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Omics Technologies for Profiling Toxin Diversity and Evolution in Snake Venom: Impacts on the Discovery of Therapeutic and Diagnostic Agents

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Abstract

Snake venoms are primarily composed of proteins and peptides, and these toxins have developed high selectivity to their biological targets. This makes venoms interesting for exploration into protein evolution and structure–function relationships. A single venom protein superfamily can exhibit a variety of pharmacological effects; these variations in activity originate from differences in functional sites, domains, posttranslational modifications, and the formations of toxin complexes. In this review, we discuss examples of how the major venom protein superfamilies have diversified, as well as how newer technologies in the omics fields, such as genomics, transcriptomics, and proteomics, can be used to characterize both known and unknown toxins.

Because toxins are bioactive molecules with a rich diversity of activities, they can be useful as therapeutic and diagnostic agents, and successful examples of toxin applications in these areas are also reviewed. With the current rapid pace of technology, snake venom research and its applications will only continue to expand.

1. INTRODUCTION

Although venomous snakes are associated with mortality and morbidity, responsible for the deaths of more than 100,000 people and disabling more than 400,000 people each year (1), compounds from their venoms have been developed into therapeutic and diagnostic agents, successfully being used to treat ailments such as hypertension and diagnose hemostatic disorders. Snake venoms are complex cocktails of many different proteins and peptides, and a single venom protein family within this mixture can exhibit a multitude of biological activities. This creates limitless opportunities for explorations into pharmaceutically interesting compounds that high-throughput and sensitive omics technologies can help to identify and characterize.

Venom profiling with omics technologies, termed venomics (2, 3), has been proceeding at a rapid rate, especially in regard to overall snake venom compositional characterization (4, 5). Venomics is an integration of the fields of proteomics, transcriptomics, and genomics. Technologies in these fields are useful tools to explore toxin diversity, which can result from variations in nucleotide sequences on a genome or transcriptome level, as well as posttranslational modifications and formations of protein complexes observable at the proteome level.

This review highlights how certain venom protein superfamilies are able to functionally diversify, what techniques are useful to study toxin diversity, and how these toxins have been repurposed as therapeutic and diagnostic agents. Snake venom proteins are the products of millions of years of evolution (6), and during this time they have developed high specificity to select receptors, channels, and substrates. In addition to being versatile, these proteins are also incredibly stable, commonly crosslinked by disulfide bonds that prevent degradation in an extracellular environment. Therefore, snake venoms provide a natural source of bioactive and stable proteins with structure and function relationships of medical relevance.

2. VENOMOUS SNAKES

Of the more than 3,700 extant snake species (7), a minority are known to inflict clinically significant bites on humans. Medically important venomous snake species are predominantly from the families Elapidae and Viperidae, with tubular fangs positioned anterior in the upper jaw (8) (**Figure 1*a*,*b***). Snakes of the families Colubridae, Homalopsidae, and Lamprophiidae (9, 10) can possess grooved or ungrooved fangs positioned posterior in the upper jaw (an exception being some snakes of the subfamily Atractaspidinae) and hence are referred to as rear-fanged (10, 11) (**Figure 1*c***), but not all species in these families are venomous, and even fewer are of medical significance (12, 13).

Although trends in venom composition are observed within each snake family, these trends are not necessarily present for all species. For example, elapid snakes have venoms largely dominated by three-finger toxins (3FTxs) and phospholipase A_2 enzymes (PLA_2s) (4, 14), whereas venoms from viperid snakes contain primarily metalloproteinases, serine proteases, and also PLA_2s (4, 14). Thus, elapid snakebites induce neurotoxic symptoms, and viperid snakebites result in tissue destruction and coagulation symptoms. Venoms from rear-fanged snakes can be either elapid-like,

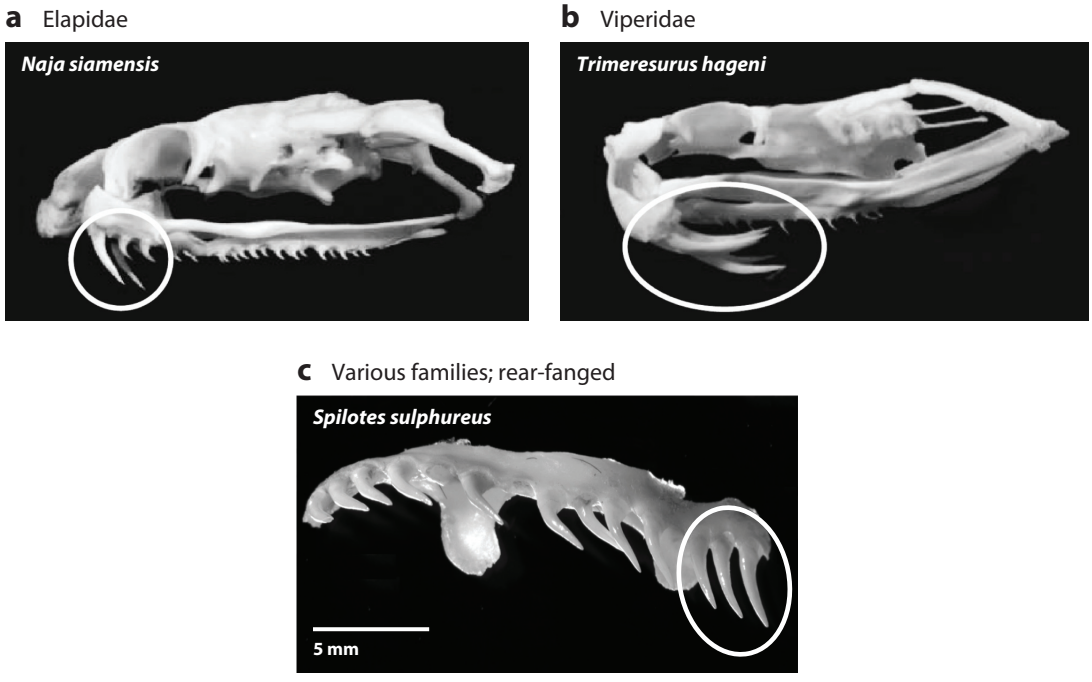


Figure 1

Fangs in venomous snakes. Snakes of the families (*a*) Elapidae and (*b*) Viperidae have fangs anterior in the upper jaw, in contrast to (*c*) rear-fanged venomous snakes with fangs positioned posterior. Elongated fangs are circled. *Naja siamensis* (Indochinese spitting cobra) and *Trimeresurus hageni* (Hagen's pit viper) images are reproduced with permission from Vonk et al. (10), and rear-fanged snake, *Spilotes sulphureus* (Amazon puffing snake), images reproduced with permission from Modahl et al. (17).

with predominately 3FTxs, or viperid-like, with metalloproteinases in greatest abundance (15). A single toxin superfamily can induce multiple pharmacological effects, and how such functional diversity is achieved within each superfamily is briefly discussed below.

3. TOXIN DIVERSITY IN SNAKE VENOM

3.1. Functional Site Differences: Three-Finger Toxins

3FTxs make up the large majority of toxins in elapid snake venoms. They are abundant in the venoms of some rear-fanged snake species (16–18), and they are expressed in the venom gland transcriptomes of some viperid snakes (19–21). These toxins are small (60–80 amino acid residues in length), nonenzymatic proteins that share a conserved structure of three β-stranded loops crosslinked by four disulfide bridges (**Figure 2*a***) (22–24). The three loops project outward, resembling three fingers of a hand (hence the name three-finger toxins). 3FTxs can be neurotoxic, acting as antagonists of nicotinic acetylcholine receptors (nAChRs) (25–27), muscarinic acetylcholine receptors (mAChRs) (28, 29), adrenergic receptors (30), and GABA receptors (31), and even binding to and altering the activation of ion channels (32–34). These toxins can also be anticoagulants (35–37). The variety of pharmacological effects of 3FTxs are due to residue differences between 3FTxs. These residue variations alter target interactions, and residue substitutions can change binding affinities to entirely different receptors or substrates.

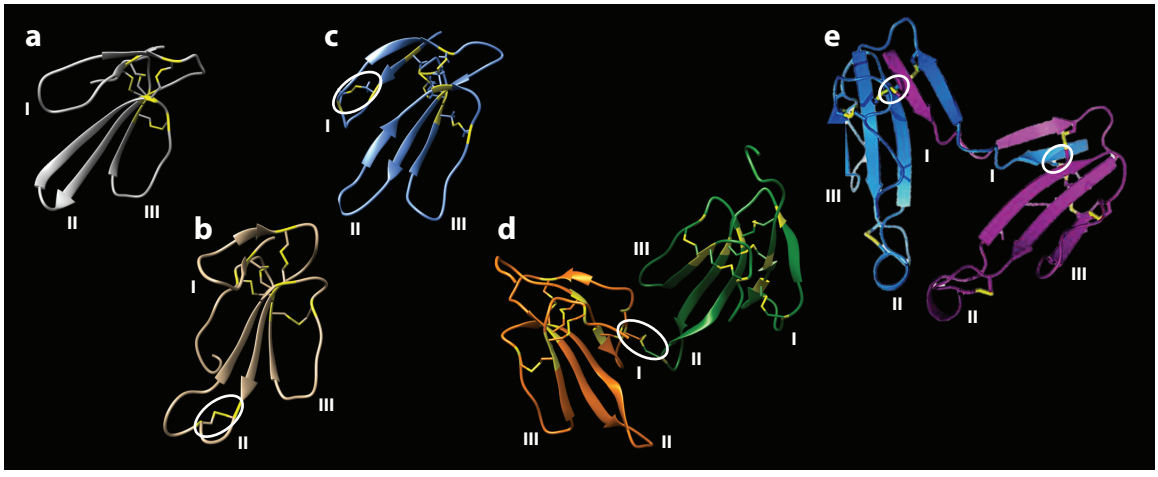


Figure 2

Structural variations in three-finger toxin (3FTx) family. (*a*) Short-chain 3FTx erabutoxin a (PDB ID: 1QKE) with four disulfide bonds. (*b*) Long-chain 3FTx, α-cobratoxin (PDB ID: 2CTX), has a fifth disulfide in loop II, and (*c*) nonconventional 3FTx candoxin (PDB ID: 1JGK) has a fifth disulfide within loop I. Additional interlinking disulfides can create 3FTx complexes, such as (*d*) heterodimeric irditoxin (PDB ID: 2H7Z) or (*e*) dimeric α-cobratoxin (PDB ID: 4AEA). Toxin structural loops are labeled and relevant disulfide bonds circled.

Additional cysteines can generate minor alterations to the 3FTx scaffold by forming disulfide bonds. Long-chain neurotoxic 3FTxs have a fifth disulfide in loop II (**Figure 2*b***), and nonconventional 3FTxs have a fifth disulfide in loop I (**Figure 2*c***). These additional disulfide bonds alter the confirmation of these loops and the toxin's binding ability to α_7 nAChRs for long-chain neurotoxins (38) and to mAChRs for nonconventional 3FTxs (39, 40). Additional cysteine residues can also interlink 3FTxs, creating covalently formed dimers (**Figure 2*d*,*e***). In rear-fanged venomous snakes, the 3FTx heterodimeric complexes irditoxin (41) and sulditoxin (17) have increased toxicity and selectivity to taxa-specific receptors (lizard nAChRs > mammalian nAChRs). Complexes formed from noncovalent interactions are also present; examples include κ-bungarotoxins (42) and haditoxin (43).

In 3FTxs, distinct residues contribute to their interactions with target proteins. For mambin, isolated from *Dendroaspis jamesoni* (Jameson's mamba), the tripeptide sequence Arg^{43}-Gly^{44}-Asp^{45} (RGD) interferes with the binding between fibrinogen and its receptor glycoprotein IIB-IIIa ($\alpha_{IIb}\beta_3$) to inhibit platelet aggregation (37). This tripeptide sequence is located in loop III. A different set of residues are responsible for α-neurotoxins' binding to nAChRs. Site-directed mutagenesis studies have identified distinct residues in erabutoxin, α-cobratoxin, and Ω-neurotoxin responsible for nAChR interactions (44–46). Thus, 3FTx structure–function relationships will help in engineering target-specific 3FTxs of pharmacological interest.

