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Chapter

Snakebite Therapeutics Based on Endogenous Inhibitors from Vipers

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Abstract

Venomous snakebite is a major human health issue in many countries and has been categorized as a neglected tropical disease by the World Health Organization. Venomous snakes have evolved to produce venom, which is a complex mixture of toxic proteins and peptides, both enzymatic and nonenzymatic in nature. In this current era of high-throughput technologies, venomics projects, which include genome, transcriptome, and proteome analyses of various venomous species, have been conducted to characterize divergent venom phenotypes and the evolution of venom-related genes. Additionally, venomics can also inform about mechanisms of toxin production, storage, and delivery. Venomics can guide antivenom and therapeutic strategies against envenomations and identify new toxin-derived drugs/tools. One potentially promising drug development direction is the use of endogenous inhibitors present in snake venom glands and serum that could be useful for snakebite therapeutics. These inhibitors suppress the activity of venom proteases, enzymatic proteins responsible for the irreversible damage from snakebite. This book chapter will focus on insights from venomous snake adaptations, such as the evolution of venom proteases to generate diverse activities and snake natural resistance to inhibit activity, and how this information can inform and have applications in the treatment of venomous snakebite.

Keywords: venomous snake, snake venom metalloprotease, hemorrhagic, nonhemorrhagic, toxin resistance, natural inhibitor, endogenous inhibitor

1. Introduction

There are over 3700 extant snake species, but only approximately 200 in 600 venomous snake species, belonging to families Viperidae, Elapidae, Atractaspididae, and some of Colubridae, are considered medically important on public health aspects of snakebite [https://www.who.int/snakebites/disease/en/; http://www.reptile-database.org/, Accessed: 2019-11-22]. The World Health Organization (WHO) has recognized snakebite envenomation as a neglected tropical disease and has characterized a subset of venomous snake species as being of higher medical importance in the four geographical areas of the world snakebite is most frequent. The definition of highest medical importance to human public health (category 1) is "highly venomous snakes which are common or widespread and cause numerous snakebites, resulting in high levels of morbidity, disability, or

mortality" [1]. These species are predominately from Elapidae and Viperidae families, but the majority of these species are from the family Viperidae (vipers and pit vipers). Viperidae species consist of approximately 50–100% of listed species in each geographical area and make up just over 60% of the entire list (**Table 1**).

Venom variation results in pharmacological and clinical symptomology differences across venomous snake species, primarily varying in the extent of snakebite tissue damage and toxicity. In recent years, venom has been investigated using comprehensive venomics approaches, combining proteomics, transcriptomics, and genomics, in an attempt to better understand venom components responsible for variation. Next-generation sequencing (NGS) has greatly accelerated the pace of venomics, making high-throughput outputs possible; several venom gland transcriptomes can be sequenced together using multiplexed barcoded libraries, with little difference in cost, and third-generation longer read technologies, such as Oxford Nanopore (minION), are now available to correct transcriptome assembly errors [2]. Venom characterization that integrates both transcriptomics with proteomics has optimized proteomics by providing a species-specific database (venom gland transcriptome) for toxin identification, an ideal method to better distinguish unique toxins present in each species [3]. Current venom gland transcriptomes, completed using NGS technologies (Illumina and Roche) and *E. coli*, generated cDNA clone libraries, and assembled genomes of venomous snakes categorized as of highest medical importance are listed in **Table 2**. In our investigation, completed transcriptomes in snake species in category 1 are only about 20% despite the

Geographical regions	Species		Total
Africa and the Middle East	Atractaspididae	*Atractaspis andersonii*	1
	Elapidae	*Dendroaspis viridis, Dendroaspis angusticeps, Dendroaspis jamesoni, Dendroaspis polylepis; Naja anchietaea, Naja annulifera, Naja asheia, Naja arabica, Naja haje, Naja katiensis, Naja melanoleuca, Naja mossambica, Naja nigricollis, Naja nigricincta, Naja nivea, Naja oxiana, Naja senegalensis*	17
	Viperidae	*Bitis arietans, Bitis gabonicaa, Bitis nasicornis, Bitis rhinocerosa; Cerastes cerastes, Cerastes gasperettii; Daboia mauritanicaa, Daboia palaestinaea; Echis borkini, Echis carinatus, Echis coloratus, Echis jogeri, Echis leucogaster, Echis ocellatus, Echis omanensisa, Echis leucogaster, Echis pyramidum; Macrovipera lebetina, Montivipera xanthina1; Pseudocerastes persicus,*	20
Asia and Australasia	Elapidae	*Acanthophis laevisa; Bungarus caeruleus, Bungarus candidus, Bungarus niger, Bungarus magnimaculatus, Bungarus multicinctus, Bungarus sindanus, Bungarus walli; Naja atra, Naja kaouthia, Naja naja, Naja mandalayensis, Naja philippinensis, Naja samarensis, Naja siamensis, Naja sumatrana, Naja sputatrix, Naja oxiana; Notechis scutatus; Oxyuranus scutellatus; Pseudonaja affinis, Pseudechis australisb, Pseudonaja mengdeni, Pseudonaja nuchalis, Pseudonaja textilis.*	25
	Viperidae	*Cryptelytrops albolabrisa, Cryptelytrops erythrurusa, Cryptelytrops insularisa, Calloselasma rhodostoma; Deinagkistrodon acutus, Daboia russeliia, Daboia siamensisa; Deinagkistrodon acutu; Echis carinatus; Gloydius blomhoffii, Gloydius brevicaudus, Gloydius halys; Hypnale hypnale; Macrovipera lebetina, Protobothrops flavoviridis, Protobothrops mucrosquamatus; Viridovipera stejnegeria,*	17
Europe	Viperidae	*Vipera ammodytes, Vipera berus, Vipera aspis,*	3
the Americas	Viperidae	*Agkistrodon bilineatus, Agkistrodon contortrix, Agkistrodon piscivorus, Agkistrodon tayloria; Bothrops asper, Bothrops atrox, Bothrops cf. atrox (Trinidad), Bothrops bilineatus, Bothrops alternatus, Bothrops brazili, Bothrops caribbaeus (St Lucia), Bothrops lanceolatus (Martinique), Bothrops diporusa, Bothrops jararaca, Bothrops jararacussu, Bothrops leucurus, Bothrops mattogrossensisa, Bothrops moojeni, Bothrops pictus, Bothrops venezuelensis; Crotalus adamanteus, Crotalus atrox, Crotalus durissus, Crotalus durissus (Aruba), Crotalus horridus, Crotalus oreganusa, Crotalus simus, Crotalus scutulatus, Crotalus totonacusa,, Crotalus viridis, Lachesis muta*	31

