Snakebites are a hazard in the tropical world. Although antivenom therapy is effective, it is beset with inherent drawbacks. A better understanding of the major components of snake venoms and their neutralisation will help in improving snakebite treatment. Snake venom metalloproteinases (SVMPs) are responsible for severe haemorrhage, the inhibition of coagulation and platelet aggregation, observed in the victims of snakebite envenoming. Inhibitors from various sources including medicinal plants, animal venoms, and sera are sought to block the pharmacological functions of SVMPs. In this review, we describe the interaction of natural inhibitors with SVMPs. To understand their inhibitory mechanisms, we focussed on the complex structures of these inhibitors and SVMPs. There are three distinct classes of inhibitors; namely, chelators, competitive inhibitors, and non-competitive inhibitors. A small number of inhibitors show their anti-haemorrhagic activity in in vivo animal models in treatment mode, but most studies evaluate either in vitro neutralisation of enzymatic activity or in vivo effects in pre-incubation protocols. We propose the distinct strategies and limitations to design either broad-spectrum or highly selective SVMP inhibitors. The goal of designing broad-spectrum inhibitors against SVMPs capable of effective treatment of snakebites without toxicity has been elusive, probably because of the narrow molecular footprint of inhibitors against a large number of SVMPs with distinct molecular surfaces. Our ability to design highly selective inhibitors is limited by the lack of information of interactions between selective inhibitors and SVMPs. Comparisons of structures of hemorrhagic and non-hemorrhagic SVMPs revealed different distributions of electric charge on the surface of SVMPs, which may be exploited to design specific inhibitors. The specific inhibitors may also be useful to identify target molecules of the SVMPs and help to understand their mechanism of action.

Introduction

Snakebite is a neglected tropical disease that leads to the deaths of 81,000 to 138,000 victims per year worldwide (https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming). To date, the only effective treatment against snakebites is intravenous administration of antivenom, the antibodies that are isolated from animals immunised with snake venom toxins (https://www.who.int/bloodproducts/snake_antivenoms/snakeantivenomguide/en/).\(^1\)\(^2\) However, such use of heterologous antibodies has numerous inherent problems, notwithstanding unaffordable high-cost.\(^3\) Therefore, many scientists are aiming to develop next-generation snakebite therapy with better efficacy and safety.\(^4\) To achieve this objective, it is important to consider the most common toxins in snake venoms and aim for their neutralisation. Snake venom metalloproteinases (SVMPs) are one of the main components of Viperidae snake venoms.\(^5\) SVMPs are responsible for severe haemorrhage and local tissue damage in the victim.\(^6\) As some of these symptoms are not adequately neutralised by antivenoms, it is appropriate to focus efforts to identify alternative solutions to resolve snakebite-induced mortalities and morbidities.\(^7\)

Here, we review recently published information on snake venom metalloproteinase inhibitors (SVMPIs) isolated from medicinal plants and animals resistant to snake venoms. As of now, there is limited clinical evidence for their use in effective snakebite treatment for humans. The review looks at structural aspects of the inhibitor–SVMP complexes and identifies hurdles in the development of SVMPI therapeutics.

Snake Venom Metalloproteinases

SVMPs cause haemorrhage by damaging the integrity of blood vessel walls due to proteolytic degradation of multiple components in the extracellular matrix and the endothelial basement membrane.\(^8\) Concurrently, inhibition of coagulation, platelet aggregation, and consumption of fibrinogen and other blood coagulation factors, enhances the effects of the physically damaged blood vessel walls, resulting in severe haemorrhage. Some snake venom components including SVMPs specifically target coagulation factors and platelet receptors.\(^9\) Therefore, an understanding of the molecular details and mechanisms of the hemorrhagic activity of SVMP is crucial for the development of
effective inhibitors and to provide effective treatment for hemorrhagic symptoms due to snakebite envenoming.

