α3β2, α4β4, α4β2, α6/α3β2β3, α2β2, α2β4, α9α10, α7, α1β1δε. ACh-evoked currents of α3β4 nAChR were blocked completely by 1 μM TxID, however 10 μM TxID showed no block at α4β4, α3β2, and α7 nAChRs. The block of α3β4 nAChR activity by TxID was reversible and could be washed out within 4 min. The IC_{50} for TxID on α4β4 and α3β2 was >1000-fold higher than α3β4 nAChR. So the amino acid residue differences between the homologous α3 and α4 subunits and between the highly homologous β2 and β4 subunits both affect TxID potency significantly. The addition of toxin TxID to α3β4 nAChR resulted in the reduction of the magnitude of the maximum ACh response suggesting a noncompetitive block. Especially, the only other α4/6 conotoxin AuIB from *C. aulicus* also targets the α3β4 nAChR with an IC50 of 750 nM (Luo et al. 1998). TxID is 60-fold more active than AuIB. Furthermore, AuIB also blocks α7 nAChR subtype and showed different features from TxID.

αB-CTx VxXXIVA (αB-VxXXIVA, VxXXIVA) from *Conus vexillum* is a novel inhibitor of α9α10 nAChRs and delineates a new conotoxin superfamily B (Luo et al. 2013a). Previously characterized conotoxins are expressed initially as prepropeptide precursors with three regions, i.e., a signal sequence, a "pro" region, and the toxin-encoding region (mature toxin). However, αB-VxXXIVA precursor consists of two regions only, i.e., signal region and mature toxin region, which lacks a "pro" region. VxXXIVA mature peptide is composed of 40 amino acid residues, which contains 4 Cys framework (C-CC-C, Table 2). There are three possible

disulfide arrangements for VxXXIVA. Its three isomers, αB-VxXXIVA[1,2], αB-VxXXIVA[1,3], and αB-VxXXIVA[1,4], were synthesized respectively (Fig. 1b). αB-VxXXIVA[1,2] with a disulfide connectivity I–II, III–IV is the most potent isomer on α9α10 nAChRs with an IC50 of 1.2 μM. αB-VxXXIVA[1,3] with I–III, II–IV was less potent than αB-VxXXIVA[1,2] on α9α10 nAChRs with an IC_{50} of 3.9 μM. αB-VxXXIVA[1,2] and αB-VxXXIVA[1,3] had little or no activity on other tested nAChR subtypes with IC_{50} >10 μM for α2β2, α2β4, α3β2, α3β4, α4β2, α4β4, α6/α3β2β3, α6/α3β4, and α7 nAChRs. αB-VxXXIVA[1,4] with I–IV, II–III is not active on α9α10 nAChRs, which blocked less than 15% of the current at the highest concentration tested of 30 μM. αB-VxXXIVA[1,4] was not active at other major nAChR subtypes either. αB-VxXXIVA[1,2] with I–II, III–IV disulfide bonds is twofold more active than the I–III, II–IV form αB-VxXXIVA[1,3]. The low affinity of αB-VxXXIVA[1,2] for α9α10 nAChRS in the micromolar range suggests the possibility that αB-VxXXIVA might eventually be found to act on other ligand- or voltage-gated ion channels besides blocking nAChRs.

αO-CTx GeXIVA (αO-GeXIVA, GeXIVA) is a very potent rat α9α10 nAChR antagonist from *Conus generalis* (Luo et al. 2015). GeXIVA consists of 28 amino acid residues, of which 9 are arginine (R) and account for a large proportion (~1/3 or 32%) of the peptide. It has four Cys residues with C-C-C-C framework. Three disulfide isomers of GeXIVA, i.e., GeXIVA[1,2] (bead form), GeXIVA[1,3] (the globular form), and GeXIVA[1,4] (ribbon form), were synthesized. GeXIVA[1,2] has a disulfide connectivity I–II, III–IV, GeXIVA[1,3] has a I–III, II–IV connectivity, and GeXIVA[1,4] has a I–IV, II–III connectivity (Fig. 1c). The bead isomer GeXIVA[1,2] was the most potent at the α9α10 nAChR with an IC_{50} of 3.8 nM in Ba^{++} ND96 buffer and 4.6 nM in regular ND96 buffer. The second potent isomer was the ribbon form GeXIVA[1,4] with an IC_{50} of 5.8 nM in Ba^{++} ND96 and 7 nM in ND96. The least active isomer was the globular form GeXIVA[1,3] with an IC_{50} of 37 nM in Ba^{++} ND96 and 22.7 nM in ND96 surprisingly. Each isomer showed similar activity on α9α10 nAChR both in Ba^{++} ND96 and regular ND96, which confirmed the toxin effect being due to blockade of the α9α10 nAChR rather than block of Ca^{++} activated chloride channels in *Xenopus* oocytes. ACh-evoked current of α9α10 nAChR was blocked completely by 100 nM GeXIVA[1,2] but the block was reversible rapidly. GeXIVA[1,2] was substantially less potent on the other nAChR subtypes than α9α10 nAChR. The IC_{50} ratios of each nAChR subtype compared to that of α9α10 ranged 86 ~ 1100-fold. The next two affinity subtypes that GeXIVA[1,2] was active at were mouse muscle nAChR (α1β1δε) and rat α7 nAChR with similar IC_{50}s of 394 nM and 415 nM respectively. The least active subtypes of GeXIVA[1,2] were α3β4 and α2β4 nAChRs with IC_{50}s >5300 nM, which were >1100-fold less active than potency of α9α10 nAChR. The amino acid residue differences between the homologous β2 and β4 subunits significantly influence GeXIVA[1,2] potency as proved by the much higher IC_{50} of GeXIVA [1,2] when a β4 rather than β2 nAChR subunit was coexpressed with the α2, α3, α4, and α6 subunit.

Mechanism of block of α9α10 nAChR by GeXIVA[1,2] was elucidated. The block of rat α9α10 nAChR by GeXIVA[1,2] was strongly voltage-dependent. an

illustration of the peptide as the first voltage-dependent antagonist of α9α10 nAChRs. The IC_{50} of GeXIVA[1,2] in ND96 at +30 mV was 24-fold bigger than that at −70 mV. Similarly the IC_{50} at +30 mV membrane potential was 32-fold bigger than that observed at −70 mV in Ba^{++} ND96. The observation in this work that more current passed through the channels at hyperpolarized potentials markedly is consistent with previous reports (Elgoyhen et al. 2001). Voltage-dependent block of GeXIVA[1,2] at α9α10 nAChR suggests the toxin binding to an allosteric site or a channel pore of the receptor rather than the ACh-binding site, which was supported by following series of competitive experiments. The block of GeXIVA[1,2] at α9α10 nAChR was rapidly reversible upon toxin washout. Therefore a slowly washout reversed toxin, RgIAm, was chosen for competitive experiments. RgIAm is an analog of α-conotoxin RgIA, a competitive inhibitor of α9α10 nAChRs with sequence GCCTDPRCCitTyr(I)QCR, where Cit = citrulline and Tyr(I) = monoiodo-Tyr (Azam and McIntosh 2012; Ellison et al. 2008). Native RgIA has rapid disassociation kinetics and was chosen as a control that has the same binding site as its analog RgIAm on α9α10 nAChR. Pre-incubation with a high concentration of GeXIVA[1,2] failed to prevent subsequent access to the nAChR by RgIAm, while pre-incubation with RgIA prevented subsequent binding of RgIAm. The concentration dependence of the effects of pre-incubation of RgIA or GeXIVA[1,2] on the washout kinetics of block by 20 nM RgIAm was tested, which showed RgIA preventing the subsequent binding of RgIAm. In contrast GeXIVA[1,2] did not prevent the subsequent binding of RgIAm as well. Increasing GeXIVA[1,2] concentrations had no effect on the recovery from block by GeXIVA[1,2] + RgIAm at any GeXIVA[1,2] concentration tested, which demonstrated GeXIVA[1,2] binding to a separate site of α9α10 nAChR from RgIAm. So GeXIVA[1,2] and RgIAm have distinct, non-overlapping binding sites on the α9α10 nAChR.

Precursor signal sequence of GeXIVA is similar to the O1-gene superfamily. Previously pharmacologically characterized O1-superfamily peptides contain six Cys residues and are antagonists of calcium, sodium or potassium channels. However, GeXIVA did not inhibit calcium channels but was the most potent antagonist of α9α10 nAChR subtype with an IC_{50} of 3.8 nM in Ba^{++} ND96. Effects of GeXIVA[1,2] on chronic constriction injury (CCI)-induced mechanical hyperalgesia were assessed (Fig. 2). GeXIVA[1,2] by an intramuscular (i.m.) injection reduced mechanical hyperalgesia induced by CCI significantly, which showed increased paw-withdrawal threshold (PWT) relative to Pre-CCI (t = 0) and relative to the saline-treated group obviously. After injection of GeXIVA[1,2], the strong increase in PWT persisted at least 6 h. Analgesic effects of 1 and 2 nmol GeXIVA[1,2] on CCI rats did not differ from the effect of 500 nmol morphine ($p > 0.05$) significantly. Furthermore, GeXIVA[1,2] with 2.5 nmol dose had no significant effect on performance of rats in the accelerating rotarod test during any of the time points 1, 2, 4, 6 h after i.m. injection. So GeXIVA[1,2] did not impair motor performance of rats, which did not interfere with hyperalgesia testing.

α-CTx Ac1.1a (α-Ac1.1a, Ac1.1a) and α-CTx Ac1.1b (α-Ac1.1b, α-Ac1.1b) are two α3/5 conotoxins from fish-hunting *C. achatinus*, both of which are potent antagonists of mouse muscle nAChRs (α1β1γδ) (Liu et al. 2007b). There is only

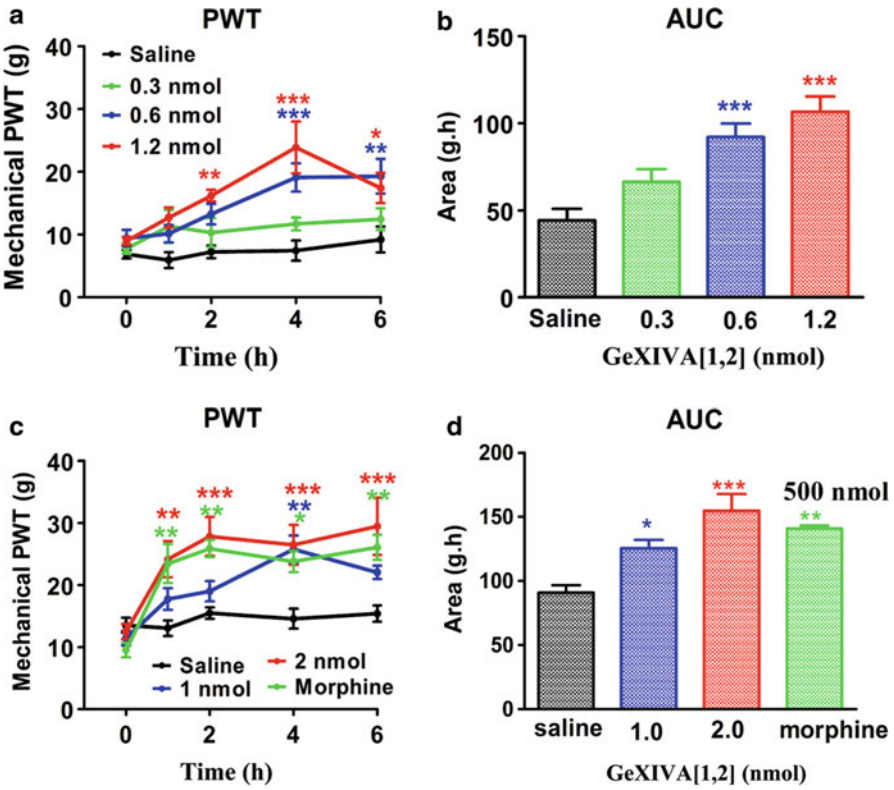

Fig. 2 Effect of αO-CTx GeXIVA[1,2] on mechanical hyperalgesia (Luo et al. 2015). (*A*) Effect of compound versus time was assessed using a series of von Frey hairs. Time point t = 0 represents mechanical PWT immediately before injection. (*B*) Corresponding dose response calculated as area under the curve (*AUC*) for data from each dose of drug in (*A*) for time points between 0 and 6 h. (*C*) PWT was assessed for a separate group of rats using an electronic von Frey anesthesia meter (*D*) (AUC) for data from each dose of drug in (*C*). $n = 8$ for *panels A* and *B*; n = 6 for *panels C* and *D*. Each point in (*A*) and (*C*) represents the mean ± SEM (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, Dunnett's post-hoc tests)

one amino acid residue difference between Ac1.1a and Ac1.1b sequences (Table 2). The two toxins showed similar potency on mouse muscle nAChRs with IC50 values of 36 nM for Ac1.1a and 26 nM for Ac1.1b. Both Ac1.1a and Ac1.1b had little or no block on tested neuronal α3β2 and α3β4 nAChR subtypes. Deletion of the N-terminus NGR of Ac1.1b did not change its potency on α1β1γδ nAChRs with an IC50 of 27 nM, which indicated that the N-terminal extension of the α3/5 conotoxins is not crucial for the muscle nAChR binding. Cone snails might use the α3/5 conotoxins to paralyze prey. Ac1.1a and Ac1.1b share high sequence homology and similar functions as other α3/5 conotoxins.

α-CTx Qc1.2 (α-Qc1.2, Qc1.2) is a new α4/4-conotoxin from vermivorous *Conus quercinus* (Peng et al. 2009). It consists of 14 amino acid residues with

two disulfide connectivities of Cys (I-III, II-IV), in which there is an N-terminal post-translationally processed pyroglutamate residue, and a free carboxyl C-terminus (Table 2). Qc1.2 was shown to be very weak inhibition on rat neuronal α3β2 and α3β4 nAChR subtypes. About 63% of the ACh-evoked currents of α3β2 and 37% of α3β4 nAChRs was blocked by 10 μM Qc1.2. Their IC50s on α3β2 and α3β4 nAChRs have not been determined and might be $\geq$10 μM. Qc1.2 exhibited no inhibition on other neuronal nAChR subtypes.

α1-CTx Pu14a (α1-Pu14a, Pu14a) is a novel α1-conotoxin subfamily member of A-superfamily from *C. pulicarius* (Peng et al. 2010). It consists of 19 amino acid residues and four Cys framework C-C-C-C with two disulfide bonds of the globular form, i.e., I–III, II–IV connectivity. The signal sequence of Pu14a precursor shares high sequence similarity with α-conotoxins. Pu14a showed inhibition on the mouse fetal muscle nAChR subtype (α1β1γδ) at 1 μM toxin concentration and blocked ~82% of the ACh-evoked current that completely recovered after 4 min of washout. 1 μM Pu14a blocked ~55% current of rat α6α3β2 nAChR with rapid reversibility. The IC50s of Pu14a were not determined for α1β1γδ and α6α3β2 nAChRs. Pu14a showed little or no block on the other rat nAChR subtypes at 10 μM high concentration. Pu14a is a weak inhibitor of mouse fetal muscle nAChR. Intramuscular injection of Pu14a to mice induced a sleeping symptom.

Conotoxin Lt14a from *Conus litteratus* is composed of 13 amino acid residues with C–C–C–C cysteine pattern and an amidated C-terminus. Lt14a behaves as a neuronal nAChRs antagonist using the whole-cell patch-clamp technique on nAChRs in rat PC12 cells. The nAChR subtypes and IC50s of Lt14a targeting remain unknown. Lt14a elevated pain thresholds in the mouse hot-plate model by intraperitoneal (i.p.) administration, which showed analgesic activity. The analgesic effects were dose-dependent (Peng et al. 2006). An analog of Lt14a, [K7A]-Lt14a, i.e., Ala substitution for Lys in position 7, elevated the pain threshold significantly after 1 ~ 5 h administration in the mice hot-plate pain model and exhibited higher activity than Lt14a as a long-lasting analgesic. MTT assay revealed that both Lt14a and [K7A]-Lt14a had low toxicity to human cells (Sun et al. 2011).

Hainan Conotoxins Active on Other Targets

Targets of four non α*-conotoxins from Hainan cone snails were investigated, which are ω-CTx SO3, cMrIA-9, κ-CTx BtX and μ-CTx LtVd (Table 3). ω-CTx SO3 (ω-SO3, SO3) is composed of 25 amino acid residues, which is from fish-hunting *C.* striatus and belongs to O-superfamily conotoxins (Table 3). SO3 is a selective antagonist of N-type calcium ion channel (Wen et al. 2005), which inhibited N-type calcium currents in cultured hippocampal neurons selectively. The high voltage-activated (HVA) calcium currents (ICa) could be inhibited by SO3 with concentration-dependence. 3 μM SO3 inhibited ~31% of the total HVA currents and had no effect on R-type currents. The block of SO3 at HVA calcium ion channel was fully reversible. Voltage-sensitive sodium currents, delayed

Table 3 Sequence, target and IC_{50} values etc. of Hainan non α*-conotoxins

Peptide	Species	Diet	Sequence	Cysteine Residues	Residue number	Disulfide Bonded	Mass (Mono)	Target	IC50 [nM]	References
ω-SO3	*C. striatus*	P	CKAAGKPCSRIAY NCCTGSCRSGKC*	6	25	3	2559.08	Nav-Kv _ Cav2.2 > Cav2.1 > Cav2.3	0.49 1.6	Lu et al. (1999), Wen et al. (2005), Yan et al. (2003)
cMrIA-9	*C. marmoreus*	M	CAGKRKSG	1	9	0	787.90	Dengue virus NS2B-NS3 protease	2200	Xu et al. (2012)
κBtX	*C. betulinus*	V	CRA(Gla)GTYC (Gla)NDSQCCLN (Gla)CCWGG CGHOCRHP*	8	31	4	3569.15	BK channel	0.7 (EC50)	Fan et al. (2003)
Lt5d	*C.litteratus*	V	DCCPAKLLCCNP	4	12	2	1274.49	Nav TTX-S	156.16nM	Liu et al. (2007a)

Notes: *Nav* voltage-dependent sodium channel, *Cav* voltage-dependent calcium channel, *Kv* voltage-dependent potassium channel, *BK channel* voltage-dependent big conductance $K(Ca^{2+})$ channel

rectifier potassium currents, and transient outward potassium currents were not affected by SO3.

cMrIA-9 is an eight-residue cyclic peptide with inhibitory activity against Dengue virus (DENV) serotype 2 DENV NS2B-NS3 protease with high potency (Xu et al. 2012). Its inhibitory constant was 2.2 μM. cMrIA-9 has good stability and cell permeability. cMrIA-9 is derived from modification of a native χ-conotoxin, MrIA, an inhibitor of neuronal noradrenaline transporter originally from the mollusk-hunting *C. marmoreus* (Sharpe et al. 2001). χ-Conotoxin MrIA showed inhibitory activity on DENV protease too with inhibitory constant of ~9 μM, which was attributed to a disulfide bond-mediated loop. cMrIA-9 has improved potency and stability compared to the parent peptide MrIA. Therefore peptide cyclization is very important for oligopeptide inhibitors cMrIA-9 against DENV protease. cMrIA-9 may be an ideal lead for a DENV protease inhibition drug design.

κ-CTx BtX (κ-BtX, BtX) is a specific voltage-dependent big conductance K(Ca^{2+}) (BK) channel modulator from *Conus betulinus* with 8 Cys framwork C-C-CC-CC-C-C (Fan et al. 2003). BtX consists of 31 amino acid residues and 4 disulfide bonds (Table 3). BtX up-modulated the Ca^{2+}- and voltage-sensitive BK channels specifically with an EC_{50} of 0.7 nM in chromaffin cells. Its time constant of wash-out at 8.3 s indicates increased open probability of BK channels with up-modulating effect. Presence of 1 μM BtX increased the peak outward current twofold when the cell was stimulated subsequently by a second ramp. The K^+ current could be recovered to the control level after the toxin removal in response to the third ramp. BtX has a single binding site on the BK channel but it had little or no effect on the voltage-gated Ca^{2+} channels.

μ-CTx Lt5d (μ-Lt5d, Lt5d) is a T-superfamily conotoxin from *Conus litteratus* targeting tetrodotoxin-sensitive (TTX-S) sodium channels of adult rat dorsal root ganglion neurons with an IC50 of 156.16 nM (Liu et al. 2007a). The first T-superfamily conotoxin identified to block TTX-S sodium channels, Lt5d is a small peptide with 12 amino acid residues and a 4-Cys framwork CC-CC (Table 3). The block of Lt5d on TTX-S sodium channels is concentration-dependent; at ~150 nM concentration it inhibited about half of the TTX-S sodium currents within 5 min or so. Lt5d did not affect the activation and inactivation kinetics of TTX-S sodium channels and had no effect on TTX-resistant sodium channels either.

Synthesis of Hainan Conotoxins

Due to small size of conotoxins consisting of 10–40 residues, usually with 2–4 disulfide bonds, chemical synthesis has been the main method to get enough conotoxin materials. Most characterized Hainan conotoxins were synthesized by one-step (Figs. 3 and 4), two-step (Fig. 5) or three-step oxidation methods (Fig. 6) using Fmoc (*N*-(9-fluorenyl) methoxycarbonyl) or t-butoxycarbonyl chemistry. More oxidation steps require more purification steps. Conotoxins are disulfide-rich peptides generally with ≥4 Cys residues. Multiple allosteric disulfide bond isomers can be produced from a synthetic linear peptide but in general only one

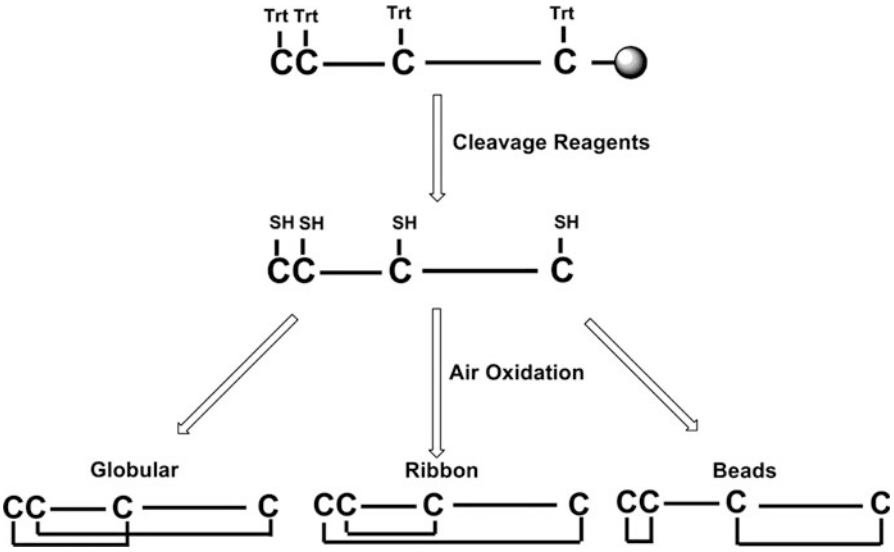

Fig. 3 Standard synthesis scheme of Hainan α-conotoxins by one-step oxidation

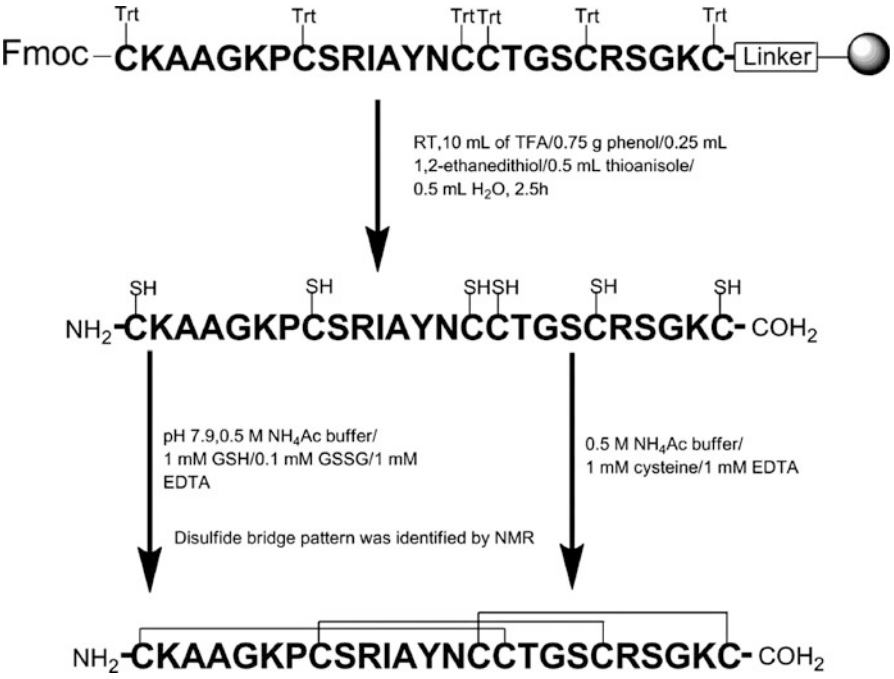

Fig. 4 Standard synthesis scheme of Hainan non α-conotoxins by one-step oxidation (SO3) (Dai et al. 2003)

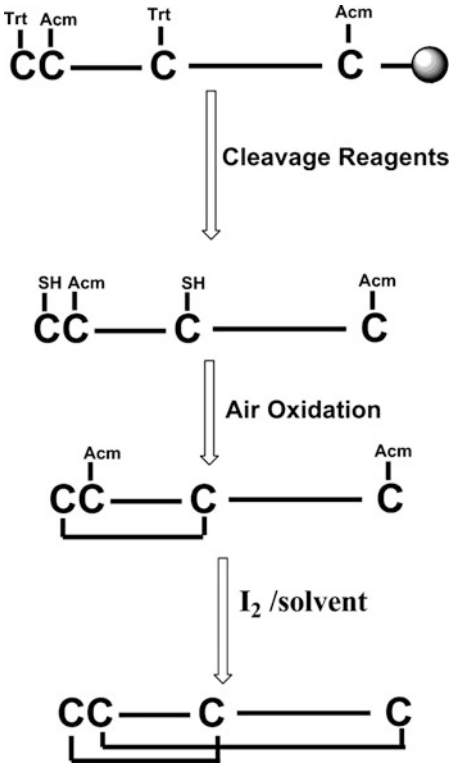

Fig. 5 Standard synthesis scheme of Hainan α-conotoxins by two-step oxidations

disulfide bond isoform is efficiently bioactive. Disulfide bonds play a unique and important role in stabilizing the structures of conotoxins (Craik 2011). There is challenge to discover ways of properly folding conotoxins, especially for peptides containing three or more disulfide bridges. Efficient oxidative folding methods remain the bottleneck in investigating cysteine-rich peptides (Muttenthaler et al. 2010; Steiner and Bulaj 2011).

Conotoxin synthesis by one-step oxidation is the easiest way for all the cysteine-rich peptides. All cysteines were protected with S-trityl groups in the synthesis of linear peptide by solid-phase methodology (Figs. 3 and 4). The reduced (linear) peptides are purified by reversed-phase HPLC after the peptides are removed from a solid support generally. After purification, the linear peptide is lyophilized and folding is carried out in a buffered solution with various components under different temperature and pH conditions. In principle one-step oxidation has lower selectivity compared to regioselective oxidation of two- or more step oxidations because random formation of possible disulfide bonds active isomer purification much harder. Three possible isomers, globular form with disulfide connectivity of CysI-CysIII and CysII-CysIV, ribbon form with disulfide connectivity of CysI-CysIV and CysII-CysIII, and bead form with disulfide connectivity of CysI-CysII and CysIII-CysIV, may formed in one-step oxidation for 4-Cys containing conotoxins,

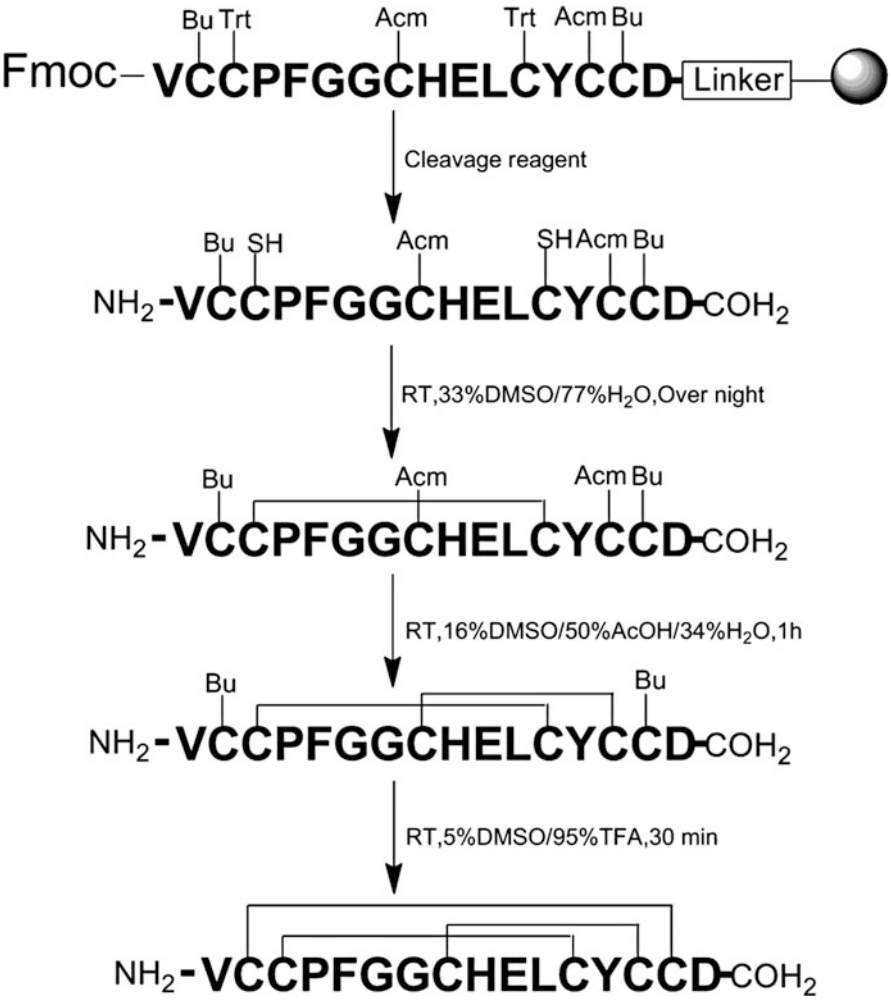

Fig. 6 Standard synthesis scheme of Hainan non α-conotoxins by three-step oxidations (mr3e) (Han et al. 2006)

such as α-conotoxins with CC-C-C pattern (Figs. 1 and 3), αB-conotoxin VxXXIVA with C-CC-C pattern, αO-conotoxin GeXIVA with C-C-C-C pattern (Fig. 1). More complex disulfide bonds isomers would be presented in the one-step oxidation for conotoxins containing ≥6 Cys residues. If 15 allosteric disulfide bonds isomers were produced in the one-step oxidation of 6-Cys containing peptides with three disulfide bonds, it would be difficult to identify and isolate the right isomer with bioactivity. However, one-step oxidation has advantages of fewer purification steps and it is less time consuming. For some conotoxins, such as α-conotoxins TxIB (Wu et al. 2013a), LtIA (Luo et al. 2010), and ω-CTx SO3 with C-C-CC-C-C framework (Dai et al. 2003), (Fig. 4), one-step oxidation could

produce better yield by experimentally optimizing oxidative folding parameters than two-step oxidation methods.

Conotoxin synthesis by two-step oxidation is a good way for Cysteine-rich peptides too, especially for 4-Cys containing peptides with two pairs of disulfide bondsbecause it could get the expected isomer with regioselective oxidation. Several Hainan α*-conotoxins and their isomers were synthesized by two-step oxidation, e.g., α-conotoxins LvIA(Luo et al. 2014), TxIA (Wu et al. 2014), αB-conotoxin VxXXIVA (Luo et al. 2013a) and αO-conotoxin GeXIVA (Luo et al. 2015) etc. Commonly linear peptides of two-step oxidation were synthesized in same way as the one-step oxidation except for cysteine residues protected with two different side chain groups, S-trityl (Trt) or S-acetamidomethyl (Acm) (Fig. 5). Each of the three possible isomers of 4-Cys containing peptides could be synthesized by two-step oxidation. For globular isomer, the first and the third Cys residues (Cys1-Cys3) are protected in pairs with S-trityl, and the second and the fourth Cys residues (Cys2-Cys4) are protected in pairs with S-acetamidomethyl. Similarly for bead isomer synthesis, either S-trityl on Cys1 and Cys2 or S-acetamidomethyl on Cys3 and Cys4 were used for Cys protected groups. For ribbon isomer synthesis, either S-trityl on Cys2 and Cys3 or S-acetamidomethyl on Cys1 and Cys4 were used for Cys protected groups. Generally the first disulfide bridge between the two Cys with S-Trt protected group is closed by air oxidation, and the monocyclic peptide was purified by reverse-phase HPLC. The second disulfide bridge between the two Cys with S-Acm protected group is closed by iodine oxidation, and the S-Acm groups are removed simultaneously. Then the bicyclic peptide is purified by reverse-phase HPLC again. The synthetic and fully folded peptides need to be confirmed by mass spectrometry.

Conotoxin synthesis by three-step oxidation is appropriate for $\geq$6 Cysteine-containing peptides, especially for peptides with no correct major peak in the folded products after the one-step oxidation. Six-Cys containing peptides with three pairs of disulfide bonds could get the expected isomer by three steps regioselective oxidation. The linear peptides of three-step oxidation could be synthesized in the same way with one-step or two-step oxidation except for cysteine residues protected with three different side chain protecting groups, S-trityl (Trt) or S-acetamidomethyl (Acm) or S-t-butyl (t-Bu). For example, M-1 conotoxins, mr3e possess a disulfide bond arrangement of Cys1-Cys5, Cys2-Cys4, Cys3-Cys6, which was synthesized by three-step oxidation (Fig. 6). To synthesize mr3e with native disulfide bonds, three different protecting groups were put on different pairs of Cysteines, i.e., Trt on Cys3 and Cys6, Acm on Cys2 and Cys4, t-Bu on Cys1 and Cys5 (isoform C1-C5, C2-C4, C3-C6) when synthesizing the linear peptide (Han et al. 2006). The synthesized linear peptides were sequentially oxidized at different folding buffers to form the first disulfide bond between free Cys residues with Trt protecting group, then to form the second disulfide bond with Acm protecting group, finally to form the third disulfide bond with t-Bu protecting group. The final fully disulfide bonded peptide was confirmed by HPLC co-elution with the natural mr3e.

Recombinant Expression of Conotoxins

Some conotoxins are very difficult to synthesize chemically, especially the bigger peptides with >40 amino acid residues and multiple disulfide-bonds. Recombinant expression technology may be a better option to produce large quantities of the bigger peptides. Thus recombinant expression of 11 conotoxins in *E. coli* or yeast cells were attempted to obtain the bioactive peptides (Table 4). The μO-CTx MrVIB from *Conus marmoreus*, a 31-amino-acid peptide containing six cysteine residues linked by three disulfide bonds is an antagonist of voltage-dependent sodium channel (Nav) 1.8 sodium channel (Ekberg et al. 2006; McIntosh et al. 1995). μO-CTx MrVIB is of great potential interest in the antinociceptive drug development field but its development has long been hindered more than 15 years by extremely difficult chemical synthesis and oxidative folding. Gao et al. described a different approach to produce recombinant MrVIB in *E. coli* by fusing MrVIB gene with pelB leader signal peptide and His-tag co-expression (Gao et al. 2013a).

A recombinant conotoxin GeXIVAWT (rCTx-GeXIVAWT) was successfully and highly expressed in *E. coli* by optimizing rare codons, which could inhibit the growth of *Spodoptera frugiperda* 9 (Sf9) cells (Gao et al. 2013b). The pET22b(+) vector with a signal protein pelB leader and a His-tag was used to express rCTx-GeXIVAWT. One-step Ni-NTA affinity chromatography could get rCTx-GeXIVAWT peptide with ~90% purity. The majority of the rCTX-GeXIVAWT fusion protein was expressed in insoluble form, which was folded by dilution into optimized folding buffer. The yield of active folded rCTX-GeXIVAWT could be increased by addition of 0.5 mM GSSG and 1 mM GSH to the folding buffer. A biologically active conotoxin PrIIIE was obtained also by recombinant expression in *E. coli* (Hernandez-Cuebas and White 2012). Other conotoxins, such as ω-conotoxin MVIIA, Mol659, Lt7a, Lt6c etc. were made in the different recombinant *E. coli* strains (Table 4). Conotoxin TxVIA from *C. textile* has insecticidal activity, which was recombinant expressed in yeast by fusing alpha factor with high yield of 10 mg/L successfully (Bruce et al. 2011). Therefore, conotoxin gene recombinant expression would be a useful way for producing a large amount of conopeptides for structure–function studies and clinical trials.

Structures of Hainan Conotoxins

The three-dimensional (3D) solution structure of most Hainan conotoxins remains unknown. So far the 3-D structures of only 12 Hainan conotoxins have been determined using NMR(nuclear magnetic resonance) spectroscopy (Figs. 7 and 8). The α-CTx TxIB solution structure with PDB accession number of 2LZ5 (Fig. 7a) determined using NMR spectroscopy represents a uniquely selective ligand to probe α6β2* nAChR structure and function (Luo et al. 2013b). The secondary structure of TxIB consists of an α-helix between residues 6–9 (−PPCR-), which is very common in α-conotoxins. TxIB has different surface features from other similar conotoxins

Table 4 Recombinant conotoxins in different expression systems. The "Host cell" column can just be replaced by a note since *E. coli* was used as host cell for all except for one where yeast was used

Conotoxin	Origin	Native peptide sequence	Fused partner	Recombinant peptide	Host cell	Yield (mg/L)	Activity/ Target	References
MVIIA	*C. magus*	CKGKGAKCSRLMYDCCTGSCRSGKC	TRX	TRX-HisTag-MVIIA	*E. coli*	40	Ca^{2+} channel	Zhan et al. (2003)
MVIIA	*C. magus*	CKGKGAKCSRLMYDCCTGSCRSGKC	GST	GS-MVIIA	*E. coli*	No data	Ca^{2+} channel	Xia et al. (2006)
Mol659	*C. monile*	FHGGSWYRFPWGY	Cytochrome b5	Mol659	*E. coli*	6-8	K^{+} channel	Kumar et al. (2005)
Lt7a	*C. litteratus*	CLGWSNYCTSHSICCSGECILSYCDIW	TRX	lt7a	*E. coli*	6	Na^{+} channel	Pi et al. (2007)
Lt6c	*C. litteratus*	WPCKVAGSPCGLVSE CCGTCNVLRNRCV	TRX	lt6c	*E. coli*	12	Na^{+} channel	Wang et al. (2008a)
PrIIIE	*C. parius*	AARCCTYHGSCLKEKCRRKYCCG	SUMO	PrIIIE	*E. coli*	1.5	α1β1δε/ α1β1γδ	Hernandez-Cuebas and White (2012)
Vn2	*C. ventricosus*	EDCIAVGQLCVFWNIG RPCCSGLCVFACTVKLP	GST	GST-HisTag-Xa-Vn2	*E. coli*	No data	Toxicity to insects	Spiezia et al. (2012)
GeXIVAWT	*C. generalis*	TCRSSGRYCRSPYDCR RRYCRRITDACV	PelB	PelB-GeXIVAWT-HisTag	*E. coli*	61.6	cytotoxicity	Gao et al. (2013b)
MrVIB	*C.marmoreus*	ACSKKWEYCIVPILGFV YCCPGLICGPFVCV	PelB	MrVIB-HisTag	*E. coli*	5.9	Analgesic activity	Gao et al. (2013a)
TxVIA	*C. textile*	WCKQSGEMCNLLDQN CCDGYCIVLVCT	Alpha factor	Pro-TxVIA	Yeast	10	insecticidal activity	Bruce et al. (2011)
Lt15a	*C. litteratus*	ECTTKHRRCEKDEECCP NLECKCLTSPDCQSGYKCKP	TRX	TRX-Lt15a-His · Tag	*E. coli*	No data	No data	Wu et al. (2014)
Lt16a	*C. litteratus*	TGEDFLEECMGGCAFDFCCKR	TRX	TRX-lt16a	*E. coli*	No data	TTX sensitive Na^{+} channel	Zhou et al. (2015)

Xa: IEGR; **HisTag**: HHHHHH; **PelB**: MKYLLPTAAAGLLLLAAQPAMA; **Pro**: DDPRNGLGNLFSNAHHEMKNPEASKLNKP; **TRX**: MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA;
GST: MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLEVLFQGPLGS. **Cytochrome b5**: GELHPPDDRSKIAKPSETL
SUMO: GGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQGKEMDSLRFLYDGIRIQADQTPEDLDMEDNDIIEAHREQIGG
Alpha factor: MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEA

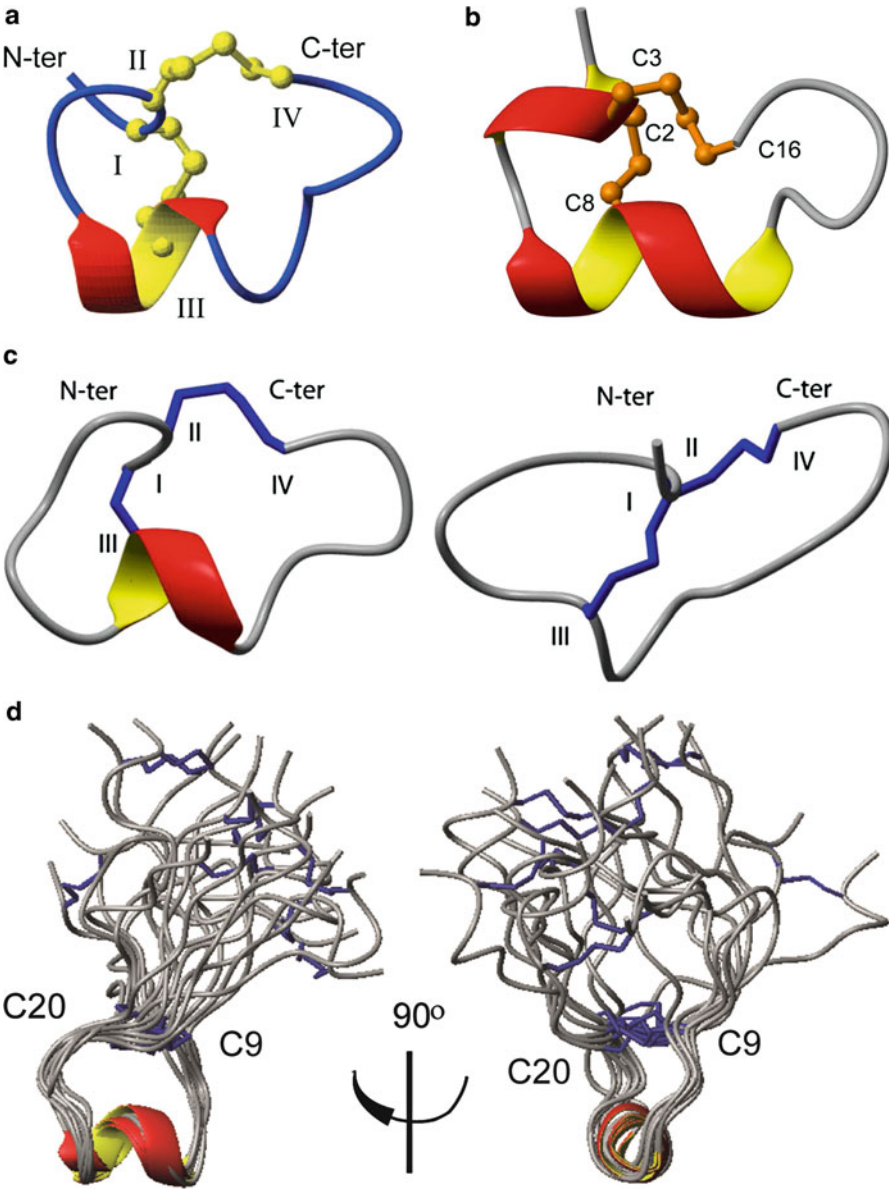

Fig. 7 Three-dimensional structures of Hainan α*-conotoxins (Luo et al. 2013b, c, 2014, 2015)

MII, PIA, BuIA and GIC in terms of the charged, polar and hydrophobic side chains of the residues. The hydrophobic patch of TxIB, obvious from its NMR structure is smaller than that in MII, PIA or BuIA and might be responsible for the higher selectivity of TxIB on α6/α3β2β3 nAChR subtype.

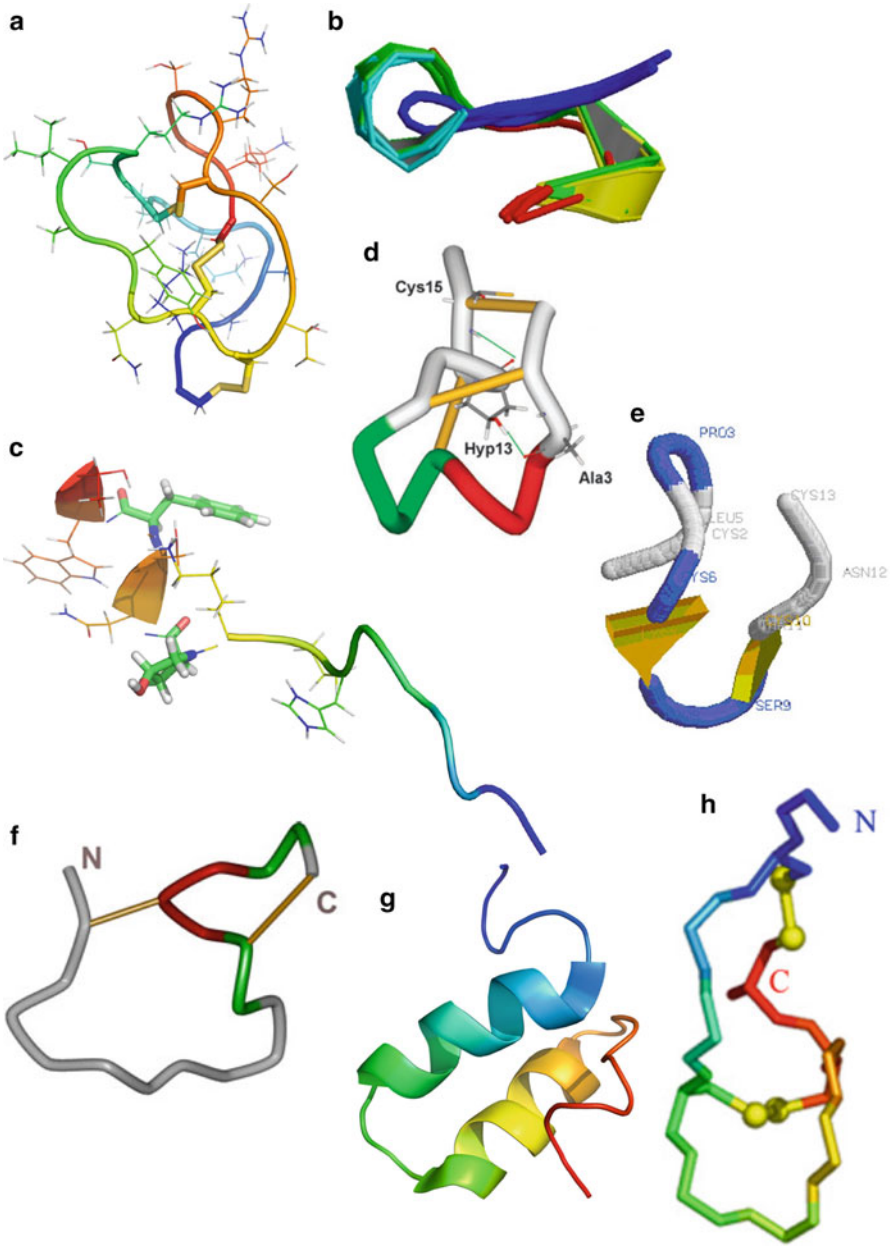

Fig. 8 Three-dimensional structures of Hainan non α-conotoxins (Du et al. 2007; Han et al. 2008; Sun et al. 2011; Xu et al. 2010; Yan et al. 2003; Ye et al. 2011, 2012; Zhang et al. 2012)

α-CTx LvIA is the first potent α3β2-subtype-selective nAChR ligand versus α6/α3β2β3 nAChR, which adopts two similarly-populated conformations in water (Luo et al. 2014). One conformation (conformer 1) assumed to be bioactive and is highly structured has been submitted to the protein databanks with accession number of PDB ID:2MQD and BMRB ID: 19501. Whereas the other conformation of LvIA (conformer 2) is mostly random-coil in nature. The two distinct conformations were observed in the NMR spectra with a near 1:1 ratio by *cis-trans* isomerization of Pro residue likely. Conformer 1 of LvIA has a compact structure with two α-helix secondary structures (Fig. 7b). One α-helical motif is across the middle of the molecule between residues Pro6-Val10 (−−PACNV–). Another α-helical turn is across residues Cys2-Ser4 (−−CCS-). It is quite common that additional conformations of conotoxins are present in solution but tend to go unreported if the minor form is less well defined in general.

The NMR analysis of α-CTx TxID, so far the most potent and selective antagonist of α3β4 nAChR subtypeshowed two major structural isomers existing in solution by *cis-trans* isomerisation of one or both of the two proline residues (Luo et al. 2013c). The TxID structures of both isomers in solution were solved successfully using a 900 MHz spectrometer (Fig. 7c). One conformational isomer (Isomer 1) of TxID comprises a common α-helical motif that is present in a majority of other α-conotoxins showing surface charge distribution different from that of other 4/6 family members. Isomer 1 was assigned as the *trans-trans* isomer, and isomer 2 was determined to be a *cis-trans* isomer due to the lack of NOEs between $H\alpha_5$–$H\delta_6$ protons and a large difference between the Cβ and Cγ chemical shifts of Pro6. Isomer 1 possess a short 3,10-helix spanning residues 8–10 (−−CSA–) (Fig. 7c left panel). In contrast, isomer 2 had no secondary structural elements identified other than the two disulfide bonds (Fig. 7c right panel). NMR structural studies of TxID were difficult and complicated by the presence of two major conformational isomers, that cannot be separated and equilibrate with each other even if they can be separated. So it is very difficult to identify which isomer possesses the bioactive conformation. Indeed α-CTx TxID is a new probe to elucidate the structure and function of α3β4 nAChRs.

αO-CTx GeXIVA has three disulfide isomers, all of which were examined using Bruker 600 and 900 MHz NMR spectrometers to determine their solution structures (Luo et al. 2015). The globular form GeXIVA[1,3] does not have a single conformation apparently. So only structures of the ribbon and bead isomers were calculated. The bead isomer GeXIVA[1,2] shows that loop C2-C9 and loop C20-C27 are internally well defined, but the 10–19 linker region has a slight helical tendency (Fig. 1c). The ribbon isomer GeXIVA[1,4] contains a better defined but short 3_{10}-helix from Y12 to R16 (−−YDRRR–), which overlays well between NMR models (Fig. 7d). Root-mean-square deviation (RMSD) of GeXIVA[1,4] helix-loop region is 0.99 ± 0.33 Å across backbone atoms. For the ribbon isomer, the central part of the peptide, from positions 12–18 (−−PYDRRRR–), is helical, but for the bead isomer, this region has a small helical propensity. The shared helical motif of the

ribbon and bead isomers reminds us of the helix displayed by α-conotoxins, which seem to adopt a conserved binding mode interacting with the ligand binding pocket of nAChRs. The differences between GeXIVA and other reported Framework 14 conotoxins is interesting particularly the loop size separating the disulfide bonds. Structural novel GeXIVA represents a valuable probe of α9α10 nAChR subtype and could be a new precious drug lead.

The three-dimensional structures of eight Hainan non α*-conotoxins we investigated are shown in Table 5 and Fig. 8. Structure of ω-CTx SO3 (Yan et al. 2003) comprises a short triple-stranded antiparallel β-sheet with four turns, which is stabilized by three disulfide bridges (Fig. 8a). The 3D solution structure of mr3e (Du et al. 2007) was determined to be well defined and contains two turns defined by residues Phe5 to Cys8 and His9 to Cys12 (Fig. 8b). The residues from Phe5 to Cys8 are characteristic of a type I β-turn. The second turn is in the region between His9 and Cys12. mr3e adopts a distinctive backbone conformation with an overall shape of a "flying bird." Conomarphin is a Cys-free peptide with a hydroxylated Pro at position 10, and a D-Phe at position 13 (Han et al. 2008). The well-defined structure of conomarphin in solution possesses a tight loop in the middle of the peptide from Ala6 to Hyp10, and a short 3_{10}-helix at the C-terminus from residues Asn11 to Trp14 (Fig. 8c). The D-Phe13 is essential for the structure of conomarphin. The conotoxin mr3c contains Hyp and three pairs of disulfide-bond linkages. The structure determined for mr3c (Xu et al. 2010) includes the secondary structure elements, i.e., a 3_{10} -helix for residues 4–6 and a type I β-turn for residues 7–10 of the peptide (Fig. 8d).

Table 5 Concise information on the three-dimensional structures of Hainan non α-conotoxins

CTx Name	Organism	Superfamily	Cys framework	Cys pattern	PDB / BMRB code	References
ω-SO3	*C. striatus*	O1 superfamily	VI/VII	C-C-CC-C-C	1FYG	Yan et al. (2003)
mr3e	*C. marmoreus*	M superfamily	III	CC-C-C-CC	2EFZ	Du et al. (2007)
Conomarphin	*C. marmoreus*	M superfamily	Cys-free	Cys-free	2YYF	Han et al. (2008)
mr3c	*C. marmoreus*	M superfamily	III	CC-C-C-CC	16785	Xu et al. (2010)
Lt14a	*C. litteratus*	L superfamily	XIV	C-C-C-C	21014	Sun et al. (2011)
Pu14a	*C. pulicarius*	A superfamily	XIV	C-C-C-C	21015	Zhang et al. (2012)
Im23a	*C. imperialis*	K superfamily	XXIII	C-C-C-CC-C		Ye et al. (2012)
Qc16a	*Cy quercinus*	unknown	XVI	C-C-CC	20128	Ye et al. (2011)

Conotoxin lt14a contains C-C-C-C cysteine pattern, which is the smallest peptide of conotoxin framework 14 (Sun et al. 2011). Lt14a solution structure comprises a short duple β-strand region and β-turn motif (Fig. 8e). The solution structure of Pu14a (Zhang et al. 2012) contains a large intercysteine loop between C2 and C13 and a 3_{10}-helix near the C-terminal. This is remarkably different from typical α-conotoxins (Fig. 8f) that have an ω-type twist in the center of typical their structures. Pu14a N-terminal is poorly converged. Except for P3 and V14, the side chains of most residues were oriented outside. The four proline residues in Pu14a adopted the trans conformation that may correlate with the large loop configuration and its biological activity. Conotoxin im23a is 42 amino acids long with C-C-C-CC-C framework (Ye et al. 2012). The solution structure of im23a adopts a novel helical hairpin fold that comprises two α-helices encompassing residues 8–17 and 22–31 connected by a loop (Fig. 8g). Residues 32–34 forms a turn leading to the C-terminal loop. The hydrophobic residues within im23a form a hydrophobic core, while charged and polar residues generally point toward the surface. Conotoxin qc16a consists of 11 amino acid residues with the sequence DCQPCGHNVCC containing the two disulfide bonds CysI-IV and CysII-III (Ye et al. 2011). The NMR structure of qc16a shows a ribbon conformation with a simple β-turn motif formed by residues Gly6, His7 and Asn8 (Fig. 8h).

Conclusion and Future Directions

Cone snail venom, a complex cocktail of many chemicals is known to contain toxins proven to be valuable drug leads. There are thousands of new peptide toxins hidden deep within the venom of just one type of cone snail. In the venom of a single cone snail of Queensland *Conus episcopatus*, 3305 novel precursor toxin sequences were discovered using biochemical and bioinformatic tools recently (Lavergne et al. 2015). Hainan cone snail venoms and most of their toxins have been overlooked in the past and need to be deep excavated in the future, and explored as tools in looking for disease patterns or discovering potential new drugs. Since conotoxins have small highly structured frameworks and are able to interact with heterologous ion channels and receptors potently and selectively, they would be promising leads for new drugs to treat pain, cancer and a range of other diseases. Characterized Hainan conotoxins with explicit target and structure are indeed valuable drug leads.

Especially nAChRs are widely used throughout the animal kingdom to enable and facilitate motor movement and sensory processing, and represent a fundamental target of predatory organisms. The importance of various nAChR subtypes in the nervous system provides a platform for translational research and drug development of related human diseases, including pain, cancer, addiction, epilepsy, Alzheimer's disease, Parkinson's disease, and schizophrenia etc. (Lebbe et al. 2014). The α9α10 nAChR subtype is a recently identified target for the development of breast cancer chemotherapeutics and analgesics, particularly to treat neuropathic pain. The αO-conotoxin GeXIVA from Hainan *C. generalis* is a

very potent and selective antagonist of the α9α10 nAChR subtype, which reduced mechanical hyperalgesia in the rat chronic constriction injury model of neuropathic pain, but had no effect on motor performance. Therefore GeXIVA represents a valuable probe of the α9α10 nAChR subtype, and a novel template for molecules active against neuropathic pain.

The nAChR subtypes containing the α3 subunit (α3β4 and α3β2) are present in autonomic ganglia and modulate cardiovascular functions, as well as pain sensation. The α3β4 nAChRs are implicated in pain sensation in the PNS and addiction to nicotine in the CNS, which affect nicotine self-administration and pain sensation. The α3β4 nAChRs are known to play important roles in the autonomic nervous system, olfactory bulb neurogenesis, amygdala, and medial habenula neurons etc. (D'Souza and Vijayaraghavan 2012; McCallum et al. 2012; Napier et al. 2012; Sharma 2013; Zhu et al. 2005). The α3β4 nAChRs mediate small cell lung carcinoma (SCLC) as well. The α3β4-selective antagonist could inhibit SCLC cell viability and growth. The α4/6-CTx TxID, the most potent antagonist of α3β4 nAChRs with low nanomolar affinity, would be a valuable tool for elucidating the diverse roles of α3β4 nAChRs and a drug lead for related diseases. The precise role of α3* nAChRs has been hampered by the paucity of specific molecular probes. The previously characterized conotoxins blocking α3β2 nAChRs are poorly selective between α3β2 versus α6β2* nAChRs. However, α6β2* nAChRs predominate in dopaminergic regions, where α3β2* nAChR expression overlaps with that of α6β2* nAChRs (McClure-Begley et al. 2012; Whiteaker et al. 2002). α-CTx LvIA from Hainan *C. lividus*, the first α-Ctx reported to show high selectivity for human α3β2 versus α6β2 nAChRs, is likely to prove very valuable for probing the function and significance of α3β2 nAChRs in normal and disease physiology.

The α6β2* nAChRs are known to modulate the release of dopamine (Quik et al. 2011). The α6 nAChR subunit is abundantly expressed in visual pathways and is also present in peripheral tissues (Hone et al. 2012; Liu et al. 2009; Mackey et al. 2012). The α6β2* nAChRs in CNS dopaminergic neurons are potential therapeutic targets for the treatment of several neuropsychiatric diseases, including substance addiction and Parkinson disease. There is a paucity of ligands that can effectively discriminate between α6β2* and α6β4* nAChRs. It is very difficult to distinguish from α6β2*, α6β4*, α3β4 and α3β2 subtypes pharmacologically. Hainan α-conotoxin TxIB is a subtype specific blocker of the α6/α3β2β3 nAChR, which showed little or no block of all the other subtypes. TxIB represents proof-of-concept that it is possible to develop compounds that selectively block α6β2* nAChRs using the peptide's framework as an initial scaffold from which to design therapeutic drugs for diseases involving α6* nAChRs, including addiction. The unique selectivity of TxIB will allow probing of nAChR function in tissues where both the α6* and other nAChR subtypes occur.

The other Hainan conotoxins targeting non-nAChRs may have therapeutic potentials too. For example, ω-CTx SO3 targeting the N-type calcium channels may have therapeutic potential as a novel analgesic agent because N-type calcium channels have important significance for pain transduction (Wen et al. 2005). Dengue virus (DENV) infection is a public health threat worldwide that requires

effective treatment. Potent and promising DENV protease inhibitors may serve as potential drug development with advantages. Therefore the cyclic peptide inhibitor cMrIA-9 with specificity against DENV protease satisfactory and cell permeability can serve as a good lead for DENV drug development (Xu et al. 2012). It is anticipated that there will be a lot more interesting conotoxins to be found in the venom of Hainan species in South China Sea for research tools and drug discovery.

Acknowledgments The authors was supported by Major International Joint Research Project of National Natural Science Foundation of China (81420108028), State High-Tech Research and Development Project (863) of the Ministry of Science and Technology of China Grant 2012AA021706, National Natural Science Foundation of China Grant 81160503 and 41366002, Changjiang Scholars and Innovative Research Team in University Grant IRT_15R15 and the Program for International Science & Technology Cooperation Program of China Grant 2011DFR31210.

Glossary

3_{10} -helix is a type of secondary structure found (often) in proteins and polypeptides. The amino acids in a 3_{10}-helix are arranged in a right-handed helical structure. Each amino acid corresponds to a 120° turn in the helix (i.e., the helix has three residues per turn), and a translation of 2.0 Å (=0.2 nm) along the helical axis, and has 10 atoms in the ring formed by making the hydrogen bond (Toniolo and Benedetti 1991).

AIDS (HIV) is Human immunodeficiency virus infection and acquired immune deficiency syndrome, which is a spectrum of conditions caused by infection with the human immunodeficiency virus (HIV). Following initial infection, a person may experience a brief period of influenza-like illness. This is typically followed by a prolonged period without symptoms. As the infection progresses, it interferes more and more with the immune system, making the person much more susceptible to common infections like tuberculosis, as well as opportunistic infections and tumors that do not usually affect people who have working immune systems. The late symptoms of the infection are referred to as AIDS (Alexander and Mirjam 2010; Kirch 2008; Sepkowitz 2001).

β-sheet is the second form of regular secondary structure in proteins. Beta sheets consist of beta strands connected laterally by at least two or three backbone hydrogen bonds, forming a generally twisted, pleated sheet. A beta strand (also β strand) is a stretch of polypeptide chain typically 3–10 amino acids long with backbone in an extended conformation (Richardson and Richardson 2002; Voet and Voet 2004).

BK channel is voltage-dependent big conductance K(Ca^{2+}) channel.

Calcium channel is an ion channel which displays selective permeability to calcium ions. It is sometimes synonymous as voltage-dependent calcium channel, (see Dorland's Medical Dictionary) although there are also ligand-gated calcium channels (Striggow and Ehrlich 1996).

Cysteine frameworks are defined by the arrangement of cysteines along the primary sequence of the conopeptide mature region in the precursor protein, which are also defined by the number of cysteines and the number of residues (none or at least one) between consecutive cysteines (Kaas et al. 2010).

EC_{50} is concentration for 50% of maximal effect.

Gene superfamilies are one of the conotoxins classification according to the similarities between the ER signal sequence of the conotoxin precursors (Kaas et al. 2010).

IC_{50} is concentration of inhibiting 50% current of ion channels or receptors.

Isomer is a molecule with the same chemical formula as another molecule, but with a different chemical structure. That is, isomers contain the same number of atoms of each element, but have different arrangements of their atoms. There are two main forms of isomerism: structural isomerism and stereoisomerism (spatial isomerism) (Petrucci et al. 2002).

Isomerization is the process by which one molecule is transformed into another molecule which has exactly the same atoms, but the atoms have a different arrangement e.g., A-B-C → B-A-C (these related molecules are known as isomers (Petrucci et al. 2002).

Neuropathic pain is pain caused by damage or disease affecting the somatosensory nervous system. (https://en.wikipedia.org/)

Neurotransmitter are endogenous chemicals that transmit signals across a synapse or junction from one neuron (nerve cell) to another "target" neuron, muscle cell or gland cell. Neurotransmitters are released from synaptic vesicles in synapses into thesynaptic cleft, where they are received by receptors on other synapses. Many neurotransmitters are synthesized from simple and plentiful precursors such as amino acids, which are readily available from the diet and only require a small number of biosynthetic steps to convert them. Neurotransmitters play a major role in shaping everyday life and functions. Their exact numbers are unknown but more than 100 chemical messengers have been identified (Kendra 2014; Lodish et al. 2000).

Nicotinic acetylcholine receptors (nAChRs) are neuron receptor proteins that signal for muscular contractions upon a chemical stimulus. They are cholinergic receptors that form ligand-gated ion channels in the plasma membranes of certain neurons and on the presynaptic and postsynaptic sides of the neuromuscular junction. As ionotropic receptors, nAChRs are directly linked to ion channels and do not use second messengers (as metabotropic receptors do) (Purvers et al. 2008).

NMR spectroscopy is nuclear magnetic resonance spectroscopy, which is a research technique that exploits the magnetic properties of certain atomic nuclei. It determines the physical and chemical properties of atoms or the molecules in which they are contained. It relies on the phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. The intramolecular magnetic field around an atom in a molecule changes the resonance frequency, thus

giving access to details of the electronic structure of a molecule (Mark Wainwright Analytical Centre 2011.; Keeler 2007; Pople et al. 1957; Shah et al. 2006).

Potassium channels are the most widely distributed type of ion channel and are found in virtually all living organisms. They form potassium-selective pores that span cell membranes (Hille 2001).

PNS The peripheral nervous system, is the part of the nervous system that consists of the nerves and ganglia outside of the brain and spinal cord (see *Dorland's Medical Dictionary*). The main function of the PNS is to connect the central nervous system (CNS) to the limbs and organs, essentially serving as a communication relay going back and forth between the brain and the extremities (http://www.mayoclinic.com/health/brain/BN00033&slide=6). Unlike the CNS, the PNS is not protected by the bone of spine and skull, or by the blood–brain barrier, which leaves it exposed to toxin sand mechanical injuries. The peripheral nervous system is divided into the somatic nervous system and the autonomic nervous system; some textbooks also include sensory systems. The cranial nerves are part of the PNS with the exception of cranial nerve II, the optic nerve, along with the retina. The second cranial nerve is not a true peripheral nerve but a tract of the diencephalon (Douglas 2008). Cranial nerve ganglia originate in the CNS. However, the remaining 12 cranial nerve axons extend beyond the brain and are therefore considered part of the PNS (White 2008).

Receptor is any structure in physiology, which is on receiving environmental stimuli and produces an informative nerve impulse. (https://en.wikipedia.org/wiki/)

Sodium channels are integral membrane proteins that form ion channels, conducting sodium ions (Na^+) through a cell's plasma membrane (Hille 2001; Jessell et al. 2000).

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Conotoxins as Tools in Research on Nicotinic Receptors

9

Elsie C. Jimenez and Lourdes J. Cruz

Abstract

Conopeptides or conotoxins, the venom peptides produced by predatory marine snails (genus *Conus*), typically consist of about 10–50 amino acid residues. They have considerably diverged and are practically classified into superfamilies. Members of conotoxin superfamilies are greatly selective antagonists of ligand-gated and voltage-gated ion channels. This chapter describes the structural and functional diversity of conotoxins that influence neurotransmission through their action on the nicotinic receptors. Conopeptide diversity has been shown to occur in members of different superfamilies with the nicotinic receptors as their molecular targets. In many cases, the conotoxins exhibit extremely high specificity towards nicotinic receptor subtypes making them useful tools for studying the structure and physiological functions of the receptors. As the functional diversity of conotoxins continues to be investigated, the pairwise combinations of conotoxins and nicotinic receptors have shown important applications in neuropharmacology and therapeutics.

Keywords

Conus • Conopeptide • Conotoxin • Nicotinic receptor

Contents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_17

Introduction

Marine cone snails belonging to the genus *Conus* are a large family of predatory gastropods comprising nearly 700 species that are classified into fish hunting, mollusc hunting, or worm hunting based on their prey type. The minority of *Conus* species use their venom to prey on fish, others prey on molluscs, and a great majority prey on polychaete worms, echiuroid worms, and hemichordates (Terlau and Olivera 2004).

Each species of cone snails possesses in its venom an unparalleled molecular diversity of pharmacologically active compounds known as conopeptides or conotoxins that are used to facilitate the immobilization of prey and also as a defense mechanism. The complexity of interactions that characterize the ecology of cone snails provides an evolutionary basis for the presence of such pharmacologically diverse gene products (Olivera 1997).

The enormous conopeptide diversity can be traced to the different superfamilies of genes that encode the peptides. The conopeptides are processed from larger precursors with different regions, such as the signal sequence, propeptide, and mature peptide regions. Precursor peptides of a superfamily have homologous signal sequence and propeptide regions but are usually highly variable in the mature peptide regions that generally retain a unique cysteine pattern (Olivera et al. 1999; Olivera 2006). Conopeptides in a superfamily are genetically related, but they achieve molecular diversity through fast divergence in amino acid sequences. This generation of structurally diverse structures has resulted in different molecular targets for conotoxins under the same superfamily or the same molecular target for those under different superfamilies (Teichert et al. 2009). The ConoServer has categorized conopeptides according to the gene superfamilies, the cysteine frameworks in the mature peptide region, and the pharmacological families based on their activity (Kaas et al. 2012).

The extreme potency and selectivity profiles for a range of molecular targets have led to a concerted effort to identify and characterize novel conotoxins that are important as research tools, drug leads, and therapeutics. The conotoxins target a wide variety of voltage-gated and ligand-gated ion channels, which make them invaluable tools for studying the properties of the receptors and ion channels in normal and disease states. Most conotoxins are rich in disulfide bonds that allow them to fold in three-dimensional structures required in binding to receptors or ion channels with high affinity and remarkable specificity (Terlau and Olivera 2004).

The first conotoxins found to act on nicotinic acetylcholine receptors (or simply nicotinic receptors) were isolated from the venom of *Conus geographus*, a fish-hunting marine snail that can paralyze and kill humans (Gray et al. 1981). The three homologous toxic peptides containing 13–15 amino acids and a disulfide bond (conotoxin GI, GIA, and GII) were found to act on the muscle endplate region with no detectable inhibition of either nerve or muscle action potential.

Conotoxins that specifically bind to the nicotinic receptors have been used to determine the physiological functions of these receptors. They have notable specificity of interactions with various isoforms of nicotinic receptors. At least one conotoxin that inhibits a nicotinic receptor subtype was identified in each of cone snail venoms examined (McIntosh et al. 1999; Jimenez 2013). Characterized members of the nicotinic receptor-targeted conotoxin families with focus on their primary structures, cysteine patterns, disulfide-bonding frameworks, and target receptor specificities are presented in this chapter.

Conotoxins bind to their target receptors or ion channels with high potency and selectivity, making them fascinating leads for drug development. The nicotinic receptors have been implicated in several neurological disorders, such as neuropathic pain, Parkinson's disease, and schizophrenia (Ishikawa and Hashimoto 2011; Quik and McIntosh 2006; Vincler et al. 2006). As selective antagonists of nicotinic receptors, conotoxins have exhibited various pharmacological functions (Olivera 1997; Olivera et al. 2008; Teichert et al. 2009). Thus, conotoxins that affect neurotransmission through their action on the nicotinic receptors have influence on the neurological and physiological states.

Nicotinic Receptors: The Molecular Targets of Many Conotoxin Families

Nicotinic receptors belong to the major ligand-gated ion channel superfamily. According to their primary sites of expression, the nicotinic receptors are generally classified into muscle type and neuronal type receptors. The muscle type nicotinic receptor is located at the neuromuscular junction, and its stimulation causes muscle contraction. The neuronal type nicotinic receptor is found in the central nervous system and is involved in neurotransmission and regulation of neuronal activity.

The functional nicotinic receptor with molecular mass of ~290 kDa is a pentameric complex composed of transmembrane subunits. Seventeen nicotinic receptor subunits, such as α1 to α10, β1 to β4, γ, δ, and ε that can assemble to produce a diverse family of nicotinic receptor subtypes have been found in vertebrates (Millar and Gotti 2009). Two to four different types of subunits often constitute native functional heteromeric nicotinic receptor complexes. Most vertebrate nicotinic receptor subtypes consist of two α-subunits and three non-α subunits. The muscle nicotinic receptor subtypes are made up of two α, one β, one δ, and either one γ or one ε that distinguishes the fetal and adult muscle subtypes: (α1)2β1γδ for fetal subtype and (α1)2β1εδ for adult subtype. The neuronal nicotinic receptor subtypes usually consist of exclusively α subunits, e.g., (α7)5, or two α and three β subunits, e.g., (α3)2(β4)3.

The nicotinic receptor forms ligand-gated ion channel in the cell's plasma membrane. The channel is permeable to Ca^{2+} whose entry through the receptor channel leads to the regulation of Ca^{2+}-dependent cellular processes including the release of neurotransmitter. Binding of two acetylcholine molecules generally on the interface of alpha and non-alpha subunits triggers a conformational change leading to the opening of a pore which then allows Ca^{+2} to enter the cell.

Nicotinic receptor antagonists that compete for binding of acetylcholine to the receptor binding site have been characterized. The classical competitive nicotinic receptor antagonists are α-bungarotoxin and curare that are important in the initial characterization of the muscle type nicotinic receptors. However, α-bungarotoxin inhibits both the muscle subtype and neuronal α_7 subtype, while curare inhibits the muscle subtype and various neuronal subtypes. Relatively few nicotinic receptor antagonists that specifically target other subtypes were previously known until the conotoxins have been systematically defined.

Nomenclature Used for Nicotinic Receptor-Targeted Conotoxins

Conotoxins exhibit remarkable diversity and are generally grouped into superfamilies that share a highly conserved preregion and a less conserved proregion followed by a highly diverse mature toxin region. Members of a superfamily are classified into families based on the common cysteine patterns and molecular targets. Divergent families and subfamilies of conotoxins targeted to nicotinic receptors have been identified and grouped on the basis of their cysteine patterns and disulfide-bonding frameworks. In accordance with this classification, different families and subfamilies of nicotinic receptor-targeted conotoxins identified in venoms of various *Conus* species have been designated.

In the conventional nomenclature, the peptide families are designated with the Greek letter α to indicate nicotinic receptor as the molecular target, followed by a capital letter indicating the superfamily, except in α-conotoxin and α1-conotoxin under the A-superfamily and ψ-conotoxin under the M-superfamily. The Roman numeral that follows the one-letter or two-letter symbol for *Conus* species stands for the class assigned to the peptide. The molecular isoform of the peptide, if there is more than one, is usually indicated by the capital letter that follows the Roman numeral.

Conotoxin Families Targeted to Nicotinic Receptors

The α-Conotoxin Family

Among the conotoxin families, the diverse targeting specificity of the α-conotoxins is so far the best characterized. The major nicotinic receptor antagonists found in cone snail venoms are usually those employed by the cone snails to disrupt neuromuscular transmission to paralyze prey. These are presumably targeted to the main

nicotinic receptor subtypes found at the neuromuscular junction. This is obviously the case for fish-hunting cone snails that produce α-conotoxins that are paralytic to fish and other vertebrates. The earlier discovered α-conotoxins from fish-hunting *Conus* species are targeted to the muscle type nicotinic receptors at the neuromuscular junction (McIntosh et al. 1999).

The α-conotoxin family (Table 1) falls under classes I and II and belongs to the A-superfamily. Members of this family have the cysteine pattern − CC − C − C− with the disulfide framework [Cys1 − Cys3, Cys2 − Cys4]. Various α-conotoxins have been characterized from fish-hunting, mollusc-hunting, and worm-hunting *Conus* species.

Some of the most characterized α-conotoxins from fish-hunting *Conus* species include α-GI and α-GIC from *C. geographus* (Gray et al. 1981; McIntosh et al. 2002), α-SI and α-SIA from *C. striatus* (Zafaralla et al. 1988; Myers et al. 1991), α-MI and α-MII from *C. magus* (McIntosh et al. 1982; Cartier et al. 1996), α-EI from *C. ermineus* (Martinez et al. 1995), α-PIA and α-PIB from *C. purpurascens* (Dowell et al. 2003; López-Vera et al. 2007), and α-BuIA from *C. bullatus* (Azam et al. 2005) (Table 2). Majority of these α-conotoxins (α-GI, α-SI, α-SIA, α-MI, α-EI, and α-PIB) target the muscle nicotinic receptor subtypes. α-MI showed $\sim 10^4$-fold higher affinity for α1/δ than α1/γ interface, while α-EI exhibited nearly the same activity for both interfaces. On the other hand, both α-GIC and α-MII inhibited the neuronal α3β2 subtype. α-PIA blocked chimeric α6/α3β2β3 and α6/α3β4, while α-BuIA inhibited the neuronal α6/α3β2 subtype.

From mollusc-hunting *Conus* species, the characterized α-conotoxins include α-PnIA and α-PnIB from *C. pennaceus* (Fainzilber et al. 1994), α-AuIA and α-AuIB from *C. aulicus* (Luo et al. 1998), α-Vc1.1 from *C. victoriae* (Sandall et al. 2003), and α-TIB and α-TxID from *C. textile* (Luo et al. 2013a, 2013b) (Table 2). The neuronal α3β2 and α7 subtypes were inhibited by α-PnIA and α-PnIB, respectively. Both α-AuIA and α-AuIB blocked the neuronal α3β4 subtype, and α-AuIB is more potent of the two. Similarly, α-TxID inhibited the α3β4 but also the α6/α3β4 subtype. On the other hand, α-TxIB blocked the α6/α3β2β3 subtype, whereas α-Vc1.1 inhibited the α9α10 subtype.

The characterized α-conotoxins from worm-hunting *Conus* species include α-ImI and α-ImII from *C. imperialis* ((McIntosh et al. 1994; Ellison et al. 2003), α-RgIA from *C. regius* (Ellison et al. 2006), and LvIA from *C. lividus* (Luo et al. 2014) (Table 2). Both α-ImI and α-ImII target the α7 nAChR subtype, but α-ImII did not act at the competitive binding site that is the high affinity target of α-ImI and α-bungarotoxin. α-RgIA potently blocked the α9α10, while LvIA blocked the α3β2 nicotinic receptor subtype.

Within the widely distributed α-conotoxin family, subfamily specialization can be detected. This family is further divided into subfamilies based on the number of amino acid residues between cysteines. The α3/5-conotoxin subfamily with the primary sequence motif − CCX3CX5C− includes those found in related fish-hunting *Conus* species; these are paralytic α-conotoxins, such as α-GI, α-SI, α-SIA, and α-MI that are targeted to the muscle nicotinic receptor subtypes in the fish prey. The α4/3-conotoxin subfamily with the sequence motif −

Table 1 Cysteine patterns and disulfide-bonding frameworks of conotoxin families/subfamilies targeted to nicotinic receptors

Family/ subfamily	Cysteine pattern	Disulfide-bonding framework	Class	Reference
α	–CC – C – C–	[Cys1 – Cys3, Cys2 – Cys4]	I, II	Gray et al. 1984
ψ	–CC – C – C – CC–	[Cys1 – Cys4, Cys2 – Cys5,Cys3 – Cys6]	III	Shon et al. 1997
αA_L	–CC – C – C – C – C–	[Cys1 – Cys5, Cys2 – Cys3, Cys4 – Cys6]	IV	Hopkins et al. 1995
αA_S	–CC – C – C – C – C–	[Cys1 – Cys3, Cys2 – Cys5, Cys4 – Cys6]	IV	Teichert et al. 2004
αS	–C – C – C – C – C – C – C – C – C – C–	Unknown	VIII	Teichert et al. 2005a
αC	–C – C–	[Cys1 – Cys2]	X	Jimenez et al. 2007
αD	–C – CC – C – CC – C – C – C – C–	Unknown	XII	Loughnan et al. 2006
α1	–C – C – C – C–	[Cys1 – Cys3, Cys2 – Cys4]	XIV	Peng et al. 2010
αO	–C – C – C – C–	[Cys1 – Cys2, Cys3 – Cys4]	XIV	Luo et al. 2015

Table 2 Amino acid sequences and nicotinic receptor specificities of selected α-conotoxins

Peptide	Amino acid sequence	Nicotinic receptor specificity	Species	Prey	Reference
α-GI	ECCNPACGRHYSC[a]	α1β1δ(γ/ε)	*C. geographus*	Fish	Gray et al. 1981
α-GIC	GCCSHPACAGNNQHIC[a]	α3β2	*C. geographus*	Fish	McIntosh et al. 2002
α-SI	ICCNPACGPKYSC[a]	α1β1δ(γ/ε)	*C. striatus*	Fish	Zafaralla et al. 1988
α-SIA	YCCHPACGKNFDC[a]	α1β1γδ	*C. striatus*	Fish	Myers et al. 1991
α-MI	GRCCHPACGKNYSC[a]	α1/δ > α1/γ	*C. magus*	Fish	McIntosh et al. 1982, Jacobsen et al. 1999
α-MII	GCCSNPVCHLEHSNLC[a]	α3β2	*C. magus*	Fish	Cartier et al. 1996
α-EI	RDOCCYHPTCNMSNPQIC[a]	α1/δ ≈ α1/γ	*C. ermineus*	Fish	Martinez et al. 1999
α-PIA	RDPCCSNPVCTVHNPQIC[a]	α6/α3β2β3, α6/α3β4	*C. purpurascens*	Fish	Dowell et al. 2003
α-PIB	ZSOGCCWNPACVKNRC	α1β1δ(γ/ε)	*C. purpurascens*	Fish	López-Vera et al. 2007
α-BuIA	GCCSTPPCAVLYC[a]	α6/α3β2	*C. bullatus*	Fish	Azam et al. 2005
α-PnIA	GCCSLPPCAANNPDYC[a]	α3β2	*C. pennaceus*	Mollusc	Fainzilber et al. 1994, Luo et al. 1999
α-PnIB	GCCSLPPCALSNPDYC[a]	α7	*C. pennaceus*	Mollusc	Fainzilber et al. 1994, Luo et al. 1999
α-AuIA	GCCSYPPCFATNSDYC[a]	α3β4	*C. aulicus*	Mollusc	Luo et al. 1998
α-AuIB	GCCSYPPCFATNPDC[a]	α3β4	*C. aulicus*	Mollusc	Luo et al. 1998
α-Vc1.1	GCCSDPRCNYDHPEIC[a]	α9α10	*C. victoriae*	Mollusc	Sandall et al. 2003, Vincler et al. 2006
α-TxIB	GCCSDPPCRNKHPDLC[a]	α6/α3β2β3	*C. textile*	Mollusc	Luo et al. 2013a
α-TxID	GCCSHPVCSAMSPIC[a]	α3β4, α6/α3β4	*C. textile*	Mollusc	Luo et al. 2013b
α-ImI	GCCSDPRCAWRC[a]	α7	*C. imperialis*	Worm	McIntosh et al. 1994, Ellison et al. 2003
α-ImII	ACCSDRRCRWRC[a]	α7	*C. imperialis*	Worm	Ellison et al. 2003
α-RgIA	GCCSDPRCRYRCR[a]	α9α10	*C. regius*	Worm	Ellison et al. 2006
α-LvIA	GCCSHPACNVDHPEIC[a]	α3β2	*C. lividus*	Worm	Luo et al. 2014

O hydroxyproline, *Z* pyroglutamate
[a]amidated C-terminus

CCX4CX3C− includes α-ImI, α-ImII, and α-RgIA that are found in worm-hunting *Conus* species and have been shown to inhibit neuronal nicotinic receptor subtypes. The α4/7-conotoxin subfamily with sequence motif − CCX4CX7C− includes α-EI, α-MII, α-PnIA, α-PnIB, α-AuIA, Vc1.1, α-TxIB, and α-LvIA obtained from any of the three groups of *Conus* species based on prey type, with any class of nicotinic receptor (muscle subtype, neuronal subtype that is either homomeric or heteromeric) as target.