Table 1.
Venomous snakes of highest medical importance (category 1); the table was modified classified sub-area in each of four broad geographical regions in tables 3–6 of Fiftyninth report/WHO Expert Committee.

	Venom gland transcriptomes		Venomous snake genomes	
	Species	**Reference**	**Species**	**Reference**
Elapidae	*Naja kaouthia,*	[75, 76]	*Notechis scutatus*	Unpublished
	Dendroaspis angusticeps,	[77]	*Pseudonaja textilis*	Unpublished
	Dendroaspis jamesoni,	[77]		
	Dendroaspis polylepis,	[77]		
	Dendroaspis viridis	[77]		
	Pseudonaja nuchalis	[78]		
	Pseudonaja textilis	[78]		
Viperidae	*Agkistrodon piscivorus*	[2, 79]	*Deinagkistrodon acutus*	[104]
	leucostoma	[80]	*Protobothrops mucrosquamatus*	[105]
	Bitis gabonica	[81]	*Protobothrops flavoviridis*	[106]
	Bothrops alternatus	[82, 83]	*Vipera berus*	[107]
	Bothrops asper	[84, 85]	*Crotalus viridis*	[108]
	Bothrops atrox	[75, 84, 86–88]	*Crotalus horridus*	[109]
	Bothrops jararaca,	[89]		
	Bothrops jararacussu	[90–92]		
	Bothrops moojeni	[90, 91, 93, 94]		
	Crotalus adamanteus	[95, 96]		
	Crotalus durissus terrificus	[83, 95]		
	Crotalus horridus	[97]		
	Crotalus oreganus helleri	[97]		
	Crotalus scutulatus,	[82, 98, 99]		
	Crotalus simus	[100]		
	Echis carinatus shochureki	[100, 101]		
	Echis coloratus	[31, 102]		
	Echis ocellatus	[100]		
	Echis pyramidum leakeyi	[103]		
	Lachesis muta	[82]		
	Protobothrops flavoviridis			

Table 2.
Venom gland transcriptomes and currently completed snake genomes of species in category 1 [75–109].

advance in increasing sequencing data. Additionally, on the completed snake genome, sequences are also less than 10% limited number.

Transcriptome and proteome analyses of Viperidae species have consistently found that snake venom metalloproteinases (SVMPs), phospholipases A_2 (PLA_2s), serine proteinases, and L-amino acid oxidases are the most abundant toxins in these venoms [4]. These toxins are all enzymatic, providing immobilization and digestive roles in prey capture. Snakebites from these species result in local tissue damage, hemorrhage, and impaired coagulation symptoms in humans, which can lead to disability and mortality [5].

To date, the only effective snakebite treatment is intravenous administration of antibodies (often called antivenom), which come from animals immunized with toxins. However, using heterologous antibodies generated from numerous venom components has inherent weaknesses, such as an increased likelihood of an allergic reaction or life-threatening anaphylactic shock. Further, antivenom does not abolish local tissue damage, as it is intravenous and is usually not administered quickly enough. Thus, there is the issue of incomplete neutralization because of geographic venom variation, and high manufacturing costs and regulations have resulted in a struggle to properly match antivenom to venomous snakes of each locality and maintain antivenom stock [5]. To alleviate these issues, specific toxin inhibitors are actively being characterized and evaluated that neutralize snake venom toxicity and would work as an alternative antivenom snakebite therapy [6–8]. The challenge still remains to investigate the safety and efficiency of these toxin inhibitors to treat snakebite envenoming in humans.