Structure and Classification of SVMPs
SVMPs are Zn$^{2+}$-dependent proteases that are divided into three structural classes (P-I, P-II, and P-III) based on domain organisation and mRNA transcription of toxin genes in the venom gland.\[13\] P-I SVMP has only a single metalloproteinase domain (MD). The P-II class has a MD and an additional disintegrin-like domain (DLD) which is homologous to the platelet aggregation inhibitors, disintegrins. The P-III class consists of three domains, MD, DLD, and a cysteine-rich domain (CRD) at the C-terminus. Previously, P-III proteins that were associated with C-type lectin-like domains were described as the fourth group of SVMP (P-IV). However, subsequent transcriptome analyses showed the lack of mRNA encoding P-IV SVMP and hence, this class of SVMPs are not synthesised as single-chain polypeptides but are post-translationally assembled. Thus, Fox and colleagues re-classified P-IV as a P-III subclass.\[13\]

The first crystal structure of P-I SVMP was determined by Gomis-Ruth et al. in 1993.\[14\] Since then more than 30 SVMP structures have been determined (Table 1). The first structure of a P-III SVMP, that of vascular apoptosis-inducing protein-1 (VAP-1), was determined by Takeda et al. in 2006\[15\] and this structure is often used as the example to understand the structural properties of SVMP (Fig. 1). Typical topology, domain architecture, and other structural features of SVMP are as follows: MD consists of six $\alpha$-helices and five stranded $\beta$-sheets (VAP-1; 209 amino acid residues) which host the catalytic site. Three important structural motifs are essential for catalytic activity: i) the conserved Zn$^{2+}$ coordinating sequence (HEXXHXXGXXH); ii) a Met-turn ( $\beta$-turn with methionine residue) located near the catalytic site; and iii) Ca$^{2+}$ binding sites.\[3,6\] The DLD has a few Ca$^{2+}$ binding sites and a disintegrin loop based on structural feature similarity (VAP-1; 137 amino acid residues). The CRD consists of three $\alpha$-helices and eight stranded $\beta$-sheets which include a hyper-variable region that shows variabilities among SVMPs (VAP-1; 122 amino acid residues).\[15\] So far there is no evidence of alternative folds or disulfide bonding patterns among the MD of SVMPs. Yet, despite the high degree of structural conservatism, SVMPs exhibit highly variable substrate specificity and hence, vastly different functions.\[13\]

Regulation of SVMP Activities in Snakes
Proteolytic activity is usually tightly regulated to ensure proper activation and inactivation through changes in pH, temperature, ions, co-factors, and physiological inhibitors. The proteolytic activity of SVMPs in the venom gland appear to be inhibited by two factors, as revealed by in vitro experiments: i) the presence of citrate (58–125 mM) that maintains an acidic pH\[16\] and ii) the tripeptide enzyme inhibitors found in the venom (described below).\[17\] Structural analysis of atragin, a P-III SVMP from Naja atra, indicates that the zinc-binding motif in the catalytic site changes based on pH. The first and third histidine residues in this motif assume distinct conformations at pH 5.0 compared with pH 7.0, although the position of Zn$^{2+}$ and the Met-turn did not change. This changes the coordination of Zn$^{2+}$ leading to a significant decrease of atragin activity in acidic conditions around pH 5.0 to 6.5.\[18\]

It is postulated that post-transcriptional and post-translational modifications generate diversity in venom compositions and contribute to the evolution and adaptation of venomous snakes.\[19\] The gene for SVMPs codes for a signal peptide of ~20 amino acid residues, followed by a prodomain of ~200 amino acid residues. This prodomain is also conserved among proteins in the ADAMs (ADAM = a disintegrin and metalloproteinase) family found in other organisms. The presence of a prodomain may play a key role in regulating activities of metalloproteinases in the venom gland. At the C-terminal end of the prodomain there is a conserved ‘cysteine switch’ (PKMCGVT) region that is commonly found in SVMPs as well as matrix metalloproteinases (MMPs). The separation of this region during activation leads to binding of Zn$^{2+}$ ions in the catalytic site.\[20\] Cyclisation of the N-terminal glutamyl residue to pyroglutamate frequently occurs in SVMPs, after post-translational cleavage of the prodomain. The prodomain

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**Dr Cho Yeow Koh** is a Research Assistant Professor in the Department of Medicine, University of Singapore. He has strong interest in drug discovery and development research related to cardiovascular and neglected tropical diseases. He explores venomous and haematophagous animals for novel molecules that may be developed into therapeutics, medical technologies or research tools. Dr Koh has written 23 peer-reviewed scientific papers and two book chapters, and has eight patents in his name.