3.2. Domain Differences: Metalloproteinases

Snake venom metalloproteinases (SVMPs) have been found in all snake venoms, with viper venoms containing at least 30% SVMPs (14, 47, 48). SVMPs are characterized by the presence of the Zn^{2+}-binding motif HEXXHXXGXXH at the catalytic site and are closely related to mammalian ADAM (a disintegrin and metalloproteinase) and ADAMTS (ADAM with thrombospondin type-1

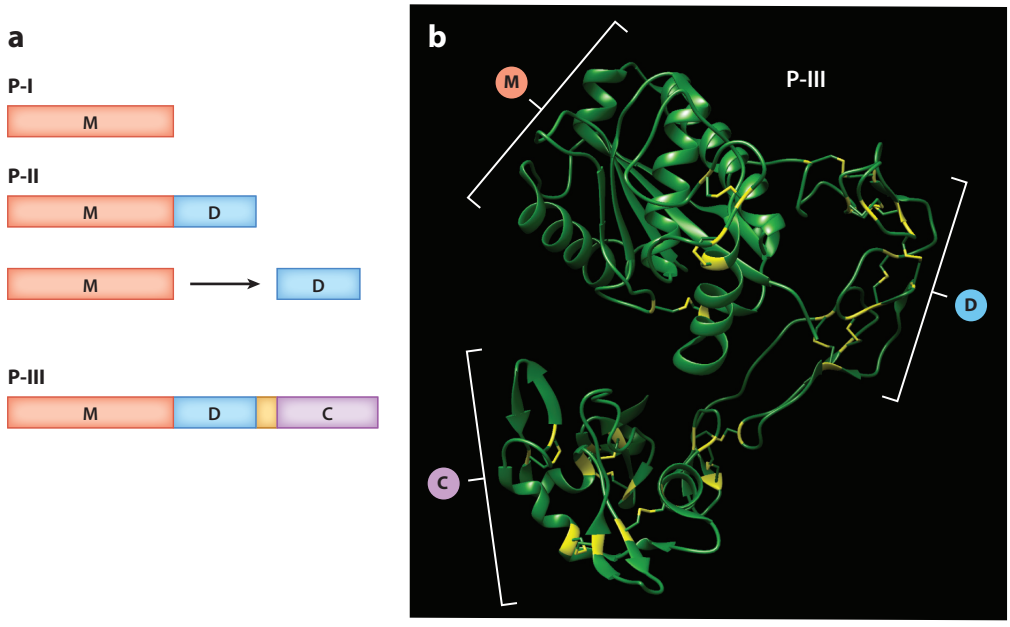


Figure 3

Snake venom metalloproteinase domain structure. (*a*) Schematic representation of domain structures of P-I, P-II, and P-III classes of metalloproteases. P-I metalloproteases contain only the catalytic metalloproteinase (M) domain, and P-II metalloproteinases have M and disintegrin (D) domains. P-III metalloproteinases have a M, D, and cysteine-rich (C) domain. The black arrow represents posttranslation processing of P-II metalloproteinases to yield venom disintegrins. (*b*) The 3D structure of AaHIV (PDB ID: 3HDB), a P-III metalloproteinase from *Deinagkistrodon acutus* (five-pacer viper) venom, is shown with labeled domains.

motif) but differ in domain organization and size, ranging from 20 to 100 kDa (48, 49). The effects of SVMPs include hemorrhage, coagulopathy, fibrinolysis, apoptosis, and the activation of factor X and prothrombin (47). SVMPs function by degrading endothelial cell membrane components or target proteins involved in coagulation, such as fibrinogen or platelet receptors (48). These enzymes are the primary factors responsible for local and systemic hemorrhage from snakebite (48).

There is an observed difference in activity depending on the presence or absence of SVMP domains. SVMPs of the P-III class are composed of a metalloproteinase domain, a disintegrin-like domain, and a cysteine-rich domain; P-IIs have metalloproteinase and disintegrin domains; and P-Is have only a metalloproteinase domain present (47) (**Figure 3*a***). Domain loss has resulted in the creation of the P-II and P-I classes (50). In comparison to P-Is, P-IIIs exhibit greater hemorrhagic activity and an overall greater diversity of biological activities (48, 49). Posttranslational processing of SVMPs has also generated additional venom activities. The P-III class of SVMP does not undergo posttranslation domain cleavage (**Figure 3*b***), but snake venom disintegrins are the products of P-II SVMP proteolytic domain processing (51) (**Figure 3*a***). Disintegrins are small proteins, 40–100 residues in length, that can inhibit platelet aggregation by binding to platelet fibrinogen integrin $\alpha_{IIb}\beta_3$ or inhibit cell migration by targeting $\alpha_v\beta_3$ and $\alpha_5\beta_1$, among other integrin targets (52). Disintegrins have also been found to aid in prey relocation for viperid snakes (53), exemplifying how one venom superfamily, SVMPs, can diversify biological functionality via domain loss and posttranslational processing (54).

3.3. Glycosylations: Serine Proteases

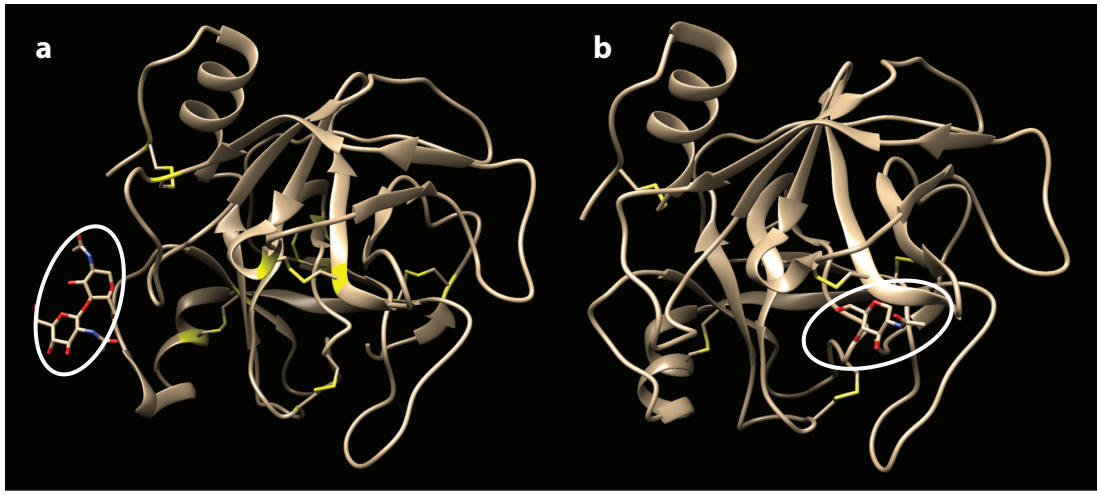
Snake venom serine proteases (SVSPs) are common components of snake venoms. In the venoms of some viperid snake species, they are the second most abundant venom protein superfamily (55). These enzymes interfere with blood clotting and can function as either procoagulants, by activating coagulation factors or inducing platelet aggregation, or anticoagulants, by, e.g., activating protein C (56, 57). SVSPs are 37–40 kDa in size, and all share the conserved catalytic triad of residues His^{57}, Asp^{102}, and Ser^{195}, with the structural loops surrounding the active site varying in both amino acid composition and length, contributing to the substrate specificity of these enzymes (58). Although SVSPs share a high degree of sequence identity (57–85%), they have been found to be specific toward a given macromolecular substrate, targeting substrates of the coagulation, fibrinolytic, and kallikrein–kinin systems and platelet surfaces to cause hemostatic system imbalance (59, 60).

A primary feature of SVSPs is that most are glycoproteins with a variable number of *N*- and *O*-glycosylation sites, resulting in differences in molecular masses and isoelectric points depending on these PTMs. Most have 5–30% carbohydrates, but some have been reported to have more than 60% (58, 61). The locations of glycosylations attached to SVSPs are variable within this venom protein superfamily (**Figure 4**). These glycosylations can inhibit or enhance activity (61). SVSP glycosylations can also hinder the binding of common protease inhibitors (61). The resistance of these proteins to inhibition has generated interest in their use for diagnostics, examples of which, such as Reptilase time, are discussed in Section 5 below. Additionally, SVSPs are not the only venom proteins with activity dependent on glycosylations; hemorrhagic properties of SVMPs have been observed to be linked to glycosylation (62).

3.4. Toxin Complexes: Phospholipase A_2 Enzymes

PLA_2s are ubiquitous to Elapidae and Viperidae snake venoms. They have also been observed in a few rear-fanged snake species (63) but rarely occur in abundance in these venoms. PLA_2s catalyze the Ca^{2+}-dependent hydrolysis of glycerophospholipid *sn*-2 fatty acyl bonds, which liberates lysophospholipids and fatty acids (64). However, PLA_2s are also functionally diverse enzymes in snake venoms, with additional activities including neurotoxicity, myotoxicity, cardiotoxicity, hemolysis, and anticoagulation (65). These enzymes mostly exist as monomers of approximately 13–15 kDa, but several have been identified as complex forming with other PLA_2s or other proteins (66).

Presynaptic neurotoxin β-bungarotoxin from the venom of *Bungarus multicinctus* (many-banded krait) (67) consists of a PLA_2 subunit covalently crosslinked by a disulfide bond to a Kunitz-type serine protease inhibitor (68) (**Figure 5*a***). PLA_2 complexes can also be noncovalently joined, such as heterodimeric PLA_2 complexes vipoxin, from *Vipera ammodytes meridionalis* (Bulgarian nose-horned viper) (69) (**Figure 5*b***), and crotoxin, the major toxic component in the venom of *Crotalus durissus terrificus* (South American rattlesnake) (70). Crotoxin is composed of one nontoxic acidic subunit named crotapotin (subunit A), which lacks PLA_2 activity, and a second basic subunit (subunit B) that is weakly toxic (71) (**Figure 5*c***). Together these subunits form a potent neurotoxin that blocks transmission of nerve signals at neuromuscular junctions. Subunit A acts as a chaperone, blocking subunit B from binding to nonspecific tissues and guiding the complex to the target site to increase potency. After binding to the synaptic membrane, the toxin complex dissociates (72). PLA_2 complexes similar to crotoxin have been isolated from multiple rattlesnake species, including *Crotalus vegrandis* (Uracoan rattlesnake) (73), *Crotalus basiliscus* (Mexican west coast rattlesnake) (74), *Crotalus viridis concolor* (midget faded rattlesnake) (75), *Crotalus horridus* (timber or canebrake rattlesnake) (74), *Sistrurus catenatus tergeminus* (Massasauga



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4E7N    IIGGDECNINEHRFLVALYTSRSRTLFCGGTLINQEWVLTAAHCDRKNFRIKLGMHSKKV    60
1OP2    VIGGNECDINEHRFLVAFFNT--TGFFCGGTLINPEWVVTAAHCDSTNFQMQLGVHSKKV    58
        :***:**:**********::.:    :******** ***:****** .**:::**:*****

4E7N    PNEDEQTRVPKEKFFCLSSKNYTLWDKDIMLIRLDSPVKNSKHIAPFSLPSSPPSVGSVC   120
1OP2    LNEDEQTRNPKEKFICPNKNNNEVLDKDIMLIKLDKPISNSKHIAPLSLPSSPPSVGSVC   118
         ******* *****:* ..:*  : ********:**.*:.*******:**************

4E7N    RIMGWGRISPTEGTYPDVPHCVNINLLEYEMCRAPYPEFELPATSRTLCAGILEGGKDTC   180
1OP2    RIMGWGSITPVKETFPDVPYCANINLLDHAVCQAGYPEL--LAEYRTLCAGIVQGGKDTC   176
        ****** *:*.: *:****:*.*****:: :*:* ***:   *  *******::******

4E7N    KGDSGGPLICNGQFQGIASWGDDPCAQPHKPAAYTKVFDHLDWIENIIAGNTDASCPP     238
1OP2    GGDSGGPLICNGQFQGIVSYGAHPCGQGPKPGIYTNVFDYTDWIQRNIAGNTDATCPP     234
         ****************.*:* .**.*  **. **:***: ***:. *******:***
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Figure 4

Snake venom glycosylated serine protease structures. *N*-acetyl-D-glucosamine posttranslational modifications are shown on the crystal structures of (*a*) Ahv-TI-I (PDB ID: 4E7N) and (*b*) Aav-SP-II (PDB ID: 1OP2). (*c*) An alignment of the two protein sequences highlights the different asparagine (N) glycosylation sites, shown in red.

rattlesnake) (74), *Crotalus oreganus helleri* (Southern Pacific rattlesnake) (76), and the well-known Mojave toxin from *Crotalus scutulatus* (Mojave rattlesnake) (77), among others (74, 78, 79). The number of PLA_2 subunits that make up these toxin complexes can vary, with most (e.g., β-bungarotoxin, crotoxin, and Mojave toxin) having only two subunits and others having three [taipoxin (80)] or five [textilotoxin (81, 82)].

PLA_2s are examples of a large venom protein superfamily that has expanded biological activity and increased toxicity by forming protein complexes, but many other venom protein superfamilies have also employed this strategy; previously mentioned were covalent and noncovalent 3FTxs, and other venom protein complexes include SVMPs, disintegrins, SVSPs, L-amino acid oxidase (LAAO), and snaclecs (snake venom C-type lectins) (66).