The ψ-Conotoxin Family

Members of the ψ-conotoxin family (Table 1) under the M-superfamily have the cysteine pattern − CC − C − C − CC− and disulfide framework [Cys1 − Cys4, Cys2 − Cys5, Cys3 − Cys6]. Unlike most other nicotinic receptor antagonists, the ψ-conotoxins are noncompetitive antagonists of the muscle type nicotinic receptors.

The ψ-conotoxin family includes ψ-PIIIE and ψ-PIIIF from *C. purpurascens* (Shon et al. 1997; Van Wagoner et al. 2003) and ψ-PrIIIE from fish-hunting *C. parius* (Lluisma et al. 2008) (Table 3). The ψ-PIIIE did not inhibit the binding of α-bungarotoxin indicating that it blocked the nicotinic receptor at a site other than the acetylcholine binding site. Both ψ-PIIIE and ψ-PIIIF inhibited muscle nicotinic receptor subtypes, although ψ-PIIIF was much less potent. ψ-PrIIIE was about 30-fold more potent than ψ-PIIIE and was more potent in $\alpha_1\beta_1\varepsilon\delta$ than in $\alpha_1\beta_1\gamma\delta$ nicotinic receptor subtype.

The αA-Conotoxin Family: αA_L- and αA_S-Conotoxin Subfamilies

The αA-conotoxin family (Table 1) under the A-superfamily has the cysteine pattern (−CC − C − C − C − C−). Members of the αA-conotoxins are classified into two subfamilies: the αA_L-conotoxin (long-chained peptide) and the αA_S-conotoxin (short-chained peptide). Both groups have the class IV cysteine pattern, but they differ in their disulfide-bonding frameworks: [Cys1 − Cys5, Cys2 − Cys3, Cys4 − Cys6] for the αA_L-conotoxin and [Cys1 − Cys3, Cys2 − Cys5, Cys4 − Cys6] for the αA_S-conotoxin.

The αA_L-conotoxins are αA_L-PIVA from *C. purpurascens* (Hopkins et al. 1995) and αA_L-EIVA and αA_L-EIVB from *C. ermineus* (Jacobsen et al. 1997) (Table 3). In competition-binding assay using α-bungarotoxin, αA_L-PIVA blocked the α/γ and α/δ interfaces. Both αA_L-EIVA and αA_L-EIVB inhibited α1-containing nicotinic receptors; αA_L-EIVA blocked α1/γ and α1/δ interfaces with equally high affinity but with different kinetics of inhibition.

Members of the αA_S subfamily include αA_S-OIVA and αA_S-OIVB from fish-hunting *C. obscurus* (Teichert et al. 2004, 2005b) and αAs-PeIVA and αAs-PeIVB from *C. pergrandis* (Teichert et al. 2006) (Table 3). The αA_S-conotoxins are selective antagonists of the α1β1γδ nicotinic receptor and compete with acetylcholine for binding at the α/γ interface.

The αS-, αC-, αD-, α1-, and αO-Conotoxin Families

The αS-conotoxin family (Table 1) in the S-superfamily has the class VIII cysteine pattern − C − C − C − C − C − C − C − C − C − C−. The αS-RVIIIA from fish-hunting *C. radiatus* (Teichert et al. 2005a) (Table 3) blocked the α1β1εδ, α1β1γδ, and the α7 nicotinic receptor subtypes.

The αC-conotoxin family (Table 1) that belongs to the C-superfamily is in class X with the cysteine pattern − C − C− forming a single disulfide bond. The αC-PrXA from *C. parius* (Jimenez et al. 2007) (Table 3) competed with α-bungarotoxin for binding at the α/γ and α/δ subunit interfaces, with higher affinity for the α/δ interface.

The αD-conotoxin family (Table 1) of the D-superfamily has the cysteine pattern − C − CC − C − CC − C − C − C − C− and is in class XII. Members include αD-VxXIIA, αD-VxXIIB, and αD-VxXIIC from worm-hunting *C. vexillum* (Loughnan et al. 2006); αD-Cp and αD-Ms are from *C. capitaneus* and *C. mustelinus*, respectively (Kauferstein et al. 2009) (Table 3). αD-VxXIIA, αD-VxXIIB, and αD-VxXIIC inhibited the α7 and α3β2 subtypes; αD-VxXIIB was the most potent of the three peptides. Both αD-Cp and αD-Ms blocked the neuronal α7, α3β2, and α4β2 subtypes.

The α1-conotoxin family (Table 1) with the − C − C − C − C− cysteine pattern and disulfide framework [Cys1 − Cys3, Cys2 − Cys4] belongs to class XIV. The α1-pu14a from worm-hunting *C. pulicarius* (Peng et al 2010) (Table 3) blocked the muscle α1β1γδ subtype and the neuronal α3β2 subtype.

The αO-conotoxin family (Table 1) of the O-superfamily has the cysteine pattern − C − C − C − C− and is classified in class XIV. The most active isomer (bead form) of αO-GeXIVA from worm-hunting *C. generalis* (Luo et al. 2015) (Table 3) has the disulfide framework [Cys1 − Cys2, Cys3 − Cys4]. This isomer potently inhibited the α9α10 nicotinic receptor.

Nicotinic Receptor-Targeted Conotoxins and Nicotinic Receptor Subtype Specificity

The substantial work on conotoxins and nicotinic receptors raises issues related to the mechanisms that may explain conotoxin specificity for the nicotinic receptor subtypes. The nicotinic receptors used as assay for the characterization of conotoxins are mostly derived from mammals. These mammalian receptors are not the natural targets of any conotoxin. The specificity seen in the mammalian nicotinic receptor subtypes may be due to the highly conserved nicotinic receptors, such that the mammalian receptor subtype is not dissimilar from the physiological target of the conotoxin. This is possible if the conotoxin is found in venom of fish-hunting *Conus* species. Given that vertebrate nicotinic receptors are conserved, it is possible that a mammalian nicotinic receptor subtype is similar in sequence at the conotoxin binding site to the corresponding receptor in fish. On the other hand, near sequence similarity of mollusc or worm nicotinic receptor subtypes to mammalian receptor

Table 3 Amino acid sequences and nicotinic receptor specificities of divergent conotoxins

Peptide	Amino acid sequence	Nicotinic receptor specificity	References
ψ-PIIIE	HOOCCLYGKCRRYOGCSSASCCQR[a]	α1β1δ(γ/ε)	Shon et al. 1997
ψ-PIIIF	GOOCCLYGSCROFOGCYNALCCRK[a]	α1β1δ(γ/ε)	Van Wagoner et al. 2003
ψ-PrIIIA	AARCCTYHGSCLKEKCRRKYCC[a]	α1β1εδ > α1β1γδ	Lluisma et al. 2008
αA_L-PIVA	GCCGSYONAACHOCSCKDROSYCGQ[a]	α1/δ, α1/γ	Hopkins et al. 1995
αA_L-EIVA	GCCGPYONAACHOCGCKVGROOYCDROSGG[a]	α1/δ ≈ α1/γ	Jacobsen et al. 1997
αA_L-EIVB	GCCGKYONAACHOCGCTVGROOYCDROSGG[a]	α1/δ, α1/γ	Jacobsen et al. 1997
αA_S-OIVA	CCGVONAACHOCVCKNTC[a]	α/γ	Teichert et al. 2004
αA_S-OIVB	CCGVONAACPOCVCNKTCG[a]	α/γ	Teichert et al. 2005b
αAs-PeIVA	CCGVONAACHOCVCTGKC	α/γ	Teichert et al. 2006
αAs-PeIVB	CCGIONAACHOCVCTGKC	α/γ	Teichert et al. 2006
αS-RVIIIA	KCNFDKCKGTGVYNCGγSCSCγGLHSCRCTYNIGS MKSGCACICTYY	α1β1εδ, α1β1γδ, α7	Teichert et al. 2005a
αC-PrXA	TYGIYDAKPOFSCAGLRGGCVLPONLROKFKE[a]	α/δ > α/γ	Jimenez et al. 2007
αD-VxXIIA	DVQDCQVSTOGSKWGRCCLNRVCGPMCCPA SHCYCVYHRGRGHGCSC	α7, α3β2	Loughnan et al. 2006
αD-VxXIIB	DDγSγCIINTRDSPWGRCCRTRMCGSMCCP RNGCTCVYHWRRGHGCSCPG	α7, α3β2	Loughnan et al. 2006
αD-VxXIIC	DLRQCTRNAPGSTWGRCCLNPMCGNFCCPR SGCTCAYNWRRGIYCSC	α7, α3β2	Loughnan et al. 2006
αD-Cp	EVQECQVDTPGSSWGKCCMTRMCGTMCCSR SVCTCVYHWRRGHGCSCPG	α7, α3β2, α4β2	Kauferstein et al. 2009
αD-Ms	DVRECQVNTPGSKWGKCCMTRMCGTMCCAR SGCTCVYHWRRGHGCSCPG	α7, α3β2, α4β2	Kauferstein et al. 2009
α1-pu14a	DCPPHPVPGMHKCVCLKTC[a]	α1β1γδ, α3β2	Peng et al. 2010
αO-GeXIVA	TCRSSGRYCRSPYDRRRRYCRRITDACV[a]	α9α10	Luo et al. 2015

O 4-hydroxyproline, *γ* γ-carboxyglutamate
[a]amidated C-terminus

subtypes is less likely. Surprisingly, α-conotoxins from mollusc-hunting and worm-hunting *Conus* species, e.g., α-AuIB, α-ImI, and α-RgIA, also showed high specificity for mammalian receptor subtypes (Luo et al. 1998; Ellison et al. 2003; Ellison et al. 2006). Thus, these α-conotoxins may be targeting some conserved regions of nicotinic receptors and may not be very sensitive to the amino acid sequence diversity in other regions of the receptors.

Interactions influencing the high affinity of α-MI for the α/δ site of the nicotinic receptor were studied by mutating some residues in α-MI, expressing the α(2)βδ(2) pentameric nicotinic receptor complex in 293 HEK cells, and measuring the competitive binding of the mutant conotoxins against α-bungarotoxin. The α-MI mutations P6G, A7V, G9S, and Y12T led to lower affinity for the α(2)βδ(2) nicotinic receptor complex. Mutations of the nicotinic receptor revealed key contributions to α-MI affinity by Tyr198 in the α subunit, and Ser36, Tyr113, and Ile178 in the δ subunit. In double mutant cycle analysis, Tyr12 of α-MI strongly interacts with Ser36, Tyr113, and Ile178 in the δ subunit and that Ser36 and Ile178 stabilize the receptor linkage with Tyr12 of α-MI. Strong interactions were also found between Pro6 and Ala7 in α-MI and Tyr198 in the α subunit and between Pro6 and Gly9 in α-MI and Ser36, Tyr113, and Ile178 in the δ subunit (Bren and Sine 2000).

Examination of the binding site of neuronal α7 nicotinic receptor utilizes α-ImI. Using cycle analysis with mutant subunits, pairs of residues that stabilize the α-ImI-receptor complex were identified. The α-ImI binding was measured by competitive binding against α-bungarotoxin. A major interaction was found between Arg7 of α-ImI and Tyr195 of α7 nicotinic receptor that anchors the toxin to the binding site. Several weak interactions were also present between Asp5 of α-ImI and Trp149, Tyr151 and Gly153 of the α7 receptor, and between Trp10 of α-ImI and Thr77 and Asn111 of the α7 receptor (Quiram et al. 1999).

Defining Nicotinic Receptor Function: Use of α-Conotoxins

The molecular complexity of nicotinic receptors makes the determination of their functions in the central nervous system thought provoking. A practical approach is to combine receptor subtype-specific α-conotoxins with knockout mutants. With the use of knockout mice, the major nicotinic receptor subtype involved in the modulation of dopamine release in the striatum was revealed. It was found that α-MII inhibited about half of nicotine-evoked dopamine release (Kulak et al. 1997). Later, it was reported that all binding of α-MII in the striatum in α6-knockout mice was eliminated (Champtiaux et al. 2002). Moreover, β3-knockout mice had much reduced α-MII binding sites expressed in the striatum (Cui et al. 2003). These studies indicate that a nicotinic receptor complex with α6β3 subunits is the major α-MII-sensitive nicotinic receptor subtype that regulates dopamine release.

The discovery of α-MII and the development of nicotinic receptor subunit null mutant mice are important in determining which nicotinic receptor subunits influence expression and function. Five nicotinic receptor subtypes that are expressed on dopaminergic nerve terminals have been identified: the α4α6β2β3, α6β2β3, and

α6β2 subtypes that bind α-MII with high affinity and α4β2 and α4α5β2 that do not have high affinity for α-MII but bind nicotine with high affinity (Grady et al. 2007). Considering the differences in sensitivity among these nicotinic receptor subtypes, it is possible that subtype-specific conotoxins could be developed that would allow therapeutic manipulation of varied nicotinic receptors that have been implicated in neurological conditions.

Conclusion and Future Directions

Much work remains to be done to understand nicotinic receptor subtype specificity of conotoxins. The disulfide framework and certain amino acid residues of conotoxins seem to be optimized for interaction with binding sites on nicotinic receptors. So far, the α-conotoxin family is the best characterized to evolve novel specificity for nicotinic receptor subtypes. During the course of the evolution of the cone snail, when ecological conditions occur so that inhibition of particular nicotinic receptor subtypes might help catch new prey or dissuade predator or competitor, the emerging *Conus* species most likely produces in its venom the nicotinic receptor antagonists with suitable targeting specificities that are necessary for its continuing existence. Insights into these evolutionary trends of cone snails have been utilized particularly in the development of unique ligands for nicotinic receptors.

The localization of distinct nicotinic receptor subtypes in specific regions of the central nervous system indicates the possibility of developing drugs for the affected regions. Some α-conotoxins were reported to have significant therapeutic potential (Table 4).

The conotoxins that target α9-containing nicotinic receptors, such as Vc1.1, α-RgIA, and αO-GeXIVA, have potential use for the treatment of neuropathic pain caused by nerve injury. Inhibition of α9α10 nicotinic receptor can relieve chronic pain resulting from peripheral nerve injury (Vincler et al. 2006). The α9α10 nicotinic receptor antagonist Vc1.1 showed potent analgesic effect in preclinical animal models of human neuropathic pain and seemed to hasten the recovery of damaged neurons (Satkunanathan et al. 2005). Conotoxin Vc1.1 (ACV-1) has reached Phase II clinical trials as treatment for neuropathic pain and is a potential treatment for

Table 4 Nicotinic-receptor-targeted conotoxins with potential for drug development

Peptide	Nicotinic receptor specificity	Possible indication	Remarks	References
α-Vc1.1	α9α10	Neuropathic pain	Phase II	Livett et al. 2006
α-RgIA	α9α10	Neuropathic pain	clinical trials	Vincler et al. 2006
αO-GeXIVA	α9α10	Neuropathic pain	–	Luo et al. 2015
α-MII	α6β2β3	Parkinson's	–	Olivera et al. 2008; Quik and McIntosh 2006
α-ImI	α7	disease	–	
		Schizophrenia	–	Ishikawa and Hashimoto 2011

diabetic neuropathy, sciatica, and shingles (Livett et al. 2006). α-RgIA caused relief in acute and chronic pain models when administered to rats after chronic constriction injury of the sciatic nerve (Di Cesare Mannelli et al. 2014). It was found that αO-GeXIVA could reduce mechanical hyperalgesia in the rat chronic constriction injury model of neuropathic pain (Luo et al. 2015).

The identification of α6-containing nicotinic receptor subtypes that are expressed in dopaminergic nerve terminals utilizes α-MII as ligand (Grady et al. 2007). The α6-containing nicotinic receptors are localized in the nigrostriatal area; thus, they represent targets for Parkinson's disease that is characterized by progressive degeneration of the nigrostriatal system (Olivera et al. 2008; Quik and McIntosh 2006).

The neuronal α7 nicotinic receptor has been implicated in the pathology of schizophrenia (Ishikawa and Hashimoto 2011). Thus, conotoxins, such as α-ImI, that target the α7 nicotinic receptor could be explored as potential therapeutic agent for schizophrenia.

The binding elements that substantially contribute to the high affinity of nicotinic receptor-targeted conotoxins have been characterized for some pairs of conotoxins and nicotinic receptors. Using similar approach, more nicotinic receptor sequences could be revealed and more binding sites could be defined. Determining which amino acid substitutions on the conotoxin will result in greater affinity for a particular nicotinic receptor subtype will be more accurate. Thus, the identification of more amino acid sequences and the growing information on the binding specificities for pairs of conotoxins and nicotinic receptors could lead to the development of unique ligands for the receptor subtypes.

Regarding the use of conotoxins, it could not be presumed that binding correlates with function; that is, a conotoxin could bind to a nicotinic receptor but not necessarily inhibit that receptor. A complication is that although the primary binding site is an interface between two subunits, the presence of other subunits in the native nicotinic receptor could affect the affinity of the conotoxin for that receptor. Nevertheless, as more conotoxins are tested on native nicotinic receptors, their binding specificities and functions could become well characterized.

The nicotinic receptors play an important role in neurotransmission. They are often involved in the pathology of certain neurological disorders. The diversity and distribution of nicotinic receptor subtypes that are the targets of divergent families/subfamilies of conotoxins offer an opportunity for the discovery of novel neuropharmacological agents.

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Part III

Peptides

Random Peptide Library for Ligand and Drug Discovery

10

Tai Kubo

Abstract

In ancient times, natural extracts that derived mainly from plants and animals were utilized for medical or religious purposes. Even after starting the drug discovery and development in the realm of modern pharmacological sciences, the traditional medicines are still reasonably valued. Moreover, the modern approaches for drug discovery are appreciably supported by the resources and the accumulated knowledge of naturally occurring ligands and the offense- and defense-related substances. Among them, bioactive peptides, peptide toxins, and antibodies have been successfully applied in therapy due to their superior target selectivity. However, such biotherapeutics have middle to large molecular weights (>500 Da) in contrast to small molecular weight drugs. Size minimization is especially a concern for membrane permeability, stability in blood, antigenicity, and production costs. Biotherapeutic molecules have been engineered and further improved in terms of their affinity, stability and humanization for more than a decade to make them suitable for biotherapeutics. In this chapter, two approaches currently being used to improve biotherapeutics are overviewed: the size reduction of immunoglobulin-based biotherapeutics and the utilization of nonimmunoglobulin scaffolds. In both approaches, random peptide libraries are constructed based on each scaffold and screened for specific binders (polypeptides) with bioactivities of interest. Natural scaffolds that have been refined during long-term evolution are focused on. Some genes encoding offense/defense or reproduction-related proteins are known to undergo unusually rapid amino acid substitutions in the mature protein-coding regions, whereas the cysteine framework and α/β structures are conserved to maintain the molecular scaffold. This mode of evolution (called accelerated evolution) inspired the utilization of the

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_2

gene as a template to construct a random peptide library for use for directed evolution in vitro. Screening and in vitro evolution technologies, including phage display, ribosome/mRNA/cDNA displays, the new technology "PERISS," and in vitro compartmentalization, are also discussed.

Keywords

In vitro evolution • Peptide library • Three finger (or 3-F) • Inhibitor cystine knot (or ICK) • Accelerated evolution • Protein-protein interaction (or PPI)

Contents

Introduction

In the living system, receptors and ion channels play essential roles to receive or to percept environmental changes/stimuli. They transduce and propagate the signals to downstream networks to respond appropriately for maintaining homeostasis or to initiate higher-order biological functions such as immune responses, cellular development, learning, and memory. Molecular recognition and selective interactions are the molecular basis of such complex but highly controlled and harmonized performances of the living system. A malfunction of the system initiates an impairment of health and leads to diseases. Receptors and ion channels are now a major target for drug discovery. Ligands, the molecules that specifically bind to and modulate the function of the receptors and ion channels, are utilized for drug development and used as a molecular tool for scientific research as well (Imming et al. 2006). The binders/modulators could be a variety of materials; organic or

Table 1 Comparison of synthetic drugs and biotherapeutics

	Synthetic Drugs	Biotherapeutics	Biotherapeutics
	Low Molecular Weight (<500 Da)	Mid-size Molecular Weight (500~10k Da)	High Molecular Weight (>10k Da)
Examples	Chemical compounds: Aspirin Atropine Colchicine Digitalis Nicotine Quinine etc.	• Antibiotics and derivatives (Cyclosporine A, FK506, Rapamycin, etc.) • Peptide hormones (ANP, CNP, Adrenomedulin, Ghrelin, etc.) • Peptide toxins and derivatives (**ω**-Conotoxin MVIIA (Ziconotide), Eptifibatide (Integrilin), Tirofiban, etc.)	• Interferons • Interleukins • Vaccines • Antibody medicines (Rituximab (anti-CD20), Bevacizumab (anti-VEGF), Trastuzumab (anti-HER2), Nivolumab (anti-PD1), etc.) • Enzymes (Amylase, Pronase, Lysozyme, Pancreatin, etc.) • Toxins (Botulinum toxins A and B)
Resources	Natural compounds (collection/library) Chemical libraries (chemical synthesis and synthetic deployment)	Natural resources or mimetics Random peptide libraries with natural/designed scaffold	Biomolecules including immunoglobulin and immune-related proteins, and functional proteins
Screening, Identification and Drug Seeds Optimization	• High-throughput screening • Experience-based development • In scilico design based on MD simulation	• Peptide library: Molecular diversity is huge, and in vitro evolution technology is applicable; flexible in selection/assay criteria	• Biological/clinical information driven • Antibody medicines: Antigen and host dependent • Humanization, PEG, Potelligent, Compligent
Membrane Permeability (Oral Administration)	Permeable (Yes)	Permeable after modification or with DDS (Yes/No)	Difficult (No)
Targeting Cytoplasm or Nucleus	Possible	Possible after modification or with DDS	Difficult
Target Specificity (Off-target Problem)	Generally low (serious in case)	High (generally low)	High (generally low)
PPI Control	Difficult (partial)	Possible	Possible
Antigenicity	Generally low	Low~Medium	Serious in case
Commercial Production	Chemical plant	Chemical/biochemical production	Bioprocessing plant
Quality Control	Regular	Regular	Higher control is required

(*continued*)

Table 1 (continued)

	Synthetic Drugs	Biotherapeutics	Biotherapeutics
	Low Molecular Weight (<500 Da)	Mid-size Molecular Weight (500~10k Da)	High Molecular Weight (>10k Da)
Production Cost	Low	Low~Medium	High
Pharmaceutical Innovations	Mega-pharma (synthetic deployment/ optimization, etc.)	Academia/institution lab or venture in seeds development and pharmaceutical companies at the later stage (production/ clinical trials/approval)	Venture (optimization) and mega-pharma (production/clinical trials/approval)

inorganic chemical compounds, peptides, DNAs/RNAs, carbohydrates or proteins (Taussing et al. 2007). In Table 1, different types of ligands and therapeutic drugs are classified based on molecular sizes; small-, medium-, and high-molecular weight. Characteristic features of each type of the drugs and pros/cons in medical usages and in drug development/production are also compared. Here in this chapter, random peptide libraries inspired by naturally occurring toxin/bioactive peptides are focused on, because of its great advantages as screening resources and in physicochemical and pharmacological features.

Chemically synthesized drugs ("Low Molecular Weight" in Table 1) have a long history of development and are widely utilized for therapeutic and diagnostic purposes. However, they are not necessarily specific to the target but instead interact with other molecule(s) unexpectedly and usually unfavorably. This phenomenon sometimes leads to serious side effects and off-target problems (Muller and Milton 2012). In contrast, biopharmaceuticals (e.g., antibody medicines; "High Molecular Weight" in Table 1) are characterized by their selectivity and specificity for the target. Multivalent intermolecular interactions make it possible to securely recognize and interact with the target molecule and to commit to the specific protein-protein interactions (PPIs) of interest (Kintzing et al. 2016).

A typical example of PPIs is specific interactions between the antibody and antigen. Due to their specificity, antibodies have been successfully utilized in therapies as "antibody medicine" and in diagnosis as probes to detect biomarkers, which are used for critical decisions concerning medical treatment and drug evaluations. Despite of their obvious advantages in specific medication, bottlenecks underlying their production process must be overcome to achieve low cost and safe medication due to particular characteristics of immunoglobulins, such as their large molecular weight, glycosylation, and humanization. Size minimization and membrane permeability are both concerns. When membrane permeability is achieved, the drug can target cytoplasmic molecules by oral administration. Besides the PPIs by antigen and antibody, selective recognition and interactions are also found in natural biological systems; e.g., protease and substrate/inhibitor, ion channel and blocker, receptor and ligand, and transcription factor and nucleotide binding sequence. Inspired by these specific PPIs observed in nature, various types of

nonimmunoglobulin binders have been developed in the past decade (Nygren and Skerra 2004; Binz et al. 2005; Wurch et al. 2012).

As compared in Table 1, biopharmaceuticals of medium size have broad advantages over small synthetic drugs or large protein medicines. Supported by recent progress in technologies and availability of high-performance computers, it is getting more precise to analyze the structure and function of drug candidates of mid-size biotherapeutics, and getting rapid to assess the druggability. Thus, the feasibility to challenge the drug discovery and development of the mid-size biotherapeutics is revolutionary increased. In this chapter, several different immunoglobulin/nonimmunoglobulin scaffolds that have been developed to construct random peptide libraries for the screening are overviewed, and recent progress in directed evolution technologies to identify specific binders from the libraries is described.

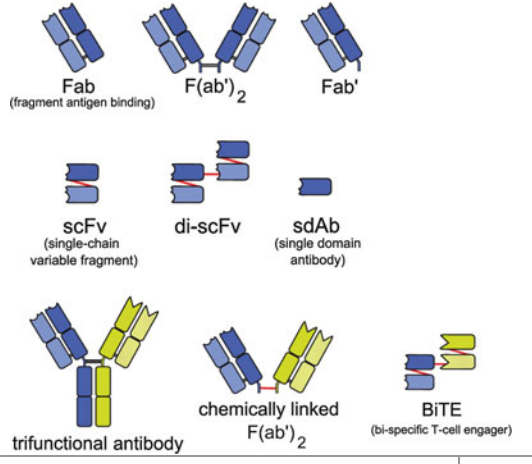
Peptide Ligands as Medium-Sized Drug Candidates: Specific Binders to Target Molecules

Medium-sized biomedicines are expected to be promising for next-generation drug development. Their advantages over small molecule chemical drugs and high molecular weight biomedicines are target specificity and selectivity, the potential multivalency of the function, a long serum half-life, low GMP (good manufacturing practice) and practical production costs (Table 1). In this section, two different approaches to develop medium-sized biomedicines are described; the first is downsizing of the immunoglobulin molecule (or its scaffold; minimized antibody) engineered without the loss of its target recognition ability, and the second is the utilization of naturally occurring nonimmunoglobulin scaffolds to create specific binders (nonimmunoglobulin binders) (Table 2).

Antibody Medicine: Immunoglobulin to Minimized Antibody

Prior to the development of monoclonal antibody technology (Kohler and Milstein 1975), antibody production and quality were totally dependent on host animals for immunization. The hybridoma and large-scale mammalian cell culture technologies have accelerated broad applications of antibodies to medicine and moved them almost within our reach. Supported by recent progress in genetic engineering, immunoglobulin molecules can be manipulated by processes such as point mutations, deletions, and replacement to modify target selectivity and affinity, posttranslational modification, and even effector coupling. Due to their elaborate target selectivity and minimal side effects, monoclonal antibodies are greatly valued as biotherapeutics (antibody medicines). However, as mentioned above, biotherapeutics also have drawbacks (e.g., they are expensive to manufacture, are limited by route of administration (injection), and are unable to target cytosolic biomolecules).

Table 2 Immunoglobulin and nonimmunoglobulin scaffolds used to construct random peptide libraries

Scaffold name	Protein and its structure features	Example of target proteins	References
1. Immunoglobulin scaffold			
Maxibody	Human bivalent scFv-Fc		
Minibody	Human scFv-CH3		
Unibody	Human IgG4		
Fab, $(Fab')_2$	Human Fab fragment(s)		
scFab	Conjugate of human Ig VH-CH1 and VL-CL		
Fv	Human Ig VH and VL		
scFv	Human Ig single chain VH linked with VL		
Diabody/tri(a)body	Two/three sets of scFv linked in tandem		
BiTE	Two different scFvs linked; bispecific diabody	CEA + CD3, CD19 + CD3, EpCAM + CD3	[1]
Nanobody	VHH domain of camel Ig	IL-6R, TNFα, vMF, etc.	[2]
dAbs, TandAb	Single variable region of VH or VL	TNFα, CD30 + CD16a	[3]
2. Non-immunoglobulin scaffold			
2.1 Protein scaffold			
Transbody	Transferrin; α/β		[4]
Tendamistat	α-Amylase inhibitor; β-sandwich	mAb, integrins	[5]
Adnectin/monobody	10th FN3 (fibronectin); β-sandwich	TNFα, ubiquitin, integrin, SH3, estrogen receptor domains, VEGFR-2	[6, 7]

Anticalin	Lipcalins; β-barrel; 0-3 S-S	Hemoglobin, CTLA-4 streptavidin, VEGF-A (digoxigenin, fluorescein, benzyl butyl phthalate)	[8, 9]
mTCR	T-cell receptor; β-sandwich	Peptide/MHC complexes	[10-12]
Affibody	Z domain of protein A; a member of the coagulation cascade; 3α	*Taq* DNA polymerase, insulin, apolipoprotein A1, protein Z, lgA, Factor Vlll, RSV protein G fragment, CD28, HER-2/neu	[13]
DARPin	Ankirin repeat (AR); 2α/2β repeated	MBP, p38, JNK2, APH, VEGF-A	[14]
Zinc finger	α/β (Zn^{2+})	mAb	[15]
Avimer	A-domain/Low density lipoprotein receptor	IL-6	[16]
Atrimer	Tetranectin; C-type lectin domain family 3; trivalency		[17]
Versabodies	High cysteine containing mini-protein/non-human origin; multi-valency		
Flitrx	Thioredoxin; α/β; 1 S-S	mAbs, CDK2, Mdm-2, E2F, E6	[18]
CBD	Cellulose binding domain; 3β; 2 S-S	Alkaline phosphatase, α-amylase(Ni-NTA beads)	[19, 20]
2.2 Scaffold refined through accelerated evolution			
Kunitz domain			
BPTl	Bovine pancreatic trypsin inhibitor; Kunitz domain; α/β; 3 S-S	Human neutrophil elastase	[21]
Ecotin	Serine protease inhibitor in the peroplasm of Gram-negative bacteria; 3β; β-sandwich	uPA, trypsin, plasma kallikrein, MT-SP1, FXllA	[22]
LACl-D1	1st domain/human lipoprotein-associated coagulation inhibitor; α/β	Plasmin, thrombin, plasma kallikrein	[23]
ICK/Knottin motif			
Scorpion toxins	Charybdotoxin; 1α/3β	Acetylcholine receptor, gp120, mAbs	[24, 25]
Spider toxin	GTx1-15; 1α/3β; 3 S-S	mAChR m2	[26]
EETl-ll	*Ecballium elaterium* (squash) trypsin inhibitor II; Coiled, 3 S-S	Elastases, chymotrypsin, trypsin, carboxypeptidase, antibody epitopes	[27]
Defensin	Insect defensin A peptide; 1α/2-3β; 3 S-S	TNFα, TNFR, mAb	[28]

(*continued*)

Table 2 (continued)

Scaffold name	Protein and its structure features	Example of target proteins	References
Three Finger	MicTx3/three-finger neurotoxin; 4β; 4 S-S	IL-6 R, trypsin, AChBP, VEGF	[29, 30, 31]
WAP motif	BufTx1; 2β; 4 S-S		[32]
2.3 Rationally designed scaffold			
Min-23	Designed based on EETI II (squash trypsin inhibitor); 3β; 2 S-S (CSB, cystine-stabilized β-sheet motif)	mAbs, AMA-1, Tom70, HIV-1 Nef	[33]
Microantibody	Helix-loop-helix/Cys-helix-loop-helix-Cys; leucine-zipper stabilized helices	GCSF-R, Aurora-A, GM1, VEGF	[34]
Aptide®	Tryptophan-zipper stabilized scaffold	VEGF, EDB	[35]
Cys-constrained 27 mer	Cys appearance in 12 % probability (3-4 Cys/27 aa)	NH_2 group	[36]
Bicycle®	Cysconstrained bicyclic peptide two variable regions flanked by Cys residues (3 Cys/two loops); bicyclic peptide formation by halomethylarene	Kallikrein	[37]

Inset figure, schematic structures of engineered immunoglobulins, was from https://commons.wikimedia.org/wiki/File:Engineered_monoclonal_antibodies.svg
Some scaffolds listed in "non-immunoglobulin scaffold" were adapted and modified from a detailed review by Binz et al. 2005

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One solution proposed to overcome the problems is to downsize the antibody from the whole molecule (~150 kDa) to the minimal elements required for function. Several innovative antibody engineering approaches have been proposed based on immunoglobulin genes (e.g., Knappik et al. 2000; Fab combinatorial library approach, Mao et al. 2010). Moreover, proteins and domains essential for the recognition of and binding to the target(s) were extracted and reconstituted to generate functional polypeptides for this purpose (see "1. Immunoglobulin scaffolds" in Table 2).

A typical example is a "single-chain variable fragment (scFv)." In scFv, antigen-binding variable regions of the immunoglobulin H- and L-chains are linked by a flexible linker (Hanes et al. 2000; Soderlind et al. 2000). The size (~30 kDa) is 1/5 of that of the immunoglobulin. When two or three sets of scFvs are assembled or tandemly linked, they are called "Diabody"/"Tri(a)body" or "tandem di-scFv"/"tandem tri-scFv," respectively (Table 2). This combination of multiple scFvs can be used to enhance affinity and to recognize different targets simultaneously. A BiTE (bispecific T cell engager) consists of two different scFvs; the first scFv functions as an antitumor antibody, and the second recognizes CD3 on the T cells. For example, a BiTE generated to target CD19 and CD3 showed very potent regression activity against lymph node tumors (Bargou et al. 2008). The BiTE can support the assembly of helper T cells around target tumors and at the same time trigger ADCC (antibody-dependent cellular cytotoxicity)-like activity by activating T cells as effectors. Because of its rational design and broad applicability, BiTE technology is expected to drive next-generation antibody medicine.

A domain antibody (dAb) or single-domain antibody (sdAb) contains a single variable region (V_H or V_L) harboring three complementarity-determining regions (CDRs 1, 2, and 3), which generate diversity in the antibody and thus are responsible for the selectivity and affinity for target molecules (Table 2, and see review Holt et al. 2003). Presentation of a variable region from a single chain is naturally found in camels, llamas (nanobody), and sharks (IgNA, new antigen receptor). These regions might be the minimum extremes of the minimized antibody for target recognition (molecular weight, 12–15 kDa, 1/10 of the immunoglobulin). Because sdAbs are short and soluble polypeptides, they possess the following advantages as biotherapeutics: (1) convenient expression in *E. coli* and subsequent purification, (2) high cellular infiltration efficiency, and (3) broad application by combination with therapeutic payloads (i.e., radionuclides, toxins, enzymes, liposomes, and viruses) (Holliger and Hudson 2005).

Nonimmunoglobulin Binders

As mentioned above (section "Antibody Medicine: Immunoglobulin to Minimized Antibody"), there are advantages in the use of engineered antibodies. However, these concepts and technologies are covered by strategic patents, which are associated with higher production costs for biotherapeutics. Similar to the CDRs in immunoglobulin molecules, spatial presentation of the sequences responsible for target recognition and binding could also be achieved using a natural nonimmunoglobulin scaffold. Inspired

by natural proteins, scientists have used many proteins to construct libraries that are then screened for selective binders. Some protein scaffolds are listed in Table 2 (the conventional or product name is in parenthesis) including fibronectin (adnectin/monobody), *Staphylococcus aureus* protein A (affibody), lipocalin (anticalin), LDL (low density lipoprotein) receptor A-domain (avimer), ankyrin (DARPin), thioredoxin (Flitrx), T cell receptor (mTCR), α-amylase inhibitor (tendamistat), transferrin (transbody), microprotein domain (versabodies), and variable lymphocyte receptor ("2. Non-immunoglobulin scaffold" in Table 2, Binz et al. 2005). These proteins are stabilized by their secondary structures (α-helices and/or β-structures). To prepare a random peptide library, randomized sequences are incorporated into the specified position(s) in the scaffold (e.g., positions in loops (fibronectin), flat surfaces (protein A), loop and helix combination (ankyrin), or a cavity (lipocalin)). All of these proteins are soluble and thermostable, and their yield is high when they are recombinantry expressed. Similar to minimized antibodies, the primarily selected proteins are usually further tuned up to improve their properties, such as affinity and specificity, by engineering called "maturation."

An ankyrin repeat (AR) is an example. Pluckthun and colleagues focused on AR, which has 33 amino acid residues arranged in a unit composed of two antiparallel α-helices and a β-turn. The authors prepared peptide libraries by introducing random sequences into the regions exposed to the surface of the AR molecule (Binz et al. 2004). They isolated specific binders to Her2 (human epidermal growth factor 2) from the AR library and then successfully improved the affinity to $K_d = 90$ pM by affinity maturation (error-prone PCR and amino acid substitutions) (Zahnd et al. 2007b). Interestingly, when two ARs were conjugated by a linker, each AR had an independent epitope against FcεR1α ($K_d = 10$ nM each) that showed bispecificity and improved affinity ($K_d = 10$ pM). Furthermore, the AR conjugate was found to inhibit the degranulation of basophilic leukocytes in a cell-based assay with a potency comparable to the known antibody medicine omalizumab (Xolair) (Eggel et al. 2009).

Some nonimmunoglobulin binders including AR have a characteristic feature of repetitive units. To design an effective nonimmunoglobulin scaffold, we can refer to peptides/proteins containing repetitive surface units (Forrer et al. 2004). Inspired by the antifreeze protein, which belongs to the β-solenoid proteins, an artificial scaffold with self-assembled flat surfaces was designed and characterized (Peralta et al. 2015). Although this type of flat surface assembly was originally proposed for use in nanoscale laminate construction (e.g., photovoltaics and scaffolding for tissue engineering), it may have potential applications in biotherapeutics upon introduction of random sequences for library construction or through attachment to functional entities.

Random Peptide Libraries Designed from Naturally Occurring Peptide Toxin Scaffolds

Disulfide bonds together with secondary structure elements (α-helix and β-structure) are important for the correct formation and maintenance of 3-dimentional structures of protein and contribute to its function. Disulfide-containing proteins often play

important roles in defense and/or offense and range from prokaryotic toxins to immunoglobulins (Gruber et al. 2007; Tsetlin 1999; Kini and Doley 2010). There has been growing interest in plant and animal toxins for pharmacological and drug template uses (Kolmar 2008; Miljanich 2004). For example, an FDA (Food and Drug Administration)-approved drug "Ziconotide" is a derivative of one of the conotoxins (the ω-conotoxin MVIIA) that is a peptide of 25 amino acid residues long with three disulfide bonds. Ziconotide was characterized as a potent and selective blocker of N-type calcium channels. Peptide toxins from venomous animals (such as cone snail, spider, scorpion, toad, snake) are small (10–80 residues), compact, resistant to proteolysis, and lowly immunogenic, and some of them are stabilized by a knotted structure by three to five disulfide linkages that imparts thermal stability. These properties also make them more suitable for drug development due to their increased bioavailability (Kolmar 2008). An interesting and potentially intriguing aspect of these proteins is their diversity within the same species; for example, snake venom contains hundreds of toxins that have diverse functions (Tsetlin 1999; Kini and Doley 2010; Jeyaseelan et al. 2003; Fry et al. 2003). The diversity is thought to be generated from the accelerated evolution of the binding or surface loops of these proteins to adapt to diverse conditions, such as changes in defense/offense targets due to dynamic changes in the environment. During evolution, several principal scaffolds with potency against multiple targets might have been selected and further refined.

Three-Finger Peptide Library from Snake Neurotoxin Scaffold

Three-finger (3-F) proteins are found in a variety of organisms, such as the elapidae snake and mammals including humans. Hundreds of 3-F proteins/toxins have been reported to date, including α-bungarotoxin, erabutoxin A, α-cobratoxin, muscarinic toxins, and lynx 1 (reviewed in another chapter of this handbook and by Endo and Tamiya 1987; Tsetlin 1999; Miwa et al. 1999; Fry et al. 2003). 3-F proteins are small proteins (Mw 7–8 kDa) with 4–5 disulfide bonds, 2–5 β-structures, and 3 protruding loops that provide the topological basis for the three-finger structure (Endo and Tamiya 1987). The striking features of the 3-F protein family are the strict conservation of the cysteine framework and high sequence diversity in the loop (corresponding to the finger) regions. These features may reasonably provide 3-F toxins with a broad spectrum of target molecules in nature, such as ion channels, receptors, and proteases (Fry et al. 2003).

Based on target diversity and structure-function studies of various types of 3-F toxins, peptides with 3-F scaffolds were expected to be utilized in drug discovery (Kini and Doley 2010). The loop regions were subjected to various amendments (e.g., point mutations, replacement, or grafting) to install a new property (i.e., target selectivity, affinity, or physicochemical property) to the original structure. New ligands for G protein-coupled receptors (GPCRs) with novel functions were generated by grafting the loop regions of the 3-F muscarinic toxins (Fruchart-Gaillard et al. 2012).

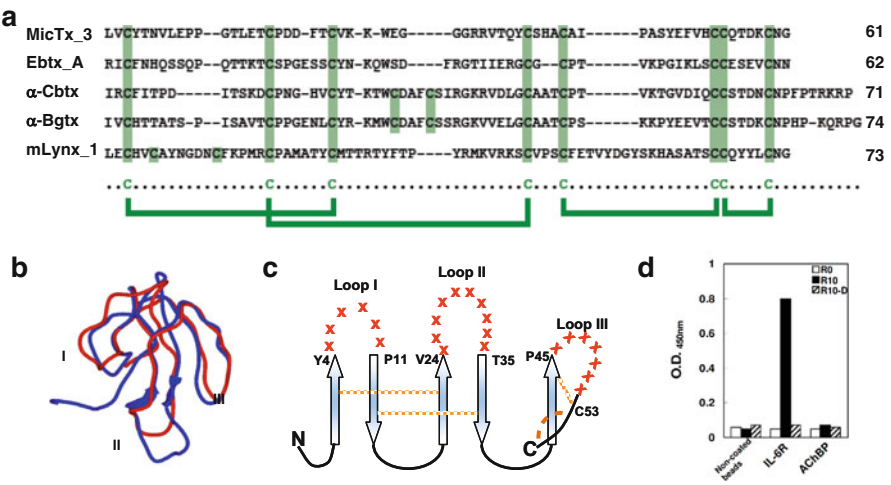

Fig. 1 Sequence and structure of MicTx3 and a three-finger peptide library constructed using the MicTx3 as a template. (**a**) Sequence alignment of MicTx3 and representative three-finger proteins. Mature forms of MicTx3, erabutoxin A (Ebtx_A; UniProt Acc#P60775), α-cobratoxin (α-Cbtx; P01391), α-bungarotoxin (α-Bgtx, P60615), and mouse lynx1 (mLynx_1, Q9WVC2) were aligned by the Clustal W method using the software Lasergene ver.7 (DNASTAR, Madison, WI, USA). Conserved cysteine residues among the toxins are highlighted in green, and the disulfide bonds expected based on the analysis of α-Bgtx are shown by lines. The number of amino acid residues is shown on the right. (**b**) Superimposition of three-dimensional structures of the MicTx3 model and the α-Bgtx experimental structure. The three-dimensional model of MicTx3 was generated by the software Internal Coordinate Mechanics (Molsoft, La Jolla, CA, USA) using the α-Bgtx structure (PDBID: 1ik8) as a modeling template. MicTx3 is shown in red, and α-Bgtx is in blue. The numbers in Roman numerals represent the loop numbers. (**c**) Schematic of the library based on the 3-F scaffold. The antiparallel β-sheets are depicted with blue arrows, and the randomized loop residues are indicated with red crosses. Numbers such as Y24 and P11 indicate residues adjacent to the randomized residues (also see **a**). N and C indicate the amino- and carboxyl-terminals, respectively. (**d**) Binding assay assessment of the quality of the libraries. The MicTx3-based 3-F library was subjected to screening for binders to the interleulkin-6 receptor (IL-6R). The initial library (R0) and round 10 library (R10) were prepared by the cDNA display method. The R10 library was reduced with dithiothreitol (DTT) to obtain the R10-D library. All three forms were incubated with noncoated beads, IL-6R-coated beads, or AChBP-coated beads and washed and detected with Penta His HRP (horseradish peroxide) (Adapted from Naimuddin et al. 2011)

When random sequences are introduced into the three fingers simultaneously, the resulting random peptide library may have profound potential to select binders to various target molecules. The pluripotency of the 3-F random peptide library was shown by Kubo and his colleagues by utilizing the library for one of the in vitro evolution technologies ("cDNA display") (Fig. 1, see section "Directed In Vitro Evolution of Peptides from a Random Peptide Library Toward Ligands and Biotherapeutics"). These authors successfully isolated 3-F peptides targeting the interleukin-6 (IL-6) receptor (Naimuddin et al. 2011), trypsin (Cai et al. 2014), acetylcholine binding protein (AChBP), and vascular endothelial

growth factor (VEGF) (Naimuddin and Kubo 2016). To prepare a random 3-F peptide library, a cDNA encoding the snake α-neurotoxin MicTx3 from the South American coral snake *Micrurus corallinus* was used as a template. MicTx3 is composed of 61 amino acids and belongs to the short α-neurotoxin family (see multiple alignment with the family; Fig. 1a). Based on the alignment and computer modeling, three finger tips containing the amino acid residues Thr5–Pro10 in loop I (six residues), Lys25–Val34 in loop II (ten residues), and Ala46–His52 in loop III (seven residues) of MicTx3 were randomized by introducing $(NNS)_6$, $(NNS)_{10}$, and $(NNS)_7$, respectively, into the corresponding positions of the cDNA (Fig. 1c). The resulting theoretical diversity of the library is 20^{23}, although the practical diversity in a test tube may reach 10^{12}–10^{13}.

As previously mentioned (Nygren and Skerra 2004), libraries with relatively large numbers (e.g., 10–24) of randomized residues (regarded as "broad and shallow") might be helpful for finding first generation binders. These libraries can be further upgraded or fine-tuned by affinity maturation via screening of "narrow and deep" libraries (Gunneriusson et al. 1999; Schlehuber and Skerra 2002). In addition to affinity maturation, selected binders should be able to perform surveillance of the region(s) responsible for affinity and selectivity. Interestingly, the 3-F binders to the IL-6 receptor, which showed antagonist- or agonist-like activity in the IL-6-dependent cell-based assay, succeeded in size minimization to one finger (24 amino acid residues) without a significant loss of activity (Naimuddin et al. 2011).

Another example of the 3-F random peptide library was a bucandin (*Bungarus candidus*)-based library. Similar to MiTx3, each fingertip was randomized, and the library was successfully used to screen binders to survivin (Nemoto et al., personal communication), which is an inhibitor of the apoptosis protein family and a valuable biomarker for lung and colon cancers.

Inhibitor Cystine Knot Library from Spider Neurotoxin Scaffold

A protein motif called inhibitor cystine knot (ICK) is also used to prepare random peptide libraries. The ICK toxins have been identified in arachnids (e.g., spiders, scorpions, ticks, and mites), mollusks, and plants. Grammotoxin, hainantoxin, hanatoxin, huwentoxin, and vanillotoxin are examples, and their major targets are ion channels, including K^+, Na^+, and Ca^{2+}channels; mechanosensitive and stretch-activated channels; and TRP (transient receptor potential) channels (see other chapter of this handbook series and Kimura et al. 2012). ICK toxins are compact and globular proteins (35–40 amino acid residues) with an antiparallel triple-stranded β-sheet and either a short or incomplete α-helix that forms a knot-like fold with three disulfide bridges. Similar to 3-F toxins, ICK motif toxins commonly maintain the principal scaffold but show sequence variations in the outer loop or surface regions that may lead to target diversity. Based on a comparison of ICK family sequences, it is obvious that the ICK family was generated by accelerated gene evolution.

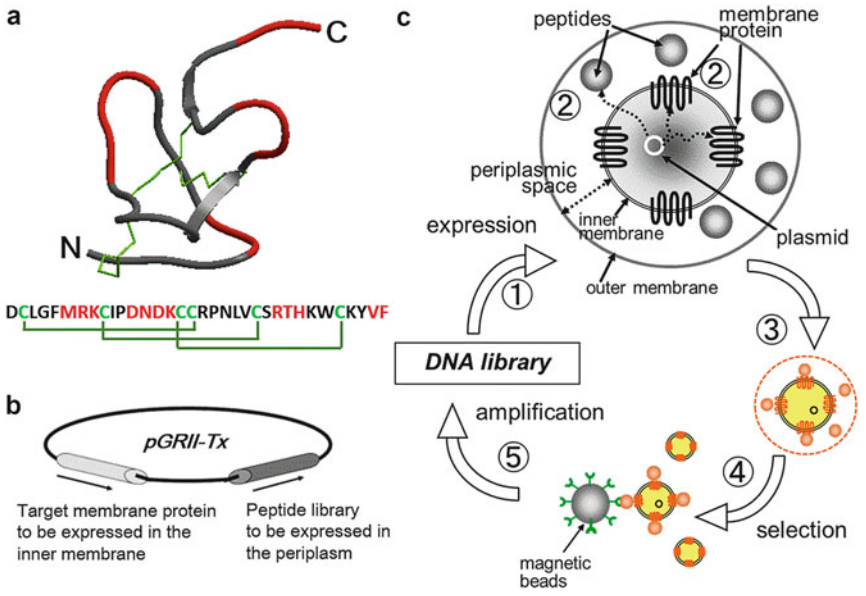

Fig. 2 Random peptide library of ICK motif peptides and the new screening system "PERISS." (**a**) 3D model of the GTx1-15 T-type Ca^{2+} channel modulator isolated from the venom of the tarantula *Grammostola rosea*. The amino acid sequence of the mature peptide is shown below. Cysteine residues and disulfide bridges are colored in green. Randomized regions are shown in red. (**b**) The designed plasmid vector pGRII-Tx harboring cDNAs encoding a target protein and a peptide library in tandem. Each polypeptide is expressed in a fusion protein so that the target proteins are expressed in the inner membrane and the peptides are expressed in the periplasm. (**c**) Schematic diagram of the PERISS method. *1*: Transformation of Gram-negative bacteria with the target- and peptide-coding cDNAs constructed in the vector pGRII-Tx. *2*: Expression of the target membrane proteins in the inner membrane and secretion and refolding of the peptides in the periplasmic space. *3*: Interaction of the peptide and the target membrane protein in the periplasm. *4*: After disruption of the outer membrane, the complex [peptide-target protein-spheroplast] is selected by magnetic beads. *5*: Amplification of the peptide cDNAs from the selected complexes. The amplified cDNA proceed to the next selection cycle (*1*), or the peptide sequence information will be decoded from the plasmid. (Adapted from JPN Patents 5717143 and 5787298; Figures in the courtesy of Seigo Ono)

Among the ICK toxins, charybdotoxin from the scorpion was the first to be engineered by introducing a metal-chelating sequence (nine amino acids) to generate carbonic anhydrase-like activity (Vita et al. 1995). Another peptide library with an ICK motif was constructed based on the spider toxin GTx1-15. The peptide GTx1-15 was originally isolated from the venom of the tarantula *Grammostola rosea* and characterized as a T-type calcium channel modulator (Ono et al. 2011). Mature GTx1-15 is 34 amino acid residues long and contains 12 randomized amino acid residues in loop I (three residues), loop II (four residues), loop IV (three residues), and the C-terminus (two residues) (Fig. 2a). The randomized regions were determined based on the multiple alignment of the family and a 3-D structural model, which was constructed using the closely related ICK peptide HnTx4 (hainantoxin-4, PDB 1njy)

as a template. This ICK-based library was subjected to screening of binders to the m2 subtype of the muscarinic acetylcholine receptor (mAChR) (Kubo et al. 2012; Ono et al. manuscript in preparation). To target the membrane protein, a novel semi in vitro evolution system named "PERISS" (intra periplasm secretion and selection) was developed; the target membrane protein was expressed in the inner membrane, and the peptide was secreted into the periplasm (see details in section "PERISS Method"). After six rounds of selection, sequence convergences were observed in the peptides selected from the ICK-based library. One of the peptides had moderate affinity (K_i^{app} ~300 nM) and subtype selectivity for the m2 receptor.

Other Peptide/Protein Scaffolds Refined Through Accelerated Evolution

From the preceding study of the 3-F and ICK peptide libraries, other toxin/bioactive peptides that evolved by accelerated evolution were identified that could also be used to construct alternative libraries to screen binders. Taking the size, number of disulfide bonds, and conformation into consideration, conotoxin and Kunitz-type protease inhibitors were identified. Conotoxins are a remarkably diverse family with unique potency and selectivity profiles (see other chapters of this handbook series and a recent review by Robinson and Norton 2014). Because the conotoxins bind to ion channels and receptors with high potency and selectivity, it will be advantageous to use the library to find the key residues for the target pharmacophore, which can lead to the production of peptidomimetics (Brady et al. 2013). A high degree of polymorphism was observed in a narrow region surrounding the reactive site in a family of serine protease inhibitors (serpins) (Borriello and Krauter 1991). The Kunitz-type protease inhibitor family including BPTI (bovine pancreatic trypsin inhibitor), PSTI (pancreatic secretory trypsin inhibitor), and Kunitz domain inhibitor of factor VIIa were used as templates to prepare random peptide libraries, of which a relatively small portion (5–8 amino acid residues) was randomized and used in phage display to generate new binders, such as elastase inhibitors (Roberts et al. 1992; Rottgen and Collins 1995; Dennis and Lazarus 1994).

Random Peptide Libraries: Rationally Designed and Cys-Constrained

Artificial Scaffold Stabilized by Leucine or Tryptophan Zipper

A peptide scaffold with a helix-loop-helix conformation, in which two α-helices each consisting of 14 amino acid residues linked by a flexible linker of 7 amino acid residues, was designed de novo (Fig. 3a). Leucine residues were placed into the heptad repeat positions to dimerize the α-helices by hydrophobic interactions; then, the solvent-exposed outside residues were randomized to generate a library (microantibody; Fujiwara and Fujii 2013). In the recent design, cysteine residues

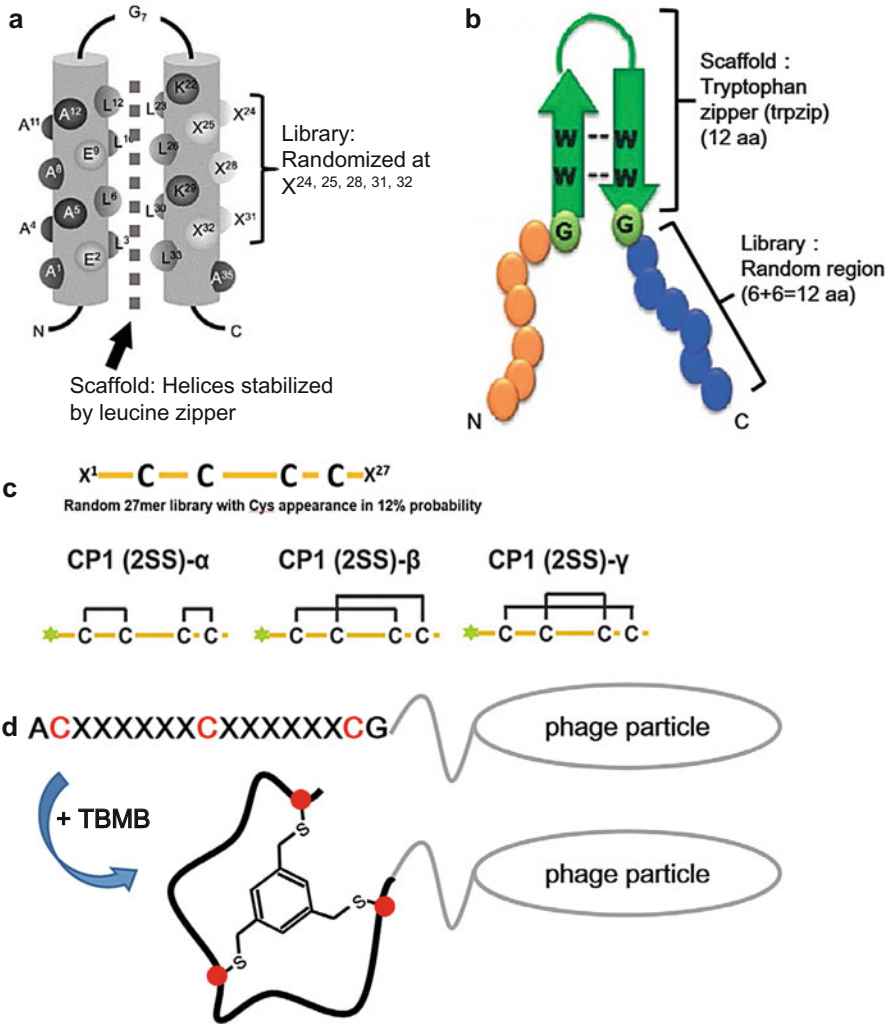

Fig. 3 *De novo* designed peptide libraries. (**a**) "Microantibody" with two α-helices. Two α-helices are firmly aligned by hydrophobic interactions generated by leucine side chain moieties. Five residues (positions 24, 25, 28, 31, and 32) located outside of the C-terminal side helix are exposed to the space to efficiently interact with target molecules. (Adapted from Fujiwara and Fujii 2013) (**b**) Schematic representation of "Aptide". The aptide consists of two randomized regions (6 amino acid residues long each) and a scaffold of 12 amino acids β-hairpin, which is stabilized by two pairs of tryptophan (W)-tryptophan (W) interaction (aromatic ring stacking). The scaffold was named tryptophan zipper or trpzip following to "leucine zipper". (Adapted from Kim et al. 2012) (**c**) Random peptide libraries were generated from 27 mer with nine codon triplets XYZ, in which each nucleotide composition at X, Y, and Z is controlled so that cysteine residues appear with 12 % probability. A linear peptide (reduced form) and three possible S-S configurations [CP1(2SS)-α, CP1(2SS)-β, and CP1(2SS)-γ] were examined for target recognition and selectivity (Adapted from Mochizuki et al. 2014) (**d**) Construction of a bicyclic peptide library. A linear peptide library with C-$(X)_6$-C-$(X)_6$-C sequence fused with the P3 phage coat protein (upper); and a library with two cyclic loops (a bicyclic peptide library) after chemical conjugation of Cys-SH by TBMB, tris (bromomethyl) benzene (lower). (Adapted from Heinis et al. 2009)

were incorporated in both the N- and C-termini and linked by disulfide bridges for stabilization. The libraries were screened by phage display, and effective binders to a cytokine receptor (granulocyte colony-stimulating factor receptor), protein kinase (Aurora-A), ganglioside (GM1), and VEGF (Fujii et al., JPN patent pending 2014-047156) were generated from the designed peptide library.

Aptide is an artificial aptamer-like peptide, which consists of a highly stable tryptophan zipper or "trpzip" in β-hairpin structure (12 amino acids) and two linear polypeptides of six randomized amino acids at each end of the trpzip scaffold (Fig. 3b; Kim et al. 2012). Two random peptide regions in the aptide library are spatially supported by a leucinezipper-like structure and are enabled to recognize and bind target molecule(s), like a tweezers. Using this aptide library in phage display screening, a VEGF-binding peptide with an affinity of approximately 30 nM was reported (Kim et al. 2012).

Cys-constrained Library

Small natural polypeptides with high target selectivity and affinity are primarily constrained by disulfide bridge(s) (e.g., venom peptides including conotoxins and polycyclic peptides). Nemoto and colleagues constructed a peptide library in which 27 amino acid residues were randomized but under some constraint of amino acid composition so that cysteine residues appeared with a frequency of 12% (i.e., 3–4 cysteines per 27 residues) (Fig. 3c; Mochizuki et al. 2014). The library was screened by the cDNA display method to find binders to a small chemical moiety amino group. After four rounds of selection, the selected peptides showed a consensus sequence containing four cysteine residues (C1 to C4). As shown in Fig. 3c three disulfide-bridge frameworks are possible: (C1-C2) + (C3-C4), (C1-C3) + (C2-C4), or (C1-C4) + (C2-C3). Among them, they found only the peptide with the S-S configuration (C1-C2) + (C3-C4) specifically bound to the amino group.

A new approach to constructing a constrained peptide library was introduced by combining chemistry and biochemistry. Heinis, Winter and colleagues used halomethylarene, tris (bromomethyl) benzene (TBMB), to chemically link the SH groups of cysteine residues allocated in a random peptide sequence (Fig. 3d; clamping three cysteine residues in a random peptide sequence by TBMB; Heinis et al. 2009). The resulting peptide complexes are expected to form a constrained scaffold and spatially independent two cyclic loops (a bicyclic peptide). When affinity selections against the serum kallikrein were performed using this bicyclic peptide library, inhibitors with IC50s between 20 and 100 nM at initial rounds of selection (later achieved 1.7 nM by affinity maturation) were selected.

Combination Library

DkTx is a bivalent toxin from the "Earth Tiger" tarantula (*Ornithoctonus huwena*) and is a single polypeptide consisting of double head-to-tail ICK unit repeats

separated by a short linker (Bohlen et al. 2010). Each ICK unit was found to activate the TRPV1 channel independently in a reversible manner. However, when combined, they synergized to produce a ligand of exceedingly high avidity that irreversibly activated TRPV1. This finding inspired us to generate a combination library by linking homo-/heteropeptide libraries of natural and/or artificial scaffolds in tandem that could be utilized to find binders of increased potency with multiple selectivities/functions toward targets.

Directed In Vitro Evolution: Utilization of Random Peptide Libraries for Screening of Ligands and Biotherapeutics

Phage Display and Other Cell Surface Display

In addition to the development of various types of peptide libraries with some constrained scaffold, selection technologies have also been developed. Phage display is a widely applied method to screen binders (polypeptides). The utilization of the filamentous phage genome as an expression vector to present an exogenous peptide sequence on the virion surface was first reported in 1985 (phage display, Smith 1985). Other display technologies such as yeast (Boder and Wittrup 1997; Feldhaus et al. 2003) and bacterial surface displays (Bessette et al. 2004) have also been established. The general concept behind these display technologies is that a peptide library with either a short stretch or embedded in some scaffold is secreted, refolded, and represented on the cell surface either in fusion with the surface protein or by anchoring.

PERISS Method

A new expression and screening technology called the PERISS (intra periplasm secretion and selection) method is based on a new concept (Fig. 2; Kubo et al. 2012; Ono et al., manuscript in preparation). In this method, cDNAs encoding a target (membrane) protein and a peptide library are constructed in tandem in a plasmid vector (pGRII-Tx, Fig. 2b). After transformation of Gram-negative bacteria (e.g., *E. coli*), the target proteins are expressed in the inner membrane, and the peptides are secreted into the periplasm. The cellular machinery necessary for oxidative folding is present in the bacterial periplasm. Thus, the periplasmic space is utilized for the expression and interaction of the target protein and peptide. After disrupting the outer membrane, the target and binder complexes are retained on the surface of the spheroplast (Fig. 2c). Sequence information of the selected binders are encoded in the plasmid DNAs in the spheroplast. It will be amplified for the next round (generation) library for screening or decoded for identifying the binders. It is noteworthy that in this PERISS method the target may not be limited to membrane protein, but soluble proteins would also be targeted by expressing them in fusion with an anchoring protein in the inner membrane (Kubo et al., JPN patents 5717143 and 5787298).

Ribosome/mRNA/cDNA Display Methods

In the in vitro selection system, linkages between genetic information (genotype) and protein function (phenotype) are achieved by chemical/biochemical/physicochemical conjugations. Several different levels of display (ribosome, mRNA, or cDNA) were developed. In ribosome display, stop codons are intentionally removed from the mRNA, and thus the translation apparatus (e.g., rRNA) is stalled without release from the mRNA. The resulting complexes of mRNA, ribosome and protein are accumulated, and are used to screen binders (see reviews Binz et al. 2004; Zahnd et al. 2007a). In mRNA display, mRNA and its translation products are linked via a puromycin-conjugated linker (Nemoto et al. 1997; Xu et al. 2002). Puromycin is an analogue of aminoacyl tRNA and is incorporated during translation. Once incorporated, puromycin prevents further peptidyl chain elongation. cDNA display is principally the same as mRNA display except that the conversion of mRNA to cDNA by reverse transcriptase is performed (Ueno and Nemoto 2012). Several modifications of the mRNA/cDNA display methods and puromycin linkers have been reported (e.g., Mochizuki et al. 2011; Naimuddin and Kubo 2016).

In every cell-surface display method, the diversity of the random peptide library depends on the efficiency of the transformation/transfection of the host cells and ranges from $10^{5–6}$. In contrast, the cell-free system or in vitro selection system has no cell-dependent process, and therefore the diversity can reach $10^{11–13}$ depending on the reaction scale.

In Vitro Compartmentalization

In addition to chemical conjugation, linkage between genotype and phenotype for in vitro evolution can be achieved by restricting the genetic information and the protein product into tiny compartments. The compartments were formed in W/O (water-in-oil) or W/O/W (water-in-oil-in-water) emulsions (Tawfik and Griffiths 1998; Miller et al. 2006). Principally, one molecule from a DNA library is entrapped in a droplet, and transcription and translation are completed in the same space. In the next step, several methods have been devised to expose the protein to the target molecule or substrate (e.g., Doi and Yanagawa 1999; Griffiths and Tawfik 2006). One of the advantages of this compartmentalization method is that the droplets are treated as cells and can be separated by a cell sorter by coupling the droplets with chromogenic/fluorogenic substrates (or antibodies).

Conclusion

Enormous efforts have been made to overcome the intrinsic problems of antibody medicines with the aim to generate better antibody alternatives or super-antibodies. The principal concern is size minimization without a loss of target diversity, selectivity, or affinity. On the basis of the accumulated knowledge of immunoglobulin

structure-function relationships, minimal essential domains (e.g., scFv and dAb) and their combinations (dAb) were successfully innovated. The CDRs, which are the most variable regions in the immunoglobulin molecule, are responsible for diverged target recognition and affinity. Nonimmunoglobulin architectures similar to CDRs or architectures advantageous for the spatial presentation of random sequences were used to construct random peptide libraries. Peptide toxins have great potential for the application of these libraries due to their compact size, stability, diversity, and evolutionally refined scaffold. Together with the development of selection technologies for a specific target molecule (directed evolution), many druggable seeds for biotherapeutics, some of which have proceeded to preclinical studies or clinical trials, are being generated from nonimmunoglobulin libraries. These middle-sized biotherapeutics are expected to overcome the bottlenecks of both antibody medicine (specific but expensive) and chemical drugs (low cost but off-target problems). Due to the diversity in family and target molecules, peptide toxins are promising candidates for middle-size biotherapeutics and are ready for further innovative evolution. Recent remarkable progress in computation technologies including AI will make the de novo design of constrained peptide scaffold more rational and accurate (see e.g. Bhardwaj et al. 2016). It is also getting realized that the next generation sequencing (NGS) in cooperation with AI is contributing to replace time-consuming and laborious library screening with logical estimation of convergence direction(s) of the sequences; a tide toward in silico screening or in silico evolution.

Cross-References

▶ Conotoxins and Drug Discovery with Special Reference to Hainan Species
▶ Conotoxins as Tools in Research on Nicotinic Receptors

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Synthetic Peptides and Drug Discovery

11

Kazuki Sato

Abstract

Venomous glands of cone snails, scorpions, and so on contain many peptide neurotoxins. They mostly act on ion channels and neurotransmitter receptors with high specificity and are promising tools for developing novel peptide drugs. For this purpose, structure-function relationship of the peptides should be studied in detail. In this chapter, the relationships will be described for several peptide neurotoxins studied in the author's laboratory such as conotoxins from cone snails and hefutoxins from scorpions.

Keywords

Structure-Function Relationship • μ-Conotoxins • ω-Conotoxins • δ-Conotoxins • κ-Hefutoxins

Contents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_14

Introduction

The essential factors for bioactive peptides to show their activity are the side-chain functional groups properly oriented in the specific three-dimensional structures. Peptide neurotoxins from cone snails and scorpions generally contain many functional amino acids and multiple disulfide bonds. Due to the multiple disulfide bonds, they take on stable globular three-dimensional structures, and this may be the reason for their high specificities. The first step of structure-function relationship study is to establish the best condition for synthesis of natural peptide. This is important to supply sufficient amount of natural peptide for biological characterization and three-dimensional structure analyses. The second step is so-called Ala scanning, in which each amino acid is stepwise replaced with single Ala. This scanning is valuable for determining the essential amino acid residues for the biological activity. Combination of the results of Ala scanning with the results of conformational analysis may give the information of the relative orientation of essential residues in the three-dimensional structure. This information gives the interaction model between peptide neurotoxins and ion channels. Based on this interaction model, it may be possible to design highly active and stable analogues for future drug development. It is a difficult problem to reveal the relationship between amino acid sequence and three-dimensional structures. In some case, single Ala substitution results in the failure of correct disulfide bond formation, suggesting that the particular amino acid is essential for the formation of correct three-dimensional structures. In this chapter, the structure-function relationships will be described for several peptide neurotoxins studied in the author's laboratory such as conotoxins from cone snails and hefutoxins from scorpions.

Conotoxins from Cone Snails

μ-Conotoxins

μ-Conotoxin GIIIA (μ-GIIIA) isolated from marine cone snail *Conus geographus* is a peptide toxin composed of 22 amino acid residues with three disulfide bridges (Nakamura et al. 1983; Gray et al. 1988; Olivera et al. 1990). This toxin was first named as geographutoxin I; however, the common name conotoxin is used throughout in this text. Amino acid sequence of μ-GIIIA was determined by two groups (Sato et al. 1983; Cruz et al. 1985). This toxin preferentially blocks skeletal muscle sodium channels (Cruz et al. 1985) and is a promising tool to investigate sodium channels (Catterall 1988). Although chemical synthesis of μ-GIIIA was achieved (Cruz et al. 1989; Becker et al. 1989), the determination of disulfide pairings was delayed because of two Cys–Cys sequences that hindered the normal method such as enzymatic fragmentation followed by amino acid sequencing or mass analysis. The disulfide bond pairing was determined by the mass analysis of cystine residues in acid hydrolyzates of synthetic μ-GIIIA specifically labeled with deuterium as shown in Fig. 1 (Hidaka et al. 1990). Based on the information of disulfide bond pairings,

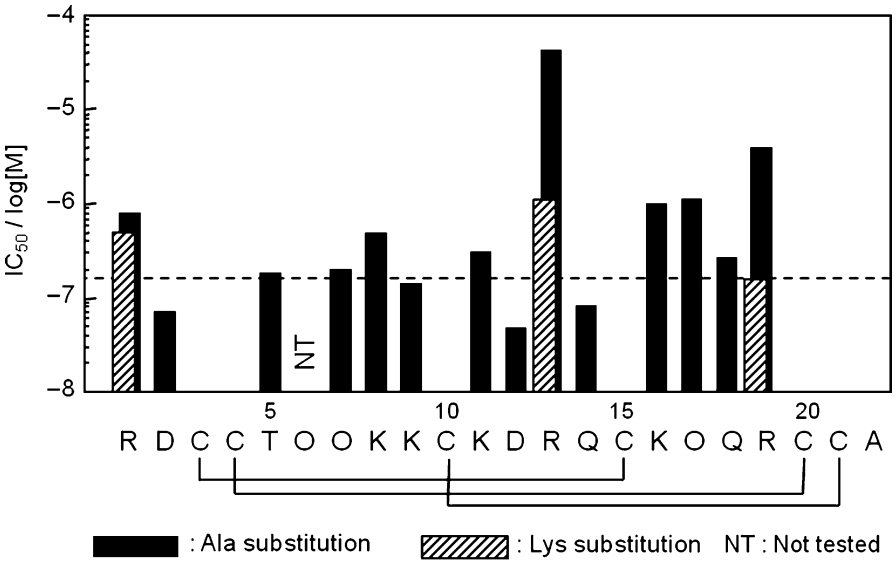

Fig. 1 IC_{50} values of synthetic analogues of μ-GIIIA (O = Hyp). *Dashed line* shows the IC_{50} value of native μ-GIIIA. μ-GIIIA has amidated C-terminus (Sato et al. 1991)

the three-dimensional structure of μ-GIIIA was determined by nuclear magnetic resonance (NMR) spectroscopy (Lancelin et al. 1991; Ott et al. 1991).

To identify the functional residues in μ-GIIIA, each amino acid (other than Cys residue) of the peptide was replaced by Ala (Sato et al. 1991). The yields of most synthesized analogues starting from 4-methylbenzhydrylamine (MBHA) resin were 2–4%. [Ala^6]μ-GIIIA could not be isolated, because the cyclization reaction did not give a major product. Synthesis of [Ala^7]μ-GIIIA was also troublesome due to very low isolation yield. The effects of replacement of amino acid residue in μ-GIIIA with Ala on IC_{50} values in the inhibition of twitch contraction of the rat diaphragm are shown in Fig. 1. When Arg, the most basic amino acid residue among the 20 standard residues in protein, was replaced, IC_{50} values increased, indicating that the substitution lowered the potency of the biological action. Ala substitution at Arg^{13} significantly reduced the potency approximately 200 fold less than the natural compound. Substitutions at Arg^{19} and Arg^1 also reduced the potency 25 and 5 fold. The replacement of another basic residue, Lys at position 8, 11, or 16, also raised the IC_{50} value to 2–6 times that of the native toxin, while substitution at Lys^9 did not. On the other hand, Ala substitutions at the 2nd and 12th Asp, an acidic residue, reduced the IC_{50} value to approximately 50% and 30% of control, indicating the increased potency with the replacement of Asp residues. These results indicate that basicity of the molecule, especially around the 13th position, is important for the biological activity. Lys is less basic than Arg. Thus, each Arg in μ-GIIIA was replaced with Lys to investigate the exchangeability between the two basic residues (Fig. 1). Replacement of Arg^{19} with Lys did not affect the activity. In contrast, IC_{50}

values for [Lys^{1}] and [Lys^{13}] analogues were 2–6 times greater than that for μ-GIIIA, indicating an appreciable loss of activity. Although it seems that Arg is more or less replaceable with Lys, the basicity around the 13th position is suggested to be more important than those of other positions for the biological activity. In addition, linear peptide [$Ala^{3,4,10,15,20,21}$]μ-GIIIA was synthesized by replacing all Cys residues with Ala. This peptide at 10^{-4} M did not exhibit any significant inhibitory effect on twitch contraction. Thus, specific conformation formed by disulfide bonds is required for the biological activity. NMR spectra of synthetic analogues were essentially identical to that of the native μ-GIIIA except for the substituted residues. The circular dichroism (CD) spectra of the analogues were also superimposable to that of native peptide. CD spectra of both native and [Ala^{13}]μ-GIIIA showed a similar negative Cotton effect around 260 nm, which reflects the disulfide bonds. This Cotton effect was absent in the spectrum of the linear analogue. These results clearly show that the observed change in inhibitory activity was due to the difference in the nature of the side chains themselves rather than a conformational change (if any) associated with the replacement.

The structure-activity relationship for μ-GIIIA showed that the basicity of the molecule is a crucial factor for activity. Especially, replacement of Arg^{13} with Ala remarkably reduced activity compared with natural μ-GIIIA, whereas replacement of other basic residues with Ala reduced activity less extensively, suggesting that Arg^{13} is mainly responsible for the inhibitory action of μ-GIIIA. The specific role of the guanidino group of Arg^{13} was supported by the finding that [Lys^{13}]μ-GIIIA also showed a large loss of activity. In contrast, without significant loss of activity, Arg^{19} can be replaced by Lys, implying that only a positive charge is required at this position. Positively charged groups other than Arg^{13} may assist in positioning of Arg^{13} in a proper binding site of sodium channels. Replacement of amino acid residues at position 5, 7, 8, or 9 with Ala did not significantly modify the inhibitory potency of twitch contractions. It seems that the sequence between Cys^{4} and Cys^{10} is not essential for the biological activity. However, a linear peptide, [$Ala^{3,4,10,15,20,21}$]-μ-GIIIA, possessed no inhibitory activity. Furthermore, Ala substitution at Hyp^{6} failed to form a proper set of disulfide bridges. Therefore, the peptide loop between Cys^{4} and Cys^{10} would be important in forming the specific conformation of the molecule for exerting the biological activity (Fig. 2).

Within the macromolecular arrangement of sodium channels, anionic sites are detected through a number of electrophysiological experiments and expected to be in the extracellular "funnel" part of the channel. Tetrodotoxin and saxitoxin, famous sodium channel blockers, contain guanidine group(s) which may bind to the anionic sites of the channels. Arg is the only amino acid residue having a guanidino group among the 20 amino acids in protein. Since study of the three-dimensional structure of μ-GIIIA by two-dimensional NMR suggests that Arg^{13} is located in the flexible segment of μ-GIIIA (Lancelin et al. 1991), the conformation of the segment may change to facilitate the subtle fit of Arg^{13} to the specific site of sodium channels. An active center of anthopleurin-A, a sea anemone peptide toxin having 45 residues with three disulfide bridges, which consequently activates sodium channels, was elucidated to be Arg^{14} (Barhanin et al. 1981). NMR study (Torda et al. 1988) showed

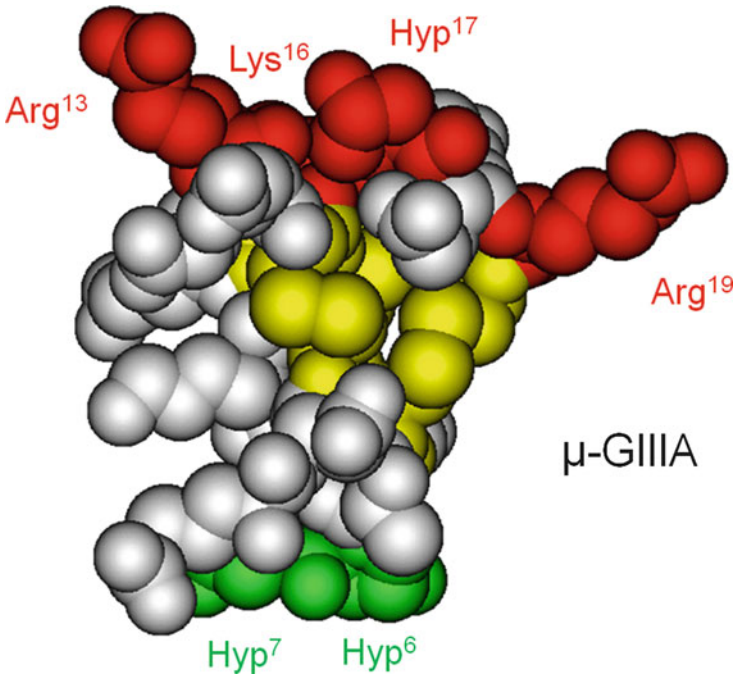

Fig. 2 Distribution of residues essential for activity (Arg^{13}, Lys^{16}, Hyp^{17}, and Arg^{19}) shown in *red* color and essential for folding (Hyp^{6} and Hyp^{7}) shown in *green* color. *Yellow* color indicates Cys residues

that the Arg^{14}-containing loop could undergo conformational changes upon binding to sodium channels. Arg may be a general residue mainly involved in peptide binding to sodium channels. Since μ-GIIIA has different dissociation constants for sodium channels of muscle and nerves, Arg^{13} may be a general active site, and other part(s) of the molecule, as yet undefined, may be specific for the binding to muscle sodium channels. In this study, a clear-cut relation was shown between the active site and the three-dimensional structure of μ-GIIIA. Its molecular architecture of a rigid core and flexible side chains shares the proposed general feature in the peptide-receptor interaction (Williams 1989). The structure-activity relationship of μ-GIIIA will provide valuable information on the structure of sodium channels and the difference between muscle and nerve subtypes of sodium channels.