**Professor R. Manjunatha Kini** of the Department of Biological Sciences, National University of Singapore, is a leading authority on structure-function relationships of proteins from venomous animals, especially snakes. He has many years of experience in protein chemistry, biophysics, protein and peptide design and engineering. He has published more than 255 original research papers, reviews and book chapters. He also has 50 patent applications and started two small biotechnology companies. He is the President of the International Society of Toxinology, Vice President of the International Proteolysis Society, and the Editor-in-Chief of Toxin Reviews.
### Table 1. Three-dimensional structures of snake venom metalloproteinases in the Protein Data Base (https://www.rcsb.org/)

<table>
<thead>
<tr>
<th>Class</th>
<th>SVMPs</th>
<th>Organism (species)</th>
<th>PDB ID.</th>
<th>Ref.</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-I</td>
<td>Acutolysin-A</td>
<td>Agkistrodon acutus</td>
<td>1BSW(pH 7.5), 1BU2(pH 5.0)</td>
<td></td>
<td>hemorrhagic MP</td>
</tr>
<tr>
<td></td>
<td>Acutolysin-C</td>
<td>Agkistrodon acutus</td>
<td>1QUA</td>
<td></td>
<td>hemorrhagic MP</td>
</tr>
<tr>
<td></td>
<td>BaP1</td>
<td>Bothrops asper</td>
<td>1ND1(free), 2W12(WR2), 2W13(WR2), 2W14(WR2) 2W15(WR2)</td>
<td></td>
<td>complex with inhibitor (WR2&lt;sub&gt;4&lt;/sub&gt;)</td>
</tr>
<tr>
<td></td>
<td>BmooMPalpha-I</td>
<td>Bothrops moojeni</td>
<td>3GBO</td>
<td></td>
<td>non-hemorrhagic MP</td>
</tr>
<tr>
<td></td>
<td>Leucurolysin-a</td>
<td>Bothrops leucurus</td>
<td>4QL1(QSW)</td>
<td></td>
<td>non-hemorrhagic MP, complex with an endogenous tripeptide (QSW)</td>
</tr>
<tr>
<td></td>
<td>Adamalysinin II</td>
<td>Crotalus adamanteus</td>
<td>1AG(free), 4AG(FLX), 3AIG(POL656), 2AIG(POL647)</td>
<td></td>
<td>hemorrhagic MP</td>
</tr>
<tr>
<td></td>
<td>TM-3</td>
<td>Protobothrops macrosquamatus</td>
<td>1KUF(free), 1KUIfEQW, 1KUkEQK, 1KUGpENW</td>
<td></td>
<td>complex with an endogenous tripeptide inhibitors</td>
</tr>
<tr>
<td></td>
<td>TM-1</td>
<td>Protobothrops flaviviridis</td>
<td>1WNI</td>
<td></td>
<td>complex with an endogenous tripeptide inhibitors</td>
</tr>
<tr>
<td>P-III</td>
<td>AaHIV</td>
<td>Agkistrodon acutus</td>
<td>3HDB</td>
<td></td>
<td>hemorrhagic MP</td>
</tr>
<tr>
<td></td>
<td>Bothropasin</td>
<td>Bothrops jararaca</td>
<td>3DSL</td>
<td></td>
<td>hemorrhagic MP, complex with inhibitor POL647</td>
</tr>
<tr>
<td></td>
<td>VAP-2B</td>
<td>Crotalus atrox</td>
<td>2DW2(free), 2DW0(GM6), 2DW1(GM6)</td>
<td></td>
<td>hemorrhagic MP, complex with inhibitor of GM6</td>
</tr>
<tr>
<td></td>
<td>VAP-1</td>
<td>Crotalus atrox</td>
<td>2ERO(free), 2ERQ(free), 2ERP(GM6)</td>
<td></td>
<td>hemorrhagic MP, complex with GM6</td>
</tr>
<tr>
<td></td>
<td>RVV-X</td>
<td>Daboia russelli</td>
<td>2E3X</td>
<td></td>
<td>complex with GM6</td>
</tr>
<tr>
<td></td>
<td>Atragin</td>
<td>Naja atra</td>
<td>3K7L</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kaouthiagin-like</td>
<td>Naja atra</td>
<td>3K7N</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>WR2 = (2R,3R)-N-((1S)-2,2-dimethyl-1-(methylcarbamoyl)propyl)-N'-hydroxy-2-(2-methylpropyl)propyl]-N-[1,3-thiazol-2-ylformamido)methyl] butanediamide.</sup>