Venomous snakes have endogenous inhibitors circulating in their plasma that provide resistance to their own venoms. These inhibitors can suppress the activity of enzymatic venom components, such as SVMPs, with high specificity. SVMPs are key venom components in viper venoms that contribute to hemorrhage and tissue damage; therefore, targeting these enzymes would greatly reduce human morbidity and mortality from snakebite. In this review, an overview of SVMPs is provided,

with a focus on structure-function relationships within the various classes of SVMPS across snake families, and is followed by insights into how snake endogenous inhibitors function to abolish SVMP activity. The goal of our group is to design peptide inhibitors that bind to hemorrhagic SVMPs with high affinity and effectively neutralize these toxins. This chapter deeply understands the target SVMP behaviors on the snakebite issue and is a summary of our current work with historical studies in endogenous inhibitor of venomous snake.

2. Metalloproteinases

Metalloproteinases (MPs) are one of the most functionally diverse proteases in more than 50 families characterized in the MEROP database [9]. MPs play a significant role in organism homeostasis, and are involved in, cell invasion, cell fertilization, self-defense, and reproduction. Metalloproteinases are classified into two subgroups known by their enzyme commission numbers (ECNs), exopeptidases (ECN 3.4.17), and endopeptidases (ECN 3.4.24). The second group are enzymes from the metzincin family and include serralysins, astacins, adamalysins (a disintegrin and metalloproteinase domain; ADAMs), and matrix metalloproteinases (MMPs). There have been many studies documenting endogenous metalloproteinase dysregulation in cancer cells [10], especially mammalian ADAMs. ADAM proteins and SVMPs belonged to the same M12 family [11], and similar domain features are present for both. For SVMPs, three-dimensional structures and well-characterized effects on animal models in vivo have been published. Thus, the functional and structural insights provided by SVMPs have been useful for human ADAM inferences.

2.1 Structural classification of snake venom metalloproteinases (SVMPs)

All SVMP genes exhibit a conserved signal peptide region and a pro-(pre) domain. The number of domains following these conserved N-terminal regions varies, and the arrangements of the domains have resulted in the categorization of SVMPs into three main classes. SVMPs of the P-III class consist of the metalloproteinase domain (MD), disintegrin-like domain (DID), and cysteine-rich domain (CRD). P-IIs have a MD and DID, and P-I has only a MD. Further, each class has subclasses classified for different representation forms (P-Ia, P-IIa-e, P-IIIa-d). These subclasses include dimeric or truncate isoforms that have only been observed within the venom and are generated mainly by post-translational modifications.

There have been many observations of gene neofunctionalization generating large families of venom proteins with multiple functionalities, and the SVMP gene family is an example of this. SVMPs originated from the gene duplication of an ancestral ADAM gene. The ADAM 28 precursor gene is the closed SVMP homolog present in nonvenomous snakes and is also present in mammalian species [12, 13]. Sequence comparisons between the lizard (*Anolis carolinensis*) ADAM 28 gene and viper (*Echis ocellatus*) SVMP gene suggested that SVMPs originated from a nonsense mutation following ADAM gene duplication. This nucleotide substitution resulted in a chain-termination codon (STOP codon) at the end of exon 12, following the CRD. The modified gene precursor produced proteins that were devoid of the C-terminal membrane anchor and cytoplasmic region present in ADAMs [14]. As more snake genome sequences have become available, it has been hypothesized that the ancestral SVMP coded for the P-III class of SVMPs. Gene duplications of P-III SVMPs resulted in P-II and P-I SVMP genes, each generated by domain loss from splicing site mutations. These last two classes, P-II and P-I, are only found in Viperidae. The reason

why a large number of different SVMPs are expressed in viper venoms is still unclear, even though some of these SVMPs are not primary lethal toxins.

SVMPs constitute more than 30% of the total venom proteins present in many Viperidae species. While these proteins are less abundant in the venoms of Elapidae, Atractaspididae, and Colubridae, they appear to be ubiquitously occurring [15, 16]. These observations suggest that P-III SVMP in Elapidae, Atractaspididae, and Colubridae venoms may have a conserve ancestral function and serve a common biological role in snake envenomation. Therefore, the functional roles of the diversified SVMPs are important for the clinical symptomology associated with Viperidae envenoming in humans. Domain structure, at least the topological shape, and sequence, especially the catalytic motif on the MD, are very similar among SVMPs, but SVMP activities vary, including their target substrates. Elucidating the structure-function relationships within this protein superfamily has applications for both protein evolution and snakebite treatment.

2.2 SVMP structure-function relationships and mechanism of action

Viperid snakebites are characterized by severe hemorrhagic, microvessel damage and inflammation, both local and systemic [17]. There is strong evidence from in vivo and in vitro studies of isolated SVMPs that these proteins are responsible for snakebite hemorrhage. Mitigating hemorrhage is critical in snakebite treatment; therefore studying the SVMP molecular mechanism of inducing hemorrhage is of critical importance. Hemorrhage results from SVMP proteolysis, targeted cleavage of extracellular matrix components, transmembrane receptors, and fibrinogen, mostly around microvessels. Interestingly, despite sharing similar catalytic activity, not all SVMPs induce hemorrhage in vivo. SVMP effects also include blood coagulation irregularities, platelet aggregation, cell infiltration, apoptosis-induced activity, and alternations in vascular permeability, even if these SVMPs do not show hemorrhagic activity [17]. These additional functionalities and targets likely result from C-terminal P-III SVMP binding, not only the catalytic activity of the N-terminally located MP domain [18]. To design inhibitors to neutralized hemorrhagic effects of SVMPs, we must first understand SVMP targets, as well as the tissue distribution and localization of SVMPs upon envenomation.