The three-dimensional structure of [Ala^{13}]μ-GIIIA was shown to be the same as that of native μ-GIIIA by NMR analysis, confirming that the loss of activity is not due to the conformational change but to the removal of essential guanidino group (Wakamatsu et al. 1992). The essential role of Arg^{13} for block of sodium channels has been reported repeatedly (Becker et al 1992; Chahine et al. 1995; Chang et al. 1998). Several analogues of μ-GIIIA were synthesized as a probe for sodium channels (Nakamura et al. 2001a, b, 2002, 2004).

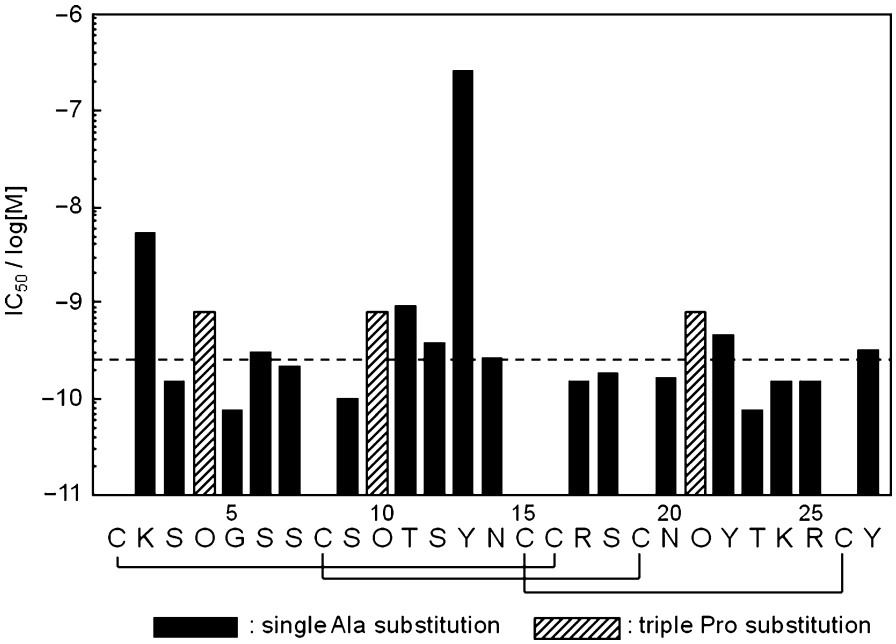

Fig. 3 IC_{50} values for ^{125}I-ω-GVIA binding of ω-GVIA analogues (O = Hyp). *Dashed line* shows the IC_{50} value of native ω-GVIA. ω-GVIA has amidated C-terminus (Kim et al. 1994)

Seven analogues of μ-GIIIA were synthesized by stepwise replacing the three cystine residues with Ala (Sato et al. 2014). The CD spectra of the analogues suggested that the deletion of disulfide bonds gradually randomized a conformation. The inhibitory effects on the twitch contractions of the rat diaphragm showed that the deletion of one disulfide bond reduced the potency to less than 1% of control. Monocyclic analogues and a linear analogue were almost inactive. Therefore, all three disulfide bridges are essential for stabilizing the specific conformation of μ-GIIIA to show the biological activity.

ω-Conotoxins

ω-Conotoxin GVIA

The N-type Ca^{2+} channel plays a critical role in regulating neurotransmitter release from wide variety of neurons (Catterall 1988). ω-Conotoxin GVIA (ω-GVIA), a peptide neurotoxin obtained from toxic marine snail *Conus geographus*, binds to a specific site in the channel and blocks its function (Olivera et al. 1987; Gray et al. 1988). The amino acid sequence of ω-GVIA was determined to contain 27 amino acid residues with three disulfide bonds as shown in Fig. 3 (Olivera et al. 1984). Chemical synthesis and determination of disulfide pairings were reported in 1986 (Nishiuch et al. 1986). The three-dimensional structure of ω-GVIA was reported by

four groups independently (Sevilla et al. 1993; Davis et al. 1993; Pallaghy et al. 1993; Skalicky et al. 1993).

Referring to the result that the basic residues were essential for the activity of μ-GIIIA, four basic residues of ω-GVIA were stepwise replaced with Ala residue (Sato et al. 1993). In contrast, it was found that 3 (Arg^{17}, Lys^{24}, and Arg^{25}) out of 4 basic residues of ω-GVIA were not critical for binding and only substitution of Ala for Lys^2 resulted in 40-fold reduction in the affinity with N-type Ca^{2+} channel. To evaluate the essential amino acid residues of ω-GVIA, analogues were further synthesized by replacing each amino acid residue except for Cys and Hyp with Ala (Kim et al. 1994). Air oxidation of the crude linear precursors of most of these ω-GVIA analogues afforded peptides having proper disulfide bonds as the major products, which was purified by gel filtration, ion exchange chromatography, and HPLC. Most ω-GVIA analogues were obtained in good isolation yields (3–10% from the starting resin), and their CD spectra except for that of a linear analogue were similar to that of native ω-GVIA, indicating that they have similar conformation to that of native ω-GVIA. The binding of ω-GVIA and their analogues to the N-type Ca^{2+} channel were examined from inhibitions of ^{125}I-ω-GVIA binding to chick brain synaptic plasma membranes. The IC_{50} values of all the synthetic analogues are summarized in Fig. 3. The replacement of Tyr^{13} with Ala (**Y13A**) resulted in the largest reduction of affinity, and the IC_{50} value (2.6×10^{-7} M) was increased to more than 1,000 times larger than that of native ω-GVIA (2.5×10^{-10} M). The conformation of **Y13A** was not significantly changed because the CD spectrum of **Y13A** was almost superimposable to that of ω-GVIA. Thus, the side chain of Tyr^{13} is essential for the binding of ω-GVIA to the N-type Ca^{2+} channel. Lys^2 is another essential residue, and the IC_{50} value was increased to 5.5×10^{-9} M when Lys^2 was replaced with Ala (Sato et al. 1993). In contrast, all the analogues except **Y13A** and **K2A** gave almost the same IC_{50} values as that of ω-GVIA. In order to know whether the phenolic hydroxyl group of the Tyr residue is important or not for the binding, **Y13F** was synthesized, in which Tyr^{13} was replaced with Phe residue. Although **Y13F** gave a CD spectrum very similar to that of ω-GVIA, the IC_{50} value of **Y13F** (4.2×10^{-8} M) was almost 100 times larger than that of ω-GVIA. This result indicates that the hydroxyl group of Tyr^{13} is essential for binding to N-type Ca^{2+} channels. In addition to the ω-GVIA analogues with single amino acid substitution, [$Pro^{4, 10, 21}$]ω-GVIA was synthesized to study the role of hydroxyl group of three Hyp residues. IC_{50} value of this analogue was 8.1×10^{-10} M, suggesting that hydroxyl groups of Hyp residues were not essential for binding. A linear analogue [$Ala^{1, 8, 15, 16, 19, 26}$]ω-GVIA was also synthesized by replacing all six Cys residues with Ala. This analogue showed a CD spectrum with a large negative Cotton effect around 196 nm typical for a random conformation. This linear analogue scarcely inhibited the ^{125}I-ω-GVIA binding, even at a concentration of 10^{-5} M, indicating that the specific conformation stabilized by three disulfide bridges was essential for binding. To examine the importance of hydroxyl group in Tyr^{13}, biological activity of **Y13F** was compared with that of native ω-GVIA using cultured rat hippocampal neurons. Intracellular Ca^{2+} concentration shows spontaneous, periodic oscillations, which are synchronized among the cells, probably due to the formation of synapses.

The oscillation is abolished after treatment with blockers for L-glutamate receptor and tetrodotoxin, indicating that release of glutamate from presynaptic terminals is involved in this phenomenon. ω-GVIA at 10^{-8} M inhibits oscillation, whereas **Y13F** did not show any inhibitory effect, even when concentration was increased up to 10^{-6} M. This result indicated that the hydroxyl group of Tyr^{13} was essential for the biological activity of ω-GVIA.

As shown previously, Lys^2 is also important for the ω-GVIA binding (Sato et al. 1993). Tyr^{13} and Lys^2 are conserved in other ω-conotoxins from *Conus magnus* and ω-conotoxin MVIIA and MVIIC, suggesting the importance of these residues for the biological activities of ω-conotoxins. Two-point binding model was proposed for ω-GVIA in which Tyr^{13} and Lys^2 interact with specific amino acid residues of the Ca^{2+} channel through the hydrogen bonding and ionic interaction, respectively.

Because the linear analogue of ω-GVIA took a random conformation and did not bind to the N-type Ca^{2+} channel, the specific conformation of the molecule is essential for the activity of ω-GVIA. The three-dimensional structure of ω-GVIA was reported based on NMR analysis and simulated annealing calculation (Sevilla et al. 1993; Davis et al. 1993; Pallaghy et al. 1993; Skalicky et al. 1993). ω-GVIA has a globular folding pattern stabilized by three short antiparallel β-strands located on one side of the molecule. Two essential residues, Tyr^{13} and Lys^2, are located on the other side of the molecule, suggesting that this side interacts with the Ca^{2+} channel. On the other hand, Ala replacement of Asn^{20} and Thr^{23} resulted in very low isolation yields, suggesting that the hydrogen bond between the side chains of these two residues was important for the folding (Kim et al. 1995c). Therefore, ω-GVIA can be separated into two domains, namely, a structural domain and a functional (binding) domain (Fig. 4). As described previously, μ-conotoxin can also be separated to structural domain containing two Hyp residues and a functional domain containing biologically essential residues (Sato et al. 1991; Wakamatsu et al. 1992). This may be the general feature for the small peptide toxins stabilized with multiple disulfide bonds and will provide a clue for the design of analogues with high specificity and activity.

Further structure-function relationship studies on ω-GVIA were reported many times (Lew et al. 1997; Flinn et al. 1999a, b; Pallaghy and Norton 1999).

ω-Conotoxin MVIIA

ω-Conotoxin MVIIA (ω-MVIIA) (CKGKGAKCSRLMYDCCTGSCRSGKC-NH_2) was isolated from cone snail *Conus magnus* and shown to block N-type neuronal Ca^{2+} channel (Olivera et al. 1985, 1987). The synthesis was reported simultaneously; however, the final yield was very low. In order to improve the synthetic yield, many different conditions were examined and finally 12% of overall yield was achieved (Kim et al. 1995a). Applying this improved condition, two analogues of ω-MVIIA were synthesized by replacing Lys^2 and Tyr^{13} with Ala. In contrast to the results of ω-GVIA, [Ala^2]ω-MVIIA showed inhibition as strong as native ω-MVIIA. Replacement of Tyr^{13} resulted in large loss of inhibition as expected from the result of ω-GVIA analogues. The three-dimensional structure of ω-MVIIA was shown to

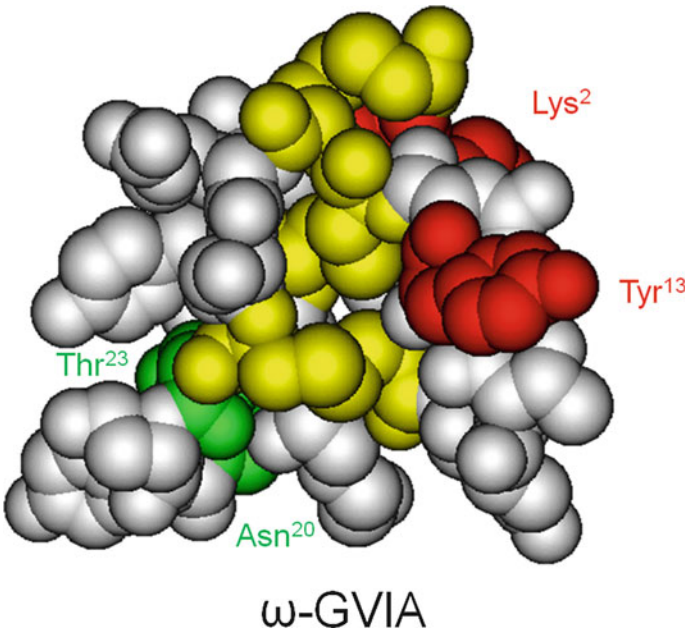

Fig. 4 Distribution of residues essential for binding (Lys^2 and Tyr^{13}) shown in *red* color and essential for folding (Asn^{20} and Thr^{23}) shown in *green* color. *Yellow* color indicates Cys residues

be similar to that of ω-GVIA (Kohno et al. 1995; Basus et al. 1995). It is well known that ω-MVIIA is now used as an analgesic drug (Miljanich 2004).

ω-Conotoxin MVIIC

ω-Conotoxin MVIIC (ω-MVIIC) was identified in the cDNA library from cone snail *Conus magnus*, synthesized chemically, and shown to block P/Q-type neuronal Ca^{2+} channels (Fig. 5) (Hillyard et al. 1992). The synthetic condition of ω-MVIIC was studied in detail (Kubo et al. 1996). The three-dimensional structure of ω-MVIIC was shown to be similar to those of ω-GVIA and ω-MVIIA (Nemoto et al. 1995; Farr-Jones et al. 1995). These three ω-conotoxins take on a similar conformation irrespective of their difference in sequences, suggesting that the conserved disulfide bonds may determine the three-dimensional structure. Replacement of Tyr^{13} in ω-MVIIC with Ala resulted in large loss of inhibition, indicating that this residue is a common active site of ω-conotoxins (Kim et al. 1995b).

Despite their high sequence homology, the peptide neurotoxins ω-MVIIA and ω-MVIIC selectively block N- and P/Q-type Ca^{2+} channels, respectively. To study the recognition mechanism of Ca^{2+} channel subtypes, two chimeric analogues of ω-MVIIA and ω-MVIIC were synthesized by exchanging their N- and C-terminal halves. Binding assay for both N- and P/Q-type Ca^{2+} channels showed that amino acid residues restricted to the N-terminal half are important for the recognition of N-type channels, whereas essential residues for P/Q-type channel recognition are

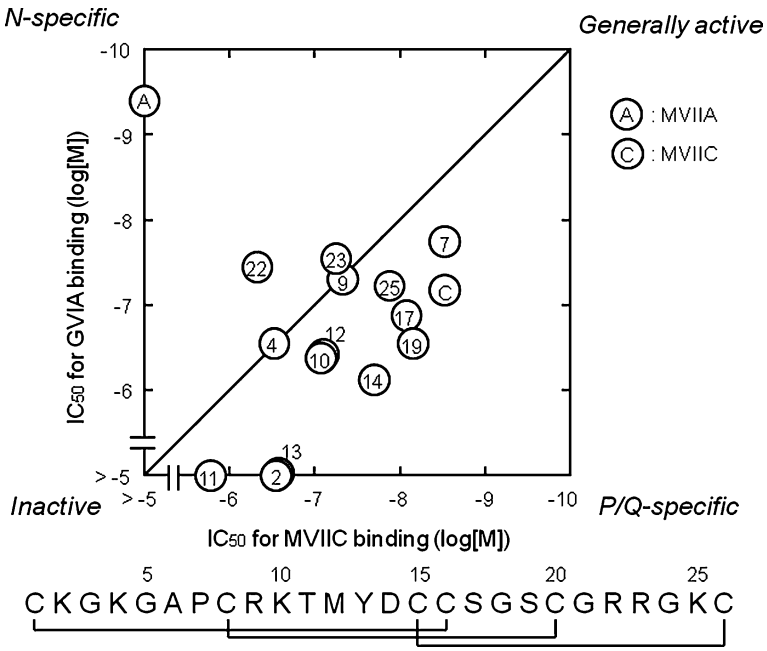

Fig. 5 Inhibition (IC_{50}) by ω-MVIIC analogues of ^{125}I-ω-GVIA or ^{125}I-ω-MVIIC binding to rat cerebellar P_2 membranes. Numbers in *circles* indicate the residues replaced with Ala. Letters A and C in *circles* indicate the IC_{50} of native ω-MVIIA and ω-MVIIC, respectively. ω-MVIIC has amidated C-terminus (Sato et al. 2000b)

widely spread over the whole ω-conotoxin molecule (Sato et al. 1997). Single reverse mutations were examined from MVIIA-type to MVIIC-type in this chimeric analogue. A reverse mutation from Lys^7 to Pro^7 decreased the affinity for both P/Q- and N-type channels, whereas that from Leu^{11} to Thr^{11} increased the affinity for P/Q-type channels and decreased the affinity for N-type channels. The roles of these two residues were confirmed by synthesizing two ω-MVIIC analogues in which Pro^7 and Thr^{11} were replaced with Lys^7 and Leu^{11}, respectively (Sato et al. 2000a).

ω-MVIIC binds to P/Q-type calcium channels with high affinity and N-type channels with low affinity. Ala-scanning analogues of ω-MVIIC were synthesized in order to reveal the residues essential for subtype selectivity (Fig. 5) (Sato et al. 2000b). Binding assays using rat cerebellar P_2 membranes suggested that Thr^{11}, Tyr^{13}, and Lys^2 are essential for binding to both N- and P/Q-type channels, whereas Lys^4 and Arg^{22} are important for binding to P/Q-type channels (Fig. 6). These results suggest that ω-MVIIC interacts with P/Q-type channels via a large surface, in good agreement with previous observations using chimeric analogues. It was desirable to obtain ω-MVIIC analogues more selective for P/Q-type calcium channels than ω-MVIIC to elucidate structural differences among the channels, which discriminate the ω-conotoxins. To prepare a number of ω-MVIIC analogues efficiently, a combinatorial method was developed which includes a random air oxidation step.

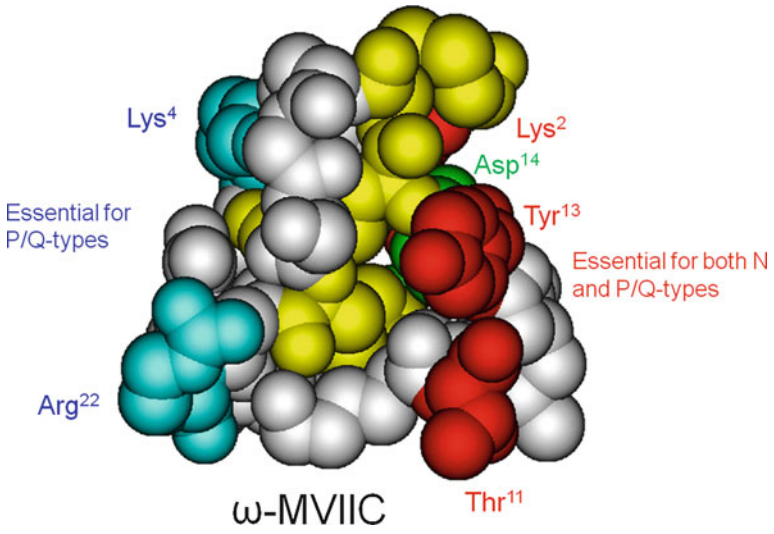

Fig. 6 Distribution of residues essential for binding to both N- and P/Q-type channels (Lys^2, Thr^{11}, and Tyr^{13}) shown in *red* color, essential for binding to P/Q-type channels (Lys^4 and Arg^{22}) shown in *blue* color, and essential for folding (Asp^{14}) shown in *green* color. *Yellow* color indicates Cys residues

Forty-seven analogues were prepared in six runs, and some of them exhibited higher selectivity for P/Q-type calcium channels than ω-MVIIC in binding assays (Sasaki et al. 2000).

As replacement of Thr^{11} of ω-MVIIC with Ala significantly reduced the affinity for both N- and P/Q-type calcium channels (Sato et al. 2000b), the effect of substitution at this position with other residues was examined in detail. Binding assays using rat cerebellar P_2 membranes showed that the affinity is in the order of Leu > Val, aminobutyric acid, Thr > Asn>> Ser, Ala, Asp, Phe, Tyr for N-type channels and Thr > Leu, Val, aminobutyric acid, Asn, Ser > Ala>> Asp, Phe, Tyr for P/Q-type channels, suggesting that aliphatic amino acids with longer side chains are favorable for block of N-type channels (Minami et al. 2001).

The binding affinities of two naturally occurring ω-MVIIA and MVIIC and a series of 14 MVIIA/MVIIC loop hybrids were studied using radioligand binding assays for N- and P/Q-type Ca^{2+} channels in rat brain tissue (Nielsen et al. 1999). In these peptides, loops 2 and 4 make the greatest contribution to voltage-sensitive Ca^{2+} channel subtype selectivity, while the effects of loops 1 and 3 are negligible. Peptide hybrids where loops 2 and 4 are from the same ω-conotoxins display clear selectivity preferences, while those where loops 2 and 4 come from different ω-conotoxins are less discriminatory.

ω-Conotoxin TxVII

ω-Conotoxin TxVII (ω-TxVII) (CKQADEPCDVFSLDCCTGICLGVCMW) was described from the venom of the molluscivorous snail *Conus textile* (Fainzilber

et al. 1996). This toxin blocks dihydropyridine-sensitive calcium channels in caudodorsal cell neurons from the mollusc *Lymnaea stagnalis* and is at that time the only conotoxin suggested to target L-type calcium channels. In contrast to other ω-conotoxins, its sequence is characterized by net negative charge and high hydrophobicity, although it retains the ω-conotoxin cysteine framework. In order to obtain structural information and to supply material for further characterization of its biological function, ω-TxVII was synthesized and its disulfide bond pairings determined (Sasaki et al. 1999). Because a linear precursor with free SH groups showed a strong tendency to aggregate and to polymerize, many different conditions were examined for air oxidation. It was found that a mixture of cationic buffer and hydrophobic solvent was the most effective for the folding of ω-TxVII. Synthetic ω-TxVII was shown to suppress the slowly inactivating voltage-dependent calcium current in cultured *Lymnaea* RPeD1 neurons, and furthermore to suppress synaptic transmission between these neurons and their follower cells. In contrast, ω-TxVII did not block calcium flux through L-type channels in PC12 cells, suggesting a phyletic or subtype specificity in this channel family. Disulfide bond pairings of ω-TxVII and its isomers were determined by enzymatic fragmentation in combination with chemical synthesis, thus revealing that ω-TxVII has the same disulfide bond pattern as other ω-conotoxins. Furthermore, the CD spectrum of ω-TxVII is similar to those of ω-MVIIA and MVIIC. The precursor sequence of ω-TxVII was determined by cDNA cloning and shown to be closest to that of δ-conotoxin TxVIA, a sodium channel inactivation inhibitor. Thus, ω-TxVII conserves the structural fold of other ω-conotoxins, and the TxVIA/TxVII branch of this family reveals the versatility of its structural scaffold, allowing evolution of structurally related peptides to target different channels.

The three-dimensional structure of ω-TxVII was determined by ^{1}H-NMR (Kobayashi et al. 2000). The structure of ω-TxVII is composed of a triple-stranded antiparallel β-sheet and four turns. The three disulfide bonds in ω-TxVII form the classical cystine knot motif of toxic or inhibitory polypeptides. The overall folding of ω-TxVII is similar to those of the N-type calcium channel blockers, ω-GVIA and MVIIA, despite the low amino acid sequence homology among them. ω-TxVII exposes many hydrophobic residues to a certain surface area. In contrast, ω-GVIA and MVIIA expose basic residues on specific surfaces. The channel-binding site of ω-TxVII is different from those of ω-GVIA and MVIIA, although the overall folding of these three peptides is similar. The gathered hydrophobic residues of ω-TxVII probably interact with the hydrophobic cluster of the α1 subunit of the L-type calcium channel, which consists of 13 residues located in segments 5 and 6 in domain III and in segment 6 in domain IV.

δ-Conotoxins

δ-Conotoxin TxVIA (δ-TxVIA) (WCKQSGEMCNLLDQNCCDGYCIVLVCT), a 27-mer peptide with three disulfide bonds, was isolated from the venom of the molluscivorous snail *Conus textile* (Hillyard et al. 1989). It is an agonist/antagonist

of sodium channels (Hasson et al. 1993). For the analysis of three-dimensional structure, δ-TxVIA was chemically synthesized and its disulfide pairings determined to be the same as ω-TxVII (Kohno et al. 2002). The three-dimensional solution structure of δ-TxVIA was determined by two-dimensional ^{1}H NMR spectroscopy with simulated annealing calculations (Kohno et al. 2002). The molecular structure of δ-TxVIA is composed of a short triple-stranded antiparallel β-sheet. The overall β-sheet topology is +2x, −1, which is the same as those for other conotoxins. However, the three-dimensional structure of δ-TxVIA has an unusual hydrophobic patch on one side of the molecule, which may play an important role in the sodium channel binding. These results provide a molecular basis for understanding the mechanism of sodium channel modulation through the toxin-channel interaction and insight into the discrimination of different ion channels.

λ-Conotoxins

A family of *Conus* peptides (λ-conotoxin CMrVIA, CMrVIB, and CMrX), which have a novel cysteine motif, were isolated from *Conus marmoreus* venom (Balaji et al. 2000). Their amino acid sequences are VCCGYKLCHOC (λ-CMrVIA), NGVCCGYKLCHOC (λ-CMrVIB), and GICCGVSFCYOC (λ-CMrX), where O represents 4-transhydroxyproline. Two of these peptides (λ-CMrVIA and λ-CMrX) have been chemically synthesized. Using a selective protection and deprotection strategy during disulfide bond formation, peptides with both feasible cysteine-pairing combinations were generated. The disulfide pattern (C1-C4, C2-C3) in native toxins was identified by their coelution with the synthetic disulfide-isomeric peptides on HPLC. Although cysteine residues were found in comparable positions with those of α-conotoxins, these toxins exhibited a distinctly different disulfide bonding pattern. λ-CMrVIA and λ-CMrX induced different biological effects when injected intracerebroventricularly in mice; λ-CMrVIA induces seizures, whereas λ-CMrX induces flaccid paralysis. The synthetic peptide with λ-conotoxin folding is about 1,150-fold more potent in inducing seizures than the mispaired isomer with α-conotoxin folding. Thus, it appears that the unique disulfide pattern, and hence the "ribbon" conformation, in λ-conotoxins is important for their biological activity (Balaji et al. 2000).

Neurotoxins from Scorpions

κ-Hefutoxins

Purification, solution NMR, and functional characterization of a novel class of weak potassium channel toxins from the venom of the scorpion *Heterometrus fulvipes* was described (Srinivasan et al. 2002). These toxins, κ-hefutoxin 1(κ-Hef-1) (GHACYRNCWREGNDEETCKERC-NH_2) and κ-hefutoxin 2 (κ-Hef-2) (GHACYRNCWREGNDEETCKERCG), exhibit no homology to any known

toxins. NMR studies indicate that κ-Hef-1 adopts a unique three-dimensional fold of two parallel helices linked by two disulfide bridges without any β-sheets. Based on the presence of the functional diad (Tyr^5/Lys^{19}) at a distance (6.0 ± 1.0 Å) comparable with other potassium channel toxins, its function was hypothesized as a potassium channel toxin. κ-Hef-1 not only blocks the voltage-gated K^+-channels, Kv1.3 and Kv1.2, but also slows the activation kinetics of Kv1.3 currents, a novel feature of κ-Hef-1, unlike other scorpion toxins, which are considered solely pore blockers. Alanine mutants (**Y5A**, **K19A**, and **Y5A**/**K19A**) failed to block the channels, indicating the importance of the functional diad.

Nine analogues of scorpion toxin peptide κ-Hef-1 were synthesized by stepwise deletion of its amino acid residues (Peigneur et al. 2013). Disulfide bond pairings of the synthetic analogues were confirmed by enzymatic digestion followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) measurements. Functional characterization shows that analogues in which N-terminal residues were deleted retained biological activity, whereas deletion of middle-part residues resulted in loss of activity. Furthermore, κ-Hef-1 and analogues were subjected to a screening on voltage-gated potassium channels in order to determine their subtype selectivity. It is shown that κ-Hef-1 is suitable as template for peptidomimetics in order to design small peptide-based therapeutic compounds.

κ-KTx1.3 (GFGCYRSCWKAGHDEETCKKECS), which shares ~60% identity with κ-Hef-1, was isolated from *Heterometrus spinifer* venom (Nirthanan et al. 2005). Interestingly, despite the presence of the functional dyad (Y5 and K19), κ-KTx1.3 failed to reproduce the K^+ blocking activity of κ-Hef-1. Since the dyad lysine in κ-KTx1.3 was flanked by another lysine (K20), it was hypothesized that this additional positive charge could hinder the critical electrostatic interactions known to occur between the dyad lysine and the Kv1 channel selectivity filter. Hence, mutants of κ-KTx1.3 substituting K20 with a neutral (**K20A**) or a negatively charged (**K20E**) amino acid were synthesized. **K20E**-κ-KTx1.3, in congruence with κ-Hef-1 with respect to subtype selectivity and affinity, produced blockade of Kv1.2 and Kv1.3 but not Kv1.1 channels. **K20A**-κ-KTx1.3 produced blockade of both Kv1.2 and Kv1.3 and in addition acquired affinity for Kv1.1 channels. These data suggest that the presence of an additional charged residue in a position adjacent to the dyad lysine impedes the functional block of Kv1 channels produced by κ-KTx1.3.

Bukatoxin

α-Toxins from scorpion venoms prolong the action potential of excitable cells by blocking sodium channel inactivation. Bukatoxin, an α-toxin with 65 amino acid residues, was purified from scorpion (*Buthus martensi Karsch*) venom (Srinivasan et al. 2001). Bukatoxin produced marked relaxant responses in the carbachol-precontracted rat anococcygeus muscle (ACM), which were mediated through the L-arginine–nitric oxide synthase–nitric oxide pathway, consequent to a neuronal release of nitric oxide. Based on the presence of proline residues in the flanking

segments of protein–protein interaction sites, the authors predicted the site between 52PP56 to be the potential interaction site of bukatoxin. A homology model of bukatoxin indicated the presence of this site on the surface. Buka11 (YKLPDKVPIRV), scrambled peptide (YPKPVRDIKVL), and the three alanine analogues (**D5A**, **K6A,** and **V7A**) were synthesized. Buka11 produced a concentration-dependent nitric oxide-mediated relaxant response in ACM. Using alanine-substituted peptides, the importance was shown for 53DKV55 flanked by proline residues in the functional site of bukatoxin.

Imperatoxin

Imperatoxin A (IpTxa) (GDCLPHLKRCKADNDCCGKKCKRRGTNAEKRCR), a 33-amino-acid peptide from the venom of the scorpion *Pandinus imperator*, was the first peptide toxin found to activate the skeletal ryanodine receptor (RyR1) with high potency and affinity, and it has been used in several biochemical and biophysical studies related to the E–C coupling (El-Hayek et al. 1995; Zamudio et al. 1997).

Both IpTxa and peptide A (TSAQKAKAEERKRRKMSRGL), derived from the II–III loop of dihydropyridine receptor (DHPR), interact specifically with RyR1, which is a Ca^{2+} release channel in the sarcoplasmic reticulum but with considerably different affinities. IpTxa activates RyR1 with nanomolar affinity, whereas peptide A activates RyR1 at micromolar concentrations. To investigate the molecular basis for high-affinity activation of RyR1 by IpTxa, the NMR solution structure of IpTxa was determined, and its functional surface was identified by using alanine-scanning analogues (Lee et al. 2004). A detailed comparison of the functional surface profiles for two peptide activators revealed that IpTxa exhibits a large functional surface area (approx. 1,900 Å2, where 1 Å = 0.1 nm), based on a short double-stranded anti-parallel β-sheet structure, while peptide A bears a much smaller functional surface area (approx. 800 Å2), with the five consecutive basic residues (Arg^{681}, Lys^{682}, Arg^{683}, Arg^{684}, and Lys^{685}) being clustered at the C-terminal end of the α-helix. The functional surface of IpTxa is composed of six essential residues (Leu^{7}, Lys^{22}, Arg^{23}, Arg^{24}, Arg^{31}, and Arg^{33}) and several other important residues (His^{6}, Lys^{8}, Arg^{9}, Lys^{11}, Lys^{19}, Lys^{20}, Gly^{25}, Thr^{26}, Asn^{27}, and Lys^{30}), indicating that amino acid residues involved in RyR1 activation make up over half of the toxin molecule with the exception of cysteine residues. Taken together, these results suggested that the site where peptide A binds to RyR1 belongs to a subset of macrosites capable of being occupied by IpTxa, resulting in differing affinity and mode of activation.

Conclusion and Future Directions

Structure-function relationship study is essential for the development of novel drugs based on peptide neurotoxins. For rational design of drugs with desired function, it is necessary to know the relative orientation of functional groups in three-dimensional structure. This can be achieved by combination of systematic amino acid substitution

and conformational analysis. However, it is still difficult to assemble the proper three-dimensional structure, since the relationship between amino acid sequence and conformation is not established. Many peptide neurotoxins contain multiple disulfide bonds which are important for the stable three-dimensional structures. For this reason, peptide neurotoxins can be a good template for future drug development.

In order to design specific blockers of ion channels, it is important to know the three-dimensional structure of target channels, particularly the groups at and around the binding site. Recently, structural models have been reviewed for sodium and calcium channels (Catterall and Swanson 2015) and potassium channels (Grizel et al. 2014; Tian et al. 2014). Based on this information on three-dimensional structures of ion channels, specific interactions of peptide neurotoxins are described for conotoxins from cone snails (Akondi et al. 2014) and scorpion toxins (Zhu et al. 2011; Bergeron and Bingham 2012). Studies on these precise interaction models may enable the rational design of peptide drugs in the future.

Cross-References

► Random Peptide Library for Ligand and Drug Discovery

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Part IV

Miscellaneous

Bee Venom and Pain

12

Jun Chen and Su-Min Guan

Abstract

In human beings, it is a common knowledgeable experience that bee stings hurt. However, pain associated with bee stings has not been well studied until the late 1990s. In the last 20 years, interests in understanding of the underlying mechanisms of bee venom (BV)-induced pain have been increased dramatically since pain-related behaviors can be identified and characterized in animals in response to BV injection. It has been stably characterized that subcutaneous BV injection into a rat's one hind paw results in an immediate, long-term period of spontaneous pain-related behaviors, followed by sustained pain hypersensitivity to thermal and mechanical stimuli. Moreover, there is no species difference from rodents to felines and to humans, suggesting a share of common nociceptive and inflammatory mechanisms among mammals in response to bee stings. Experimentally, it has been demonstrated that melittin, the major pain-producing peptide of bee venom, can depolarize and sensitize the primary nociceptor cells through opening of TRPV1 channels by phospholipase A2-lipoxygenase/cyclooxygenase metabolites, leading to production of spontaneous pain and hyperalgesia/allodynia. The pain signals produced at the peripheral terminals of the primary nociceptor cells by bee venomous pain-producing substances can further activate and sensitize the spinal dorsal horn pain-signaling neurons that relay and send the enhanced nociceptive information to the cerebral cortices, resulting in spatiotemporal

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_1

synaptic plasticity and/or metaplasticity of the "pain matrix" (e.g., primary somatosensory cortex, anterior cingulate cortex, and hippocampal formation), leading to alterations of animal behaviors. This chapter will address the questions of what, how, and why BV causes pain.

Keywords

Bee venom • Nociception • Pain-signaling pathways • Peripheral sensitization • Central neural plasticity

Contents

Introduction

Scientific knowledge consists in the search for truth, but it is not the search for certainty.
Karl Popper (a philosopher of science)

Although it is a common experience and knowledge for human beings that bee stings are naturally harmful due to painful and anaphylactic reactions, understandings of the biological, pharmacological, and toxicological effects and the underlying mechanisms of bee venom-induced pain have not been well gained due to lack of scientific investigations until the end of the last century (Chen and Lariviere 2010). Empirically, the bee venom injection, referred to as bee venom therapy (BVT), has been used as a complementary and alternative therapeutic method for thousands of years to treat many diseases, including rheumatoid arthritis, bursitis, tendinitis,

shingles (herpes zoster), multiple sclerosis, wounds, gout, burns, and infections (Son et al. 2007; Chen and Lariviere 2010). Recently, attempts have also been made to use bee venom and its chemical components for potential cancer treatment (Son et al. 2007; Chen and Lariviere 2010). Historically, the use of BVT in patients can be traced back to ancient Egypt, Greece, and China. In literature, Philip Terc is believed to be the first user of BVT for treatment of rheumatic patients (American Apitherapy Society Inc. 1989; Chen and Lariviere 2010). The BVT was then introduced to the United States of America by one of Philip Terc's followers (Beck 1935; Mraz 1981; American Apitherapy Society Inc. 1989). Although BVT is likely to be a promising therapeutic alternative for treatment of chronic pain and other diseases (Son et al. 2007), so far its efficacy and safety have not been approved by food and drug authorities worldwide (Chen and Lariviere 2010).

Interests in understanding of the bee venom-induced pain and its underlying mechanisms have been dramatically increased since the initial characterizations of the altered pain-related behaviors and inflammation in rats (Lariviere and Melzack 1996, 2000; Chen et al. 1999b; Chen and Chen 2000). It has been stably characterized that subcutaneous (s.c.) bee venom injection into one hind paw of rats results in an immediate, long-term spontaneous alterations in behaviors relevant to pain and nociception lasting at least more than 1 h for quantitative observation (Lariviere and Melzack 1996; Chen et al. 1999b). The bee venom-induced spontaneous pain-related behaviors have been later identified as a spinally processed nonvoluntary nociceptive reflex (paw flinches) and a supraspinally processed voluntary self-caring and favoring behaviors (paw lifting, licking, and biting) in rats (Ren et al. 2008). Moreover, it has been further identified that pain hypersensitivity evoked by thermal or mechanical stimulus modality can also occur spatially at the site of bee venom injection (primary thermal and mechanical hyperalgesia), at an area remote to the bee venom injection site (secondary thermal hyperalgesia), and at symmetrical paw contralateral to the bee venom injection site (mirror-image thermal hyperalgesia) lasting for 48–96 h (Chen et al. 1999b; Chen and Chen 2000). Unlike other algogens (pain-producing substances) (Chen and Lariviere 2010), the nociceptive and inflammatory effects of s.c. bee venom have no species difference in mammals ranging from mice (Lariviere et al. 2002), to rats (Lariviere and Melzack 1996; Chen et al. 1999b; Chen and Chen 2000), to cats (Chen et al. 1998), and to human beings (Koyama et al. 2000, 2003; Sumikura et al. 2003, 2006), suggesting a share of common nociceptive and inflammatory mechanisms among mammals in response to bee stings.

In the past two decades, nociceptive and inflammatory effects of bee venom and its pain-producing components on the somatosensory system have been well studied (Chen 2003, 2007, 2008; Chen and Lariviere 2010). Furthermore, the anti-nociceptive and anti-inflammatory effects of bee venom have also been reported and reviewed (Son et al. 2007; Chen and Lariviere 2010). In this chapter, the scientific questions of what, how, and why bee venom causes pain and sensory hypersensitivity will be fully addressed, while the mechanisms of the BVT will not be dealt with in the current review because it is beyond the scope of this topic.

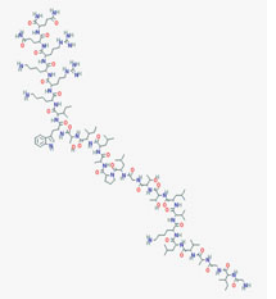
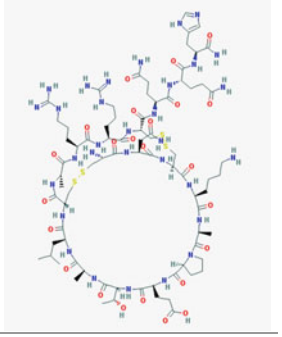
Bee Venom and Its Biological Components

As listed in Tables 1 and 2, (honey) bee venom (of *Apis mellifera*) is a complex composition of polypeptides, enzymes, amines, lipids, and amino acids (Habermann 1972; Gauldie et al. 1976, 1978; Lariviere and Melzack 1996; Chen and Lariviere 2010). Among the various chemical constituents of bee venom, the most unique biologically active substances are some polypeptides, including melittin, apamin, mast-cell degranulating (MCD) peptide, mastocytolytic peptide, minimine, secapin, melittin F, cadiopep, and adolapin (Tables 1 and 2). In general, the bee venom polypeptides are likely to act on the nervous system in both the peripheral nervous system and the central nervous system (CNS) as activators and/or modulators of ion channels and other molecular targets, leading to various biological, pharmacological, and toxicological actions (Tables 1 and 2). However, so far the specifications of these toxic peptides in terms of biological actions, pharmacological and toxicological effects, and structure–function relationships remain mostly unknown. The key effects of some polypeptides and enzymes found in greatest abundance in bee venom are briefly described below.

Melittin

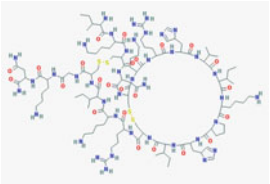
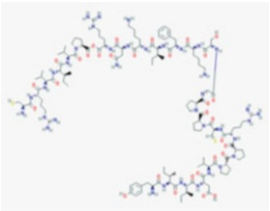
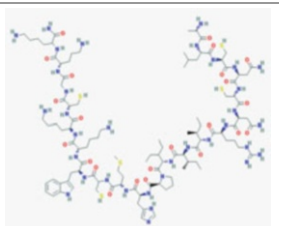
Melittin is a strongly basic 26 amino acid polypeptide which constitutes 40–60% of the whole dry bee venom (Chen and Lariviere 2010). Its physical and chemical properties are listed in Table 1. It has various biological, pharmacological, and toxicological actions including strong surface activity on cell lipid membranes, hemolyzing activity, antibacterial and antifungal activities, and antitumor properties (Table 2; also see Habermann 1972; Son et al. 2007; Chen and Lariviere 2010). Recently, it has become known that melittin is also a strong pain-producing substance able to activate and sensitize nociceptor cells in the periphery via opening of nonselective cation channels – transient receptor potential vanilloid receptor 1 (TRPV1) mediated by the activation of phospholipase A2 (PLA2) cascade pathways (Du et al. 2011). In this process, the activation of ATP P2X and P2Y receptors and phosphorylation of protein kinase A (PKA) and mitogen-activated protein kinases (MAPKs) are also believed to be involved (Hao et al. 2008; Lu et al. 2008; Yu et al. 2009; Du et al. 2011; for details, see below). Moreover, tetrodotoxin-resistant subunit Nav1.9 of voltage-gated sodium channel (VGSC) is upregulated in small cells of the dorsal root ganglia (DRG) by s.c. injection of melittin (Yu et al. 2013). Contrarily, melittin is also believed to be the active substance of bee venom in producing antinociceptive effects when applied to the acupoint of a subject ("apipuncture") (Son et al. 2007). However, the molecular and cellular mechanisms underlying the antinociceptive effects of melittin remain unclear.

Table 1 Chemical and physical properties of some major peptides derived from honeybee (*Apis mellifera*) venom[a]

Name	Molecular formula	Amino acid sequence (amino acid residue number)	Molecular weight	InChI key[b]	2D structure[c]
Melittin	$C_{131}H_{229}N_{39}O_{31}$	Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-GlnNH2 (26)	2846.46266 g/mol	VDXZNPDIRNWWCW-UHFFFAOYSA-N	
Apamin	$C_{79}H_{131}N_{31}O_{24}S_4$	Cys-Asn-Cys-Lys-Ala-Pro-Glu-Thr-Ala-Leu-Cys-Ala-Arg-Arg-Cys-Gln-Gln-HisNH2 (18)	2027.33874 g/mol	YVIIHEKJCKCXOB-STYWVVQQSA-N	

(*continued*)

Table 1 (continued)

Name	Molecular formula	Amino acid sequence (amino acid residue number)	Molecular weight	InChI key[b]	2D structure[c]
Mast-cell degranulating peptide (also known as peptide 401)	$C_{110}H_{192}N_{40}O_{24}S_4$	Ile-Lys-Cys-Asn-Cys-Lys-Arg-His-Val-Ile-Lys-Pro-His-Ile-Cys-Arg-Lys-Ile-Cys-Gly-Lys-AsnNH2 (22)	2587.21508 g/mol	QMBRLNFAEHGOLY-UHFFFAOYSA-N	
Secapin (also known as LS-144697)	$C_{131}H_{213}N_{37}O_{31}S_2$	Tyr-Ile-Ile-Asp-Val-Pro-Pro-Arg-Cys-Pro-Pro-Gly-Ser-Lys-Phe-Ile-Lys-Asn-Arg-Cys-Arg-Val-Pro-ValNH2 (24)	2866.45222 g/mol	CJARBYBELTXTGO-WBHDHAESSA-N	
Tertiapin (also known as 58694-52-3)	$C_{106}H_{180}N_{34}O_{23}S_5$	Ala-Leu-Cys-Asn-Cys-Asn-Arg-IIe-IIe-IIe-Pro-His-Gln-Cys-Trp-Lys-Lys-Cys-Gly-Lys-LysNH2 (21)	2459.1024 g/mol	QWKNGDWCZWKGME-NPHAJLTCSA-N	

[a]Data obtained from the website of PubChem: http://pubchem.ncbi.nlm.nih.gov/ which is a database of chemical molecules and their activities against biological assays

[b]InChI, the International Union of Pure and Applied Chemistry (IUPAC) International Chemical Identifier

[c]3D structures of the honeybee venom-derived peptides are not available because conformer generation is disallowed since there are too many atoms, they are too flexible, and there are too many undefined stereo centers

Table 2 Ingredients of honey bee (*Apis mellifera*) venom and their biological effects

Ingredients	Biological effects	Molecular target	Activity level
Peptides			
Adolapin (1%)	Antinociceptive and anti-inflammatory effects	Cyclooxygenases	ED50: 0.016 mg/kg by acetic acid writhing test. ED50: 0.013 mg/kg by the Randall–Selitto test
Apamin (2–3%)	Selective blocker of the small-conductance calcium-dependent potassium channel	$K_{ca}2.2$ (SK2, KCNN2)	Unspecified
		$K_{ca}2.3$ (SK3, KCNN3)	IC50: 0.000168 μM by thallium flux assay in transfected HEK293 cells IC50: 0.000064 μM by electrophysiological assay in transfected HEK293 cells
Cardiopep (0.7%)	Cardioactive and antiarrhythmic effects	β-adrenergic receptor	Unspecified
Mast-cell degranulating peptide (2–3%)	Histamine release	Mast-cell degranulation	ED50: 0.1 mg/kg assessed in turpentine- and carrageenin-induced inflammatory models of rats
	Induction of LTP in the hippocampus Inhibition of epilepsy	Pertussis toxin-sensitive G protein and voltage-gated potassium channel	Activation of pertussis toxin-sensitive G protein and inhibition of voltage-gated potassium channel in brain membranes
MCL peptide	A selectively mastocytolytic factor	Unspecified	Unspecified
Melittin (40–60%)	Pore-forming activity	Lipid bilayer membrane	Dependent upon lipid composition and vesicle size
	Hemolyzing activity	Red blood cells	Active toxicity in RBC assessed as hemolysis
	Activation of phospholipase A2 (PLA2)	PLA2	Assessed by measuring calcium influx in Ras-transformed cells Prostaglandin biosynthesis in cell culture and in vivo
	Antifungal and antibacterial activity by induction of membrane damage	Phospholipids	1.56–100 μM by Sytox green-based fluorescence assay in fungus (*Aspergillus fumigatus*) Antimicrobial activity against *Penicillium digitatum* PHI-26 assessed by orange fruit decay assay

(*continued*)

Table 2 (continued)

Ingredients	Biological effects	Molecular target	Activity level
	Antiangiogenic activity Antitumor activities	Antiangiogenic activity	IC50: 13 μM against VEGFA-stimulated capillary differentiation in HUVEC by Matrigel assay IC50: 18 μM against VEGFA-stimulated cell proliferation in HUVEC by BrdU incorporation assay
Melittin F (<1%)	Unspecified		
Minimine	Unspecified		
Procamine	Unspecified		
Promelittin	Unspecified		
Secapin (<1%)	High-affinity binding sites in rat brain	Unspecified	Unspecified
Tertiapin (<1%)	High-affinity binding sites in rat brain Inhibition of the enzyme-activating capacity of calmodulin Inhibition of activity of soluble phosphodiesterase	Unspecified	Unspecified
Others			
Acetylcholine	Agonist at muscarinic and nicotinic acetylcholine receptors		
Amino acids	Unspecified		
Carbohydrates (2%)	Unspecified		
Dopamine	Agonist at dopamine receptors		
Esterase	Unspecified		
Histamine (1.5%)	Agonist at histamine receptors		
Hyaluronidase (1.5–2.0%)	Hyaluronic acid hydrolyzing enzyme Allergenicity Histamine release		
Lipids (5%)	Unspecified		
Noradrenaline	Agonist at adrenoceptors		
Phospholipase A (10–12%)	Antigenicity and allergenicity Inflammatory and nociceptive Interaction with		

(*continued*)

Table 2 (continued)

Ingredients	Biological effects	Molecular target	Activity level
	melittin Activation of neurons and glial cells Nerve regeneration Facilitation of neurotransmitter release		
Phospholipase B	Unspecified		
Protease inhibitor	Unspecified		

Values in parentheses for ingredients (left) indicate percent by weight of dry bee venom. *LTP* long-term potentiation

Apamin

Apamin is another important bee venom neurotoxic polypeptide of 18 amino acids comprising 2–3% of whole dry bee venom (Chen and Lariviere 2010). Its physical and chemical properties are listed in Table 1. It possesses a selective inhibitory action on calcium-dependent potassium channels (CDPC) that are involved in regulation of the after-hyperpolarization period that affects the frequency of action potentials in the nervous system (Table 2). Based upon its selective action, apamin has been widely used as a tool drug for studying the characteristics of CDPC.

Mast-Cell Degranulating (MCD) Peptide

MCD peptide, also known as peptide 401, is a bee venom polypeptide with 22 amino acids and constitutes 2–3% of whole dry bee venom (Chen and Lariviere 2010). Its physical and chemical properties are listed in Table 1. It was originally named due to its biological action of causing release of histamine from mast cells (Chen and Lariviere 2010). MCD peptide has specific binding sites in the hippocampus, and application of this peptide onto hippocampal slices was shown to result in the production of long-term potentiation (LTP) in the CA1 area that is distinct from the LTP evoked by conditioning electrical stimulation (Table 2). MCD peptide may also be involved in the pathogenesis of epilepsy (Table 2). The molecular and cellular mechanisms underlying MCD peptide-evoked LTP in the hippocampus are not clear, but some reports showed that it has selective binding sites and actions on voltage-dependent potassium channels (Table 2).

Adolapin

Adolapin is a basic polypeptide with 103 amino acid residues and comprises 1% of whole dry bee venom (Chen and Lariviere 2010). Its physical and chemical

properties remain unclear. It is the only one that has been shown to have anti-nociceptive, anti-inflammatory, and antipyretic effects primarily (Table 2). Adolapin can inhibit prostaglandin synthesis via inhibition of cyclooxygenase (COX) activity (Table 2).

Enzymes

There are two major enzymes in the bee venom. One is PLA2 that constitutes 10–12% of whole dry bee venom and has various pharmacological and toxicological effects (Chen and Lariviere 2010). Cellular PLA2 is a membrane-associated phospholipid-converting enzyme that is important in the production of arachidonic acid (AA), which is further metabolized to prostaglandins by COX and to leukotrienes and eicosanoids by lipoxygenase (LOX). PLA2 exhibits complex interactions with melittin that can result in potentiation or in inhibition of secretory PLA2 effects depending on the peptide/phospholipid ratio (Table 2). PLA2 has effects in a range of cells related to nociception including astrocytes and neurons and possibly microglial cells. PLA2 is also involved in pronociceptive glutaminergic neurotransmission in the substantia gelatinosa of the dorsal horn of the spinal cord. The other major enzyme in the bee venom is hyaluronidase that constitutes 1.5–2% of whole dry bee venom (Chen and Lariviere 2010). Bee venom hyaluronidase may share the same property as the endogenous hyaluronidase that breaks down hyaluronic acid in tissues (Table 2).

Nociceptive and Inflammatory Effects of Subcutaneous Bee Venom and Its Pain-Producing Constituents

Experimental Human Studies

Pain sensation produced by a bee sting is a common unpleasant experience of human beings. However, due to the risk of anaphylaxis and systemic reactions to its allergens, so far there has been no experimental report of whole bee venom injection into humans for the primary purpose of studying pain. At the beginning of the twenty-first century, due to increased interest in animal studies on bee venom-induced pain, the first experimental study on pain and inflammatory responses of healthy adults (two women and five men) to intradermal (i.d.) injection of melittin was carried out (Koyama et al. 2000). The pain intensity was rated using a 0–10 visual analog scale (VAS) in which scores of 0, 5, and 10 indicated "no pain," "moderate pain," and "intolerable pain," respectively. A sharp pain sensation (score > 8.0) was reported in all 7 subjects immediately after i.d. injection of melittin (5 μg in 50 μl saline) into the volar aspect of one forearm. The pain sensation declined gradually and totally disappeared at 3 min after melittin injection. It was clearly described that there was no itch sensation following melittin injection, suggesting that significant histamine might not be released. Meanwhile, melittin produced a

visual flare surrounding a wheal near the injection site that disappeared within 2 h. This local inflammatory response was also characterized by an increase in skin temperature monitored by a computer-assisted infrared thermograph. The melittin-induced peak increase in skin temperature was at least 10 min delayed compared to the peak pain sensation, and this process was sustained for at least 1 h. Topical lidocaine gel administration markedly blocked the melittin-induced visual flare and the increased skin temperature but not the pain sensation, suggesting that the local inflammatory response induced by melittin is neurogenic and mediated by dorsal root reflex and/or axonal reflex and sympathetic regulation (Koyama et al. 2000, 2002). The painful effects of two higher doses of melittin (10 and 50 μg in 50 μl saline) have been also observed in two other experimental studies on healthy human volunteers (Sumikura et al. 2003, 2006). The peak pain intensity was similar to the abovementioned report; however, the duration of the pain sensation was much longer with the higher doses used and demonstrated a dose-related increase in the duration of the pain. A similar relationship has been reported in rats following whole bee venom injection (Lariviere and Melzack 1996; Chen and Lariviere 2010; see below). The doses of melittin used in the human experiments are far less than the amount delivered by one bee sting, which contains about 140 μg of dried bee venom per sting and 40–60% melittin (Schumacher et al. 1992, 1994). Moreover, i.d. injection of melittin results in primary heat and mechanical hyperalgesia as well as a secondary heat hyperalgesia identified in an area remote from the injection site (Sumikura et al. 2003, 2006). There is no anaphylaxis and systemic reactions in the human subjects receiving i.d. melittin injection.

Experimental Animal Studies

Nociceptive and Inflammatory Effects of Bee Venom Injection

The primary purpose of the first experimental animal study on the nociceptive and inflammatory effects of bee venom was to establish a new rat model for the study of pain (Lariviere and Melzack 1996). Subsequently, the bee venom-induced pain-related behavioral responses have been well studied in different species including cats, rats, and many strains of mice (Chen et al. 1998, 1999b; Chen and Chen 2000, 2001; Lariviere et al. 2002; for details see Chen and Lariviere 2010). A comparative study of the effects of six venoms from honeybee, bumblebee, yellow jacket and paper wasps, and yellow- and white-faced hornets on rats showed that honeybee venom was the most potent to produce pain in animals (Lariviere and Melzack 2000). Generally, animals respond behaviorally to a given dose of s.c. bee venom injection in a similar pattern and time course. They display painful spontaneous behaviors by a bout of self-caring and favoring actions such as flinching, licking, and biting the injected paw robustly in the first 5–10 min, followed by a slow decline in pain scores over 1 h of observation. Dose–effect investigation shows that the intensity and time course of the bee venom-induced spontaneous pain-related behaviors are dose dependent (Fig. 1). The bee venom-induced pain-related behaviors are shown to be sensitive to pharmacological intervention by morphine and

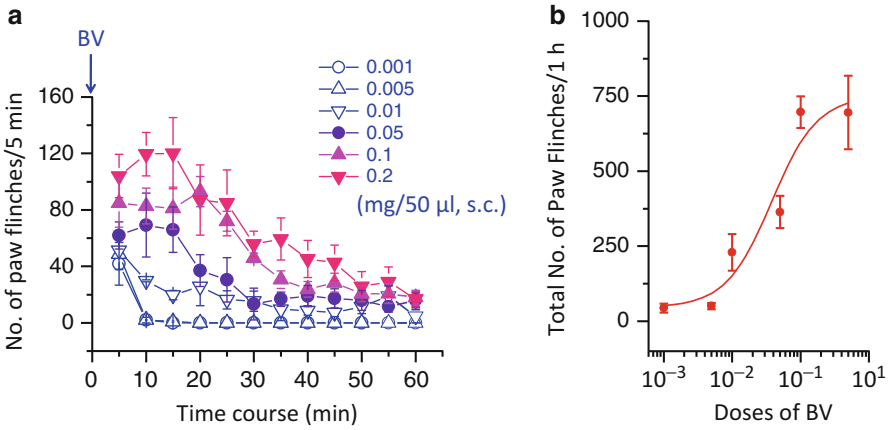


Fig. 1 Dose effect of subcutaneous injection of bee venom (*BV*) on spontaneous pain-related behaviors in rats. (**a**) Time courses of spontaneous paw flinches induced by subcutaneous injection of six doses of BV. (**b**) A fitted curve of dose effect following BV injection

nonsteroidal anti-inflammatory drugs (NSAIDs), demonstrating that the bee venom-induced behaviors are nociceptive or painful. The lower mammalian response pattern and time course are similar to the human response, and thus, there is no evidence for species differences. The consistency of behavioral responses to bee venom among different species reflects common, natural biological processes in mammals in response to bee stings.

Hyperalgesia is referred to as an enhanced painful sensation evoked by painful stimulation (mechanically or thermally nociceptive), while allodynia is referred to as a painful sensation evoked by non-painful stimulation (mechanically or thermally non-nociceptive) under the conditions of tissue and/or nerve injury (McMahon and Koltzenburg 2006; Chen et al. 2013). In animals, pain hypersensitivity (hyperalgesia and allodynia) can be assessed and quantified by measuring changes in paw withdrawal thermal latency (PWTL) or paw withdrawal mechanical threshold (PWMT) between pre- and posttreatment of bee venom. The phenomena of pain hypersensitivity in rats following s.c. bee venom injection were first identified by Chen and his colleagues (Chen et al. 1999b; Chen and Chen 2000, 2001). Following at least 1 h after bee venom injection when spontaneous pain-related behaviors disappear, radiant thermal or mechanical von Frey filament stimuli can be applied to the injection site and its remote surrounding area. Dramatic reductions in PWTL or PWMT can be consistently identified during the period between 2 and 96 h after bee venom injection, indicating the occurrence of primary heat and mechanical hyperalgesia in the injured area of the paw (Chen et al. 1999b, 2000, 2001, 2003; Chen and Chen 2000). This result was consistent with what was seen in experimental human subjects in response to melittin injection (Sumikura et al. 2003). Moreover, a secondary and a mirror-image heat, but not mechanical, hyperalgesia can also be identified in rats, which can also be seen in the same human subjects (Sumikura et al. 2006). This is an interesting clinical phenomenon referred to as mirror-image pain

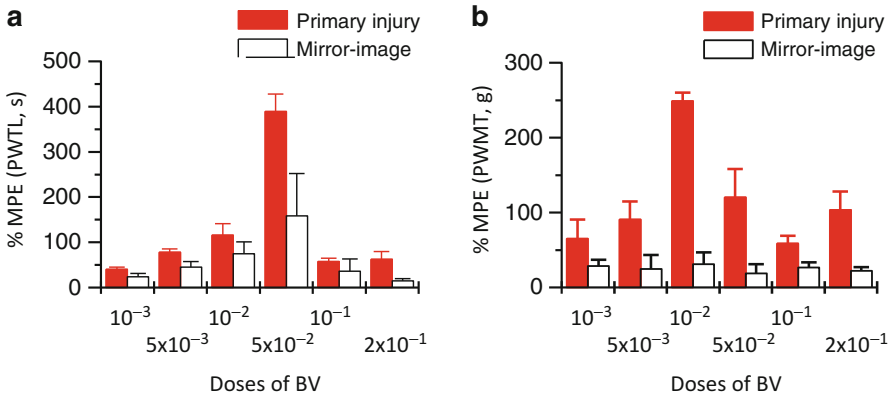


Fig. 2 Dose effect of subcutaneous injection of bee venom (*BV*) on thermal or mechanical pain hypersensitivity in rats. (**a**) Percent maximal possible effect (% MPE) of six doses of BV injections on thermal pain hypersensitivity. Dose-dependent increase in % MPE can be clearly established between 0.001 and 0.05 mg of BV; however, dose effect disappears when the dose is higher than 0.1 mg. (**b**) Percent MPE of six doses of BV injections on mechanical pain hypersensitivity. Dose-dependent increase in % MPE can be clearly established between 0.001 and 0.01 mg of BV; however, dose effect disappears when the dose is higher than 0.05 mg. *PWTL* paw withdrawal thermal latency, *PWMT* paw withdrawal mechanical threshold. Primary injury indicates s.c. BV injection site; *mirror image* indicates symmetrical site on the contralateral paw to the BV injection

that occurs on the side of the body contralateral to the injured side (Chen et al. 2013; Chen and Lariviere 2010). It is also intriguing to note that the time course of hyperalgesia to thermal and mechanical stimulus modalities in the primary injury area is different. Primary mechanical hyperalgesia lasts more than 96 h, while primary heat hyperalgesia lasts 48 h (Chen 2003, 2007, 2008; Chen and Lariviere 2010). Dose–effect investigation also reflects difference in percent maximal possible effect (% MPE) between thermal and mechanical pain hypersensitivities induced by s.c. bee venom in rats (Fig. 2). These results implicate distinct underlying mechanisms of the bee venom-induced pain hypersensitivity evoked by different stimulus modalities.

The bee venom-induced local inflammatory responses have also been scored by measurement of increased volume of the injected paw (Lariviere and Melzack 1996). It has been shown that edema develops immediately and reaches a maximal plateau at about 15–20 min after injection. The time course of edema outlasts the spontaneous pain-related behaviors and gradually disappears by 48 h after bee venom injection. The dose-dependent effect of bee venom on paw edema is well established between the doses ranging from 0.001 to 0.1 mg (in 50 μl saline), while good dose-dependent effect cannot be seen for plasma extravasation (Fig. 3). Indomethacin, an NSAID, could effectively inhibit the bee venom-induced paw edema. There is no overt necrosis developed in response to bee venom injection, and no rat shows signs of allergic reaction, even when retested within 1 month after the first testing (Lariviere and Melzack 1996).

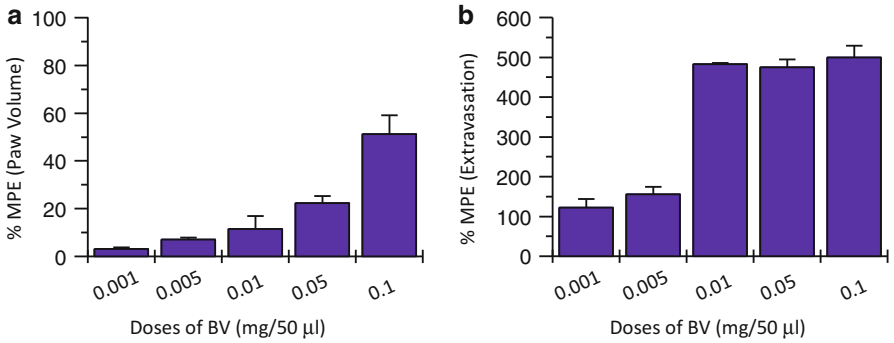


Fig. 3 Dose effect of subcutaneous injection of bee venom (*BV*) on inflammatory responses in rats. (**a**) Percent maximal possible effect (% MPE) of six doses of BV injections on paw volume (edema). (**b**) Percent MPE of six doses of BV injections on plasma extravasation. Paw edema reflected by paw volume induced by s.c. BV injection has a great dose-dependent effect; however, plasma extravasation does not

Nociceptive and Inflammatory Effects of Bee Venom Peptide Constituents

As listed in Tables 1 and 2, bee venom is a complex composition of more than 20 constituents or ingredients. Four major polypeptides including melittin, apamin, MCD peptide, and a novel PLA2-related peptide have been separated, purified, and structurally identified from whole dried bee venom so as to search for active long-term pain-producing components (Chen et al. 2006). In animal studies, it has been revealed that all four of the polypeptides could produce marked local inflammatory responses (edema) when measured 1 h after s.c. injection. However, the nociceptive and hyperalgesic effects differ from substance to substance. Among the four polypeptides tested subcutaneously, melittin, MCD peptide, and PLA2-related peptide are able to produce distinct nociceptive paw flinches; however, only melittin causes pain-related behaviors lasting for nearly 1 h. Because all three polypeptides induce nociception that peaks shortly after injection, it has been proposed that they activate nociceptors more quickly (Chen et al. 2006). Upon examining the effects of the four polypeptides, only melittin and apamin are shown to result in heat as well as mechanical hypersensitivity at the primary injury site, and only melittin-induced primary mechanical hypersensitivity lasts over 48 h. Moreover, the melittin-induced number of rat paw flinches is surprisingly and particularly similar to the number induced by bee venom during the late period (20–60 min after injection), but less than the bee venom-induced paw flinches during the early period (0–19 min after injection). Thus, among the components of bee venom, melittin has been demonstrated to be the major polypeptide responsible for the prolonged painful stimulation of bee venom injection, leading to both tonic nociception and hypersensitivity, while the other polypeptides contribute only to the early nociceptive responses within 10–20 min after injection (Chen and Lariviere 2010).

It has been also surprisingly revealed that the melittin-induced nociceptive responses can be partially inhibited by both pre- and posttreatment with capsazepine

(CPZ), a potent antagonist of the thermal nociceptor TRPV1, suggesting the involvement of this molecular target in the melittin-induced nociception (Chen et al. 2006). Since CPZ can reverse the melittin-induced primary heat hypersensitivity, but has no effect on the melittin-induced primary mechanical hypersensitivity, it highly supports the notion that the activation of TRPV1 by melittin specifically contributes to persistent nociception and primary heat hyperalgesia (Chen et al. 2006). Altogether, it becomes clear that during the symphony of bee venom-produced inflammatory pain and hypersensitivity, different components of bee venom play different roles in the entire process; however, melittin is likely to play a central role in the production of the long-lasting pain, hyperalgesia, and local inflammation following bee sting in mammals.

Neuronal Activities in Response to Bee Venom and Melittin Along the Pain Pathways

Activation and Sensitization of Primary Nociceptive Neurons

To understand how primary nociceptive neurons respond to bee venom, direct effects of melittin, the major pain-producing polypeptide of bee venom, were examined using in vitro whole-cell patch-clamp recordings of primary sensory neurons acutely dissociated from rat's DRG (Du et al. 2011). Topical application of melittin resulted in $[Ca^{2+}]i$ rise in 69% of the cells tested (20–35 μm in diameter) (Fig. 4a, b). In current clamp mode, 55% of the recorded DRG neurons were shown to respond to melittin with tonic discharge of action potentials (APs) following a slow membrane depolarization (Fig. 4c). The duration of melittin-induced firing ranged from 70 to 1200 s and was most intense during the 200–800 s time period (Fig. 4d, e). The melittin-responsive DRG cells were defined as nociceptors because the APs exhibited typical electrophysiological characteristics of nociceptive cells: (1) a long AP duration and a prolonged after-hyperpolarization; (2) an inflection on its falling phase, which is generally considered a characteristic of nociceptors (Fig. 4c′); (3) capsaicin sensitive; and (4) IB4-positive cell sensitive. Accordingly, the majority of the melittin-sensitive cells recorded are likely to be primary nociceptive neurons.

Using voltage clamp recordings at a holding potential of −70 mV, it was found that melittin could evoke large inward currents in 42% of the recorded DRG neurons in a concentration-dependent manner (Fig. 5a, b; Du et al. 2011). Moreover, repeated application of a given concentration of melittin (2 μM for 200–300 s duration, 20 s intervals) had sensitizing effects on the inward currents, as evidenced by increased current amplitude following the second and the third melittin application compared to the first response (Fig. 5c, d). TRPV1, also known as the capsaicin receptor and proton-activated ion channel, is well known to be a thermal nociceptor transducing nociceptive heat (>42 °C) stimuli into a series of pain "signals" (action potentials) at the peripheral terminals of a population of primary nociceptive neurons (Chen et al. 2013). Co-application of CPZ, a selective TRPV1 antagonist, could completely

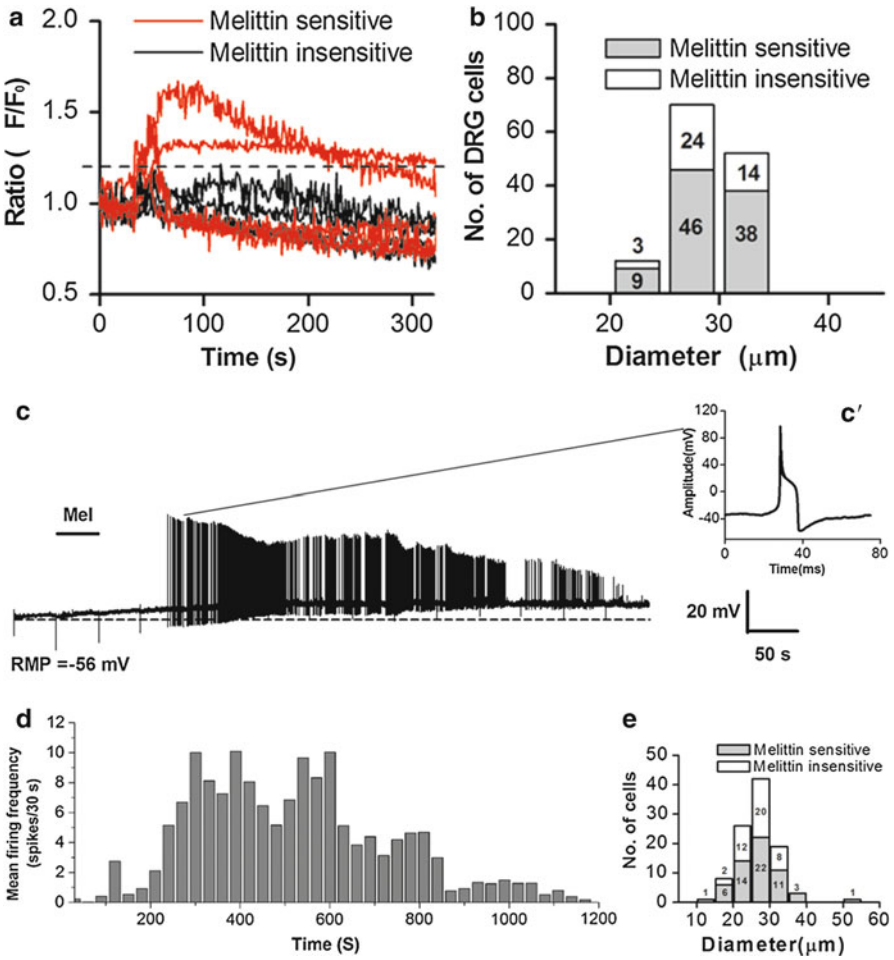


Fig. 4 Melittin (Mel)-induced rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) and action potential firing in primary sensory (DRG) neurons of rats. (**a**) *Graph* shows that a subpopulation of acutely isolated rat DRG neurons was able to respond to Mel (2.0 μM) with a $[Ca^{2+}]i$ rise (*red* vs. *black*). Typical traces of $[Ca^{2+}]i$ changes were simultaneously recorded during the application of Mel for 30 s. (**b**) Distribution histogram of the cell body sizes for Mel-sensitive ($n = 93$) and Mel-insensitive ($n = 41$) DRG neurons. (**c**) Application of Mel (2.0 μM, for 50 s) to a DRG cell evoked a slow membrane depolarization (resting membrane potential, RMP $= -56$ mV before Mel application; *dotted line*) and a period of tonic action potential (*AP*) discharge. *Inset* **c′** shows the waveform of the AP recorded at the indicated time, 133 s after the Mel application. (**d**) Histogram showing time course of firing frequency averaged from 45 Mel-sensitive DRG neurons. (**e**) Distribution histogram of cell body diameters for Mel-sensitive ($n = 56$) and Mel-insensitive ($n = 46$) DRG neurons as measured in current clamp experiments (From Fig. 1 of Du et al., Melittin activates TRPV1 receptors in primary nociceptive sensory neurons via the phospholipase A2 cascade pathways. Biochem Biophys Res Commun 2011; 408:32–37. With permission from Elsevier)

block the occurrence of the melittin-induced inward currents (Fig. 5e, f) and $[Ca^{2+}]i$ rise (Fig. 5g–i) in a reversible manner (Du et al. 2011), suggesting a selective action rather than nonselective pore-forming effects of melittin on the primary sensory neurons (Chen and Lariviere 2010). In this study, further experiments showed that: (1) inhibitions of PLA2, but not phospholipase C (PLC), could suppress the melittin-induced inward currents; (2) inhibitors of COX and LOX, two key components of the AA metabolism pathway, each partially suppressed the inward current evoked by melittin; (3) inhibitor of protein kinase A (PKA), but not of PKC, also abolished the melittin-induced inward currents. These results indicate that melittin can excite primary nociceptive neurons at least in part by activating TRPV1 receptors via PLA2-COXs/LOXs cascade pathways (Fig. 5j; Du et al. 2011). In another two experiments, roles of TRPC in mediation of the melittin-induced activation of primary nociceptive neurons and pain-related behaviors were also studied (Ding et al. 2011, 2012). It was revealed that direct application of SKF-96365, a potent antagonist of TRPC3/6/7, could block the melittin-induced inward current and $[Ca^{2+}]i$ rise (Ding et al. 2011) and the melittin-induced spontaneous pain-related behaviors as well as thermal pain hypersensitivity (Ding et al. 2012). Similar to TRPV1, TRPC family is also a nonselective cation channel that is highly permeable to Ca^{2+} when activated. Because it is known that TRPC1, TRPC3, and TRPC6 are the major subunits localized in the rat DRG and intracellular diacylglycerol (DAG) is an endogenous activator of TRPC3/6/7 channels that are sensitive to SKF-96365, it can be proposed that melittin, which can insert itself into the lipid membrane (Chen and Lariviere 2010), causes pore formation that leads to the release of ATP and the activation of P2X channels and P2Y G-protein-coupled receptors (GPCRs) (Lu et al. 2008). The activation of GPCRs by melittin results in the production of DAG and inositol 1,4,5-triphosphate (IP3), and this may serve as another route for melittin to activate the subpopulation of nociceptor cells containing IB4, but not TRPV1 (Ding et al. 2011, 2012).

More recently, it was revealed that s.c. injection of melittin caused upregulation of tetrodotoxin-resistant VGSC subunits Nav1.9 and Nav1.8 in the DRG cells (Yu et al. 2013). However, antisense-mediated knockdown of Nav1.9, but not Nav1.8, in the DRG resulted in inhibition of melittin-induced pain-related behaviors (Yu et al. 2013). Contrarily, Nav1.8, but not Nav1.9, was shown to be involved in complete Freund's adjuvant (CFA)-induced inflammatory pain (Yu et al. 2011). Patch-clamp recordings of DRG cells dissociated from rats 2 h after s.c. melittin injection revealed that the melittin-responsive cells were tonic type, but not phasic type, in terms of electrophysiological characteristics (Yu et al. 2014). This suggests that the tonic subpopulation of the DRG cells studied is likely to be a key primary nociceptor cellular type in production and conduction of spike firing induced by melittin and bee venom.

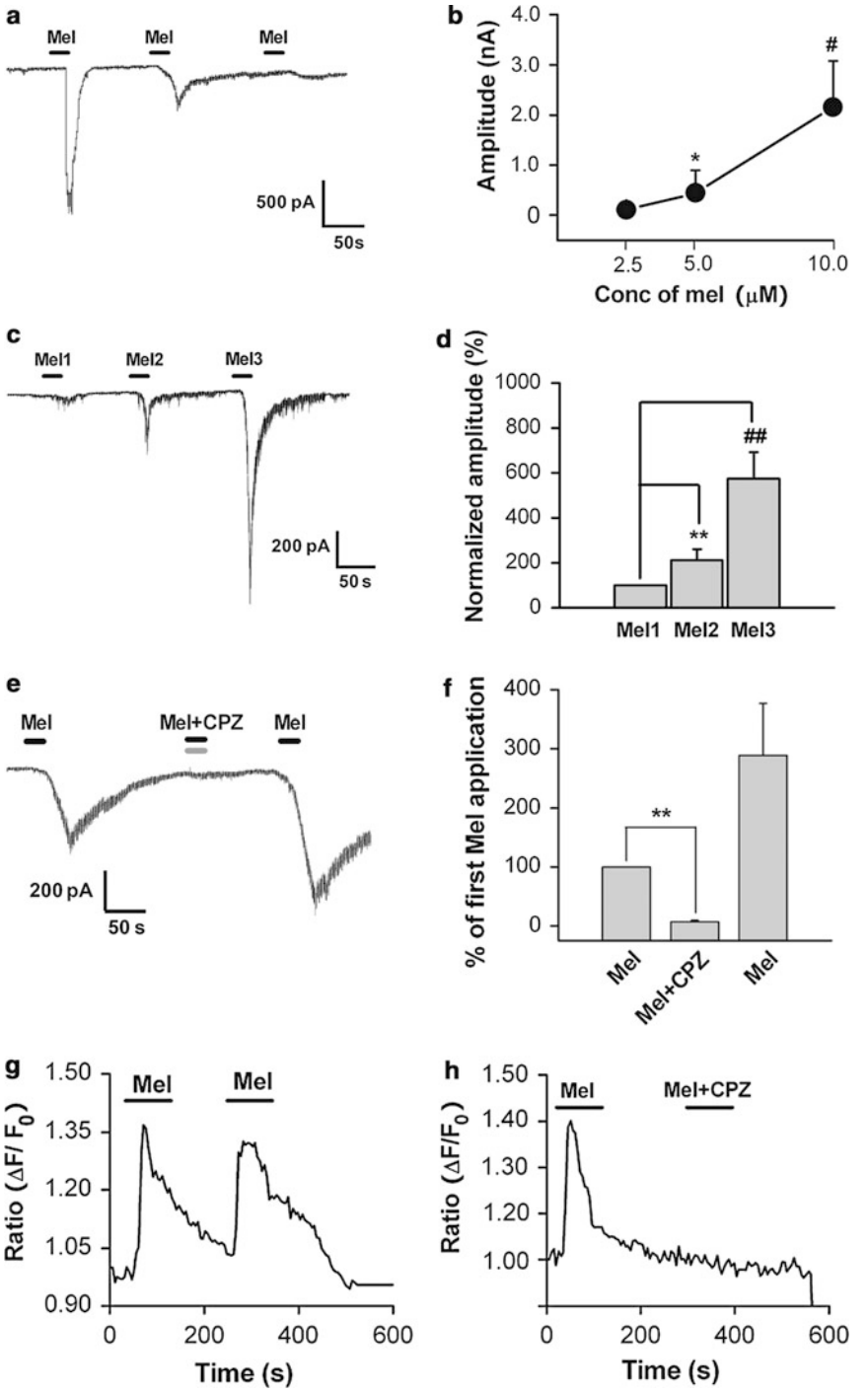


Fig. 5 (continued)

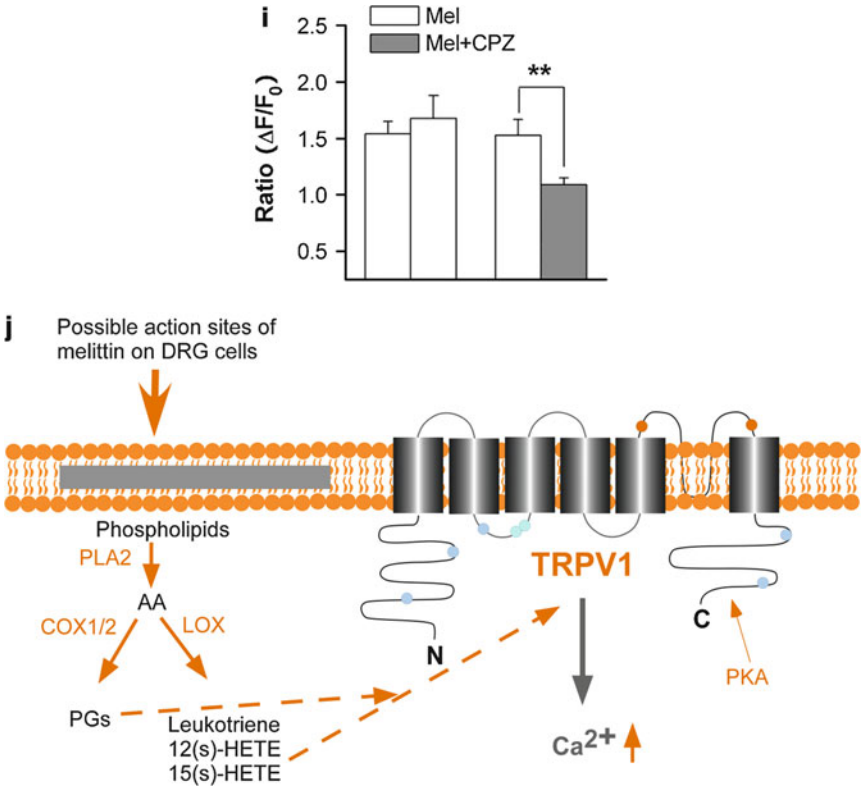


Fig. 5 Effects of topical application of melittin (Mel) on the current responses of primary sensory (DRG) neurons. (**a**) Amplitude of current evoked from a DRG cell following successive applications of Mel (10 μM, 5 μM, 1 μM) showed dose-dependent increase in evoked current. (**b**) Plot of mean inward current amplitude evoked by different doses of melittin (2.5 μM, 5 μM, 10 μM) in DRG cells ($n = 17, 15, 7$, respectively). $^{*\#}P < 0.05$ compared to lowest dose (Kruskal–Wallis ANOVA on ranks with Dunn's post test). For this experiment each cell was exposed to only one dose. (**c**) Sample trace of a current recording from a DRG cell following three repeated applications of 2 μM Mel (Mel1, Mel2, and Mel3). There was a clear sensitization of the Mel-evoked inward current following repeated applications. (**d**) Average responses to successive applications of 2 μM Mel, normalized to the amplitude of the first response. $^{**\#\#}P < 0.01$ vs. Mel1 (Wilcoxon signed rank test with Bonferroni correction, $n = 20$). Cells with no response to the first application are excluded from the analysis. (**e**) Co-application of capsazepine (CPZ, 5 μM), a TRPV1 antagonist, resulted in a significant blockade of the 2.0 μM Mel-evoked inward currents from a holding potential of −70 mV ($n = 5$). (**f**) Statistical analysis of inhibitory effects of CPZ on the Mel-induced inward current. $^{**}P < 0.01$, Mel + CPZ vs. Mel (first application) (Student's paired *t*-test). (**g**) Repeated application of Mel-evoked rises in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) measured by calcium imaging. (**h**) Blocking effects of CPZ on melittin-induced rise in $[Ca^{2+}]i$ measured by calcium imaging. (**i**) Statistical analysis of inhibitory effects of CPZ on the Mel-induced rises in $[Ca^{2+}]i$. $^{**}P < 0.01$, Mel + CPZ vs. Mel (paired *t*-test). (**j**) A proposed scheme showing direct action of Mel on the DRG cells where it activates and sensitizes TRPV1 by PLA2-LOX/COX metabolites (**a**–**i** merged from Figs. 2 and 3 of Du et al. Melittin activates TRPV1 receptors in primary nociceptive sensory neurons via the phospholipase A2 cascade pathways. Biochem Biophys Res Commun 2011; 408:32–37. With permission from Elsevier)

Activation and Sensitization of Spinal Dorsal Horn Neurons

The dorsal horn of the spinal cord is structurally and functionally involved as the first synaptic relay in the mediation of nociceptive information from the periphery to the CNS (McMahon and Koltzenburg 2006; Chen et al. 2013). To see how the spinal dorsal horn neurons respond to s.c. bee venom and melittin injection is of particular importance for understanding of the spinal mechanisms of the bee venom-induced spontaneous pain and hypersensitivity.