<sup>BLX = N-(furan-2-yl)carbonyl-(S)-leucyl-(R)-[1-amino-2(1H-indol-3-yl)ethyl]phosphonic acid.</sup>

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**Fig. 1.** Catalytic site of SVMP. (a) The crystal structure of P-III SVMP, VAP1 (PDB ID: 2ERP) rendered using PyMOL molecular viewer. MD is coloured cyan, and the Zn<sup>2+</sup>-binding region and Met-turn (H<sup>335</sup>EMGHNLGMDH<sup>345</sup> and Met<sup>357</sup>) in MD is highlighted in magenta. DLD is orange, CRD is sky-blue, and the Hyper Variable Region (HVR, 562–583 residues) in CRD is highlighted in blue. Zn<sup>2+</sup> and Ca<sup>2+</sup> are shown as spheres in grey and green, respectively. Inhibitor GM6001 (GM6) is shown as a stick model in green. (b) The atomic model of the catalytic site of VAP1 depicts the coordination of Zn<sup>2+</sup> (white sphere with dots) by three histidine and glutamate of the conserved region (HExxHxxxxH). The water molecule in the catalytic site is omitted.
can be detected in high abundance in secretory vesicles using anti-prodomain antibody through immunohistochemistry and immunogold labelling electron microscopy.[21,22] These results suggest that SVMPs are stored in secretory vesicles after synthesis and the prodomain is either proteolytically processed during secretion or as soon as it reaches the lumen of the venom. An intact SVMP prodomain has not been isolated and characterised from crude venom. Peptides from the SVMP prodomain are rarely identified in the proteomes of viper venoms.[21–23] These processing and activation mechanisms are different in MMPs and ADAMS.[24,25] Detailed molecular analyses are needed for insights into the processing mechanism of SVMPs.

**Natural Inhibitors**

Several inhibitors have been investigated for their effects against crude snake venom or isolated individual venom components. These natural inhibitors are extracted from plants, mammals, marine animals, and even snakes.[25] Natural inhibitors of SVMP can be divided into i) small organic compounds extracted from plants and ii) peptide/proteinaceous inhibitors isolated from venoms and animals resistant to venom, especially snakes and mammals. We will briefly describe some of these natural inhibitors and their inhibitory mechanism.

**Inhibitors from Plants**

Some plants are used in traditional treatment for snakebite injuries. There are more than 1000 of these medicinal plants, most of them from Asia (71%), the Americas (23%), and Africa (4%), and described to be effective in reducing local tissue damage caused by snakebite envenoming according to the provided lists of the reviews.[27,28] This may be because the people who live in the tropical areas in these continents are victims of snakebites and have frequently sought alternative first-aid treatments and complementary therapies due to inaccessibility and other difficulties in the use of venom antiserum therapy. Out of 150 plant families, plants from nine families (Fabaceae, Asteraceae, Apocynaceae, Lamiaceae, Rubiaceae, Euphorbiaceae, Araceae, Malvaceae, and Acanthaceae) are most frequently used to treat snakebites. These medicinal plants are used as treatments against Elapidae and Viperidae snakebite envenoming, and they have included compounds that were found to demonstrate in vitro and in vivo activities in a dose-dependent manner with half-maximal (50%) inhibitory concentration (IC50) values between 0.15 and 0.95 mM for different SVMPs.[24,35] In addition, polyHis-polyGly peptides (EDDH4GVG10) from E. ocellatus and Atheris sp. also inhibit SVMP-induced haemorrhage in vivo.[37,39]

**Inhibitory Mechanisms Based on the Structures of Inhibitor–SVMP Complexes**

**Plant Inhibitors (Small Molecular Weight Compounds)**

We classified the plant inhibitors into flavonoid analogues (Fig. 3a) and others (Fig. 3b). Utilising the structural information available (Table 1), models of the complexes between inhibitors and SVMPs have been constructed by molecular dynamics and docking simulation. Docking models of SVMP with these inhibitors (Flavonoid Myricetin,[42] apigenenin,[43] quinolinolone Q8 (Q8 = 2-hydroxymethyl-6-methoxy-1,4-dihydro-4-quinolinolone);[44] ascorbic acid;[45] and PCT[46] (PCT = triacontyl p-coumarate)) were analysed to identify inhibitory mechanisms of these compounds.