Both of P-III and P-I SVMPs have hemorrhagic activities, but P-III SVMPs tend to show greater hemorrhagic activity than P-I SVMPs [19, 20]. It has been proposed that the occurrence of hemorrhage results from the degradation of the vascular basement membrane of capillaries. Immunofluorescence confocal microscopy, immunochemical and proteomic analyses of tissue, and exudate in vivo have revealed a distinct pattern of P-I and P-III SVMP distribution in tissue. Observations from SVMPs labeled with Alexa Fluor 647 have found that P-III SVMP co-localized with capillary collagen IV, especially in those of microvesicles. P-I SVMPs applied to whole tissues appear to function primarily in the degradation of basement membrane components [21]. Hemorrhagic P-I and P-III SVMPs show a preference for type IV collagen in targeted degradation. J.M. Gutiérrez et al. provided a two-step hypothesis for SVMP mechanism of hemorrhage [21]. First, SVMPs hydrolyze type IV collagen and perlecan at the basement membrane components of capillaries and surrounding endothelial cells, resulting in a weakening of the mechanical stability of the basement membrane and microvessel wall. The second step occurs when the biophysical hemodynamic forces operating in microcirculation induce a distention in the wall, causing capillaries to disrupt, followed by consequent extravasation.

SVMP hemorrhage disrupts capillary networks, facilitating toxin dispersion. In 2016, reviews by Sanhajariya and colleagues investigated snake venom pharmacokinetics using an ELISA time course, varying venom concentrations mixed with the

plasma of laboratory animals (rat, rabbit, and sheep) and humans [22]. In laboratory animals, two phases were observed in the metabolism of snake venom by intravenous injection of the venoms or toxins: the first phase consisting of rapid distribution with half-lives of 5–48 min and a second a slow elimination phase with half-lives of 0.8–28 h. Half-lives of the second phase did not show a significant difference among the species (*Bothrops alternatus*, *Vipera aspis*, and *Naja* sp.) explored under intravenous injection, but *Naja* sp. did show a twofold shorter phase time than *Vipera aspis* 32 hours after intermuscular injection. For humans, venom concentrations in plasma were examined from 24 pharmacokinetic studies in humans that used similar ELISA criteria. Venom concentrations of the patients bitten by vipers were typically higher than those of by elapids. Eventually, total 218 timed concentration data of 145 patients bitten by snakes of Viperidae and Elapid were used for the computational analysis within a nonlinear mixed-effects modeling framework with NONMEM. The result provided an estimated venom elimination half-life of 9.71 ± 1.29 h. Interestingly, these data also show that there is no big difference between Viperid and Elapid. It is very important to understand the certain pharmacokinetic of venom for post-treatment of inhibitor to neutralize toxicity of venoms.

3. Toxin resistance in venomous snakes

Given the toxic and proteolytic nature of venom, it is of the utmost importance that venomous snakes are protected against the activity of their own venom. This is an interesting area of research because venoms, especially viper venoms, have high concentrations of proteases that must be stored in an inactive state in the venom gland to prevent degradation of both the snake's own tissue and other proteins present in the venom. These proteases must then be readily activated when delivered into prey, requiring a finely tuned on/off switch. Here, we briefly summarize how these toxins are regulated in snake venom glands, with a focus on endogenous inhibitors, especially SVMP inhibitors, as SVMPs are one of the major venom compounds in Viperid snake venoms.

3.1 Mechanisms of toxin resistance

Three toxin resistance mechanisms have been proposed for venomous snakes: (1) target receptor mutations, (2) venom gland physiological conditions, and (3) inhibitors present in the venom gland or blood circulation. For the first mechanism, limited mutations on target receptors in snakes prevent the binding of their own toxins [23–25]. An example of this has been described by Takacs et al., where resistance against conspecific α-neurotoxins, major lethal components of Elapidae venoms, has been shown to be mediated by a unique N-glycosylation of the nicotinic acetylcholine receptor ligand binding domain of Elapidae snakes [23]. This observation has only been made for a few Elapidae species, but there is currently a limited amount of research in this area. The second mechanism involves the physiological conditions within the venom gland and those required for enzymatic toxins to be active. A high concentration of citrate is present in venom, and this results in a low pH environment. It is estimated that 25% of dried crude venom (30–150 mM) from *Crotalus* sp., *Agkistrodon* sp., *Bothrops* sp. *Dendroaspis* sp., *Sistrurus miliarius barbouri*, *Bitis gabonica gabonica*, *Vipera russellii russellii*, and *Lachesis muta* is citrate. Citrate concentrations of 18 or 27 mM exogenously added to whole venom have been shown to inhibit protease activity in vitro [26]. Secreted whole venom of *Crotalus* sp. has been observed to be acidic (pH 5.25–5.75), suggestive of an acidic