3.5. Minor Toxin Complexity

Other protein families present in snake venom include snaclecs, cysteine-rich secretory proteins (CRiSPs), LAAO, acetylcholinesterase, nucleases and nucleotidases, growth factors,

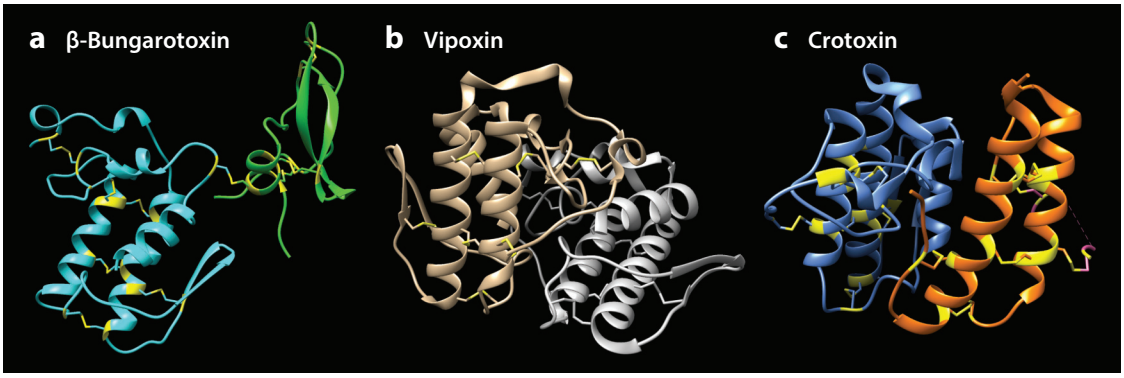

Figure 5

Structures of phospholipase A_2 (PLA_2) complexes. (*a*) In β-bungarotoxin (PDB ID: 1BUN), a PLA_2 is linked to a Kunitz-type serine protease inhibitor through a disulfide bond, whereas (*b*) vipoxin (PDB ID: 1JLT) is composed of two PLA_2 subunits and (*c*) crotoxin (PDB ID: 3R0L) is a complex of a PLA_2 and crotapotin.

hyaluronidases, cobra venom factors, and Kunitz protease inhibitors (83); these are not covered here, but see Reference 83 for a complete review in this area. The diversity of activities exhibited by all toxins adds to the overall complexity observed in snake venoms, and the outcome is greater venom functional versatility.

4. OMICS TOOLS TO IDENTIFY TOXIN DIVERSITY

4.1. Proteomics

Snake venoms are primarily composed of proteins; hence, proteomic methods have been fundamental in characterizing venom composition. More than 200 studies have been published on snake venom proteomes, and researchers have used several different strategies for proteomic analyses of venom. As complex mixtures, separation methods such as electrophoresis and high-performance liquid chromatography (HPLC) are usually the first steps carried out to fractionate venom components. These methods are then followed by mass spectrometry (MS) for protein or peptide identifications or by Edman degradation (N-terminal sequencing) to obtain information regarding amino acid sequence (3). Examples of electrophoretic techniques include 1D and 2D polyacrylamide gel electrophoresis, isoelectric focusing (IEF), gel elution liquid fraction entrapment electrophoresis (GELFrEE), capillary zone electrophoresis, and capillary isoelectric focusing (84–86). Popular HPLC fractionation methods include reverse-phase liquid chromatography, hydrophobic interaction chromatography, hydrophilic interaction, ion-exchange chromatography, size exclusion chromatography, and mixed-mode chromatography (87). Proteomic analyses of snake venom usually use several of these strategies in combination, such as liquid chromatography tandem mass spectrometry (LC-MS/MS), to fractionate and then directly identify peptides. Collision-induced dissociation is the most popular MS/MS technique, and it relies heavily on a complete public database for identifications, but peptide sequences can also be predicted de novo, which can greatly increase the number of identifiable peptides in a sample (88).

The two gold-standard strategies are (*a*) bottom-up proteomics (BUP) and (*b*) top-down proteomics (TDP). Methods based on BUP are most commonly used for proteomic analysis and identification of toxins and have been the classical venomics approach (2, 3, 89, 90). For BUP, proteins are first digested with proteases such as trypsin (most commonly used), chymotrypsin, or Glu-C, and then MS/MS-produced spectra are used for peptide mass fingerprinting or de novo

sequence determination (2, 91). The development of sample preparation methods (nano-UHPLC, multidimensional HPLC, 2D electrophoresis) with soft-ionization MS has greatly aided in investigating the complexity and composition of several snake venoms using various BUP strategies (92–94). TDP, in which protein cleavage is avoided to preserve accurate protein sizes, distinguish proteoforms, and also inform about complexes and PTMs, has gained popularity in more recent years (95). Native TDP can identify large proteins (>50 kDa) and even noncovalent complex interactions (95). A TDP study by Melani and colleagues (96) identified 131 proteins and 184 proteoforms from 14 toxin families in *Ophiophagus hannah* (king cobra) venom using GELFrEE and solution IEF fractionation followed by LC-MS/MS analysis. This study generated detailed information about two of the largest venom glycoproteins: the homodimeric LAAO (~130 kDa) and the multichain toxin cobra venom factor (~147 kDa) (96). TDP completed on *Boiga irregularis* (brown tree snake) identified 25–30 full-length 3FTx isoforms (16), exemplifying how TDP technology can be used to characterize multiple isoforms from one venom protein superfamily. In addition, TDP can identify proteins that are produced from the natural processing of larger proteins, as in the case of disintegrins, which is not possible with BUP.

However, these methods have limitations. For example, with BUP, tryptic fragments may be too short or too long for MS detection, or trypsin cleavage may be blocked by glycosylated regions of the protein (97). Other limitations lie in the mass discrepancy in proteoforms and the limited number of toxins (only known and/or characterized) in the proteome databases. Even methods such as Edman degradation for the N-terminal sequencing of proteins are limited in the number of residues that can be determined. Therefore, proteomic analyses must be complemented with comprehensive species-specific genome and/or venom gland transcriptomic database searches (98) to obtain the validated, accurate proteoforms or toxicoforms in a venom (16).

4.2. Transcriptomics

Since the first attempt to characterize toxin genes by isolating polyadenylated messenger RNAs (mRNAs) (99), transcriptomics analysis of snake venom glands has greatly advanced with the continuous development of new technologies. The first venom gland complementary DNA (cDNA) library was compiled using cloning technology and revealed that toxin genes constitute a large percentage of the expressed genes in the venom gland and that toxin diversity was higher than previously known (100). In recent years, many transcriptomes have been produced using both traditional cloning techniques and next-generation sequencing (NGS) platforms. There are researchers that still favor a cloning-based technique, as it is relatively inexpensive and can be performed in most molecular biology laboratories using readily available equipment and reagents (5, 101). However, with the decrease in costs of the less labor-intensive, high-throughput NGS technologies, the paradigm has shifted more toward the latter. One of the first uses of NGS for cataloging toxin genes from venom glands was done with 454 technology to catalog toxins from *Crotalus adamanteus* (eastern diamondback rattlesnake) (102). This study and others since have shown how toxins can be detected in very low abundance in the mRNA pool using NGS technologies, which is difficult to achieve with cDNA cloning and low-throughput BigDye sequencing (102, 103).

The use of high-throughput NGS approaches and computational methods to identify and quantify transcripts present in an RNA preparation is generally termed RNA-seq. RNA-seq methodology has perpetually advanced owing to the development of DNA sequencing technologies and, thus, the increase in throughput, accuracy, and read length of transcripts (104). NGS technologies have aided in the construction of snake venom transcriptomes with greater coverage in a shorter period of time (105). Illumina, one of the most commonly used methods currently,

has become an affordable and rapid way to obtain the venom profile of animals (106). It is even possible to sequence several venom gland transcriptomes together using multiplexed barcoded libraries, for very little difference in cost. This technology has already been used to generate large data sets through transcriptomic sequencing of several snake venom glands (**Table 1**). These more extensive data sets enable us to explore many species and address research questions such as the influence of lineage diversification on venom gene expression and venom phenotype (107–109). More recently, Oxford Nanopore (MinION) longer-read technology has been used to generate venom gland transcriptomes, and these longer–read length technologies are able to avoid issues that arise from de novo transcriptome assemblies (110).

There are a few limitations to using transcriptomics to characterize snake toxins. In the absence of reference genomes or transcriptomes, important transcripts involved in toxin production may be missed during assembly and annotation-based metrics for nonmodel species because of a reliance on distant species, in which chances are high that orthologs have been duplicated, changed, or lost (137). Therefore, transcriptomic data must be validated using proteomic databases or proteomic analysis of the venom proteome and vice versa, where transcriptome assemblies can provide

Table 1 Venom gland transcriptomes (next-generation sequencing technologies only)

Family and species	Platform[a]	Reference
Elapidae		
Acanthophis wellsi (Pilbara death adder)	1	111
Brachyurophis roperi (shovel-nosed snake)	1	111
Cacophis squamulosus (golden-crowed snake)	1	111
Dendroaspis angusticeps (eastern green mamba)	2	112
Dendroaspis jamesoni (Jameson's mamba)	2	112
Dendroaspis polylepis (black mamba)	2	112
Dendroaspis viridis (western green mamba)	2	112
Denisonia devisi (De Vis's banded snake)	1	111
Echiopsis curta (bardick)	1	111
Furina ornata (orange-naped snake)	1	111
Hemiaspis signata (marsh snake)	1	111
Hoplocephalus bungaroides (broad-headed snake)	1	111
Hydrophis platurus (yellow-bellied sea snake)	2	113
Micrurus corallinus (painted coral snake)	2	108
Micrurus fulvius (eastern coral snake)	2	108, 114
Micrurus lemniscatus (South American coral snake)	2	108
Micrurus paraensis (Pará coral snake)	2	108
Micrurus spixii (Amazon coral snake)	2	108
Micrurus surinamensis (aquatic coral snake)	2	108
Naja kaouthia (monocled cobra)	2	115, 116
Ophiophagus hannah (king cobra)	2	117, 118
Pseudonaja aspidorhyncha (strap-snouted brown snake)	1	119
Pseudonaja modesta (ringed brown snake)	1	111
Pseudonaja nuchalis (western brown snake)	1	119
Pseudonaja textilis (eastern brown snake)	1	119
Suta fasciata (Rosen's snake)	1	111
Vermicella annulata (bandy-bandy)	1	111

(*Continued*)

Table 1 (*Continued*)

Family and species	Platform[a]	Reference
Viperidae		
Atropoides mexicanus (Central American jumping pit viper)	1	120
Atropoides picadoi (Picado's pit viper)	1	120
Bothriechis lateralis (side-striped palm pit viper)	1	120
Bothriechis schlegelii (eyelash pit viper)	1	120
Bothrops asper (terciopelo)	1	120
Bothrops atrox (common lancehead)	2	121
Bothrops jararaca (jararaca)	1	122
Bothrops moojeni (Brazilian lancehead)	2	115
Cerrophidion godmani (Godman's montane pit viper)	1	120
Crotalus adamanteus (eastern diamondback rattlesnake)	1; 2	102, 123
Crotalus cerastes (sidewinder)	2	107
Crotalus culminatus (northwestern neotropical rattlesnake)	1	120
Crotalus durissus terrificus (South American rattlesnake)	2	124
Crotalus horridus (timber or canebrake rattlesnake)	2	125
Crotalus oreganus helleri (Southern Pacific rattlesnake)	2	126
Crotalus scutulatus (Mojave rattlesnake)	2	127
Crotalus simus (Middle American rattlesnake)	1; 2	120, 128
Crotalus tzabcan (Yucatán neotropical rattlesnake)	1; 2	120, 128
Daboia russelii (Russell's viper)	2	129
Echis coloratus (painted saw-scaled viper)	3	130
Ovophis okinavensis (Okinawa pit viper)	2	131
Protobothrops flavoviridis (habu)	2	131
Rear-fanged (various families)		
Ahaetulla prasina (Oriental whip snake)	2	138
Boiga cynodon (dog-toothed cat snake)	2	109
Boiga dendrophila (mangrove cat snake)	2	109
Boiga irregularis (brown tree snake)	2	15, 16
Boiga nigriceps (black-headed cat snake)	2	109
Borikenophis portoricensis (Puerto Rican racer)	2	138
Dispholidus typus (boomslang)	2	132
Erythrolamprus miliaris (military ground snake)	2	133
Hypsiglena spp. (night snakes)	2	15
Macropisthodon rudis (false viper)	2	134
Oxyhopus guibei (false coral snake)	2	133
Phalotris mertensi (false coral snake)	2	103
Psammophis mossambicus (olive grass snake)	1	135
Spilotes sulphureus (Amazon puffing snake)	2	17
Xenodon merremi (Wagler's snake)	2	133
Lamprophiidae		
Atractaspis aterrima (slender burrowing asp)	1	136

[a]Platform 1, 454; Platform 2, Illumina; Platform 3, Oxford Nanopore.

databases for protein identifications when current databases are insufficient or missing data (138). However, a transcriptome cannot be used to obtain accurate quantitative data, and it is difficult to predict PTMs, which include glycosylation, disulfide bond formation, and side chain or N- and C-terminal modifications (54, 139, 140). Transcriptomics data should be supported by proteomics data to validate toxin transcripts and also to fill knowledge gaps caused by the inability to identify PTMs. Integration of multiple omics technologies has been shown to be ideal for proper toxin diversity profiling (2).