Local administration of 1600 µM myricetin immediately following injection of B. atrox crude venom (6 µg) or Batx-I (30 µg; P-I type SVMP from B. atrox), resulted in a 30% reduction of the hemorrhagic lesion in vivo. Based on the docked model, myricetin binds to the active site of SVMP through formation of hydrogen bonds between the 3’, 4’, and S’ hydroxy groups of the benzyl moiety and the amino acid Glu143 of BaP1, a SVMP from B. asper. It also binds to the Zn2+ in the catalytic site (Fig. 4a).[42]

An Apigenin derivative is able to suppress the pharmacological effect induced by SVMP in vivo when pre-incubated with toxin in a 25-fold (w/w %) excess (this is in the µM range). This compound inhibition of the hematological effects, and systemic alterations induced by newwiedae, a P-I SVMP from the pit viper Bothrops neuwiedei pauloensis. These extracts are usually highly complex and have poorly defined compositions, confounding the study of their mechanism of action. Different components present in the extracts may also act synergistically or inhibit toxins with redundancy that may not be replicated with isolated components.

**Peptide and Protein Inhibitors Isolated from Animals Resistant to Venom**

Some protein inhibitors against SVMPs have been discovered from animals resistant to envenoming, including some mammals, birds, and reptiles. The details of natural SVMP inhibitors from resistant animals was summarised by Bastos et al.[33] These endogenous inhibitors are found in their venom or serum. Tripeptide inhibitors (pEKW, pENW, pEQW, and pERW) have been isolated from venoms of Proteobothrops mucrosquamatus,[34,35] Bothrops asper,[36] Echis ocellatus, Cerastes cerastes cerastes,[37] and some rattlesnakes.[38] These tripeptides inhibit the hemorrhagic SVMP (Fig. 2a, Table S1, Supplementary Material). Their inhibitory activity is not strong, with half-maximal (50%) inhibitory concentration (IC50) values between 0.15 and 0.95 mM for different SVMPs.[34,35] In addition, polyHis-polyGly peptides (EDDH4GVG10) from E. ocellatus and Atheris sp. also inhibit SVMP-induced haemorrhage in vivo.[37,39]
does not chelate either Zn\(^{2+}\) or Ca\(^{2+}\), as revealed by UV-vis analyses, and did not show toxicity to the animal. Docking studies show that the flavone group of Apigenin interacts with the active site Glu146 of Bothropasin (PIII SVMP) with a hydrogen bond, which in turn connects with Ile142 and His145 and the other key hydrophobic amino acid residues Val141, Ile168, Gly170, Pro171, Thr172, and Ile173 (Fig. 4b). This resulted in the re-arrangement of Zn\(^{2+}\) coordinating residues and stripping of the metal ion off the active site.

Q8 efficiently inhibits the proteolytic and hemorrhagic activity of B. jararacussu, B. moojeni, and B. alternatus crude venoms and BjussuMP-I (P-III SVMP from B. jararacussu) in vivo. The potential of Q8, an anti-snake venom drug, was demonstrated by the effect against haemorrhage when the inhibitor is post-injected 15 min after introduction of toxins, which was considered for envenomation treatment. The BaP1 structure from B. asper was used as a template to build the BjussuMP-II (P-I SVMP from B. jararacussu) model, followed by docking simulations to construct the Q8/BjussuMP-II complex. The docked Q8 is inserted deep into the S1\(_0\)-pocket of BjussuMP-II (Fig. 4c).