storage condition for venom enzymes, which then when delivered into prey or human tissue at a pH of 7.2–7.4, these enzymes become activated [27]. In addition to the acidic storage conditions of the venom gland, four tripeptide inhibitors of venom metalloproteinases, pEKW, pENW, pEQW, and pERW, have been documented in the venoms of *Protobothrops mucrosquamatus* [28, 29], *Bothrops asper* [30], *Echis ocellatus*, *Cerastes cerastes cerastes* [31], and some rattlesnakes [32] and are likely present in the venom gland lumen. These inhibitors have been found in relatively large amounts (approximate concentrations in *P. mucrosquamatus* venom were reported greater than 5.0 mM), but their inhibitory activity is not strong, with IC_{50} values between 0.15 and 0.95 mM for different SVMPs in vitro [28, 32]. The X-ray crystal structure of a SVMP complexed with a pExW inhibitor revealed the indole ring of Trp in the pExW inhibitor stacked against the imidazole in the first histidine residue of the SVMP Zn^{2+} binding site [29]. In 2007, Philippe and colleagues discovered the 2–3 kDa polyHis-polyGly peptides in venom of *Atheris squamigera* by mass spectrometry-based strategies, and it was identified as a new class of peptides with clusters of histidine and glycine residues [33]. Similar peptides were found coded in C-type natriuretic peptide (CNP) and bradykinin-potentiating peptide (BPP)–CNP transcript precursors and were isolated from *E. ocellatus* and *Atheris* sp. venoms. Interestingly, these pHpGs have shown stronger inhibitory effect against SVMP in vivo than tripeptides [31].

In addition to inhibitors present in the venom and venom gland, serum proteins in some venomous snakes have also been found to bind toxins with high affinity and neutralize toxin pathophysiological effects [34]. These serum proteins and those found in the venom/venom gland are referred to as "endogenous inhibitors," but kinds of their inhibitor are different. Serum inhibitors circulate in the blood to effectively bind and neutralize host toxins, but they are different than immune antibodies. The roles of these endogenous inhibitors and their classifications will be discussed in the next sections.

3.2 Endogenous inhibitor protein families

There are three main endogenous inhibitor classes; these are phospholipase A2 inhibitors (PLI) [35, 36], anti-hemorrhagic factors [37], and small serum proteins [38–41], which have predominately been found in the blood of vipers. All endogenous inhibitors are stable at high temperatures and in acidic conditions and have been purified by reverse-phase high-performance liquid chromatography (RP-HPLC).

3.2.1 Phospholipase A_2 inhibitors (PLIs)

Snake venom PLA_2s are ubiquitous to Viperidae and Elapidae venoms and one of the primary components in viper venoms. These toxins are versatile and can induced a variety of effects, including neurotoxicity, myotoxicity, cardiotoxicity, hemolysis, and anticoagulation [42]. Inhibitors of PLA_2s, PLIs, have been identified and characterized from the blood sera of both venomous and nonvenomous snakes. PLI genes have been found uniquely expressed in snake liver tissues [43]. This suggests that the secretion of PLIs into blood circulation could be to provide protection against accidental self-envenomation in venomous snakes.

PLIs are divided into three groups (PLIα, PLIβ, and PLIγ) based on structural characteristics. PLIαs are glycoproteins with molecular masses ranging from 75 to 120 kDa and more than three non-covalently associated subunits. Their structural features demonstrate sequence homology to the carbohydrate-recognition domain of Ca^{2+}-dependent lectins (C-type lectin-like domain), but they lack the

carbohydrate-binding ability. A highly conserved region (residues 49–143) shares 80–90% sequence identity between PLIαs and appears to be responsible for PLA_2 binding [44]. PLIβs are 150–160 kDa glycoproteins composed of three non-covalently bonded subunits and have nine tandem leucine-rich repeats. A homology analysis by BLAST shows similarity to human leucine-rich α_2-glycoproteins, which structurally forms horseshoe-shaped molecules, as observed in Toll-like receptors [45]. PLIβs have been purified and characterized from only one venomous snake, *Gloydius brevicaudus* [46], whereas there are a couple examples of these proteins that have been found in nonvenomous colubrids (*Elaphe quadrivirgata* and *Elaphe climacophora*) [47, 48]. The presence of a PLIβ gene in *Lachesis muta* was discovered by Lima et al., but the exact function of this inhibitor has not been identified [49]. PLIβs from *G. brevicaudus* specifically inhibited only group-II basic PLA_2s, forming stable toxin-inhibitor complexes at a 1:1 molar ratio [45]. PLIγs are acidic glycoproteins consisting of oligomers with 20–30 kDa subunits, the primary structure of which consists of conserved patterns of cysteine residues to form two units of repeats known as three-finger motifs. Structurally related proteins belong to the urokinase activating plasminogen receptor (u-PAR)/ly-6 superfamily [50]. This gamma class inhibitor comprises the greatest number of endogenous PLIs and has been isolated from the sera of many snake species, including those from Elapidae, Viperidae, Hydrophidae, Boidae, and Colubridae families [35].