4.3. Genomics

Advances in NGS technology and concomitant decreases in costs have allowed for complete genome sequencing of many nonmodel organisms, including snakes. Newer sequencing technologies, such as PacBio and Oxford nanopore, have greatly improved genome assemblies by increasing read lengths, and positional information to better assemble sequences into longer scaffolds and chromosomes can now be obtained via Hi-C techniques. The first two snake genomes were published in 2013 (118, 141), and 19 additional genomes have become publicly available since (**Table 2**).

Snake genomes are interesting for studying extreme organism adaptations. These adaptations include (but are by no means limited to) heteromorphic and homomorphic sex chromosomes (149, 151), absence of limbs (145), unique digestive processes (141), survival in extreme environmental conditions [i.e., snakes of the genus *Thermophis* (hot spring snakes) (150)], and of course the

Table 2 Currently completed snake genomes

Snake species	Genome size	Scaffold N50[a]	GenBank accession	Reference
Ophiophagus hannah (king cobra)	1.59 Gb	241 Kbp	AZIM00000000	118
Python bivittatus (Burmese python)	1.43 Gb	213 Kbp	AEQU00000000	141
Boa constrictor (red-tailed boa)	1.6 Gb	-	-	142
Pantherophis guttatus (corn snake)	1.40 Gb	4.3 Kbp	JTLQ00000000	144
Vipera berus (common adder)	1.3 Gb	126 Kbp	JTGP00000000	Unpublished
Crotalus mitchellii pyrrhus (speckled rattlesnake)	1.12 Gb	5.2 Kbp	JPMF00000000	143
Deinagkistrodon acutus (hundred-pace viper)	1.47 Gb	2,120 Kbp	-	145
Protobothrops mucrosquamatus (brown-spotted pit viper)	1.67 Gb	424 Kbp	BCNE00000000	146
Protobothrops flavoviridis (habu)	1.41 Gb	467 Kbp	BFFQ00000000	147
Thamnophis sirtalis (common garter snake)	1.12 Gb	647 Kbp	LFLD00000000	148
Thermophis baileyi (hot-spring snake)	1.74 Gb	2,414 Kbp	QLTV00000000	150
Crotalus viridis (prairie rattlesnake)	1.34 Gb	179,897 Kbp	PDHV00000000	149
Crotalus horridus (timber rattlesnake)	1.52 Gb	23 Kbp	LVCR00000000	Unpublished
Notechis scutatus (tiger snake)	1.66 Gb	5,997 Kbp	ULFQ00000000	Unpublished
Pseudonaja textilis (eastern brown snake)	1.59 Gb	14,685 Kbp	ULFR00000000	Unpublished
Hydrophis melanocephalus (slender-necked sea snake)	1.40 Gb	59.8 Kbp	BHFS00000000	Unpublished
Hydrophis cyanocinctus (annulated sea snake)	1.38 Gb	7.4 Kbp	RSAE00000000	Unpublished
Hydrophis hardwickii (spine-bellied sea snake)	1.29 Gb	5.3 Kbp	RSAD00000000	Unpublished
Emydocephalus ijimae (Ijima's sea snake)	1.62 Gb	18.5 Kbp	BHEV00000000	Unpublished
Laticauda laticaudata (blue-banded sea krait)	1.55 Gb	39.3 Kbp	BHFT00000000	Unpublished
Laticauda colubrine (yellow-lipped sea krait)	2.02 Gb	3,139 Kbp	BHFR00000000	Unpublished

[a]N50 numbers are from the most recent genome assembly versions, which might vary from those reported in the publications.

evolution of venom genes (118, 145, 146, 149). As one would expect, snake genomes experience positive selection for metabolism, olfactory receptor, and venom genes and relaxed selection for limb-patterning *Hox*, vision, and auditory genes (118, 141, 145). Snake genomes contain high levels of repetitive elements; in fact, they have the highest microsatellite content of any known eukaryote (152). These genomes also have an abundance of transposable elements (141, 153); up to 47% of the *Deinagkistrodon acutus* (hundred-pace viper) genome was discovered to consist of such elements (145). This high abundance of repetitive elements may contribute to venom gene duplications or deletions, because nonhomologous recombination is more likely to occur in these regions (153).

Many toxin-encoding genes originate from gene duplications and are thought to then evolve following duplication by positive Darwinian selection (154–159). Snake venom proteins undergo rapid adaptive evolution owing to high mutation rates in toxin genes, known as "accelerated evolution" (160). High-coverage sequencing of whole snake genomes can more confidently identify single-nucleotide polymorphisms (SNPs) within toxin genes. These mechanisms increase genome complexity, as well as generate new gene functions (neofunctionalization) (118, 161). The genome of *Protobothrops flavoviridis* (habu) has provided support for accelerated evolution of venom genes (147), but the genome of *Protobothrops mucrosquamatus* (brown-spotted pit viper) indicated that although some venom gene families exhibit positive selection, others are evolving under neutral processes (146).

Documented toxin evolutionary phenomena include (*a*) more frequent mutation of exons than introns (147, 154, 155, 160, 161); (*b*) alterations in intron–exon boundaries (162); (*c*) accelerated segment switch in exons to alter targeting (ASSET) (163, 164); (*d*) point mutations in unstable nucleotide triplets, which are more frequent in exons than in introns (165); (*e*) exon deletions (166); (*f*) domain recombination or loss (50, 167); (*g*) rapid accumulation of variations in exposed residues (RAVERs) (168); and (*h*) gene exonization and intronization (169). These evolutionary mechanisms all contribute to generation of residue or domain differences between venom proteins in the same superfamily. Further, genome sequencing has revealed that both toxin gene gain and loss have generated venom phenotypic variation between species. The *O. hannah* (king cobra) genome revealed expansion of venom protein gene families, especially 3FTxs (118). However, in the case of several rattlesnakes, *Crotalus atrox* (western diamondback rattlesnake), *C. adamanteus* (eastern diamondback rattlesnake), and *C. scutulatus* (Mojave rattlesnake), which have had select genome regions sequenced, the loss of neurotoxic PLA_2 genes has resulted in venom variation between these different crotalid species (170).

In the absence of a complete gene record, it can even be difficult to determine putative toxins owing to limited tissue sampling (171). Genome sequences provide a reference to map transcriptome and proteome data, determining whether toxin variation originates from gene loss or gain, SNPs, alternative splicing, or false positives from venom gland transcriptome assembly inaccuracies. Soon, many high-quality snake genomes will be available for large-scale toxin gene comparisons across species and families.

5. THERAPEUTIC AND DIAGNOSTIC APPLICATIONS

5.1. Antihypertensive Therapeutics

Toxins represent a rich source of inspiration for discovery and development of therapeutic and diagnostic agents, especially related to cardiovascular diseases (172–175). One of the greatest successes is the development of the angiotensin-converting enzyme (ACE) inhibitor captopril, used mainly for hypertension treatments. ACE is a zinc metalloprotease that cleaves bradykinin, an endogenous molecule that increases vascular permeability. In addition, ACE also cleaves angiotensin I to angiotensin II, the latter being a potent vasoconstrictor (176). BPPs from the venom of the pit

viper *Bothrops jararaca* (jararaca) are inhibitors of ACE. Structural and functional studies into BPPs eventually resulted in the design and development of captopril, the first-in-class ACE inhibitor (177, 178).

Other classes of snake venom hypotensive toxins have also been developed as therapeutics. For example, venom natriuretic peptide from *Dendroaspis angusticeps* (eastern green mamba), DNP, was found to have diuretic and vasodilatory effects similar to human atrial natriuretic peptides (ANPs) (179). However, DNP has a longer plasma half-life than endogenous NPs because it is resistant to degradation by human neutral endopeptidases (180). Human C-type NP has strong antiproliferative and low diuretic effects compared with other NPs. Cenderitide, a chimera between CNP and DNP, combined the diuretic effect and plasma stability of DNP with the antiproliferative effect of CNP (181). Cenderitide demonstrated safety and improved renal functions in heart failure patients in phase I and II trials (182, 183). Recently, studies into krait NP from *Bungarus flaviceps* (red-headed krait) revealed molecular switches that enable the dissociation of diuretic and vasodilatory effects of ANP. This would aid in the development of targeted and personalized treatments for heart failure patients (184, 185).

5.2. Platelet-Targeting Therapeutics

Two antiplatelet drugs used to prevent and treat thrombosis originated from studies into snake venom disintegrins. These drugs, tirofiban and eptifibatide, mediate their antiplatelet effect by inhibiting integrin $\alpha_{IIb}\beta_3$, thus preventing aggregation between platelets. Tirofiban and eptifibatide were designed based on the disintegrins echistatin and barbourin, from *Echis carinatus* (saw-scaled viper) and *Sistrurus miliarius barbouri* (pygmy rattlesnake), respectively (see 172 and references therein). In addition, ^{99m}Tc-labeled recombinant bitistatin, a disintegrin initially isolated from *Bitis arietans* (puff adder), was developed as an imaging agent for acute thrombi and emboli for its affinity to integrin $\alpha_{IIb}\beta_3$ (186). Results of a phase I trial of ^{99m}Tc-bitistatin were reported (187), but the phase II trial appeared to have been withdrawn owing to lack of funding (188).

Many snaclecs also target platelet surface receptors. Binding of snaclecs to platelet glycoprotein receptors may result in induction or inhibition of aggregation (189). Many of these toxins may be used as diagnostic agents and research tools for studying various blood disorders (190). For example, botrocetin, isolated from *B. jararaca* (jararaca), binds to blood glycoprotein von Willebrand factor (VWF) and its platelet receptor GPIb. The stabilization of interaction between VWF and GPIb promotes platelet aggregation (191, 192). Botrocetin is used to diagnose VWF disorders such as Bernard–Soulier disease and type IIa von Willebrand disease (190).

5.3. Anticoagulants

Anticoagulant proteins from snake venoms may be enzymatic or nonenzymatic. For enzymatic anticoagulants, enzymatic function may or may not be responsible for their anticlotting effects (see 193 and references therein). Among all, two defibrinogenating agents, ancrod (from *Calloselasma rhodostoma*, Malaysian pit viper) and batroxobin (from *Bothrops moojeni*, Brazilian lancehead), have been most extensively investigated in clinical trials. These are SVSPs that degrade fibrinogen into soluble fibrin clots, which in turn can be easily removed from the circulation by plasmin. Therefore, they act as anticoagulants in vivo through fibrinogen depletion (194). Despite a favorable outcome in an earlier trial (195), phase III trials of ancrod for acute ischemic stroke were terminated for lack of efficacy and because it increased bleeding when given within 6 h of stroke onset (196). Ancrod has also been tested in humans for sudden sensorineural hearing loss, although the outcome has yet to be reported (197). Batroxobin has been reported to be effective in limb salvage

of deep vein thrombosis patients (198). Batroxobin has shown good results for stroke prevention in patients with hyperfibrinogenemia (199) and as a perioperative anticoagulant in spinal fusion surgery (200). However, the clinical benefit of batroxobin has remained controversial, as most of these studies have involved a small number of subjects and demonstrated limited improvement over nontreatments (201). Because batroxobin and ancrod cleave fibrinogen and are not inhibited by heparin, they are useful for clotting assays. This type of clotting blood test (Reptilase time) is used to diagnose fibrinogen disorders, especially in heparinized samples (190).

5.4. Procoagulants

Ecarin from *E. carinatus* (saw-scaled viper) venom is a prothrombin activator that produces meizothrombin and is insensitive to heparin, warfarin, or lupus anticoagulants (antibodies against phospholipids). Thus, Ecarin time is used to monitor the plasma level of direct thrombin inhibitors such as lepirudin and dabigatran (190). Another prothrombin activating metalloprotease from *E. carinatus* venom, carinactivase-1, specifically recognizes the Ca^{2+}-bound conformation of the Gla domain in prothrombin for activation. It is therefore used to assay normal prothrombin levels in warfarin-treated patients (202). *Daboia russelii* (Russell's viper) venom factor X activator directly activates factor X (203). It is used for the Stypven time assay to help diagnose deficiencies in FX and FVII and in lupus anticoagulant assay (190).

5.5. Natural Inhibitors for Snakebite

Some venomous snakes are resistant to their own venom. So far, two possible mechanisms for this resistance have been reported. One mechanism involves the occurrence of limited mutations on the receptor in the resistant snake that prevents binding of the toxin to its target (204). For example, Takacs et al. (205) described that the resistance against conspecific α-neurotoxins, the major lethal components of Elapidae venoms, was mediated by a unique *N*-glycosylation of the nAChR ligand-binding domain in Elapidae snakes. The other mechanism involves serum proteins that bind the toxins with high affinity and neutralize the toxin's pathophysiological effects (206). These proteins that provide natural resistance are called endogenous inhibitors.

Endogenous inhibitors circulate in blood and effectively bind and neutralize the venom toxins from the host. However, they differ from antibodies. The three main endogenous inhibitor classes are phospholipase inhibitors (PLIs) (207, 208), antihemorrhagic factors (209), and small serum proteins (210–213) found in the blood of vipers. Numerous studies have described the highly effective inhibition of PLA_2 toxicity in vitro and in vivo by PLIs purified as a soluble protein from snake serum (214–217). The molecular mechanism of toxin neutralization by these endogenous inhibitors remains unclear owing to the lack of structural information on the binding of these inhibitors to toxins.