Spatiotemporal Properties of Spinal Dorsal Horn Neural Activities

Neural activity biomarker c-Fos, an immediate early proto-oncogene protein, has been widely used for the study of spinal dorsal horn neural activities in response to painful stimuli (Luo et al. 1998; Chen and Lariviere 2010). It has been clearly shown that neuronal activities associated with pain in the spinal dorsal horn can be localized mainly in the superficial layers (laminae I–II) and the deep layers (laminae IV–VI) (Chen et al. 2013). The superficial and deep layers contain pain-related neurons that receive input from primary nociceptive afferents (Chen et al. 2013). Thus, localization of c-Fos-like immunoreactivity can reflect spatiotemporal characteristics of the dorsal horn functional state. Briefly, 30 min after bee venom injection into the ipsilateral hind paw, expression of c-Fos protein became localized within laminae I–II of lumbar spinal cord. The spatial range of neuronal activities extended parallel to the number increase of c-Fos-positive neurons in both superficial and deep layers at 1 h and reached peak level at 2 h after bee venom injection (Luo et al. 1998). The c-Fos-positive neurons began to decline in number in both superficial and deep layers at 4 h and completely disappeared at 96 h after bee venom injection (Luo et al. 1998). In comparison with the time course of the bee venom-induced behavioral nociceptive responses and hyperalgesia, it is likely that c-Fos expression reflects establishment of a sensitized state at the spinal dorsal horn which requires at least 30 min of persistent primary afferent input to the central site. The sensitized state of the spinal dorsal horn is responsible for the development and maintenance of pain hypersensitivity that disappears with the disappearance of spinal c-Fos expression (Luo et al. 1998; Chen et al. 1999b; Chen and Chen 2000).

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases found in a variety of cells, transducing a broad range of extracellular stimuli into diverse intracellular responses by producing changes in transcriptional modulations of key genes as well as posttranslational modifications of target proteins. The roles of three MAPK family members, extracellular signal-regulated kinases (ERKs), p38 MAPK, and c-Jun N-terminal kinase (JNK), in mediation of the bee venom and melittin-induced neural activities and pain-related behaviors have been well studied (Yu and Chen 2005; Cao et al. 2007; Guo et al. 2007; Cui et al. 2008; Hao et al. 2008; Liu et al. 2007, 2011; Li et al. 2008; Yu et al. 2009). Temporal and spatial features of activated ERK and p38 MAPK in the spinal dorsal horn in response to s.c. bee venom injection were examined using immunocytochemical staining in rats (Cui et al. 2008). Subcutaneous injection of bee venom resulted in very quick phosphorylation of ERKs in superficial layer neuronal cell bodies within

2 min that gradually declined within 1 day; however, the phosphorylation of p38 MAPK in the superficial layer neurons began 1 h later than ERKs, reached peak at about 2 h, and was maintained active until the end of 7 days of observation. There were very few ERK- and p38-labeled neurons observed in the deep layers of the dorsal horn, suggesting that only neurons in the superficial layers use these two types of MAPKs in response to bee venom injection. A dramatic phenomenon was that phosphorylated p38 MAPKs began to be localized in microglia across laminae III–IV 1 day after bee venom challenge and reached peak on the 3rd day when bee venom-induced pain hypersensitivity almost disappeared in behavioral observations, implicating that appearance of microglia with p38 MAPK phosphorylation in the dorsal horn is not likely to be involved in the maintenance of pain and hyperalgesia, instead, probably as a scavenger to remove "dead" cells and repair injury. Throughout the observation period, there was neither ERKs nor p38 MAPK detected in astrocytic cells, suggesting that astrocytes may use other protein kinases in the dorsal horn in response to peripheral injury. Actually, there are region-related differences in distribution between different isoforms of a given subtype of MAPKs along the somatosensory system (Guo et al. 2007; Liu et al. 2007). For example, under normal state ERK1 and JNK54 were highly expressed in the spinal dorsal horn with very low level of ERK2 and JNK46 signal; however, in the primary somatosensory cortex (S1 area) or the hippocampal formation, ERK2 and JNK46 were highly expressed. However, under the bee venom-induced inflammatory pain state, ERK2 phosphorylation and JNK46 phosphorylation were distinctly increased in the dorsal horn, suggesting that spinal ERK1 and JNK54 are constitutive isoforms, whereas ERK2 and JNK46 are inducible by peripheral nociception.

In summary, s.c. injection of bee venom indeed induces long-term activation of spinal dorsal horn neural activities that expands from superficial layers to the deep layers in a synchronized way. As a response to the peripheral ongoing input induced by bee venom, ERKs are quickly phosphorylated and peaked at 2 min in the superficial neurons, followed by appearance of p38 MAPK with peak timing at 2 h in the superficial neurons as well. In contrast, the activation of microglia is much later than the activation of neurons, and phosphorylation of microglial p38 MAPK peaks on the 3rd day after bee venom injection. The modulation of the bee venom-induced nociceptive processes at the spinal dorsal horn is likely more complex in nature than previously thought and needs to be systematically studied further.

In Vivo Electrophysiological Recordings of Spinal Dorsal Horn Neuronal Activities

There are at least three classes of neurons, low-threshold mechanoreceptive (LTM) neurons, wide-dynamic-range (WDR) neurons, and nociceptive-specific (NS) neurons in the spinal dorsal horn serving as transducer, encoder, and probably filter of various somatosensory information (Chen et al. 2013). The functional classification of dorsal horn neurons is based upon their neuronal response characteristics to natural mechanical stimuli applied to their cutaneous receptive fields (cRF). LTM neurons are driven mostly by innocuous stimulation and located mainly in laminae III–IV; WDR neurons are driven by noxious as well as non-noxious

stimulation and located mainly within lamina V; NS neurons are driven only by noxious stimulation and located mainly in lamina I (Chen et al. 2013). Among the three classes of dorsal horn neurons, there are reliable lines of evidence showing that the majority of WDR neurons, but not NS neurons, are intercalated in the circuitry responsible for the nociceptive withdrawal reflex (Chen et al. 2013). Thus, spinal WDR neurons may play very important roles in the mediation of nociceptive responses and hypersensitivity observed in pain-related behaviors following s.c. bee venom injection. The first electrophysiological recording of the response to bee venom injection was made by Chen and his colleagues on the WDR neurons located mainly within laminae IV–VI of the spinal dorsal horn in anesthetized cats (Chen et al. 1998, 1999a). Similar to what was observed in the behavioral assays, s.c. injection of bee venom into the center of the cRF resulted in an immediate sustained increase in neuronal firing that reached peak within 5 s and declined until the end of the 60-min observation period. The bee venom-induced spike discharges of WDR neurons could be suppressed by systemic morphine and lidocaine locally applied onto the sciatic nerve that resulted in blockade of primary afferent input. In these studies, responsiveness of spinal WDR neurons to both non-nociceptive and nociceptive mechanical stimuli was also significantly enhanced 1–2 h after bee venom injection, suggesting that spinal WDR neurons that mediate and encode spinal nociceptive reflex are sensitized by peripheral bee venom injection. Because the response pattern and time course of spinal WDR neuronal activities are quite similar to those of behavioral manifestation induced by s.c. bee venom injection and because blockade of altered spinal neuronal activities results in parallel disappearance of the bee venom-induced pain-related behaviors (see Chen and Lariviere 2010), it is rational to believe that the sensitized dorsal horn WDR neurons play a key role in the mediation of paw withdrawal reflex facilitation, displayed as persistent paw flinches and hyperalgesia. Recordings made of the same class of neurons in the anesthetized rats show similar altered neuronal responses in the dorsal horn in response to bee venom treatment (You and Chen 1999; Zheng et al. 2002, 2004).

In an excellent electrophysiological study, it was found that injection of either bee venom or melittin into the cRF of spinal WDR neurons resulted in a dose-dependent increase in spontaneous spike discharges in terms of both frequency and duration in anesthetized rats (Li and Chen 2004). The melittin-induced spike firing of spinal dorsal horn WDR neurons can be completely blocked by local peripheral injection of CPZ into the ipsilateral cRF, but not the contralateral paw, suggesting that melittin has local nociceptive effects rather than systemic effects (Fig. 6). Similar to the bee venom effects, local peripheral injection of melittin into the same WDR neuronal cRF resulted in increase in responsiveness to both thermal and mechanical stimuli compared to the baseline controls (Fig. 7a, b). This functionally sensitized state of the spinal nociceptive neurons following melittin injection can be reflected by a clear leftward shift of the functional curves of the stimulus intensity−responsiveness relationship for both thermal and mechanical hypersensitivities (Fig. 7c, d).

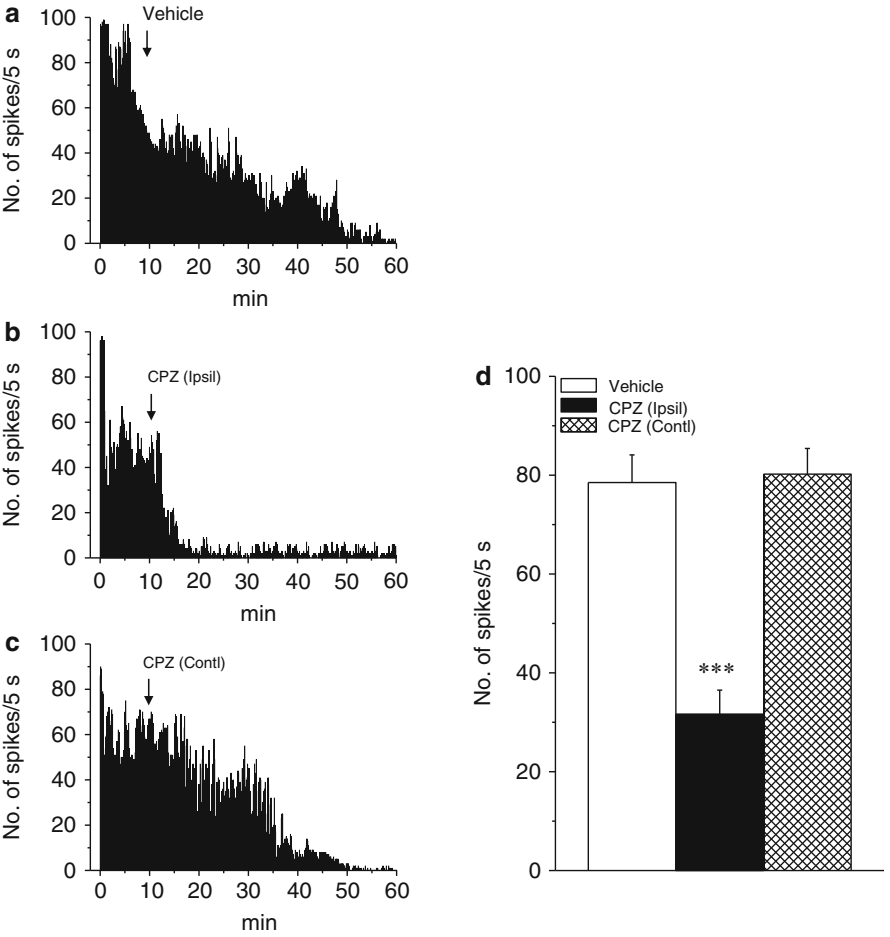


Fig. 6 Blocking effect by local peripheral TRPV1 antagonism on the spike responses of spinal dorsal horn pain-signaling neurons to subcutaneous injection of melittin in anesthetized rat. (**a**) The melittin-induced sustained period of spike responses of a spinal dorsal horn wide-dynamic-range (*WDR*) neuron cannot be influenced by local peripheral application of saline (vehicle). (**b**) Local peripheral application of capsazepine (0.3 mg CPZ in 50 μl 50% dimethysulphoxide), a selective TRPV1 antagonist, into the melittin injection site (Ipsil, ipsilateral) resulted in a dramatic reduction in spike responses of a spinal WDR neuron. (**c**) Contralateral (Contl) administration of CPZ had no effect on the melittin-induced spike firing of a spinal WDR neuron. (**d**) Averaged blocking effects of CPZ on the melittin-induced spike discharges of spinal WDR neurons. $^{***}P < 0.001$ CPZ (Ipsil) vs. vehicle or CPZ (Contl) ($n = 11$ for each group). *Arrows* indicate the timing for injection of CPZ or vehicle 10 min after subcutaneous injection of melittin (Partially from the original data of Li and Chen. Altered pain-related behaviors and spinal neuronal responses produced by s.c. injection of melittin in rats. Neuroscience 2004; 126:753–762. With permission from Elsevier)

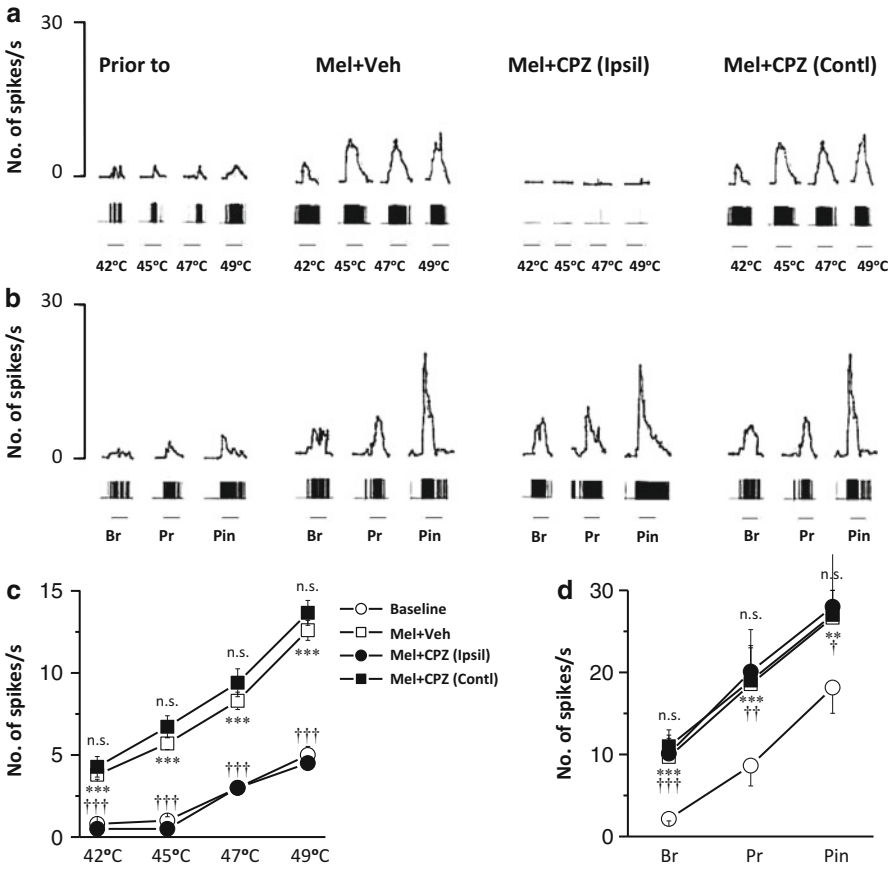


Fig. 7 Blocking effect by local peripheral TRPV1 antagonism on the melittin (Mel)-induced thermal hypersensitivity of spinal dorsal horn pain-signaling neurons in anesthetized rat. (**a**) Subcutaneous Mel injection enhanced the responsiveness of a spinal WDR neuron to suprathreshold nociceptive thermal stimuli (>42 °C) applied to its cutaneous receptive field (cRF). Local peripheral application of CPZ (0.3 mg in 50 μl 50% dimethysulphoxide) into the Mel injection site [Mel + CPZ (Ipsil)] resulted in a dramatic inhibition of spinal WDR neuronal responsiveness to thermal stimuli, whereas contralateral administration of CPZ [Mel + CPZ (Contl)] had no effect. (**b**) Local application of CPZ did not influence the Mel-induced mechanical hypersensitivity of a spinal WDR neuron regardless of either route of administrations. (**c**) Intensity-responsiveness functional curves of spinal WDR neurons plotted from rats ($n = 11$ for each group). $^{\dagger}P < 0.05$; $^{***\dagger\dagger\dagger}P < 0.01$; $^{******\dagger\dagger\dagger}P < 0.001$ Mel + Vel vs. Baseline; Mel + CPZ (Ipsil) vs. Mel + Vel or Mel + CPZ (Contl). *Br* brush, *Pr* pressure, *pin* pinch, *n.s.* no significance (Partially from the original data of Li and Chen. Altered pain-related behaviors and spinal neuronal responses produced by s.c. injection of melittin in rats. Neuroscience 2004; 126:753–762. With permission from Elsevier)

Intriguingly, the leftward shift of the thermal functional curves was completely reversed by local peripheral injection of CPZ into the ipsilateral cRF of spinal WDR neurons, whereas that of the mechanical functional curves was not affected by the same treatment, strongly suggesting existence of different underlying

mechanisms between thermal and mechanical hypersensitivities (Fig. 7a–d). To demonstrate the roles of spinal dorsal horn WDR neurons in the mediation of bee venom-induced withdrawal reflex facilitation, simultaneous pairwise recordings were made in both WDR neurons and single motor units (SMU) along the spinally organized nociceptive reflex circuitry (You et al. 2003a). The result showed an immediate parallel increase in spike discharges of the paired WDR neuron and SMU that lasted for 1 h in response to injection of bee venom in their common cRFs (Chen 2003; You and Arendt-Nielsen 2005). These paired recordings also showed a parallel enhancement of mechanically nociceptive responsiveness as well as wind-up responses 1–2 h after bee venom treatment (Chen 2003; Chen and Lariviere 2010). These data reliably demonstrate the neuronal basis underlying thermal and mechanical hypersensitivity observed in behavioral studies. In another study, responses of lamina VII multi-receptive nociceptive neurons were studied in anesthetized, spinalized rats (You et al. 2008). In contrast to the long-term response patterns of dorsal horn WDR neurons, s.c. bee venom injection evoked a short-term (<10 min) firing of this population of neurons. However, the neurons were switched to give a long-term biphasic firing with an early phase (4–13 min) that was followed by a late tonic firing phase (28–74 min) after a quiescent period (4–11 min) in the spinalized rats. The data is interesting and suggests the existence of two separate modulatory systems in the spinal dorsal horn. Spinal WDR neurons that serve as encoders of spinal nociceptive withdrawal reflex are not likely tonically controlled by descending antinociceptive systems, while the deeper-layer nociceptive neurons that send nociceptive information along the spinoreticulothalamic tract to the medial thalamus are likely tonically controlled by a descending antinociceptive system. The response characteristic of lamina I NS neurons to s.c. bee venom injection remains uninvestigated, and thus the precise role of NS neurons needs to be determined.

In summary, in vivo electrophysiological recordings of spinal dorsal horn neurons in both cats and rats reveal similar results of a centrally sensitized state following s.c. bee venom or melittin injection which is responsible for animal paw withdrawal reflex facilitation and altered pain sensation such as hyperalgesia and allodynia.

Spinal Release of Excitatory and Inhibitory Amino Acids in Response to Bee Venom Injection

Given that melittin and other algogenic components of bee venom are able to activate nociceptors upon diffusion to peripheral free nerve endings when injected subcutaneously, pain "signals" (APs) would be generated and conducted anterogradely to the central terminals of primary nociceptive neurons, leading to release of neurotransmitters at the dorsal horn of the spinal cord (Chen et al. 2013). This hypothesis was tested by measuring the release of both excitatory amino acids (EAAs) and inhibitory amino acids (IAAs) and other amino acids involved in recycling of metabolites of those neurotransmitters (Yan et al. 2009). It is well known that glutamate and aspartate are important excitatory neurotransmitters used by the primary afferent to affect the spinal dorsal horn in addition to neuropeptides such as substance P and CGRP (Chen and Lariviere 2010; Chen et al. 2013). Correspondingly, glycine and γ-aminobutyric acid (GABA) are important inhibitory neurotransmitters used by local inhibitory

interneurons in the dorsal horn (Chen and Lariviere 2010; Chen et al. 2013). Thus, simultaneous monitoring of both EAAs and IAAs at the spinal level in awake animals experiencing painful stimulation is of particular importance for the understanding of the basic neurotransmitters mediating long-lasting pain.

Cerebrospinal fluid (CSF) samples were collected by microdialysis in conscious rats every 20 min for a period of 120 min when spontaneous nociceptive responses were induced by s.c. bee venom injection (Yan et al. 2009). At least nine amino acids including excitatory (e.g., glutamate, aspartate), inhibitory (glycine, GABA, taurine), and other metabolites were analyzed by high-pressure liquid chromatography. The results showed an immediate increase in the concentration of all the nine amino acids at 20 min; however, in the remaining period of 100 min, EAAs remained above the baseline levels, while IAAs soon decreased to the baseline levels and thereafter to a level much lower than the baseline for the remaining time. The bee venom-induced imbalance between EAAs and IAAs at the spinal cord could be reversed by pre-blockade with bupivacaine at the injection site during which the bee venom-induced pain-related behaviors disappeared completely. These results suggest that the imbalance between EAAs and IAAs at the spinal cord may be associated with the maintenance of persistent pain-related behaviors induced by bee venom (Yan et al. 2009). These results directly support the idea that pain-related behaviors are caused by persistent activation of postsynaptic pain-related neurons driven by ongoing activation of primary nociceptors that use glutamate and aspartate as neurotransmitters in the central terminals projecting to the dorsal horn of the spinal cord (Yan et al. 2009). The results that intrathecal pre- and post-blockade of glutamate NMDA and non-NMDA receptors can inhibit bee venom-induced spontaneous pain-related behaviors provide more strong supporting evidence for the roles of EAAs in mediation of the bee venom-induced pain (You et al. 2002, 2003b; Yan et al. 2009).

Cortical Activation and Synaptic Metaplasticity in "Pain Matrix" Induced by Bee Venom Injection

There are tremendous amounts of brain imaging studies in both human and animal subjects supporting that pain is a complex experience consisting of sensory-discriminative, affective-motivational, and cognitive-evaluative dimensions (McMahon and Koltzenburg 2006; Liu and Chen 2009, 2014; Zhao et al. 2009; Chen et al. 2013). Now it has been gradually known that noxious information caused by tissue or nerve damage is processed by a widely distributed, hierarchically interconnected neural network, referred to as neuromatrix, in the brain ("pain matrix") (Melzack 2005). To study the effects of s.c. bee venom on the cortical responses, three areas, including the primary somatosensory cortex (S1), the anterior cingulate cortex (ACC), and the hippocampal formation, have been investigated using multiple experimental approaches (Guo et al. 2007; Liu et al. 2007, 2011, 2012; Chang et al. 2008; Ren et al. 2008; Zhao et al. 2009; Gong et al. 2010; Lyu et al. 2013; Lu et al. 2014; for details, see review by Liu and Chen 2014).

The Primary Somatosensory Cortex

The S1 area is believed to be involved in perceiving pain sensation at the cortical level (McMahon and Koltzenburg 2006; Chen et al. 2013). To investigate how the S1 area responds to bee venom injection spatiotemporally, c-Fos immunoreactive neuronal activities in the hindlimb representative of the S1 area were observed 1 h, 2 h, 3 h, and 4 h after s.c. bee venom injection into one hind paw (Chang et al. 2008). Compared to naïve and saline-treated rats, c-Fos-labeled neurons became densely increased in superficial layers (II–III), but less increased in deep layers (IV–VI), following s.c. bee venom injection. The mean number of c-Fos-positive neurons double labeled with selective neuronal biomarker NeuN began to increase at 1 h and reached peak at 2 h within the layers II–III after bee venom treatment that was followed by gradual decrease afterward. The time course of c-Fos expression in the layers IV–VI was in parallel with that of the superficial layers, but with a much lower density and magnitude. This result demonstrates that bee venom in the periphery can evoke increased neuronal activities in the S1 area with predominant localization in layers II–III.

In other two studies, subtypes of two isoforms of MAPKs including ERK1/ERK2 and JNK46/JNK54 were also examined following s.c. bee venom injection (Guo et al. 2007; Liu et al. 2007). In the S1 area of the cortex, ERK2 was expressed more abundantly than ERK1, while there was a larger amount of ERK1 than ERK2 in the spinal cord, suggesting existence of a region-specific expression of different subtypes of ERKs along the pain pathway. Moreover, phosphorylated ERK2 (pERK2), not phosphorylated ERK1 (pERK1), was normally expressed with a high level in the S1 area, but neither pERK1 nor pERK2 was detectable in normal spinal cord, suggesting that under normal condition the S1 cortices use pERK2 in processing non-nociceptive information from dorsal column–medial lemniscus system. However, under condition following bee venom injection, ERK1 was more remarkably phosphorylated than ERK2 in the S1 area, while pERK2 exhibited stronger response than pERK1 in the spinal cord, implicating that the S1 uses pERK2 while the spinal dorsal horn uses pERK1 as a mechanism in processing nociceptive information from the dorsal horn–spinothalamocortical tract (Guo et al. 2007). Furthermore, pJNK46 was shown to be normally expressed in the S1 area, but not in the spinal cord, while neither of the two structures contained pJNK54 (Liu et al. 2007). Subcutaneous bee venom resulted in a significant increase in the phosphorylation of both JNK isoforms in the S1 area for a long period (lasting at least 48 h). Nevertheless, JNK46 exhibited a much higher activation than JNK54 in the spinal cord, whereas the same noxious stimulation elicited evident activation of JNK54 in the S1 area, leaving JNK46 less affected (Liu et al. 2007).

Multielectrode array (MEA) recordings in vivo enable experimenters to identify ensemble WDR and NS neurons at the S1 area in freely moving rats (Wang et al. 2011; Chen's unpublished data). Subcutaneous injection of bee venom into the cRFs of both WDR and NS neurons resulted in a parallel increase in spike discharges and pain-related behaviors lasting for at least 1 h in a similar time course pattern to the spinal dorsal horn responses (Chen and Lariviere 2010; Liu and Chen 2014).

Moreover, slice recordings using MEA (8×8 channels) probe and patch-clamp technique in vitro enable experimenters to evaluate the effects of s.c. bee venom on the synaptic plasticity at the cortical level (Zhao et al. 2009; Liu et al. 2011, 2012; Lyu et al. 2013; Lu et al. 2014; for review see Liu and Chen 2014). The results showed that s.c. bee venom caused a dramatic switch in synaptic efficacy from the LTP-resistant state to the LTP-inducible state (Chen's unpublished data), implicating a great impact of s.c. bee venom on the neural plasticity in the S1 area.

The Anterior Cingulate Cortex

The medial prefrontal cortex including the prelimbic, sublimbic, and the ACC is believed to be a critical brain structure involved in mediation of direct pain-related emotion and vicarious empathy for pain (Ren et al. 2008; Li et al. 2014; Liu and Chen 2014). The synaptic mechanisms of the ACC underlying different types of pain have been well studied in several animal models of pain (Liu and Chen 2014). Briefly, s.c. bee venom injection resulted in dramatic spatial enlargement of synaptic connections as well as leftward shift of input–output (I-O) functional curves of synaptic transmission in comparison with naïve and saline controls (Lu et al. 2014); however, the induction of LTP could not be altered in the same experimental preparation (Lu et al. 2014). Patch-clamp recordings of the slices containing the ACC harvested 2 hours after s.c. bee venom injection also showed a hyperexcitable state under which enhanced firing rate can be seen (Gong et al. 2010). The bee venom-induced hyperexcitable state of the ACC neurons is largely due to disinhibition of tonic control of excitatory synaptic transmission by inhibitory synaptic input, because enhanced excitatory postsynaptic currents (EPSCs) and reduced inhibitory postsynaptic currents (IPSCs) could be seen following s.c. bee venom injection, implicating that the balance between the excitatory and inhibitory synaptic functions is disrupted by s.c. bee venom injection (Gong et al. 2010).

The Hippocampal Formation

The hippocampal formation, traditionally believed to be a key brain structure in learning and memory, has also been demonstrated to be involved in processing of pain information (Liu and Chen 2009; 2014). Similar to the S1 area, in the hippocampus (Guo et al. 2007), (1) there was a larger amount of ERK2 than ERK1 in naïve state; (2) pERK2, not pERK1, was normally expressed with a high level in the hippocampus; (3) following bee venom injection, however, ERK1 was more remarkably activated than ERK2 in the hippocampus. These results implicate that, similar to the S1 area, the hippocampus uses ERK2 to process information under the naïve condition; however, it uses ERK1 to process nociceptive information under the peripheral inflammatory pain state.

Using 8×8 MEA recordings of brain slices containing the hippocampal formation, entorhinal–dentate gyrus (DG)/CA1 synaptic responses can be observed following electrical stimulation of the perforant path (PP) through which direct axonal projections from the entorhinal cortical (EC) neurons are sent to the DG or the CA1 (Zhao et al. 2009). The EC-DG/CA1 pathways are believed to be major projections mediating information from other cortical areas to the hippocampus via relays in the

EC (Liu and Chen 2009, 2014). Following s.c. bee venom injection, there were great changes in both synaptic connections spatially and synaptic efficacy temporally. For example, the EC-DG/CA1 synaptic connections were significantly increased in number when MEA recordings were made on the hippocampal slices from rats with bee venom injection (Zhao et al. 2009). Meanwhile, LTP, an electrical phenomenon reflecting temporal synaptic plasticity (enhancement of synaptic efficacy) induced by theta-burst stimulation of the PP, can also be significantly enhanced by preconditioning with s.c. bee venom injection (Zhao et al. 2009). This suggests that metaplasticity, a concept coined by Abraham and Bear in 1996 indicating a plasticity of synaptic plasticity (Abraham and Bear 1996; Abraham 2008), occurs in the hippocampus of rats following preconditioning with bee venom-induced peripheral inflammatory pain.

Pharmacological inhibition of activations of either ERK by U0126 or of JNK by SP600125 significantly reduced the bee venom-enhanced LTP in both EC-DG and EC-CA1 synaptic transmissions, while bath application of the p38 MAPK inhibitor SB239063 resulted in a further increase in the LTP magnitude in the same samples (Liu et al. 2011). In a similar pattern, the bee venom-enhanced LTP could be reversed by antagonism of metabotropic glutamate receptor 5 (mGluR5) with 2-methyl-6-(phenylethynyl)-pyridine; however, the bee venom-enhanced LTP could be further augmented by antagonism of mGluR1 by a selective antagonist 7-hydroxyiminocyclopropan [b] chromen-1α-carboxylic acid ethyl ester perfusion (Liu et al. 2012). These data implicate that mGluR5 and ERK/JNK signaling link is likely to be involved in facilitating the hippocampal metaplasticity (enhancement of LTP) induced by bee venom injection, whereas mGluR1 and p38 MAPK signaling link is likely to be involved in counteracting the processes of hippocampal metaplasticity. More interestingly, s.c. injection of bee venom resulted in sustained (>8 h) phosphorylation of mammalian target of rapamycin (mTOR) and its downstream target p70 S6 kinase (S6K) in the hippocampus (Lyu et al. 2013). The bee venom-induced metaplasticity (enhanced LTP) and the increased phosphorylation of mTOR-S6K signaling pathway in the hippocampus were able to be suppressed by systemic administration of rapamycin, an mTOR inhibitor (Lyu et al. 2013). These data suggest that the bee venom-induced mTOR activation is involved in hippocampal metaplasticity that may affect behaviors of rats. As a supporting line of evidence, rats injected with bee venom exhibited markedly reduced ambulation and exploratory activity in the open field due to over self-caring and favoring behaviors in a corner (signs of anxiety). Systemic administration of rapamycin could completely reverse the bee venom-induced anxiety-like behaviors (Lyu et al. 2013).

Since spatial reorganization of synaptic connections is likely a consequence of metaplasticity, it can be deduced that pharmacological modulation of metaplasticity would result in a parallel reversal of the bee venom-induced spatial enlargement of synaptic connections in the hippocampus. As lines of supporting evidence, antagonisms or blockades of mGluR5, ERK/JNK, and mTOR-S6K signaling links result in the reversal of the bee venom-induced spatial synaptic reorganization; however, antagonism or blockade of mGluR1 and p38 MAPK signaling link results in enhancing effects of the bee venom-induced spatial synaptic reorganization.

In summary, peripheral inflammatory pain state induced by s.c. bee venom can cause a form of the LTP-enhanced metaplasticity in the hippocampal formation that may result in spatial reorganization of synaptic connections at network level, leading to anxiety-like behaviors seen in the bee venom test. Moreover, there exists an inter-counteracting system required to maintain the balance or homeostasis of neural network in the hippocampal formation that can be interrupted by peripheral persistent pain. The signaling link between mGluR1 and p38 MAPK may exist as a counteracting factor against the LTP-enhanced form of metaplasticity; however, the signaling links between mGluR5 and ERK/JNK and mTOR-S6K may act as facilitating factors mediating the LTP-enhanced form of metaplasticity.

Proposed Mechanisms of Bee Venom-Induced Pain

Peripheral Mechanisms

The underlying peripheral mechanisms of bee venom-induced nociception and pain hypersensitivity have been proposed according to many experimental data (for review see Chen and Lariviere 2010). In general, two sets of mechanisms are likely to be involved: (1) direct actions of the bee venomous pain-producing constituents on the primary nociceptors and (2) indirect actions of the bee venomous constituents on the primary nociceptors through algogens and proinflammatory and inflammatory mediators released from mast-cell degranulation, tissue damage, and immune responses. As a consequence, pain-producing substances directly activate cation channel nociceptors leading to pain sensation, while pain-enhancing substances regulate or modify cation channel nociceptors via intracellular cascades leading to hyperalgesia and/or allodynia (pain hypersensitivity).

As shown in Fig. 8, following s.c. injection of bee venom, melittin, a major pain-producing substance, activates TRPV1 receptors by hydroxyeicosatetraenoic acids (HETEs), endogenous ligands of TRPV1 receptor, through catalyzing phospholipids by PLA2 and then AA by LOX that finally produce 5-, 12-, or 15-HETEs, leading to depolarization of primary nociceptor cells (Du et al. 2011). Meanwhile, melittin, MCD peptide, bee venom PLA2, and hyaluronidase may cause tissue damage, leading to releases of adenosine triphosphate (ATP) and photon (H^+) that may activate P2-purinoceptor X3 (P2X3) and P2Y, TRPV1, and acid-sensing ionic channel (ASIC) (Chen and Lariviere 2010). Indirect actions of melittin, MCD peptide, and bee venom PLA2 cause degranulation of mast cells, leading to releases of histamine, bradykinin (BK), and 5-hydroxytryptamine (5-HT) that may activate H1 receptor, 5-HT3 receptor, and BK1/2 receptors. Because TRPV1, P2X3, 5-HT3, and ASIC are selective or nonselective cation channels and opening of these cation channels may lead to depolarization of primary nociceptor cells, the activators of these cation channels such as melittin, ATP, 5-HT, and H^+ are believed to be pain-producing substances which are responsible for the production of spontaneous pain sensation in humans and pain-related behaviors in animals (Chen and Lariviere 2010). Melittin can also cause production of pain-enhancing substances such as

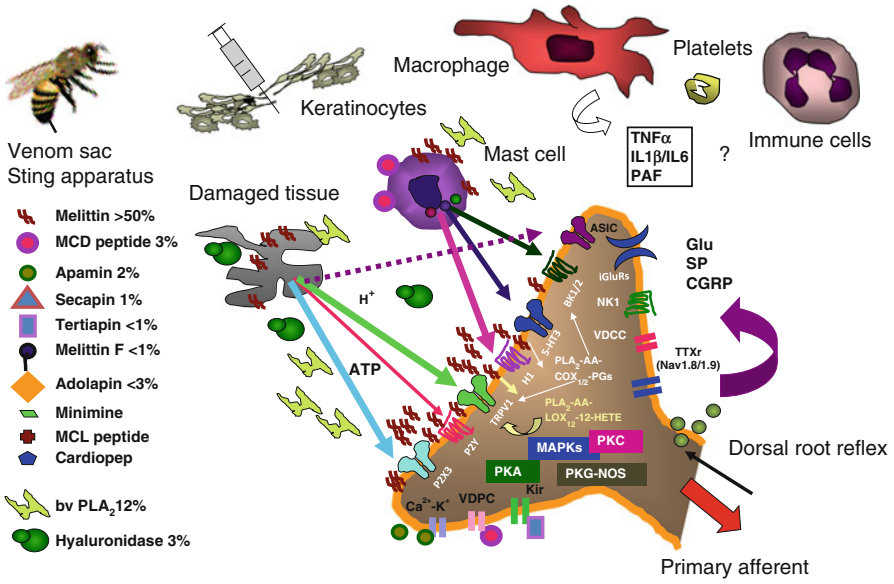


Fig. 8 A schematic drawing of proposed underlying mechanisms of the bee venom-induced peripheral neural plasticity (activation and sensitization). On the left column, venom sac and sting apparatus is shown at the tip of a honeybee abdomen. The major polypeptides and enzymes of the bee venom are listed below (also see Table 1). Subcutaneous injection of bee venom by a syringe is shown on the *left top*, while a nerve terminal of primary afferent is shown on the *bottom right*. The direct and indirect actions of each ingredients of the bee venom are proposed. Color symbols representing each ingredient of the bee venom (*left*) can be clearly seen on the *right* where melittin (*dark red double strand*), MCD peptide (*red-colored circle*), apamin (*green-colored circle*), and tertiapin (*light blue rectangle*) bind directly to the membrane of a nociceptor cell leading to the activation of it. Meanwhile, melittin, MCD peptide, bv PLA2 (*light green* "H"), and hyaluronidase (*dark green double balls*) cause tissue damage (*gray*) and release of ATP and H+ that activate P2X3 (*thick blue arrow* and paired channel)/P2Y (*thin red arrow* and paired channel), TRPV1 (*green arrow* and paired channel), and ASIC (*purple dashed arrow* and paired channel). Indirect actions of melittin, MCD peptide, and bv PLA2 cause degranulation of mast cells (*purple*) and release histamine, BK, and 5-HT that activate H1 receptor (*pink thick arrow* and paired receptor), 5-HT3 receptor (*blue arrow* and paired receptor), and BK1/2 receptors (*dark green arrow* and paired receptor). The firing of nociceptor terminals will be mediated by voltage-dependent sodium channels (TTXr Nav1.8/1.9), VDCC, VDPC, Kir, and Ca2 + −K+. Dorsal root reflex and axon reflex may cause the release of glutamate and neuropeptides (SP and CGRP) that further activate their autoreceptors on the nociceptor terminals or blood vessels causing inflammatory extravasation (neurogenic) with infiltration of macrophage, immune cells and platelets, and many cytokines (TNF-α, IL1β, PAF, etc.). The *syringe* indicates transcutaneous injection of bee venom. Abbreviations: *5-HT3* 5-hydroxytryptamine receptor 3, *12-HETE* 12-hydroxyeicosatetraenoic acids; *AA* arachidonic acid, *ASIC* acid-sensing ionic channel, *ATP* adenosine triphosphate, *BK1/2* bradykinin receptors 1/2, *bv PLA2* bee venom phospholipase A2, $Ca^{2+}-K^{+}$ calcium-dependent potassium channel, *CGRP* calcitonin-gene-related peptide, *COX-1/COX-2* cyclooxygenases 1/2, *Glu* glutamate, *H1* histamine receptor type 1, *iGluRs* ionotropic glutamate receptors, *IL1b* interleukin 1b, *IL6* interleukin 6, *Kir* inward-rectifier potassium channel, *LOXs* lipoxygenases, *MAPKs* mitogen-activated protein kinases, *MCD peptide* mast-cell degranulating peptide, *MCL peptide* mastocytolytic peptide, *NK1* neurokinin 1, *NOS* nitric oxide synthase, *P2X3* P2-purinoreceptor X3, *P2Y* P2-purinoreceptor Y, *PAF* platelet-activated factor, *PGs* prostaglandins, *PKA* protein

prostaglandin E2 through catalyzing phospholipids by PLA2 and then AA by COX that finally produce various prostanoids, leading to sensitization of primary nociceptors via sensitizing TRPV1 receptors (Chen and Lariviere 2010; Du et al. 2011). Other G-protein-coupled receptors (GPCRs) such as P2Y, H1, and BK1/2 are also likely to be involved in the sensitization of primary nociceptors through PLC- or adenylyl cyclase (AC)-signaling pathways (McMahon and Koltzenburg 2006; Chen et al. 2013). Because MCD peptide, apamin, and tertiapin are blockers of VGPC, calcium-dependent potassium channel (Ca^{2+}–K^{+}), and inward-rectifier potassium channel (Kir), respectively, inactivation of VGPC, Ca^{2+}–K^{+}, and Kir channels may facilitate depolarization and increase firing rate of primary nociceptor cells (Chen and Lariviere 2010). Further findings support the important roles of TTX-resistant VGSC subunit (Nav1.8 and Nav1.9) in mediation of the bee venom-induced long-term spontaneous pain-related behaviors and pain hypersensitivity because s.c. injection of melittin can induce sustained upregulation of both Nav1.8 and Nav1.9, but not TTX-sensitive, subunits in the small DRG cells (Yu et al. 2013). However, it is intriguing to see that only antisense-mediated knockdown of Nav1.9, but not Nav1.8, in the DRG cells can reverse the melittin-induced pain hypersensitivity. The roles of Nav1.8 cannot be completely excluded in the bee venom-induced nociception because CFA-induced pain hypersensitivity can be suppressed by antisense-mediated knockdown of Nav1.8, but not Nav1.9, suggesting differential roles of TTX-resistant VGSC subunits in different processes of inflammatory pain (Yu et al. 2011).

Neurogenic inflammation is also proposed to play important roles in mediation of bee venom-induced inflammatory pain processes by releasing glutamate and neuropeptides (SP and CGRP) from the peripheral terminals of primary nociceptor cells due to dorsal root reflex and axon reflex, leading to inflammatory extravasation with infiltration of macrophage, immune cells and platelets, and many cytokines (TNF-α, IL1β, platelet-activated factor, etc.) that exacerbate pain and hypersensitivity (Fig. 8; see Chen and Lariviere 2010; Chen et al. 2013).

Spinal Mechanisms

The spinal dorsal horn is the first relay of synaptic transmission for nociceptive information between primary afferent input and pain-related central neuronal cells (McMahon and Koltzenburg 2006; Chen et al. 2013). The functional state of the spinal dorsal horn has been shown to be activity dependent and is changed by

Fig. 8 (continued) kinase A, *PKC* protein kinase C; *PKG* protein kinase G; *SP* substance P, *TNF-a* tumor-necrosis factor-*a*, *TRPV1* transient receptor potential vanilloid receptor 1, *TTXr* tetrodotoxin resistant, *VDCC* voltage-dependent calcium channel, *VDPC* voltage-dependent potassium channel (From Fig. 3 of Chen and Lariviere. The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword. Prog Neurobiol 2010; 92:151–183. With permission from Elsevier)

peripheral persistent neural sensitization or plasticity induced by bee venom injection (Chen 2003, 2007, 2008; Chen and Lariviere 2010). The spinal neural mechanisms of bee venom-induced pain and hyperalgesia have also been proposed based upon many experimental data (Chen and Lariviere 2010).

As shown in Fig. 9a, the induction and maintenance of bee venom-induced persistent spontaneous pain-related behaviors are dependent upon the functional state of the synaptic connections between the primary nociceptive nerve terminals (presynaptic component) and dorsal horn pain-signaling neuronal cell bodies (postsynaptic component) (Chen 2007, 2008; Chen and Lariviere 2010). Astrocytes and microglial cells can also be activated by long-term impulse barrages from the periphery induced by s.c. bee venom, forming a dynamic tripartite synaptic composition (Fig. 9a–c; Chen et al. 2013). Following s.c. bee venom injection, the levels of EAAs (glutamate and aspartate) and IAAs (glycine and GABA) at the spinal cord are initially elevated, followed by a sustained increase in EAAs release and decrease in IAAs release (Yan et al. 2009). The sustained release of EAAs from primary afferents plays a key role in coactivation of both ligand-gated ionotropic glutamate receptors (iGluRs), including AMPA/KA and NMDA receptors, and, as a consequence, leads to increase in intracellular Ca^{2+} concentration (You et al. 2003b; Yan et al. 2009). On the other hand, release of EAAs and SP also activates mGluR group I and neurokinin 1 (NK1) receptors, but not mGluR groups II and III (Zheng and Chen 2001; Yan et al. 2009). This transsynaptic signal transduction via G-protein-mediated signaling results in the phosphorylation of various MAPKs (e.g., ERK and p38 MAPK) (Yu and Chen 2005; Cao et al. 2007; Cui et al. 2008; Li et al. 2008) and protein kinases (PKC, PKA, and PKG) (Li et al. 2000; Li and Chen 2003; Chen 2007, 2008; Chen and Lariviere 2010) as well as enzymes COX-1/COX-2 (Chen and Lariviere 2010). ATP P2X receptors are activated by extracellular ATP leakage in the spinal dorsal horn in response to s.c. bee venom (Zheng and Chen 2000). Meanwhile, at presynaptic component, TRPV1, P2X3, VGCC, Nav1.8, and Nav1.9 are also likely to be activated or upregulated by tonic persistent primary afferent that in turn facilitate neurotransmitter release. Presynaptic localization of NK1, as autoreceptors of SP released from primary afferent terminals, might be further activated and has been demonstrated to enhance both TRPV1- and TTX-resistant Nav1.8 through PKCε (Chen and Lariviere 2010). However, the ongoing spinal dorsal horn neural activities are peripherally dependent, and the establishment of the centrally sensitized state in the spinal cord has been shown to require a certain minimum time window for the accumulation of peripheral input before dorsal horn sensitization can be established (Chen et al. 1998, 1999a, 2000, 2001; You et al. 2002). The maintenance of this centrally sensitized state is also shown to require the activation of peripheral EAAs receptors, P2X and P2Y receptors, protein kinases, and MAPKs (Chen et al. 1999a, 2008; You et al. 2002; Hao et al. 2008; Lu et al. 2008; Yan et al. 2009; Yu et al. 2009).

The spinal mechanisms underlying the bee venom-induced hyperalgesia and/or allodynia (pain hypersensitivity) are much more complex than spontaneous pain due to its different response properties to different stimulus modalities (chemical, thermal, and mechanical) and its spatial location in relation to the primary injury site

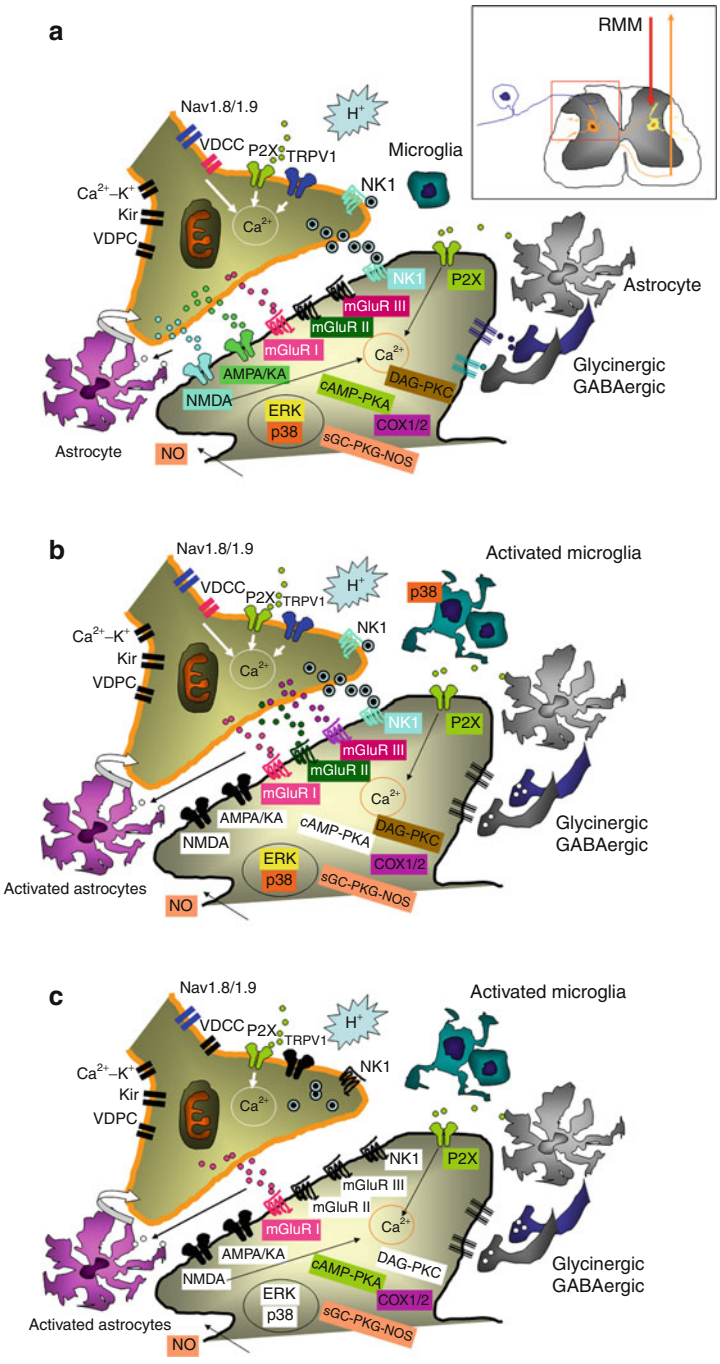


Fig. 9 Schematic drawings of proposed underlying mechanisms of the bee venom (*BV*)-induced sustained spontaneous pain, primary thermal hyperalgesia, and primary mechanical hyperalgesia in

(primary, secondary, and mirror-image hyperalgesia) (Fig. 9b, c; also see Chen and Lariviere 2010).

Briefly, the iGluRs (NMDA and AMPA/KA) in the dorsal horn are not likely to be involved in the bee venom-induced primary thermal hypersensitivity (Fig. 9b; Chen and Chen 2000; Yan et al. 2009). Instead, GPCRs including mGluR groups I, II, and III and NK1 play important roles in the induction and maintenance of the primary thermal hypersensitivity (Zheng and Chen 2001; Yan et al. 2009). Consequently, intracellular DG-PKC, but not cAMP-PKA, is activated (Li et al. 2000; Li and Chen 2003). Meanwhile, ERK, p38 MAPK, soluble guanylyl cyclase (sGC)-PKG-NOS, and COX-1/COX-2 in the spinal cord are also recruited as important factors in the mediation of the primary thermal hypersensitivity (Yu and Chen 2005; Cao et al. 2007; Cui et al. 2008; Chen 2008; Li et al. 2008).

Fig. 9 (continued) the spinal cord dorsal horn. (**a**) Shown is a synaptic structure between a presynaptic component (central terminal of primary afferents, *left*) and a postsynaptic component (membrane of a pain-signaling neuron, *right*). Astrocytes and microglia are not fully activated at this stage. At the synaptic cleft, in response to the coming of tonic firing action potentials, excitatory amino acids (EAAs, glutamate and aspartate, small colored clear vesicles) are selectively activating ionotropic glutamate receptors (NMDA and AMPA/KA) and metabotropic glutamate receptor group I (*mGluR I*), while substance P (*SP*, large dense-cored vesicles) is activating neurokinin 1 (*NK1*) receptors. At this process, mGluRs II and III are not activated. At presynaptic component, vanilloid receptor TRPV1, voltage-dependent calcium channel (VDCC), and NK1 are activated. ATP (*green* clear vesicles) released from glial cells or damaged cells due to cytotoxic effects are activating ATP P2X receptors. On the other hand, glycinergic and GABAergic modulations become weak due to lack of inhibitory amino acids. Intracellularly, extracellular signaling-regulated kinase (*ERK*) and p38 MAPK protein kinases (PKA and PKC and PKG) are phosphorylated and recruited as enhancing modulators of membrane receptors and ion channels. Cyclooxygenases 1/2 (*COX-1/COX-2*) are also recruited as enzymes catalyzing arachidonic acids to prostaglandins. The *inset* on the *right top* shows involvement of descending nociceptive facilitatory pathway from rostral medial medulla (*RMM*) in the development and maintenance of mirror-image thermal pain hypersensitivity due to the centrally sensitized state. (**b**) Shown is a tripartite synaptic structure with a presynaptic component (*left*) and a postsynaptic component (*right*) and astrocytes or microglia. At the synaptic cleft, in response to thermally nociceptive heat stimuli, EAAs are selectively activating groups I, II, and III of mGluRs but without the activation of ionotropic glutamate receptors. SP is also activating NK1 receptors. At presynaptic component, TRPV1, VDCC, and NK1 are activated. ATP is activating ATP P2X receptors. Intracellularly, ERK and p38 MAPK, PKC, and PKG, but not PKA, are phosphorylated and recruited as enhancing modulators of membrane receptors and ion channels. COX-1/COX-2 is also recruited as enzymes catalyzing arachidonic acids to prostaglandins. (**c**) Shown is also a tripartite synaptic structure. At the synaptic cleft, in response to mechanically von Frey filament stimuli, EAAs are selectively activating mGluR group I, but without the activation of ionotropic glutamate receptors and mGluR groups II and III. NK1 receptors are not activated. At presynaptic component, TRPV1, VDCC, and NK1 are inactivated. ATP is still activating ATP P2X receptors. Intracellularly, only PKA and PKG, but not PKC, are phosphorylated. COX-1/COX-2 is also involved in this process (Merged from Figs. 4, 5, and 6 of Chen and Lariviere. The nociceptive and anti-nociceptive effects of bee venom injection and therapy: A double-edged sword. Prog Neurobiol 2010; 92:151–183. With permission from Elsevier)

Through intensive investigations on the spinal mechanisms underlying the bee venom-induced primary mechanical hypersensitivity, it is surprising to note that few pharmacological targets are effectively involved (Fig. 9c; Chen 2007, 2008; Chen and Lariviere 2010). Group I of mGluRs has been demonstrated to be involved in the induction and maintenance of the primary mechanical hypersensitivity, while other membrane receptors including iGluRs (NMDA and AMPA/KA), groups II and III of mGluRs, and NK1 are not likely to be involved (Fig. 9c; Zheng and Chen 2001; Yan et al. 2009). Because inhibition of ATP receptors P2X3/P2X2 and P2Y in the periphery is shown to be effective (Lu et al. 2008), involvement of P2X receptors is highly possible. Intracellular cAMP-PKA and sGC-PKG-NOS, but not DG-PKC, are phosphorylated and responsible for the induction and maintenance of the primary mechanical hypersensitivity (Li et al. 2000; Li and Chen 2003).

The bee venom-induced secondary and mirror-image hypersensitivity share quite similar spinal mechanisms that have been proposed for the bee venom-induced spontaneous nociception (Fig. 9a; Chen 2007, 2008; Chen and Lariviere 2010). However, the mirror-image hypersensitivity has a unique mechanism involving the descending pain facilitatory pathway from the rostral medial medulla to the spinal dorsal horn (see right upper inset of Fig. 9a; Chen et al. 2000, 2001, 2003; You and Arendt-Nielsen 2005).

In summary, the bee venom-induced multiple symptomatic "phenotypes" of nociception and hyperalgesia have separate spinal mechanisms. The spinal mechanisms of these nociception and hyperalgesia are highly associated with the stimulus modalities applied in the periphery.

Cortical Mechanisms

So far, the cortical mechanisms underlying the bee venom-induced pain and hyperalgesia/allodynia (pain hypersensitivity) have not been fully elucidated. However, activations of the S1 area (Guo et al. 2007; Liu et al. 2007; Chang et al. 2008) and the ACC (Ren et al. 2008; Gong et al. 2010; Lu et al. 2014) and the hippocampal formation (Guo et al. 2007; Zhao et al. 2009; Liu et al. 2011, 2012; Lyu et al. 2013) have been consistently induced by s.c. bee venom injection. Moreover, the primary motor cortex has also been shown to be activated by s.c. bee venom (Wang et al. 2011). The activations of the cerebral cortices are likely to be dependent upon the ascending nociceptive input from the peripheral bee venom injection site, because some cortical activations have been demonstrated to be completely eliminated by local peripheral co-injection of bee venom and bupivacaine that can block VGSCs mediating generation and conduction of action potentials (nociceptive impulses) (Zhao et al. 2009; Lu et al. 2014). However, the functional roles of these cortical areas in the production and maintenance of the bee venom-induced pain are not clear due to limited available data from either human subjects or animals. In animals, it has been shown that bilateral complete ACC chemical lesions caused by kainic acid microinjection significantly decrease the bee venom-induced paw lifting and licking behavior (self-caring and favoring behaviors) but produce no influence upon spinally

processed spontaneous paw-flinching reflex (involuntary withdrawal reflex) (Ren et al. 2008). Moreover, the bilateral ACC lesions can also relieve the bee venom-evoked primary thermal or mechanical hypersensitivity compared with the sham control group (Ren et al. 2008). These results implicate that the bee venom-induced self-caring and favoring anxiety-like behaviors are mediated in part by the activation of the ACC. The ACC is also likely to be involved in maintaining the bee venom-induced primary thermal or mechanical hypersensitivity through descending facilitatory effects. Furthermore, the bee venom-induced self-caring and favoring anxiety-like behaviors can also be relieved by systemic administration of rapamycin that is shown to inhibit the mTOR-S6K signaling phosphorylation and the LTP-enhancing form of metaplasticity in the hippocampal formation (Lyu et al. 2013; for details also see section "The Hippocampal Formation").

Based upon the existing human neuroimaging and animal studies associated with the functions of the S1 area, the ACC, and the hippocampal formation (McMahon and Koltzenburg 2006; Chen et al. 2013; Li et al. 2014), it is proposed that the activation of the S1 area is responsible for the spontaneous pain sensation, while the ACC and the hippocampal formation are responsible for the emotional responses and cognitive evaluation in response to s.c. bee venom injection. However, when pain becomes persistent following s.c. bee venom injection, animals would become annoyed and anxious due to LTP-enhancing metaplasticity in the hippocampal formation, and this may reflect the adverse effects of honeybee sting in human beings (Liu and Chen 2014; unpublished data from Chen's Lab).

Conclusion and Future Directions

Subcutaneous bee venom injection in both humans and animals has been used as a surrogate to study the underlying mechanisms of (honey) bee sting-induced pain and inflammation. Following s.c. bee venom injection, an immediate, sustained period of spontaneous pain and hyperalgesia/allodynia can be induced. Melittin, a major component of bee venom, acts predominantly as an algogen (pain-producing substance) on the primary nociceptor cells to evoke membrane depolarization and firing via the activation of TRPV1 receptors by PLA2-LOX/COX metabolites and activations of P2X3 receptors by ATP or TRPC receptors by GPCRs-PLC-IP3 pathway, leading to peripheral sensitization. The melittin-induced persistent pain signals are conducted to the spinal dorsal horn via mediation of VGSC (Nav1.9), leading to the release of EAAs and SP that activates postsynaptic glutamate and NK1 receptors of spinal WDR neurons, resulting in long-term synaptic plasticity or central sensitization. The melittin-induced peripheral and spinal sensitization of pain-signaling neurons is responsible for the production of spinally processed spontaneous nociceptive reflex and pain hypersensitivity to thermal and mechanical stimuli. Meanwhile, the melittin-induced long-term activation and sensitization of pain-signaling neurons result in synaptic plasticity and metaplasticity of the cerebral cortices (S1 area, ACC, and hippocampus), leading to pain sensation and pain-related emotional responses such as anxiety-like behaviors. During the processes associated

with the bee venom-induced plasticity at the levels of the primary nociceptor cells, the spinal dorsal horn, and the cerebral cortical neurons, many extracellular and intracellular molecular events are detected by multiple experimental approaches and are thought to be involved in the bee venom-induced pain and hyperalgesia/allodynia.

The bee venom injection model is one of the most carefully studied animal models of acute inflammatory pain. The use of the bee venom model can be helpful for understanding of the underlying neural mechanisms of naturally occurring pain and hyperalgesia in humans. It can also be used as a tool for screening of novel analgesics through evaluations of both behaviors and neural plasticity and metaplasticity at different levels of the pain pathways. Unraveling of the underlying molecular and cellular mechanisms of the bee venom-induced anxiety-like behaviors at the cortical level would be challenging and of particular importance for understanding of the transition from persistent pain to its comorbidity with emotional disorders and cognitive deficits (see Liu and Chen 2014).

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L-Amino Acid Oxidase from Venoms

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Payel Bhattacharjee, Jyotirmoy Mitra, and Debasish Bhattacharyya

Abstract

L-Amino acid oxidase (LAAO), a flavoenzyme, is a component of many animal venoms of which the enzyme from snake venom is particularly well characterized. Since the oxidation of L-amino acids produces hydrogen peroxide, the enzyme induces toxicity. Presumably, a glycan part of the enzyme anchors the molecule to cell surface and thereby generates high concentration of hydrogen peroxide locally on cell surface leading to cytotoxicity. Depending on localization of LAAO on normal cells or unwanted cells, the enzyme may be toxic or possess therapeutic potential. The mechanism of action of the enzyme is known, and the crystal structure of snake venom LAAOs has been solved. They are structurally well-conserved enzymes. Therefore, inhibitors of the enzyme may be synthesized using the template of the substrate and features of its catalytic site. Though snake venom LAAO is viewed as toxic, several beneficial activities of the enzyme are known. The physiological actions of the enzyme are not clearly understood, e.g., some contradictory results exist on its platelet aggregation activity. This article updates present knowledge on the enzyme covering various aspects of its toxicity along with its therapeutic potential.

Keywords

L-Amino acid oxidase • Venoms • Toxicity • Enzyme inhibition • Therapeutic applications

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_11

Contents

Introduction

L-Amino acid oxidase (LAAO, E.C. 1.4.3.2) is a flavoenzyme that catalyzes stereo-specific oxidative deamination of an L-amino acid to form corresponding α-keto acid along with ammonia and hydrogen peroxide (H_2O_2) (Fig. 1). This enzyme is involved in the metabolic pathways of eight amino acids, e.g., alanine, aspartate, methionine, valine, leucine, tyrosine, phenylalanine, and tryptophan; degradation of isoleucine and phenylalanine; and biosynthesis of tyrosine, tryptophan, and some alkaloids (Mason et al. 2004). LAAO is potentially useful for quantification of amino acids because a product of the enzyme-catalyzed reaction is H_2O_2, which can be easily quantified by colorimetric assays (Kameya et al. 2013).

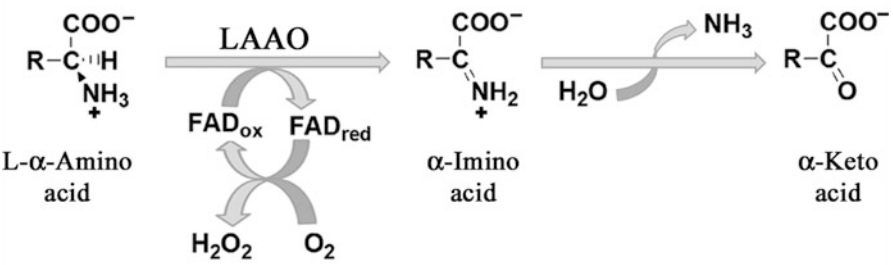

Fig. 1 LAAO-catalyzed oxidation of L-α-amino acid

LAAOs are widely distributed in several taxa. In bacteria, fungi, and green algae, LAAO appears to use amino acids for nitrogen fixation (Vallon et al. 1993). LAAOs found in the venoms of snakes, insects, and marine animals act as an effective deterrent against enemies and/or predators. LAAO activity has been reported in peroxisomes and mitochondria of rat kidney and liver (Mason et al. 2004). The antibacterial activity of mammalian milk is owing to the presence of LAAO (Nagaoka et al. 2009). Mason et al. first characterized the mammalian LAAO (IL4I1) that has significant sequence similarity with snake venom LAAO family (Mason et al. 2004). The functional diversity of LAAO suggests that this enzyme has undergone significant changes through evolution. Snake venom LAAO is possibly the most well-studied enzyme within these groups, which show a high degree of sequence homology with conservation of at least 13 of the 24 N-terminal amino acids that are involved in substrate binding as per X-ray crystallographic structure (Zuliani et al. 2009). The N-terminal sequence contains a highly conserved domain of the motif β-α-β that is involved in flavin adenine dinucleotide (FAD) binding (Du and Clemetson 2002). Among the snakes, venoms from Viperidae, Crotalidae, and Elapidae are rich sources of LAAO constituting 1–4% of the dry weight of the venom. In snake bite victims, the LAAO induces toxicity by platelet aggregation, formation of edema, hemorrhage, and cytotoxicity. These toxic effects are primarily attributed to H_2O_2 produced during catalysis, and therefore, it is speculated that inactivating the enzyme should attenuate the adverse effects thereby conferring partial relief from envenomation.

LAAO was first discovered in 1944 from the venom of the snake "moccasin" (*Agkistrodon piscivorus*) and later identified as a flavoprotein (Kearney and Singer 1951). Since then, investigation on LAAO progressed rapidly and an increasing number of LAAO were reported from different sources. In 1960, Wellner and Meister first prepared the crystals of LAAO from *Crotalus adamanteus* and studied the properties of the enzyme including prosthetic groups, electrophoretic fractions, stability, and pH dependency. Subsequently, the details of its mechanism of catalysis were elucidated (Wellner 1966). Initial studies were mostly centered on purification and enzymatic characterization of LAAO with emphasis on the spectral and redox properties of the non-covalently bound cofactor FAD. With advancement of technology, the research objective gradually shifted toward structural and mechanistic studies to explain the varied biological functions of the enzyme and also to explore its different biomedical applications. Marcotte and Walsh identified vinylglycine to be a suicide substrate/inhibitor of LAAO (Marcotte and Walsh 1976). Till date, investigators are continuing to investigate different inhibitors of LAAO, either synthetic or derived from natural sources, to prevent LAAO-induced oxidative damage to cells. Skarnes first observed bactericidal activity of LAAO from *C. adamanteus* venom (Skarnes 1970). Later, other beneficial properties of LAAO have been worked out by various groups; however, structural knowledge of the enzyme was fairly limited. In 2000, the X-ray crystallographic structure of LAAO was first resolved from the venom of *Calloselasma rhodostoma* (Malayan pit viper). At present, the search for LAAO structure in protein data bank hits only four snake venom LAAOs and three bacterial LAAOs. All these LAAOs have high topological

similarities, but they may differ from one another in exerting biological effects. The sequence of 2.8-kb cDNA encoding LAAO from *C. adamanteus* was determined (Raibekas and Massey 1998). The heterologous expression of various LAAOs was carried out for understanding the phylogenetic relationship of the enzyme from different sources and to explore the potential of the enzyme as a therapeutic agent. Expression of LAAOs from several animal species in various expression hosts has been attempted, but the levels of expression were usually very low. A major limitation in producing recombinant LAAO is that the enzyme itself produces H_2O_2, which in turn generates harmful reactive oxygen species and induces cytotoxicity. Further, the expression of a protein in the absence of secondary modifications could lead to an insoluble variety of protein, e.g., LAAO carries glycan moieties that presumably help in its solubilization. Therefore, recombinant LAAOs without these moieties become insoluble after purification, once the level of expression is fairly improved. As the active site of LAAO maintains complex configuration containing FAD, alteration in pH, temperature, presence of specific ions, and repeated freeze-thaw cycles lead to its inactivation (Tan and Fung 2009).

At present, venom research is not confined to the development of means for neutralizing the toxins but also to determine their mechanism of action. This will eventually facilitate the development of pharmaceutical agents based on the structure of the toxins. These objectives are of immense importance, challenging, and demanding (▶ Chap. 15, "Computational Approaches for Animal Toxins to Aid Drug Discovery," this handbook). LAAOs have found applications as a part of biosensors in monitoring L-glutamate in mammalian brain and to evaluate L and D forms of amino acids in food products such as fruit juices, wines, beers, vinegars, etc., for quality control (Arima et al. 2009). They have also been used as catalysts in biotransformations (Takahashi et al. 1997). Further, LAAO exhibits beneficial biological effects like antiviral, anti-HIV, anti-leishmaniasis, and reduction of tumor cell proliferation. This vivid functional diversity of the enzyme is believed to be mediated by H_2O_2, though the underlying mechanisms are unclear in many cases. This article summarizes recent advancements in venom LAAO research and particularly focuses on their structures, activity, inhibition, and biomedical applications. During the period 2011–2013, LAAOs from six varieties of snakes have been reported that were not characterized earlier (Table 1).

Enzymatic Features

LAAOs are highly stereospecific to L-enantiomer of α-amino acids and do not hydrolyze the D-isomer. The enzymes from snake venom exhibit high preference for aromatic and hydrophobic amino acids such as L-phenylalanine, L-leucine, L-methionine, and L-isoleucine (Du and Clemetson 2002; Guo et al. 2012). There are exceptions too, e.g., LAAO from *Ophiophagus hannah* shows highest preference for L-lysine, while L-aspartic acid and L-glutamic acid are more readily hydrolyzed by LAAO from *Bungarus fasciatus* (Wei et al. 2009). Substrate preference for different amino acids arises from differences in side chain of the amino acids present

Table 1 Summarized profile of snake venom LAAO as referred in recent works[a]

Species	Physical properties	Substrate specificity	Platelets aggregation	Apoptosis	Anti-leishmaniasis	Antimicrobial	Other biological activity	References
Bothrops pirajai	Homodimer of 66 kDa monomeric entity, pI 4.9	L-Phe, L-Leu, and L-Ile	Induce	Yes	Yes	Yes	Cytotoxic, potentiates effect of imatinib mesylate drug on leukemic cells, antiparasitic	Izidoro et al. 2011; Burin et al. 2013
Bothrops leucurus	60 kDa monomer, pI 5.8–6.1	L-Met, L-Norleu, L-Leu, L-Phe, and L-Trp	Inhibit	Yes	Yes	ND	Cytotoxic	Naumann et al. 2011
Crotalus durissus cumanensis	55 kDa, monomer, pI 8.0	L-Leu	ND	No	ND	Yes	Non-cytotoxic	Vargas et al. 2013
Daboia russelii russelii	62 kDa, monomer, pI 6.8–8.8	L-Phe, L-Tyr, L-Met, L-Leu, L-Trp, and L-Ile	Induce	ND	ND	ND	ND	Chen et al. 2012
Lachesis muta	Homodimer of 60 kDa, monomeric entity, pI 5.1	L-Leu	ND	No	Yes	ND	Cytotoxic, myotoxic, antitumor	Bregge-Silva et al. 2012
Naja naja (Indian cobra)	62 kDa, monomer, pI 8.12	L-Leu	ND	ND	ND	ND	ND	Dineshkumar and Muthuvelan 2012

[a]Previous reports on snake venom LAAOs which were already been reviewed have not been included in this table

in the substrate binding site of the enzymes (Ponnudurai et al. 1994). The side chain of hydrophobic amino acids is perfectly incorporated in the hydrophobic binding pocket located in the catalytic cavity of the enzyme (Moustafa et al. 2006). The difference in substrate affinity indicates that in spite of structural similarity among different LAAOs, they are distinguishable.

The oxidation of L-amino acid by the enzyme proceeds in two steps (Fig. 1). During the reductive half-reaction, the α-hydrogen atom of the amino acid is abstracted by FAD to produce α-imino acid as an intermediate which in turn is hydrolyzed by an enzyme bound water molecule to form the α-keto acid. An oxidative half-reaction completes the catalytic cycle after reoxidizing the FAD with molecular oxygen producing H_2O_2. Two alternative mechanisms are proposed for the reductive half-reaction: the carbanion mechanism, in which the proton is transferred leaving a negative charge on the alpha carbon atom, or a hydride-transfer mechanism, in which the hydrogen atom and the two electrons are transferred simultaneously. Recent structural and mechanistic studies on D-amino acid oxidase (DAAO), a functionally related enzyme that carries out the same redox reaction but with specificity to D-enantiomer of amino acids, support the hydride-transfer mechanism (Fitzpatrick 2004).

Earlier studies focusing on characterization of the redox activity of snake venom LAAO showed that the enzyme forms a ternary complex constituting the enzyme, substrate, and oxygen and that reduction of the flavin involves formation of a semiquinone (Massey and Curti 1967). The presence of FAD is responsible for the yellow color of snake venom and the typical absorption maxima of LAAO at 380 and 465 nm. LAAOs particularly from snake venom have unique properties of inactivation and reactivation upon temperature and pH variations. Inactivation is accompanied by changes in spectral properties of the enzyme, which get restored upon reactivation. This suggests that the inactivation is reversible in nature and is due to conformational changes in protein structure, particularly in the vicinity of the FAD cofactor. Heat-mediated inactivation of the enzyme is pH dependent where inactivation increases with increase in pH (Kearney and Singer 1951; Wellner 1966). Curti et al. (1968) showed that the enzyme is inactivated when stored under frozen conditions with maximal inactivation at −20 °C. However, the frozen enzyme maintained at −60 °C did not undergo any inactivation. Heat- and freeze-inactivated LAAO could be reactivated by reheating the enzyme after adjusting the pH to 5 (Kearney and Singer 1951; Wellner 1966; Curti et al. 1968). This illustrates conformational flexibility and stability of the native state of the enzyme.

Activity of LAAOs is usually estimated by coupled assay using horseradish peroxidase (HRP). In this protocol, H_2O_2 generated by LAAO oxidizes o-dianisidine to color products in the presence of HRP which is monitored at 436 nm (Bergmeyer 1983). Based on the same principle, a spectrophotometric microplate and an in-gel assay method have been developed for processing large number of samples (Kishimoto and Takahashi 2001; Rau and Fischer 2011). Prussian blue agar assay coupled with SDS-PAGE has been acclaimed to be a simple, rapid, sensitive, and cost-effective method for quantitative in-gel determination of LAAO (Yu et al. 2013).

Structural Features

Basic Structure

LAAOs are usually homodimeric FAD-binding glycoproteins of molecular mass 110–150 kDa when measured by gel filtration under non-denaturing conditions, while the monomeric subunits are of 50–70 kDa as detected by mass spectrometry and SDS-PAGE under reducing and nonreducing conditions (Du and Clemetson 2002; Bregge-Silva et al. 2012). However, LAAOs from *Bungarus fasciatus* and *Bothrops leucurus* are monomeric (Wei et al. 2009; Naumann et al. 2011). LAAO has a wide range of isoelectric points (pI) from 4.4 to 8.8 (Zhong et al. 2009). Interestingly, like snake venom phospholipase A_2 (PLA_2), there are acidic, neutral, and basic LAAOs and these too exist in the same venom. The presence of LAAO isoforms in venoms of *Bothrops alternatus*, *Pseudechis australis*, *Vipera berus berus*, *Daboia russelii russelii*, *Daboia russelii siamensis*, *Bothrops jararaca*, and *Agkistrodon blomhoffii ussurensis* has been reported, and such phenomenon is a consequence of protein glycosylation or protein synthesis from different genes (Stabeli et al. 2004; Samel et al. 2008; Mandal and Bhattacharyya 2008; Ciscotto et al. 2009; Sun et al. 2010). These LAAO isoforms are thought to possess different pharmacological properties similar to those of PLA_2 isoforms, which originate from different substrate binding sites. This prediction appears to be true for two LAAO isoforms from *D. russelii* venom (Mandal and Bhattacharyya 2008).

Amino Acid Sequence

LAAOs cloned from *A. halys blomhoffii*, *Bothrops moojeni*, *C. rhodostoma*, *C. adamanteus*, *Crotalus atrox*, and *Trimeresurus stejnegeri* showed around 80% of sequence homology (Rodrigues et al. 2009). The N-terminal amino acid sequences of many LAAOs are very similar, where a highly conserved glutamic acid-rich motif is observed. The structure of LAAO from *C. rhodostoma* revealed that residues 5–25 constitute a part of the substrate-binding domain. The conserved amino acids may, therefore, play an important role in the substrate binding. LAAOs share sequence homology with human monoamine oxidase and also with bacterial, fungal, and mouse interleukin 4-induced Fig1-protein (Raibekas and Massey 1998). Though all these proteins have low sequence homology with LAAOs, they share a similar topological architecture, except the highly preserved catalytic site (Du and Clemetson 2002; Rodrigues et al. 2009).

Three-Dimensional Structure

The X-ray crystallographic structure of the LAAO from *C. rhodostoma* was the first to be solved that provided important insights into its mechanism of substrate binding and catalysis (Pawelek et al. 2000). Recently 3-D structures of LAAOs from the

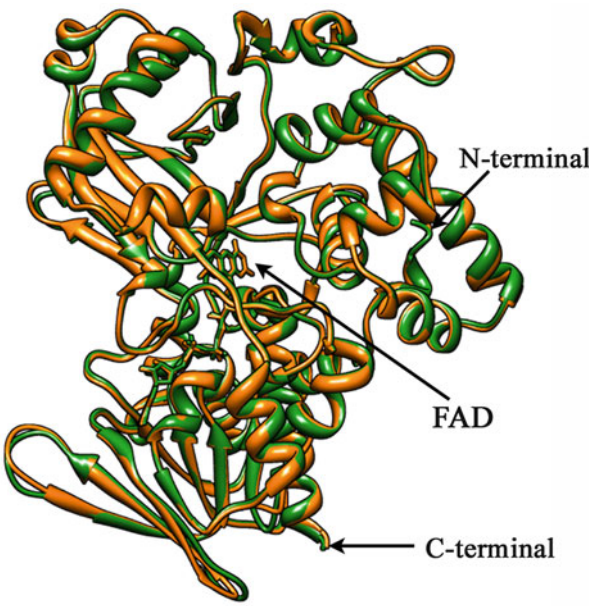

Fig. 2 LAAOs from snake venoms are structurally conserved. The ribbon models derived from X-ray crystallographic structures of LAAOs from Malayan pit viper (*C. rhodostoma*) (*orange*, PDB 1F8S) and cotton mouth viper (*A. halys pallas*) (*green*, PDB 1REO) show high extent of overlapping

venoms of *Agkistrodon halys pallas*, *Vipera ammodytes ammodytes*, and *Bothrops jararacussu* have also been reported (Zhang et al. 2004; Georgieva et al. 2011; Ullah et al. 2012). These enzymes share an average of 85% of sequence identity with *C. rhodostoma* along with high structural similarity (Fig. 2; Zhang et al. 2004; Ullah et al. 2012). The high-resolution X-ray structure of LAAO from *C. rhodostoma* indicated that it is a functional dimer where each subunit consists of 15 α-helices and 22 β-stands that fold into three well-defined domains: an FAD-binding domain (residues 35–64, 242–318, and 446–471), a substrate-binding domain (residues 5–25, 73–129, 233–236, and 323–420), and a helical domain (residues 130–230). This helical domain contributes to a Y-shaped entrance to the enzyme's active site, which is located at the base of this long funnel extending 25 Å from the surface into the interior of the protein. The isoalloxazine ring of the FAD cofactor, essential for catalysis, lies at the base of this funnel. In fact, there are two access routes to the active site which makes the funnel Y-shaped (Fig. 3). It is proposed that one route is for the entry of the substrate while the other is for the exit of H_2O_2. The common stem of this Y-shaped funnel is approximately 9 Å in length and extends from the substrate-binding cavity to the bifurcation point. Although L-glutamate oxidase from *Streptomyces* sp. and polyamine oxidase (PAO) have high topological similarity with snake venom LAAO, their catalytic funnels differ greatly from that of snake venom LAAO (Arima et al. 2009). The shape of the funnel and length of L-glutamate oxidase which is %30 Å resemble PAO. PAO has a U-shaped catalytic funnel which is narrower than the funnel of snake venom LAAO but broader than that of L-glutamate oxidase (Arima et al. 2009).