Numerous studies have described highly effective inhibition of PLA_2 toxicity in vitro and in vivo by PLIs purified as a highly soluble protein from snake serum. However, binding sites, as well as inhibitory mechanism, have not been fully elucidated for these proteins because each PLIs' group targets different PLA_2s. Currently, there are two review articles that have attempted to determine the PLA_2 targets of PLI classes based on structural predictions [35, 36]. In 2015, Zhen and colleagues successfully established recombinant expression of PLIγ in *Escherichia coli*. After expression optimization, the amount of recombinant PLIγ achieved was 23 mg/l of culture, and the recombinant PLIγ demonstrated inhibitory activity against *Deinagkistrodon acutus* venom purified PLA_2s, and *D. acutus*, *Naja atra*, and *Agkistrodon halys* crude venoms in vitro and in vivo. This type of experimental work will make it possible in the future to determine the inhibitory mechanism by inhibitor mutant analysis and/or obtaining the three-dimensional structure of the inhibitor and PLA_2 complexes.

3.2.2 Anti-hemorrhagic factors

As previously detailed, hemorrhage, one of the main symptoms of viper envenomation, is induced by snake venom metalloproteinases (SVMPs). The first anti-hemorrhagic factor, habu serum factor (HSF), was identified from the serum of *Protobothrops flavoviridis* by Omori-Satoh et al. in 1972 [51]; in 1992, the complete amino acid sequence was determined for this protein [52]. To date, anti-hemorrhagic factors habu serum factor (HSF), BJ46a [53], and mamushi serum factor (MSF) [54] have been purified and characterized from the venomous snakes *P. flavoviridis* (habu), *Bothrops jararaca,* and *Gloydius blomhoffii*, respectively (**Table 3**). These anti-hemorrhagic factors belong to the fetuin family, part of the cystatin superfamily, consisting of two cystatin-like domains and a His-rich domain. These anti-hemorrhagic factors show high sequence identity and are all single-chain, acidic glycoproteins. They also all demonstrate strong anti-hemorrhagic activity in vivo against the crude venom of the snake species they are isolated from. HSF and MSF showed relatively broad range inhibitory activity against both nonhemorrhagic and hemorrhagic SVMPs, as well as both P-I and P-III

Inhibitor name	Species	pI		Molecular Weight	Target MP	Family	reference	Database accession numbers of sequence
venomous snake 1 (neutralized own toxin)		calculated*	observed	by SDS-PAGE** (kDa)				
Habu serum factor; HSF	*Protobothrops flavoviridis*	5.9	4	55	(Crude venom) Protobothrops flavoviridis (SVMPs from Protobothrops flavoviridis) HR1A (P-III), HR1B (P-III), HR2a (P-III), HR2b (P-III), H2 proteinase (P-I) (SVMP from *Gloydius blomhoffi brevicaudus*) Brevilysins H3, Brevilysins H4, Brevilysins H6, Brevilysins L4.	Cystatin super family (Fetuin family)	[52, 74]	AB058635
BJ46a	*Bothrops Jararaca*	5.7	5.2	55.4	(Crude venom) *B. jararaca* (SVMP) jararhagin (P-III from B. jararaca) atrolysin C (P-I from *Crotalus atrox*)		[53]	AF294836.1
Mamushi serum factor; jMSF (Japanese MSF)	*Gloydius blomhoffii blomhoffii*	5.6	N.D	48	(Crude venom) *Gloydius blomhoffi brevicaudus* (SVMP from *Gloydius blomhoffi brevicaudus*) brevilysin H2, brevilysin H3, brevilysin H4, brevilysin H6 (P-III SVMP from *Protobothrops flavoviridis*) HR2a (weak)		[54]	AB200169
cMSF (Chainese MSF)	*Gloydius blomhoffi brevicaudus*	5.6	N.D	48	(Crude venom) *Gloydius blomhoffi brevicaudus* (SVMP from *Gloydius blomhoffi brevicaudus*) brevilysin H3, brevilysin H4, brevilysin H6 (P-III SVMP from *Protobothrops flavoviridis*) HR1A, HR1B		[54]	Q5KQS4.1
BaSAH	*Bothrops asper*	N.D	5.2	66	(Crude venom) *Bothrops asper* (10 microgram) (SVMP) BaH1 (*Bothrops asper*, 0.18 microgram)	N.D	[63]	N.D
XX	*Agkistrodon contortrix mokasen*	N.D	4.6	64–68	(Crude venom) *Agkistrodon c. mokassen* *Agkistrodon piscivorus conanti*	N.D	[64]	N.D
XX	*Crotalus atrox*	N.D	N.D	65–80	(Crude venom) *Crotales atrox* (SVMP) hemorrhagic toxin-e (from *Crotales atrox*)	N.D	[65]	N.D
XX	*Vipera palaestinae*	N.D	4.7	80	(Crude venom) *Vipera palaestinae* *Echis colorata* *Cerasies cerastes.*	N.D	[66]	N.D
TMI	*Protobothrops mucrosquamatus*	N.D	N.D	47	(Crude venom) *P. mucrosquamatus* *P. flavoviridis* *P. stejnegeri* (SVMP from *P. mucrosquamatus*) TM-1, TM-2, TM-3	N.D	[67]	N.D
venomous snake 2 (neutralized toxin from other species)								
BaltMPI	*Bothrops alternatus*	N.D	5.27	60.5 and 42.4	(SVMP) Batroxase (P-I MP from *B. atrox*) BjussuMP-I (P-III MP from *B.jararacussu*)	N.D	[58]	N.D
Non-venomous								
NtAH (serum)	*Natrix tessellata*	N.D	N.D	880, 70, 100, 150	(Crude venom) *Bothrops asper* (SVMP from *Bothrops asper*) BaH1	N.D	[69]	N.D
XX	*Dinodon semicarinatu*	N.D	N.D	59 and 52	(Crude venom) *Trimeremrus flavoviridis*	N.D	[70]	N.D
XXX	*Drymarchon couperi*	N.D	N.D	Whole serum	(Crude venom) *Agkistrodon contortrix*	N.D	[71]	N.D