To understand the molecular evolution of endogenous inhibitors, genes encoding small serum proteins were investigated. Interestingly, as with toxin genes, the number of nonsynonymous substitutions was significantly greater in these inhibitor genes compared with synonymous substitutions (218, 219). These mutation hotspots are found on the molecular surface, whereas the protein scaffold structure is highly conserved. Endogenous inhibitor genes also appear to have evolved via gene duplication and rapid diversification, facilitating the neutralization of various toxins. Thus, these studies help in deciphering the evolution of endogenous natural resistance in venomous snakes. The molecular interaction of endogenous inhibitors with respective toxins may help elucidate the specificity and selectivity of these endogenous inhibitors and aid in the design of

better therapeutic agents for the treatment of snakebite, where a rapidly evolving diversity of snake venom toxins are present.

6. CONCLUSIONS

Each toxin superfamily can exhibit activity ranging from neurotoxic effects, with affinities for distinct receptors and channels, to those that affect the cardiovascular system and blood coagulation. The increasing affordability and sensitivity of technologies in this omics era, such as those of the proteomic, transcriptomic, and genomic fields, have allowed us to characterize venoms and their toxins on a level not possible a decade ago and, additionally, to now address advanced research questions. Mechanisms responsible for toxin superfamily diversification through amino acid residue substitutions or PTMs, as well as domain structures and complex formations, can be evaluated through omics tools. Studies into snake venom toxins have provided many opportunities and inspirations for the development of therapeutics and diagnostic agents. New and highly sensitive approaches in discovery, characterization, and drug development will continue to bring more molecules into the pipeline.

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LITERATURE CITED

1. Gutiérrez JM, Calvete JJ, Habib AG, Harrison RA, Williams DJ, Warrell DA. 2017. Snakebite envenoming. *Nat. Rev. Dis. Prim.* 3:17063
2. Calvete JJ. 2014. Next-generation snake venomics: protein-locus resolution through venom proteome decomplexation. *Expert Rev. Proteom.* 11:315–29
3. Calvete JJ, Sanz L, Angulo Y, Lomonte B, Gutiérrez JM. 2009. Venoms, venomics, antivenomics. *FEBS Lett.* 583:1736–43
4. Tasoulis T, Isbister GK. 2017. A review and database of snake venom proteomes. *Toxins* 9(9):290
5. Brahma RK, McCleary RJR, Kini RM, Doley R. 2015. Venom gland transcriptomics for identifying, cataloging, and characterizing venom proteins in snakes. *Toxicon* 93:1–10
6. Fry BG, Vidal N, Norman JA, Vonk FJ, Scheib H, et al. 2005. Early evolution of the venom system in lizards and snakes. *Nature* 439:584
7. Uetz P, Freed P, Hosek J. 2018. *The Reptile Database*. **http://www.reptile-database.org/**
8. Kochva E. 1987. The origin of snakes and evolution of the venom apparatus. *Toxicon* 25:65–106
9. Pyron RA, Burbink FT, Wiens JJ. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC Evol. Biol.* 13:93
10. Vonk FJ, Admiraal JF, Jackson K, Reshef R, de Bakker MA, et al. 2008. Evolutionary origin and development of snake fangs. *Nature* 454:630–33
11. Jackson K. 2003. The evolution of venom-delivery systems in snakes. *Zool. J. Linn. Soc.* 137:337–54
12. Weinstein SA, White J, Keyler DE, Warrell DA. 2013. Non-front-fanged colubroid snakes: a current evidence-based analysis of medical significance. *Toxicon* 69:103–13
13. Kardong KV. 2002. Colubrid snakes and Duvernoy's "venom" glands. *J. Toxicol. Toxin Rev.* 21:1–19
14. Mackessy SP. 2010. Field of reptile toxinology: snakes, lizards, and their venoms. In *Handbook of Venoms and Toxins of Reptiles*, ed. SP Mackessy, pp. 2–23. Boca Raton, FL: CRC Press/Taylor & Francis Group
15. McGivern JJ, Wray KP, Margres MJ, Couch ME, Mackessy SP, Rokyta DR. 2014. RNA-seq and high-definition mass spectrometry reveal the complex and divergent venoms of two rear-fanged colubrid snakes. *BMC Genom.* 15:1061

16. Pla D, Petras D, Saviola AJ, Modahl CM, Sanz L, et al. 2017. Transcriptomics-guided bottom-up and top-down venomics of neonate and adult specimens of the arboreal rear-fanged Brown Treesnake, *Boiga irregularis*, from Guam. *J. Proteom.* 174:71–84
17. Modahl CM, Mrinalini Frietze S, Mackessy SP. 2018. Adaptive evolution of distinct prey-specific toxin genes in rear-fanged snake venom. *Proc. Biol. Sci.* 285:20181003
18. Pawlak J, Mackessy SP, Fry BG, Bhatia M, Mourier G, et al. 2006. Denmotoxin, a three-finger toxin from the colubrid snake *Boiga dendrophila* (mangrove catsnake) with bird-specific activity. *J. Biol. Chem.* 281:29030–41
19. Pahari S, Mackessy SP, Kini RM. 2007. The venom gland transcriptome of the Desert Massasauga Rattlesnake (*Sistrurus catenatus edwardsii*): towards an understanding of venom composition among advanced snakes (Superfamily Colubroidea). *BMC Mol. Biol.* 8:115
20. Junqueira-de-Azevedo ILM, Ching ATC, Carvalho E, Faria F, Nishiyama MY, et al. 2006. *Lachesis muta* (Viperidae) cDNAs reveal diverging pit viper molecules and scaffolds typical of cobra (Elapidae) venoms: implications for snake toxin repertoire evolution. *Genetics* 173:877–89
21. Makarova YV, Kryukova EV, Shelukhina IV, Lebedev DS, Andreeva TV, et al. 2018. The first recombinant viper three-finger toxins: inhibition of muscle and neuronal nicotinic acetylcholine receptors. *Dokl Biochem. Biophys.* 479:127–30
22. Tsetlin V. 1999. Snake venom α-neurotoxins and other 'three-finger' proteins. *Eur. J. Biochem.* 264:281–86
23. Utkin YN. 2013. Three-finger toxins, a deadly weapon of elapid venom—milestones of discovery. *Toxicon* 62:50–55
24. Kini RM, Doley R. 2010. Structure, function and evolution of three-finger toxins: mini proteins with multiple targets. *Toxicon* 56:855–67
25. Bourne Y, Talley TT, Hansen SB, Taylor P, Marchot P. 2005. Crystal structure of a Cbtx-AChBP complex reveals essential interactions between snake α-neurotoxins and nicotinic receptors. *EMBO J.* 24:1512–22
26. Nirthanan S, Gwee MCE. 2004. Three-finger α-neurotoxins and the nicotinic acetylcholine receptor, forty years on. *J. Pharmacol. Sci.* 94:1–17
27. Chang CC, Lee CY. 1963. Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their modes of neuromuscular blocking action. *Arch. Int. Pharmacodyn. Ther.* 144:241–57
28. Chung C, Wu BN, Yang CC, Chang LS. 2002. Muscarinic toxin-like proteins from Taiwan banded krait (*Bungarus multicinctus*) venom: purification, characterization and gene organization. *J. Biol. Chem.* 383:1397–406
29. Karlsson E, Jolkkonen M, Mulugeta E, Onali P, Adem A. 2000. Snake toxins with high selectivity for subtypes of muscarinic acetylcholine receptors. *Biochimie* 82:793–806
30. Rajagopalan N, Pung YF, Zhu YZ, Wong PT, Kumar PP, Kini RM. 2007. β-Cardiotoxin: a new three-finger toxin from *Ophiophagus hannah* (king cobra) venom with beta-blocker activity. *FASEB J.* 21:3685–95
31. Rosso J-P, Schwarz JR, Diaz-Bustamante M, Céard B, Gutiérrez JM, et al. 2015. MmTX1 and MmTX2 from coral snake venom potently modulate $GABA_A$ receptor activity. *PNAS* 112:E891–900
32. Rivera-Torres IO, Jin TB, Cadene M, Chait BT, Poget SF. 2016. Discovery and characterisation of a novel toxin from *Dendroaspis angusticeps*, named Tx7335, that activates the potassium channel KcsA. *Sci. Rep.* 6:23904
33. Yang DC, Deuis JR, Dashevsky D, Dobson J, Jackson TNW, et al. 2016. The snake with the scorpion's sting: novel three-finger toxin sodium channel activators from the venom of the long-glanded blue coral snake (*Calliophis bivirgatus*). *Toxins* 8:303
34. de Weille JR, Schweitz H, Maes P, Tartar A, Lazdunski M. 1991. Calciseptine, a peptide isolated from black mamba venom, is a specific blocker of the L-type calcium channel. *PNAS* 88:2437–40
35. Kini RM, Haar NC, Evans HJ. 1988. Non-enzymatic inhibitors of coagulation and platelet aggregation from *Naja nigricollis* venom are cardiotoxins. *Biochem. Biophys. Res. Commun.* 150:1012–16
36. Banerjee Y, Mizuguchi J, Iwanaga S, Kini RM. 2005. Hemextin AB complex, a unique anticoagulant protein complex from *Hemachatus haemachatus* (African Ringhals cobra) venom that inhibits clot initiation and factor VIIa activity. *J. Biol. Chem.* 280:42601–11

37. McDowell RS, Dennis MS, Louie A, Shuster M, Mulkerrin MG, Lazarus RA. 1992. Mambin, a potent glycoprotein IIb-IIIa antagonist and platelet aggregation inhibitor structurally related to the short neurotoxins. *Biochemistry* 31:4766–72
38. Servent D, Winckler-Dietrich V, Hu HY, Kessler P, Drevet P, et al. 1997. Only snake curaremimetic toxins with a fifth disulfide bond have high affinity for the neuronal α7 nicotinic receptor. *J. Biol. Chem.* 272:24279–86
39. Mordvintsev DY, Polyak YL, Rodionov DI, Jakubik J, Dolezal V, et al. 2009. Weak toxin WTX from *Naja kaouthia* cobra venom interacts with both nicotinic and muscarinic acetylcholine receptors. *FEBS J.* 276:5065–75
40. Mordvintsev DY, Rodionov DI, Makarova MV, Kamensky AA, Levitskaya NG, et al. 2007. Behavioural effects in mice and intoxication symptomatology of weak neurotoxin from cobra *Naja kaouthia*. *Basic Clin. Pharmacol. Toxicol.* 100:273–78
41. Pawlak J, Mackessy SP, Sixberry NM, Stura EA, Le Du MH, et al. 2009. Irditoxin, a novel covalently linked heterodimeric three-finger toxin with high taxon-specific neurotoxicity. *FASEB J.* 23:534–45
42. Chiappinelli VA, Weaver WR, McLane KE, Conti-Fine BM, Fiordalisi JJ, Grant GA. 1996. Binding of native κ-neurotoxins and site-directed mutants to nicotinic acetylcholine receptors. *Toxicon* 34:1243–56
43. Roy A, Zhou X, Chong MZ, D'Hoedt D, Foo CS, et al. 2010. Structural and functional characterization of a novel homodimeric three-finger neurotoxin from the venom of *Ophiophagus hannah* (king cobra). *J. Biol. Chem.* 285:8302–15
44. Antil S, Servent D, Menez A. 1999. Variability among the sites by which curaremimetic toxins bind to *torpedo* acetylcholine receptor, as revealed by identification of the functional residues of α-cobratoxin. *J. Biol. Chem.* 274:34851–58
45. Antil-Delbeke S, Gaillard C, Tamiya T, Corringer PJ, Changeux JP, et al. 2000. Molecular determinants by which a long chain toxin from snake venom interacts with the neuronal α7-nicotinic acetylcholine receptor. *J. Biol. Chem.* 275:29594–601
46. Hassan-Puttaswamy V, Adams DJ, Kini RM. 2015. A distinct functional site in Ω-neurotoxins: novel antagonists of nicotinic acetylcholine receptors from snake venom. *ACS Chem. Biol.* 10:2805–15
47. Fox JW, Serrano SM. 2010. Snake venom metalloproteinases. In *Handbook of Venoms and Toxins of Reptiles*, ed. SP Mackessy, pp. 95–114. Boca Raton, FL: CRC Press/Taylor & Francis Group
48. Takeda S, Takeya H, Iwanaga S. 2012. Snake venom metalloproteinases: structure, function and relevance to the mammalian ADAM/ADAMTS family proteins. *Biochim. Biophys. Acta* 1824:164–76
49. Fox JW, Serrano SM. 2005. Structural considerations of the snake venom metalloproteinases, key members of the M12 reprolysin family of metalloproteinases. *Toxicon* 45:969–85
50. Casewell NR, Wagstaff SC, Harrison RA, Renjifo C, Wuster W. 2011. Domain loss facilitates accelerated evolution and neofunctionalization of duplicate snake venom metalloproteinase toxin genes. *Mol. Biol. Evol.* 28:2637–49
51. Kini RM, Evans HJ. 1992. Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. *Toxicon* 30:265–93
52. Calvete JJ, Marcinkiewicz C, Monleón D, Esteve V, Celda B, et al. 2005. Snake venom disintegrins: evolution of structure and function. *Toxicon* 45:1063–74
53. Saviola AJ, Chiszar D, Busch C, Mackessy SP. 2013. Molecular basis for prey relocation in viperid snakes. *BMC Biol.* 11:20
54. Moura-da-Silva AM, Almeida MT, Portes-Junior JA, Nicolau CA, Gomes-Neto F, Valente RH. 2016. Processing of snake venom metalloproteinases: generation of toxin diversity and enzyme inactivation. *Toxins* 8(6):E183
55. Ohler M, Georgieva D, Seifert J, von Bergen M, Arni RK, et al. 2010. The venomics of *Bothrops alternatus* is a pool of acidic proteins with predominant hemorrhagic and coagulopathic activities. *J. Proteome Res.* 9:2422–37
56. Braud S, Bon C, Wisner A. 2000. Snake venom proteins acting on hemostasis. *Biochimie* 82:851–59