The oxygen atom from the Q8 carbonyl group was observed to predominantly interact with the Zn\(^{2+}\) in the catalytic site. The two other inhibitors, ascorbic acid and PCT, are not flavonoid analogues (Fig. 3b). With pre-incubation, ascorbic acid (1 : 10 w/w) completely inhibited the proteolytic activity of E. carinatus venom (4 μg) in vitro. Ascorbic acid binds to ecarin (P-III SVMP) with a dissociation constant (\(K_d\)) of 26 μM and a 1 : 1 stoichiometry as determined by isothermal titration calorimetry experiments. For the docking of ascorbic acid, the Russell’s viper venom factor X activator (Protein Data Bank

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**Fig. 2.** Peptide and peptidomimetic inhibitors of SVMP. (a) Tripeptide inhibitors isolated from snake venom as endogenous inhibitor. (b) Commercially available inhibitors of matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase (ADAMs). 2D structures of these inhibitors were drawn using Chemdraw.

**Fig. 3.** Natural inhibitors of SVMP from plant and its derivatives. (a) Natural inhibitors with a flavonoid group. (b) Natural inhibitors without a flavonoid group. Rosmarinic acid is listed in Table S1 (Supplementary Material) with their biological activity. The 2D structures of these inhibitors were drawn using Chemdraw.
PDB ID: 2E3X, 65 % identity to ecarin) was used as a template. PCT binds to the active site cleft while the Zn$^{2+}$ remains in the active site. The oxygen atom on C9 of PCT participated in Zn$^{2+}$ coordination along with the three histidine residues (His142, His146, and His152) and Glu143 in the active site (Fig. 5b). The model also showed the displacement of a water molecule required for catalytic activity by the inhibitor. In addition, the hydrophobic long tail of PCT also interacts with the extensive surface of the SVMP, anchoring the inhibitor in the active site pocket. These docked models showed that ascorbic acid and PCT target the active site and bind to Zn$^{2+}$ through an oxygen similar to the flavonoid analogues described above. The Apigenin analogue is the only exception among these inhibitors to have a binding mode that is incompatible with the presence of Zn$^{2+}$.
Peptidomimetic Inhibitors from Venom or Chemical Synthesis

The structure of pExW (tripeptide inhibitors isolated from venom) variants in complex with TM-3, a P-1 SVMP isolated from Trimeresurus microscutatus, revealed details of the interactions. The indole ring of Trp of the pExW was stacked against the imidazole in His143 of the SVMP at the S1' site (Fig. 6a). In addition, a water molecule essential for catalysis was displaced by the C-terminal carboxylic group of the inhibitor. The second residue was buried in a relatively large pocket of the S2' site and the ring of pyroglutamate was bound in a hydrophobic pocket in the S3' site. The complex structure of pENW/TM-1 refined to 1.8 Å resolution revealed that the S1' substrate-binding pocket in the active site of TM-1 is deeper than that of TM-3. In contrast to Glu174 in TM-3, Pro174 in TM-1 lined the S1' pocket, allowing for a larger space to comfortably accommodate the indole ring of the Trp residue in pENW. This observation is in agreement with the binding preference whereby TM-1 is 3-fold more sensitive to inhibition by pENW, pEQW, and pEKW than TM-3.

Although these inhibitors are not natural inhibitors, the commercially available peptidomimetic inhibitors, namely peptidomimetic hydroxamate inhibitor (WR2), Batimastat, Marimastat, and Ilomastat, are well established broad-spectrum, nanomolar inhibitors of mammalian ADAMs. WR2 and Batimastat are inhibitors of SVMPs. The three-dimensional structures of their complexes with SVMP have been determined and their PDB IDs are listed in Table 1. WR2 showed an inhibitory effect with an IC50 value of 22.2 µM against BaP1, a P-I SVMP from Bothrops asper venom, when pre-incubated with BaP1 in vitro, and using an azocasein solution assay. WR2 has four functional groups (hydroxamate, isobutyl, tert-butyl, and methyl, Fig. 2b). The two oxygen atoms of the hydroxamate group coordinate Zn2+ with three histidine residues located in the catalytic site of SVMP (Fig. 6b). In addition, WR2 was stabilised mainly through hydrophobic contacts and cation–π interactions between isobutyl, tert-butyl, and methyl groups with residues of 106–110 and 168–170 in the hydrophobic pocket of BaP1. Recently, the commercially available agent Batimastat was shown to be an effective treatment against toxicities, local hemorrhagic, lethality, and defibrinogenation induced by Echis ocellatus venom at 500 µM in mice. In this study, Batimastat was injected through the intraperitoneal route at different time points up to 60 min post-administration of venom. The target of Batimastat in E. ocellatus venom is not clear. However, WR2 and Batimastat are largely similar (Fig. 2b), it is possible that Batimastat inhibits SVMPs. Therefore, structural studies between Batimastat and SVMPs would be helpful for its development as antivenom therapeutics.