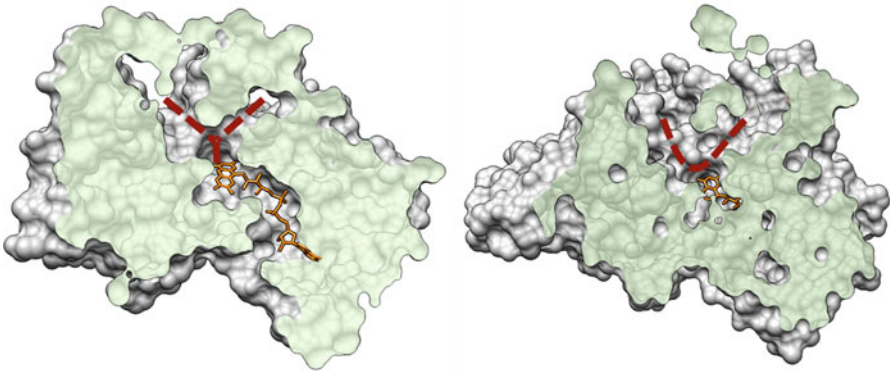

Fig. 3 Comparison of the shape of the funnels constituting the catalytic site of LAAO from snake venom (*C. rhodostoma*) (PDB ID: 2IID) (*left*) and bacteria (*Streptomyces* sp. X-119-6) (PDB ID: 2E1M) (*right*). Snake venom LAAO has a Y-shaped funnel whereas that from bacteria has a U-shaped funnel. *Brown dotted lines* indicate entry of substrate on the *left* and exit of products on the *right*

Crystallographic structures of LAAO complexes with the reversible inhibitor o-aminobenzoate and the substrate L-Phe provided important insights into the mode of substrate binding and the possible mechanism of catalysis (Moustafa et al. 2006; Pawelek et al. 2000). The substrate L-Phe remains bound to the reface of the cofactor FAD, and the side chain of L-Phe is extended away from the cofactor. It is accommodated in a subpocket which is composed of the side chains of Ile374, His223, and Arg322 residues. The carboxylate group of the substrate is involved in a salt-bridge interaction with the guanidinium group of Arg90 and a hydrogen bond with the OH group of Tyr372. The α-C atom of the substrate, representing the site of oxidative attack, is positioned over the isoalloxazine ring at a distance of 3.1 Å from N5 atom of FAD. The amino group of the substrate forms hydrogen bond with the carbonyl oxygen atom of Gly464. Conformational changes of the key components of the active site residues (cf. His223, Arg322, and FAD) can be related to the mechanism of direct transfer of hydride ion. His223 alters its side-chain conformation in a fashion to activate the substrate by deprotonating its α-amino group. Thereafter, the lone pair of electrons from the nitrogen atom of the amino group moves to the α-carbon atom followed by hydride transfer to FAD and forms the imine compound. The conserved water molecule at Lys326 plays a role in the reductive half-reaction, thereby assisting the formation of H_2O_2. Thus, conformational change of His223 is important for substrate entry as well as for hydride transfer and is highly conserved in LAAOs. The presence of Gly316 in place of His223 in L-glutamate oxidase indicates that catalysis in bacterial LAAO may occur through a different route or may be through the involvement of other residues which need clarification. Structural superposition of several LAAOs showed that the key residues for amino acid recognition are fully conserved including Tyr372, Arg90, Trp465, ILe430, His223, and Arg322. This conservation indicates a highly specific function of LAAOs during the process of envenomation. The volume of the active

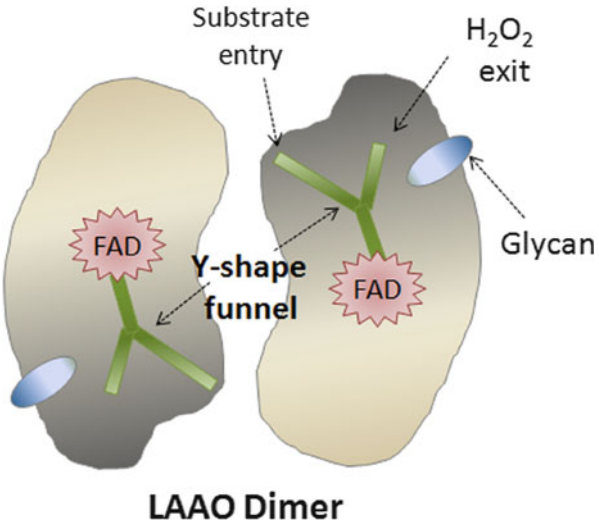

Fig. 4 Simplified two-dimensional representation of LAAO dimer. Functional dimeric form of the protein is stabilized by opposite charges on the surface. This is indicated by two color shades. The catalytic funnel with probable substrate entry and product (H_2O_2) exit paths are marked. FAD is extended in opposite direction in the dimeric form. The glycan attachments at Asn172 are exposed on the surface, are located almost diagonally, and are close to the H_2O_2 exit path

site also influences the substrate specificity. Ullah et al. (2012) reported that in *B. jararacussu* LAAO, the presence of Ile in place of Trp430 leads to increased volume of active-site cavity which increases the specificity of the enzyme toward bulky hydrophobic amino acids. It was observed that the key residues involved in dimerization of *B. jararacussu* LAAO are Lys191, Arg317, His314, Arg317, Ser318, Arg300, Arg301, Tyr436, Asp376, Asp349, Asp210, Asp201, H320, Thr182, Asp205, Asp388, Lys186, Arg297, and His440. Electrostatic surface analysis of monomers of LAAO from *V. ammodytes* and *B. jararacussu* indicates that dimerization is mediated by Coulombic forces between negatively charged substrate-binding domains and positively charged FAD-binding domain (Fig. 4; Ullah et al. 2012). Furthermore, the entrance of the substrate to the active site for each protomer is located on opposite surface of the dimer. Also the adenine dinucleotide part of FADs is extended in opposite directions.

V. ammodytes LAAO is tetrameric with a zinc ion present in the interface of the tetramer coordinating with His75 and GLy279 residues. It was observed that the presence or absence of zinc ion did not interfere with the catalytic activity and the absence of zinc in LAAO from *C. rhodostoma* and *B. jararacussu* strongly suggest that the zinc ion plays an important role in the stabilization of the tetrameric structure.

Glycan Residues

There are good indications that LAAO-induced cytotoxicity is not due to the sole effect of H_2O_2 that is generated during catalysis but to some extent also related to its ability to interact with the cell surface through its glycan moiety (Suhr and Kim 1996; Ande et al. 2006). This glycan moiety consists of only 3–5% of LAAOs by

mass. Fluorescence microscopy using FITC (fluorescein isothiocyanate)-labeled LAAO revealed direct attachment of the enzyme to the cell surface which varies depending on the cell line (Suhr and Kim 1996). Deglycosylation of the enzyme isolated from *C. atrox* (Apoxin1) abolished its catalytic activity, whereas the activity is reduced by 75% in case of *A. halys pallas* LAAO (Torii et al. 2000). However, removal of the glycan had no effect on enzymatic activities of LAAOs from *Bothrops pauloensis*, *B. jararaca*, *B. alternatus*, and *B. moojeni* (Stabeli et al. 2004; Ciscotto et al. 2009; Rodrigues et al. 2009). The structure of LAAO from *C. rhodostoma* reveals two glycosylation sites, Asn172 and Asn361, where the glycan is linked to the amide nitrogen atom of the residues (Pawelek et al. 2000). Glycosylation at Asn361 is absent in LAAO from *V. ammodytes*. The oligosaccharide at Asn172 is exposed on the protein surface irrespective of its multimericity. Isolated glycan from LAAO of *C. rhodostoma* venom using N-glycosidase F (PNGase F) and subsequent analysis using 2-D NMR and MALDI mass spectrometry identified the glycan as bis-sialylated, biantennary, core-fucosylated dodecasaccharide (Geyer et al. 2001). The glycan moiety at Asn172 lies near to the proposed H_2O_2 exit channel. It was hypothesized that putative binding of LAAO to cell surface through the glycan moiety results in production/release of locally high concentration of H_2O_2 near the binding interface, which in turn may damage the structural elements of the cells through oxidative stress leading to cell apoptosis (Geyer et al. 2001). In contrast to snake venom LAAO, PAO has only one glycosylation site at Asn77, whereas no such glycosylation is observed in LAAO from bacterial sources (Arima et al. 2009). So the precise role of glycosylation in snake venom LAAOs could not be predicted with certainty.

Toxicology of LAAO

LAAO is an interesting molecule for research because of its hemolytic, hemorrhagic, cytotoxic, anticoagulant, genotoxic, and other physiological effects. All these effects are thought to be mediated by H_2O_2 generated during catalysis because H_2O_2 scavenger such as catalase attenuates the effects. However, some studies imply that H_2O_2 production is not the only factor responsible for all these biological effects. Several LAAOs were reported to exhibit moderate toxicity having LD_{50} of 5–9 μg/g in mouse (i.v.). In fact, LD_{50} of snake venom LAAO is usually higher than that of the corresponding venom (Tan and Saifuddin 1989). The structure and function of LAAOs from different sources have been extensively reviewed by several authors (Du and Clemetson 2002; Tan and Fung 2009; Guo et al. 2012). Here, recent progresses in the toxicological studies of LAAO will be discussed.

Edema-Inducing and Hemorrhagic Activities

LAAOs are involved in the pathogenesis of snakebite-induced inflammation. Wei et al (2007) reported that LAAO could cause pneumorrhagia, pulmonary interstitial

edema, fusion of pulmonary alveoli, cardiac interstitial edema, and liver cell necrosis. It could also stimulate lymphocytes and monocytes to release IL-6, IL-2, IL-12, and T cells. Later, the same group demonstrated that LAAO from *B. fasciatus* venom causes local inflammation by evoking the cytotoxicity and pro-inflammatory activities in mice (Wei et al. 2009). LAAO from *O. hannah* exhibited "delayed type" strong edema-inducing activity, which indicates that the edema formation caused by this enzyme was not mediated through release of amines (Tan and Choy 1993). The edema-inducing activity of the enzyme was not inhibited by the anti-allergic and anti-inflammatory drugs, diphenhydramine or dexamethasone. Izidoro et al. (2006) suggested that edema formation is due to activation of the inflammatory response by the H_2O_2 generated, as administration of glutathione (an antioxidant) to the mouse paw inhibited the edema-inducing activity of the enzyme (Izidoro et al. 2011). All these facts point toward toxicity of LAAOs that may cause serious pathogenesis in snakebite victims.

Anticoagulant Effects

Sakurai et al. (2003) initially reported factor IX-specific anticoagulant activity of LAAO from *Agkistrodon halys blomhoffii* venom. But the molecular mechanism of this action is not clear. Later, Fujisawa et al. (2009) purified LAAO from the venom of *Gloydius blomhoffii* (previously known as *A. halys blomhoffii*) that contains a 39 kDa fibrinogenolytic metalloproteinase. It induces anticoagulant activity through the degradation of Aα- chain of fibrinogen, and this activity is independent of the enzymatic activity of LAAO or generation of H_2O_2.

Genotoxic Effect

Marcussi et al. (2013) evaluated the genotoxic potential of venoms from *B. jararacussu*, *B. atrox*, *Bothrops moojeni*, *B. alternatus*, and *B. brazili* snakes and some isolated toxins (MjTX-I, BthTX-I and BthTX-II myotoxins, BjussuMP-II metalloprotease, and Batx LAAO) on human lymphocytes. BthTX-I, BthTX-II, and LAAO caused significant DNA damage and enhanced the formation of micronuclei (Marcussi et al. 2013).

Strategies for Inhibition of LAAO

Currently, administration of polyvalent antivenom serum (AVS) is the only and effective means of treatment for snake envenomation. Although monovalent antivenom has often been considered more efficacious, the production of polyvalent AVS is preferred in many countries as identification of the invading snake species is generally not possible by the attending physician. Polyvalent AVS that is prepared by raising antibody in horse against four to five different species of snakes often

causes allergic reactions in victim's body due to the presence of nonspecific components. Apart from this, poor availability, improper maintenance of storage conditions, and high price limit the application of the AVS (Gutierrez et al. 2010; Deshpande et al. 2013). Moreover, the success of AVS treatment varies greatly with the geographical area as the venom composition and the clinical manifestation of envenomation vary with geographical location (Warrell 1989). The search for an alternative antidote against envenomation that can be used in case of emergency, when AVS is not readily available or not sufficiently effective, is always in demand. Such an antidote should allow victims to survive and also to prevent severe local inflammatory effects of envenomation, e.g., muscle necrosis, abnormal bleeding that often lead to gangrenous limbs, etc., until established and efficacious treatment is available. The situation may be compared with the application of anticholinesterase drugs such as edrophonium that can partly overcome blockade by postsynaptic neurotoxins and have shown good results in cobra envenomation (Warrell 1999). The discovery of specific inhibitors of snake venom toxins promises great therapeutic application in snakebite envenomation.

So far, all known LAAOs are flavoenzymes except lysine oxidase from *Marinomonas mediterranea*. In parallel, an increasing number of other flavoenzymes have been inhibited by substrate analogs. However, reports on inhibitors of LAAO are limited (Yu and Qiao 2012). In general, substrate analogs are well recognized by the catalytic residues of an enzyme and hence are exploited for both reversible and irreversible inhibitions of enzymes. The binding mode of *o*-amino benzoic acid, a reversible inhibitor of LAAO, was demonstrated using crystallographic structure of the enzyme-inhibitor complex (Pawelek et al. 2000). Moreover, kinetic studies of other reversible inhibitors predicted the presence of different electrostatic and hydrophobic patches within the catalytic funnel of two LAAO isoforms of *D. russelii* venom (Mandal and Bhattacharyya 2008). Similar to other flavoenzymes, irreversible inactivation of LAAOs could be achieved by "mechanism-based inhibitor" or "suicide substrate." These are a special class of irreversible inhibitors having structural and chemical features resembling those of the normal substrate of an enzyme. In addition, the inhibitors contain a functional group at the enzyme binding site which is converted to a highly reactive moiety within the active site of the enzyme during catalysis. This reactive moiety generated at the transition state either attacks an essential amino acid residue or prosthetic group of the enzyme to form a covalent linkage leading to irreversible inactivation of the enzyme. Hence, the inhibitors are highly specific and suitable for designing drugs based on enzyme inhibition. A notable example is the inhibition of monoamine oxidase (MAO) by drugs such as clorgiline, selegiline, and rasagiline. These drugs have great potency against Parkinson's disease (Youdim 2013). The mechanism of modification of the flavin residue by this class of drugs is shown in Fig. 5. Vinylglycine has been described as a suicide substrate of *C. adamanteus* LAAO (Marcotte and Walsh 1976). Later, L-propargylglycine was also shown to inhibit the enzyme from the same venom in a similar fashion, where inactivation of the enzyme occurs through covalent modification of the active site His223 (Mitra and Bhattacharyya 2013). Additional endeavors are likely to yield more potent inhibitors of LAAO.

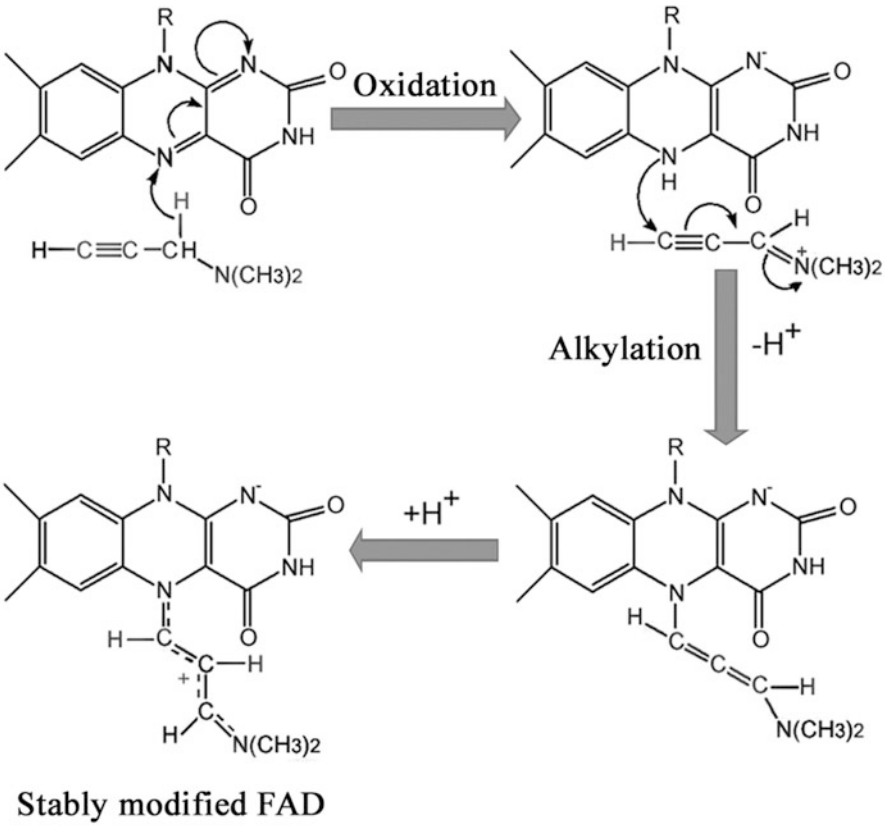

Fig. 5 Mechanism of irreversible inactivation of flavoenzymes through modification of FAD by suicide substrate. Though formation of adducts through N atom is most favored, involvement of C atom of the isoalloxazine ring of FAD in adduct formation is also reported. The mechanism is very similar to normal catalysis process; the first step is oxidation of the inhibitor followed by alkylation instead of product release as in the case of enzyme-substrate complex formation

Medicinal plants are known to neutralize toxicity of snake venoms (▶ Chap. 4, "Natural Inhibitors of Snake Venom Metallopeptidases," this handbook). Few of them possess inhibitory activity against LAAO. For example, the major phenolic components of the ethanolic extract of the seed kernels of Thai mango (*Mangifera indica*) are 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (61.28%), methyl gallate (0.68%), and gallic acid (0.44%), of which the first component inhibits LAAO of *C. rhodostoma* and *Naja naja kaouthia* venoms in a dose-dependent manner in vitro. This compound forms hydrogen bonds with amino acid residues in the substrate-binding domain (Arg90, His223, Asp224, Phe227, Ala228, Lys345, Phe354, Tyr356, Tyr372, and Ile374) of the LAAO from *C. rhodostoma* venom and inhibits the enzyme with high specificity. Since the N-terminal sequences of LAAOs from *N. kaouthia* and *C. rhodostoma* venoms are highly homologous, it is possible that

this compound also binds to the substrate-binding pocket of LAAO of *N. kaouthia* venom and thereby inhibits its activity (Leanpolchareanchai et al. 2009). An extract of *Tamarindus indica* seed inhibits LAAO activity of *Vipera russelii* (also called *D. russelii*) venom in a dose-dependent manner (Ushanandini et al. 2006). The methanolic extract of fresh tea leaves of *Camellia sinensis* inhibits local tissue damage induced by *N. kaouthia* and *C. rhodostoma* venom by inhibiting LAAO along with other hydrolytic enzymes (Pithayanukul et al. 2010). The author's laboratory is also involved in the designing and characterization of inhibitors of LAAO from natural and synthetic sources. A compound from an Indian medicinal plant has been isolated that could inhibit LAAO activity from snake venom. Few derivatives of this compound have been synthesized to increase inhibitory potential with reduced toxicity as compared to the original one (Bhattacharjee 2014).

Therapeutic Potentials of LAAO

In the last two decades, LAAO drew the attention of more researchers because of its multifunctional effects on different biological systems. This section summarizes information regarding the antiprotozoal, antibacterial, antiviral, and apoptosis-inducing effects of LAAO including its ambiguous action on platelet aggregation. Through judicious modification of the structure of LAAO, one could reduce its toxicity on host cells retaining its beneficial function for therapeutic applications.

Antiprotozoal Property

Leishmaniasis is an endemic tropical disease with human infections ranging from self-healing cutaneous ulceration to lethal visceral infection. Approximately 350 million people are at risk from the disease, and an estimated 1.6 million new cases occur annually, with its prevalence in about 88 nations (Stockdale and Newton 2013).

Many reports suggest that LAAO possesses strong antileishmanial activity (Toyama et al. 2006). H_2O_2 generated by the enzyme induces apoptosis in promastigotes of *Leishmania* sp. by enhancing oxidative stress, activating heat shock proteins leading to disorganization of its cell membrane and cytoplasmic vesicles. In other words, the H_2O_2 produced by LAAO breaks the antioxidant system of the promastigote cells to induce apoptosis. LAAO from *Bothrops marajoensis* could inhibit the growth of promastigotes of *L. chagasi* and *L. amazonensis* in a dose-dependent manner (Costa Torres et al. 2010). LAAO from *B. moojeni* significantly inhibits the growth of promastigotes of *L. amazonensis*, *L. chagasi*, and *L. panamensis* exhibiting IC_{50} of 1 mg/ml. The enzyme also inhibits the growth of *Trypanosoma cruzi* and *L. major* promastigotes by causing cytoplasmic vacuolization and increasing the volume of mitochondria. It causes the death of promastigotes by disrupting the respiratory chain of the parasite leading to reduced production of ATP. The antileishmanial activity is negatively correlated with the

levels of catalase and glutathione peroxidase. It has been demonstrated that catalase and glutathione peroxidase are present three and 14 times more in amastigotes of *L. donovani* than in promastigotes, which potentially makes the former four times more resistant to LAAO than the latter (Toyama et al. 2006). Bregge-Silva et al. (2012) reported that LAAO from *Lachesis muta* demonstrates antiparasitic activity on *Leishmania braziliensis* (IC_{50} 2.22 μg/mL) but is ineffective for *T. cruzi* infection.

At present, only a few drugs are available for the treatment of leishmaniasis, although they are highly toxic. As LAAOs can produce high concentrations of H_2O_2 in certain localized areas of the cell, a small amount of the enzyme can target toward the parasitophorous vacuole, and this would represent a highly specific treatment not only for leishmaniasis but also for other intracellular parasites. Insightful expedition into the mode of action of LAAO upon parasites, other than the effect exerted by H_2O_2, may trigger the design of new drugs or improved therapeutic approaches for leishmaniasis.

Antibacterial Properties

The emergence of drug-resistant strains of bacteria has increased the risk of morbidity and mortality worldwide. Therefore, the discovery of new powerful therapeutics is necessary for treatment of infections caused by the resistant microorganisms. Recently, powerful antibacterial components against gram-positive and gram-negative bacteria from natural sources have drawn attention owing to their fewer side effects (Tõnismägi et al. 2006; Samel et al. 2008; Costa Torres et al. 2010; Lee et al. 2011; Vargas et al. 2013). Venoms are rich sources of antimicrobial compounds such as peptides. The antibacterial effect of LAAO is attributed to the generation of H_2O_2, since addition of catalase completely inhibits the activity. Increased exogenous H_2O_2 production and accumulation of reactive oxygen species (ROS) induce rupture of bacterial membrane leading to cell death (Lee et al. 2011). Few LAAOs are known to exhibit higher bactericidal potency than the other well-established antibacterial drugs; for example, under identical conditions, *A. blomhoffii ussurensis* venom LAAO (Akbu-LAAO) exhibits 18-fold higher bactericidal effect on *Staphylococcus aureus* than that of cephalosporin C (Sun et al. 2010). Ciscotto et al. (2009) studied the antibacterial activity of the venom from bee, caterpillar, scorpion, spider, and snakes and found that the venoms from *B. jararaca* and *B. jararacussu* showed the highest potency against gram-positive and gram-negative bacteria.

Antiviral Properties

Snake and insect venoms are beneficial for the treatment of drug-resistant human immunodeficiency virus (HIV) infection (Wachinger et al. 1998; Alrajhi and Almohaizeie 2008). LAAO from *Trimeresurus stejnegeri* exhibited dose-dependent inhibition of HIV-I infection at concentrations that showed little effect on cell

viability. The presence of catalase causes fivefold decrease in its antivirus activity but results in an increase of its antiviral selectivity. Most probably, the mechanism of the anti-HIV-I effect of LAAO involves specific binding of the enzyme to the cell membrane, which helps to generate high local concentrations of H_2O_2. High concentration of H_2O_2 may trigger certain signal reactions and subsequently activate the host cells, resulting in the inhibition of HIV infection. This mechanism, though might not be exclusive, is supported by the fact that exogenous H_2O_2 exhibits no anti-HIV activity. Further, the authors suggested that the dosages of H_2O_2 and relative molecular pathways mediating the suppression of viral infection and replication are independent and/or different from those causing cell deaths. Viral load is associated with the level of expression of p24 antigen. LAAO present in the venom of *C. atrox*, *Pseudechis australis*, and *T. stejnegeri* prevents HIV infection through inhibition of expression of p24 antigen in a dose-dependent manner (Zhang et al. 2003; Du and Clemetson 2002). LAAO from *B. jararaca* venom showed antiviral activity against the dengue type 3 virus (Sant'Ana et al. 2008).

Apoptosis-Inducing Activity

Apoptosis is a highly regulated form of cell death in which the cell contains necessary information to die on its own and is characterized by phenotypic features such as exposure of phosphatidylserine on the cell surface followed by chromatin condensation, DNA fragmentation, and formation of membrane-enclosed cell fragments that are collectively known as apoptotic bodies. Apoptosis plays a crucial role in the development and maintenance of equilibrium in the body. Defect in apoptosis program or dysregulation in apoptotic process results in cancer, autoimmune diseases, and spreading of viral infections. Therefore, elucidation of the antiproliferative activity of LAAO is an important step toward their potential application as anticancer agents. LAAOs have been shown to induce cell death in yeast and several mammalian cancer cell lines like vascular endothelial cells, mouse lymphocytic leukemia cells, human T cells, human promyelocytic leukemia cells, human embryonic kidney cells, glioma cell lines, HeLa cell lines, and Jurkat cells (Araki et al. 1993; Suhr and Kim 1996; Torii et al. 1997, 2000; Sun et al. 2003; Zhang et al. 2004; Ande et al. 2006; de Melo Alves Paivaa et al. 2011). Lee et al. showed that the LAAO isolated from *O. hannah* venom selectively kills cancer cells via apoptotic pathway by regulating the caspase-3/7 activity but is nontoxic to normal cells. This study is important toward development of the enzyme as an anticancer agent. The authors summarized the antiproliferative activities of some LAAOs reported so far (Lee et al. 2014).

The LAAOs from other animal toxins have also been associated with apoptosis-inducing activity. AIP (apoptosis-inducing protein) purified and cloned from *Anisakis simplex* (a larval nematode)-infected chub mackerel shows induction of apoptosis in various mammalian cells including human tumor cell lines (Murakawa et al. 2001). Dolabellanin, a LAAO from an ocean mollusk, induces cytotoxicity on EL-4 murine lymphoma cells (Iijima et al. 2003). Achacin, belonging to the family

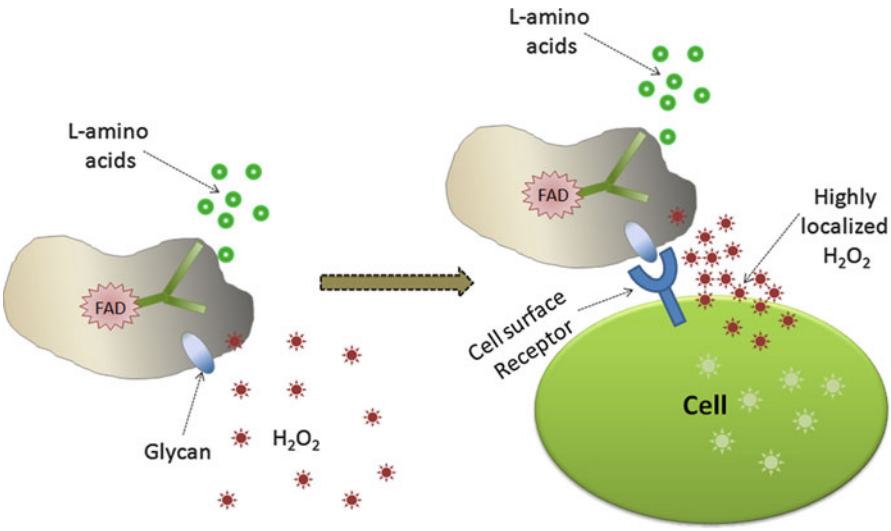

Fig. 6 Schematic representation of LAAO-mediated cytotoxicity where the glycan part of the enzyme helps in binding at the cell surface and produces localized high concentration of H_2O_2

of LAAO, can induce *cytotoxic effects* on HeLa cell lines (Kanzawa et al. 2004). Butzke et al. (2004) showed that APIT (*Aplysia punctata* ink toxin), a 60 kDa LAAO, triggers cell death with profound tumor specificity (Butzke et al. 2004). Although the generation of H_2O_2 is necessary for induction of apoptosis, it is speculated that glycosylation of LAAO contributes to this process by interaction of the bis-sialylated N-glycan moiety with structures on the cell surface (Fig. 6; Suhr and Kim 1996; Ande et al. 2006). LAAO also induces apoptosis via caspase- and mitochondrial-dependent pathways. As zVAD-fmk, a broad-spectrum caspase inhibitor, and Bcl-2, an antiapoptotic protein, completely abolish the apoptotic effects induced by LAAO, it is hypothesized that LAAO depletes hydrophobic and aromatic amino acids or alters the composition of serum supplement in the medium, which might result in apoptosis (Ande et al. 2006).

Regulation of Platelet Aggregation

Aggregation of platelet plays a key role in the development of thrombosis, an important phenomenon for hemostatic plug formation. Platelet aggregation also plays a pivotal role in the development of cardiovascular diseases (Bhatt and Topol 2003). As a consequence, inhibition of platelet aggregation has become increasingly important for the prevention and treatment of cardiovascular disorders (Jackson 2007).

The effects of LAAOs on platelet functioning are inconclusive. Some LAAOs induce platelet aggregation, whereas other possess inhibitory activity. Interestingly,

both effects were inhibited by catalase, a H_2O_2 scavenger (Du and Clemetson 2002). This indicated that H_2O_2 generated during catalysis is involved in both effects. LAAO from different snake venoms exhibiting either of these two effects has been recently reviewed (Guo et al. 2012). Those works that contribute to the understanding of the mechanism by which LAAO affects the functioning of platelets have been emphasized. Akbu-LAAO isolated from the venom of *A. blomhoffii ussurensis* shows apparent anti-aggregation effects on human and rabbit platelets (Sun et al. 2010). *V. lebetina* LAAO inhibits ADP or collagen-induced platelet aggregation in a dose-dependent manner (Tõnismägi et al. 2006).

Platelet aggregation is not affected solely by H_2O_2. Platelet aggregation is amplified by H_2O_2 in the presence of agonist like collagen and arachidonic acid but not ADP (Pratico et al. 1992). It is interesting that catalase inhibits platelet aggregation induced by collagen in a dose-dependent manner whereas it does not affect the aggregation by other agonists like ADP or thrombin (Pignatelli et al. 1998). Catalase inhibits thromboxane A_2 production and release of arachidonic acid from platelet membrane. This study showed that collagen-induced platelet aggregation is associated with the production of H_2O_2 that acts as a second messenger by stimulating the arachidonic acid metabolism and phospholipase C pathway. Toyama et al. (2006) reported that the platelet aggregation-inducing activity of *Crotalus durissus cascavella* venom LAAO (Casca LAAO) was inhibited by cyclooxygenase pathway inhibitors such as indomethacin and aspirin. It was suggested that H_2O_2 generated by LAAO was involved in the production of inflammatory enzyme thromboxane A_2, which consequently resulted in platelet aggregation (Du and Clemetson 2002; Toyama et al. 2006). However, thromboxane A_2 was not produced by washed human platelets when stimulated by H_2O_2, collagen, or arachidonic acid alone but by H_2O_2 in the presence of sub-threshold concentration (non-aggregating concentration) of the collagen or arachidonic acid (Pratico et al. 1992). So it remains unclear how H_2O_2 produced by LAAO induces platelet aggregation. Naumann et al. (2011) reported that *Bothrops leucurus* LAAO does not interact with endothelial cells. Therefore, the other possibility that LAAO may activate platelet in a receptor-dependent manner similar to the varied association of the enzyme with different cell lines needs further investigation.

The inhibitory potency of LAAO over platelet aggregation may be connected with reduced binding affinity for ADP in platelet exposed to H_2O_2 or the interference of H_2O_2 in the interaction between the activated platelet integrin αIIb/β3 and fibrinogen in the platelet-rich plasma (PRP) system (Suhr and Kim 1996). It should be considered that physiological conditions of platelet aggregation are quite different from what is measured in an aggregometer. In the instrument, platelet clumping occurs under low, nonlaminar shear conditions, whereas platelets are exposed to a broad range of hemodynamic conditions ranging from relatively low flow situations in venules and large veins (typical wall shear rates $<500\ s^{-1}$) or small arterioles (shear rates up to $5000\ s^{-1}$) to stenosed arteries with shear rates as high as $40{,}000\ s^{-1}$ (Jackson 2007). Moreover, the experimental conditions and procedures for platelet poor preparation (PPP) are vital for subsequent aggregation experiments. So the contradictory results on induction/inhibition of platelet aggregation by LAAO and

the underlying mechanism need to be revisited using the recent advancement of technology like improved live-cell imaging techniques which enable high-resolution visualization of the platelet aggregation processes operating in vitro and in vivo.

Industrial Applications

The use of LAAOs as biosensors has great potential. Biosensors utilizing bacterial L-glutamate oxidase are commercially available and are a useful tool for in vitro and in vivo monitoring of mammalian brain L-glutamate (Ryan et al. 1997). LAAO-based biosensors are used in food industries for quality assurance of edible products through evaluation of L and D enantiomers of amino acids which enter into foodstuff during fermentation of yeast and bacteria (Lee and Huh 2009; Lata and Pundir 2013). Biotransformation is another application of LAAOs which includes industrial production of α-keto acids and separation of enantiomers from racemic mixture of amino acids; particularly for the preparation of D-isomers (Takahashi et al. 1997). H_2O_2 and its precursors are often used as bleaching agent in detergents. LAAO may be advantageously incorporated, together with a substrate, into detergent compositions for generation of H_2O_2. The formulation also comprises a peroxidase system to regulate the activity of H_2O_2; otherwise, the dye may be transferred from dyed fabric to other fabrics during washing (Schneider 1994). LAAO from *Rhodococcus* sp. is successfully used in the synthesis of aminoadipic derivatives that are precursors for β-lactam antibiotics (Isobe et al. 2008). All industrial applications of LAAO are limited to microbial enzymes as they are easy to produce. Whether venom LAAO is superior remains unknown as their commercial applications require overproduction in microorganisms.

Conclusion and Future Direction

LAAOs from snake venoms and other organisms have overall structural similarity. The biological functions of LAAOs especially from snake venom on platelet aggregation, cell apoptosis, antimicrobial activity, antileishmanial activity, antitumor, and anti-HIV activity are mostly induced by H_2O_2 generated during catalysis. Enzyme-cell membrane receptor interaction seems to play certain role in inducing many of these biological effects, which need to be further established. The exact mechanism of toxicity and different means of neutralization of LAAOs await further studies. The enzyme holds great potential application in cancer therapy. Better understanding of the mode of action of the enzyme upon parasites may lead to development of new therapeutic approaches for the treatment of various bacteria, virus, and protozoa-borne diseases. This multifunctional enzyme has also gained popularity in diagnostics and industrial applications. Strong antimicrobial activity of LAAO may be utilized for future application in food and cosmetics.

Acknowledgment PB and JM were supported by UGC-NET and CSIR-NET senior research fellowships, respectively. We thank Dr. Debashree De for critical reading of the manuscript.

Cross-References

► Computational Approaches for Animal Toxins to Aid Drug Discovery
► Natural Inhibitors of Snake Venom Metallopeptidases

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Myotoxin Inhibitors

14

Norival Alves Santos-Filho, Lucas Blundi Silveira, and Johara Boldrini-França

Abstract

Myonecrosis is an important medical complication in snake envenoming and, in severe cases, can lead to drastic sequelae, such as inability and permanent tissue loss. Although the traditional serum therapy effectively neutralizes the systemic effects induced by snake venoms, there are some limitations in reversing the local symptoms induced by myotoxins, since they are small- and medium-sized proteins of low immunogenicity. Due to this impairment in current snakebite treatment, there has been an increasing interest in the search for naturally occurring molecules, as well as in the design of synthetic compounds, which are able to inhibit the main pathophysiological effects induced by snake venom PLA_2 and other myotoxins. These inhibitory molecules are mainly obtained from animal blood and plant extracts; however, a diversity of other sources of myotoxin inhibitors has also been described. This chapter aims to provide current information regarding the diversity of natural and synthetic PLA_2 inhibitors described so far. Moreover, a better understanding of the mechanisms of action involved in PLA_2 inhibition may generate perspectives to the use of these molecules leading to the development of novel therapeutic approaches for snakebite management.

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_12

Keywords
Myotoxins • Phopholipases A_2 • Myotoxin inhibitors

Contents

Introduction

Several compounds, both chemical and natural, are known to inhibit animal toxins and are promising agents for the treatment of envenomings. Some of these compounds have been extensively studied for several years, such as p-bromophenacyl bromide (BPB), phenylmethylsulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), benzamidine, and rosmarinic acid, while others are still under investigation.

The present chapter aims to report the most commonly studied myotoxin inhibitors focusing on those known to neutralize phospholipases A_2 (PLA_2s) and PLA_2-like myotoxins.

Phospholipases A_2 (PLA_2s)

Phospholipases A_2 (PLA_2s, EC3.1.1.4) are enzymes involved in metabolism of phospholipids. These enzymes catalyze the hydrolysis of fatty acids from the sn-2 position of glycerophospholipids, releasing lysophospholipids and fatty acids, in a calcium-dependent reaction (Santos-Filho et al. 2008). PLA_2s present important roles in various cellular functions, including maintenance of cellular phospholipids, generation of prostaglandins (PG) and leukotrienes, cell proliferation, and muscle contraction. Furthermore, it is known that these enzymes are involved in human inflammatory processes (Schaloske and Dennis 2006).

PLA_2s are of great medical and scientific interest due to their involvement in several human inflammatory diseases and envenoming by snakes and bees. These enzymes are widely distributed in nature and have been found in a variety of biological fluids and cells, such as bacteria, plants, mammalian tissues (the lung,

liver, spleen, and heart), erythrocytes, platelets, and leukocytes. However, the most widely known and studied PLA_2s are those found in mammalian pancreatic tissues and in reptiles and insect venoms.

Nowadays, the superfamily of phospholipase A_2 enzymes consists of 16 groups and their subgroups, including six distinct types of enzymes: secreted PLA_2s ($sPLA_2$), cytosolic PLA_2s ($cPLA_2$), Ca^{2+}-independent PLA_2s ($iPLA_2$), platelet-activating factor acetylhydrolases (PAF-AH), lysosomal PLA_2s ($LPLA_2$), and adipose-PLA_2 (AdPLA). This classification is based on PLA_2s source, amino acid sequence, catalytic mechanisms, and other biochemical, functional, and structural features (Dennis et al., 2011).

Snake venoms are rich sources of PLA_2s, which are responsible for a wide variety of pharmacological and/or toxic effects, such as myotoxicity, neurotoxicity, cardiotoxicity, inhibition of platelet aggregation, and anticoagulant, hypotensive, and edema-inducing activities (Santos-Filho et al. 2008). Snake venom PLA_2s ($svPLA_2$s) belong to groups I and II, while the enzymes from Viperidae venoms are classified in group IIA (Schaloske and Dennis 2006). The $svPLA_2$s belonging to group IIA are subdivided into subgroups of which the more studied are: (i) catalytically active Asp49 PLA_2s and (ii) PLA_2-like enzyme Lys49 that has low or no enzymatic activity but high myotoxicity.

Myotoxins

Myotoxins can be defined as natural components (usually small proteins and peptides), which induce irreversible damage to the skeletal muscle fibers (myonecrosis) after injection in animals. Some myotoxins act locally, damaging muscle fibers at the injection site, while others act systemically, causing muscle injury in distant locations (Lomonte et al. 2003).

Myonecrosis is an important medical complication of snakebites, and in severe cases, damages in the bite site can lead to drastic consequences, such as permanent tissue loss, disability, or even amputation of the limb. On the other hand, systemic myotoxicity (rhabdomyolysis) may lead to myoglobinuria and acute renal failure that is a frequent cause of death by envenomings (Lomonte et al. 2003).

Myotoxins from animal venoms can be classified into three main groups, including (i) small myotoxins, e.g., crotamin from *Crotalus durissus terrificus* venom; (ii) three-finger toxin (3FTx) cytolysins, also referred to as cardiotoxins; and (iii) PLA_2 enzymes, e.g., bothropstoxin II (BthTX-II) and the Lys49 PLA_2-like bothropstoxin I (BthTX-I) from *Bothrops jararacussu* venom. PLA_2 and PLA_2-like myotoxins form the largest group of myotoxins from animal venoms (mainly snake) (Lomonte and Rangel 2012). It is important to highlight that the focus of this chapter is to approach some structural and functional features about organic and inorganic molecules that are able to inhibit PLA_2 myotoxins.

As previously mentioned, snake venom PLA_2s comprise catalytically active enzymes and PLA_2-like myotoxins with no enzymatic activity. The latter are of great interest for scientific research because their myotoxic properties are

independent of phospholipid hydrolysis and are probably due to a pharmacologic site other than the catalytic site.

Site-directed mutagenesis assays were conducted in order to elucidate the sites that are responsible for the myotoxicity induced by Lys49 PLA_2s. The resulting mutants demonstrated the importance of a heparin-binding site in the C-terminus of the toxin, which is located in a region that comprises the amino acid residues in positions 115–129 (Lomonte and Rangel 2012). This site is mainly composed of hydrophobic and cationic amino acid residues (e.g., sequence KKYRYYLKPLCKK in myotoxin II from *Bothrops atrox*) and is responsible for its cytotoxicity (Lomonte et al. 2010) and in vivo myonecrosis effects (Lomonte et al. 2003).

Phospholipase A_2 Inhibitors (PLIs)

Snake venom toxins are known to be inhibited by various molecules, including monoclonal and polyclonal antibodies, synthetic chemicals, and compounds from animal and vegetable source.

Two well-known examples of chemical agents that are able to inhibit PLA_2 myotoxins are the low-molecular-weight heparin and p-bromophenacyl bromide (BPB). Heparin is a naturally anionic polysaccharide that binds to cationic and hydrophobic amino acid residues located in the C-terminal region of myotoxins (heparin-binding site), inhibiting the myotoxic and cytotoxic effects induced by these macromolecules. On the other hand, BPB acts by binding covalently to His48 on the catalytic site of these enzymes, thereby inhibiting their activity (Marcussi et al. 2007).

Furthermore, several plants are popularly used in the treatment of envenoming by snakes. The use of plant extracts as antidote for snakebites is an ancient option for many underserved communities, located far from public health centers. Based on popular knowledge, some scientists are now studying various plant extracts in the search for new compounds that neutralize snake venom toxins.

In addition to these neutralizing agents, there are also examples of inhibitors from animal serum, such as the antivenom complex (CAB) from *Didelphis albiventris* and *D. marsupialis* (Rocha et al. 2002), inhibitors from snakes plasma (Santos-Filho et al. 2011), and other inhibitory molecules from various sources, which will be further discussed in this chapter.

Phospholipase A_2 Inhibitors from Natural Sources

Phospholipase A_2 Inhibitors from Snake Blood

Venomous and nonvenomous snakes present PLA_2 inhibitory proteins, called PLIs, in their blood serum or plasma. A hypothesis that could explain the presence of these PLIs in serum of venomous snakes would be self-protection against their own toxins, which could eventually reach the circulatory system. However, this hypothesis does

not explain the occurrence of these inhibitory proteins in the plasma of nonvenomous snakes. The presence of PLIs in nonvenomous snakes suggests that their physiological role might not be restricted to protection against PLA_2 toxins but could be extended to other still unknown functions.

PLIs are produced in the liver of snakes, as indicated by the Northern blot and RT-PCR analysis of the genetic material extracted from different tissues (Lizano et al. 2000; Okumura et al. 2002). The production of PLIs in snake liver allows these inhibitors to fall into the bloodstream, since the liver is the main producer of plasma proteins, accelerating the mechanism of protection against accidental self-envenoming. The best explanation for the presence of these inhibitors in the bloodstream of both venomous and nonvenomous snakes is that these molecules are part of a natural resistance against predation by other snakes.

A diversity of PLIs was previously purified from plasma of different species of snakes, and their structures have been determined. These PLA_2 inhibitors are found in the blood of these animals even before possible exposure to the venom components. The blood used for PLI isolation is typically collected by cardiac puncture, through the tail vein or after decapitation of the snake. It is noteworthy that, in recent years, concerns about ethics in animal use have been growing, and for this reason, blood collection through the tail vein has been the method of choice of many researchers.

Snake plasma PLIs are acid oligomeric and globular glycoproteins which form soluble complexes with PLA_2 myotoxins, thereby inhibiting their enzymatic activity. These inhibitors are classified into α, β, and γ, according to their structural features.

Alpha-Type Phospholipase A_2 Inhibitors

Alpha-type inhibitors (αPLIs) from snake plasma are acid glycoproteins that present a region of high similarity to the carbohydrate recognition domain (CRD) of Ca^{2+}-dependent lectins (C-type lectin) and pulmonary surfactant protein (Fig. 1). This class of PLI has showed inhibition against enzymatic, myotoxic, edema-forming, and cytotoxic activities of different PLA_2s (Lizano et al. 2003; Oliveira et al. 2008; Santos-Filho et al. 2011). According to some authors, αPLIs are found in solution as oligomers, but these inhibitors are most commonly found as trimeric structures (Lizano et al. 2003; Oliveira et al. 2008; Nishida et al. 2010). The carbohydrate recognition domain (CRD) comprises approximately 67% of identity with primary sequences of each αPLI monomers and is the most conserved region in these molecules (Lizano et al. 2000). In contrast to C-type lectins, the interaction of the CRD of αPLIs with their respective ligands is Ca^{2+} independent. This is also reflected by the lack of amino acid residues involved in Ca^{2+} binding in CRD (Santos-Filho et al. 2011).

It has been reported that αPLIs from American Crotalinae snakes are selective to basic PLA_2s, while αPLIs from Asiatic snakes inhibit preferentially acid PLA_2s (Oliveira et al. 2008). αPLIs, especially those from bothropic snakes, present specificity for Lys49 PLA_2-like enzymes, which are composed of a large number of cationic and hydrophobic residues. However, the reason for these specificities and the mechanism of interaction between αPLIs and PLA_2s have not yet been elucidated (Santos-Filho et al. 2011). Interestingly, no obvious difference in amino acid

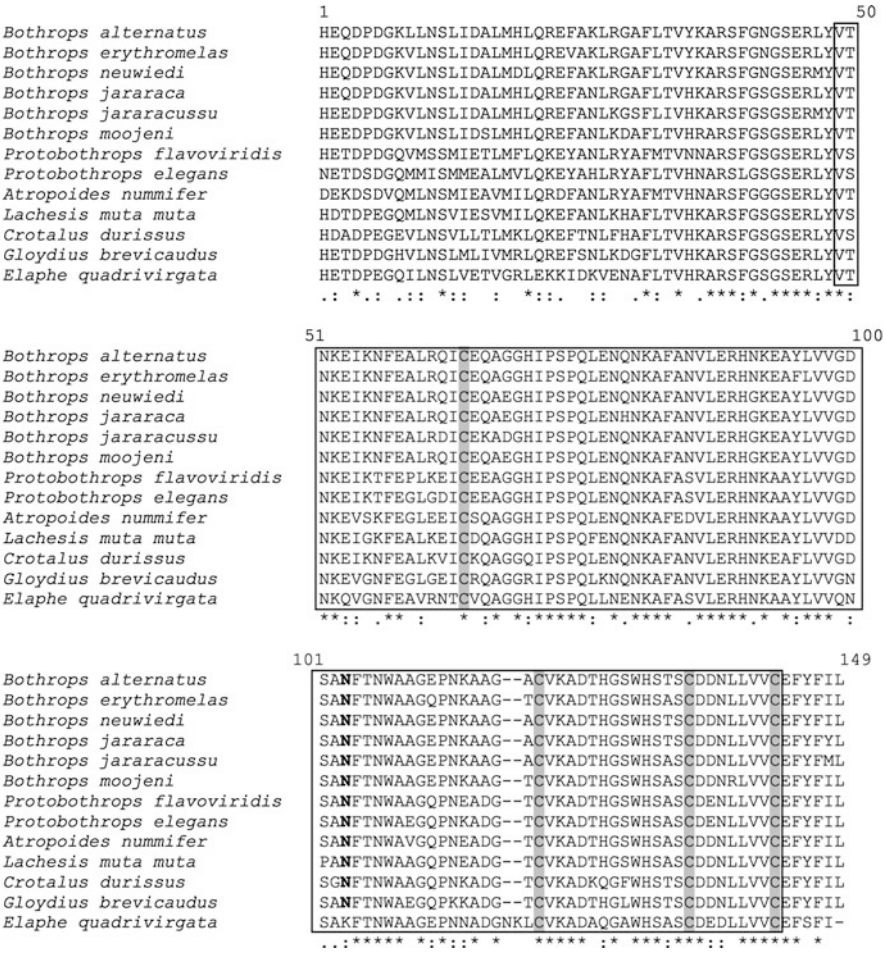

Fig. 1 Multiple sequence alignment of αPLI from GenBank database. Multiple sequence alignment was performed using CLUSTAL *X*2.1. Residues 49–143 correspond to the CRD region (*boxed* region). The residue Asn103 (in *bold*) corresponds to the glycosylation site and is preserved in all sequences. αPLIs: *Bothrops alternatus* (gi:215275473), *Bothrops erythromelas* (gi:167547095), *Bothrops neuwiedi* (gi:218546740), *Bothrops jararaca* (gi:167547111), *Bothrops jararacussu* (gi:167547115), *Bothrops moojeni* (gi:218546738), *Protobothrops flavoviridis* (gi:160357989), *Protobothrops elegans* (gi:302698922), *Atropoides nummifer* (gi:109690029), *Lachesis muta muta* (gi:218546742), *Crotalus durissus terrificus* (gi:218546744), *Gloydius brevicaudus siniticus* (gi:6467183), Elaphe *quadrivirgata* (gi:82133672)

composition that justifies these variations in specificity can be observed (Lizano et al. 2000). Thus, a hypothesis is that these inhibitors coevolved with their target toxins in a process of intraspecific specialization.

Although the molecular mechanism of interaction between αPLIs and PLA_2s is not yet completely known, some efforts have been conducted in order to elucidate

the structure of the formed complex. Some authors have suggested at least two mechanisms by which αPLIs neutralize PLA_2: (i) αPLIs bind to PLA_2 by its CRD domain, blocking the interaction of the enzyme with cell membrane by a physical impediment, and (ii) αPLIs bind directly to other regions of these myotoxins, avoiding direct contact with the membrane.

An analysis of partial deletion of amino acid residues in αPLIs was carried out with the aim of elucidating the domain responsible for PLA_2 inhibitory activity. This analysis revealed a strong dependence of inhibitory activity on CRD, especially the region comprising the residues 135–147. The importance of the residue Y144 and two hydrophobic tripeptides to PLA_2 binding was also demonstrated (Quirós et al. 2007).

Further evidences of the importance of CRD in PLA_2 binding were observed in the investigation of other PLA_2 ligands, such as mammal M-type receptor, which also contains CRD and binds to several classes of PLA_2s. Studies with M-type PLA_2 receptors from rabbits demonstrated that CRD is responsible for binding to PLA_2. It was also shown that the pulmonary surfactant protein A (SP-A) from mammals, which is another soluble protein with a CRD domain, inhibits a PLA_2 enzyme from *Trimeresurus flavoviridis* venom (Lizano et al. 2003).

Moreover, other authors suggest different regions responsible for the inhibitory activity of PLIs. Okumura and co-workers (2005) conducted a study with an αPLI from *Agkistrodon blomhoffii sinaiticus* venom and an inactive protein, similar to αPLI (αPLI-like protein). Based on chimeras between these two proteins, combined with a site-directed mutagenesis study, it was possible to suggest that αPLIs form a trimeric structure in which the N-terminal region positioned above the CRD is responsible for the trimerization, resulting in a central pore in the complex, which would be the site responsible for PLA_2 binding (Okumura et al. 2005; Nishida et al. 2010).

Beta-Type Phospholipase A_2 Inhibitors

Beta-type PLA_2 inhibitors (βPLIs) are acidic globular glycoproteins of about 160 kDa. These inhibitors are found as trimeric structures composed of monomeric subunits of about 50 kDa and 308 amino acids, presenting four *N*-glycosylation sites each (Fig. 2). It is interesting to note that the βPLI, purified from *A. blomhoffii sinaiticus*, is composed by only one kind of subunit. On the other hand, Okumura and co-workers (2002) characterized a βPLI from *Elaphe quadrivirgata* (EqPLI) that is composed of two highly homologous monomeric structures (subunits A and B). Each monomeric unit of βPLI binds to PLA_2 molecules with 1:1 stoichiometry, inhibiting mainly basic PLA_2s from group II.

βPLIs also inhibit other PLA_2s from group II, particularly those that contain residues His1, Arg6, Glu17, and Trp70 located at the interfacial binding surface of the PLA_2 molecule, suggesting that inhibition may entail binding to PLA_2 surface to prevent access to its catalytic center (Lizano et al. 2003).

The primary structure of βPLIs is characterized by an N-terminal proline-rich region, nine tandem leucine-rich repeats, and a C-terminal cysteine-rich region. Furthermore, βPLIs show 33% of homology with human α-2-glycoprotein (LRR),

Fig. 2 Multiple sequence alignment of βPLI from GenBank database. Multiple sequence alignment was performed using CLUSTAL *X*2.1. Cysteine residues are shown in *gray*. N-glycosylation sites are in *bold*. βPLIs: *Elaphe quadrivirgata* (gi:19911060), *Elaphe climacophora* (gi:226235137), *Elaphe quadrivirgata* (gi:19911058), *Gloydius brevicaudus siniticus* (gi:7994665)

a Leu-rich serum protein (Shirai et al. 2009). Since its discovery, Leu-rich repetitions have been found in more than 6,000 proteins in different organisms, with a wide array of biological functions, such as hormone receptors, enzymes, enzyme inhibitors, cell adhesion proteins, and ribosome-binding proteins (Lizano et al. 2003). These repeats are thought to play an important role in protein-ligand interactions (Lima et al. 2011). Although no in-depth study on this type of inhibitor has been performed yet, some authors believe that LRR motifs are directly involved in PLA_2 binding.

βPLIs have been identified in two distinct snake species: the venomous snake *Gloydius brevicaudus* and the nonvenomous snake *E. quadrivirgata* (Okumura et al. 2002), which is known to prey venomous snakes, including *G. brevicaudus*. According to Lima and co-workers (2011), in these cases, the presence of βPLIs in snake plasma may be a self-protection mechanism against an eventual presence of toxic venom PLA_2 in the snake circulating blood. Interestingly, a βPLI was isolated from *Elaphe climacophora* blood plasma (Shirai et al. 2009), which is a non-venomous and non-ophiophagous snake, bringing up other possible functions to PLIs in snakes beyond self-protection, such as clearance of cytochrome c released from dead cells in the circulatory system (Lima et al. 2011).

Gamma-Type Phospholipase A_2 Inhibitors

All three types of PLIs have been identified in viperid snakes, whereas only gamma-type PLA_2 inhibitors (γPLIs) have been identified in elapid and hydrophid snakes. To date, γPLIs were found in five families of snakes (Elapidae, Hydrophiidae, Viperidae, Colubrida, and Boiidae), inhibiting the pharmacological properties of Asp49 PLA_2s, such as phospholipase, inflammatory, anticoagulant, myotoxic, edema-inducing, cytotoxic, bactericidal, and lethal activities. Differences in the genes that encode the αPLIs and γPLIs suggest that these two inhibitory molecules probably have distinct evolutionary origin (Nobuhisa et al. 1997).

Gamma-type PLA_2 inhibitors comprise a growing number of plasma proteins from snakes, and, in contrast to αPLIs and βPLIs, γPLIs are rather nonspecific inhibitors, showing a broad spectrum of inhibitory activities toward PLA_2s from groups I, II, and III. These inhibitors are found as multimeric molecules composed of two homologous subunits, γPLI-A (Fig. 3) and γPLI-B (Fig. 4), except Crotalus neutralizing factor (CNF) from *C. d. terrificus* (dos Santos et al. 2005) and PLA_2 inhibitory peptide (PIP) from *Python reticulatus* (Thwin et al. 2000) that have been found in monomeric conformation, composed of only γPLI-A subunit.

Lizano and co-workers (2003) observed fundamental differences in γPLIs by biochemical analysis that may warrant further subclassification of this group into two subclasses: γPLI-I and γPLI-II. Sequence analyses of these γPLI-I indicate a heteromeric structure composed of two subunits (γPLI-A and γPLI-B). On the other hand, γPLI-IIs are oligomeric molecules composed of only one type of subunit.

Studies conducted with these PLIs revealed that γPLI-Is inhibit all classes of snake venom PLA_2s (I, II, and III), while γPLI-II seems to be specific to PLA_2 from class II. This apparent lack of specificity of γPLI-Is may result in important pharmacological implications, such as the possible neutralization of secreted human PLA_2 from class II, which is involved in inflammatory processes.

Another important feature of γPLIs is a tandem pattern of cysteine residues, characteristic of the three-finger motifs, as found in Ly-6-related proteins, such as the cell surface antigens Ly-6A/E, Ly-6C, ThB, and CD5, urokinase-type plasminogen activator receptor (u-PAR), and the snake venom α-neurotoxins (Lizano et al. 2003; Thwin et al. 2010). The three-finger motif is present in each subunit of γPLIs, which typically assemble into oligomeric structures.

It is interesting to note that three-finger proteins have been involved in some PLA_2-related physiological processes. An example is RoBo-1 (rodent bone-1), a putative modulator of bone growth and remodeling, which bears homology to γPLI inhibitors (Lizano et al. 2003). Since PLA_2 is a key modulator of prostaglandin synthesis, RoBo-1 could conceivably act as a PLA_2 inhibitor and provide a highly tissue-restricted inhibitory effect to control local prostaglandin-mediated bone formation. Alternatively, RoBo-1 could perhaps regulate the PLA_2 activity associated with the extracellular matrix vesicles produced by chondrocytes and osteoblasts; these vesicles play an important role in the mineralization process of bones (Lizano et al. 2003).

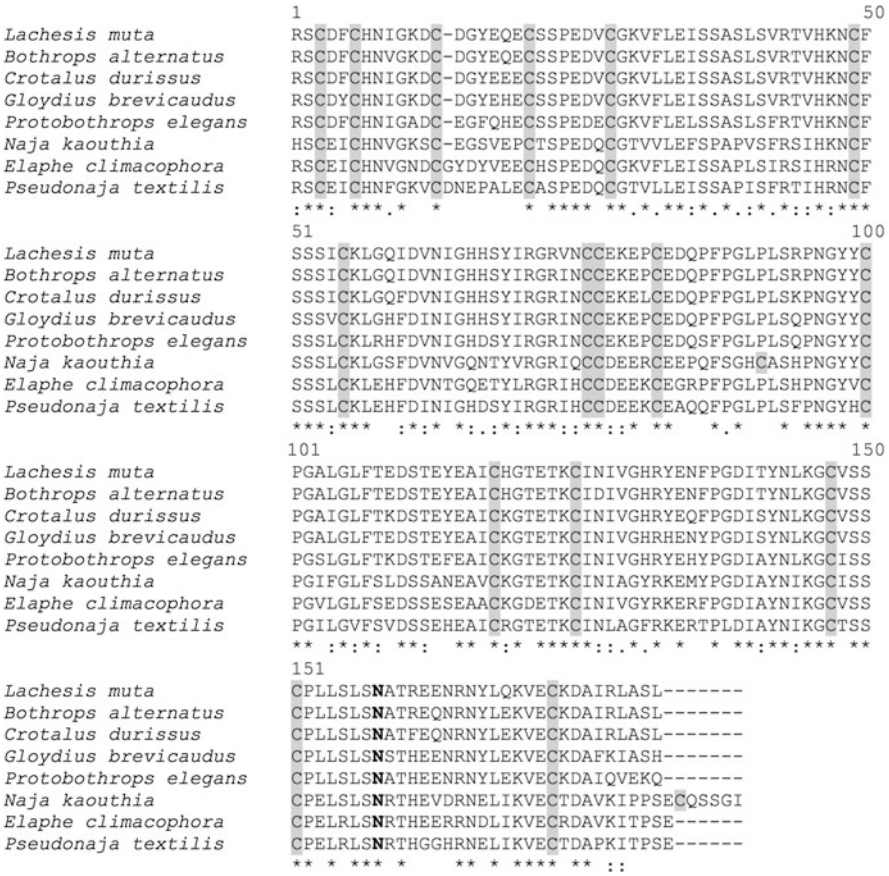

Fig. 3 Multiple sequence alignment of γPLI-A from GenBank database. Multiple sequence alignment was performed using CLUSTAL *X*2.1. Cysteine residues are shown in *gray*. N-glycosylation sites are in *bold*. γPLIs-A: *Lachesis muta muta* (gi:45477024), *Bothrops alternatus* (gi:157885058), *Crotalus durissus terrificus* (gi:45477157), *Gloydius brevicaudus siniticus* (gi:26454644), *Protobothrops elegans* (gi:302698902), *Naja naja kaouthia* (gi:47117137), *Elaphe climacophora* (gi:226235139), *Pseudonaja textilis* (gi:6685096)

As well as αPLIs and βPLIs, γPLIs apparently neutralize PLA_2 activity by forming complexes with these enzymes. An example is CNF (Fortes-Dias et al. 1999) that forms a complex with crotoxin by interacting only with crotoxin B subunit, forming a stable enzymatically inactive CNF-CB complex between one CNF subunit and CB (1:1 molar ratio). Some authors suggest that the direct binding of PLA_2 to γPLIs occurs in the well-conserved γPLI-A subunit, since the direct binding of PLA_2 to the γPLI-A subunit has been observed for the *P. reticulatus* PIP (Thwin et al. 2000).

Despite the importance of γPLIs as potent PLA_2 inhibitory agents, the potential of these inhibitors in venom neutralization therapy has been poorly explored.

Fig. 4 Multiple sequence alignment of γPLI-B from GenBank database. Multiple sequence alignment was performed using CLUSTAL *X*2.1. Cysteine residues are shown in gray. N-glycosylation sites are in bold. γPLIs-B: *Elaphe climacophora* (gi:226235141), *Elaphe quadrivirgata* (gi:23396801), *Naja naja kaouthia* (gi:47117138), *Pseudonaja textilis* (gi:6644406), *Gloydius brevicaudus siniticus* (gi:26423285)

A potential neutralizing molecule is γPLI-II from *Cerrophidion godmani* (CgMIP-I), which has been shown to inhibit the myotoxic properties of myotoxin I (Lizano et al. 2000). PIP has also been able to neutralize the toxic and edematogenic properties of several PLA_2s from snake and bee venoms, especially those from class II. Furthermore, a peptide based on PIP sequence (amino acid residues 89–109) also exhibited antitoxic and anti-inflammatory properties and was able to bind to various venom PLA_2s in a similar way to that of native PIP (Thwin et al. 2010). Thus, these reports show the potential of γPLIs as leads to the design of new therapeutic agents for treatment of some inflammatory process and envenoming by venomous animals.

Phospholipase A_2 Inhibitors from Other Animal Sources

It is known that some animals, such as certain mammals (e.g., hedgehog, opossum, and mongoose), are immune to snake venoms. This can be explained by the presence of neutralizing factors in their blood, which inhibit important toxic components. Many studies have been carried out in search for these natural snake PLA_2 inhibitors (Marcussi et al. 2007).

The first report of a myotoxin inhibitor with an immunoglobulin-like structure from animal blood is DM64, an antimyotoxic protein from opossum (*Didelphis marsupialis*) serum. It is an acidic protein showing 15% of glycosylation. Its amino acid sequence was found to be similar to DM43, a metalloproteinase inhibitor also from *D. marsupialis* serum, and to human α_1B-glycoprotein, indicating the presence of five immunoglobulin-like domains. DM64 neutralized both the in vivo myotoxicity and in vitro cytotoxicity of myotoxins I (MT-I/Asp49) and II (MT-II/Lys49) from *Bothrops asper* venom. Accordingly, DM64 did not neutralize the anticoagulant effect of MT-I nor its intracerebroventricular lethality, effects that depend on its enzymatic activity, and which demonstrate the dissociation between the catalytic and toxic activities of this Asp49 myotoxic PLA_2 (Rocha et al. 2002).

Furthermore, hyperimmunization of animals with myotoxins or peptides from these molecules has been used to produce PLA_2 inhibitors. Previous studies have identified a stretch of cationic and hydrophobic amino acid residues near the C-terminus region (115 ± 129 in the numbering system of Renetseder et al. 1985) of PLA_2s, which is associated with toxicity. With that in mind, Calderon and Lomonte (1999) used a synthetic peptide comprising residues 115 ± 129 of myotoxin II from *B. asper*, which is bactericidal and cytotoxic to cultured endothelial cells, as an immunogen in mice, to explore the possible protection against in vivo myotoxic activity induced by myotoxin II.

After the immunization, mice were challenged with myotoxin II, and the extent of myonecrosis was estimated and compared to non-immunized mice. After the challenge, it was observed a significant reduction of myonecrosis, which demonstrates that region 115 ± 129 of myotoxin II constitutes a neutralizing epitope and is important to its in vivo myotoxic effect (Calderón and Lomonte 1999).

Myotoxin I from *B. asper*, the first myotoxic PLA_2 isolated from *Bothrops* sp. venom, was utilized to isolate its specific antibodies from equine antivenom, as well as to prepare a monospecific antiserum in rabbits. Both the equine and rabbit antibodies were able to neutralize the myotoxic activity of this protein in mice and also to prevent about 70–80% the muscle damage induced by crude *B. asper* venom. Interestingly, quantitative neutralization studies using anti-myotoxin antibodies first revealed the separation between the PLA_2 activity and the myotoxic effect of this protein. Its myotoxicity persisted at antiserum/toxin ratios around which its enzymatic activity was completely abolished (Lomonte et al. 2009).

Antibodies against myotoxin II, another myotoxin from *B. asper* venom, were isolated from the therapeutic antivenom produced in Costa Rica and utilized to demonstrate the high antigenic cross-reactivity between the catalytically active (Asp49) myotoxins and the catalytically inactive (Lys49) ones. Remarkably, antibodies to myotoxin II, which lack catalytic activity, neutralized the PLA_2 activity of myotoxin I, confirming their high antigenic similarity (Lomonte et al. 1990a). Neutralization studies on myotoxin II showed that a complete inhibition of its myotoxic activity occurs at an antibody:toxin molar ratio of 2:1 or higher, implying the existence of both neutralizing and non-neutralizing epitopes in its structure. At least one neutralizing epitope in myotoxin II was subsequently mapped at its regions 115–129, near its C-terminus, given the fact that antibodies raised against a synthetic

peptide containing this sequence inhibited its in vitro and in vivo toxic actions (Lomonte et al. 2009).

Another PLA_2 inhibitor was found in the water extract of *Pavo cristatus* feathers (PCF). Its inhibitory properties were checked against different PLA_2 enzymes from *Vipera russelii*, *Naja naja*, and *Trimeresurus malabaricus* snake venoms; inflammatory fluids, like pleural and Ehrlich ascites fluids; and acid-extracted serum samples. The water extract of PCF inhibited all PLA_2 enzymes in a dose-dependent manner. The least inhibited PLA_2 enzyme was that of *Naja naja* snake venom (55% inhibition). All other snake venom, inflammatory fluids, and serum PLA_2 enzymes are $>$80% inhibited (Murari et al. 2005).

The feather extract presented positive results for the presence of macromolecules and small molecules, like proteins and steroids, respectively, besides several other metal ions. The nature of inhibitory compounds was checked using several parameters. The feather extract was subjected to extensive dialysis, ammonium sulfate precipitation, acid hydrolysis, and boiling. The inhibitory compound loses its activity on heating and on acid hydrolysis. On the other hand, the inhibitory activity was retained after dialysis, indicating that the inhibitor is a macromolecule or an aggregation product of micromolecules. On 100% ammonium sulfate precipitation, the inhibitory activity of the extract was retained in the supernatant and not in the precipitate fraction, ensuring that the inhibitory molecule is not proteic.

Phospholipase A_2 Inhibitors from Plants

Medicinal plants are largely used for the treatment of snakebites since ancient times. Nowadays, this kind of therapeutic is specially applied in folk medicine by populations with no easy access to serum therapy. Beyond their use in folk medicine, a growing number of scientific studies have reported the neutralizing activity of crude plant extracts against some local and systemic effects of snake venoms, thus demonstrating their potential use as antivenoms or as supplements for conventional serum therapy. Despite the recognized antiophidian activity displayed by diverse vegetal species, only few active molecules have been isolated and characterized.

The active compounds present in crude extracts are mainly generated by plant secondary metabolism and include flavonoids, terpenoids, alkaloids, proteins, coumarin, coumestans, caffeic acid derivatives, among others (Fig. 5). These compounds may act as enzymatic inhibitors, immunomodulators, and chemical inactivators or can block the interaction of toxins with receptors, resulting in the neutralization of some pathophysiological effects induced by snake venom toxins.

Some plant extracts or isolated molecules are capable of inhibiting PLA_2 activity, leading to suppression of the biological effects caused by these toxins, such as inflammation, myonecrosis, and neurotoxicity. The mechanism of action of most PLA_2 inhibitors from plants has not been elucidated yet, although many studies demonstrate that the blockage of the hydrophobic channel, responsible for substrate binding, is an efficient way to inhibit some pharmacological effects induced by this class of toxins.

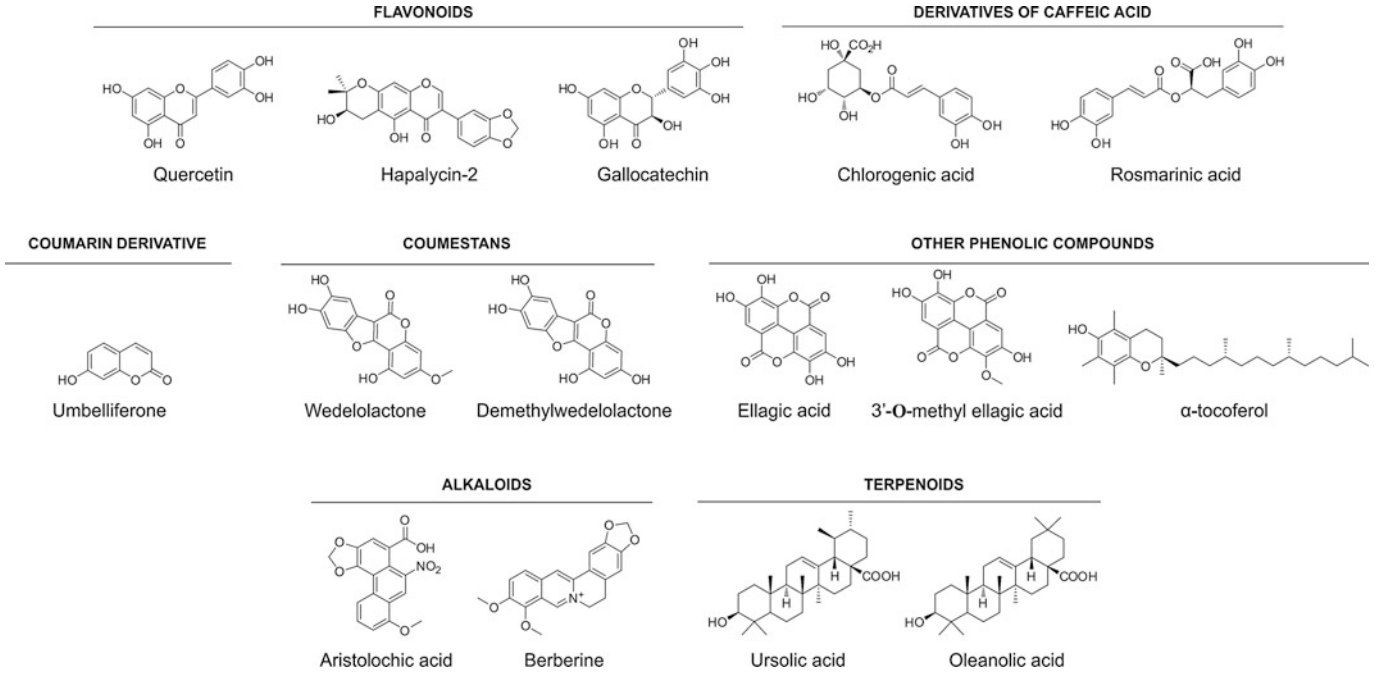

Fig. 5 Structure of PLA_2 inhibitors from plants. Structures were drawn using the ChemBioDraw® Ultra 13.0 software. Molecular structures were kindly provided by Lucas Cunha Dias de Rezende

Plant Extracts

Several plant extracts have been reported to inhibit the enzymatic activity of snake venom PLA_2, as well as the pathophysiological effects caused by this class of toxins. Most of these extracts are obtained from the leaves, but there are also active extracts obtained from other parts of the plant, such as bark, root, seeds, fruits, exudates, and whole aerial and subterraneous parts. These plant extracts are commonly prepared as infusions, macerations, teas, and decoction and may be administered orally or applied directly in the bite site.

The extract from *Casearia sylvestris* is an important source of PLA_2 inhibitors. In a study conducted by Borges and colleagues (2000), the crude aqueous extract from *C. sylvestris* leaves partially neutralized PLA_2 activity of Elapidae and Viperidae snake venoms and effectively inhibited myotoxic activity of both purified Asp49 and Lys49 PLA_2s from *Bothrops asper*, *Bothrops jararacussu*, and *Bothrops pirajai*. Moreover, the extract inhibited anticoagulant activity of several PLA_2 and reduced the edema-inducing activity of *Bothrops moojeni* and *B. jararacussu* venoms and isolated MjTX-II and BthTX-I myotoxins. The authors also demonstrated the capacity of the extract in enhancing the survival of mice injected with lethal doses of several snake venoms and purified PLA_2s. In a more recent study, the *C. sylvestris* demonstrated protective activity against neuromuscular blocking induced by Asp49 PLA_2 (crotoxin A from *C. durissus terrificus*) and Lys49 PLA_2s (bothropstoxin I from *B. jararacussu*, piratoxin-I from *B. pirajai*, and myotoxin II from *B. moojeni*) in mouse phrenic-diaphragm preparations (Cavalcanti et al. 2007). The extract was also able to inhibit the muscle damage caused by Lys49 PLA_2s, thus reinforcing the potential of *C. sylvestris* as a source of PLA_2 inhibitors (Borges et al. 2000).

The aqueous extract of the subterranean system of *Mandevilla velutina* was shown to be a PLA_2 inhibitor even more potent than *C. sylvestris* (Biondo et al. 2003). *M. velutina* is found in Brazilian savanna, and its rhizome infusion has been traditionally used in the treatment of snakebites and common inflammatory processes in some regions of Brazil. The extract completely inhibits the PLA_2 and myotoxic activity of *C. d. terrificus* crude venom and purified crotoxin B. On the other hand, the extract showed partial inhibition of *Bothrops* venoms and was almost ineffective against isolated bothropic PLA_2.

The *Morus alba* leaf aqueous extract has been assayed against local and systemic effects caused by the Indian *Daboia russelii* venom (Chandrashekara et al. 2009). The venom proteolytic and hyaluronolytic in vitro activities were completely neutralized by the plant extract, as well as edema and hemorrhage effects induced in experimental animals. The *Morus alba* extract also effectively inhibited the venom-induced myonecrosis, indicating an anti-PLA_2 property.

In a recent study, Strauch and colleagues (2013) demonstrated the phospholipase A_2 inhibitory activity of ethanolic root extract of *Humirianthera ampla* against venoms of *B. atrox*, *B. jararacussu*, and *B. jararaca* snakes. *H. ampla* belongs to the Icacinaceae family and is popularly used in treatment of snakebites by rural communities in Amazon, but the therapeutic properties of this extract had not been scientifically proven so far. In this study, the ethanolic extract from its roof was able

to partially inhibit in vitro and in vivo myotoxicity induced by those venoms in a dose-dependent manner. The PLA_2 inhibitory effect was obtained both by pre-incubation with venoms and posttreatment, what gives support to the folk treatment carried out in Amazon. The root extract from *H. ampla* also showed inhibitory activity against edema induced by *Bothrops* venoms, particularly by the pre-incubation protocol, indicating an anti-inflammatory effect (Strauch et al. 2013).

Bark extracts from *Dipteryx alata*, obtained with different polar and apolar solvents, were tested against neurotoxic and myotoxic effects caused by *B. jararacussu* and *C. d. terrificus* venoms (Nazato et al. 2010). Prior to testing of the neutralization properties of the bark extracts, the authors analyzed the hydroalcoholic extracts from *D. alata* by thin layer chromatography in order to monitor the presence of some compounds in the samples. They observed that the presence of tannins in the hydroalcoholic extracts leads to an enhancement in the neutralization of neuromuscular blockage promoted by *B. jararacussu* venom. Furthermore, when extracts obtained by different solvents were tested, it was possible to note a high neuromuscle blockage and myotoxicity protection of methanolic extract and a partial protection of dichloromethane extract by preincubation with *B. jararacussu* venom. On the other hand, none of the extracts were able to protect against neuromuscular paralysis induced by *C. d. terrificus* venom, indicating that the antivenom properties of plant extracts may be specific to some classes of snakes.

Musa paradisiaca is a widely distributed tropical plant and is a source of various biologically active compounds, such as dopamine, noradrenaline, serotonin, and antihyperglycemic factors. The *M. paradisiaca* juice or exudate, when previously incubated with Crotalidae venoms, effectively inhibited the PLA_2, myotoxic, and hemorrhagic activities and lethality in mice. However, when the exudate and venom were administered by separate routes or at different times, no protection was observed, indicating that the neutralizing properties of *M. paradisiaca* exudate is due to interspecific association of its compounds with venom proteins (Borges et al. 2005).

A diversity of other plant extracts has demonstrated the ability to neutralize PLA_2 catalytic and pharmacologic effects, including myotoxicity. In the last two decades, a number of reviews have been published in an attempt to report several plant species around the world that may represent great sources of PLA_2 inhibitors. These reports highlight the importance of ethnopharmacological studies that may drive the discovery and design of new inhibitory molecules and the development of improved therapeutic approaches for the treatment of snakebites.

Phenolic Compounds

Flavonoids

Flavonoids are polyphenolic compounds that belong to a widely distributed group of plant secondary metabolites. These natural compounds are among the most versatile ones due to the possibility of making different arrangement of hydrogen and/or glycoside bonds around the carbon skeleton. Therefore, flavonoids are able to

interact with a variety of biological polymers, which makes these molecules good candidates for pharmacologically active agents. Flavonoids are known to interfere in the pathways related to inflammation and immune response and are also capable of inhibiting PLA_2 activity and preventing the arachidonic acid release. Some examples of flavonoid PLA_2 inhibitors are rutin, hipolaetin-8-glucoside, quercetin, genistein, gallocatechin, harpalycin 2, tectoridin, curcumin, and morin. Quercetin and harpalycin 2 are known to inhibit the enzymatic activity of PLA_2s probably by binding in the proximity of His48 and Asp49 residues in the catalytic site, thus affecting the ability of these enzymes to interact with the substrates and bind to Ca^{2+} ion (Cotrim et al. 2011; Ximenes et al. 2012). Gallocatechin presents similar inhibitory mechanism to Lys49 PLA_2s, binding next to His48 and Lys49 residues into active site. In this case, the residue Lys69 apparently plays important role, assuming a different conformation in order to allow the ligand binding (Vale et al. 2011). Moreover, the binding of flavonoid inhibitors to PLA_2s induces secondary conformational changes and the modification of some amino acids in the molecule, leading to the decrease of its functionality (Cotrim et al. 2011; Ximenes et al. 2012).

The binding of these flavonoid inhibitors to PLA_2s leads to an effective inhibition of its enzymatic, myotoxic, edematogenic, platelet aggregation, and antibacterial activities. However, in some cases, certain PLA_2 pharmacological effects do not seem to be neutralized by flavonoids, such as inflammation and neurotoxicity (Iglesias et al. 2005; Cotrim et al. 2011). These evidences indicate that PLA_2 pharmacological effects depend not only on the catalytic site but also on another active domain, probably located near to the C-terminus of the molecule, which is not inhibited by flavonoids.

Caffeic Acid and Its Derivatives

Caffeic acid and its derivatives are phenolic compounds produced by many species of plants. Caffeic acid is derived from cinnamic acid and is found in some medicinal plants traditionally used in snakebites, such as *Prestonia coalita* and *Strychnos nux-vomica*. This compound inhibits Lys49 PLA_2 by interacting with its C-terminus region, which is believed to be responsible for the myotoxic effect induced by non-catalytically active PLA_2 (Shimabuku et al. 2011).

Chlorogenic acid, which is the ester of caffeic acid and quinic acid, is considered a good candidate for PLA_2 inhibitor due to its ability to interact with Asp49, Lys69, and Trp31 into the active site of Russell's viper PLA_2, forming a stable complex (Nirmal et al. 2008).

Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxylphenyl lactic acid and is one of the active compounds of plants from Boraginaceae and Lamiaceae families. Rosmarinic acid inhibits both muscle damage and neuromuscular blockage induced by snake venom PLA_2. Unlike caffeic acid, rosmarinic acid does not interact with the C-terminus region of PLA_2s but binds at the entrance of the hydrophobic channel in the active site of these molecules, resulting in a physical blockade that prevents the access of substrate (Dos Santos et al. 2011).