57. Kini RM. 2005. Serine proteases affecting blood coagulation and fibrinolysis from snake venoms. *Pathophysiol. Haemost. Thromb.* 34:200–4
58. Serrano SM, Maroun RC. 2005. Snake venom serine proteinases: sequence homology versus substrate specificity, a paradox to be solved. *Toxicon* 45:1115–32
59. Serrano SM. 2013. The long road of research on snake venom serine proteinases. *Toxicon* 62:19–26
60. Ullah A, Masood R, Ali I, Ullah K, Ali H, et al. 2018. Thrombin-like enzymes from snake venom: structural characterization and mechanism of action. *Int. J. Biol. Macromol.* 114:788–811
61. Soares SG, Oliveira LL. 2009. Venom-sweet-venom: N-linked glycosylation in snake venom toxins. *Protein Pept. Lett.* 16:913–19
62. García LT, Parreiras e Silva LT, Ramos OH, Carmona AK, Bersanetti PA, Selistre-de-Araujo HS. 2004. The effect of post-translational modifications on the hemorrhagic activity of snake venom metalloproteinases. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 138:23–32
63. Hill RE, Mackessy SP. 2000. Characterization of venom (Duvernoy's secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins. *Toxicon* 38:1663–87
64. Kini RM. 1997. *Venom Phospholipase A2 Enyzmes: Structure, Function and Mechanism*. Chichester, UK: John Wiley and Sons Ltd.
65. Kini RM. 2003. Excitement ahead: structure, function and mechanism of snake venom phospholipase A_2 enzymes. *Toxicon* 42:827–40
66. Doley R, Kini RM. 2009. Protein complexes in snake venom. *Cell. Mol. Life Sci.* 66:2851–71
67. Abe T, Alema S, Miledi R. 1977. Isolation and characterization of presynaptically acting neurotoxins from the venom of *Bungarus* snakes. *Eur. J. Biochem.* 80:1–12
68. Kwong PD, McDonald NQ, Sigler PB, Hendrickson WA. 1995. Structure of β_2-bungarotoxin: potassium channel binding by Kunitz modules and targeted phospholipase action. *Structure* 3:1109–19
69. Tchorbanov B, Grishin E, Aleksiev B, Ovchinnikov Y. 1978. A neurotoxic complex from the venom of the Bulgarian viper (*Vipera ammodytes ammodytes*) and a partial amino acid sequence of the toxic phospholipase A2. *Toxicon* 16:37–44
70. Santos KF, Murakami MT, Cintra ACO, Toyama MH, Marangoni S, et al. 2007. Crystallization and preliminary X-ray crystallographic analysis of the heterodimeric crotoxin complex and the isolated subunits crotapotin and phospholipase A2. *Acta Crystallogr. F Struct. Biol. Cryst. Commun.* 63:287–90
71. Aird SD, Kaiser II, Lewis RV, Kruggel WG. 1985. Rattlesnake presynaptic neurotoxins: primary structure and evolutionary origin of the acidic subunit. *Biochemistry* 24:7054–58
72. Bon C, Changeux JP, Jeng TW, Fraenkel-Conrat H. 1979. Postsynaptic effects of crotoxin and of its isolated subunits. *Eur. J. Biochem.* 99:471–81
73. Kaiser II, Aird SD. 1987. A crotoxin homolog from the venom of the Uracoan rattlesnake (*Crotalus vegrandis*). *Toxicon* 25:1113–20
74. Chen YH, Wang YM, Hseu MJ, Tsai IH. 2004. Molecular evolution and structure-function relationships of crotoxin-like and asparagine-6-containing phospholipases A2 in pit viper venoms. *Biochem. J.* 381:25–34
75. Pool WR, Bieber AL. 1981. Fractionation of midget faded rattlesnake (*Crotalus viridis concolor*) venom: lethal fractions and enzymatic activities. *Toxicon* 19:517–27
76. French WJ, Hayes WK, Bush SP, Cardwell MD, Bader JO, Rael ED. 2004. Mojave toxin in venom of *Crotalus helleri* (Southern Pacific Rattlesnake): molecular and geographic characterization. *Toxicon* 44:781–91
77. Ho CL, Lee CY. 1981. Presynaptic actions of Mojave toxin isolated from Mojave rattlesnake (*Crotalus scutulatus*) venom. *Toxicon* 19:889–92
78. Weinstein SA, Minton SA, Wilde CE. 1985. The distribution among ophidian venoms of a toxin isolated from the venom of the Mojave rattlesnake (*Crotalus scutulatus scutulatus*). *Toxicon* 23:825–44
79. Powell RL, Lieb CS, Rael ED. 2004. Identification of a neurotoxic venom component in the tiger rattlesnake, *Crotalus tigris*. *J. Herpetol.* 38(1):149–52
80. Fohlman J, Lind P, Eaker D. 1977. Taipoxin, an extremely potent presynaptic snake venom neurotoxin. Elucidation of the primary structure of the acidic carbohydrate-containing taipoxin-subunit, a prophospholipase homolog. *FEBS Lett.* 84:367–71

81. Pearson JA, Tyler MI, Retson KV, Howden MEH. 1991. Studies on the subunit structure of textilotoxin, a potent presynaptic neurotoxin from the venom of the Australian common brown snake (*Pseudonaja textilis*). 2. The amino acid sequence and toxicity studies of subunit D. *Biochim. Biophys. Acta* 1077:147–50
82. Su MJ, Coulter AR, Sutherland SK, Chang CC. 1983. The presynaptic neuromuscular blocking effect and phospholipase A2 activity of textilotoxin, a potent toxin isolated from the venom of the Australian brown snake, *Pseudonaja textilis*. *Toxicon* 21:143–51
83. Boldrini-França J, Cologna CT, Pucca MB, Bordon KC, Amorim FG, et al. 2017. Minor snake venom proteins: structure, function and potential applications. *Biochim. Biophys. Acta* 1861:824–38
84. Fonslow BR, Kang SA, Gestaut DR, Graczyk B, Davis TN, et al. 2010. Native capillary isoelectric focusing for the separation of protein complex isoforms and subcomplexes. *Anal. Chem.* 82:6643–51
85. Tran JC, Zamdborg L, Ahlf DR, Lee JE, Catherman AD, et al. 2011. Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature* 480:254–58
86. Zhou Y, Zhang X, Fornelli L, Compton PD, Kelleher N, Wirth MJ. 2017. Chromatographic efficiency and selectivity in top-down proteomics of histones. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 1044–45:47–53
87. Capriotti AL, Cavaliere C, Foglia P, Samperi R, Lagana A. 2011. Intact protein separation by chromatographic and/or electrophoretic techniques for top-down proteomics. *J. Chromatogr. A* 1218:8760–76
88. Chapeaurouge A, Silva A, Carvalho P, McCleary RJR, Modahl CM, et al. 2018. Proteomic deep mining the venom of the red-headed krait, *Bungarus flaviceps*. *Toxins* 10(9):373
89. Calvete JJ. 2013. Snake venomics: from the inventory of toxins to biology. *Toxicon* 75:44–62
90. Calvete JJ. 2012. Venomics, what else? *Toxicon* 60:427–33
91. Escoubas P, Quinton L, Nicholson GM. 2008. Venomics: unravelling the complexity of animal venoms with mass spectrometry. *J. Mass Spectrom.* 43(3):279–95
92. Fox JW, Serrano SM. 2008. Exploring snake venom proteomes: multifaceted analyses for complex toxin mixtures. *Proteomics* 8:909–20
93. Serrano SM, Shannon JD, Wang D, Camargo AC, Fox JW. 2005. A multifaceted analysis of viperid snake venoms by two-dimensional gel electrophoresis: an approach to understanding venom proteomics. *Proteomics* 5:501–10
94. Sousa LF, Nicolau CA, Peixoto PS, Bernardoni JL, Oliveira SS, et al. 2013. Comparison of phylogeny, venom composition and neutralization by antivenom in diverse species of *Bothrops* complex. *PLOS Negl. Trop. Dis.* 7:e2442
95. Melani RD, Nogueira FCS, Domont GB. 2017. It is time for top-down venomics. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 23:44
96. Melani RD, Skinner OS, Fornelli L, Domont GB, Compton PD, Kelleher NL. 2016. Mapping proteoforms and protein complexes from king cobra venom using both denaturing and native top-down proteomics. *Mol. Cell. Proteom.* 15:2423–34
97. Choudhury M, McCleary RJR, Kesherwani M, Kini RM, Velmurugan D. 2017. Comparison of proteomic profiles of the venoms of two of the 'Big Four' snakes of India, the Indian cobra (*Naja naja*) and the common krait (*Bungarus caeruleus*), and analyses of their toxins. *Toxicon* 135:33–42
98. Petras D, Heiss P, Sussmuth RD, Calvete JJ. 2015. Venom proteomics of Indonesian king cobra, *Ophiophagus hannah*: integrating top-down and bottom-up approaches. *J. Proteome Res.* 14:2539–56
99. Vandenplas ML, Vandenplas S, Brebner K, Bester AJ, Boyd CD. 1985. Characterization of the messenger RNA population coding for components of viperid snake venom. *Toxicon* 23:289–305
100. Junqueira de Azevedo IL, Farsky SH, Oliveira ML, Ho PL. 2001. Molecular cloning and expression of a functional snake venom vascular endothelium growth factor (VEGF) from the *Bothrops insularis* pit viper: a new member of the VEGF family of proteins. *J. Biol. Chem.* 276:39836–42
101. Leonardi A, Sajevic T, Pungerčar J, Križaj I. 2019. Comprehensive study of the proteome and transcriptome of the venom of the most venomous European viper: discovery of a new subclass of ancestral snake venom metalloproteinase precursor-derived proteins. *J. Proteome Res.* 18:2287–309