N.D; not determined, XX; not named or not determined,
*Theoretical Isoelectric point (pI) were calculated on the ProtParam of ExPASy server (https://web.expasy.org/protparam/),
**sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition

Table 3.
Antihemorrhagic proteins from snake sera (or plasma).

SVMP classes, but preferentially inhibited P-III SVMPs. These observations were also exhibited in the differences in the degree and specificity of inhibition against individual SVMP [54, 55]. HSF strongly inhibited the proteolytic and hemorrhagic activities in vivo and in vitro of HR1 and HR2 (P-III SVMPs), the main toxins in *P. flavoviridis* venom. Similarly, BJ46a is a potent inhibitor of atrolysin C (P-I SVMP) and jararhagin (P-III SVMP) proteolytic activities and the overall hemorrhagic activity of *B. jararaca* venom [53]. HSF and MSF did appear to be specific to SVMPs and were found to not inhibit cysteine proteases, such as papain and cathepsin B, serine proteases trypsin and chymotrypsin, or thermolysin, a bacterial MP. Binding studies of these factors suggest that they are forming noncovalent complexes with the MD of SVMPs; this has been hypothesized because they interacted with P-I SVMPs, but did not bind to the C-terminal region of SVMP jararhagin-C. Interestingly, the molar ratios for complex formation vary between inhibitors; for HSF and brevilysin H6 (P-III SVMP), a 1:1 ratio is required, but complex formation between BJ46a and jararhagin (or atrolysin C) was found in a 1:2 ratio. The N-terminal region of HSF (residues 5–89) has been found to be responsible for anti-hemorrhagic activity [56], and sequence comparisons between HSF and a HSF-like protein (HLP), which does not show SVMP inhibition, identified a substitution difference in the first cystatin-like domain [57]. These results suggested that N-terminal region of HSF is potentially responsible for SVMP binding.

Recently, BaltMPI [58] was found as a hemorrhagic inhibitor in *Bothrops alternatus* serum. BaltMPI should also be of the fetuin family as the N-terminal region consists of 60 amino acid residues (determined by Edman degradation) that showed high homology (97%) with BJ46a. BaltMPI has potent anti-hemorrhagic

activity and inhibited the proteolytic activity of Batroxase and BjussuMP-I but has yet to be investigated against the crude venom *Bothrops alternatus*, the snake species it originates from.

3.2.3 Small serum proteins (SSPs)

Since 2007, a new class of endogenous inhibitors, named small serum proteins (SSP-1 to SSP-5), has been characterized from Japanese vipers, *P. flavoviridis* (habu) and *G. b. blomhoffii*, that have been found to effectively neutralize various snake toxins [38, 40]. SSP-1, SSP-2, and SSP-5 consist of two domains of approximately 90 amino acid residues, while SSP-3 and SSP-4 have only a 60 residue N-terminal domain. All serum proteins have conserved cysteine residues and belong to the prostatic secretory protein of 94 amino acid (PSP94) family, despite only limited sequence identity to any mammalian PSP94 [59]. These inhibitors target different toxins; SSP-2 and SSP-5 have high affinity for CRISP family toxins [38, 60], while SSP-1, SSP-4, and SSP-3 inhibit distinct SVMPs. These results suggested SSP-1 to SSP-5 contributes to a snake's natural resistance against toxins. SSP-1 and SSP-4 complex with HSF, inhibiting apoptosis induced by HV1, a P-III SVMP from *P. flavoviridis* venom [39]. Each SSP alone could bind to target SVMPs, but SSP-1 and SSP-4 inhibit HV1 through a ternary complex involving HSF, SSPs, and HV1 (SSP-4, data has not published). In contrast, SSP-3 alone inhibits flavorase, a P-III SVMP from *P. flavoviridis* [41], and is not dependent on other proteins. These results suggested that the inhibition mechanisms of the SSP group are different, but they are noncompetitive. Recently, the crystal structure of complex SSP-2 and triflin, an ion channel-blocking CRISP toxin, was determined [61]. The interface between these two proteins consists of the two short β-strands of SSP-2 binding to the concave region centrally located in the N-terminal domain of triflin. Interestingly, the key β-strand on the N-terminal of SSPs is a hypervariable region, which might correspond to the ability to bind and target different venom toxins. This is in agreement with the molecular evolution of SSP genes, where the number of non-synonymous nucleotide substitutions is significantly greater than those of synonymous substitutions in N-terminal regions. Additionally, these mutational hotspots are found on the molecular surface, specifically located on the toxin interaction interface, while the protein scaffold structure is highly conserved [62].