Coumestans and Coumarin Derivatives

Coumarins are phenylpropanoid compounds found naturally in many plants and in some bacteria and fungi. A diversity of pharmacological activities has been attributed to coumarin and its derivatives, such as anticoagulant, antihypertensive, anti-inflammatory, antimicrobial, antifungal, antimyotoxic, and analgesic, among other effects. Hydroxycoumarin (7-HOC), also known as umbelliferone, is a coumarin derivative that inhibits enzymatic and in vivo myotoxic and inflammatory activities of a PLA_2 from *Crotalus durissus collilineatus* venom. The interaction of 7-HOC with the PLA_2 leads to a significant alteration in the secondary structure of the enzyme, decreasing the alpha-helix content in its active site. Moreover, the 7-HOC induces the dissociation of the dimeric PLA_2, thus inhibiting its enzymatic activity (Toyama et al. 2009).

Coumestans represent a group of naturally occurring aromatic compounds similar to coumarin. Wedelolactone and its demethylated form, demethylwedelolactone, are found in *Eclipta alba* and are the main responsible for the antivenom property of this plant extract. These coumestans efficiently inhibit the myotoxic activity of two basic PLA_2s from *C. d. terrificus* and *B. jararacussu*, although the inhibitory mechanism of these compounds remains unknown (Diogo et al. 2009).

Other Phenolic Compounds

Ellagic acid is a phenolic secondary metabolite found in many plants, especially red fruits and berries. Four ellagic acid derivatives from *Casearia sylvestris* (ellagic acid, 3′-*O*-methyl ellagic acid, 3,3′-di-*O*-methyl ellagic acid, and 3-*O*-methyl-3′, 4′-methylenedioxy ellagic acid) present inhibitory properties against an Asp49 PLA_2 from *Bothrops jararacussu* (BthTX-II), neutralizing the enzymatic, edematogenic, and myotoxic activities induced by this toxin. Ellagic acid and 3′-*O*-methyl ellagic acid inhibited PLA_2 activities in a more effective way, probably due to the presence of hydroxyls at position C3 or C3′. These polar groups may favor the interaction of benzopyrene rings with the hydrophobic amino acids Phe5, Trp31, and Phe98 in the active site and can also allow hydrogen bond formation between the hydroxyls at C4 and C4′ with the amino acids His48 and Asp49. On the other hand, 3,3′-di-*O*-methyl ellagic acid and 3-*O*-methyl-3′,4′-methylenedioxy ellagic acid present methoxyl groups in positions C3 and C3′ that are located in polar regions in the active site, generating repulsive forces that destabilize the formed complex, decreasing the inhibitory properties of these compounds (Da Silva et al. 2008).

Another phenolic compound known to present inhibitory activity against snake venom PLA_2 is α-tocopherol (α-TP), which is a form of vitamin E. This phenolic compound acts as a competitive inhibitor of PLA_2, and the interaction of α-tocopherol with a dimeric PLA_2 from *Daboia russelii* venom was investigated by the co-crystallization of the formed complex. α-tocopherol binds specifically to one of the molecules that forms the dimer by the interaction of an OH group from the aromatic structure of α-TP with the residues His48 and Asp49 in the active site of PLA_2. The inhibitor also makes interaction with the residue Trp31, resulting in an alteration on its side chain that is oriented toward the hydrophobic channel, obstructing the passage of substrate. The α-TP also makes a diversity of hydrophobic

interactions with the PLA_2 molecule, what enhances the stability of the formed complex, thus improving the inhibitory properties of this phenolic compound (Chandra et al. 2002b).

Alkaloids

Alkaloids are potent pharmacologically active molecules generally found in higher plants and in a diversity of other living organisms. Aristolochic acid (9-hydroxy-8-methoxy-6-nitrophenanthro(3,4-d)-1,3-dioxole-5-carboxylic acid), from *Aristolochia* sp., is the most studied alkaloid with anti-PLA_2 activity. This alkaloid inhibits the enzymatic and edema-inducing activities of *Daboia russelii* (Russell's viper) PLA_2s but fails to neutralize other pharmacological effects of these enzymes, such as necrosis and hemorrhage in the liver and kidney, internal bleeding, and lethality (Vishwanath and Gowda 1987). The co-crystallization of the inhibitor with an asymmetric dimer of *D. russelii* PLA_2 revealed that aristolochic acid interacts with the entrance of hydrophobic channel of one of the two molecules of the complex by its aromatic domain, so that its OH group forms two hydrogen bonds with His48 and Asp49 in PLA_2 active site. Aristolochic acid also forms hydrogen bond with Trp31, resulting in a conformational alteration in its flexible side chain that moves it toward the entrance of hydrophobic channel (Chandra et al. 2002a).

Berberine is an isoquinoline alkaloid from the herbaceous climber *Cardiospermum halicacabum*, which is a plant traditionally used in Ayurveda for the treatment of snakebites. As well as aristolochic acid, this compound binds in the active site of PLA_2 from Russell's viper venom, forming a hydrogen bond to His48. However, unlike the sort of interaction typically accomplished by other PLA_2 inhibitors, berberine also makes hydrogen bond to Gly30 of the enzyme. Beyond these chemical bonds, the inhibitor establishes many van der Waals contacts with the hydrophobic moieties of amino acid residues in its vicinity, indicating that berberine is a high affinity PLA_2 inhibitor. Moreover, the interaction of this inhibitor to PLA_2 results in a conformational change in the calcium-binding loop that constitutes one of the walls of the hydrophobic channel (Chandra et al. 2011).

Terpenoids

Terpenoids are naturally occurring organic chemicals similar to terpene. These molecules are composed of an arrangement of isoprene units and differ from one another by functional groups and carbon skeleton. Based on the number of isoprene units, terpenoids are classified as hemiterpenoids (1 unit), monoterpenoids (2 units), sesquiterpenoids (3 units), diterpenoids (4 units), sesterterpenoids (5 units), triterpenoids (6 units), tetraterpenoids (8 units), and polyterpenoids (>8 units). Terpenoids are largely used in traditional medicine and present a diversity of pharmacological applications, such as anticancer, anti-malaria, anti-inflammatory, and antimicrobial.

Diterpenoids and triterpenoids are the classes of plant terpenoids known to act as PLA_2 inhibitors. Studies indicate that the triterpenoids bind directly in the catalytic site of PLA_2s, acting as an irreversible and kinetically competitive inhibitor. The triterpenoids 1 and 2 from berries of *Schimus terebinthifolius* (pink peppercorn)

make hydrogen bond to His48 of PLA_2 by their carboxyl group, located in the side chain, preventing both Ca^{2+} binding and substrate interaction (Jain et al. 1995). Ursolic acid is another triterpenoid that inhibits PLA_2 from both groups I and II by directly interacting with the enzymes to result in the formation of enzyme-ursolic acid complex, leading to its unfolding (Nataraju et al. 2007). Similar action mechanism was reported for the triterpenoid oleanolic acid, which inhibited secretory PLA_2s activity probably by binding to the enzymes, leading to its conformational change (Dharmappa et al. 2009). On the other hand, the diterpenoid columbin inhibits PLA_2 in a noncompetitive manner, suggesting a binding site other than the catalytic domain (Nok et al. 2002).

Proteins

PLA_2 inhibitors are generally compounds of low molecular mass, with some exception, such as inhibitors from snake plasma (PLIs) that are large multimeric glycoproteins. Although less common, two proteic compounds from plants have been reported to present PLA_2 inhibitory activity: tumerin from *Curcuma longa* L. and WGS from *Withania somnifera*. Tumerin is a protein of about 14 kDa and is known to inhibit the enzymatic activity of a PLA_2 from *Naja naja* venom and is also able to neutralize some pharmacological effects induced by this protein, such as cytotoxicity, edema, and myotoxicity (Chethankumar and Srinivas 2008). WGS is an acid glycoprotein of about 27 kDa, similar to the α-chain of PLIs. This glycoprotein neutralizes the enzymatic and pharmacological properties of multifunctional PLA_2s from *Naja naja* venom. Although WGS is glycoprotein, its carbohydrate moieties are not involved in the inhibitory activity. As well as tumerin, WGS inhibits the cytotoxicity, myotoxicity, and edema-inducing activity of PLA_2 from cobra venom but failed to neutralize its neurotoxicity. The WGS is ineffective to inhibit acid PLA_2s and demonstrates selective inhibitory activity to basic enzymes, indicating that this inhibitor binds to PLA_2 by electrostatic interactions between negatively charged groups of the acid glycoprotein and positively charged groups of the basic PLA_2. However, the selectivity of WGS to some basic PLA_2 suggests the importance of specific conformation for the groups involved in inhibitor binding (Machiah and Gowda 2006).

Marine Phospholipase A_2 Inhibitors

The ocean is also a source of PLA_2 inhibitors. An example of that is fucoidan, a complex sulfated polysaccharide extracted from marine algae. Although sharing some biological properties with heparin, sulfated fucans constitute alternative polyanionic molecules with a range of different pharmacological profiles.

Fucoidan efficiently inhibits the cytotoxic (in vitro) and myotoxic (in vivo) effects induced by a group of myotoxic PLA_2s purified from different Crotalinae venoms. Regarding two catalytically active (Asp49) PLA_2s, fucoidans inhibit their toxic actions without affecting their ability to hydrolyze phospholipids, demonstrating once more the dissociation between toxicity and enzymatic activities in Asp49

myotoxins (Angulo and Lomonte 2003). The inhibitory mechanism of fucoidan appears to be the rapid formation of complexes, probably mediated through multivalent, electrostatic interactions between the anionic sulfates of the polysaccharide and the numerous cationic residues of PLA_2, which have highly basic isoelectric points.

The possible binding site of fucoidan on these myotoxins was investigated using short synthetic peptides that represent the membrane-damaging region (residues 115–129) of three toxins, and it was observed that fucoidan clearly inhibited the cytolytic activity of all synthetic peptides, demonstrating its ability to interact with the C-terminal myotoxic region of these phospholipases A_2 (Angulo and Lomonte 2003). The region comprising residues 115–129 of Lys49 myotoxic PLA_2s, such as *B. asper* myotoxin II, was previously shown to constitute a heparin-binding site, and to be involved in its in vitro cytolytic action, where fucoidan appears to act in a similar mode as heparin.

Recent interest in marine natural products has led to the isolation of several new terpenoids from sponges, which are probably secondary metabolites. Among them, manoalide was shown to be analgesic and to antagonize phorbol-induced inflammation, but not that induced by arachidonic acid, suggesting that manoalide may act before the cyclooxygenase step in prostaglandin synthesis. Lombardo and Dennis (1985) demonstrated that manoalide is a potent inhibitor of cobra venom phospholipase A_2, and it is shown to react irreversibly with lysine residues in the enzymes (Lombardo and Dennis 1985).

Scalaradial (SLD), a 1,4-dialdehyde marine terpenoid that was isolated from sponge (*Cacospongia mollior*), possesses in vivo and in vitro anti-inflammatory properties. SLD is a potent inactivator of bee venom PLA_2 in a time-dependent manner. The rate of inactivation was reduced markedly in the presence of excess of phosphatidylcholine, suggesting that modification of the enzyme occurs at or near the substrate-binding site. Due to its peculiar dialdehyde structural feature, it has been proposed that scalaradial exerts its enzymatic inactivation by means of an irreversible covalent modification of its target (Perumal Samy et al. 2012).

Synthetic Phospholipase A_2 Inhibitors

Suramin is a highly charged polysulfonated compound. One of the first successful synthetic therapeutic agents clinically used in the treatment of African trypanosomiasis and onchocerciasis. It binds to a wide variety of proteins of different function, structure, and size due to its torsional flexibility and ability to stretch or compress itself.

Through X-ray modeling, Murakami et al. 2005 analyzed the binding of suramin to Basp-II from *B. asper* venom, indicating that this binding results in a rigid-body shift of the motif formed by the calcium-binding loop and C-terminus. The C-terminal region does not bind to suramin but contributes significantly to the maintenance of the positive charge on the interfacial recognition face (i-face), which is important for the binding of myotoxin to phospholipid bilayers. This

structural change inhibits the in vitro and in vivo myotoxic activity of Basp-II, when preincubated or administered alone after injection of the toxin (Murakami et al. 2005).

Lactones are esters formed from the cyclization reaction between a hydroxyl group and another acid in the same molecule. Lactones with five or six carbons are more stable due to their low-tension energy in the ring. Some studies have demonstrated the capacity of different lactones to inhibit phospholipase A_2. Alvarenga and co-workers (2011) synthesized eight sesquiterpene lactone compounds and evaluated their ability to inhibit some of the toxic effects of both whole venom and PLA_2 isolated from *B. jararacussu* venom. Compounds Lac01 and Lac02 reduced myotoxicity by approximately 70%. Compounds Lac03 and Lac04 reduced the myotoxic activity by approximately 56%, while compounds Lac05–Lac08 did not demonstrate any activity against myotoxic effects. The kinetic parameter value shows that the four active synthetic lactones inhibit PLA_2 in a noncompetitive manner, meaning that the binding site of these inhibitors might be different from the active site of the enzyme (de Alvarenga et al. 2011).

Chemical Phospholipase A_2 Inhibitors

PLA_2s can undergo chemical modifications at different amino acid residues, according to the used reagent. His48 is alkylated by p-bromophenacyl bromide (BPB) or methylated by methyl-p-nitrobenzenesulfonate. Lys residues can be carbamylated by potassium cyanide (KCN), guanidinated by o-methylisourea, trinitrophenylated by trinitrobenzene sulfonic acid (TNBS), or acetylated by acetic anhydride (AA). Tyr can be sulfonated by 2-nitrobenzenesulfonyl fluoride (NBSF), while Trp is sulfonated by 2- nitrobenzenesulfonyl chloride (NPSC) or alkylated by 2-hydroxy-5-nitrobenzyl bromide (HNB-Br). Met can be oxidized by chloramide or carboxymethylated by iodoacetic acid. Cyanogen bromide (CNBr) is responsible for cleavage of PLA_2s releasing the N-terminal octapeptide as previously described for piratoxins I and II from *B. pirajai* (Marcussi et al. 2007).

Two widely studied examples of chemical agents capable of inhibiting the action of PLA_2 and myotoxins are low-molecular-weight heparin and p-bromophenacyl bromide (BPB). Heparin is a natural anionic polysaccharide that binds to a cationic hydrophobic region (heparin-binding site) located in the C-terminal portion of myotoxins, thus inhibiting the myotoxic and cytotoxic activity of these macromolecules. By a different mechanism, BPB binds covalently to the His48 present on the catalytic site of these enzymes, thereby inhibiting its activity. The alkylation of residue His48 by BPB promotes total or partial loss of the phospholipids' hydrolysis capability and additionally can induce reduction of toxic effects of these enzymes (Soares and Giglio 2003; Marcussi et al. 2007).

It was shown by Marchi-Salvador and co-workers (2009) that PrTX-I, a Ly49 PLA_2 from *B. pirajai*, when complexed with BPB, suffered important tertiary and quaternary structural changes. The reduction of its myotoxicity after the ligand interaction may be related to these structural changes (Marchi-Salvador et al. 2009).

When PhTX-I form *Porthidium hyoprora* was treated with different chemical modifiers, such as p-bromophenacyl bromide (BPB), p-nitro benzenesulfonyl fluoride (NBSF), o-nitrophenylsulfenyl chloride (NPSC), and 4-nitro-3-(octanoyloxy) benzoic acid, it was possible to observe that acetylation of Lys residues significantly reduced the enzymatic activity; however, a 26% residual activity was detected, as observed also for MT-III (*B. asper*) and MT-I (*B. godmani*). The mode of specific acetylation is not clear, but there are some evidences of reduction of the calcium-binding capacity, thereby reducing the enzymatic activity of PLA_2. On the other hand, both myotoxic and cytotoxic effects were totally abolished, whereas a residual edematogenic effect remained. These observations are in accordance with previous reports (Soares and Giglio 2003), where modification of the Lys residues of PrTX-I, PrTX-III, and BnSP-7 PLA_2s by acetylation drastically decreased myotoxic, edema-inducing, and bactericidal activities.

Treatment with ethylenediaminetetraacetic acid (EDTA) also affected myotoxicity and edematogenic activities, supporting the hypothesis that phospholipid enzymatic hydrolysis is involved in these effects. Similarly, neurotoxic and myotoxic activities were inhibited almost completely after alkylation of His48 of PLA_2 Basp-III (*B. asper*), PrTX-III (*B. pirajai*), BthTX-II (*B. jararacussu*), Cdc-9, and Cdc-10 (*Crotalus durissus cumanensis*), showing that these pharmacological effects are dependent on the catalytic activity. After sulfonylation (NBSF) of Tyr residues, a residual enzymatic activity about 38% was observed. Residues Tyr52 and Tyr73 are in the catalytic site of PLA_2s, and changes in this system would affect the enzymatic activity. Tyr modification of PhTX-I (*Porthidium hyoprora*) decreased myotoxic and neurotoxic activities more than the enzymatic activity (Huancahuire-Vega et al. 2013) (Table 1).

Conclusion and Future Directions

After over a century of investigations, the antivenom serum therapy remains the unique specific and effective treatment for envenoming by snakes. The antibody production protocols involve a pool of crude venoms as the immunization mixtures, making no attempt to direct the immune response to the most clinically relevant toxins; hence, quality and efficacy of antiserum are often impaired by immunoglobulins against nontoxic but highly immunogenic proteins. Therefore, in many cases, for an effective neutralization of the main effects of snakebites, it is necessary to administer high dose of antivenoms, thereby enhancing the risk of anaphylaxis and serum reactions.

Furthermore, antiophidic serum is inefficient in reversing local symptoms, such as myonecrosis, dermonecrosis, edema, and hemorrhage. For this reason, even when the victim survives, this impairment in immunotherapy can lead to permanent physical and psychological sequelae. Given the fact that most victims are young and in productive age, sequelae generated by snakebites may lead to disability of the victim and cause considerable impacts on the world economy.

Table 1 Natural and synthetic PLA_2 inhibitors

Source	Compound type		Inhibitory activity	Examples of known inhibitors
Animals	Snake blood proteins	αPLI	Enzymatic, myotoxicity, edema inducing, and cytotoxicity	αBjussuMIP, and αBaltMIP
		βPLI	Enzymatic	EqPLIβ
		γPLI	Enzymatic, inflammation, anticoagulant, myotoxicity, edema inducing, and cytotoxicity	CNF, and PIP
	Immunoglobulin-like		Myotoxicity and cytotoxicity	DM64
	Immunoglobulins		Enzymatic and myotoxicity	Anti-myotoxins I and II (*B. asper*)
Plants	Flavonoids		Enzymatic, myotoxicity, edema inducing, and platelet aggregation	Rutin, hipolaetin-8-glucoside, quercetin, genistein, gallocatechin, harpalycin 2, tectoridin, curcumin, and morin
	Caffeic acid and its derivatives		Myotoxicity and neuromuscular blockage	Chlorogenic acid and rosmarinic acid
	Coumestans and coumarin derivatives		Myotoxicity and inflammation	Hydroxycoumarin, wedelolactone, and demethylwedelolactone
	Ellagic acid		Enzymatic, edema inducing, and myotoxicity	Ellagic acid, 3′-*O*-methyl ellagic acid, 3,3′-di-*O*-methyl ellagic acid, and 3-*O*-methyl-3′,4′-methylenedioxy ellagic acid
	Alkaloids		Enzymatic and edema inducing	Aristolochic acid and berberine
	Terpenoids		Inflammation	Triterpenoids 1 and 2, ursolic acid, and columbin
	Proteins		Enzymatic, cytotoxicity, edema inducing, and myotoxicity	Tumerin and WGS
Marine organisms	Sulfated fucans		Cytotoxicity and myotoxicity	Fucoidan
	Terpenoids		Inflammation and pain inducing	Manoalide and scalaradial

(*continued*)

Table 1 (continued)

Source	Compound type	Inhibitory activity	Examples of known inhibitors
Synthetic	Highly charged polysulfonated compound	Myotoxicity	Suramin
Chemical	p-Bromophenacyl bromide (BPB)	Enzymatic	–
	2-Nitrobenzenesulfonyl fluoride (NBSF)	Enzymatic	–
	2-Nitrobenzenesulfonyl chloride (NPSC)	Enzymatic	–
	Ethylenediaminetetraacetic acid (EDTA)	Myotoxicity, enzymatic, and edema inducing	–
	Heparin	Myotoxicity and cytotoxicity	–

Myotoxins are mainly responsible for the severe local effects induced by Viperidae venoms. These toxins are typically small- and medium-sized molecules of low immunogenicity. Due to the importance of myotoxins in Viperidae envenoming, the abovementioned features represent a problem in generating antivenoms against these toxins, since low titration of antibody is generally obtained, which leads to suboptimal neutralization of the clinical effects of envenomation.

Due to these deficiencies in snakebite treatment, there has been an increasing interest in developing new therapeutic approaches, as well as in searching for natural and synthetic products that neutralize the most toxic components in snake venoms. The most important sources of naturally occurring molecules with inhibitory properties against myotoxins, especially phospholipases A_2, are animal sera, plants, and marine animals. Moreover, a number of compounds have been designed and synthesized for the inhibition of the catalytic and pharmacological activities of these toxins. Additionally, these natural and synthetic compounds may serve as leads for development of novel potent and safe inhibitory molecules.

It is noteworthy that these inhibitory compounds may be important not only in neutralizing the local effects induced by animal venoms but may also present therapeutic potential as anti-inflammatory agents, since PLA_2s are closely involved in some pathophysiological conditions related to inflammatory processes.

Despite the great potential of myotoxin inhibitors in snakebite management, few studies have been conducted to evaluate if the supplementation of antiophidic serum with these molecules may improve antivenom efficacy without impairing its safety. In order to enable the therapeutic application of myotoxin inhibitors, some important issues related to these compounds must be further investigated, such as their production, availability, immunogenicity, distribution, and adverse effects.

Probably the main difficulty in developing new therapeutic approaches based on myotoxin inhibitors is the limitations in obtaining these compounds in sufficient

amounts for a detailed investigation and the impossibility of handling native molecules. Problems related to production and availability of PLA_2 inhibitors may be solved by developing techniques of chemical synthesis and protein heterologous expression. Many inhibitors from snake plasma have been cloned, and some of them were already expressed in diverse heterologous systems.

However, the process of producing heterologous proteins involves multiple steps and variables that may result in changes in the final product, thus modifying their primary function. Therefore, the control and validation of biotechnological processes by which these proteins of therapeutic interest are produced are essential to assess whether the recombinant molecules attend to the basic requirements needed for its use in clinical trials. Moreover, the development of biotechnological strategies for production of myotoxin inhibitors, allied to analysis of their structural features and mechanism of action, may generate perspectives for the design of new inhibitory molecules and the manipulation of known compounds in order to enhance the neutralizing properties and minimize the undesirable effects.

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Computational Approaches for Animal Toxins to Aid Drug Discovery

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Priyadarshini P. Pai and Sukanta Mondal

Abstract

Organisms are endowed with mystical strategies for survival in nature. These strategies help them strike a unique balance in driving and combating forces of life and death. What exists as an integral part one organism's defense may be capable of consuming the life of others. History of research in toxinology suggests that mankind has been long in fascination of animal toxins for simulating natural phenomena in attempts to design biological interventions. These comprise both preventive and therapeutic approaches targeted at gaining knowledge and developing novel drugs. With advances in technology and high-throughput screening, the development of toxin-based drugs is being facilitated by computational methods in tandem with experimental studies. In this chapter, these widely employed techniques including docking, molecular dynamics, Brownian dynamics, and structure–activity relationship studies have been discussed. Their specific contributions in deciphering toxin–target interactions as well as their effects have been elucidated using a range of animal toxin examples with some future directions. Altogether, this comprehensive-yet-compact organization of information on toxinology is aimed at bringing forth the readers a panoramic view of computational approaches for drug discovery.

Keywords

Docking • Brownian dynamics • Molecular dynamics • Quantitative structure–activity relationships • Pharmacophores

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_20

Contents

Introduction

Toxins or venom, through different taxonomical kingdoms such as plants, animals, and bacteria, helps organisms in procuring their food and protecting themselves from predators. Their intertwined interactions play a key role in natural selection and flourishing of vivid species. Enticed by the very idea of being able to redirect natural interactions, mankind in its attempt to simulate natural phenomena for biological interventions has been long in awe of toxins. Of the range of toxins available in nature, toxins from animals have been widely used for gaining an understanding of their remarkable biological properties and effects on organisms, in search of their potential real-life applications (Kini and Fox 2013; Kularatne and Senanayake 2014).

Venomous animals, such as snakes, scorpions, spiders, jellyfish, hymenopterans (ants, wasps, and bees), cone snails, sea anemones, lizards, centipedes, and the platypus, are equipped with injection devices (spines, fangs, stingers, hypostomes, spurs, harpoons) that permit the active use of venom in predation (Jungo et al. 2010). Poisonous animals, such as some mammals, toads, ticks, or worms, which only produce toxic substances for defensive purposes, lack such organs. However, both venomous and poisonous animals have developed a wide-ranging treasure trove of substances designed to imperil potentially threatening organisms in their environment (Jungo and Bairoch 2005).

The modes of action of these toxins and venomous substances are extremely diverse. Some act on the nervous system, affecting ion channels, perturbing glutamatergic transmissions, stimulating transmitter release, etc. Others disrupt host cell membrane, behaving as antibacterial, cytolytic, or hemolytic peptides. A wide variety of enzymes, such as phospholipase A2, proteases, oxidases, sphingomyelinases, etc., are also found in animal venom in different taxonomic groups catalyzing vital body metabolic reactions. These have been studied through various physiological and pharmacological experiments, giving valuable leads for drug development such as interesting insights into the molecular excitability in a nerve, skeletal muscle, and cardiac cells (Nirthanan and Gwee 2004).

However, for their use in medicine and therapy, toxins showing resemblance to important physiological properties of organisms, in various complex mixtures of lethal and pharmacologically active proteins and polypeptides or other non-proteinaceous compounds, have been isolated from organisms such as snakes, lizards, snails, scorpions, spiders, sea anemones, frogs, jellyfishes, etc., as briefly described in the following.

The first angiotensin-converting enzyme (ACE) inhibitor captopril (Cushman and Ondetti 1991) administered for hypertension and in heart failure after myocardial infarction was obtained from the venom of the poisonous Brazilian viper, *Bothrops jararaca*. Exendin-4 (Göke et al. 1993), a peptide isolated from the venom of a Gila monster, *Heloderma suspectum*, a species of poisonous lizard from North America that exhibits a significant amino acid homology, in its synthetic form (exenatide), was licensed in 2005 for the treatment of diabetes with metformin/sulfonylureas in various combinations. MVIIA, 25 amino acid peptide ω-conotoxin isolated from venom of *Conus magus*, artificially synthesized ziconotide (PRIALT), is approved by the Food and Drug Administration of United States of America for the treatment of severe pain (Hama and Sagen 2009). Some other applications of toxins in medicine and therapy include designer toxins that employ whole scorpions, scorpion stings (Guan et al. 2001), or their extracts for ameliorating cancer (Ding et al. 2014) and neurologic dysfunction (Kularatne and Senanayake 2014). These potent drug applications along with many others have turned the spotlight on toxins and stimulated new research, leading to an explosion in the number of characterized venom proteins.

Several experimental and computational approaches have been endeavored to provide intensive information on toxins for developing a sound knowledge base guiding their diverse biomedical applications. Scientists from clinical medicine, pharmacology, physiology, biochemistry, evolutionary biology, molecular biology, structural biology, enzymology, protein chemistry, biophysics, and toxinology, both in isolation and in collaboration, are studying venoms (i) to determine the mode and mechanism of action of the toxins, (ii) to find ways and means to neutralize the toxicity and adverse effects of bites from the poisonous organisms, (iii) to develop specific research tools that are useful in understanding normal physiological processes at both cellular and molecular levels, and (iv) to develop prototypes of pharmaceutical agents based on the structure of toxins (Kini and Fox 2013).

A plethora of information on toxins and their effects including source organisms, number of identified proteins and peptides, toxicity, and mechanism of actions is available today. Employing this information, several computational approaches are being explored at different stages of drug design and discovery. This is essentially targeted at expediting the process of translating research in laboratories and knowledge of mechanisms of natural phenomenon to the drugs available in the market. In the following section, the role of computational approaches toward gaining and utilizing knowledge of toxins and their effects for biomedicine is discussed in some detail with an elaboration on the different techniques used in the computer-aided drug discovery process.

Toxin Antidotes Using Computational Approaches: A Bird's Eye View

Devising novel counters from nature's bestowment on organisms is an art, most certainly, a fine one. The tools and techniques by far play an important role in drawing innovative perspectives from the knowledge of toxins to therapeutics.

Experiments in tandem with the rapid and intuitive computational methods become an all-encompassing approach to this fine art. Computational approaches can provide rapid predictive scope for scientific investigations, for example, in the binding affinity and specificity of a given toxin and its target. Further, computational modeling can reveal in exquisite detail the interactions, mechanisms, and structural contacts involved in toxin–target complex formation. This is important because such information can help deduce about the toxin–target binding in combination with the findings of experiments. Currently, no single available computational method can possibly achieve all the idealized aims. There are, however, a number of computational techniques that can perform some of these tasks with varying degrees of efficiency. Each of these methodologies has advantages and disadvantages and is specifically employed based on the understanding of computational speed, accuracy, and drug discovery stage. With advances in genomics and proteomics data, these methods have proved to be handy in high-throughput formats for studying different biomolecular behavioral aspects (Gordon et al. 2013).

In order to study toxin–target interactions and preserve the findings for future knowledge grounds and in biomedical applications, several web resources have been created. In the following, first toxin annotation and web resources are described. Following this, the four most commonly used techniques for computer-aided drug discovery have been reviewed, i.e., docking, molecular dynamics, Brownian dynamics, and structure–activity studies.

Toxin Annotation and Web Resources

Biological databases are libraries of life sciences information, collected from scientific experiments, published literature, high-throughput experiment technology, and computational analyses. They contain information from research areas including genomics, proteomics, metabolomics, microarray gene expression, and phylogenetics. Information contained in biological databases includes gene function, structure, localization (both cellular and chromosomal), clinical effects of mutations, as well as similarities of biological sequences and structures. These databases are important tools in assisting analysis and interpretation of a host of biological phenomena from the structure of biomolecules and their interaction to the whole metabolism of organisms and to understanding the evolution of species. This knowledge helps facilitate the fight against diseases and assists in the development of medications, in predicting certain genetic diseases, and in discovering basic relationships among species in the history of life.

Biological databases have to respond to the needs of its various users. A certain biological data often means very different things to different researchers. For example, a physicist, a biochemist, and a biologist sitting in the same room would be interested in different aspects of the same protein. Even two biologists would be interested in looking at the protein from different perspectives.

Similarly, the databases of toxin–target interactions are developed for forming a knowledge base, conducting analysis, addressing queries, and interpreting various

Table 1 Selected toxin web resources

Database name	Brief description	URL or web address
Tox-Prot	A wealth of information including many structure–function–location details	http://www.uniprot.org/program/Toxins
Animal Toxin DataBase (ATDB)	Metadatabase that collects and compiles information from several databases providing toxin ontology	http://protchem.hunnu.edu.cn/toxin/
Toxin and Toxin-Target Database (T3DB)	Comprehensive information about common or ubiquitous toxins and their targets into a single electronic repository with multiple enlisted attributes	http://www.t3db.org/
ConoServer	A database that gives access to protein sequences, nucleic acid sequences, and structural information on conopeptides derived from cone snails	http://www.conoserver.org/
ArachnoServer	Manually curated custom-built database containing information on the sequence–structure–biological activity of protein toxins derived from spider venom	http://www.arachnoserver.org/mainMenu.html

experimental findings. Based on the information stored, three broad categories of web resources are available: general toxin related, organism-specific toxin related, and toxin-target related (Table 1). In the following are discussed in brief the general web resources such as Tox-Prot (Jungo and Bairoch 2005) and Animal Toxin Database (ATDB) (He et al. 2010), organism-specific ones such as the ConoServer (Kaas et al. 2008) and ArachnoServer (Herzig et al. 2011), and toxin–target interaction database such as toxin and Toxin-Target Database (T3DB) (Lim et al. 2010).

General web resources: Tox-Prot program aims to systematically annotate venom proteins and proteins produced by poisonous animals that act as toxins. The first step consisted of the annotation of small (<10 kDA) toxin proteins. Over stages, currently it houses information on a variety of toxins from snakes, snails, scorpions, sea anemones, and many other organisms. The most recent release of Tox-Prot, as of April 16, 2014, contains over 500,000 reviewed entries with a total number of over 5,000 reviewed animal toxin and venom protein entries (Fig. 1). Another database, the ATDB, is a metadatabase that collects and compiles information from several databases, general as well as from organism-specific. It mainly focuses on construct a globe-scale animal toxin–channel interaction network based on literatures and database annotations (He et al. 2010).

Organism-specific web resources: Organism-specific databases have also been established such as ConoServer, ArachnoServer, etc. ConoServer specializes in toxins from marine cone snails (*Conus* species). ArachnoServer focuses on spider venom proteins.

ConoServer is a database that gives access to protein sequences, nucleic acid sequences, and structural information on conopeptides derived from cone snails. Cone snails are carnivorous gastropods, which hunt their prey using a cocktail of toxins that potently subvert nervous system function. The ability of these toxins to

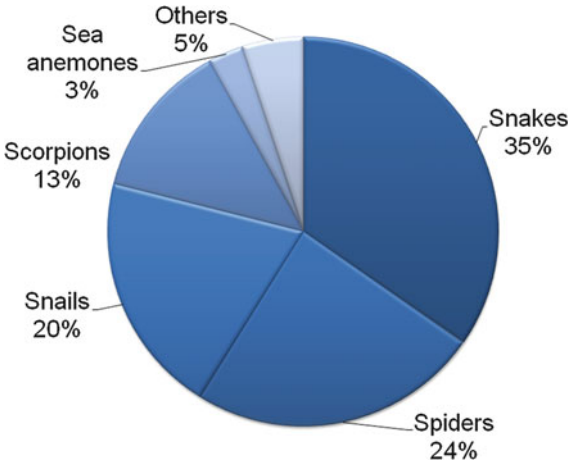

Fig. 1 Distribution of animal toxins in Tox-Prot as of April 16, 2014

specifically target receptors, channels, and transporters of the nervous system has attracted considerable interest for their use in physiological research and as drug leads. The database is updated several times a year providing a variety of statistics on classification schemes, three-dimensional structures, conopeptide-bearing species and endoplasmic reticulum signal sequence conservation trends, etc.

ArachnoServer, on the other hand, is a manually curated database containing information on the sequence, three-dimensional structure, and biological activity of protein toxins derived from spider venom. Spiders are the largest group of venomous animals, and they are predicted to contain by far the largest number of pharmacologically active peptide toxins (Escoubas et al. 2006). It allows advanced searches, browsing, and analysis of entries facilitating customization. A wide range of biological scientists, including neuroscientists, pharmacologists, and toxinologists, can readily access key data relevant to their discipline without being overwhelmed by extraneous information.

Toxin and toxin–target interaction-based web resources: Yet another unique bioinformatic resource, the Toxin and Toxin-Target Database (T3DB), currently houses over 3,000 toxins described by 32,000 and more synonyms, including pollutants, pesticides, drugs, and food toxins, which are linked to beyond 1,600 corresponding toxin target records. Altogether there are more than 37,000 toxins and toxin target associations. Each toxin record (ToxCard) contains many data fields and holds information such as chemical properties and descriptors, toxicity values, molecular and cellular interactions, and medical information. This information has been extracted from over 5,000 sources, which include other databases, government documents, books, and scientific literature.

The focus of the T3DB is on providing mechanisms of toxicity and target proteins for each toxin. This dual nature of the T3DB, in which toxin and toxin target records are interactively linked in both directions, makes it unique from existing databases. It is also fully searchable and supports extensive text, sequence, chemical structure, and relational query searches. Potential applications of T3DB include toxin

metabolism prediction, toxin/drug interaction prediction, and general toxin hazard awareness by the public, making it applicable to various fields. Overall, the variety and accessibility of the T3DB make it a valuable resource for both the casual user and the advanced researcher.

With the help of available knowledge and analysis resources, such as those mentioned above, toxins can be employed for translating research into therapeutically interesting target-specific drug discovery. Both experimental and computer-aided approaches can be deployed in tandem for learning about toxin–target interactions. Toxins have broadly three types of targets: (i) ion channels which may be voltage gated or transmitter gated; (ii) receptors of two kinds 7TM (e.g., muscarinic acetylcholine receptor) and 1TM (e.g., vascular endothelial growth factor receptor), as per the number of transmembrane (TM) helices in the receptors; and (iii) transporters such as noradrenaline and glutamate transporter proteins. Depending on the toxin–target interaction, various counters for effects of venoms such as in endothelial cell lesions, cytotoxicity, neurotoxicity, hemorrhage, myotoxicity, antitumoral, antiangiogenic, cellular signaling, and antimicrobial receptor recognition are being fostered.

Though updated, database maintenance is a key challenge faced by the investigators. These challenges may be addressed by securing adequate and reliable sources of funding and technical support. For this, contribution could firstly be validated for their data or information quality according to accepted standards and continuously updated, both at the level of material or data and inclusion of novel biological resources. Possibilities of a framework of interoperable databases with concomitant added value to the scientific community may be explored. However, to achieve a constantly developing infrastructure, support from biologists or curators, bioinformaticians, and others involved, altogether, is essential.

Computational Techniques

Toxin-to-drug discovery can be aided by computational approaches at many stages. Experimentally guided strategies as well as de novo methods have been integrated to gain insights of toxin–target interactions as well as their effects from multifaceted perspectives. Basic science fields of physics, chemistry, and computation can play different strokes in biology. This is shown below in the topics of docking, molecular dynamics, Brownian dynamics, and structure–activity studies. First, an introduction of the technique is provided followed by the examples of toxin (Table 2) and the objectives behind scientific investigations conducted on them along with specific insights for drug development. In addition to this, a list of selected software packages that could be used for computational studies on toxins is provided in Table 3.

Docking

About the technique: Molecular docking is a key tool in structural molecular biology and computer-assisted drug design. The primary goal of this technique is to predict the predominant binding mode(s) of a ligand or protein and a receptor of known

Table 2 Selected toxins and their effects

Toxin	Source organism	Description	UniProtKB[a] accession
PcTx1	*Psalmopoeus cambridgei* (Trinidad chevron tarantula)	Secreted from tarantula venom gland, potently and selectively blocks the acid-sensing ion channel in a rapid and reversible manner	P60514
Scyllatoxin	*Leiurus quinquestriatus hebraeus* (yellow scorpion)	Secreted by scorpion venom gland blocking the small conductance calcium-activated potassium channels (SK_{Ca})	P16341
Atrolysin-E	*Crotalus atrox* (Western diamondback rattlesnake)	Metalloproteinase atrolysin-E secreted by the venom glands causes hemorrhage by provoking the degradation of the subendothelial matrix proteins	P34182
PLA-2	*Naja naja* (Indian cobra)	Secreted by venom glands of snakes catalyzes the calcium-dependent hydrolysis of the 2-acyl groups in 3-sn-phosphoglycerides	P15445
Charybdotoxin	*Leiurus quinquestriatus hebraeus* (yellow scorpion)	Potent selective inhibitor of high conductance (maxi-K), different medium and small conductance calcium-activated potassium channels (K_{Ca}), as well as a voltage-dependent potassium channel ($K_v1.3$). It appears to block channel activity by a simple bimolecular inhibition process	P13487
Margatoxin	*Centruroides margaritatus* (scorpion)	Secreted by venom gland acts as potent selective inhibitor of voltage-dependent potassium channels such as $K_v1.3$	P40755
TsTX-IV	*Tityus serrulatus* (Brazilian scorpion)	Secreted by venom gland, toxin inhibits high conductance calcium-activated potassium channels. Weakly inhibits Shaker B potassium channels	P59936
κ-Hefutoxin1	*Heterometrus fulvipes* (Indian black scorpion)	Secreted by venom gland, slows the activation kinetics of $K_v1.3$ currents, and blocks $K_v1.3$ and $K_v1.2$ potassium channels. This block is dose dependent, voltage independent, and reversible	P82850
μ-Conotoxin PIIIA	*Conus purpurascens* (purple cone)	Expressed by venom duct, μ-conotoxins block voltage-gated sodium channels in a reversible manner (Na_v). This peptide causes flaccid paralysis in both mice and fish	P58925

(*continued*)

Table 2 (continued)

Toxin	Source organism	Description	UniProtKB[a] accession
α-Bungarotoxin A31	*Bungarus multicinctus* (many-banded krait)	Binds with high affinity to muscular and neuronal nicotinic acetylcholine receptors. Produces peripheral paralysis by blocking neuromuscular transmission at the postsynaptic site	P60615
α-Elapitoxin-Nk2a	*Naja kaouthia* (monocled cobra)	The monomeric form binds with high affinity to muscular, Torpedo, and neuronal α7 nAChR. Has no effect on α3/β2 nAChR. Causes paralysis by preventing acetylcholine binding to the nAChR	P01391
Pardaxin-1	*Pardachirus marmoratus* (finless sole)	Exhibits unusual shark repellent and surfactant properties. Forms voltage-dependent, ion-permeable channels in membranes. At high concentration causes cell membrane lysis	P81863
5′ Nucleotidase (5′ NUC)	*Demansia vestigiata* (lesser black whip snake)	Causes dysregulation of physiological homeostasis in humans by inducing anticoagulant effects and by inhibiting platelet aggregation	A6MFL8
Tertiapin	*Apis mellifera* (honeybee)	Secreted by venom gland, presynaptic neurotoxin that blocks the inwardly rectifying potassium and calcium channels. Interacts specifically with calmodulin in the presence of calcium	P56587
OsK1	*Orthochirus scrobiculosus* (Central Asian scorpion)	Blocks voltage-gated potassium channels, calcium channels, and Shaker IR	P55896

[a]Available at http://www.uniprot.org/

three-dimensional structure. Receptors for which the structures are unknown can be obtained first by homology (comparative) modeling and then used for docking purposes. Successful docking methods search high-dimensional spaces effectively and use a scoring function that correctly ranks candidate dockings. Details of various searching and scoring algorithms along with their limitations, most recent advances toward considering protein flexibility, water molecules, metal ions, and covalent drugs have been very recently reviewed elaborately. Information on selection of docking programs, available databases, necessary preparative steps, and analytic phases are available from a user's perspective for theory and applications (Morris and Lim-Wilby 2008).

Table 3 Selected software packages for computational studies on toxins

Software	Brief description	URL or web address
Visualization		
PyMOL	Molecular graphics system for communicating structural results	http://www.schrodinger.com/pymol/
UCSF Chimera	Extensible program for interactive visualization and analysis of molecular structures and related data	http://www.cgl.ucsf.edu/chimera/
VMD	Visual Molecular Dynamics (VMD) is designed for modeling, visualization, and analysis of biological systems	http://www.ks.uiuc.edu/Research/vmd/
Homology modeling		
MODELLER	Protein tertiary structure prediction using satisfaction of spatial restraints	http://salilab.org/modeller/
I-TASSER	Protein structure and function predictions based on multiple-threading alignments	http://zhanglab.ccmb.med.umich.edu/I-TASSER/
SWISS-MODEL	Automated protein structure homology-modeling server	http://swissmodel.expasy.org/
Docking		
AutoDock Vina	Molecular docking and virtual screening using a sophisticated gradient optimization method in its local optimization procedure	http://vina.scripps.edu/
DOCK	Anchor-and-grow-based docking program	http://dock.compbio.ucsf.edu/
GOLD	Genetic Optimization for Ligand Docking (GOLD) is based on a genetic algorithm that mimics the process of evolution	http://www.ccdc.cam.ac.uk/Solutions/GoldSuite/Pages/GOLD.aspx
HADDOCK	HADDOCK (High Ambiguity Driven Biomolecular Docking) uses biochemical and/or biophysical interaction data such as chemical shift perturbation data resulting from experimental data or bioinformatic predictions	http://www.nmr.chem.uu.nl/haddock/
PatchDock	Web server for structure prediction of protein–protein and protein–small molecule complexes based on shape complementarity principles	http://bioinfo3d.cs.tau.ac.il/PatchDock/
Structure–activity relationship		
cQSAR	A regression program that has dual databases of over 21,000 quantitative structure–activity relationship (QSAR) models	http://www.biobyte.com/bb/prod/cqsar.html
QSAR with CoMFA	Builds statistical and graphical models that relate the properties of molecules (including biological activity) to their structures such as comparative molecular field analysis (CoMFA)	http://www.certara.com/products/molmod/sybyl-x/qsar/

(*continued*)

Table 3 (continued)

Software	Brief description	URL or web address
Molecular dynamics		
GROMACS	Groningen Machine for Chemical Simulations (GROMACS) is a versatile package to perform molecular dynamics, i.e., simulate the Newtonian equations of motion for systems with hundreds to millions of particles	http://www.gromacs.org/
NAMD	Nanoscale Molecular Dynamics (NAMD) program is a parallel molecular dynamic code designed for high-performance simulation of large biomolecular systems	http://www.ks.uiuc.edu/Research/namd/

A comprehensive list of computer-aided drug design (*CADD*) software, databases, and web services is available at http://www.click2drug.org/

Briefly, the search process generates possible binding orientations and/or conformations (or modes) between two molecules. They involve studies as rigid-body sampling of binding orientations and conformational sampling of molecules, whereby rigid-body sampling is performed by the orientational search algorithm and conformational sampling is achieved by explicit protein flexibility consideration. The scoring process involves measurement of the binding tightness and/or score between two molecules in a binding mode which are then ranked for final selection of docking solutions.

The search and scoring processes can be coupled together during the docking process or can occur in different docking stages, the so-called later scoring stage and the post-docking processes. Based on the different types of interactions, docking can be classified as protein–protein, protein–peptide, protein–ligand, etc. Respective searching algorithms required for docking may vary. However, for scoring, both protein–protein docking and protein–ligand docking use similar types of scoring method, which can normally be grouped into three basic categories: (i) force field based, (ii) knowledge based, and (iii) empirical, as well as a combination of two or all of them.

Docking has attracted attention in drug discovery for a long time since its inception. Applications of docking methods have been widely explored for not only hit identification but also to de novo drug design, fragment-based drug discovery, lead optimization, metabolism prediction, off-target binding, selectivity, protein structure prediction, and drug–drug interaction. Structure-based drug design has brought new horizon to pharmaceutical development (Chen et al. 2012). Therefore, needless to say, it has been a coveted aspect of toxin-based drug discovery.

In the following, docking studies used for gaining insights into various facets of toxin–target interactions have been described. Some included findings have translated all the way from experiments in the laboratory and information on computers to the drug markets. Various issues of toxin-induced adverse effects in organisms have been addressed using molecular docking studies such as identifying their peptide selectivity profiles (Chen and Kuyucak 2012), discovery of potential toxin inhibitors (Lu et al. 2011; Ambure et al. 2014) as agents of Alzheimer's disease, identification of appropriate

toxin target models validating a rational design of specific and potent peptidomimetic compounds that may be useful for the treatment of pain or ischemic stroke (Qadri et al. 2009), detection of binding mode of toxin with ion channel (Mondal et al. 2007), etc.

Examples One of the early studies on spider toxins was based on the interaction of the tarantula toxin PcTx1 with the hASIC1a ion channel (Pietra 2009) elucidating preliminary computational chemistry aspects of spider toxins and their ion channel targets. Around the same time, some scorpion toxins were also studied for analysis of their binding residues and D2 dopamine receptors (Sudandiradoss et al. 2008). Previous to this study, the interaction between scorpion toxin ScyTx and small conductance calcium-activated potassium channel was simulated by docking and molecular mechanics Poisson–Boltzmann surface area (MM-PBSA) (Wu et al. 2004). These studies mentioned above and their like helped gain an understanding of toxin–target interaction site, binding residues, and mechanisms of action.

Molecular Dynamics

About the technique: Molecular dynamics (MD) has now become one of the important computational tools for simulating biomolecular systems, and no different is the case with research on toxins. With the availability of different user-friendly packages, the ease of understanding toxin–target interactions has grown considerably.

Essentially in MD simulations, the trajectories of N particles interacting via a many-body potential using Newton's equation of motion are followed. The atoms and molecules are allowed to interact for a period of time, giving a view of the motion of the atoms. In this context, implicit solvent and course graining are sometimes employed besides the atomistic molecular dynamic-based approach. MD simulation is briefly described next.

The molecular dynamics (MD) method and its applications to molecular systems are largely based on classical mechanics and statistical mechanical theories. Intensive calculations are required in this method; hence, the efficiency of MD simulations is heavily dependent on algorithmic developments in mathematics and computer science as well as on advancements in computer hardware. In a typical atomistic MD simulation, interactions are calculated between atoms using a set of parameters that define a potential energy function, representing a "force field." The derivation of force field parameters is typically a painstaking iterative process, in which initial parameters obtained from experimental data and quantum mechanical calculations are optimized to reproduce structure and vibrational modes, as well as thermodynamic properties of the molecular systems of interest. Various force fields are available for biomolecular simulations, with minor differences in their potential energy functions and corresponding differences in parameters. A typical potential energy function for biomolecular simulations, U, includes terms that describe bonded (bonds (U_{bond}), bond angles (U_{angle}), dihedral angles (U_{dihedral}), etc.) and nonbonded (van der Waals (U_{vdW}) and electrostatic (U_{elec})) interactions:

$$U = U_{\text{bond}} + U_{\text{angle}} + U_{\text{dihedral}} + U_{\text{vdW}} + U_{\text{elec}}$$

Nonbonded interactions often play an important role in describing interactions between different molecules or even between different functional groups within the same molecule, e.g., side chain interaction within a protein.

During an MD simulation, at each time step, the total force $\boldsymbol{F}$ acting on each atom is calculated as the negative derivative of the potential energy U with respect to its coordinates $\boldsymbol{r}$:

$$\boldsymbol{F} = -dU/d\boldsymbol{r}$$

Using these forces, the Newtonian equations of motion are then integrated to determine changes in the position $\boldsymbol{r}$ and velocity $\boldsymbol{v}$ of individual atoms in time, t:

$$\boldsymbol{F} = m\boldsymbol{a}; \boldsymbol{a} = d\boldsymbol{v}/dt; \boldsymbol{v} = d\boldsymbol{r}/dt$$

where m is the mass and $\boldsymbol{a}$ is acceleration. The result of an MD simulation is, therefore, a trajectory file that includes an ensemble of configurations of the system over a period of time, typically ranging from tens to hundreds of nanoseconds for atomistic simulations of biomolecular systems. These trajectories contain information about motions and interactions that can occur in a system, under given conditions, and may also be used to calculate macroscopic properties for prediction of or comparison to experimental observables.

MD has the advantage of providing dynamical information at high spatial and temporal resolutions. It allows the researcher not only to monitor the natural motion of a molecular system in real time but also to test out various conditions, including those that are not accessible experimentally. While extremely powerful in generating and testing hypotheses, MD methods are severely limited in time scale because of the short time steps (on the order of femtoseconds for atomistic simulations) required for accurate integration of the trajectory and proper description of the molecular system.

MD has been successfully employed in studying a wide range of biomolecular systems and phenomena, including toxins and their target's interactions. Furthermore, with continuous algorithmic improvement, the availability of faster hardware, and refinement of the available force fields addressing these limitations, the gap between simulations and experiments is rapidly closing, as evidenced by recent studies reporting simulations reaching time scales on the order of microseconds to sub-milliseconds.

Several attempts to understand the dynamics of toxin–target interactions have been made over the past few decades. Some examples toward learning more about the stability and energetics of toxin–target complexes, as well as, for refining docked poses that may have been produced manually or with the help of docking programs are described below. The description is based on toxin effects and toxin–target interactions, i.e., venom enzymes, toxin–ion channel, and toxin receptor.

Examples Studying venom enzymes in search of antivenom therapies: One of the most recent examples of molecular dynamic-based study is that of the interaction

between a small synthetic peptide component (LT10) derived from the N-terminal of the lethal toxin-neutralizing factor (LTNF) isolated from Opossum (*Didelphis virginiana*) and two snake venom enzymes, namely, PLA2 from *Naja naja* and atrolysin C from *Crotalus atrox* (Chavan and Deobagkar 2014). This in silico study aimed at providing molecular basis that might account for the interactions of LT10 peptides toward these two snake venom toxins for the design and development of anti-snake venom therapies.

Understanding mechanisms of action of toxin–ion channel interaction: Previous to the abovementioned studies, an exclusive MD simulation work was done on ion channel type of toxin targets, described in a review article (Gordon et al. 2013). Toxins from scorpions (Chen and Chung 2013), spiders (Pietra 2009), marine snails (Hama and Sagen 2009), snakes (Nirthanan and Gwee 2004), etc., have been studied so far.

Briefly, in these examples, the research involved molecular recognition, structural and functional characterization, and mechanisms of actions were explored. Such as, Scorpion toxin recognition by the Ca^{2+}-activated potassium channel $K_{Ca}3.1$ was simulated (Chen and Chung 2013) using MD for understanding the mechanism of this toxin–ion channel interaction. Specific studies on understanding potential implications of potassium channels in midbrain dopamine neurons and their suggested role in depolarization block (Ji et al. 2012) were conducted, findings revealing the potential scope of search for antipsychotic drug targets. Further, molecular approaches for structural characterization of a new potassium channel blocker from *Tityus stigmurus* venom using a combination of cDNA cloning, homology modeling, dynamic simulations, and docking (Almeida et al. 2013) were used to propose amino acid residues and their interaction involved in the blockade. Besides this, charybdotoxin and margatoxin acting on the human voltage-gated potassium channel $hK_v1.3$ and its H399N mutant and $mK_v1.3$ were also studied using an experimental and computational comparison (Nikouee et al. 2012). Differential molecular information of maurotoxin peptide recognizing IK_{Ca} and $K_v1.2$ channels was explored by computational simulation (Yi et al. 2011), and interactions of κ-hefutoxin1 with the voltage-gated potassium channels (Zarrabi and Naderi-Manesh 2008) were investigated. Additionally, spider toxins have also been explored in the past. MD simulations and docking of the interaction of the tarantula peptide psalmotoxin-1 with ASIC1a channels using a homology model (Pietra 2009) were performed. Mechanism of μ-conotoxin PIIIA binding to the voltage-gated Na^+ channel $Na_V1.4$ (Chen et al. 2014) was explored.

Drug discovery through insights of toxin–receptor interaction: Besides ion channels, receptors with seven transmembrane helices and one transmembrane helix, 7TM and 1TM types, respectively, have also been extensively investigated. Muscarinic acetylcholine receptor, adrenoreceptor, bradykinin receptor, vasopressin/oxytocin receptor, endothelin receptor, vasoactive intestinal polypeptide receptor, glucagon-like peptide receptor, somatostatin receptor, cholecystokinin receptor, neurotensin receptor, vascular endothelial growth factor receptor, and epidermal growth factor receptors have been studied (Jungo and Bairoch 2005).

The discovery, about 40 years ago, of α-bungarotoxin, a three-finger α-neurotoxin from *Bungarus multicinctus* venom, enabled the isolation of the nicotinic acetylcholine receptor (nAChR), making it one of the most thoroughly characterized receptors today. Since then, the sites of interaction between alpha-neurotoxins and nAChRs have largely been delineated, revealing the remarkable plasticity of the three-finger toxin fold that has optimally evolved to utilize different combinations of functional groups to generate panoply of target specificities to discern subtle differences between nAChR subtypes. New facets in toxinology have now broadened the scope for the use of α-neurotoxins in scientific discovery. For instance, the development of short, combinatorial library-derived, synthetic peptides that bind with sub-nanomolar affinity to α-bungarotoxin and prevent its interaction with muscle nAChRs has led to the in vivo neutralization of experimental α-bungarotoxin envenomation, while the successful introduction of pharmatopes bearing "α-bungarotoxin-sensitive sites" into toxin-insensitive nAChRs has permitted the use of various α-neurotoxin tags to localize and characterize new receptor subtypes. More ambitious strategies can now be envisaged for engineering rationally designed novel activities on three-finger toxin scaffolds to generate lead peptides of therapeutic value that target the nicotinic pharmacopoeia (Nirthanan and Gwee 2004).

Brownian Dynamics

About the technique: In 1827, botanist Robert Brown examined under his microscope pollen of plants suspended in water and observed that the pollen, about 10^{-6} m in diameter, performed stochastic motions of the order of 10^{-5} m. The cause and behavior of such processes were later explained and also known as Brownian dynamics which are unlike the deterministic molecular dynamics.

Ever since the dawn of Brownian dynamics, it has been used to describe the motion of molecules, for example, in molecular simulations of protein–protein interaction, computation of their association rates, and the nature of the physical environment (e.g., as a function of ionic strength or viscosity) (Gabdoulline and Wade 1998). Over a period of time, they have found use in studies of ion channels and their blockers. Some examples of interest are described here with regard to venom enzymes and toxin–ion channel interactions.

Examples Understanding catalytic behavior of venom enzyme for therapeutics: In the late 1990s, computer simulations were performed to investigate the kinetics and role of electrostatic interactions in promoting the association of the enzyme acetylcholinesterase with its peptide inhibitor, the neurotoxin fasciculin. Distinctions between active center ligands and fasciculin were explored (Radić et al. 1997). Study findings using Brownian dynamics also revealed the possible effects of electrostatic interactions in promoting the kinetics of binding and advantages of using electrostatic desolvation effects (Elcock et al. 1999).

Conductance of ions and behavioral aspects of toxin–ion channel interactions: On another line, Brownian dynamics have been extensively used for simulations of toxin–ion channels. Many findings have been reported from scorpion toxins and their targets such as Lq2 and potassium ion channel (Cui et al. 2001); P05 and small

conductance calcium-activated potassium (SK) channels rsk1, rsk2, and rsk3 (Cui et al. 2002); and maurotoxin with voltage-gated potassium ion channels $K_v1.1$, $K_v1.2$, and $K_v1.3$. These studies were targeted at providing insights into the toxin–target recognition and interactions. Additionally, Brownian dynamic simulations have also provided help in understanding the action of inhibitors. For example, a branched analogue of spermine interaction with the voltage-gated potassium channel was studied where simulations revealed the mechanism of action of this inhibitor and ways of modulation. Some salient features of ion channels have been replicated such as the current–voltage concentration profiles, the binding affinity and mechanisms using Brownian dynamics in combination with molecular dynamics, clearly emphasizing the use of more than one technique in combination for understanding toxin–target interactions at greater depth.

Structure–Activity Relationship Studies

About the technique: In order for drugs to possess maximum potency and minimal side effects, the relationship between a drug's chemical structure and biological activity is extensively studied. These studies are termed structure–activity relationship (SAR) studies, and they enable the determination of the chemical groups responsible for evoking a target biological effect in the organism allowing modification of the effect or the potency of a bioactive compound (typically a drug) by changing its chemical structure. Medicinal chemists use the techniques of chemical synthesis to insert new chemical groups into the biomedical compound and test the modifications for their biological effects. Computational approaches in this context help illustrate common motifs, structural features, and pharmacophores that define interesting aspects of peptide-based drugs. Additionally, their synthesis, chemical modifications, and the development of more selective and stable analogues such as peptidomimetic agents with ample therapeutic potential can be aided using computational methods (Muttenthaler et al. 2011).

Quantitative structure–activity relationship (QSAR)-based study represents a very high level of modeling involving quantification of a number of attributes such as hydrophobicity, polarizability or refractivity, or spatial structural information. The findings of QSAR modeling can be used to predict the activities of as yet untested compounds. So is the case with toxins. Attempts to decipher important chemical components from toxins of fish (Kolusheva et al. 2008), sea anemone (Hellberg and Kem 1990), scorpion (Cohen et al. 2005), marine snails (Verdier et al. 2005) and snakes (Bende et al. 2013), etc., have been made and are briefly described in the following.

Examples Quantitative structure–activity relationships in explaining toxin-target specificity: The fish toxin, pardaxin, used for developing a biomimetic membrane assay to gain insights into the toxin-cell and toxin-lipid artificial membrane interactions, has been found to have broad applications for studying membrane interactions of peptides in general and pore-forming toxins in particular. Researchers hope that it could become an important tool for evaluating QSAR (Kolusheva et al. 2008). Using few other bioassays of organisms and QSAR, the diverse toxin-target specificity and

pharmacological properties of sea anemone toxins have been explained (Hellberg and Kem 1990).

Deciphering dramatic-yet-important chemical groups in toxin–target interactions: Further, specific pharmacologically important groups, the pharmacophores, have been determined with the assistance of experimental data such as in the case of development of an antibody against *Tityus serrulatus* scorpion venom (Alvarenga et al. 2005), in developing pharmacophore model for proving the existence of diverse binding modes of peptidic toxins of marine snails (Verdier et al. 2005), in determining modes of action of insecticidal spider venoms containing knottin peptides (Bende et al. 2013), in explaining the potent presynaptic and postsynaptic neurotoxicity of Australian pygmy copperhead snake toxin (Van Der Haegen et al. 2011), and more. Concepts of target specificity (Van Der Haegen et al. 2011) and intraspecific variation (Romeo et al. 2008) have been shed light upon.

The overall progress of the drug discovery process is a combination of structure–activity studies with docking-dynamic approaches rather than each of the abovementioned techniques in isolation. A few of them illustrating the variety of roles of the docking-dynamic and SAR studies have been described in the next section.

Integrative Approach Is the Key

Complexities in biology are such that for any natural phenomena, in order for mankind to derive insights, any technique or approach in isolation usually falls inadequate. Similarly, to establish different perspectives for successful drug development, integrating various computational approaches has become the key.

Toxin research has also seen many examples of integrative computational approaches in tandem with experimental data ably providing explanations for their biomedical applications. A few examples are elaborated here for illustrating the stages of computational involvement in the drug discovery process. They are on snake toxin, honeybee toxin, and scorpion toxins. However, there are many others.

Snake Toxin to Therapeutics

Venoms of snakes contain a ubiquitously distributed enzyme known as 5′ nucleotidase (5′ NUC) that is responsible primarily for causing dysregulation of physiological homeostasis in humans by inducing anticoagulant effects and by inhibiting platelet aggregation. It is also known to act synergistically with other toxins to exert a more pronounced anticoagulant effect during envenomation. Its structural and functional role had not been ascertained clearly. The 3D structure of snake venom 5′ nucleotidase (SV-5′ NUC) had not been known. Few scientists predicted these for the first time using a homology-modeling approach using *Demansia vestigiata* protein sequence. The accuracy and stability of the predicted SV-5′ NUC structure were validated using several computational approaches. Key interactions of SV-5′ NUC were studied using experimental studies/molecular docking analysis of the inhibitors vanillin, vanillic acid, and maltol. All these inhibitors were found to

dock favorably following pharmacologically relevant absorption, distribution, metabolism, and excretion (F) profiles. Further, atomic level docking interaction studies using inhibitors of the SV-5′ NUC active site revealed amino acid residues Y65 and T72 as important for inhibitor–(SV-5′ NUC) interactions. Further, the in silico analysis was found to be in concordance with experimental inhibition results of SV-5′ NUC with vanillin, vanillic acid, and maltol. The findings of this study, in addition to the newly identified pharmacophoric features, are hoped to be of use in the discovery of putative antivenom agents of therapeutic value for snake bite management (Arafat et al. 2014).

Honeybee Toxin to Healing

Honeybee has a polypeptide toxin called tertiapin which is known to block several subtypes of their targets, the inwardly rectifying ion channels. To explain its differential affinities toward the target subtypes, a combination of computational methods was employed. Its unveiled crystal structure along with molecular and Brownian dynamics simulations was used to elucidate several conductance properties of the targets. Integrative approach showed that the pore is closed by a hydrophobic gating mechanism similar to that observed in another similar channel. Further, the critical residues in the toxin and channel involved in the formation of a stable complex were identified. Their interaction and insights on mechanism of selective-yet-diverse affinity are implicated in cardiac and neurological disorders (Hilder and Chung 2013).

Scorpion Toxin in the Saving Mode

Small peptides isolated from scorpion venom, charybdotoxin (ChTx), and OSK1, targeting the human Ca^{2+}-activated channel of intermediate conductance ($K_{Ca}3.1$), have potential as anti-sickling and immunosuppressant agents. They inhibit $K_{Ca}3.1$ with nanomolar affinities and are promising drug scaffolds. Although the inhibitory effect of peptide toxins on $K_{Ca}3.1$ has been examined extensively, the structural basis of toxin–channel recognition had not been understood in detail. Scientists then explored binding modes of two selected scorpion toxins, in atomic detail using MD simulations. Employing a homology model of $K_{Ca}3.1$, the conduction properties of the channel were first determined using Brownian dynamics and ascertained with experimental data. The model structures of ChTx–$K_{Ca}3.1$ and OSK1–$K_{Ca}3.1$ complexes were then constructed using MD simulations biased with distance restraints. The ChTx–$K_{Ca}3.1$ complex predicted from biased MD was consistent with the crystal structure of ChTx bound to a voltage-gated K^+ channel. The dissociation constants (K_d) for the binding of both ChTx and OSK1 to $K_{Ca}3.1$ determined experimentally were reproduced within fivefold using potential of mean force calculations. Making use of the knowledge gained by studying the ChTx–$K_{Ca}3.1$ complex, enhancing of the binding affinity of the toxin was attempted, by carrying out a theoretical mutagenesis. Findings of this study hoped to provide insight into the key molecular determinants for the high-affinity binding of peptide toxins to $K_{Ca}3.1$ demonstrating the power of computational methods in the design of novel toxins (Chen and Chung 2013).

Conclusion and Future Directions

Over past few decades, efforts toward understanding toxins at greater depth and exploring their potential in therapeutics have gathered impetus. In this context, the most striking features of toxins, i.e., their high specificity toward a wide range of ion channels, receptors, and transporters, have been found to play an important role in rendering them as an excellent prospective of pharmacological probes and lead molecules in drug design. Studies with computational and experimental approaches in tandem have been ventured to gain insights into the mode of action of toxins with a hope of unraveling their function. Rapid advances in toxinology and community-wide endeavors have resulted in construction of extremely large libraries of probable therapeutic toxin derivatives, increasing our ability to efficiently and effectively screen against these targets in high-throughput formats.

This chapter presents the computational approaches applied in studying animal toxins for drug discovery. With details of techniques and examples, a broad impression of computational approaches as an extensive process involving several stages of possible contribution is put forth. Objectives achieved through various studies conducted on a variety of animal toxins are explained briefly with highlights of application such as in preparation of toxin-target libraries; representation of involved biomolecules; preparation of target structure by target identification, alignment, model building, refinement, and evaluation; binding site detection and characterization using geometric methods; energy-based approaches; pocket matching; molecular dynamic-based detection; evaluation of simulations using different scoring functions; high-throughput screening; pharmacophore modeling, etc. Further, developing an understanding of influencing factors such as the knowledge base, experimental support, available web resources, and toxin-target effects is suggested, since there are no fundamentally superior techniques.

Future directions based on an understanding of the present routine experiment-guided approaches require collaborative and interdisciplinary efforts both in terms of knowledge acquisition and supporting resources. For example, annotations in the toxin databases can help improve our knowledge resource and avenues for engineering various physiological and pharmacological phenomena in organisms. Gaining knowledge of toxins–target interactions using suitable combinations of computational tools with existing experimental strategies can provide a rapid and promising scope for obtaining important leads in drug design and discovery. These leads may help not only in toxin-induced medical conditions such as snake bite toxicity and scorpion or bee sting toxicity but also provide pharmacophores for disorders such as Alzheimer's disease and tumors. Applications of these leads have also been found further, in a wide range of therapeutic agents such as anticoagulants, anti-inflammatory agents, analgesics, bactericidal agents, and in skin formulations for causing antiaging effects.

Though the expanse of knowledge about the deadly animal toxins for healing life has gathered momentum over the past many years, there are numerous polypeptide toxins yet to be discovered and characterized from venoms of arachnids, reptiles, and marine invertebrates. The advent of new and powerful computational approaches

will enable us to rule out rapidly a very large number of untested toxins with inferior blocking characteristics so that in vitro or in vivo experimental testing can be more focused. In a nutshell, well-integrated strategies founded on reliable knowledge base, upgraded technology, and computational strength are required to boost community-wide research and development efforts for unfolding novel healing sides of the toxins.

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Toad Poison and Drug Discovery

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Abstract

In this chapter, the potential medical-pharmaceutical use of molecules derived from toad skin secretions is discussed. Over the past decades, numerous bioactive components from toad poisons, with diverse biological activities, have been studied. They present diversified chemical structures, such as steroids, alkaloids, peptides, proteins, and biogenic amines. Toad poison components are the result of pharmacological and biochemical refinement that occurred during the process of evolution, which made them highly effective. They present a broad spectrum of physiological and defense functions. The dried secretion from the skin glands of toads has been used clinically for over a millennium as an anodyne cardiotonic, antimicrobial, local anesthetic, and antineoplastic agent. The discovery of poison components with biological activity, such as anticancer, antimicrobial, antiviral, antiparasitic, modulators of blood coagulation, neurotransmission, analgesic, and anti-inflammatory, among others, aiming to discover new drugs, is a challenge. However, despite the difficulties, these studies have been conducted and many poison components with therapeutic potential have been identified.

Keywords

Toad poison • Antimicrobial peptides • Antitumor activity • Cardiovascular activity • Bufotoxins

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_16

Contents

Introduction

Natural products have been demonstrated to contain an array of biologically active molecules which can provide important tools to investigate different physiological systems and can be useful for the development of new drugs. Some of these molecules have been shown therapeutical potential due to their antitumor, cardiovascular, antimicrobial, antiviral, and antiparasitic activities. The most common sources of these molecules are plants and venomous and poisonous animals and their secretions. Venomous animals have specialized cells or glandular structures producing venoms and apparatuses (e.g., stings, teeth, harpoon) capable to inoculate these secretions. Poisonous animals can produce poison or acquire toxins from the environment, plants, other animals, or microorganisms and accumulate them in glands or tissues, but these animals are unable of active inoculation since they have no apparatuses for this purpose (Mebs 2002).

In the animal kingdom, there is an incredible arsenal of molecules which are important for their survival. These compounds are used for defense against predators or to catch food. In hunting, they give them a unique ability to paralyze and/or kill their prey by activating specific structures of different organ systems of the prey (Mortari et al. 2007).

One of the most important sources of molecules from animals is derivate from amphibians. These animals had their evolution adjustments in their skin due to the transition between water and land. The skin of these animals was modified according to their new lifestyle and was based on the adaptation (Toledo and Jared 1995).

Today, the total number of species of amphibians catalogued is 7044, and these are divided in three orders: **Anura** (6,200 species), **Caudata** (652 species), and **Gymnophiona** (192 species) (Frost 2013). Toads are among the Anura, and the Bufonidae family is termed as true toads (Frost 2013; Bocxlaer et al. 2010).

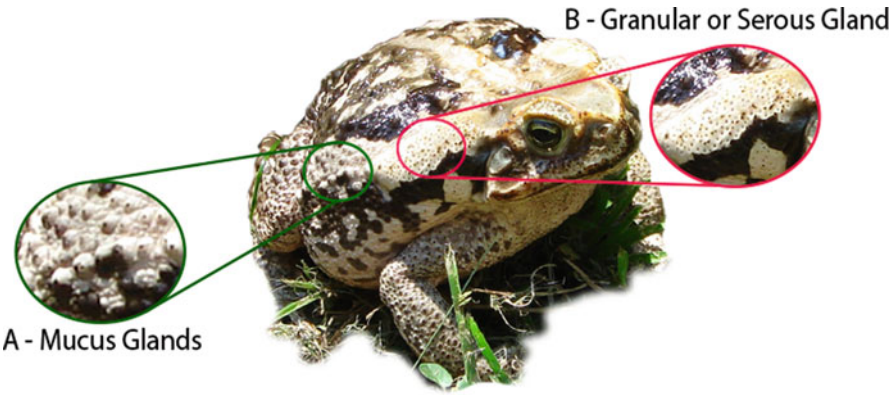


Fig. 1 Toad glands of the genus *Bufo* (*Rhinella schneideri*): mucus (**a**), granular or serous (**b**). Animal Toxins Laboratory – University of São Paulo at Faculty of Pharmaceutical Sciences of Ribeirão Preto, Brazil

Toad adaptation had allowed this animal to be cosmopolitan species, because it has glands (serous and mucous) that produce an array of molecules able to defend itself and poison the predators (Toledo and Jared 1995; Bocxlaer et al. 2010).

Amphibian skin presents very complex morphology, physiology, and biochemistry, related to the survival of the animal (Clarke 1997). The amphibian skin glands (Fig. 1) are represented by two types: mucous glands and granular or serous glands (Toledo and Jared 1995; Clarke 1997). The mucous glands are numerous, smaller than granular glands, and related to the control of body surface pH, keeping the skin moist as well as producing substances with antimicrobial effects (Clarke 1997). The granular glands are directly related to the defense of the animal against predators, besides possessing compounds against bacteria and fungi. These glands may be spread through the skin surface or grouped to form clusters, such as parotoid glands found as paired protuberances, in postorbital position among the Anura (Clarke 1997; Jared et al. 2009). The genus *Bufo* has parotoid glands located on the sides of the head (previously mentioned) and on the neck or shoulder regions (Perry 2000).

Toad Poison Composition

Toad poisons, excreted by both types of glands, mucous or granular, are composed of a variety of chemical compounds (Fig. 2), including proteins and peptides, biogenic amines, steroids, and alkaloids, depending on species and the environment in which they live (Sciani et al. 2013a).

The food is also very important for the composition of the toad poison. The kind of diet depends on factors like the location they inhabit and the structural size of their

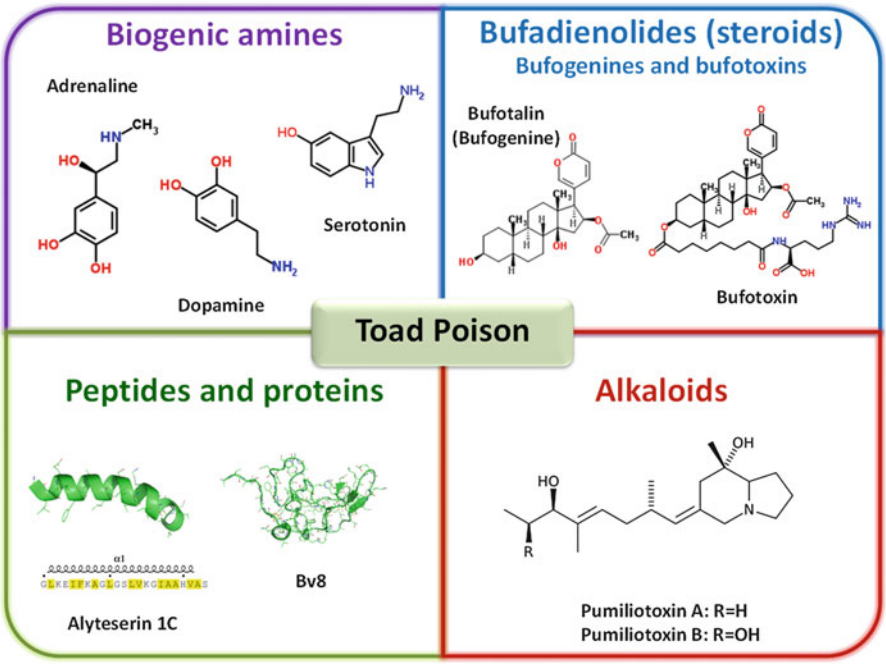


Fig. 2 Toad poison compounds: biogenic amines, peptides, alkaloids, and bufogenines and bufotoxins (steroids). The structures presented in the figure are examples of each class of toxin, but there is a wide structural diversity among these molecules. Structures of Bv8 (PDB ID 2KRA) and alyteserin 1C (PDB ID 2L5R) were generated by PyMOL

body. Usually their food contains small insects, but may be varied including other invertebrates (Batista et al. 2011).

Other factors besides the diet are of utmost importance for the toad poison composition. Some molecules like bufadienolides can be derived from metabolism of bacteria colonizing the parotoid glands of toads. These bacteria perform transformations that bring a large structural diversity in this class of metabolites (Hayes et al. 2009).

Poisoning by contact with the animal is extremely rare, except when the secretion or the animal itself is ingested, because it only has an effect on contact with wounds or mucous membranes of the aggressor (as the mouth and eyes), considering they only release their poison when they feel threatened or provoked. However, born second-generation captive amphibian loses its ability to produce toxin, and the animals become completely nontoxic, because all the toxins are produced by precursors found in the animal's diet (Daly et al. 1997) which explains the considerable variation in the toxicity of toads in different geographical locations. Therefore, it can be seen that the constitution of the poison depends on several factors, and in this chapter its four main constituents (Fig. 2) will be discussed below in greater detail.

Bufogenines and Bufotoxins: Steroids

Bufogenines and bufotoxins (esters of suberylarginine and the corresponding bufogenine) are derived from cholesterol and belong to the steroid group of toad poison components. Bufadienolides (bufogenines and bufotoxins) are cardiotonic glycosides and possess both pharmacological and toxicological effects, as shown by in vitro and in vivo studies, such as cardiotoxic, cardiotonic, anesthetic, and anti-tumor effects.

Cardiotonic glycosides are molecules found in nature and are present in both plants and animal poisons. They are divided into cardenolides and bufadienolides; this division occurs according to their molecular arrangement. The bufadienolides present in toad poisons are also named cardiotonic glycosides (Table 1) (Stein and Heerden 1998).

The difference between the cardenolide and bufadienolide is in the nature of the lactone moiety in the C-17 substituent core cyclopentanoperhydrophenanthrene (Fig. 3), also known as the steroidal nucleus which is considered as the pharmacophore of the molecule grouping. The cardenolide will present butyrolactone ring which is a five-membered ring, while bufadienolide will contain one six-membered ring called also the unsaturated pyrone ring (Prassas and Diamandis 2008). These molecules receive this name due to their capacity to bind to the extracellular portion of the enzyme sodium/potassium ATPase (Na^+/K^+ ATPase). This interaction causes the inhibition of the enzyme, which stops performing the transport of sodium to the outside of the cell and potassium to the inside of the cell, activating the calcium pump that enhances the heart contraction (Mijatovic et al. 2007).

Bufadienolides are present in the body of the toad genus *Rhinella* (Bufonidae), and these molecules do not occur only in aglucone form, but multiple connections in C-3 are known, such as sulfates, esters, and dicarboxylic amino acids. There is a wide variety of molecules generated by chemical modification of the substituents on the steroid core (Stein and Heerden 1998).

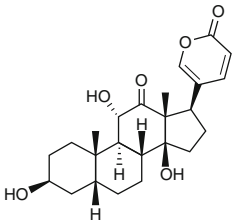
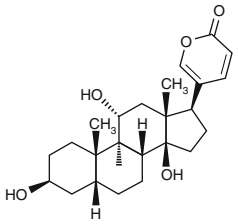
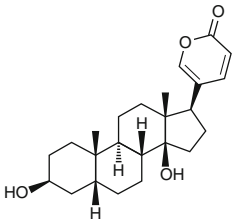
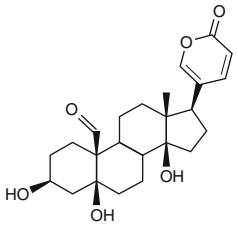
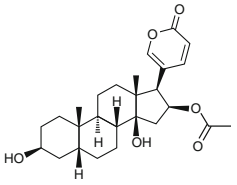
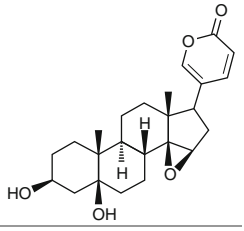
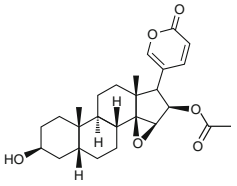
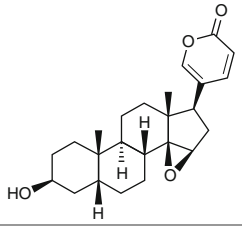
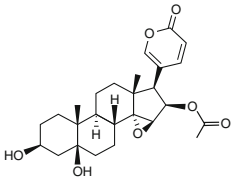
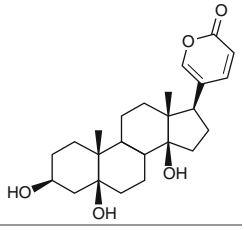
The structure of cardiotonic glycosides (Fig. 3) has a steroid core with four rings denominated A, B, C, and D, with cis-connection of rings A/B as well as C/D (Kamano et al. 1998). The C-17 position has an α-pyrone ring (unsaturated lactone ring, named bufadienolide ring).

The study of structure-activity relationship of cardiotonic glycosides shows that radicals or spatial configuration changes are able to modulate their activities. For example, some structural changes in the bufalin molecule (Fig. 4) can increase or decrease its cytotoxic activity in primary liver carcinoma cells PLC/PRF/5 (Kamano, et al. 1998).

Proteins and Peptides

Several proteic compounds are present in toad's gland secretions. The discovery of proteins and peptides in these secretions propelled new studies about these compounds. Among them, the largest class is the peptides of dermal glands. Most of

Table 1 Some examples of cardiotonic steroids from toad poisons and their structures

Cardiotonic steroids	Structure	Cardiotonic steroids	Structure
Arenobufagin		**Gamabufotalin**	
Bufalin		**Hellebrigenin**	
Bufotalin		**Marinobufagin**	
Cinobufagin		**Resibufogenin**	
Cinobufotalin		**Telocinobufagin**	

Based on Stein and Heerden (1998)

them present antimicrobial activity and are responsible for the immediate defense against many microorganisms (Table 2).