SVMPs have structurally similar catalytic domains but showed differential binding preferences to inhibitors. Three-dimensional structural information from crystallographic studies and computational models suggested that the simultaneous presence of an oxygen atom in the inhibitor to coordinate with Zn2+ alongside a planar hydrophobic region is crucial for binding to SVMP. In addition, the S1' pockets of different SVMPs are of different sizes depending on the amino acids lining the base of the pocket, thereby deriving selectivity for different inhibitors.

Protein Inhibitors

Habu serum factor (HSF, antihemorrhagic factor) and Small serum protein-1 and -3 (SSP-1 and -3) isolated from Japanese viper (Protobothrops flavoviridis) are proteinaceous endogenous inhibitors of SVMPs found in the serum of venomous snakes (45, 10, and 6.5 kDa respectively). Investigation into inhibitory activities of these inhibitors in vitro suggested that they non-competitively inhibited the peptidase activity for their target P-III SVMP. Based on our preliminary binding studies, these inhibitors can interact with P-III SVMP without MD. These results are in good agreement with the non-competitive inhibition mechanism. Another proteinaceous inhibitor is DM43 from D. marshalli serum, an acidic glycoprotein classified...
into the immunoglobulin supergene family. DM43 inhibits proteolytic activity of the SVMP (jararhagin) in mice.\textsuperscript{[51,55]} DM43 showed high affinity for jararhagin (~1 nM). It binds to the MD of this SVMP, as it was not found to bind to the 28 kDa disintegrin-like domain of jararhagin (jararhagin-C). Glycosylation of DM43 is important to the inhibition.

Taken together with all of the SVMP inhibitors described in the previous section, there are three main mechanisms for inhibiting SVMP: i) chelation of catalytic Zn\textsuperscript{2+}; ii) competitive inhibition; and iii) non-competitive inhibition through binding to the allosteric site.

These findings suggested that almost all of the low molecular weight inhibitors and tripeptide inhibitors are competitive inhibitors. Their selectivity depends on the size and hydrophobicity of the binding pocket in the active site. On the other hand, proteinaceous endogenous inhibitors might act as non-competitive inhibitors of SVMP. However, these inhibitors selectively inhibit a limited number of SVMPs and details of the interaction sites of these proteinaceous endogenous inhibitors have not been identified.

Fig. 7. Comparison of (a) hemorrhagic and (b) non-hemorrhagic metalloproteinases. Coordinates of the seven P-I SVMPs were downloaded from the PDB (PDB IDs are 1ND1; BaP1, 1QUA; Acutolysin-C, 1BSW; Acutolysin-A, 4Q1L; Leucurolysin-a, 3GBO; BmooMPalpha-I, 1IAG; Adamalysin II, 1WNI; H2-protease). Positive, negative, and neutral charges on the surfaces are represented in blue, red, and grey, respectively.

The Prospect of SVMP Inhibitors as an Antidote for Snakebites

There are two possible approaches to the design of inhibitors for SVMPs, either to develop broad-spectrum or highly specific inhibitors. A broad-spectrum inhibitor that can target multiple SVMPs across different species of snakes would be crucial to limit damages caused by snakebite envenoming. As described above, small molecular weight compounds generally act by perturbing the coordination of Zn\textsuperscript{2+}, and have anti-hemorrhagic and anti-proteolytic potency in vitro and in vivo. For example, the well known metal chelator, ethylenediaminetetraacetic acid (EDTA), is capable of neutralising venom-induced lethality \textit{in vivo}.\textsuperscript{[56]} One of the most important considerations in the development of broad-spectrum inhibitors is the toxicity such a treatment would cause, as promiscuity in the mechanism of action would generally lead to unpredictable off-target effects. Therefore, investigation of the efficacy and toxicity of such inhibitors should always carefully consider the dosing regimen. Unfortunately, toxicity issues arising from off-target effects are difficult to overcome. Hydroxamate inhibitors, Marimastat, and Batimastat, have all failed phase III clinical trials for the above reason.