102. Rokyta DR, Wray KP, Lemmon AR, Lemmon EM, Caudle BS. 2011. A high-throughput venom-gland transcriptome for the Eastern Diamondback Rattlesnake (*Crotalus adamanteus*) and evidence for pervasive positive selection across toxin classes. *Toxicon* 57:657–71
103. Campos PF, Andrade-Silva D, Zelanis A, Paes Leme AF, Rocha MM, et al. 2016. Trends in the evolution of snake toxins underscored by an integrative omics approach to profile the venom of the colubrid *Phalotris mertensi*. *Genome Biol. Evol.* 8:2266–87
104. Lowe R, Shirley N, Bleackley M, Dolan S, Shafee T. 2017. Transcriptomics technologies. *PLOS Comput. Biol.* 13:e1005457
105. Wang Z, Gerstein M, Snyder M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10:57–63
106. Liu L, Li Y, Li S, Hu N, He Y, et al. 2012. Comparison of next-generation sequencing systems. *J. Biomed. Biotechnol.* 2012:251364
107. Hofmann EP, Rautsaw RM, Strickland JL, Holding ML, Hogan MP, et al. 2018. Comparative venom-gland transcriptomics and venom proteomics of four Sidewinder Rattlesnake (*Crotalus cerastes*) lineages reveal little differential expression despite individual variation. *Sci. Rep.* 8:15534
108. Aird SD, da Silva NJ, Qiu L, Villar-Briones A, Saddi VA, et al. 2017. Coralsnake venomics: analyses of venom gland transcriptomes and proteomes of six Brazilian taxa. *Toxins* 9(6):187
109. Dashevsky D, Debono J, Rokyta D, Nouwens A, Josh P, Fry BG. 2018. Three-finger toxin diversification in the venoms of cat-eye snakes (Colubridae: *Boiga*). *J. Mol. Evol.* 86(8):531–545
110. Holding ML, Margres MJ, Mason AJ, Parkinson CL, Rokyta DR. 2018. Evaluating the performance of de novo assembly methods for venom-gland transcriptomics. *Toxins* 10(6):249
111. Jackson TNW, Sunagar K, Undheim EAB, Koludarov I, Chan AHC, et al. 2013. Venom down under: dynamic evolution of Australian elapid snake toxins. *Toxins* 5:2621–55
112. Ainsworth S, Petras D, Engmark M, Sussmuth RD, Whiteley G, et al. 2018. The medical threat of mamba envenoming in sub-Saharan Africa revealed by genus-wide analysis of venom composition, toxicity and antivenomics profiling of available antivenoms. *J. Proteom.* 172:173–89
113. Durban J, Sasa M, Calvete JJ. 2018. Venom gland transcriptomics and microRNA profiling of juvenile and adult yellow-bellied sea snake, *Hydrophis platurus*, from Playa del Coco (Guanacaste, Costa Rica). *Toxicon* 153:96–105
114. Margres M, Aronow K, Loyacano J, Rokyta D. 2013. The venom-gland transcriptome of the eastern coral snake (*Micrurus fulvius*) reveals high venom complexity in the intragenomic evolution of venoms. *BMC Genom.* 14:1–18
115. Xu N, Zhao HY, Yin Y, Shen SS, Shan LL, et al. 2017. Combined venomics, antivenomics and venom gland transcriptome analysis of the monocoled cobra (*Naja kaouthia*) from China. *J. Proteom.* 159:19–31
116. Tan KY, Tan CH, Chanhome L, Tan NH. 2017. Comparative venom gland transcriptomics of *Naja kaouthia* (monocled cobra) from Malaysia and Thailand: elucidating geographical venom variation and insights into sequence novelty. *PeerJ* 5:e3142
117. Tan CH, Tan KY, Fung SY, Tan NH. 2015. Venom-gland transcriptome and venom proteome of the Malaysian king cobra (*Ophiophagus hannah*). *BMC Genom.* 16:687
118. Vonk FJ, Casewell NR, Henkel CV, Heimberg AM, Jansen HJ, et al. 2013. The king cobra genome reveals dynamic gene evolution and adaptation in the snake venom system. *PNAS* 110:20651–56
119. Reeks T, Lavergne V, Sunagar K, Jones A, Undheim E, et al. 2016. Deep venomics of the *Pseudonaja* genus reveals inter- and intra-specific variation. *J. Proteom.* 133:20–32
120. Durban J, Juárez P, Angulo Y, Lomonte B, Flores-Diaz M, et al. 2011. Profiling the venom gland transcriptomes of Costa Rican snakes by 454 pyrosequencing. *BMC Genom.* 12:259
121. Amazonas DR, Portes-Junior JA, Nishiyama-Jr MY, Nicolau CA, Chalkidis HM, et al. 2018. Molecular mechanisms underlying intraspecific variation in snake venom. *J. Proteom.* 181:60–72
122. Junqueira-de-Azevedo ILM, Bastos CMV, Ho PL, Luna MS, Yamanouye N, Casewell NR. 2015. Venom-related transcripts from *Bothrops jararaca* tissues provide novel molecular insights into the production and evolution of snake venom. *Mol. Biol. Evol.* 32:754–66
123. Rokyta DR, Lemmon AR, Margres MJ, Aronow K. 2012. The venom-gland transcriptome of the eastern diamondback rattlesnake (*Crotalus adamanteus*). *BMC Genom.* 13:312

124. Wiezel GA, Shibao PYT, Cologna CT, Morandi Filho R, Ueira-Vieira C, et al. 2018. In-depth venome of the Brazilian rattlesnake *Crotalus durissus terrificus*: an integrative approach combining its venom gland transcriptome and venom proteome. *J. Proteome Res.* 17:3941–58
125. Rokyta DR, Wray KP, Margres MJ. 2013. The genesis of an exceptionally lethal venom in the timber rattlesnake (*Crotalus horridus*) revealed through comparative venom-gland transcriptomics. *BMC Genom.* 14:394
126. Sunagar K, Undheim EA, Scheib H, Gren EC, Cochran C, et al. 2014. Intraspecific venom variation in the medically significant Southern Pacific Rattlesnake (*Crotalus oreganus helleri*): biodiscovery, clinical and evolutionary implications. *J. Proteom.* 99:68–83
127. Strickland JL, Mason AJ, Rokyta DR, Parkinson CL. 2018. Phenotypic variation in Mojave rattlesnake (*Crotalus scutulatus*) venom is driven by four toxin families. *Toxins* 10(4):135
128. Durban J, Sanz L, Trevisan-Silva D, Neri-Castro E, Alagon A, Calvete JJ. 2017. Integrated venomics and venom gland transcriptome analysis of juvenile and adult Mexican rattlesnakes *Crotalus simus*, *C. tzabcan*, and *C. culminatus* revealed miRNA-modulated ontogenetic shifts. *J. Proteome Res.* 16:3370–90
129. Yee KT, Tongsima S, Ngamohiw C, Vasieva O, Rojnuckarin P. 2019. Analysis of snake venom metalloproteinases from Myanmar Russell's viper transcriptome. *Toxicon* 158:S46–S47
130. Hargreaves AD, Mulley JF. 2015. Assessing the utility of the Oxford Nanopore MinION for snake venom gland cDNA sequencing. *PeerJ* 3:e1441
131. Aird SD, Watanabe Y, Villar-Briones A, Roy MC, Terada K, Mikheyev AS. 2013. Quantitative high-throughput profiling of snake venom gland transcriptomes and proteomes (*Ovophis okinavensis* and *Protobothrops flavoviridis*). *BMC Genom.* 14:790
132. Pla D, Sanz L, Whiteley G, Wagstaff SC, Harrison RA, et al. 2017. What killed Karl Patterson Schmidt? Combined venom gland transcriptomic, venomic and antivenomic analysis of the South African green tree snake (the boomslang), *Dispholidus typus*. *Biochim. Biophys. Acta* 1861:814–23
133. Junqueira-de-Azevedo IL, Campos PF, Ching AT, Mackessy SP. 2016. Colubrid venom composition: an -omics perspective. *Toxins* 8:E230
134. Zhang Z, Zhang X, Hu T, Zhou W, Cui Q, et al. 2015. Discovery of toxin-encoding genes from the false viper *Macropisthodon rudis*, a rear-fanged snake, by transcriptome analysis of venom gland. *Toxicon* 106:72–78
135. Brust A, Sunagar K, Undheim EA, Vetter I, Yang DC, et al. 2013. Differential evolution and neofunctionalization of snake venom metalloprotease domains. *Mol. Cell. Proteom.* 12:651–63
136. Terrat Y, Sunagar K, Fry BG, Jackson TNW, Scheib H, et al. 2013. *Atractaspis aterrima* toxins: the first insight into the molecular evolution of venom in side-stabbers. *Toxins* 5:1948–64
137. O'Neil ST, Emrich SJ. 2013. Assessing de novo transcriptome assembly metrics for consistency and utility. *BMC Genom.* 14:465
138. Modahl CM, Frietze S, Mackessy SP. 2018. Transcriptome-facilitated proteomic characterization of rear-fanged snake venoms reveal abundant metalloproteinases with enhanced activity. *J. Proteom.* 187:223–34
139. Andrade-Silva D, Zelanis A, Kitano ES, Junqueira-de-Azevedo IL, Reis MS, et al. 2016. Proteomic and glycoproteomic profilings reveal that post-translational modifications of toxins contribute to venom phenotype in snakes. *J. Proteome Res.* 15:2658–75
140. Degueldre M, Echterbille J, Smargiasso N, Damblon C, Gouin C, et al. 2017. In-depth glycopeptidomics approach reveals unexpected diversity of glycosylated peptides and atypical post-translational modifications in *Dendroaspis angusticeps* snake venom. *Int. J. Mol. Sci.* 18:2453
141. Castoe TA, de Koning APJ, Hall KT, Card DC, Schield DR, et al. 2013. The Burmese python genome reveals the molecular basis for extreme adaptation in snakes. *PNAS* 110:20645–50
142. Bradnam KR, Fass JN, Alexandrov A, Baranay P, Bechner M, et al. 2013. Assemblathon 2: evaluating *de novo* methods of genome assembly in three vertebrate species. *GigaScience* 2:2047-217X-2-10
143. Gilbert C, Meik JM, Dashevsky D, Card DC, Castoe TA, Schaack S. 2014. Endogenous hepadnaviruses, bornaviruses and circoviruses in snakes. *Proc. Biol. Sci.* 281:20141122
144. Ullate-Agote A, Milinkovitch MC, Tzika AC. 2014. The genome sequence of the corn snake (*Pantherophis guttatus*), a valuable resource for EvoDevo studies in squamates. *Int. J. Dev. Biol.* 58:881–88

145. Yin W, Wang Z-j, Li Q-y, Lian J-m, Zhou Y, et al. 2016. Evolutionary trajectories of snake genes and genomes revealed by comparative analyses of five-pacer viper. *Nat. Commun.* 7:13107
146. Aird SD, Arora J, Barua A, Qiu L, Terada K, Mikheyev AS. 2017. Population genomic analysis of a pitviper reveals microevolutionary forces underlying venom chemistry. *Genome Biol. Evol.* 9:2640–49
147. Shibata H, Chijiwa T, Oda-Ueda N, Nakamura H, Yamaguchi K, et al. 2018. The habu genome reveals accelerated evolution of venom protein genes. *Sci. Rep.* 8:11300
148. Perry BW, Card DC, McGlothlin JW, Pasquesi GIM, Adams RH, et al. 2018. Molecular adaptations for sensing and securing prey and insight into amniote genome diversity from the garter snake genome. *Genome Biol. Evol.* 10:2110–29
149. Schield DR, Card DC, Hales NR, Perry BW, Pasquesi GM, et al. 2019. The origins and evolution of chromosomes, dosage compensation, and mechanisms underlying venom regulation in snakes. *Genome Res.* 29:590–601
150. Li J-T, Gao Y-D, Xie L, Deng C, Shi P, et al. 2018. Comparative genomic investigation of high-elevation adaptation in ectothermic snakes. *PNAS* 115:8406
151. Vicoso B, Emerson JJ, Zektser Y, Mahajan S, Bachtrog D. 2013. Comparative sex chromosome genomics in snakes: differentiation, evolutionary strata, and lack of global dosage compensation. *PLOS Biol.* 11:e1001643
152. Pasquesi GIM, Adams RH, Card DC, Schield DR, Corbin AB, et al. 2018. Squamate reptiles challenge paradigms of genomic repeat element evolution set by birds and mammals. *Nat. Commun.* 9:2774
153. Castoe TA, Hall KT, Guibotsy Mboulas ML, Gu W, de Koning APJ, et al. 2011. Discovery of highly divergent repeat landscapes in snake genomes using high-throughput sequencing. *Genome Biol. Evol.* 3:641–53
154. Juárez P, Comas I, González-Candelas F, Calvete JJ. 2008. Evolution of snake venom disintegrins by positive Darwinian selection. *Mol. Biol. Evol.* 25:2391–407
155. Deshimaru M, Ogawa T, Nakashima K, Nobuhisa I, Chijiwa T, et al. 1996. Accelerated evolution of crotalinae snake venom gland serine proteases. *FEBS Lett.* 397:83–88
156. Aird SD, Aggarwal S, Villar-Briones A, Tin MM, Terada K, Mikheyev AS. 2015. Snake venoms are integrated systems, but abundant venom proteins evolve more rapidly. *BMC Genom.* 16:647
157. Gibbs HL, Rossiter W. 2008. Rapid evolution by positive selection and gene gain and loss: PLA_2 venom genes in closely related *Sistrurus* rattlesnakes with divergent diets. *J. Mol. Evol.* 66(2):151–66
158. Margres MJ, Walls R, Suntravat M, Lucena S, Sanchez EE, Rokyta DR. 2016. Functional characterizations of venom phenotypes in the eastern diamondback rattlesnake (*Crotalus adamanteus*) and evidence for expression-driven divergence in toxic activities among populations. *Toxicon* 119:28–38
159. Pegueroles C, Laurie S, Alba MM. 2013. Accelerated evolution after gene duplication: a time-dependent process affecting just one copy. *Mol. Biol. Evol.* 30:1830–42
160. Nakashima K, Ogawa T, Oda N, Hattori M, Sakaki Y, Kihara H, et al. 1993. Accelerated evolution of *Trimeresurus flavoviridis* venom gland phospholipase A2 isozymes. *PNAS* 90(13):5964–68
161. Ogawa T, Kitajima M, Nakashima K, Sakaki Y, Ohno M. 1995. Molecular evolution of group II phospholipases A2. *J. Mol. Evol.* 41:867–77
162. Fujimi TJ, Nakajyo T, Nishimura E, Ogura E, Tsuchiya T, Tamiya T. 2003. Molecular evolution and diversification of snake toxin genes, revealed by analysis of intron sequences. *Gene* 313:111–18
163. Doley R, Mackessy SP, Kini RM. 2009. Role of accelerated segment switch in exons to alter targeting (ASSET) in the molecular evolution of snake venom proteins. *BMC Evol. Biol.* 9:146
164. Doley R, Pahari S, Mackessy SP, Kini RM. 2008. Accelerated exchange of exon segments in Viperid three-finger toxin genes (*Sistrurus catenatus edwardsii*; Desert Massasauga). *BMC Evol. Biol.* 8:196
165. Kini RM, Chinnasamy A. 2010. Nucleotide sequence determines the accelerated rate of point mutations. *Toxicon* 56:295–304
166. Pawlak J, Kini RM. 2008. Unique gene organization of colubrid three-finger toxins: complete cDNA and gene sequences of denmotoxin, a bird-specific toxin from colubrid snake *Boiga dendrophila* (Mangrove Catsnake). *Biochimie* 90:868–77