A classic peptide known as buforin I is an antimicrobial peptide (AMP) of 38 amino acids that resides isolated from the stomach tissue of the Asian toad

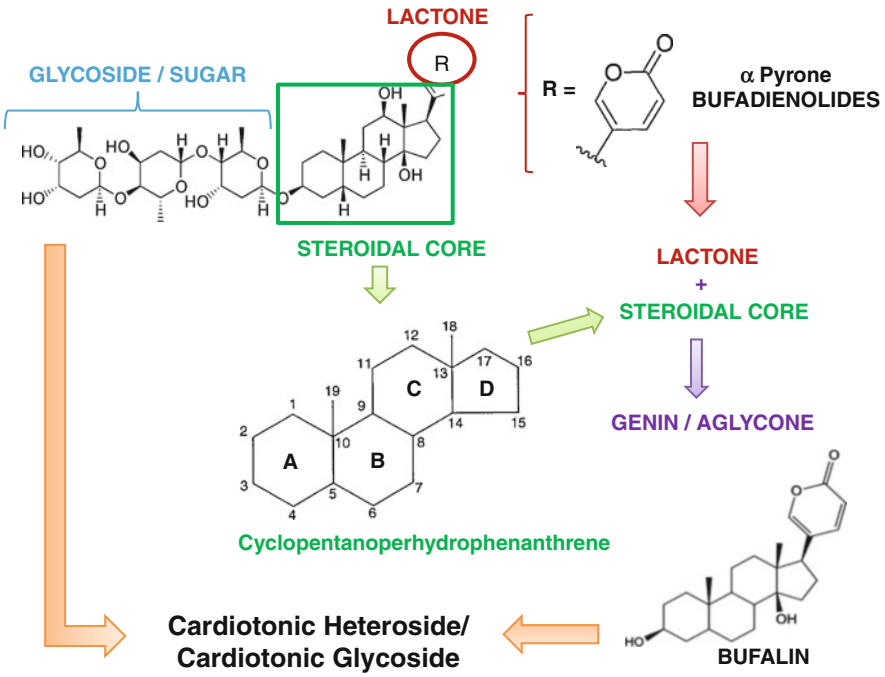
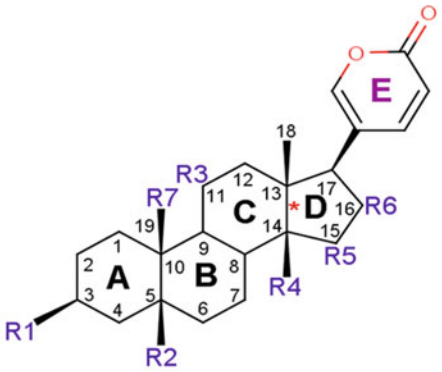


Fig. 3 Structure of cardiotonic steroids from toad poison. In *blue* is represented a sugar/glycoside moiety at position 3 of a glycosylated cardiotonic steroid. In *green* is represented the steroidal core. In *red* is represented a radical named α-pyrone ring, characteristic of bufadienolide compounds. In purple is represented the moiety aglycone or genin that is composed of steroidal core and R group that, in this case, is α-pyrone ring (Based on Mijatovic et al. 2007)

Bufo bufo gargarizans and was described firstly by Park and collaborators (1996). This peptide has shown strong antimicrobial activities against a broad spectrum of microorganisms such as gram-positive and gram-negative bacteria and fungi. Today there are other similar molecules described in the literature such as buforin II (twice more potent that buforin I) (Cho et al. 2009) and many others, identified due to the increase of molecular biology techniques currently employed. The buforin is a classic example of the existence of important toxins in other parts of the animal body (stomach tissue) and not just in poisons.

The alyteserins constitute an important group of antimicrobial peptides present in skin secretions of the midwife toad (*Alytes obstetricans*). Within this group, there are two families: alyteserin 1 and alyteserin 2. Both families have C-terminal α-amidated and broad antimicrobial activity. Currently, with the study of granular gland transcriptome and other molecular biology techniques, several alyteserin-like peptides have been identified (Conlon et al. 2009; König et al. 2012).

Nowadays, there are 56 different antimicrobial peptide cDNA sequences from two skin cDNA libraries of two specimens of the Chinese redbelly toad (*Bombina maxima*), evidencing its rapid diversification driven by Darwinian selection



Cis C/D ring junction increse the cytotoxic activity
E: 17-α-pyrone ring contributed to increse of the cytotoxic activity

Radical	Carbon Atom	Substituents	Activity
R1	3	3β-hydroxy	DECREASE
R1	3	Acetylation 3β-hydroxy	INCREASE
R1	3	3-esterified and 3-keto derivatives	DECREASE
R2	5	5β-hydroxy	DECREASE
R3	11	11α-hydroxy	INCREASE
R4	14	14β-hydroxy	DECREASE
R5	15	15α-hydroxy	DECREASE
R5	15	β-substituents	INCREASE
R5	15	α-hydroxyl, ester and epoxide group	DECREASE
R6	16	16β-acetoxy	DECREASE
R6	16	16β-hydroxy or ester group	DECREASE
R7	19	19-aldehyde	INCREASE
R7	19	19-CH_2OH group	DECREASE

Fig. 4 Bufalin molecule, which may be subjected to some modifications in its structure, which could increase or decrease its cytotoxic activity in primary liver carcinoma cells PLC/PRF/5. The letters A, B, C, and D correspond to steroid skeleton of the molecule (Based on Kamano et al 1998)

(Lee et al. 2005). A novel antimicrobial peptide named maximin H5 was obtained from a skin cDNA library of *B. maxima*. This peptide is the first example of anionic antimicrobial peptide from amphibians, and it was active against only gram-positive strain *Staphylococcus aureus* (Lai et al. 2002b).

Some high molecular weight proteins have also been described in toad secretions (Table 3). Gomes and colleagues (2011) characterized a protein (BMP1, MW-79kDa) isolated from the skin extract from an Indian toad (*Bufo melanostictus*) that showed anticancer activity. There are other proteic compounds present in toad poisons, such as the lysozyme BA-lysozyme of 15 kDa (Zhao et al. 2006) and

Table 2 Some examples of peptides from toad

Peptide	Sequence	Activity/ action	Species	References
Alyteserin 1a	GLKDIFKAGL[10] GSLVKGIAAH[20] VAN-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 1b	GLKEIFKAGL[10] GSLVKGIAAH[20] VAN-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 1c	GLKEIFKAGL[10] GSLVKGIAAH[20] VAS-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 1d	GLKDIFKAGL[10] GSLVKNIAAH[20] VAN-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 2a	ILGKLLSTAA[10] GLLSNL-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 2b	ILGAILPLVS[10] GLLSNKL-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Alyteserin 2c	ILGAILPLVS[10] GLLSSKL-NH_2	Antimicrobial	*Alytes obstetricans*	Conlon et al. (2009)
Buforin I	AGRGKQGGKV[10] RAKAKTRSSR[20] AGLQFPVGRV[30] HRLLRKGNY	Antimicrobial	*Bufo bufo gargarizans*	Park et al. (1996)
Buforin II	TRSSRAGLQF[10] PVGRVHRLLR[20] K	Antimicrobial	*Bufo bufo gargarizans*	Park et al. (1996)
Buforin IIb	RAGLQFPVGR[10] LLRRLLRRLL[20] R	Antimicrobial	*Bufo bufo gargarizans*	Cho et al. (2009)
Maximin H5	ILGPVLGLVS[10] DTLDDVLGIL[20]-NH_2	Antimicrobial	*Bombina maxima*	Lai et al. (2002b)

lactose-binding lectin (Riera et al. 2003). Some serine protease inhibitors were also isolated from toad skin secretions, for example, BMSI 1 and BMSI 2, from *Bombina microdeladigitora*, both composed of 60 amino acids (Lu et al. 2008); BTMI from the Chinese redbelly toad *Bombina maxima*, composed also of 60 amino acids (Lai et al. 2002c); BOTI and BVTI from the Oriental fire-bellied toad (*Bombina orientalis*) and the European yellow-bellied toad (*Bombina variegata*) (Chen and Shaw 2003), respectively; BATI from the skin extracts of toad *Bufo andrewsi* (Zhao et al. 2005a); and baserpin, an irreversible serine protease inhibitor from the toad *Bufo andrewsi* (Zhao et al. 2005b).

Besides all these compounds described above, an interesting protein designed as BAS-AH was purified and partially characterized from skin secretions of *Bufo andrewsi*. BAS-AH is a single-chain protein with an apparent molecular weight of 63 kDa that showed potent anti-HIV-1 activity, inhibiting both HIV-1 infection and

Table 3 Some examples of proteins from toad poisons

Protein	Action/activity	Molecular weight	Species	References
BA-lysozyme	Antimicrobial	15kDa	*Bufo andrewsi*	Zhao et al, (2006)
Lactose-binding lectins	Antimicrobial	50kDa/ 56kDa	*Bufo arenarum*	Riera et al. (2003)
BMPI	Anticancer activity	79 kDa	*Bufo melanostictus*	Gomes et al. (2011)
BMSI 1	Serine protease inhibitors	9.130 kDa[a]	*Bombina microdeladigitora*	Lu et al. (2008)
BMSI 2	Serine protease inhibitors	9.261 kDa[a]	*Bombina microdeladigitora*	Lu et al. (2008)
BTMI	Serine protease inhibitors	9.080 kDa[a]	*Bombina maxima*	Lai et al. (2002c)
BATI	Serine protease inhibitors	22 kDa	*Bufo andrewsi*	Zhao et al. (2005a)
Baserpin	Irreversible serine protease inhibitor	60 kDa	*Bufo andrewsi*	Zhao et al. (2005b)
Bv8	Hyperalgesia	8 kDa	*Bombina variegata* and *Bombina bombina*	Mollay et al. (1999)
BAS-AH	Anti-HIV-1 activity	63 kDa	*Bufo andrewsi*	Zhao et al. (2005c)

[a]Molecular weight by UniProt (www.uniprot.org)

HIV-1 replication as well as revealing low toxicity on cell viability assay (Zhao et al. 2005c).

Important studies had shown a diversity of applications to these peptides/proteins from toad poisons. One example is the study about the small protein named Bv8 from skin secretions of *Bombina variegata* and *Bombina bombina* that induced hyperalgesia in rats (Mollay et al. 1999) evidencing other possible applications as in signaling pathway that triggers the development of neuropathic and inflammatory pain.

There are few studies about peptides and proteins from toads when compared with the studies of these same compounds in frogs. In the future, it will be necessary to better differentiate frogs and toads, since many authors do not distinguish between these animals.

Biogenic Amines

Biogenic amines are small molecules that play key roles in neurotransmission and several signaling functions that include, according to Gerald (2009), three catecholamines (dopamine, norepinephrine, and epinephrine) and tyrosine metabolites,

octopamine and tyramine, beyond serotonin (indoleamine) and histamine (imidazoleamine). These compounds can act as neurotransmitters in various physiological responses. Generally, they have multiple sites of action in the body and are able to affect functions related to appetite, blood pressure, and body temperature (Singewald and Philippu 1996; Zeisberger 1998; Toninello et al. 2004; Frampton et al. 2010).

In the toad skin, aromatic amines were found which are divided in three groups: (1) the indolealkylamines, including 5-hydroxytryptamine and N-methylated derivatives (e.g., bufotenin and bufotenidine), (2) the imidiazolealkylamines and related histamine and hydroxyphenylalkylamines present mainly in frogs, (3) and adrenaline and its derivate noradrenaline found in *Bufo* spp. of toads (Erspamer 1971; Clarke 1997).

The biosynthesis of adrenaline, noradrenaline, dopamine, and epinine (N-methyldopamine) starts from phenylalanine, which is oxidized via tyrosine to yield dioxphenylalanine. The decarboxylation of dioxphenylalanine produces dopamine, which is either methylated to epinine or hydroxylated to noradrenaline. Methylation of noradrenaline yields adrenaline (Habermehl 1981).

The indolealkylamine derivatives, such as bufotenin and bufotenidine, have hallucinogenic, hypertensive, and vasoconstrictor properties beyond some that can trigger convulsions (Clarke 1997). Among the biogenic amines found in toads (cyclized and/or conjugated amines), the majority have low activity or are inactive on blood pressure, respiration, and smooth muscle preparations, but it might be active on other systems or influence biochemical events yet unknown (Erspamer 1971).

Alkaloids

Alkaloids are compounds found in nature, which are derived from amino acids. They have nitrogen as an electron acceptor and are defined as cyclic nitrogen-containing molecules. This class of secondary metabolites exhibits a distribution in nature that encompasses both plant and animal kingdom, notably in amphibians (Clarke 1997).

Amphibians have the ability to sequester alkaloids from dietary arthropods for chemical defense. They present in the skin a very broad range of apolar alkaloids that exhibit activity in molecular targets. Among the alkaloids from amphibians, samandarines, batrachotoxins, histrionicotoxins, gephyrotoxins, epibatidine, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, decahydroquinolines, a diversity of izidines (pyrrolizidines, indolizidines, quinolizidines, lehmizidines), pyrrolidines, piperidines, various tricyclics, and spiropyrrolizidines can be cited (Daly et al. 2005).

As stated before, the aim of this chapter is to present molecules provided with toads, such as those described above as true toads, the Bufonidae family. Hence, the immense wealth of alkaloids derived from frogs will be explored on another occasion.

Alkaloids that were previously molecules regarded as Dendrobatidae family were also discovered in skin secretions of genus *Melanophryniscus* that belongs to the Bufonidae family. These alkaloids are part of a class of **pumiliotoxin-A** (Garraffo et al. 1993). *Melanophryniscus* can be found in South America particularly in countries like Brazil, Uruguay, Paraguay, Argentina, and Bolivia. In this genus there are 25 species, and only three of them (M. devincenzii, M. montevidensis, M. stelzneri) had their secretions studied and showed the presence of alkaloids such as pumiliotoxins, decahydroquinolines, indolizidinas, pyrrolizidines and quinolizidines (Mebs et al. 2005).

The presence of these alkaloids in toad secretions may be related to their feeding constituted by arthropods. However, these substances can also be produced by metabolic pathways independent of food, which have not yet been clarified (Mebs et al. 2005; Daly et al. 2005).

Activities of Poison Components with Biotechnological Potential

Numerous bioactive components from toad poisons, with diverse biological activities, have been studied (Fig. 5). Toads and their skin secretion properties may be found throughout several cultures and folklores around the world. This traditional knowledge led to the concept that the secretions of toads can be a rich source of biologically active compounds (Sciani et al. 2013b). Traditional Chinese medicine (TCM) is currently practiced worldwide, and Chansu, the dried secretion from the skin glands of toads, has been used clinically for over a millennium as an anodyne cardiotonic, antimicrobial, local anesthetic, and antineoplastic agent (Qi et al. 2011). It has been used for centuries in various prescriptions, recipes, and readily available remedies, such as Liushenwan (anti-inflammation), Shexiangbaoxinwan (anti-arrhythmia), and Niuhuangxiaoyanwan (anti-inflammation and anesthesia) in China, Japan, and other Southeast Asian countries (Hong et al. 1992 *apud* Li et al. 2013).

Antitumor Activity

Cancer is a complex multifactorial disease of the cell. Transformed cells are not constrained by growth controls, which regulate the proliferation of normal cells. Nowadays, chemotherapy is one of the most important and fundamental tools for the treatment of cancer. However, the current chemotherapeutic agents have been shown ineffective for certain types of cancer and have caused many side effects to patients (Lu et al. 2010). Thus, the use of complementary and alternative medicine has become increasingly popular among cancer patients over the past few years.

Traditional Chinese medicine (TCM) is currently practiced worldwide and is frequently used to treat cancer, either alone or in combination with other medicines. Chansu, initially recorded in TCM for more than 1,000 years, is the dried toad

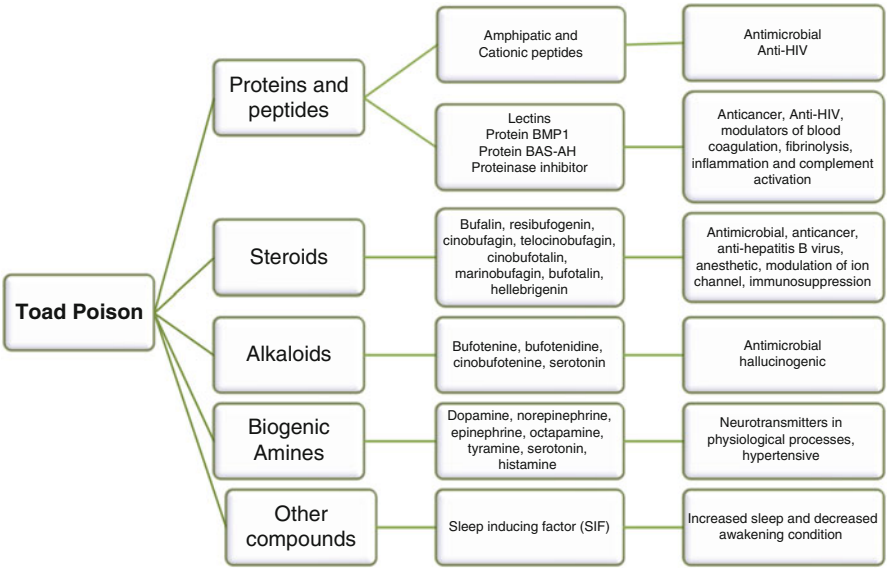


Fig. 5 Toad poison composition and activities

venom secreted by the skin glands of *Bufo bufo gargarizans* Cantor or *Bufo melanostictus* Schneider (Qi et al. 2011).

Huachansu (Cinobufacini) which contains Chansu is a sterilized water extract of dried toad skin that has been prepared for injection and has been widely used to treat patients with various types of cancer in oncological clinics in China (Qi et al. 2011). Huachansu is approved by the Chinese State Food and Drug Administration (ISO 9002). Its major biologically active chemical components are steroidal cardiac glycosides (such as bufalin, resibufogenin, cinobufagin, cinobufotalin, marinobufagin, and bufotalin) and indole alkaloids (bufotenine, bufotenidine, cinobufotenine, and serotonin) (Su et al 2003). Several studies in vitro have demonstrated that Huachansu exerts antitumor activity on various tumor cell lines and it is able to inhibit cell proliferation and induces cell differentiation, apoptosis, and disruption of the cell cycle. In vivo studies demonstrated its ability to inhibit cancer angiogenesis, to reverse multidrug resistance, and to regulate the immune response (Zhu and Liu 2006).

In vitro studies demonstrated that Huachansu inhibited cell proliferation in human hepatocellular (SMMC-7721), gastric (MKN45), and colon cancer (LoVo) cell lines and induced apoptosis of gastric carcinoma MGC-80-3 cells (Zhang et al. 1999 *apud* Meng et al 2012; Zuo et al 2003a). Huachansu inhibited the growth of human cell lines MGC-80-3 and SMMC-7721 through S-phase arrest and inhibition of bcl-2 expression (Zuo et al 2003b). It also inhibited the biosynthesis of DNA and RNA in ascitic hepatoma cells (H22 AH) in a dose-dependent manner (Guan et al. 1993). Furthermore, when human breast cancer cells (BCF-7) and lung cancer cells (A549) were treated with Huachansu, the drug significantly inhibited the growth of A549

cells, but not BCF-7 cells, suggesting that the antiproliferative activity of Huachansu might be cell type specific (Liu et al. 2002).

The toxicity of Huachansu was evaluated by a Phase I clinical study, conducted by Meng et al. (2009). The typical dose used in China is approximately 15 mL of drug per meter squared of body mass (mL/m^2). In this study, 15 patients with stage III or IV hepatocellular (liver) carcinoma, non-small cell lung cancer, or pancreatic cancer received one of five dose levels ranging from 10 up to 90 mL/m^2 for 18 months. The results showed that six hepatocellular carcinoma patients had stable disease for a median of 6 months and one patient had a 20% reduction in tumor mass that lasted for more than 11 months, and no significant cardiac toxicity was observed. Thus, this study showed that Huachansu has tolerable toxicity levels, even at doses six times those normally administered, and may slow disease progression in some cancer patients.

Another study examined 15 randomized clinical trials, using clinically relevant outcome measures, and focused on direct comparisons of the efficiency and safety on Huachansu combined with chemotherapy to simple chemotherapy in advanced gastric cancer (Xie et al. 2013). The results showed that Huachansu combined with chemotherapy is superior to simple chemotherapy treatment for increasing the total response rate and Karnofsky score, reducing the occurrence of gastrointestinal side effects and leukocytopenia in advanced gastric cancer patients.

Researchers also evaluated the efficacy and safety of gemcitabine-oxaliplatin (GEMOX) combined with Huachansu in patients with advanced gallbladder carcinoma to assess the quality of life of the patients (Qin et al. 2008). This cancer has a very poor prognosis. There is no generally accepted standard, and the median survival time of advanced cancer patients who receive best supportive care is approximately 6 months. The results of this study indicated that combined GEMOX and Huachansu chemotherapy is an effective regimen for metastatic gallbladder carcinoma. The patients can tolerate it well, and the toxicities are mostly hematological and easily manageable. If this combined regimen could be applied in clinic practice, the patients would have a longer survival time.

Bufalin, resibufogenin, and cinobufagin are the three major cardiac glycosides to which the anticancer activity of Huachansu can be attributed (Su et al 2003). The enzyme Na^+/K^+ ATPase appears to play a significant role in inhibiting the proliferation of tumor cells induced by cardiac glycosides. Na^+/K^+ ATPase is composed of three subunits: α-catalytic subunit, containing the biding sites for Na^+/K^+ ATPase and the cardiac glycoside, that has four different isoforms, namely, α-1, α-2, α-3, and α-4; β-regulatory subunit, required for the biogenesis and activity of the enzyme complex; and γ-subunit, another regulator of the Na^+/K^+ ATPase that can increase the apparent affinity of the enzyme for ATP (Kaplan 2002 *apud* Li et al. 2011). Some studies demonstrated that ATPase isoform α-3 may be an important therapeutic target for cardiac glycosides to treat cancer (Newman et al. 2008; Li et al. 2011).

Bufalin is the major digoxin-like immunoreactive component of Chansu, and it has been demonstrated to be able to induce growth inhibition, cell cycle arrest, and apoptosis on tumor cells. Bufalin inhibited cell proliferation in vitro of endometrial cancer and ovarian cancer cells by G0/G1 arrest of the cell cycle and by inducing the

apoptosis. It demonstrated little effect on normal human endometrial epithelial cells, which suggests that the effects of bufalin might be cell type specific and could be weaker on the normal endometrium (Takai et al. 2008). Bufalin also inhibited the proliferation of gastric cancer MGC803, so that at lower concentrations it induced M-phase cell cycle arrest, whereas at higher concentrations it induced apoptosis (Li et al. 2009). Bufalin has significant antitumor activities in vitro in hepatocellular carcinoma, leukemia, lung cancer, breast cancer, colon cancer, osteosarcoma, and bladder cancer, inducing apoptosis in the cells by different pathways (Takai et al. 2012).

Cinobufagin accounts up to 6–7% of Chansu and is capable to inhibit cancer cell growth by induction of apoptosis through different pathways in human cancer cell lines: HCT116 (colon), HT29 (colon), A431 (skin), PC3 (prostate), A549 (lung), Spc-A1 (lung), and MCF-7 (breast) (Li et al. 2013).

Resibufogenin inhibited the proliferation of human liver cancer cells SMMC-7721 and BEL-7402 in a time-dependent manner and induced G2-/M-phase cell cycle arrest in SMMC-7721 cells (Xie et al. 2012).

The protein BMP1 (MW-79 kDa) isolated from *Bufo melanostictus* was also tested to evaluate its antitumor activity. This protein demonstrated to be anti-proliferative and apoptogenic against mouse Ehrlich ascites carcinoma with limited toxicity (Bhattacharjee et al. 2011). It inhibited cell proliferation and induced apoptosis on leukemic (U937, K562) and hepatocellular carcinoma (HepG2) cells with minimum toxicity toward normal cell indicating its specificity (Gomes et al. 2011).

Taking into account the studies conducted to date using toad poison, it can be concluded that the compounds present in secretions possess potent antitumor activity, showing proapoptotic functions and an inhibitive effect on a wide range of cancer cells. Huachansu and its active bufadienolides should be studied more deeply and are excellent candidates to become a new clinical therapy to treat cancer and may improve the quality of life of patients.

Antimicrobial, Antiviral, and Antiparasitic Activities

Toad defense mechanism is mainly provided by the cutaneous secretion, and the production of a large number of antimicrobial peptides ensures its defense against various pathogens. Currently, there are many natural peptides and several synthetic peptide possibilities available to studies, but only few of these compounds are in clinical trial phase. Several of these bioactive components may be used to solve problems faced by the pharmaceutical industry, agriculture, and livestock, due to the increase of resistant pathogens to antimicrobial agents commercially available (Gordon and Romanowski 2005). Among the applications of the toad compounds, the antimicrobial activity is the most studied.

In general, the mechanism of action of antimicrobial peptides from toad poisons involves disturbance of membrane functions. They can adopt an amphipathic α-helical structure in hydrophobic environments, and oligomers of such molecules

would form pores in the phospholipid bilayer of target microorganisms, resulting in cell death or inhibition of cell growth.

Amphibians are rich sources of antimicrobial compounds, particularly peptides. In the last years, the number of peptides/proteins with antimicrobial activity isolated from different toads has increased. Among the peptides, the designed maximins 1, 2, 3, 4, and 5 showed potent antimicrobial activity. These peptides have similar structure to bombinin-like peptides (BLPs) and demonstrated a broad spectrum of antibacterial activities against bacteria, fungi, and yeasts. These maximins injected intraperitoneally on mice induced typical poisoned symptoms, such as gasp, jerk, and tension. In addition, the subcutaneous (s.c.) injection of them induced widespread hyperemia of s.c. capillary vessel. The LD_{50} value obtained to maximin 1 was 8.2 mg/kg and to maximin 3 was 4.3 mg/kg (Lai et al. 2002a). Peptides from the stomach tissue of *Bufo bufo gargarizans* named buforin I and buforin II have also shown important antimicrobial activity against several gram-positive and gram-negative bacteria and fungi. It was observed that the buforin I fragment, 21-amino acid long and named buforin II, produced by treatment of buforin I with endoproteinase Lys-C, showed more potent antimicrobial activity than buforin I (39-amino acid long) (Park et al. 1996). As possible mechanism of action of buforin II, it was suggested that this antimicrobial peptide permeates the membrane and inhibits the cellular functions, by binding to DNA and RNA, resulting in the rapid cell death (Park et al. 1998).

There are several other peptides with antimicrobial activity. The alyteserins are good examples of peptides with a broad-spectrum antimicrobial activity. They are active against *E. coli*, *K. pneumonia*, *P. aeruginosa*, *A. baumannii*, *S. aureus*, and *C. albicans*. The antimicrobial potency of peptides and its cytotoxicity is determined by complex interactions among hydrophobicity, amphipathicity, α-helicity, and cationicity (Conlon et al. 2009; Subasinghage et al. 2011; König et al. 2012).

Among the lysozymes, the BA-lysozyme exerted antimicrobial activity against *S. aureus* and *E. coli* (Zhao et al 2006). The lysozyme class lyses the cell wall of some gram-positive bacteria by splitting β-1,4-glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan (Hasselberger et al. 1978 *apud* Zhao et al. 2006; Hughey and Johnson 1987). Other examples of proteins with antimicrobial activity are the lectins. They are a class of multivalent carbohydrate-binding proteins of nonimmune origin. Lectins recognize several sugar structures with a high degree of specificity and are involved in diverse biological processes as cellular signaling, host-pathogen interactions, target to cellular compartments, and others (Gallegos et al. 2013). In the skin of the *Bufo arenarum* toad, Riera and colleagues (2003) isolated two β-galactoside-binding lectins (molecular weights of 50 and 56 kDa) with strong bacteriostatic activity against gram-negative bacteria (*E. coli* K12 4100, *E. coli*, and *Proteus morganii*) and gram-positive bacteria (*Enterococcus faecalis*) and significant hemagglutination activity.

With the increasing use of molecular biology techniques and organic synthesis, numerous studies have discovered several peptide/protein sequences and, subsequently, synthesized them. In this sense, as an example, there is the maximin H5, an anionic antimicrobial peptide isolated from a skin cDNA library of *B. maxima* that

showed activity against *Staphylococcus aureus*. Maximin H5 was chemically synthesized in the C-terminal amidated form and characterized by three remarkable aspartate residues at positions 11, 14, and 15. It has a limited antimicrobial spectrum on tested bacterial and fungal stains, but *S. aureus* was sensitive to it, with an MIC of 80 μM. Maximin H5 does not induce hemolysis on rabbit red cells, and it was the first evidence that anionic antimicrobial peptides may exist naturally as part of the innate defense system of toads (Lai et al. 2002b).

Besides the proteins and peptides, other compounds such as steroids demonstrate significant antimicrobial activity among others. The bufadienolides known as marinobufagin and telocinobufagin are present in the skin secretion of many toads. These compounds are focus of numerous studies to several applications, mainly antitumor. Both steroidal compounds are active against *Staphylococcus aureus* and *Escherichia coli* (Cunha filho et al. 2005).

There are already several alkaloids identified which demonstrated an important antimicrobial activity in many microorganisms. The quantity of alkaloid present in the skin of toads is highly related to both animal diet and the availability of these compounds in the area that the animal inhabits. Among the several lineages, anurans, the bufonid toads (genus *Melanophryniscus*), are able to sequester alkaloids from dietary arthropods (Daly et al. 2007 *apud* Daly et al. 2008).

Few studies demonstrated the use of toad compounds in activities against parasites and viruses revealing an area with little data in the literature. There are many studies of base, and few studies have actually provided compounds with possible commercialization or even used in clinical medicine.

Among the cardioactive steroids, the bufadienolides have been utilized against parasitic diseases. In the study of Tempone and colleagues (2008), two bufadienolides (telocinobufagin and hellebrigenin) isolated from *Rhinella jimi* parotoid macrogland secretion were tested against *Leishmania* (L.) *chagasi* promastigotes and *Trypanosoma cruzi* trypomastigotes. Both bufadienolides demonstrated activity against *Leishmania* (L.) *chagasi* promastigotes, but only hellebrigenin was active against *Trypanosoma cruzi* trypomastigotes. Telocinobufagin caused cellular damage of promastigotes inducing the loss of nucleolar structures with amastigote-like forms, probably due to an increased cell permeation. A possible action mechanism the activity in *Leishmania* might involve is the disturbance of the parasite plasma membrane and mitochondrial metabolism, causing a cellular edema and death.

The antiparasitic activities of secretions of toads *Rhinella ictericus* and *Rhinella schneideri* were performed against *L. chagasi* promastigotes, *L. chagasi* intracellular amastigotes, *T. cruzi* trypomastigotes, and *Toxoplasma gondii* intracellular tachyzoites. The secretions of both toads were toxic to *L. chagasi* and *Toxoplasma gondii* intracellular forms, and only the secretion of the toad *Rhinella schneideri* presented antileishmanial activity against the promastigotes of *Leishmania* (L.) *chagasi*. For the antitrypanosomal activity, both toad secretions showed promising activities (Tempone et al. 2007).

The traditional Chinese medicine uses dried toad skin in clinical therapy against various diseases in China. The *Bufo bufo gargarizans* Cantor skin secretions are

used in the traditional Chinese medicine such as Chansu and Huachansu (Cinobufacini). The Huachansu is widely used in several cancer therapies and treatment of hepatitis B in China. The Huachansu was tested in the human HBV-transfected cell line HepG2.2.15 to examine the anti-hepatitis B virus (HBV) activities. Beyond the Huachansu, bufalin and cinobufagin were also tested. The results showed that Huachansu had more potent anti-HBV properties than bufalin and cinobufagin and that its inhibitory activities were not induced by cytotoxicity in vitro. The compounds evaluated were more potent in lower concentrations than in higher concentrations regarding the inhibition of HBV antigen secretion, and this effect was attributed to the specific inhibition of HBV mRNA expression (Cui et al. 2010). Despite presenting great therapeutic potential, the Huachansu will need further studies to a greater adhesion in the world. A peptide named maximin 3, isolated from skin secretions of *Bombina maxima*, has shown a significant anti-HIV activity besides showing other important activities as antimicrobial, spermicidal, and cytotoxic against tumor cells (Lai et al. 2002a), but its action mechanism remains unclear.

Just as referred above, a protein designed as BAS-AH was purified and partially characterized. This protein with an apparent molecular weight of 63 kDa was able to inhibit both HIV-1 infection and HIV-1 replication. Its N-terminal sequence (NAKXKADVIGKISILLGQDNLSNIVAAM) showed little identity with other known anti-HIV proteins. This protein has the heme cofactor that contributes to the reverse transcriptase inhibition. In this study, the heme is not the principal reason for inhibition of the reverse transcriptase, and there are other unknown elements involved in this action. The antiviral potency showed by this protein is very similar to an L-amino acid oxidase from *Trimeresurus stejnegeri* snake venom (Zhao et al 2005c).

This information has contributed to drug design and tools to the development of the new therapeutics against several pathologies.

Cardiovascular Activity

Bufogenines and bufotoxins present inotropic effects similar to digitalis toxins, increasing the strength of contraction of the heart muscle. Poisoning by toad toxins is primarily manifested by digitalis-like symptoms, cardioactive effects resulting in bradycardia, different degrees of atrioventricular block, ventricular tachycardia, ventricular fibrillation, and sudden death (Chi et al. 1998).

Among the compounds present in the toad poison, the bufogenines and bufotoxins, derived from cholesterol, have properties that alter the normal functioning of the heart, increasing the strength of the heartbeat rhythm and then decreasing it (Clarke 1997). These steroids possess similar effect to those found in plant cardiac glycosides. A single drop of poison from *Bufo* sp. or toads of the genus *Leptodactylus*, deposited directly over the heart, immediately provokes violent contractions of the ventricle (Brazil and Vellard 1926).

Extracts from the parotoid glands of *Bufo marinus* toad, containing endogenous molecules similar to digoxin, present cardioactive effects, for example, vasoconstriction via noradrenergic mechanism and direct inhibition of sodium and potassium pump in the vascular system (Bagrov et al 1993).

Toad poisons are used in traditional Chinese medicine even as aphrodisiacs, and according to Gowda et al. (2003), a man died after ingesting pills containing the extract of parotoid glands due to cardiac arrest. The death occurred because this poison has symptoms similar to digoxin and soon developed symptoms after ingestion of severing unrelenting bradycardia, hyperkalemia, acidosis, and sever life-threatening arrhythmias.

Among the components of the toad poison, cardiac glycosides are responsible for the symptoms of cardiac toxicity. These molecules are able to bind to the extracellular domain of the Na^+/K^+ ATPase, causing its inhibition and, consequently, increased intracellular Na^+ concentration ($[Na^+]_i$) (Mijatovic et al. 2007). The Na^+/Ca^{2+} exchanger (NCX) is reversible and can extrude Ca^{2+} as an inward ionic current ($I_{Na/Ca}$) or bring Ca^{2+} into the cell as outward $I_{Na/Ca}$. The amount of Ca^{2+} influx can be increased greatly if $[Na^+]_i$ is elevated. Therefore, the high $[Na^+]_i$ caused by Na^+/K^+ ATPase inhibition favors outward $I_{Na/Ca}$ enhancing the intracellular Ca^{2+} concentration, which is available for the contractile proteins, resulting in contraction (Bers 2002) (Fig. 6).

In rats, the toad poison showed potential to cause constriction in isolated aortic rings, and the inhibition of Na^+/K^+ ATPase in both rat aorta and erythrocytes could be observed (Bagrov et al. 1993). Some works had been shown that the poison causes vasoconstriction, and this occurs because of vascular smooth muscle enzyme inhibition. The glycoside poisoning also manifests electrocardiographic changes associated with atrioventricular blocks and flattened T-waves (Lim et al. 1997).

Although these drugs present a very low therapeutic window, there are reports of the use of these molecules for treatment of heart disease and that cases were successful (Mijatovic et al. 2007; Stein and Heerden 1998).

Other Activities

Toad poisons present a lot of peptides with diverse biological activities, among them peptides related to mammalian hormones or neurotransmitters. Some bradykinin-like peptides have been isolated from amphibian skin secretions. They are structurally and functionally related with mammalian bradykinin. Bombinakinin M, purified from skin secretions of the Chinese redbelly toad *Bombina maxima*, is an example of this class of peptide (Lai et al. 2001), with molecular mass of 2,179 Da. It is composed of 19 amino acid residues (DLPKINRKGPRPPGFSPFR), and its C-terminal 9-amino acid residue segment corresponds to the mammalian bradykinin. Bombinakinin M induced contractile activity isolated from guinea pig ileum, but its physiological role is not entirely clear.

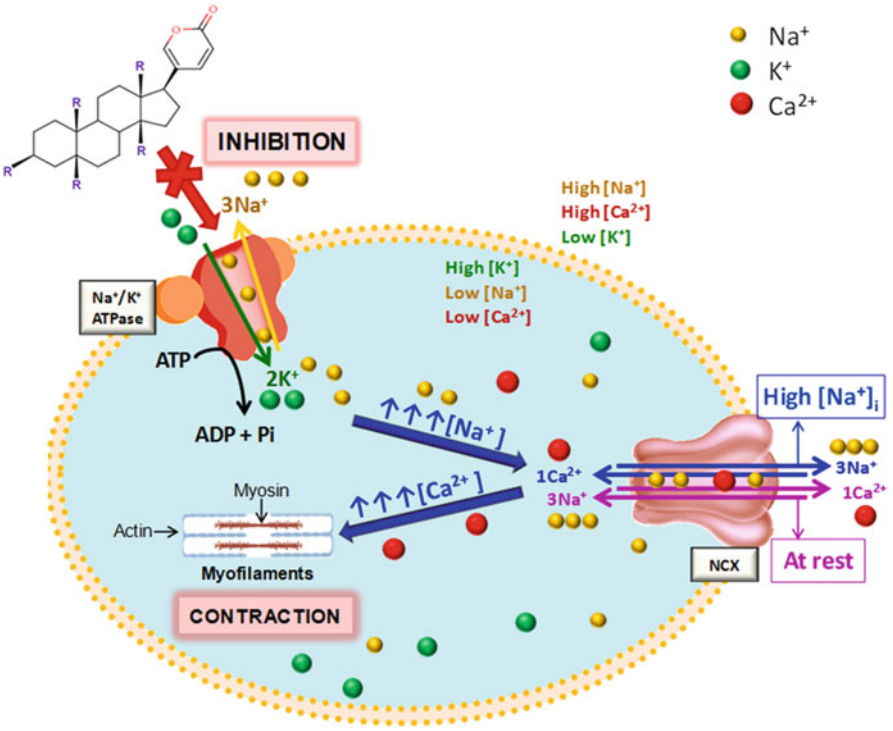


Fig. 6 Cardiotonic glycosides are inhibitors of Na^+/K^+ ATPase that is responsible for translocating Na^+ and K^+ ions across the cell membrane using ATP. For every molecule of ATP hydrolyzed, three Na^+ ions from the intracellular space and two K^+ ions from the external medium are exchanged. The Na^+/K^+ ATPase inhibition induces an increase in $[Na^+]_i$ that stimulates a reverse ion flux ($3Na^+/1Ca^{2+}$) held by NCX, which increases $[Ca^{2+}]_i$, enhancing the force of contraction of the heart

The serine protease inhibitors BMSI 1 and BMSI 2, isolated from the skin secretions of the toad *Bombina microdeladigitora* (Lu et al. 2008), are proteins (60 amino acids including 10 half cystines) that are of broad interest because they can act as modulators in a variety of physiological functions such as blood coagulation, fibrinolysis, inflammation, and complement activation. These molecules can play a key role in resistance to pathogen invasion, justifying its accumulation in the toad skin. BMSI 1 potently inhibited trypsin (K_i 0.02 μM) and thrombin (K_i 0.15 μM).

Other serine protease inhibitors have also been isolated from the skin secretions of toads of the genus *Bombina* (BSTI, 60 amino acids), which are also potent inhibitors of thrombin and trypsin (Chen and Shaw 2003). A Kunitz-type serine protease inhibitor (57 amino acids) was also isolated from *Dyscophus guineti* skin secretions (Conlon and Kim 2000).

A sleep-inducing factor (SIF), an 880 Da conjugated aromatic compound with a hydroxyl and carbonyl functional group, was isolated from the skin extract of *Bufo melanostictus* (Das et al. 1996). SIF increased sleep and decreased awakening condition of freely moving rats and induced significant alteration of

brain biogenic amine levels, monoamine oxidase (MAO), and tryptophan hydroxylase (TH) activity. This may be the reason of SIF-induced sleep.

Two bufadienolides, named resibufogenin (RBG) and cinobufagin (CBG), are able to inhibit potassium channels of hippocampal neurons (Hao et al. 2011a). RBG inhibited both the outward delayed rectifier potassium current (I(K)) and outward transient potassium current (I(A)), whereas CBG inhibited I(K) without noticeable effect on I(A). RBG was also able to activate voltage-gated sodium channel currents (I(Na)) of hippocampal neurons in a concentration-dependent manner (Hao et al. 2011b). These results suggest that the inhibition of I(K) is probably the mechanisms of bufadienolides' pathological effects on the central nervous system.

Chansu has long been used for the treatment of chronic inflammation in the Chinese folk medicine. In a study performed in BV2 microglial cells, it was shown that Chansu inhibited the secretion of NO, decreased the protein level of inducible nitric oxide synthase (iNOS), and inhibited production of prostaglandin E2 (PGE2) and the expression of cyclooxygenase (COX)-2, without affecting cell viability. Additionally, it was able to inhibit proinflammatory cytokines (tumor necrosis factor (TNF)-α, interleukin (IL)-1-β and IL-12) and the degradation of IκB-α (inhibitor of nuclear factor (NF)-κB). Since NF-κB is considered a potential transcription factor from the expression of iNOS, COX-2, and proinflammatory cytokines, these results suggest that Chansu could exert its anti-inflammatory effects by suppressing the synthesis of NO, COX-2, PGE2, and ILs through inhibition of NF-κB activity (Kim et al. 2008).

Many studies show that cardenolides and bufadienolides present strong local anesthetic action (HABERMEHL 1981) and immunoregulatory properties. Terness et al. (2001) analyzed the in vitro effects of 52 bufadienolides in human T-cells stimulated with mitogens or alloantigens. They show that bufadienolides inhibited preactivated T-cell activity at low concentrations and that suppressed viable T-cells could not be restimulated. The replacement of 14b-OH with an epoxy group abolishes the cardiac activity of the bufadienolide, but slightly decreased its immunosuppression activity. This finding is relevant for therapeutic applications, since the molecule keeps the immunoregulatory properties without the risk of cardiotoxicity.

Bufotenin (bufotenine or dimethyl serotonin) is an alkaloid found in the skin secretions of some species of toads (e.g., *Bufo alvarius*), mushrooms, higher plants, and mammals. It is related to the neurotransmitter serotonin and presents activity similar to LSD (lysergic acid diethylamide) and other known hallucinogens (Emanuele et al. 2010). Endogenous bufotenin may play a role in the pathogenesis of severe mental disorders, such as autism and schizophrenia, which are known to be linked to the methylation of serotonin. Urine bufotenine levels are significantly higher in subjects with autistic spectrum disorder and patients with schizophrenia, compared with healthy control subjects. Thus, the presence and levels of bufotenin in urine seem to be important markers for the diagnosis of autism (Emanuele et al. 2010). The psychoactivity of bufotenin is probably due to its ability to bind and activate the purported hallucinogenic serotonin (5-HT) receptors, 5-HT2A and 5-HT2C (Mcbride 2000). Toad venom preparations containing bufotenine, such as *Chansu* and *love stone*, have been used as supposed aphrodisiac (they are not

aphrodisiac) and as a psychedelic. This use as a street drug has resulted in several cases of poisoning, since they are lethal poisons.

Conclusions and Future Directions

The knowledge of the toxicity of the toads dates back to early times. Chansu, a dried and powdered toad secretion, has been used as a heart drug in traditional Chinese and Japanese medicine since antiquity (3,000 years). Over the past several decades, numerous studies have focused on the biologically active components existing in toad poisons. They are a rich source of peptides, proteins, steroids, biogenic amines, and alkaloids that present diverse physiological and defense functions. These compounds present a broad spectrum of activities, such as anticancer, antimicrobial, antiviral, antiparasitic, modulators of blood coagulation, neurotransmission, analgesic, and anti-inflammatory, among others. Therefore, these natural molecules isolated from toad poisons with selective activity could provide lead structures for the development of novel therapeutic drugs. Additionally, molecules with selective activity to target receptors, many of which play important physiological roles or are associated with specific pathologies, can be used as biological tools for the study of the function of these receptors.

As can be seen throughout this chapter, researchers worldwide have identified several bioactive molecules from toad secretions that possess medicinal properties. However, they need to be properly harnessed and exploited to the fullest, so that they are ready to enter the stage of clinical trials. Currently, new drug delivery systems have been used to improve toxin bioavailability. As example, wheat germ agglutinin-grafted lipid nanoparticles were developed for oral delivery of bufalin (Liu et al. 2010). The development of nano-conjugated venom toxins can provide an option of delivering toxins through different routes, improving their therapeutic properties. However, extensive research is necessary before these products can be considered for clinical trial.

Cross-References

► Random Peptide Library for Ligand and Drug Discovery
► Synthetic Peptides and Drug Discovery

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Venoms as Sources of Novel Anti-Parasitic Agents

17

Camila M. Adade and Thaïs Souto-Padrón

Abstract

Parasitic diseases continue to take an enormous toll on human health, particularly in developing tropical regions, where more than 200 millions people are infected with protozoan parasites belonging to the genera *Plasmodium*, *Trypanosoma*, and *Leishmania*, responsible for Malaria, African trypanosomiasis, and Chagas disease, and the different forms of leishmaniasis. Current antiprotozoan chemotherapeutic agents are toxic and have many decades of clinical use, with ever decreasing efficacy due to drug resistance. Therefore, there is an urgent need for the development of novel agents against protozoan parasites. In recent years, research into animal venom toxins and other molecules isolated from natural sources has intensified, focusing on the potential of these compounds to interfere with parasite physiology and/or vector biology. Venom components and their derivatives already represent more than 50% of pharmaceuticals in clinical use, and novel drugs to treat parasitic diseases could be developed from these compounds. This chapter examines in detail the research into numerous animal venom toxins with potential to become novel clinical agents to treat protozoan-borne diseases. Also, some of these compounds are capable of inhibiting parasite establishment in the insect vector, representing good candidates to be used in the development of strategies for disease transmission control. Overall, the studies discussed here correspond to significant advances in the discovery of new antiprotozoan molecules, and highlight the potential of venoms and toxins as rich sources of relevant biologically active compounds.

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_4

Keywords
Parasitic diseases • Antiparasitic chemotherapy • Protozoan parasites • Animal venoms

Contents

Introduction

Protozoan parasites comprise a very diverse group of unicellular eukaryotes from the Protista kingdom. Parasitic diseases caused by protozoa continue to take an enormous toll on human health, particularly in tropical regions (Pink et al. 2005), where sustainable protection can only be achieved by a combination of improved sanitary conditions, vaccination, and chemotherapy (Müller and Hemphill 2013). The main protozoan infections in humans are caused by parasites from the genera *Plasmodium*, *Trypanosoma*, and *Leishmania*, although other agents with medical importance exist, such as the intestinal protozoa *Toxoplasma gondii* and *Trichomonas vaginalis* (Monzote and Siddiq 2011).

Protozoan diseases cause great suffering throughout developing countries, with high rates of morbidity and mortality, constituting the most widespread global health problem. Millions of individuals worldwide suffer from one or more parasitic infections, with the greatest causes of morbidity attributed to trypanosomatid and apicomplexan parasites (Tempone et al. 2007; Utzinger et al. 2012) (Table 1). Some of these diseases are classified as Neglected Tropical Diseases (NTDs), which refers to a group of mainly chronic, debilitating, and often stigmatizing conditions that primarily affect the poorest individuals living in remote rural and deprived urban settings, in tropical and subtropical countries (Utzinger et al. 2012). They are classified as "neglected" due to the limited amount of research and control funding allocated to them, both by developing world governments and by other donors. Less than 10% of research funds are dedicated to NTDs compared with malaria, HIV/Aids, and tuberculosis (All-Party Parliamentary Group on Malaria and Neglected Tropical Diseases- APPMG, 2008/9). In 2000, only about 0.1% of global investment in health research was devoted to drug discovery for selected tropical diseases (malaria, leishmaniasis and trypanosomiasis) and tuberculosis, which

Table 1 Overview of the main parasitic diseases for human health addressed in this chapter. This summarizes general features, current estimates for the global burden, currently used drugs and their approximate time of introduction

Disease and[a] etiological agents	Estimated cases[b] ($x10^6$/ year)	Death[c] ($x10^3$/ year)	Geographical[d] localization	Clinical symptons[d]	Drugs[b,d]	Introduction[b, d]
Malaria (*Plasmodium* spp., particularly *P. falciparum* and *P. vivax*	250	889–1,272	Over 100 countries in tropic and subtropics	Fever, shivering, cough, respiratory distress, pain in the joints, headache, watery diarrhea, vomiting, convulsions, severe anemia	Quinine Quinacrine Chloroquine Mefloquine Artemisine and derivates Doxycycline Proguanil (+Atovaquone)	seventeenth century 1930 Around 1945 1985 1972 <1990 1946
Leishmaniasis (*Leishmania* spp., particularly *L. donovani, L. infantum* and *L. chagasi*)	1–2	70	Over 88 countries in tropic and subtropics	Skin ulcers, mucocutaneous complications, and visceral diseases (hepatosplenomegaly)	Pentamidine Liposomal amphotericin B Antimony-(V)-compounds Miltelfosine	1939 1990 1950 2002
Human african trypanosomiasis (*Trypanosoma brucei gambiense*, *T. b. rhodesiense*)	0.01	<10	36 countries in sub-Sahara Africa	Initial haemolytic phase (fever, joint pains followed by neurological disorder, somnolence)	Suramin Pentamidine Melarsoprol Nifurtimox-eflornithine	1920 1930 1949 2009
Chagas disease (*T. cruzi*)	Ten currently infected (chronic disease)	14	From northern Mexico to South Argentina	Acute phase (fever and splenomegaly) Chronic phase (irreversible damage to heart, esophagus, and colon)	Benznidazole Nifurtimox	1974 1970

Data from: [a]Pink et al. 2005; [b]Müller and Hemphill 2013; [c]Utzinger et al. 2012; [d]Monzote and Siddiq 2011

together contribute with approximately 5% of the global disease burden (Pink et al. 2005). In developed countries, these diseases have now been eradicated, but some have reappeared as opportunistic infections in immunodeficient patients, or as emerging diseases related to tourist exchange, immigration flows from endemic areas, and climate changes linked to global warming (Zucca and Savoia 2011).

The control of protozoan diseases requires a complex interplay of activities in the fields of public health, education, politics, and medical sciences. The epidemiological control of protozoan diseases is unsatisfactory due to the difficulties in accomplishing vector and reservoir control, while progress in the development of vaccines is slow and arduous. Currently, chemotherapy remains essential for clinical management and for disease control programs in endemic areas (Monzote and Siddiq 2011). The chemotherapy agents commonly used to treat parasitic diseases are "dated" and have limited efficacy (Table 1). During the past decades, the development of novel chemotherapy agents to treat infectious diseases has regained importance because of increasing resistance against existing drugs, and due to the emergence of novel, previously unnoticed infectious agents (Müller and Hemphill 2013).

Global Burden of the Main Parasitic Diseases

Malaria is a life-threatening disease caused by parasites of the genus *Plasmodium*, with an annual death toll of 655,000, and more than 200 million cases of the disease recorded in the 106 countries where transmission occurs (Burrows et al. 2014) (Table 1). The World Malaria Report (2014) estimate 3.3 billion people in 97 countries and territories are at risk of being infected with *Plasmodium* and developing malaria, and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year). Human malaria is caused by five species of *Plasmodium: P. falciparum*, *P. vivax*, *P. malariae, P. ovale*, and *P. knowlesi* (Autino et al. 2012b). Transmission is achieved through the bite of female *Anopheles* mosquitoes. The emergence of *Plasmodium* resistance to antimalarial drugs and *Anopheles* resistance to insecticides has hampered traditional approaches for malaria treatment and control.

All clinical symptoms of malaria are consequences of merozoite infection of human erythrocytes. Most fatal cases are due to severe anemia or cerebral malaria, but other clinical manifestations also exist and vary in severity and outcome, depending on the parasite species, the organ involved, and the quality and availability of patient care (Autino et al. 2012a). Artemisinin combination therapies (ACTs), used as first-line treatment for uncomplicated malaria, are extremely safe and effective after 3 days of treatment (Burrows et al. 2014). The drugs used in these combinations are synthetic variants of the natural product quinine, including mefloquine, lumefantrine, and chloroquine. Quinine, a quinoline with an (8S, 9R) quinuclidinemethanol group, was the first antimalarial, and its recorded use dates back to the seventeenth century (Burrows et al. 2011). Chloroquine is no longer in use for falciparum malaria, due to reduced parasite sensitivity, and has now been replaced by more recent analogs without cross-resistance: amodiaquine, piperaquine, and the "quinacrine-amodiaquine" hybrid pyronaridine (Burrows

et al. 2014). In addition, combinations of two or more medicines are essential in the fight against resistance, since it is estimated that 1 in 1010 *P. falciparum* parasites become resistant during treatment with ACTs (Burrows et al. 2011).

Pathogenic trypanosomatids from the genera *Trypanosoma* and *Leishmania* infect over 20 million people worldwide, with an annual incidence of ~3 million new infections, in at least 88 countries. An additional 400 million people are at risk of infection by exposure to parasite-infected insects (Schmunis and Yadon 2010) (Table 1 and Fig. 1). *Leishmania* and *Trypanosoma* species have complex life cycles where parasites alternately infect blood-feeding insects and mammalian hosts. Transmission of *Leishmania* and African trypanosomes initiates by injection of infective metacyclic parasites into the skin of the mammalian host, during feeding by insect vectors (sandflies or tsetse flies, respectively). In the case of *T. cruzi* infection, infective metacyclic forms found in the hindgut of triatomine bugs are expelled with feces into or near the feeding site and are introduced into the wound by contamination (McGwire and Kulkarni 2010).

Leishmania parasites cause five distinct disease forms – cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous leishmaniasis (DCL), post-kala-azar dermal leishmaniasis (PKDL) and visceral leishmaniasis (VL) (Rodrigues et al. 2014). VL (also known as "black fever" or "kala-azar,"in India) is the most severe leishmaniasis form and is fatal if left untreated. It is characterized by prolonged fever, enlarged spleen and liver, substantial weight loss, and progressive anemia. Also, VL severity is often increased by patient coinfection with other diseases, such as malaria or HIV (Rodrigues et al. 2014). First-line treatment of leishmaniasis is based on pentavalent antimonials such as meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostan), while second-line drugs are amphotericin B or pentamidine (Alvar et al. 2006). For VL, miltefosine (Impavido) has been employed recently by oral route in India as a first-line treatment, in combination therapy regimes (Alvar et al. 2006). Toxic side effects and increasing resistance severely limit the use of most current antileishmaniasis therapy.

HAT (human African trypanosomiasis), known as sleeping sickness, is caused by two subspecies of *T. brucei* parasites, *T. b. gambiense* or *T. b. rhodesiense* (Table 1). Sleeping sickness has a neurological phase, where the parasite crosses the blood–brain barrier and invades the central nervous system. Treatment is stage specific, and the currently available therapeutic agents for HAT treatment – melarsoprol, eflornithine, pentamidine, and suramin – are few and their use is limited due to age, high toxicity, and drug resistance in several African regions. Also, most drugs are "dated," difficult to administer (especially in resource-limiting conditions) and by no means always successful (Barrett et al. 2007; Jacobs et al. 2011). Few novel compounds have been developed, but none has the potential to dramatically change either the treatment or the control options for this disease.

Chagas disease, caused by *T. cruzi* (Fig. 2), affects the cardiovascular, gastrointestinal, and nervous systems. Besides insect-mediated transmission in areas of endemic disease, protozoan parasites can also be transmitted through contaminated blood transfusions, organ transplants, contaminated food and drink, laboratory accidents, and via the transplacental route (Dias 2009; Schmunis and Yadon 2010). Moreover, in the past

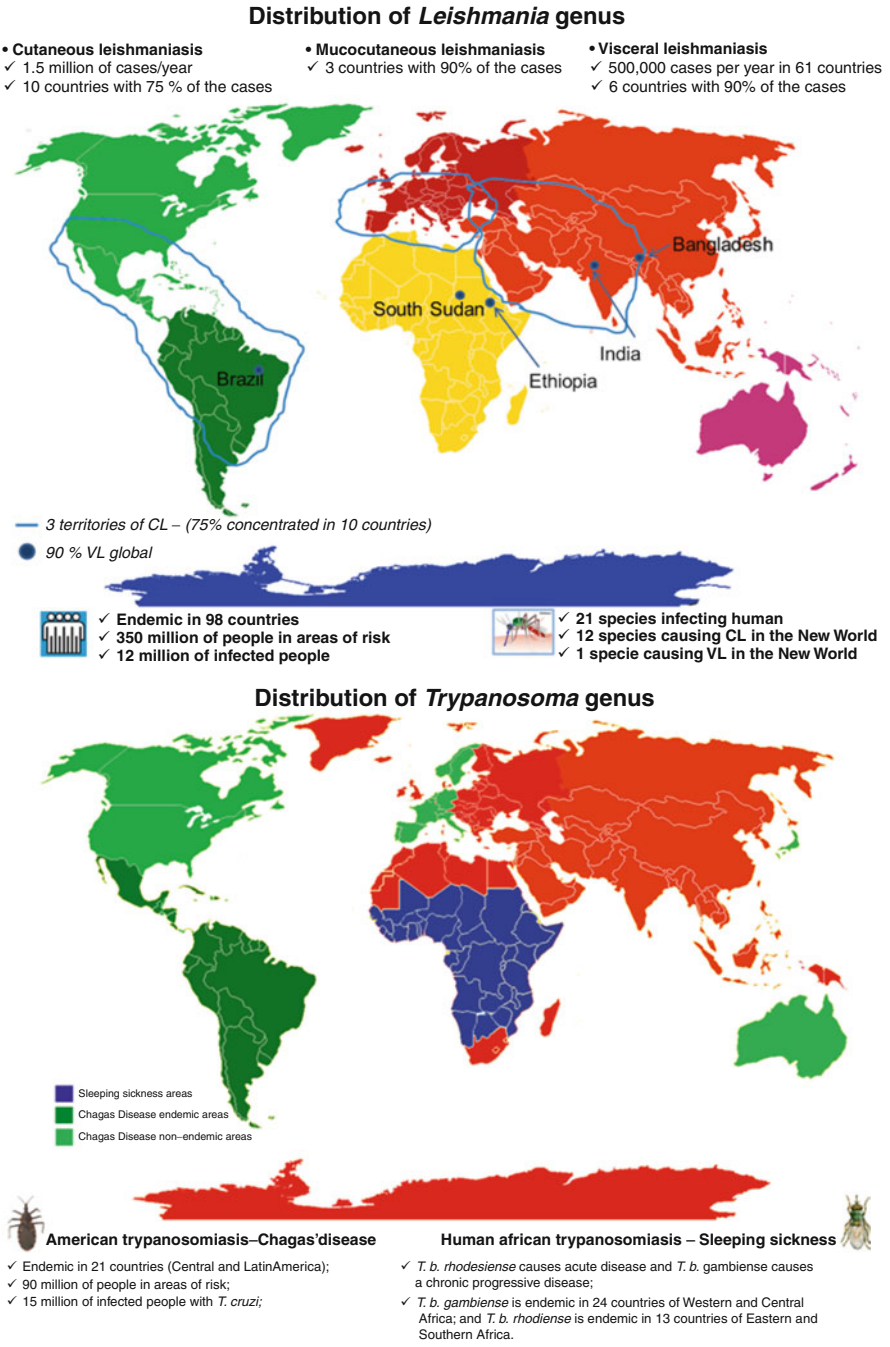

Fig. 1 World distribution of the different clinical manifestations of leishmanias, Chagas disease, and human African trypanosomiasis (*HAT*) (Image reproduced with kind permission from Ref. Rodrigues et al. © (2014) Springer Science and Business Media)

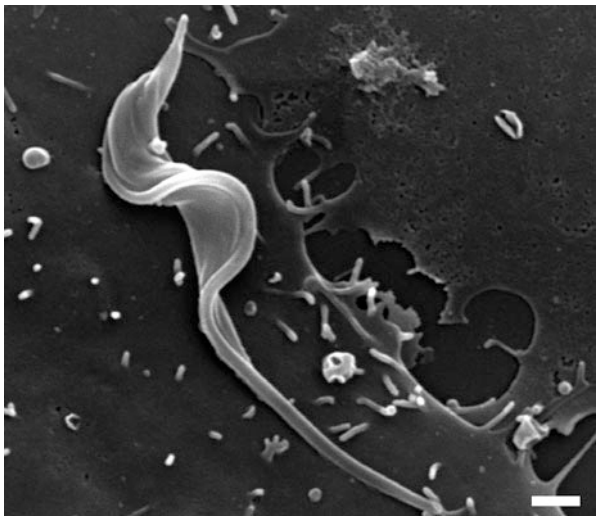

Fig. 2 Scanning electron microscopy of a *T. cruzi* trypomastigote adhered on LLC-MK_2 cells. Bar: 1 μm

few decades, increases in traveling and immigration has turned Chagas' disease into a worldwide public health problem (Schmunis and Yadon 2010), with 15–18 million people at risk. Chagas' disease chemotherapy employs two nitroheterocyclic compounds, nifurtimox and benznidazole, both of which have limited efficacy (particularly against chronic disease), high toxicity and several side effects, and are also prone to drug resistance (Dias 2009). In parallel with clinic treatment, parasite eradication from the insect vector has also been studied as a measure for disease control.

Animal Venoms and Their Therapeutic Properties

The venomous function is present in a wide variety of invertebrates – including sea anemones, corals, jellyfish, marine mollusks, spiders, scorpions, hymenoptera insects, and marine worms – as well as in vertebrates such as snakes, frogs, and lizards. Animal venoms represent libraries of pharmacologically active, receptor-targeted molecules that have been "preoptimized" by nature for use in medicinal chemistry (Escoubas and King 2009). Animal venom toxins are highly complex chemical mixtures of proteins, neurotoxins, ions and peptides, many of which are bioactive, ranging from small organic molecules (<1 kDa), up to high-molecular-weight proteins of more than 100 kDa, including enzymes (Escoubas and King 2009; King 2011).

Initial interest in snake venom aimed at determining the nature and mode of action of venom toxins and developing strategies to revert the effects of snakebites in humans. Also, crude venoms were used in traditional medicine for treatment of human diseases. With the advent of biotechnology, the efficacy of such treatments has improved, with the purification of venom components and refined identification of their therapeutic properties (King 2011; McCleary and Kini 2013). The modern era of venom-based drug discovery started in 1970s with the development of

Table 2 Some drugs derived from venom proteins approved by the US Food and Drug Administration

Drug	Toxin/source of venom protein	Indication	Molecular target	Year	FDA website links
Captopril (Capotenl®)	Bradykinin-potentiating peptides from *B. jararaca* venom	Hypertension	Inhibition of Angiotensin Converting Enzyme	1981	www.fda.gov/cder/foi/anda/97/74532ap.pdf
Tirofiban (Aggrastat®)	Nonpeptide structure based on RGD sequence from snake venom disintegrins	Treatment of acute coronary syndromes	Reversible antagonist of the platelet glycoprotein (GP) IIb/IIIa receptor; inhibits platelet aggregation	1999	www.fda.gov/cder/foi/label/1998/209121b1.pdf
Ziconotide (Prialt®)	ω-conotoxin MVIIA from the cone snail *Conus magus*	Chronic pain	Block N-type Ca2+ channels	2004	www.fda.gov/cder/foi/nda/2004/21-060_Prialt_pharmr.PDF
Eptifibatide (Intergrilin®)	Structure based on the KGD sequence of disintegrin barbourin from *Sistrurus barbouri*	Treatment of acute coronary syndromes	Antagonist of the platelet receptor glycoprotein (GP) IIb/IIIa of human platelets; inhibits platelet aggregation	1998	www.fda.gov/cder/foi/nda/98/20718_INTEGRILIN_pharmr_P1.pdf
Exenatide (Byetta®)	Exendin-4 (39 amino acid peptide) is a incretin mimetic from the saliva of the lizard *Heloderma suspectum* (Gila monster)	Type 2 diabetes	Binding and activation of GLP-1 receptor to reduce plasma glucose and lower HbA1c	2005	www.fda.gov/cder/foi/nda/2005/021773_Byetta_pharmr.PDF

Data from King (2011) and Fox and Serrano (2007)

captopril, a blockbuster antihypertensive drug, whose design was based on the structure of bradykinin-potentiating peptides (BPPs) isolated from the snake venom of the Brazilian viper *Bothrops jararaca* (Ferreira 1965) (Table 2). The last decades have witnessed increasing interest in "mining" libraries of venom proteins as natural sources of molecularly-targeted bioactive compounds with potential use in pharmacotherapeutics or diagnostics (Fox and Serrano 2007). The drug Prialt® (ziconotide, a synthetic version of the peptide ω-conotoxin MVIIA, from the

venom of the marine snail *Conus magus*) exemplifies the potential of venom toxins as selective, molecularly-targeted drugs with cell or tissue specificity. Ziconotide has strong analgesic activity and is used in the treatment of chronic pain mediated by blockage of N-type calcium channels (Fox and Serrano 2007) (Table 2). For detailed information on this issue, see ▶ Chap. 11, "Synthetic Peptides and Drug Discovery."

Development of antiprotozoan chemotherapy could emerge from the screening of venom components, since these natural products and their derivatives already represent more than 50% of pharmaceuticals currently in clinical use (Paterson and Anderson 2005). Animal venoms have been screened as potential sources of treatment for neglected diseases caused by protozoan parasites, and this subject will be discussed here in detail.

Animal Venoms and Their Potential Antiparasitic Activity

Spider Venoms

Spider venoms are complex mixtures comprised of low molecular weight components and larger polypeptide toxins. The former include inorganic ions and salts, free acids, glucose, free amino acids, biogenic amines, and neurotransmitters. They also include acylpolyamines, which act as voltage-dependent open-channel blockers, and perhaps as pore blockers of glutamate receptor-associated ion channels (Corzo and Escoubas 2003). Polypeptide toxins are the main components of spider venoms and comprise mainly ion channel blockers, pore-forming peptides, and enzymes, corresponding to >1000 unique peptides with molecular masses ranging from 2 to 8 kDa (Corzo and Escoubas 2003; Escoubas et al. 2006).

Spider venoms have known neurotoxic effects and spider venom-derived peptides with well-characterized pharmacological activity include antiarrhythmic, antimicrobial, analgesic, antiparasitic, cytolytic, hemolytic, and enzyme inhibitory compounds (Saez et al. 2010).

The antimicrobial actions of spider venom toxins are due to membrane-acting antimicrobial peptides (AMPs) whose biological activity was tested mainly on bacteria and fungi (Garcia et al. 2013; Kuhn-Nentwig et al. 2011). However, some studies have focused on the parasitic activities of spider venoms on the pathogenic protozoa *P. falciparum, T. brucei rhodesiense* and *T. cruzi* (Table 3).

Choi et al. (2004) first isolated the U1-TRTX-Pc1a (Psalmopeotoxin I) and U1-TRTX-Pc1a (Psalmopeotoxin II) from the venom of the Trinidad chevron tarantula *Psalmopoeus cambridgei*. These peptides are effective against the intraerythrocyte stage of *P. falciparum*, with IC_{50} values of 1.1–1.6 μM. Importantly, these agents do not have hemolytic activity. Psalmopeptoxins I and II belong to the inhibitor cysteine knot (ICK) superfamily, with structural determinants common to several neurotoxins that act as ion channel effectors, and their mode of action could be related to that of ion channel targeting proteins (Pimentel et al. 2006; Bastianelli et al. 2011).

Cupiennin 1a (Cu 1a), a linear, cationic and cytolytic peptide isolated from the *Cupiennius salei* spider venom, displayed strong activity against *P. falciparum*,

Table 3 Arthropod Venom Components with Antiparasitic Activity

Animal	Venom component	Chemical character	Activity/Specificity	Cytolytic activity in animals	References
SPIDERS (Class Arachnida, Order Araneae)					
Psalmopoeus cambridge	Psalmopeotoxins I & II	Peptides belonging to cysteine knot (ICK) superfamily	*P. falciparum* (intra-erythrocyte stage)	No hemolytic activity	Choi et al. (2004) Pimentel et al. (2006) Bastianelli et al. (2011)
Cupiennius sali	Cupiennin 1a (Cu 1a)	Linear, cationic peptide	*P. falciparum, T. cruzi, T. brucei rhodesiense*	Cytolytic activity over human primary and line cells	Kuhn-Nentwig et al. (2011)
SCORPIONS (Class Arachnida, Order Scorpiones)					
Pandinus imperator	Scorpine	75-AA peptide, 3 -S-S-bridges, 8350 Daltons	*P. berghei* fertilization and ookinete formation	Not specified	Conde et al. (2000)
	Recombinant scorpine peptide		98 % mortality in sexual stages of *P. berghei*; 100% reduction in *P. falciparum* parasitemia	Did not cause hemolysis of cell lines and intact insects (Drosophila)	Carballar-Lejarazú et al. (2008)
Tityus discrepans	Whole venom		*Leishmania* spp. promastigotes	No cytotoxic effect over PC12 (pheochromocytoma) and human umbilical endothelial cells	Borges et al. (2006)
Mesobuthus eupeus	Meucin −24; meucin-25	Linear cationic peptides	Inhibited development of *P. berghei* Killed intraerythrocytic *P. falciparum*	No hemolytic activity on mouse erythrocytes	Gao et al. (2010)

Bees (Class Insecta, Order Hymenoptera)					
Apis mellifera	Whole venom		All development forms of *T. cruzi*	No cytotoxic effects in mammalian cells (LLC-MK_2 and mouse peritoneal macrophages)	Adade et al. (2012)
	PLA_2		*T. brucei brucei* Toxic to intraerythrocytic stages of *P. falciparum* and reduced *P. berghei* oocyst formation	Low hemolytic activity	Boutrin et al. (2008) Deregnaucourt and Schrével (2000) Guillaume et al. (2004) Moreira et al. (2002)
	Melittin	Basic 26-residue peptide	*L. major* promastigotes. *T. cruzi* all developmental forms	Hemolytic and cytotoxic effects on human dendritic cells Low toxicity in LLC-MK_2 cells and mouse peritoneal macrophages	Adade et al., (2013) Fieck et al. (2010) Pérez-Cordero et al. (2011)
	Melittin hybrids		*Leishmania* spp. promastigotes and amastigotes *P. falciparum* bloodstream forms	Minimal cytotoxicity to red blood cells Safe and effective against naturally acquired canine leishmaniasis	Alberola et al. (2004) Boman et al. (1989) Chicharro et al. (2001) Diaz-Achirica et al. (1998) Luque-Ortega et al. (2003)

T. brucei rhodesiense and *T. cruzi* (Kuhn-Nentwig et al. 2011). The peptide exhibited cytolytic activity in the low micromolar range, with IC_{50} values of 0.029–0.034 μM for *P. falciparum*, 0.055–0.061 μM for *T. brucei rhodesiense* and 0.658–1.182 μM for *T. cruzi*. However, Cu 1a displayed some degree of cytolytic activity for a range of human primary cells and cell lines (Kuhn-Nentwig et al. 2011).

Scorpion Venoms

Scorpion venoms are a complex mixtures of inorganic salts, free amino acids, heterocyclic components, peptides, and proteins (mainly enzymes) and are classified based on four different criteria: (1) the type of ion channel involved (sodium, potassium, calcium, or chlorine); (2) the specific receptor inhibited by the main active component; (3) the three-dimensional structure of the toxin; and (4) the type of response induced by the venom (activation/inactivation of the receptor) (Quintero-Hernández et al. 2013). Some scorpion peptides have considerable potential for therapeutic application in the treatment of cancer, as well as autoimmune and infectious diseases (Lewis and Garcia 2003; Heinen and da Veiga 2011). Since the discovery of the first scorpion venom-derived antibacterial peptide (hadrurin, from *Hadrurus aztecus*), other such peptides with activity against bacteria and fungi have been identified (Cao et al. 2012; Corzo et al. 2001).

The work of Conde et al. (2000) is the first report of a promising antiprotozoan effect for an isolated scorpion venom peptide, scorpine, purified from *Pandinus imperator* venom. Scorpine is a 75-amino acid peptide with three disulfide bridges, a molecular mass of 8350 Da and an N-terminal segment similar to cecropins. Scorpine displayed antibacterial activity and completely inhibited both fertilization and ookinete formation in *P. berghei*, at 50 and 3 μM concentrations, respectively. The peptide was also toxic to gametes and ookinete stages of parasite development, with ED_{50} of 10 and 0.7 μM for gametes and ookinets, respectively. The authors speculated that scorpine incorporation into the genome of anophelines could be used to generate genetically engineered malaria-resistant mosquitoes. To develop this strategy, Carballar-Lejarazú et al. (2008) produced a recombinant version of the scorpine peptide (RScp) that has considerable potential for use in the development of transgenic mosquitoes incapable of transmitting dengue fever and/or malaria. Similarly to scorpine, RScp produced 98% mortality in the sexual stages of *P. berghei* at 15 μM, and 100% reduction in *P. falciparum* parasitemia at 5 μM. It was also capable of strongly inhibiting dengue 2 virus (DENV-2) infection. RScp did not cause hemolysis and does not seem to be toxic for survival and division/reproduction when expressed in cell lines and insects (*Drosophila*).

Borges et al. (2006) reported that crude venom from *Tityus discrepans* and fractions from this fluid inhibited the growth of *Leishmania spp*. promastigote forms, eventually leading to parasite death. Treated parasites displayed drastic morphological alterations (Fig. 3), and venom toxicity was different depending on the *Leishmania* species, with the most susceptible being *L. Mexicana*, followed by *L. braziliensis* and *L. chagasi*.

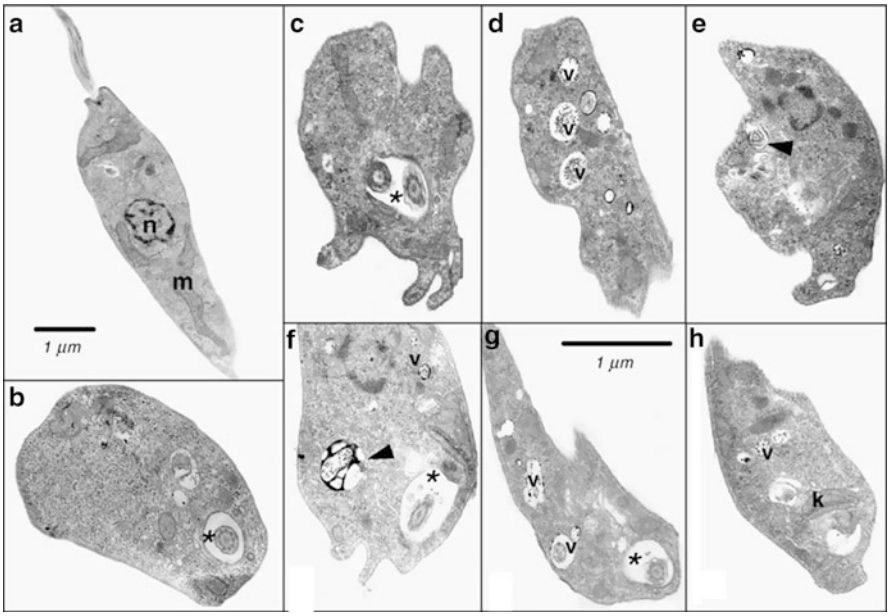

Fig. 3 Ultrastructural alterations *in Leishmania (L.) m. mexicana* promastigotes exposed to *T. discrepans* venom. (**a**, **b**) Control (saline-incubated) parasites (n nucleus; m mitochondrion; *flagellar pocket). (**c**–**h**) Ultrastructural gallery of parasites treated with venom (35 μg/ml) for 15 min showing vacuoles filled with glycogenlike material, resembling autophagosomal structures (v), enlarged flagellar pocket(*),and vacuoles filled with electrondense material forming myelin like figures (◀). Bar (1 μm) in field (**g**) applies to promastigotes shown in (**b**–**h**) (Image reproduced with kind permission from Ref. Borges et al. © (2006) Springer)

Gao et al. (2010) isolated and characterized two linear cationic peptides with antimalarial properties and no obvious cytotoxicity, named meucin-24 and meucin-25, from the venom glands of the scorpion *Mesobuthus eupeus*. These peptides share sequence similarity to magainin 1 and 2, two cationic antimicrobial peptides from frog skin. Both meucin-24 and meucin-25 inhibited the development of *P. berghei* and killed intraerythrocytic *P. falciparum* parasites at micromolar concentrations, did not affect the viability of human GC-2 cells even at 50 μM concentrations, and had no haemolytic effect on mouse erythrocytes at 100 μM. All these results are summarized in Table 3.

Insect Venoms

Among insect venoms, only those produced by bees from the species *Apis mellifera* have been evaluated for their activity against pathogenic protozoan parasites (Table 3).

The *A. mellifera* venom contains at least 18 active components with a wide variety of pharmaceutical properties, including enzymes such as Phospholipase A_2

(PLA_2) and hyaluronidase, peptides (such as melittin, apamin, and MCD), biogenic amines (such as histamine and epinephrine), and nonpeptide components (Son et al. 2007). The two major bioactive components in bee venom are melittin and PLA_2, which correspond to 40–50% and 10–12% of the venom dry weight, respectively (Raghuraman and Chattopadhyay 2007).

The therapeutic application of honeybee venom for the treatment of various diseases is known as "bee venom therapy" (Son et al. 2007) and has mainly been used to treat arthritis, rheumatism, back pain, and cancerous tumors (Park et al. 2004, 2011). As antiparasitic agent, *A. mellifera* venom displayed trypanocidal activity against all *T. cruzi* developmental forms, (Fig. 4), at concentrations 15- to 100-fold lower than those required to cause toxic effects in mammalian cells (Adade et al. 2012). Moreover, the distinct parasite life cycle stages seemed to die via different programmed cell death pathways, with predominance of autophagy in epimastigotes, apoptosis in trypomastigotes, and a heterogeneous cell death profile for intracellular amastigotes (Fig. 5) (Adade et al. 2012).

Honeybee venom PLA_2 ($bvPLA_2$) disrupts cell membrane integrity by hydrolyzing the 2-acyl bonds of membrane phospholipids, releasing lysophospholipids and fatty acids that may, themselves, cause secondary membrane damage (Habermann 1972). This enzyme also displays bactericidal activity (against Gram-negative enterobacteria), and is capable of lysing *T. brucei brucei* parasites in vitro, at a concentration of 1 mg/ml (Boutrin et al. 2008).

Deregnaucourt and Schrével (2000) demonstrated $bvPLA_2$ activity towards *P. falciparum* in vitro, with lower doses been required for cell death of young trophozoite stage parasites, and IC_{50} values much lower than the minimum enzyme concentration required for 1% hemolysis. These specific effects were reproduced by using human serum previously incubated with the corresponding PLA_2, and purified lipoproteins lipolyzed by $bvPLA_2$ were inhibitory to parasite growth. The results demonstrated that $bvPLA_2$ modified serum lipoproteins in different ways, generating specific lipoprotein by-products toxic to intraerythrocytic *Plasmodium* (Deregnaucourt and Schrével 2000). This feature was also confirmed by the enzymatic pretreatment of human serum with $bvPLA_2$ and others $sPLA_2$s (Guillaume et al. 2004). In addition, no effect was observed when parasites were grown in a semi-defined medium (AlbuMAX II) lacking lipoproteins and containing 10 fold less phospholipids than medium containing human serum (Guillaume et al. 2004), confirming that the anti-*Plasmodium* properties of sPLA2s are due to toxic lipid by-products of sPLA2 enzymatic activity, rather than to a direct antiparasitic effect of $sPLA_2$. Importantly, transgenic $bvPLA_2$ expression in *A. stephensi* mosquitoes reduced *P. berghei* oocyst formation by 87% on average, and greatly impaired transmission of the parasite to naive mice (Moreira et al. 2002). These results indicate that PLA_2 may be used as an additional effector gene to block the development of the malaria parasite inside mosquitoes, and that vector genetic manipulation is a promising strategy for reducing malaria transmission.

Melittin is an AMP consisting of a highly basic 26-residue peptide that is almost entirely hydrophobic (but for a short hydrophilic Lys-Arg-Lys-Arg sequence, near the C-terminus), and damages cell membrane enzyme systems (Habermann 1972).