4. Undetermined proteinous inhibitors

Isolated proteins from the serum of *Bothrops asper* [63], *Agkistrodon contortrix* [64], *Crotalus atrox* [65], and *Vipera palaestinae* [66] have shown effective neutralization of hemorrhagic activity in vivo from crude venom corresponding to each species or species-specific toxins (**Table 3**). The SVMP inhibitor isolated from *Protobothrops mucrosquamatus* serum, named TMI, demonstrated a 1000-fold stronger in vitro inhibitory activity than endogenous tripeptides (IC_{50} = 0.2–1.0 μM) and additionally inhibited venom proteolytic activity from other species [67]. Despite in vivo and in vitro experimental evidence, the sequence of these inhibitors has not yet been determined. A novel group of endogenous inhibitors may be responsible for this activity, as the molecule sizes of these proteins are different than what has been previously reported for other characterized inhibitors.

Venom resistant has been discovered in some species in mammals, birds, and reptiles, which are either predators or prey of venomous snakes. Natural inhibitors isolated from resistant animals are detailed in reviews by Domont et al. [34, 68] and

Bastos et al. [37], which summarized toxin resistance corresponding to several snake species from the plasma, serum, and muscle of mammals. There are a few reports of natural inhibitors isolated from nonvenomous snakes, such as NtAH from *Natrix tessellate* [69] and a 59 kDa protein from *Dinodon semicarinatus* [70]. Recently, the whole serum of *Drymarchon couperi* [71] has also shown anti-hemorrhagic activity to venom, but the protein responsible for this activity has yet to be determined. These natural inhibitors in nonvenomous snakes are potentially protective for a diet that consists of venomous snakes, while resistance to SVMPs may be relatively widespread among snake species.

Endogenous inhibitor genes are expressed in the liver of venomous snakes, and these genes appear to be evolving by gene duplication and rapid diversification. This facilitates the neutralization of various toxins within venoms, which also are evolving under similar mechanism [62, 72]. Thus, a detailed characterization of inhibitors against species-specific toxins may help to decipher the evolution of endogenous natural resistance in venomous snakes. Unfortunately, the structural features that govern the inhibitor interaction are still unknown. Recently, there are reports that making a computational analysis predicted three-dimensional structure available [46], and one paper demonstrated that recombinantly expressed BJ46 was able to produce using the expression system of the methylotrophic yeast *Pichia pastoris* [73]. The ability to recombinantly produce these inhibitors will provide material for future work deciphering complex formations between inhibitors and toxins from mutation and structural analyses, providing insight into the molecular mechanisms behind toxin activity inhibition. With current technologies, it is not difficult to comprehensively evaluate venomous snake sera components or the sera from resistant animals. However, we have to be informed from structure-function studies to correlate amino acid sequence to the physiological activity of an inhibitor. It is possible that different inhibitors are operating under different mechanisms, even if they show high similarity, as has been the case for various venomous snake toxins. Thus, understanding in depth how toxin inhibitors function may aid in identifying novel inhibitors and new strategies for snakebite treatment.

5. Conclusions and remaining challenges

There are still many current challenges in the field of toxin inhibitors. Systemic effects of envenomation in humans by snakebite are often mitigated by antivenom therapy, the medically accepted treatment to date. Inhibitors have yet to gain acceptance in clinical use. However, local tissue damage is not neutralized by antivenom and results in permanent morbidity and disability in patients [74]. Local tissue damage is incurred by enzymatic toxins and thus is one of the reasons that Viperidae species occupy just over 60% of venomous snakes listed as Category 1 of highest importance to human health (**Tables 1** and **2**). Endogenous inhibitors isolated from almost all Viperidae show potent inhibition against their own venom and have also demonstrated to be selective toward highly lethal enzymes within these venoms. The serum inhibitor genes of venomous snakes might have evolved by gene duplication and rapid diversification to facilitate the neutralization of various venom toxins. These serum inhibitors are very stable (resistant to acidic, alkaline, and high temperature environments), selective in inhibitory activity against snake toxins, and are nontoxic, given they exist in blood serum and consist of amino acids (**Table 3**). However, the molecule mechanism of toxin neutralization involving endogenous inhibitors remains unclear due to a lack of three-dimensional structures detailing toxin and inhibitor complexes. By exploring molecular mechanisms

responsible for natural toxin resistance in snakes, we may begin to understand the specificity and selectivity of endogenous inhibitors and use these insights in the design of better therapeutic agents for the treatment of snakebite victims.

Conflict of interest

The authors declare no conflict of interest.

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