On the other hand, SVMP inhibitors found from resistant animals are typically specific against selected SVMPs. There might be extensive interactions between the inhibitors and targeted SVMPs over a large surface area, resulting in a distinctive recognition mechanism which minimises off-target bindings and hence lowers the chance of toxicity issues. We postulate that there are enough differences among SVMPs to allow for the design of selective inhibitors. For example, there are hemorrhagic and non-hemorrhagic SVMPs and these proteins have important differences near the active site. Some of the main structural differences among SVMPs include a long loop consisting of residues 149 to 177 (atroxlysin-I from Bothrops atrox), and the surface electrostatic potential around the active site region, as revealed by computational models (Fig. 7).\textsuperscript{[57]} By considering charge–charge interactions and moderate but distinctive effects of polar interactions, it may be possible to design peptide-based inhibitors to target the pockets around the catalytic site of the SVMP. Moreover, the most potent hemorrhagic SVMPs belong to the P-III class. These SVMPs have additional domains such as DLD, CRD (including a non-conserved region (hyper-variable region, HVR)), and C-type lectin-like domains. These domains might contribute to the recognition of their macromolecular substrates. Changes in the molecular surface topology and charges in toxins can bring about drastic changes in biological activities, hypothesised as a major mechanism of accelerated evolution known as the Accelerated Segment Switch in Exons to alter Targeting (ASSET). A segment identified based on ASSET in SVMPs, represented by VGEESCGTPE, is a part of the shoulder domain containing one of the calcium-binding regions, and the other segment is the surface of HVR (15 amino acid residues) of CRD.\textsuperscript{[58,59]} Targeting these differences on respective SVMP surfaces could be a promising approach to the design of a specific inhibitor of SVMP.

Conclusions

Consequently, this review discussed how natural inhibitors interact with the catalytic domain of SVMP, because the majority of the demonstrated inhibitors are predicted to interact with the active site, or close, of SVMP as described above.
Despite many reports of natural inhibitors such as extracts of medicinal plants being effective for the treatment of snakebite envenoming, no drug has been successfully demonstrated to have consistent efficacy and safety through clinical trials. A majority of SVMP inhibitors have been evaluated by pre-incubating venoms or SVMPs with the inhibitor before in vitro or in vivo assessments. Such experimental strategies do not replicate clinical situations of snakebite envenoming. It is important for in vivo experiments to be designed vigorously to model the snakebite. For example, a post-injection of inhibitor shows a neutralisation effect after the injected toxin even if a few minutes to hours.\[47,60\] In addition, pharmacodynamics and pharmacokinetics, mechanism of action, target toxins or venom components, molecular interactions between inhibitor and target, duration of action, biodistribution, metabolism, and clearance might be also necessary. The development of effective and low-cost antivenom remains a challenge but with the advent of new technologies, collaborations, and organisation, the future is particularly promising.

At present, a couple of phospholipases A2 (PLA2) inhibitors, including an orally available prodrug are being advanced into clinical trials. These molecules showed efficacy when administered as delayed oral and intravenous antivenom, hence have potential for both field and hospital use for treatment of snakebite.\[60,61\] Several other solutions are also being proposed and developed as next-generation snakebite therapy.\[2\] These include biosynthetic oligoclonal antibodies (BOA) based on recombinantly expressed oligoclonal mixtures of human monoclonal antibodies, as well as repurposing of small molecule enzyme inhibitors targeting snake toxins. BOA may solve the problem of SVMP inhibitors, incorporation of structural information, especially those derived from inhibitors isolated from animals naturally resistant to envenoming, continues to be essential. These endogenous inhibitors bind to SVMPs over a large area of the protein, providing interaction details that could be utilised to design specific and high affinity inhibitors.

Supplementary Material
A summary of the natural and synthesised compounds that have inhibitory activity against SVMP and their effects are available on the Journal’s website.

Conflicts of Interest
The authors declare no conflicts of interest.

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