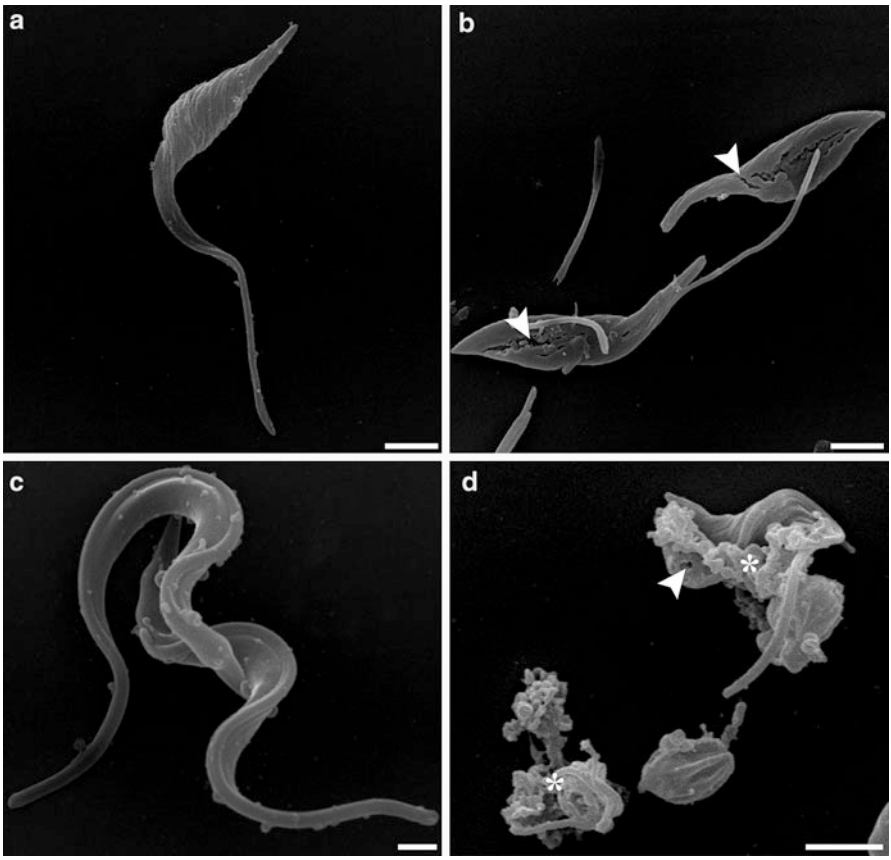

Fig. 4 Scanning electron microscopy of control epimastigotes (**a**), trypomastigotes (**b**) and treated epimastigotes (**c**) and trypomastigotes **(d)** displaying remarkable alterations. Loss of cell body membrane integrity with cracks and pores (*arrowheads*) and abnormal conformation of the cell body (*asterisks*). Bars: 1 μm

Melittin induces membrane permeabilization by pore formation, lysing prokaryotic and eukaryotic cells in a nonselective manner (Raghuraman and Chattopadhyay 2007; Papo and Shai 2003). This mechanism of action is responsible for the hemolytic, antimicrobial, and antitumor activities of melittin. (Blondelle and Houghten 1991; Chicharro et al. 2001; Diaz-Achirica et al. 1998; Gajski and Garaj-Vrhovac 2013; Han et al. 2009; Luque-Ortega et al. 2003; Pérez-Cordero 2011).

The melittin peptide exhibits strong inhibitory activity against *L. major* promastigotes, with an EC_{50} of ~74 μg/ml (Pérez-Cordero et al. 2011); however, it also has hemolytic and cytotoxic effects on human dendritic cells. Interestingly, cecropin A–melittin hybrid peptides display remarkable in vitro leishmanicidal activity (*L. donovani* and *L. pifanoi* promastigotes and amastigotes), with LD_{50}

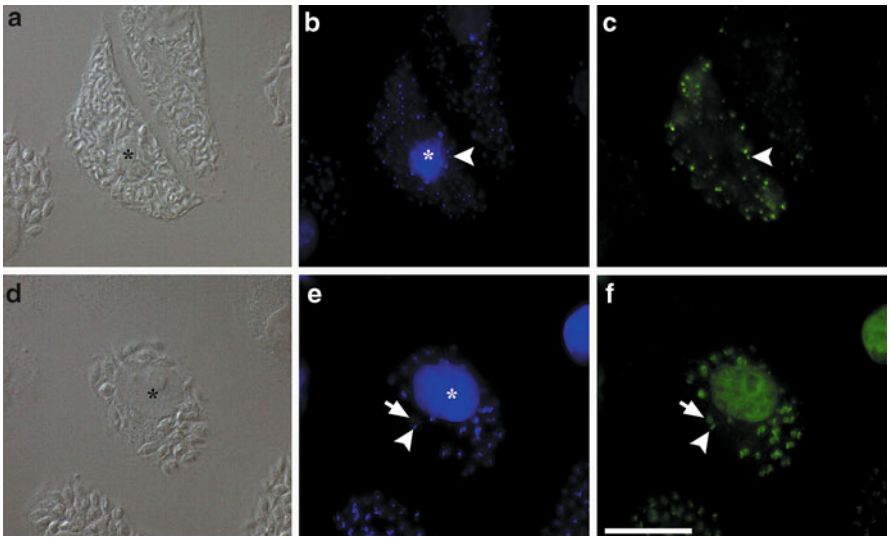

Fig. 5 Fluorescence microscopy of TUNEL labeling in *T. cruzi*-infected and *A. mellifera* venom-treated LLC-MK_2 cells. DAPI (**b**, **e**, *blue*) stained the DNA of the host cells nuclei (asterisks) and intracellular parasites kinetoplast (*arrowheads*) and nuclei (*arrows*). (**a**–**c**) Infected cells incubated with DNAse I, showing a large number of TUNEL positive parasites (*green*). (**d**–**f**) *A. mellifera* treated cells, presenting positive staining at nuclei and kinetoplast of intracellular amastigotes (*green*). Bars: 10 μm

values in the micromolar range, and minimal cytotoxicity to red blood cells (Chicharro et al. 2001; Diaz-Achirica et al. 1998; Luque-Ortega et al. 2003). Moreover, in vivo testing showed that the acylated cecropin A-melittin hybrid AP Oct-CA(1–7)M(2–9) is safe and effective against naturally acquired canine leishmaniasis (Alberola et al. 2004), since 7/8 *L. infantum* infected dogs treated with Oct-CA(1–7)M(2–9) appeared to be in good health, 6 months after the end of treatment, with no adverse events detected. Cecropin A–melittin hybrid peptides also displayed anti-*Plasmodium* activity, and the cecropin A(l-13)-melittin(l-13) hybrid was the most active against the bloodstream forms of the parasite (Boman et al. 1989).

Few studies have addressed the antiparasitic effects of melittin on *T. cruzi* (Adade et al. 2013; Fieck et al. 2010). Melittin treatment is not toxic to host cells (Adade et al. 2013), and is toxic to all *T. cruzi* developmental forms, including intracellular amastigotes (Adade et al. 2013). Similarly to that observed for *A. mellifera* venom-treated parasites, melittin treatment appeared to trigger different cell death pathways, with predominance of autophagic cell death in epimastigotes and apoptosis in trypomastigotes. Fieck et al. (2010) tested the melittin peptide in search for candidates to be used in a Chagas's disease paratransgenic system (similar to that tested with magainin 2 peptide), with a trypanocidal LD_{100} of 30 μM. As cited previously, the treatment of *T. cruzi* epimastigotes with melittin in pair-wise combinations with others AMPs revealed a variable pattern of trypanosomes growth inhibition. All these results are summarized in Table 3.

Toad/Frog Venoms

The anuran skin has cutaneous glands controlled by sympathetic nerves that discharge their contents on the dorsal surface of the animal in response to a variety of stimuli (Barra and Simmaco 1995). The fluid secreted by these glands contain wide variety of noxious or toxic substances that play essential roles in the regulation of physiological functions such as respiration and desiccation prevention, and in the defense against predators and unwanted microorganism proliferation (Barra and Simmaco 1995; Azevedo Calderon et al. 2011).

Since bombinin, was first isolated from skin secretion of the yellow-bellied toad, *Bombina variegata* and demonstrated to have antimicrobial activity (Csordas and Michl 1970), several AMPs peptides from different amphibian species have been described. Some authors have demonstrated the action of natural amphibian AMPs in pathogenic protozoa (Table 4), with most activities in the micromolar range. Studies on protozoan targets focus mainly on dermaseptins (DSs), and to a lesser extent on magainins, phylloseptins, bombinins, temporins, PGLa, and the skin peptide YY (Azevedo Calderon et al. 2011).

Dermaseptins (DSs)

DSs are a family of peptides isolated from the skin secretion of frogs from the *Phyllomedusa* genus (Azevedo Calderon et al. 2011; Brand et al. 2006; Zampa et al. 2009). They are 24–34-amino acid long cationic molecules that fold into amphiphilic helices when in hydrophobic media. Just like other AMPs, they exert a citolytic effect on a broad spectrum of microorganisms, including bacteria, fungi, and viruses, with low or no toxic effects on mammalian blood cells (Brand et al. 2002; Chinchar et al. 2004; Dagan et al. 2002; Feder et al. 2000; Mor et al. 1994a; Mor and Nicolas 1994a, b; Pinto et al. 2013; Savoia et al. 2008).

Most AMPs that have been tested against pathogenic protozoa are DSs (Azevedo Calderon et al. 2011) (Table 4). The first study showing that DSs have antiprotozoal activity were performed by Hernandez et al. (1992), where *L. major* promastigotes were incubated with different concentrations of the 34-amino-acid dermaseptin 1 (DS1, isolated from *P. sauvagii*), and 50% and 100% growth inhibition were observed at 3 and 10 μM, respectively. This study also showed, by electron microscopy, alterations on the parasite's cell membrane after treatment with DS1, and concluded that parasite death was due to compromised surface membrane permeability.

Mor and Nicolas (1994b) tested the activity of DSs first isolated from *P. sauvagii*, and of a series of ten truncated synthesized DS analogs, against bacteria, fungi, mammalian cells, and *L. mexicana*, evaluating drug effects on cell viability and proliferation. In this work, minimal inhibitory concentrations (MICs) were defined as those resulting in 100% growth inhibition after 48 h of drug treatment. DS leishmanicidal activity had a MIC of 15 μg/ ml. Treatment with DS did not affect the viability of human KJ3 cells or guinea pig lymphocytes, but some erythrocyte hemolysis was detected. The same authors also isolated and characterized four DSs (DS2, DS3, DS4, and DS5) derived from *P. sauvagii* skin (Mor and Nicolas 1994a)

Table 4 Frogs and Toads Venom Components with Antiparasitic Activity

Animal	Venom component	Chemical character	Activity/Specificity	Cytolytic activity in animals	References
Phyllomedusa sauvagii	Dermaseptin 1 (DS1)	34-amino-acid peptide	*L. major* promastigotes	Not specified	Hernandez et al. (1992)
	Ten truncated synthesized DS1 analogs		*L. mexicana* promastigotes	Did not affect the viability of human KJ3 cells or guinea pig lymphocytes, but some erythrocyte hemolysis was detected	Mor and Nicolas (1994b)
	DS3 DS4		Intraerythrocytic *P. falciparum* stages	Only DS4 was toxic to host erythrocytes	Gosh et al. (1997)
	DS4 and synthesized analogs		*L. major* promastigotes Intraerythrocytic stages *and* trophozoites of *P. falciparum*	Synthetic analogs displayed weak cytotoxic effect over erythrocytes	Feder et al. (2000) Krugliak et al. (2000) Dagan et al. (2002) Efron et al. (2002) Kustanovich et al. (2002)
P. oreades *P. hypocondrialis*	DS01	29-residue-long peptide with a molecular mass of 2.8 kDa	*T. cruzi* epimastigotes and trypomastigotes *Leishmania* spp	Low cytotoxic effects to erythrocytes and leukocytes.	Brand et al. (2006) Savoia et al. (2008) Zampa et al. (2009)
P. hypocondrialis	DShypo 01, 02, 03, 04, 06, and 07		*L. amazonensis* promastigotes	DShypo 01 had efficient leishmanicidal activity, with no detectable hemolytic activity	Brand et al. (2006)
P. nordestina	Dermaseptins 1 and 4		*L. infantum* promastigotes *T. cruzi* trypomastigotes	No toxicity to mammalian cells at the highest tested concentrations	Pinto et al. (2013)
Not reported	Dermaseptin		*L. panamensis* and *L. major promastigotes and amastigotes*	Hemolytic and cytotoxic effects on human dendritic cells	Pérez-Cordero et al. (2011)

P. oreades	Phylloseptin-4 (PS-4) Phylloseptin-5 (PS-5)	Peptides of 19–21 residues (1.7–2.1 kDa)	*T. cruzi* trypomastigotes	Toxicity to mammalian cells at high concentrations only	Leite et al. (2005)
P. nordestina	PS-7 PS-8 PS-9	Peptides of 19–21 residues (1.7–2.1 kDa)	*T. cruzi* trypomastigotes *L. infantum* promastigotes	Only PS-7 displayed toxicity over mouse peritoneal macrophages	Pinto et al. (2013)
P. azurea	PS-1	Peptides of 19–21 residues (1.7–2.1 kDa)	*L. amazonensis* promastigotes *P. falciparum* intraerythrocytic stages	Toxic to mammalian cells only at high concentrations	Kückelhaus et al. (2009)
Rana temporaria	Temporins A and B	α-helical 8–17-amino-acid peptides, amidated at the C-terminus and with a low net positive charge	*L. donovani* promastigotes *L. pifanoi* amastigotes	No cytolytic activity against human erythrocytes	Mangoni et al. (2005)
Pelophylax (Rana) saharica	Temporin-1Sa, −1Sb, and -1Sc		*L. infantum* promastigotes and amastigotes	No cytotoxic activity against peritoneal mouse macrophages	Abbassi et al. (2008)
P. saharicus	Temporin-SHd	17-residue peptide with a net charge of +2	*T. cruzi* epimastigotes *T. brucei gambiense* procyclic forms *Leishmania* spp. promastigotes and amastigotes	No cytotoxicity towards erythrocytes and macrophages	Abbassi et al. (2013)
Xenopus laevis	Magainin 2 and synthesize analogs		Arresting the development of oocysts of *P. falciparum*, *P. knowlesi*, and *P. cynomolgi* parasites in different mosquito hosts *P. falciparum* bloodstream forms	Not specified	Gwadz et al. (1989) Boman et al. (1989)

(*continued*)

Table 4 (continued)

Animal	Venom component	Chemical character	Activity/Specificity	Cytolytic activity in animals	References
	PGLa	Polypeptide of 21 amino acids	*P. falciparum* bloodstream forms	Not specified	Boman et al. (1989)
	Magainin 2 and synthesized analogs (magainin B, G, and H)		*T. cruzi* epimastigotes	Not specified	Huang et al. (1990) Fieck et al. (2010)
	Magainin 2 and synthesize analogs (MG-H1, MG-H2, F5W-magainin 2)		*L. donovani* promastigotes	Not specified	Guerrero et al. (2004)
	MSI-94 (magainin 2 analog)		*T. cruzi* trypomastigotes	Hemolytic at high concentrations	Löfgren et al. (2008)
Bombina variegata	bombinins H2 and H4		*L. donovani* promastigotes *L. pifanoi* amastigotes	Bombinins have no appreciable haemolytic activity Bombinins H have higher haemolytic activity	Mangoni et al. (2006)
Phyllomedusa genus	Synthetic Skin polypeptide YY (SPYY)	Peptide pharmacologically and structurally related to the Neuro Peptide Y (NPY) family (36 residues in length)	*L. major* promastigotes and amastigotes	Not toxic to erythrocytes or to macrophages	Vouldoukis et al. (1996)
Rhinella jimi	Bufadionelides (Telocinobufagin and hellebrigenin)	C-24 steroids	*L. chagasi* promastigotes and amastigotes *T. cruzi* trypomastigotes	No cytotoxicity against mouse peritoneal macrophages or erythrocytes	Tempone et al. (2008)

with high sequence similarity with DS1 (53–94% similarity), and which displayed antifungal and antibacterial activity (Mor and Nicolas 1994a; Mor et al. 1994a).

Following the isolation of new DS1-related peptides, several studies addressed the biological activity and mode of action of these molecules, especially that of DS4 and related analogs, since DS4 is unique amongst natural DSs in displaying high cytotoxicity towards erythrocytes as well as against parasites. Ghosh et al. (1997) investigated the cytotoxicity of DS4 toward human erythrocytes infected with *P. falciparum*, and compared it with that of the nonhemolytic peptide DS3. Both DS compounds were capable of affecting the viability of intraerythrocytic parasites, although DS4 was toxic both to parasites and to host erythrocytes, while DS3 was toxic to intracellular parasites only. An investigation on the molecular basis for this selective cytotoxicity showed that, although both molecules are capable of permeabilizing phosphatidylserine/phosphatidylcholine vesicles to a similar extent, NMR, and fluorescence data indicated that DS4 was found in higher order aggregate in aqueous solutions when compared with DS3. Feder et al. (2000) investigated the possibility that aggregation is important for DS4 cytotoxicity, by using DS4 and synthesized analogs. All compounds displayed leishmanicidal activity in the micromolar range, while monosubstitutions did not affect dramatically the antileishmanial activity. Some DS4-derived peptides also displayed both enhanced antibacterial potency and reduced hemolytic activity. These results support the notion that peptide aggregation in solution affects considerably the cytotoxic properties of DS compounds. Kustanovich et al. (2002) showed that DS4 C-terminal truncations, combined with increasing the positive charge of the N-terminal domain, resulted in short DS4 peptides with binding affinity similar to that of DS4, but displaying selective activity against bacteria, fungi, and *L. major* promastigotes, with reduced toxicity towards human red blood cells. This study showed that, whereas potency appears to be a direct function of the electrostatic potential field, selectivity (the spectrum of action) is affected primarily by the hydrophobic properties of the peptides.

DS4 derivatives also display antimalarial activity. Among them, K4-S4(1–13)a and K4K20-S4 inhibited *P. falciparum* growth by lysis of infected erythrocytes, but still retained a weak cytotoxic effect, with lysis of noninfected erythrocytes (Krugliak et al. 2000). These peptides were more effective at inhibiting trophozoite growth, and were less effective against the parasite ring stage. Nevertheless, the K4-S4(1–13) derivatives C3-P, iC4 -P, and NC7-P affected parasite viability without inducing host cell lysis. Also, none of these compounds was stage selective, displaying similar inhibitory activities both towards young, ring-stage parasites and against more mature trophozoites (Dagan et al. 2002; Efron et al. 2002).

Other DSs have been isolated and purified from *P. oreades* and *P. hypocondrialis* and have had their cytolytic activities tested against bacteria and protozoa (Brand et al. 2002, 2006; Savoia et al. 2008). DS 01, a 29-residue-long peptide with a molecular mass of 2.8 kDa, was first isolated from *P. oreades* and was later found in *P. hypocondrialis*. This peptide displayed trypanocidal activity (against *T. cruzi* epimastigote and trypomastigote forms; Brand et al. 2002) and also leishmanicidal activity (against *L. amazonensis*, *L. major*, and *L. chagasi* promastigotes; Brand et al. 2006; Savoia et al. 2008; Zampa et al. 2009) in the

micromolar range, with low cytotoxic effects to erythrocytes and leukocytes. DS 01 molecules were also immobilized in nanostructural thin films to serve as sensors for the detection of *Leishmania* cells, which may be interesting for diagnostic purposes (Zampa et al. 2009). Six novel dermaseptins from *P. hypocondrialis* were isolated and characterized by Brand et al. (2006) (named DShypo 01, 02, 03, 04, 06, and 07). Since DShypo 01 was the most abundant peptide, it was the only peptide used for bactericidal and leishmanicidal activity evaluation, and to investigate the cytolytic effects on mammalian blood cells. Similar to DS 01, DShypo 01 had efficient leishmanicidal activity, with no detectable hemolytic activity (Brand et al. 2006).

Dermaseptins 1 and 4 were isolated from *P. nordestina* secretion and Pinto et al. (2013) studied their effectiveness against *L. infantum* and *T. cruzi* (Pinto et al. 2013). Both peptides displayed promising trypanocidal activity, killing trypomastigotes at concentrations ranging from 0.25 to 0.68 μM. In contrast, no leishmanicidal activity was detected for dermaseptins 1 and 2. The peptides also showed no toxicity to mammalian cells at the highest tested concentrations, with selectivity indexes above 29 and 40, respectively, for DS1 and DS2. Based on IC_{50} values, dermaseptins were at least 648 fold more effective than benznidazole against *T. cruzi* trypomastigotes.

Pérez-Cordero et al. (2011) showed that a dermaseptin peptide and other AMPs isolated from several venoms inhibited the growth of *L. panamensis* and *L. major*, with EC_{50} values between 18 and 64 μg/ml for promastigotes, and 1.3 and 12.4 μg/ml for amastigotes. The DSs tested showed a selectivity index of 8 against intracellular forms of *L. major*. Unfortunately, this peptide also displayed hemolytic and cytotoxic effects on human dendritic cells.

Phylloseptins

Phylloseptins (PSs) were first isolated from *P. oreades* and *P. hypochondrialis* skin secretions by Leite et al. (2005). They are also present in the skin secretions of *P. azurea*, *P. bicolor*, *P. burmeinsteri*, *P. rohdei*, *P. tarsius*, *P. nordestina*, and *P. tomopterna* (Azevedo Calderon et al. 2011; Pinto et al. 2013). These peptides are AMPs of 19–21 residues (1.7–2.1 kDa) and their common structural features include a highly conserved sequence FLSLI[L]P in the N-terminal region, as well as C-terminal amidation (Leite et al. 2005). Their antimicrobial activity has been demonstrated against bacteria, fungi, and protozoa, with considerable toxicity to mammalian cells at high concentrations only (Kückelhaus et al. 2009; Leite et al. 2005; Pinto et al. 2013).

To date, only PS-4 and −5 from *P. oreades* (Leite et al. 2005), and PS-7 and −8 from *P. nordestina* (Pinto et al. 2013) have been tested against *T. cruzi* trypomastigotes. All of these molecules displayed lytic activity in the micromolar range, with IC_{50} values of 5.1 and 4.9 μM for PS-4 and −5, respectively, and 0.34 and 0.48 μM, for PS-7 and −8, respectively. PS-7 and PS-8 were also tested against *L. infantum* promastigotes, but only PS-7 demonstrated leishmanicidal activity, with an IC_{50} of 10.06 μM (Pinto et al. 2013). Thus, *Leishmania* promastigotes were approximately 30-fold more resistant to PS-7 than *T. cruzi* trypomastigotes. The authors of this work suggested that the lack of antileishmanial activity for PS-8 is

due to two amino acid substitutions relative to PS-7 (H7T and I15L). Kückelhaus et al. (2009) reported that PS-1 from the skin secretion of *P. azurea* inhibited *L. amazonensis* promastigote growth at concentrations as low as 0.5 μg/ml, which are comparable to those reported for N-methylglucamine antimoniate. PS-1 also showed activity against intraerythrocytic *P. falciparum* parasites at the concentration of 16 μg/ml, and reached an activity comparable to that of artesunate, at the concentration of 64 μg/ml. These doses are much lower than those required to cause toxicity to mammalian cells.

Temporins

Temporins constitute a large family of α-helical AMPs produced by the skin of Eurasian and New World ranid frogs (Abbassi et al. 2013; Mangoni et al. 2000a). They are short, 8–17-amino-acid peptides, amidated at the C-terminus and with a low net positive charge (from 0 to +3, with only one positively charged amino acid). Temporin sequences are highly variable and rich in hydrophobic amino acids.

The efficacy and selectivity of temporins against microbial pathogens is sensitive to peptide length variations, amino acid sequence, and subtle variations in the net charge, where peptides with charges of 0 or +1 are inactive or weakly active, whereas those with charges of +2 or +3 have higher potency (Abbassi et al. 2008; Conlon et al. 2009). Temporins display activity against viruses, Gram-positive bacteria (including vancomycin-resistant *Enterococcus faecium* and *E. faecalis*) and methicillin-resistant *Staphylococcus aureus*, with MICs ranging from 2.5 to 25 μM (Chinchar et al. 2004; Conlon et al. 2006; Giacometti et al. 2005). However, only a few temporins with a net charge of +3 are active against Gram-negative bacteria, fungi and yeasts (Abbassi et al. 2008, 2013; Conlon et al. 2006; Rollins-Smith et al. 2003). A common feature of temporins is the absence (or near absence) of toxicity to mammalian cells (Abbassi et al. 2008, 2013; Conlon et al. 2007; Mangoni et al. 2005).

Only three reports evaluated the antiprotozoal activity of temporins to date (Table 4). Temporins A and B, 13-amino-acid AMPs secreted from the skin of the European red frog *Rana temporaria*, display anti-*Leishmania* activity (against *L. donovani* promastigotes and *L. pifanoi* amastigotes) at micromolar concentrations, with no cytolytic activity against human erythrocytes and preserved biological function in serum (Mangoni et al. 2005). Temporins A and B appear to trigger parasite cell death through plasma membrane permeabilization. Temporin-1Sa, -1Sb, and -1Sc, from the skin of the Saharan frog *Pelophylax (Rana) saharica*, displayed a broad activity spectrum against bacteria, filamentous fungi, and yeasts, but Temporin-1Sa only displayed activity against *L. infantum* promastigotes and amastigotes, with complete inhibition of parasite viability (in MTT assays) at 15–25 μM (Abbassi et al. 2008). Temporin-1Sa had no harmful effect on macrophages (the amastigote host cells) at doses lethal to parasites. Temporin-SHd from *P. saharicus*, a 17-residue peptide with a net charge of +2, had a broad spectrum of antimicrobial activities – including toxicity to *Leishmania* spp. promastigotes and amastigotes, *T. cruzi* epimastigotes, and *T. brucei gambiense* procyclic forms – with IC_{50} values ranging between 6.7 and 23.5 μM. At these concentration values,

cytotoxicity (towards erythrocytes and macrophages) was either low or absent (Abbassi et al. 2013).

Magainins and PGLa

Magainins are 23-amino-acid peptides first isolated from the skin of the African clawed frog *Xenopus laevis* and belonging to the large family of amphibian amphipathic and α-helical antimicrobial peptides (Zairi et al. 2009; Zasloff 1987). These compounds have a wide spectrum of antimicrobial activities against viruses, Gram-positive and Gram-negative bacteria, and fungi, with low to negligible cytotoxicity to host cells (Boman et al. 1989; Chinchar et al. 2004; Fieck et al. 2010; Soravia et al. 1988; Zasloff 1987; Zasloff et al. 1988).

The antimicrobial PGLa (peptidyl-glycine-leucine-carboxyamide) was also isolated from the skin of *X. laevis*, and consists of a polypeptide of 21 amino acids (Andreu et al. 1985). Although PGLa has little homology to other peptides found in skin secretions of *X. laevis*, it is often included in the magainin family due to overall (albeit low level) sequence similarity to these proteins (Lohner and Prossnigg 2009). Like others AMPs, PGLa also displays antimicrobial activity (Chinchar et al. 2004; Lohner and Prossnigg 2009; Soravia et al. 1988).

The antiprotozoal activity of magainins and PGLa was first described by Zasloff (1987) and Soravia et al. (1988). Treatment of *Paramecium caudatum*, *Amoeba proteus*, and *Euglena gracilis* with magainin 2, and of *P. caudatum, Acanthamoeba castellani* and *Tetrahymena pyriformis* with PGLa, perturbed membrane function, leading to alterations in protozoan osmotic balance, with progressive parasite swelling and eventual rupture. Gwadz et al. (1989) showed that magainin 2 was capable of arresting the development of oocysts of *P. falciparum*, *P. knowlesi*, and *P. cynomolgi* parasites in different mosquito hosts. Magainin 2 and PGLa also displayed antiprotozoal activity in the micromolar range against bloodstream forms of *P. falciparum* (Boman et al. 1989).

Magainin 2 was tested together with other AMPs as part of a screen for candidates to be used in a Chagas paratransgenic system for disease prevention (Fieck et al. 2010). In this system, the obligate *Rhodnius prolixus* midgut symbiont *Rhodococcus rhodnii* is transformed with a "shuttle" DNA plasmid that expresses a protein product toxic to *T. cruzi* or capable of interfering with parasite establishment in the vector hindgut. Magainin 2 exhibited a trypanocidal LD_{100} of 33 μM. Subsequent treatments of *T. cruzi* epimastigotes in pair-wise combinations of different AMPs were at least capable of inhibiting the growth of trypanosomes. Magainin 2 interacted with other drugs in varying patterns, when used in combined treatments (Fieck et al. 2010).

The in vitro activities of the magainin 2 analogs magainin B, G, and H were assayed against *Blastocystis hominis*, *Entamoeba histolytica*, and *T. cruzi* (Huang et al. 1990). Magainin B had the highest antiprotozoal activity, followed by magainin G, whereas magainin H had no appreciable antiprotozoal activity. Guerrero et al. (2004) studied the leishmanicidal mechanisms of two hydrophobic magainin 2 analogs, MG-H1 and MG-H2, as well as that of their parental peptide, F5W- magainin 2. These three peptides showed activity against *L. donovani* promastigotes in the micromolar range, and MG-H2 was the most active, with an

LD_{90} value of 1.0 μM. Another magainin 2 analog, MSI-94, had trypanocidal activity against *T. cruzi* trypomastigotes, with an LD_{50} (concentration to cause 50% of death lethality) of 64.8 μM (Löfgren et al. 2008); however, the peptide was markedly hemolytic at high concentrations.

Bombinins and Bombinins H

The bombinins and bombinins H are antimicrobial peptides found in the skin secretions of *Bombina* species (Simmaco et al. 2009). Bombinins are active against Gram-positive and Gram-negative bacteria, as well as against *C. albicans*, with no appreciable haemolytic activity, whereas bombinins H have, in general, lower antibacterial and higher haemolytic activity (Mangoni et al. 2000b; Simmaco et al. 1991, 2009).

Mangoni et al. (2006) demonstrated that bombinins H2 and H4 were active against *L. donovani* promastigotes and *L. pifanoi* amastigotes at micromolar concentrations, although H4 LC_{50} values were significantly lower than those of H2. The mechanism of action of both peptides seems to involve plasma membrane permeabilization and/or disruption. The increased activity of H4 is probably due to its higher hydrophobicity, which increases its binding affinity to the parasite membrane.

Skin Polypeptide YY

Skin polypeptide YY (SPYY) is present in the *Phyllomedusa* genus, and is the only peptide pharmacologically and structurally related to the Neuro Peptide Y (NPY) family (36 residues in length) (Azevedo Calderon et al. 2011). A broad spectrum of important regulatory functions are mediated by these peptides (e.g., sympathetic vascular control, central regulation of endocrine and autonomic function, and circadian rhythm regulation) and are believed to induce various biological effects, by activating specific membrane-bound receptors (Azevedo Calderon et al. 2011; Mor et al. 1994b; Vouldoukis et al. 1996).

SPYY displays antimicrobial activity against fungi, Gram-negative, and Gram-positive bacteria (Mor et al. 1994b; Vouldoukis et al. 1996). *L. major* promastigotes and amastigotes also had their growth inhibited irreversibly by SPYY, and the peptide was not toxic to erythrocytes or to macrophages, at concentrations of up to 100 μg/ml (Vouldoukis et al. 1996).

Bufadienolides

Bufadienolides are cardioactive C-24 steroids from animals and plants with reported antitumor and antimicrobial activities (Gao et al. 2011; Krenn and Kopp 1998). The bufadienolide, telecinobufagin, and hellebrigenin, isolated from the parotoid macroglands of the *Rhinella jimi* toad, displayed antiparasitic activity (Tempone et al. 2008). Telocinobufagin was capable of inhibiting the growth of *L. chagasi* promastigotes and amastigotes, but did not exert any effect on *T. cruzi* trypomastigotes. In contrast, hellebrigenin had trypanocidal and leishmanicidal activities. No cytotoxicity against mouse peritoneal macrophages or erythrocytes was detected at concentrations higher than the IC_{50} values, for either steroid. All these results are summarized in Table 4.

Snake Venoms

Snake venoms are complex mixtures of proteins, nucleotides, and inorganic ions, with a high degree of target specificity. Venomous toxins often cause several disturbances in preys, including disturbing the activity of ion channels and enzymes, inhibiting blood coagulation, as well as disturbing neural and cardiovascular tissues and the neuromuscular system (Calvete et al. 2009). These features of snake venoms have made them increasingly popular as pharmacological tools and prototypes for drug discovery efforts, including the development of novel cancer chemotherapy (Papo and Shai 2003; Vyas et al. 2013). Similarly to the venom of others animals, snake venoms displays a broad spectrum of antimicrobial activities (Gomes et al. 2005; Samy et al. 2012).

Among their antiprotozoal activity, several papers have been published testing the whole venom and/ or purified molecules, with most reports focusing on L-amino acid oxidases (L.A.A.O) and PLA_2 (Table 5). These authors have evaluated snake whole venoms over *T. cruzi* and *Leishmania* spp. parasites: Fernandez-Gomez et al. (1994) (*Cerastes cerastes* and *Naja haje*), Gonçalves et al. (2002) (*B. jararaca*), Passero et al. (2007) (*Crotalus durissus terrificus*, *C. durissus cascavella*, and *C. durissus collineatus*), Adade et al. (2011) (*C. viridis viridis*), Peichoto et al. (2011) (*Philodryas baroni, P. olfersi olfersi*, *P. patagoniensis*, *Hypsiglena torquata texana*, and *Trimorphodon biscutatus lambda*), and de Menezes et al. (2012) (*Bothropoides lutzi*). All of them displayed promising results with antiprotozoal activity in micromolar range. Deolindo et al. (2005) described that the crude venom of *B. jararaca* triggers a programmed cell death process similar to metazoan apoptosis, in *T. cruzi* epimastigotes (Table 5).

L.A.A.Os have also been targeted for isolation and purification from snake venoms, and have long been used in clinical trials against pathogenic parasites. In a pioneering work, Tempone et al. (2001) demonstrated that the *B. moojeni* L.A.A.O caused a high mortality of *Leishmania* spp. promastigote forms and had its leishmanicidal activity completely abolished using catalase as a scavenger for hydrogen peroxide, demonstrating that H_2O_2-induced toxicity was responsible for parasite death upon L.A.A.O treatment. LAAOs from *C. durissus cascavella* (Toyama et al. 2006), *B. jararaca* (Ciscotto et al. 2009; Deolindo et al. 2010), *B. marajoensis* (Costa Torres et al. 2010), *B. atrox* (Paiva et al. 2011), and *Lachesis muta* (Bregge-Silva et al. 2012) inhibited *Leishmania* spp. promastigote and *T. cruzi* epimastigote growth (Table 5). All trypanocidal and leishmanicidal activities were inhibited by catalase, corroborating the hypothesis that these effects of L.A.A.Os are due to H_2O_2 production.

Interestingly, some of the L.A.A.Os active against *Leishmania* spp. did not have the same activity against *T. cruzi* trypomastigotes (Paiva et al. 2011; Bregge-Silva et al. 2012). The EC_{50} of the Batrox L.A.A.O was 62.8 μg/ml against *T. cruzi* trypomastigotes, whereas EC_{50} values for *Leishmania* were 23.3 (*L. braziliensis*), 4.3 (*L. donovani*), and 4.5 μg/ml (*L. major*) (Paiva et al. 2011). Bregge-Silva et al. (2012) reported that the median LD_{50} of LmL.A.A.O for *T. cruzi* trypomastigotes in vitro is above 32 μg/ml. In contrast, Deolindo et al. (2005, 2010) reported that the

Table 5 Snake Venom Components with Antiparasitic Activity

Animal	Venom component	Chemical character	Activity/Specificity	Cytolytic activity in animals	References
Cerastes cerastes *Naja haje* *Bothrops jararaca* *Bothropoides lutzi* *Crotalus durissus terrificus* *C. d. cascavella* *C. d. collineatus* *C. d. cumanensis* *C. viridis viridis* *Philodryas baroni*, *P. olfersi olfersi*, *P. patagoniensis* *Hypsiglena torquata texana* *Trimorphodon biscutatus lambda*	Whole venom		*T. cruzi* *Leishmania* spp. *P. falciparum*	Variable toxicity according to the concentrations tested and the cell model	Adade et al. (2011) de Menezes et al. (2012) Fernandez-Gomez et al. (1994) Gonçalves et al. (2002) Passero et al. (2007) Peichoto et al. (2011) Quintana et al. (2012)
B. moojeni *C. d. cascavella* *B. jararaca* *B. marajoensis* *B. atrox* *Lachesis muta*	L-amino acid oxidases (L.A.A.O)	Homodimeric FAD binding glycoproteins with molecular mass around 50–70 kDa	*Leishmania* spp. promastigotes *T. cruzi* epimastigotes and trypomastigotes	Variable toxicity according to the concentrations tested and the cell model	Bregge-Silva et al. (2012) Ciscotto et al. (2009) Costa Torres et al. (2010) Paiva et al. (2011) Deolindo et al. (2010) Tempone et al. (2001) Toyama et al. (2006)
N. mossambica mossambica, *Agkistrodon halys*, *Notechis scutatus scutatus* and *Vipera ammodytes*) *C. adamanteus* *B. asper*	Phospholipase A_2 (PLA_2)		*P. falciparum* trophozoite and schizont stages *P. gallinaceum* and *P. falciparum* oocyst formation	Not Specified Cytotoxic activity on peripheral blood mononuclear cells	Castillo et al. 2012 Guillaume et al. (2004) Peichoto et al. (2011) Zieler et al. (2001)
C. durissus cumanensis	Crotoxin B	PLA_2 with 14,197.6 Da	*P. falciparum*	Cytotoxic activity on peripheral blood mononuclear cells	Quintana et al. (2012)
T. biscutatus lambda	PLA_2 (trimorphin)	PLA_2 with 13,962 Da	*L. major* promastigotes	Not specified	Peichoto et al. (2011)
Eristocophis macmahoni	Spermine	Polyamine	*Tn brucei* bloodstream forms	Not specified	Merkel et al. (2007)

crude venom of *B. jararaca* triggers an apoptosis-like programmed cell death in *T. cruzi* epimastigotes, and showed the L.A.A.O activity present in the venom was responsible for epimastigote "apoptosis" (Deolindo et al. 2010).

Similarly to that described for bvPLA$_2$, Guillaume et al. (2004) reported that pretreatment of human serum with sPLA$_2$ molecules from different snakes species (*N. mossambica mossambica*, *Agkistrodon halys*, *Notechis scutatus scutatus*, and *Vipera ammodytes*) had antiparasitic activity against intraerythrocitic *Plasmodium*. All PLA$_2$s were toxic against both trophozoite and schizont stages, contrasting with the specific antitrophozoite effect of bvPLA$_2$. The *N. mossambica* sPLA$_2$ was, together with bvPLA$_2$, the most effective compound, with an IC$_{50}$ of 2.3 pM. Zieler et al. (2001) demonstrated that a PLA$_2$ isolated from *C. adamanteus* snake venom inhibited *P. falciparum* and *P. gallinaceum* oocyst formation (Table 5) when added to infected chicken blood and fed to mosquito vectors. These results validate the potential use of PLA$_2$ expression in transgenic mosquitoes as a tool to inhibit malaria transmission.

Recently, Castillo et al. (2012) tested the antiplasmodial activity of whole venom from the snake *B. asper*, as well as that of two venom fractions: fraction V, containing catalytically-active phospholipases A2 (PLA2s) and fraction VI, containing a PLA2 homologue devoid of enzymatic activity. These authors reported IC$_{50}$ values against *P. falciparum* of 0.13 (whole venom), 1.42 (fraction V), and 22.89 (fraction VI) μg/ml. Interestingly, fraction V showed higher cytotoxicity toward PBMC than whole venom. Nevertheless, the isolated fractions were cytotoxic only at concentrations higher than those necessary for parasite death. Whole venom showed the highest toxicity levels in mice, with clear lethal effect, while no lethality was observed for fractions V and VI. Similarly, Quintana et al. (2012) tested the antiplasmodial activities of *C. durissus cumanensis* whole venom, a venom fraction containing a crotoxin complex (fraction II), and that of isolated Crotoxin B. All tested substances were active against *P. falciparum*, with IC$_{50}$ values of 0.17 (whole venom), 0.76 (fraction II), and 0.6 (Crotoxin B) μg/ml. In acute cytotoxicity assays against mouse PBMCs, Crotoxin B exhibited the highest toxicity levels, although no mouse lethality was induced by this molecule (Table 5).

Peichoto et al. (2011) showed that trimorphin, a PLA$_2$ isolated from *Trimorphodon biscutatus lambda*, exhibited potent cytotoxicity over *L. major* promastigotes, with an IC$_{50}$ of 0.25 μM (3.6 μg/mL). Interestingly, the amount of purified trimorphin required for 100% reduction in parasite cell viability corresponded to a mere 3.3% of the amount of crude venom required to accomplish the same task.

Merkel et al. (2007) evaluated the activity of the whole venom of the leaf-nosed viper *Eristocophis macmahoni*, as well as that of the most active venom compound, polyamine spermine, on *T. brucei* bloodstream forms. These authors reported IC$_{50}$ values of 186 ng/ml and 3.5 μM, for whole venom and spermine, respectively, and showed that the polyamine oxidase activity in the fetal calf serum used for parasite cultivation was responsible for the trypanocidal effect of spermine, whereas the lytic activity of the crude venom was virtually independent from serum. Also, spermine seemed to induce autophagy in treated parasites. All these results are summarized in Table 5.

Conclusions and Future Directions

Protozoan parasites cause debilitating human diseases worldwide, particularly in areas of extreme poverty and neglected by governments, and where chemotherapy in patients is not economically attractive to pharmaceutical companies. This situation is aggravated by the lack of effective vaccines, parasite resistance and the toxicity of currently used therapies, which have variable efficacy and require parenteral administration and long treatment periods. This chapter focuses on the antiprotozoal properties found in animal venoms, which represent vast libraries of pharmacologically active molecules and, thus, rich sources of compounds for the development of novel antiprotozoal chemotherapy, urgently required for effective control of malaria, HAT, Chagas' disease, and the leishmaniasis. Here, are displayed that several venom-derived compounds with promising activity against protozoan parasites (and low cytotoxicity to human cells) have already been identified and had their mode of action partly characterized. Further efforts are now required to extend these findings in the path to drug development, and to explore further the antiparasitic potential of animal venoms.

Cross-References

- ▶ Antiproliferative Effects of Snake Venom Phospholipases A_2 and Their Perspectives for Cancer Treatment
- ▶ Bee Venom and Pain
- ▶ Inflammatory Action of Secretory Phospholipases A_2 from Snake Venoms
- ▶ L-Amino Acid Oxidase from Venoms
- ▶ Synthetic Peptides and Drug Discovery
- ▶ Toad Poison and Drug Discovery

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Intriguing Cystine-Knot Miniproteins in Drug Design and Therapeutics

18

Priyadarshini P. Pai and Sukanta Mondal

Abstract

Cystine-knot miniproteins (CKMPs) are members of cystine-rich protein families, typically comprising of less than 50 amino acids, widespread among various living organisms, including toxins of venomous animals such as spiders, scorpions, and marine cone snails. They have a characteristic knotted structure formed by three intramolecular disulphide bonds which provide for remarkable stability against extreme temperature, pH, chemical denaturation, and proteolysis. Evolution over years has yielded in structural and functional diversity of CKMPs across different organisms performing functions such as defense or modulation of cell growth and development. These biological roles have found therapeutic relevance over years such as in chronic pain, heart, and neurodegenerative diseases. With advances in protein engineering, the robust knotted scaffold of CKMPs has been used in the field of drug design and therapeutics for developing novel protease inhibitors, integrin binders, anti-HIV compounds, growth factor mimetics, multiepitope vaccine, and much more. This chapter provides a brief overview of the CKMPs and provides glimpses on why they have become increasingly attractive in drug design and therapeutics.

Keywords

Knottin • Cyclotide • Inhibitory cystine knot • Scaffold • Loop grafting

Contents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1_25

Introduction

Mankind has largely plunged many of its endeavors into understanding of complex biological mechanisms for promoting health and developing therapies against diseases. Often drawn into nature, into the design and working of vivid biological agents fulfilling their roles in the intertwined pathways, being orchestrated in the larger picture of life-sustaining processes, faced by the challenges that each of these pursuits present, many a researchers have been attracted to venture into the oblivious. Many of their missions have unveiled interesting aspects of how structures have been naturally crafted and constituted to yield specificity in biological functioning, spanning from gene to protein to organism level and beyond. Among the many achievements, one of the most fascinating areas of discovery still is the one that deals with manipulating and taming the wild and toxic for developing cure. This chapter deals with one such robust biological agents of natural defense – the cystine-knot miniproteins (CKMPs) and the journey of its application in drug design and therapeutics.

CKMPs are widespread in living organisms such as fungi, plants, insects, molluscs, mammalia, and viruses. Depending upon the context, members of CKMPs are popular as knottins, cyclotides, inhibitory cystine knots, etc. They are brought under the same umbrella term CKMPs in this chapter, typically consisting of a unique three-dimensional structure, as shown in Fig. 1. CKMPs are characterized by three intramolecular disulphide bonds forming a cystine knot, performing a variety of biological functions (Kolmar 2011). In plants, they are often involved in resistance to

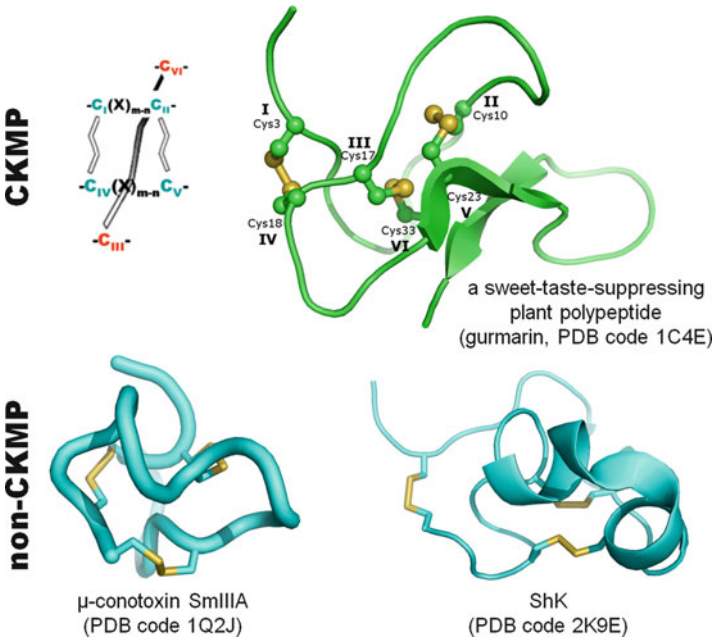

Fig. 1 Cystine-knot miniprotein (CKMP) scaffold. Example of CKMP scaffold in the top panel with schematic representation of knotted disulfide bonds in the left; cysteine (I-VI) pairing for knot formation is highlighted in ball and stick in the right. Examples of non-CKMP with disulfide bonds not forming the knotted structure in stick representation in the bottom panel

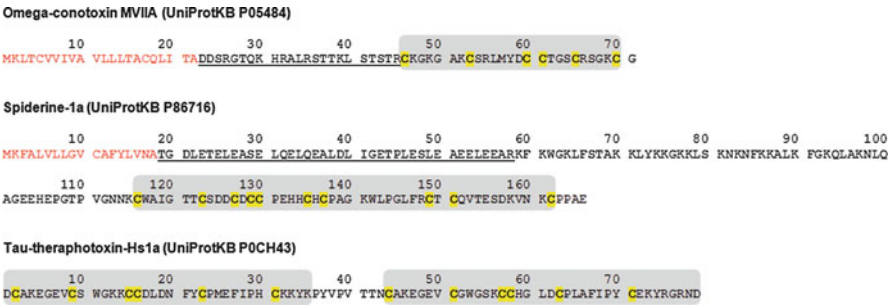

Fig. 2 Cystine-knot domain. CKMP domain is marked in gray, the signal peptide is marked in red, and the propeptide is underlined

pathogens as protease inhibitors and or as elicitors of defense response. In animals, they occur as peptides that function as extracellular ligands and regulate cellular functions such as cell growth and development (Cavallini et al. 2011). By means of research through years, it has been established that the cystine knot acts as a structural scaffold for the whole protein or for its domains as shown in Fig. 2. This

confers to the CKMPs a compact and remarkably stable structure against extreme pH, chemical and thermal denaturation, and proteolytic attack owing to their roles in nature (Kolmar 2011).

Some of the natural activities of CKMPs have been found to have diagnostic and therapeutic relevance. For example, many CKMPs have been isolated from the toxins of spiders (King and Hardy 2013; Sachkova et al. 2014; Berkut et al. 2015), scorpions (Santibáñez-López and Possani 2015), and marine cone snails (Williams et al. 2008; Clark et al. 2010), where they perform various crucial functions. Among them, ω-conotoxin MVIIA, a potent voltage-gated calcium channel blocker derived from the mollusc *Conus magus* has been approved by the Federal Drug Administration (FDA) in December 2004 (commercial name Prialt) for the treatment of severe chronic pain (Williams et al. 2008). Further, there is accumulating evidence that conotoxin-mediated ion channel blockade may be of broader therapeutic value, and this class of CKMPs is also under consideration for treatment of brain injury due to ischemic stroke (Kolmar 2011). Nevertheless, this is just one area, and CKMPs have been isolated from plethora of natural sources for their extraordinary properties relevant in therapeutic spectrum and other applications such as in cancer, asthma, obesity, diabetes, lymphatic filariasis, and environmental-friendly biopesticides.

In the following sections, the biological occurrence of CKMPs, their architecture, evolution, natural activities, and applications in drug design and therapeutics are discussed. A brief on the informative resources available that house experimental structures and predictive models for identifying protein knots is provided for readers. Examples of research and development in this field are shown for providing the impetus toward utilizing natural sources that support various biological functions.

Diversity and Evolution: Unfolding Interesting Facets of CKMPs

The structural craftsmanship of CKMPs is fascinating in the sense it has less than about 50 amino acid residues weaved in a way to form conserved signatures of this diverse family.

CKMP Architecture

CKMPs are small proteins characterized by a very special "disulfide through disulfide knot." Their scaffold has a unique disulfide through disulfide knot, in which the III-VI disulfide bond crosses the macrocycle formed by two other disulfide bonds (I-IV and II-V) and the interconnecting backbone segments, where I-VI indicates the highly conserved six cysteine residues starting from the N-terminus (Gelly et al. 2004). Some examples of CKMPs and non-CKMPs are provided here for clarity in Fig. 1. Structural aspects of linear and cyclic CKMPs are shown by means of examples in Fig. 3. Further details of the CKMP scaffold can be obtained in the review by Gracy and Chiche (2011).

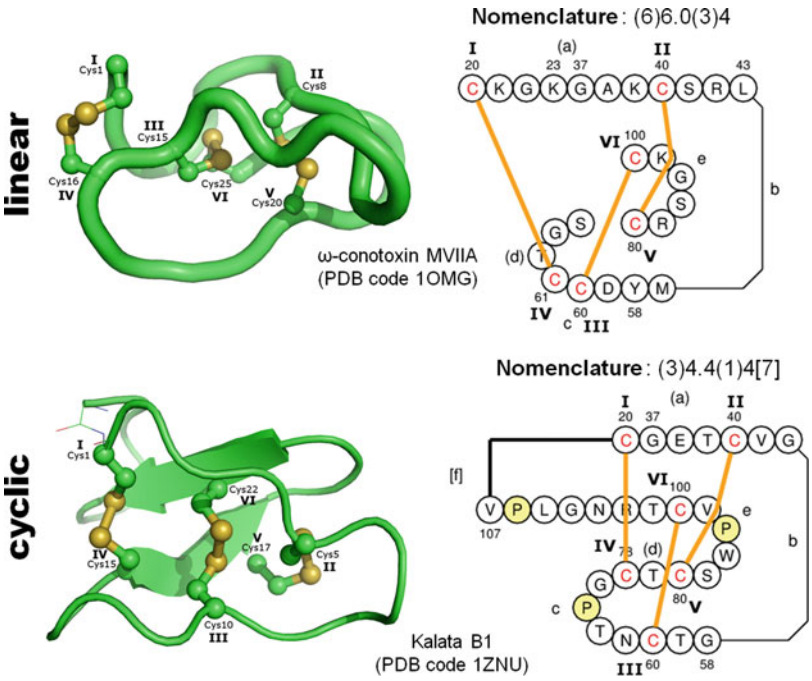

Fig. 3 Architecture and nomenclature of CKMPs. Top panel shows an example of linear CKMP, and bottom panel shows an example of cyclic CKMP with relevant KNOTTIN (Gracy et al. 2008) database-based nomenclature (a-f representing intercysteine loop length) and Collier de Perles image in the corresponding right

Resources

Information about various types of CKMP can be found in general as well as specific databases. General databases of proteins include Protein Data Bank (PDB) (Berman et al. 2000), UniProtKB (The UniProt Consortium 2015), etc., and CKMP specific databases include KNOTTIN database (Gelly et al. 2004; Gracy et al. 2008), CyBase (Wang et al. 2008). etc. CKMPs derived from toxins can be found in toxin databases such as those mentioned in ► Chap. 15, "Computational Approaches for Animal Toxins to Aid Drug Discovery" of this handbook. Since this chapter deals mainly with CKMPs, a brief on the examples of CKMP-specific databases (Table 1) and predictive tools (Table 2) is provided here. This is just to give an idea of what kind of information can be availed about CKMPs and what aspects can be predicted for experimental validation and applications.

KNOTTIN Database

The main history and results about knottin discoveries, sequences, structures, folding, functions, applications, and bibliography are presented in the database as static pages. Dynamic search reports providing tabular reports or tools for sequence

Table 1 Examples of databases for cystine-knot miniproteins

Database	Description	URL	Reference
KNOTTIN	Standardized data on the knottin structural family (also referred to as the "inhibitor cystine knot (ICK) motif/family/fold")	http://knottin.cbs.cnrs.fr/	Gracy et al. 2008
CyBase	Cyclic protein sequences and structures data, includes cyclotides	http://www.cybase.org.au/	Wang et al. 2008

Table 2 Examples of computational tools developed for prediction of cystine-knot miniproteins

Tools	Description	URL	Reference
PredSTP	Support vector machines based model to predict sequential cystine stabilized peptides	http://crick.ecs.baylor.edu/	Islam et al. 2015
CyPred	High-throughput sequence-based prediction of cyclic proteins	http://biomine-ws.ece.ualberta.ca/CyPred/	Kedarisetti et al. 2014
Knoter1D	Predicts if a protein sequence is a knottin	http://knottin.cbs.cnrs.fr/Tools_1D.php	Gracy and Chiche 2011
Knoter3D	Determines if a protein structure is a knottin	http://knottin.cbs.cnrs.fr/Tools_3D.php	Gracy and Chiche 2011
Knoter1D3D	Builds knottin 3-D models from sequences	http://knottin.cbs.cnrs.fr/Tools_models.php	Gracy and Chiche 2011

alignments for knottin three-dimensional structures or sequences are provided for the users. Further, to enable detailed studies and comparative analysis, a simple nomenclature, based on loop lengths between cysteines, is proposed and is complemented by a uniform numbering scheme (Gelly et al. 2004; Gracy et al. 2008). Figure 3 is an illustration of the same.

CyBase

Database comprises of cyclotides, cyclic CKMPs with special emphasis on the macrocyclic feature. CyBase provides a number of tools dedicated to CKMP cyclization such as a specific diversity wheel viewer, a tool to predict cyclization free energy, or a tool to predict linkers if one wants to make linear proteins cyclic (Wang et al. 2008).

Predictive Models

The vast diversity of CKMP and their potential therapeutic relevance as bioinsecticides, antimicrobial peptides, and peptide drug candidates have attracted researchers into their inherent structure as well as applications. However, the wide sequence variation, sources, and modalities of group members impose serious

limitations on our ability to rapidly identify potential CKMP members. Existing relevant approaches use sequence and/or structure information to predict CKMPs by means of homology and automated high-throughput member classification approaches. Prediction and homology modeling for gaining insights into the stability and dynamics of CKMPs have been reviewed extensively by Gracy and Chiche (2011). Essentially, approaches utilize sequence-structure similarity; cysteine number and alignment; intercysteine loop lengths; taxonomic, functional, and keyword annotations for prediction of CKMPs; and disulfide connectivity. These are evolving, and newer approaches are also reported such as predSTP (Islam et al. 2015).

The vastness of the KNOTTIN database and limitations faced by predictive models clearly indicate the prowess of combination of small numbers of amino acids in contributing to the CKMPs diversity. How these miniproteins originated and evolved have widely attracted the attention of scientists. Many attempts in understanding the evolution of various CKMPs resulted in the inception of some theories. Evolutionary origin-related studies initially suggested that the gene characterized by three well-delineated exons respectively coding for three structural and functional domains in the CKMP toxin precursors illustrates the correlation between exon and module as suggested by the exon theory of genes. Based on the analysis of precursor organization and gene structure combined with the 3-D fold and functional data, findings highlighted a common evolutionary origin for CKMP from animals, and CKMP from plant and fungus might be independently evolved from another ancestor. Thus, it was proposed that the structural similarity observed between animal and plant CKMPs could be a result of convergent evolution. Meanwhile, studies also found CKMPs in viruses. It was seen that these CKMPs were different from those derived from cells and lacked a propeptide. They also were devoid of introns. Since these viruses belonged to the Baculoviridae family, that only infect invertebrates, and their CKMP structure is more related to animal CKMPs, it was hypothesized that a gene transfer event might have occurred between an ancestor virus and its host, by which the virus acquired an CKMP gene from the genome of the infected host cells and subsequently lost the two introns and the exon encoding propeptide in the course of evolution (Zhu et al. 2003). Divergence (Zhu et al. 2003), evolutionary parallelism (Berkut et al. 2015), and many other theories have also been proposed to account for the one-domain, two-domain structure (Sachkova et al. 2014) and functional diversity observed in CKMPs.

Owing to their functionally diverse-yet-innately robust nature, cystine-knot proteins have been widely studied. They have been naturally extracted, chemically synthesized, and produced in lucrative yields using recombinant methods for various applications. These are discussed in some detail in the upcoming section.

CKMP Mining: Unveiling Natural Roles and Therapeutic Relevance

Studies reveal that the systematic research in the field of CKMPs arose in the 1970s when a cyclotide kalata B1 was identified in Congo, where women were reported to brew tea from the leaves of the plant *Oldenlandia affinis* to accelerate childbirth

(Gran 1973). And gradually within few decades, the structural properties of CKMPs were deciphered and its various roles were studied from natural resources. Since evolutionary optimization of CKMP synthesis in nature is toward high yields, for facilitating studies, extraction of wild-type sequences have been commonly done. Wild-type CKMP sequences are known to share the same mode of three disulfide bond connectivity but have different interspacing amino acid sequences resulting in different biological activities (Reinwarth et al. 2012). The following is a panoramic view of this interesting journey where therapeutic relevance of CKMP (Kolmar 2011) has been drawn.

Chronic Pain and Brain Injury

Toxins isolated from spiders, scorpions, and marine cone snails often contain CKMPs, where they mainly target ion channels. Studies have suggested that CKMPs account for over a hundred of unique toxin sequences participating in natural defense. Some CKMPs have found direct applications in therapeutics.

Conotoxin CKMP: As already mentioned in the introduction of this chapter, one of the most prominent example of therapeutic implications of CKMPs is ω-conotoxin MVIIA, a potent voltage-gated calcium channel blocker derived from the mollusc *Conus magus*, which has been approved for the treatment of severe chronic pain (Williams et al. 2008). Besides this, there is an increasing evidence for consideration of conotoxin-mediated ion channel blockade CKMPs in treatment of brain injury due to ischemic stroke (Perez-Pinzon et al. 1997; Massote et al. 2008).

Spider CKMP: There are other CKMPs that have been described to act as excitatory channel agonists rather than antagonists. For example, the venom from *Psalmopoeus cambridgei*, the Trinidad chevron tarantula, contains vanillotoxins, CKMPs that target the capsaicin receptor TRPV1, an excitatory channel expressed by sensory neurons of the pain pathway. They activate excitatory channels on somatosensory neurons to elicit pain and inflammation in mammals (Siemens et al. 2006). Besides this, a miniprotein from Earth Tiger tarantula has been isolated that serves as a specific and irreversible TRPV1 agonist. The toxin is an initial natural example for a bis-headed knottin that contains two independently folded CKMP domains, endowing it with an antibody-like bivalency that results in extremely high avidity for its multimeric channel target, making it a powerful biochemical tool for probing TRP channel structure and function (Bohlen et al. 2010).

Cancer

CKMPs have been found to have important applications in various phases of cancer development of different types. Their potential has been explored, for example, as probes in tumor imaging and as agents of tumor suppression.

Scorpion CKMP: Another very interesting CKMP with four disulfide bridges is *chlorotoxin* (*CTX*), a 36 amino acid peptide from *Leiurus quinquestriatus*, a scorpion

of the Buthidae family that preferentially binds to a lipid raft-anchored complex on glioma (Lyons et al. 2002). Thus, labeled chlorotoxins have been used as tumor imaging probes (Veiseh et al. 2005).

Potato carboxypeptidase inhibitor CKMP: An interesting miniprotein that has been found to act as a potent antagonist of epidermal growth factor (EGF) receptor binding is the potato carboxypeptidase inhibitor (PCI). This 39-residue miniprotein not only competitively inhibits several metallocarboxypeptidases but has also a suppressive effect on tumor cell growth via binding to EGF (Hass and Derr 1979).

Signal peptides CKMP: ASIP (Agouti signaling protein) and AgRP (Agouti-related protein) are human proteins that contain a carboxyterminal cystine-knot motif. They act as inverse agonists that control pigmentation and metabolic function. Studies on tumor-bearing mouse models suggested crucial implications of these CKMPs in reducing the negative impact of cachexia. Since the AgRP-derived cystine-knot motif is of human origin which reduces the risk of unwanted immune response upon administration to patients, it has been used extensively as a scaffold for the introduction of novel bioactivities (Joppa et al. 2007).

Psychiatric and Neurodegenerative Diseases

Psychotria CKMP: Cyclotides are well known for their diverse bioactivities such as anti-human immunodeficiency virus (HIV) and immunosuppressive properties. However, another study described the potential of a cyclotide isolated from *Psychotria solitudinum* as an inhibitor of another serine-type protease, namely, the human prolyl oligopeptidase (POP). The enzyme POP is well known for its role in memory and learning processes, and it is currently being considered as a promising therapeutic target for the cognitive deficits associated with several psychiatric and neurodegenerative diseases, such as schizophrenia and Parkinson's disease (Hellinger et al. 2015).

Atherosclerosis

Synthetic CKMP: A bivalent integrin $\alpha_v\beta_3$ CKMP binder 3-4A which was conjugated at its N-terminus to 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) and radiolabeled with 64Cu has been successfully used for positron emission tomography (PET) of integrin $\alpha_v\beta_3$ expression associated with carotid atherosclerotic plaques (Jiang et al. 2015).

Antiangiogenesis

PCI CKMP: Some CKMPs display antithrombotic or antiangiogenic properties. For example, potato PCI inhibits mammalian pancreatic carboxypeptidase and can act as antithrombotic agent (Wang et al. 2006). Subsequently, two other CKMPs from

tomato fruit have been reported to inhibit angiogenesis in vitro at nanomolar concentrations without exerting toxic effects on endothelial cell proliferation and viability (Cavallini et al. 2011).

Biopesticides

Pea CKMP: Many natural miniproteins from certain plant species have interesting insecticidal properties. For example, PA1b, a 37 amino acid miniprotein isolated from pea seeds (*Pisum sativum*) adopts a cystine-knot fold and acts as a potent insecticidal agent against major pests in stored crops and vegetables, making it a promising bioinsecticide that could become a valuable alternative to chemical pesticides (Da Silva et al. 2010).

Spider-toxin CKMP: Spider venoms contain a plethora of insecticidal peptides that act on neuronal ion channels and receptors. Because of their high specificity, potency, and stability, these peptides have attracted much attention as potential environmental-friendly insecticides. CKMPs from yellow sac spider toxin CpTx1, from tarantulas Psalmopeotoxin I and II, tube-web spider Sf1a, and other spiders have been used. For further details of structure and pharmacology, an exclusive review by King and Hardy (2013) may be referred.

Several naturally occurring CKMPs have received approval for treating chronic pain and irritable bowel syndrome, while others are under development (Ackerman et al. 2014). To expand beyond nature's gamut, rational and combinatorial protein engineering methods are generating specialized CKMPs for use as medical diagnostics and therapeutics. These are described in brief in the following sections.

Synthesis and Recombinant Expression

Natural extraction from various sources of CKMPs is not straightforward. Thus, in order to utilize CKMPs for research and development, their production by artificial means is essential. This is achieved broadly by two kinds of approaches – the chemical synthesis and recombinant expression, summarized below.

Chemical Synthesis

CKMP synthesis for various large-scale studies and applications has been done using chemical methods. Usually, for achieving the desired yield and quality, multiple crucial steps are included such as chain assembly, backbone cyclization, and oxidative folding. Since the late 1980s, which marked early reports of solid-phase syntheses of the CKMPs CMTI-III and EETI-II to now, many advances have been achieved in terms of strategies and methodological techniques (Boulegue et al. 2006), for example, in the choice of solid support and induction of disulfide connectivity using modified cysteine. Studies suggest that for small peptides

(~30–40 residues) the solid-phase synthesis is recommendable, while for longer peptides, the chemical ligation of prepurified fragments is more efficient. For many years the native chemical ligation (NCL) approach (Dawson et al. 1994) has been widely used for the synthesis of moderate-size proteins in general, and CKMPs in particular (Reinwarth et al. 2012). The key concept of NCL consists in assembling an unprotected synthetic peptide thioester with another unprotected segment containing an N-terminal cysteine residue to form a native peptide bond. Many modifications have also been proposed in various studies for achieving different properties in CKMPs. For examples:

Cystine-knot trypsin inhibitor with enhanced helicity: Upon inserting a guaninyl nucleoamino acid as a conformationally restricted and less basic arginine isostere or homoarginine and aminoisobutyric acid, the helicity of cystine-knot trypsin inhibitor was enhanced.

Omega-selenoconotoxin GVIA with improved folding yields: Insertion of nonnatural elements like selenocysteines in conserved cysteine regions of ω-conotoxin GVIA, a well-characterized CKMP motif peptidic antagonist of voltage-gated calcium channels, showed improvement of folding yields. Introducing such nonnatural functional aspects not only provides additional option for structural diversity but also allows for the implementation of coupling sites for backbone cyclization or oligomerization.

Increasing repertoire of reagents, methodologies, and instruments has enabled elimination of many synthesis-related problems. Various steps and modifications for production of CKMPs in large quantities can be obtained in an elaborate review article presented by Reinwarth et al. (2012).

Recombinant Expression

In addition to the chemical synthesis, a cost-effective alternative approach that has also been widely sought after for production of CKMPs without posttranslational modifications is the recombinant expression system. This includes use of both prokaryotic and eukaryotic expression systems, opening the route toward large combinatorial libraries by using the classical tools of molecular biology.

Prokaryotic expression system: One of the most used expression system to produce CKMPs is *Escherichia coli*, as it involves relatively low cost and short culturing times. In order to improve the expression level, facilitate purification, and/or obtain soluble expressed proteins, disulfide-rich miniproteins have been frequently produced in combination with larger proteins such as maltose-binding protein, thioredoxin, and glutathione S-transferase.

Eukaryotic expression system: Other expression systems are also adopted for CKMP production allowing intracellular folding and expression into the surrounding medium such as in yeast *Pichia pastoris* or insect cells with *Baculovirus* system.

Despite the cost and culture-time advantages, there are many issues faced during recombinant production of some CKMPs, especially cyclotides, where cyclization posttranslational modification mediated via an arginyl endopeptidase in vivo is

particularly challenging. However, the setbacks faced during recombinant expression of cyclic CKMPs have not deterred their artificial production. For example, a cyclotide MCoTI-I has been successfully produced in living bacterial cells using an intramolecular version of the NCL using an enzyme intein (Austin et al. 2009). In another attempt, a cheap and high-yielding recombinant production of folded and oxidized linear precursors is combined with efficient chemical linkage of the termini to yield cyclic CKMPs (Avrutina et al. 2008). These advances have elevated the horizon of endeavors for exploring the potential of CKMPs in drug design and therapeutics over the years. Efforts have been directed toward constructing CKMPs with novel specificities and much more. These efforts are outlined in the following section.

Engineering CKMPs

Advances in strategies for natural extraction, chemical synthesis, and recombinant production have enhanced the availability of CKMPs, which in turn, have boosted scientific investigations on the scope of their biotechnological engineering. There is accruing experimental evidence that the cystine-knot scaffolds are able to tolerate additions, removals, and substitutions of individual residues at least within the β-strand connecting loops without loss of structural integrity. Thus, modifications and grafting of various aspects of the cystine-knot scaffolds are being attempted for facilitating studies and applications of CKMPs in various fields. Examples are described under broad categories of loop grafting, scaffold modification, and integration.

Loop Grafting

The variable loop of the CKMP scaffold, described in the earlier sections, can be targeted for engineering of specific properties. This approach is called loop grafting where a bioactive loop is transferred onto a miniprotein scaffold. One of the initially reported examples of loop grafting was the generation of a CKMP with curare-like activity.

Transplantation of snake toxin loop in scorpion toxin CKMP scaffold: In this study (Drakopoulou et al. 1996) as shown in Fig. 4, protein engineering was done by transplantation of a central loop of a snake toxin onto the scaffold of the scorpion toxin charybdotoxin and the resulting 31-residue chimeric sequence was synthesized by solid-phase peptide synthesis and folded in vitro. Though its potential application of binding with the nicotinic acetylcholine receptor was reported to be relatively inefficient as compared to other neurotoxins, it provided initial experimental evidence of loop grafting feasibility in making a peptide sequence rigid. Beneficial effects of rigidifying a peptide sequence via miniprotein incorporation on in vitro and in vivo activity and stability stemmed from such initial efforts, and many milestones have been achieved since then. Some successful outcomes are outlined in the next section covering the therapeutic relevance exclusively.

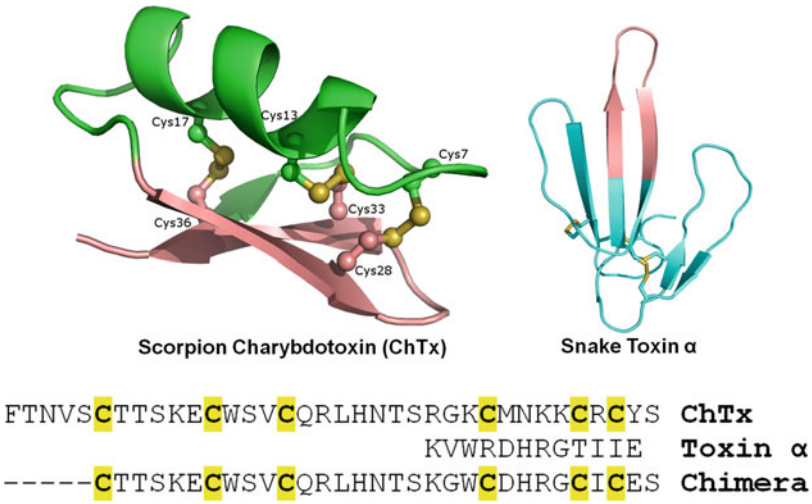

Fig. 4 Loop grafting in CKMP. Engineering of curaremimetic snake toxin loop in the cystine knot containing scorpion charybdotoxin scaffold to yield chimera. The transplanted loop (Toxin α, PDB code 1NEA, residues 26–37) and structurally similar region in CKMP scaffold (ChTx, PDB code 2CRD) are highlighted in salmon

Chemical Cross-Linking

Various studies have reported use of chemical cross-linking for engineering CKMPs. An example of multimerization of CKMPs through oxime formation between an introduced aminooxy functional group and a dialdehyde-functionalized cross-linker has been reported by Kim et al. (2015) is shown in Fig. 5. Using this strategy, a dimeric CKMP was created with promising integrin binding affinity by strongly inhibiting tumor cell migration and proliferation. Thus, a nonnatural amino acid can be incorporated at different locations within CKMPs without perturbing protein folding or function. This chemical function also enables site-specific orthogonal conjugation of small molecules or imaging probes for targeted drug delivery or diagnostic applications.

Drug Design and Therapeutic Applications

Drug designing for combating diseases by prevention and suitable therapy is a very demanding task. This is because, diseases develop from disruption of crucial life-sustaining metabolic pathways which are coordinated by complex-intertwined interactions among biological agents, and understanding their natural roles is not easy. However, nature and its complexities has not only perplexed mankind but also mesmerized them into embarking on novel explorations. These are mostly in quests of knowledge, of how nature works, and addresses for general well-being. A classic

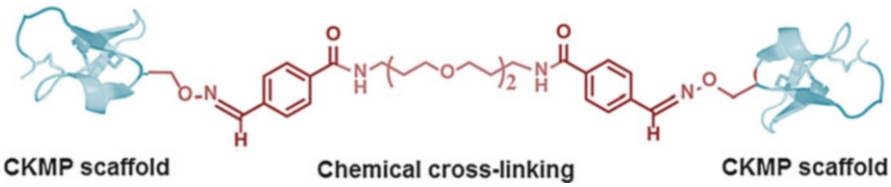

Fig. 5 Chemical cross-linking in CKMP

case of the mankind's venture into the wild, understanding nature and seeking solutions for biological disorders is CKMP. After years of efforts for understanding natural architecture and functioning of these robust biological agents of defense, potential applications of therapeutic relevance have been sought after. Studies reveal interesting progress in CKMP engineering with findings with improved properties for selective enzyme inhibition or protein interaction under research for cancer, asthma, and others; some examples are summarized in Table 3. An important part of engineering scaffolds have been derived from toxins and along with scaffolds from plants, they are also mentioned below.

Protease Inhibitors

Plant CKMP scaffold: A variety of plants such as *Momordica cochinchinesis*, *Spinacia oleracea,* and *Ecballium elaterium* have been sought after for extraction of CKMP scaffold engineering to develop various protease inhibitors. The natural open-chain cystine-knot trypsin inhibitors of the MCoTI (*Momordica cochinchinensis* trypsin inhibitor) and SOTI (*Spinacia oleracea* trypsin inhibitor) families served as starting points for the generation of inhibitors of matriptase-1, a type II transmembrane serine protease with possible clinical relevance in cancer and arthritic therapy. Another engineered analogue of McoTI-II has been produced that is a low-micromolar inhibitor of foot-and-mouth disease virus 3C protease, the initial peptidic inhibitor of this viral enzyme (Thongyoo et al. 2008). Studies have further showed development of miniproteins that inhibit human mast cell tryptase which plays an important role in allergic asthma (Sommerhoff and Schaschke 2007). Therefore, these CKMPs have increasingly garnered attention in this field of research.

Integrin Binders

Spider-toxin, squash plant, and Agouti-related protein scaffold: CKMPs have shown a promising potential as molecular scaffolds for the development of targeted therapeutics and diagnostic agents. Initial protein engineering efforts have focused on CKMPs based on the *Ecballium elaterium* trypsin inhibitor (EETI) from squash

Table 3 Examples of cystine-knot miniproteins applications in drug design and therapeutics

Sources	Applications	References
Trypsin inhibitors from *Momordica*, spinach, and squash plants	Cancer, arthritis, allergic asthma	Thongyoo et al. 2008; Sommerhoff and Schaschke 2007
Trypsin inhibitor from squash plant, Agouti-related protein (AgRP) neuropeptide from mammals, Agatoxin from spider venom	Integrin binders for tumor imaging, cancer radiotherapy	Moore et al. 2013; Kolmar 2011.
Toxins from cone snails, spiders, and sponges	Fast drug actions through oral delivery, neuropathic pain	Clark et al. 2010; Li et al. 2014; Kikuchi et al. 2015
Toxin from scorpion venom	Anti-HIV compounds	Martin et al. 2003
Squash plant and AgRP	Growth factor mimetics for activation of thrombopoeisis in low platelet conditions	Krause et al. 2007; Kimura et al. 2011
Cyclotide kalata B1 from *Oldenlandia* plant	Immunosuppressive peptides for overreactive immune response as is the case in autoimmune diseases, allergic reactions, and following organ transplantation with reduced toxicity effects	Thell et al. 2014
Nematode abundant larval transcript	Multiepitope vaccine for human lymphatic filariasis	Madhumathi et al. 2016

seeds, the AgRP neuropeptide from mammals, or the Kalata B1 uterotonic peptide from plants. Over years, it was demonstrated that Agatoxin (AgTx), an ion channel inhibitor found in spider venom, can be used as a molecular scaffold to engineer CKMPs that bind with high affinity to a tumor-associated integrin receptor. Considering the critical roles that integrins play in tumor cell survival, invasion, metastasis, and angiogenesis, molecular imaging agents that selectively target tumor-associated integrins have potential diagnostic applications in disease staging and management, and monitoring response to therapy (Moore et al. 2013). Integrin-specific peptides using CKMP scaffold have potential applications as imaging tools in glioblastoma and other malignancies. However, insufficient tumor targeting efficacy and suboptimal pharmacokinetics have limited clinical use of engineered CKMPs as molecular imaging agents. Besides this, engineered CKMPs such as AgRP are also potential antiplatelet aggregating agents. One such potent example is the FDA-approved cyclic heptapeptide eptifibatide (integrilin) (Silverman et al. 2011). Furthering from the diagnostic and characterization, reports also indicate that the integrin binding EeTI-II variants are suitable for radionuclide therapy of small tumor and metastasis, especially in integrin positive tumors types. Details of emerging tools for cancer imaging and therapy are reviewed exclusively by Ackerman et al. (2014).

Pharmacophoric Scaffolds for Oral Peptides

Conotoxin, spider-toxin, and sponge-toxin scaffold: As discussed in earlier sections, the CKMP fold exhibits high proteolytic stability and has a promising scope in the context of oral delivery of peptides where proteolytic degradation by enzymes of the gastrointestinal tract is a major threat. Studies by Craik and coworkers showed that a cyclic version of α-conotoxin Vc1.1, a 16-mer peptide with exciting potential for the treatment of neuropathic pain that contains two disulfide bonds is orally active (Clark et al. 2010). Linear CKMPs also came to fore from marine sponges showing considerable stability against luminal proteolysis because of the presence of *N*-terminal pGlu and the absence of basic residues (Li et al. 2014). Subsequently, proteolytic stability was also marked in spider toxins (Kikuchi et al. 2015). Study findings suggest that since CKMPs inhibit various ion channels which are involved in pathology of many diseases, oral delivery using these scaffolds can provide an alternate site of drug absorption for fast onset of drug action. However, further studies are required for validation of open chain and head-to-tail cyclization knot scaffolds in in vivo conditions.

Anti-HIV Compounds

Scorpion toxin scaffold: Various drugs are currently being investigated both in vitro and in vivo for potential use as HIV microbicides to prevent sexual HIV transmission. Of the many classes of compounds, the nonnucleoside reverse transcriptase inhibitors are attractive because they show high antiviral activity with a relatively low toxicity. A mimetic of this class was generated by transplanting the conserved domain (CD) 4-binding surface of the HIV-1 glycoprotein (gp) onto a scorpion toxin scaffold. Study findings suggested that the mimetic was capable of inhibiting infection of both immortalized and primary cells by HIV-1 and can form a potential component of vaccine formulations (Martin et al. 2003).

Growth Factor Mimetics

Squash and AgRP scaffold: Receptors of the growth factor can become activated by dimerization via a ligand, and this property of the growth factor (GF) superfamily has been articulated using CKMP scaffolds for developing growth factor mimetics. The fact that a GF receptor can be converted into agonist from antagonist in principle encouraged a study on thrombopoietin receptor. Basically, a dimeric agonist was generated by transplanting a 14-mer peptide sequence with known high affinity to the thrombopoietin receptor into the structural scaffold of a CKMP from squash and also into human AgRP (87–132) followed by chemical dimerization using a bifunctional linker. Study findings indicated that this engineered CKMP acted as activators of thrombopoiesis and displayed a remarkably low subnanomolar mean effective concentration close to the natural agonist thrombopoietin. They were able to

stimulate growth and colony formation of megakaryocytes from human bone marrow mononuclear cells. This strategy may have considerable potential for the future development of high-affinity bispecific binders and desirable receptor agonists that mediate receptor dimerization (Krause et al. 2007; Kimura et al. 2011).

Immunosuppressive Peptides

Cyclotide [T20K] kalata B1 scaffold: Myelin oligodendrocyte-glycoprotein-derived peptide fragments were grafted onto a cyclotide scaffold, which did not only improve their stability, but one engineered molecule displayed potent ability to prevent disease development in a mouse model of experimental autoimmune encephalomyelitis. Study findings suggest that cyclotides suppress T cell polyfunctionality and arrest the proliferation of immune-competent cells through inhibiting interleukin (IL) 2 biology at more than one site. Various signaling pathways and associated canonical messenger molecules, such as IL2 signaling molecules, offer drug targets using cyclotides for the treatment of an overreactive immune response as is the case in autoimmune diseases, allergic reactions, and following organ transplantation with reduced toxicity effects (Thell et al. 2014)

Multiepitope Vaccine

Nematode scaffold: Abundant larval transcript (ALT) is a protein that plays a crucial role in host pathogen interaction in one of the tropical diseases called human lymphatic filariasis. It has a multifaceted ability in host immune regulation resulting in permanent and long-term disability with severe immunopathology in the hosts. In a study based on epitope mapping and structural model of *Brugia malayi* ALT-2, that has a knotted fold, a recombinant multiepitopic gene comprising of the epitopic domains was engineered and the protective efficacy of recombinant ALT epitope protein (AEP) was tested. Study findings suggested that engineering protective epitopes to produce multiepitope protein may be a novel vaccine strategy for complex parasitic infections (Madhumathi et al. 2016).

Conclusion and Future Directions

With increasing discoveries of natural cystine-knot miniproteins in various living organisms, newer avenues for developing a deeper understanding of their roles in nature has become possible. Various perspectives on their evolution and diversification have come to fore revealing the fact that the structural integrity and functional roles played by CKMPs have greater intricacies that can facilitate novel applications for the benefit of mankind. Though efforts are being put to house the accruing information, it would be nice to see community-wide participation in maintenance of CKMP-related resources. Growing research in this domain can be facilitated with

accelerating combination and ensembles of molecular biology and chemistry, in order to support greater availability of CKMPs as well as their libraries. It is well-recognized that within few decades, several milestones have been achieved in utilizing the natural gifts of these miniproteins with therapeutic relevance such as in chronic pain, ischemic stroke, vasculogenesis, and cancer. However, their potential in other associated contexts such as in treatment of animal bites causing hemorrhage, receptor-mediated host pathogen interaction leading to disease development, and much more has ample scope of exploration.

Cross-References

► Computational Approaches for Animal Toxins to Aid Drug Discovery
► Venoms as Sources of Novel Anti-parasitic Agents

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P. Gopalakrishnakone et al. (eds.), *Toxins and Drug Discovery*, Toxinology,
DOI 10.1007/978-94-007-6452-1