167. Moura-da-Silva AM, Furlan MS, Caporrino MC, Grego KF, Portes-Junior JA, et al. 2011. Diversity of metalloproteinases in *Bothrops neuwiedi* snake venom transcripts: evidences for recombination between different classes of SVMPs. *BMC Genet.* 12:94
168. Sunagar K, Jackson T, Undheim E, Ali S, Antunes A, Fry BG. 2013. Three-fingered RAVERs: Rapid Accumulation of Variations in Exposed Residues of snake venom toxins. *Toxins* 5:2172–208
169. Kini RM. 2018. Accelerated evolution of toxin genes: exonization and intronization in snake venom disintegrin/metalloprotease genes. *Toxicon* 148:16–25
170. Dowell NL, Giorgianni MW, Kassner VA, Selegue JE, Sanchez EE, Carroll SB. 2016. The deep origin and recent loss of venom toxin genes in rattlesnakes. *Curr. Biol.* 26:2434–45
171. Hargreaves AD, Swain MT, Logan DW, Mulley JF. 2014. Testing the Toxicofera: Comparative transcriptomics casts doubt on the single, early evolution of the reptile venom system. *Toxicon* 92:140–56
172. Koh CY, Kini RM. 2012. From snake venom toxins to therapeutics—cardiovascular examples. *Toxicon* 59:497–506
173. Koh CY, Modahl CM, Kulkarni N, Kini RM. 2018. Toxins are an excellent source of therapeutic agents against cardiovascular diseases. *Semin. Thromb. Hemost.* 44:691–706
174. Pennington MW, Czerwinski A, Norton RS. 2018. Peptide therapeutics from venom: current status and potential. *Bioorg. Med. Chem.* 26:2738–58
175. Harvey AL. 2014. Toxins and drug discovery. *Toxicon* 92:193–200
176. Gainer JV, Morrow JD, Loveland A, King DJ, Brown NJ. 1998. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. *N. Engl. J. Med.* 339:1285–92
177. Smith CG, Vane JR. 2003. The discovery of captopril. *FASEB J.* 17:788–89
178. Ferreira SH. 2010. From the *Bothrops Jararaca* bradykinin potentiating peptides to angiotensin converting enzyme inhibitors. In *Toxins and Hemostasis: From Bench to Bedside*, ed. RM Kini, KJ Clemetson, FS Markland, MA McLane, T Morita, pp. 13–17. Dordrecht: Springer Neth.
179. Schweitz H, Vigne P, Moinier D, Frelin C, Lazdunski M. 1992. A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J. Biol. Chem.* 267:13928–32
180. Chen HH, Lainchbury JG, Burnett JC Jr. 2002. Natriuretic peptide receptors and neutral endopeptidase in mediating the renal actions of a new therapeutic synthetic natriuretic peptide *Dendroaspis* natriuretic peptide. *J. Am. Coll. Cardiol.* 40:1186–91
181. Lisy O, Huntley BK, McCormick DJ, Kurlansky PA, Burnett JC Jr. 2008. Design, synthesis, and actions of a novel chimeric natriuretic peptide: CD-NP. *J. Am. Coll. Cardiol.* 52:60–68
182. Ichiki T, Dzhoyashvili N, Burnett JC Jr. 2019. Natriuretic peptide based therapeutics for heart failure: Cenderitide: a novel first-in-class designer natriuretic peptide. *Int. J. Cardiol.* 281:166–71
183. Kawakami R, Lee CYW, Scott C, Bailey KR, Schirger JA, et al. 2018. A human study to evaluate safety, tolerability, and cyclic GMP activating properties of cenderitide in subjects with stable chronic heart failure. *Clin. Pharmacol. Ther.* 104:546–52
184. Sridharan S, Kini RM. 2018. Decoding the molecular switches of natriuretic peptides which differentiate its vascular and renal functions. *Biochem. J.* 475:399–413
185. Sridharan S, Kini RM. 2015. Tail wags the dog: Activity of krait natriuretic peptide is determined by its C-terminal tail in a natriuretic peptide receptor-independent manner. *Biochem. J.* 469:255–66
186. Knight LC, Baidoo KE, Romano JE, Gabriel JL, Maurer AH. 2000. Imaging pulmonary emboli and deep venous thrombi with ^{99m}Tc-bitistatin, a platelet-binding polypeptide from viper venom. *J. Nucl. Med.* 41:1056–64
187. Knight LC, Romano JE, Bright LT, Agelan A, Kantor S, Maurer AH. 2007. Platelet binding and biodistribution of [^{99m}Tc]rBitistatin in animal species and humans. *Nucl. Med. Biol.* 34:855–63
188. Temple Univ. 2015. *Phase II investigation of 99mTc-rBitistatin for imaging venous thrombosis*. ClinicalTrials.gov, ID NCT00808626. **https://clinicaltrials.gov/ct2/show/NCT00808626**
189. Matsui T, Hamako J, Titani K. 2010. Structure and function of snake venom proteins affecting platelet plug formation. *Toxins* 2:10–23
190. Perchuc AM, Wilmer M. 2010. Diagnostic use of snake venom components in the coagulation laboratory. In *Toxins and Hemostasis: From Bench to Bedside*, ed. RM Kini, KJ Clemetson, FS Markland, MA McLane, T Morita, pp. 747–66. Dordrecht: Springer Neth.

191. Brinkhous KM, Read MS, Reddick RL, Griggs TR. 1981. Pathophysiology of platelet-aggregating von Willebrand factor: applications of the venom coagglutinin vWF assay. *Ann. N.Y. Acad. Sci.* 370:191–204
192. Fukuda K, Doggett T, Laurenzi IJ, Liddington RC, Diacovo TG. 2005. The snake venom protein botrocetin acts as a biological brace to promote dysfunctional platelet aggregation. *Nat. Struct. Mol. Biol.* 12:152–59
193. Kini RM. 2006. Anticoagulant proteins from snake venoms: structure, function and mechanism. *Biochem. J.* 397:377–87
194. Castro HC, Zingali RB, Albuquerque MG, Pujol-Luz M, Rodrigues CR. 2004. Snake venom thrombin-like enzymes: from reptilase to now. *Cell. Mol. Life Sci.* 61:843–56
195. Sherman DG, Atkinson RP, Chippendale T, Levin KA, Ng K, et al. 2000. Intravenous ancrod for treatment of acute ischemic stroke: the STAT study: a randomized controlled trial. Stroke Treatment with Ancrod Trial. *JAMA* 283:2395–403
196. Levy DE, del Zoppo GJ, Demaerschalk BM, Demchuk AM, Diener HC, et al. 2009. Ancrod in acute ischemic stroke: results of 500 subjects beginning treatment within 6 hours of stroke onset in the ancrod stroke program. *Stroke* 40:3796–803
197. Nordmark Arzneimittel GmbH & Co. KG. 2018. *Efficacy, safety, and tolerability of ancrod in patients with sudden hearing loss*. ClinicalTrials.gov, ID NCT01621256. **https://clinicaltrials.gov/ct2/show/NCT01621256**
198. Lei Z, Shi Hong L, Li L, Tao YG, Yong Ling W, et al. 2011. Batroxobin mobilizes circulating endothelial progenitor cells in patients with deep vein thrombosis. *Clin. Appl. Thromb. Hemost.* 17:75–79
199. Xu G, Liu X, Zhu W, Yin Q, Zhang R, Fan X. 2007. Feasibility of treating hyperfibrinogenemia with intermittently administered batroxobin in patients with ischemic stroke/transient ischemic attack for secondary prevention. *Blood Coagul. Fibrinolysis* 18:193–97
200. Hu HM, Chen L, Frary CE, Chang CC, Hui H, et al. 2015. The beneficial effect of Batroxobin on blood loss reduction in spinal fusion surgery: a prospective, randomized, double-blind, placebo-controlled study. *Arch. Orthop. Trauma Surg.* 135:491–97
201. Zeng Z, Xiao P, Chen J, Wei Y. 2009. Are batroxobin agents effective for perioperative hemorrhage in thoracic surgery? A systematic review of randomized controlled trials. *Blood Coagul. Fibrinolysis* 20:101–7
202. Yamada D, Morita T. 1999. CA-1 method, a novel assay for quantification of normal prothrombin using a Ca^{2+}-dependent prothrombin activator, carinactivase-1. *Thromb. Res.* 94:221–26
203. Kini RM, Koh CY. 2016. Metalloproteases affecting blood coagulation, fibrinolysis and platelet aggregation from snake venoms: definition and nomenclature of interaction sites. *Toxins* 8(10):284
204. Takacs Z, Wilhelmsen KC, Sorota S. 2001. Snake α-neurotoxin binding site on the Egyptian cobra (*Naja haje*) nicotinic acetylcholine receptor is conserved. *Mol. Biol. Evol.* 18:1800–9
205. Takacs Z, Wilhelmsen KC, Sorota S. 2004. Cobra (*Naja* spp.) nicotinic acetylcholine receptor exhibits resistance to Erabu sea snake (*Laticauda semifasciata*) short-chain α-neurotoxin. *J. Mol. Evol.* 58:516–26
206. Perales J, Neves-Ferreira AG, Valente RH, Domont GB. 2005. Natural inhibitors of snake venom hemorrhagic metalloproteinases. *Toxicon* 45:1013–20
207. Dunn RD, Broady KW. 2001. Snake inhibitors of phospholipase A2 enzymes. *Biochim. Biophys. Acta* 1533:29–37
208. Campos PC, de Melo LA, Dias GLF, Fortes-Dias CL. 2016. Endogenous phospholipase A_2 inhibitors in snakes: a brief overview. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 22:37–37
209. Bastos VA, Gomes-Neto F, Perales J, Neves-Ferreira AGC, Valente RH. 2016. Natural inhibitors of snake venom metalloendopeptidases: history and current challenges. *Toxins* 8:250
210. Aoki N, Sakiyama A, Deshimaru M, Terada S. 2007. Identification of novel serum proteins in a Japanese viper: homologs of mammalian PSP94. *Biochem. Biophys. Res. Commun.* 359:330–34
211. Shioi N, Ogawa E, Mizukami Y, Abe S, Hayashi R, Terada S. 2013. Small serum protein-1 changes the susceptibility of an apoptosis-inducing metalloproteinase HV1 to a metalloproteinase inhibitor in habu snake (*Trimeresurus flavoviridis*). *J. Biochem.* 153:121–29
212. Shioi N, Deshimaru M, Terada S. 2014. Structural analysis and characterization of new small serum proteins from the serum of a venomous snake (*Gloydius blomhoffii*). *Biosci. Biotechnol. Biochem.* 78:410–19

213. Shioi N, Nishijima A, Terada S. 2015. Flavorase, a novel non-haemorrhagic metalloproteinase in *Protobothrops flavoviridis* venom, is a target molecule of small serum protein-3. *J. Biochem.* 158:37–48
214. Lizano S, Lomonte B, Fox JW, Gutiérrez JM. 1997. Biochemical characterization and pharmacological properties of a phospholipase A2 myotoxin inhibitor from the plasma of the snake *Bothrops asper*. *Biochem. J.* 326(3):853–59
215. Lizano S, Angulo Y, Lomonte B, Fox JW, Lambeau G, et al. 2000. Two phospholipase A2 inhibitors from the plasma of *Cerrophidion* (*Bothrops*) *godmani* which selectively inhibit two different group-II phospholipase A2 myotoxins from its own venom: isolation, molecular cloning and biological properties. *Biochem. J.* 346(3):631–39
216. Nobuhisa I, Inamasu S, Nakai M, Tatsui A, Mimori T, et al. 1997. Characterization and evolution of a gene encoding a *Trimeresurus flavoviridis* serum protein that inhibits basic phospholipase A2 isozymes in the snake's venom. *Eur. J. Biochem.* 249:838–45
217. Perales J, Villela C, Domont GB, Choumet V, Saliou B, et al. 1995. Molecular structure and mechanism of action of the crotoxin inhibitor from *Crotalus durissus terrificus* serum. *Eur. J. Biochem.* 227:19–26
218. Aoki N, Matsuo H, Deshimaru M, Terada S. 2008. Accelerated evolution of small serum proteins (SSPs)—the PSP94 family proteins in a Japanese viper. *Gene* 426:7–14
219. Tanaka Y, Oyama S, Hori S, Ushio K, Shioi N, et al. 2013. Accelerated evolution of fetuin family proteins in *Protobothrops flavoviridis* (habu snake) serum and the discovery of an L1-like genomic element in the intronic sequence of a fetuin-encoding gene. *Biosci. Biotechnol. Biochem.* 77:582–90