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REVIEW

Snake venoms: attractive antimicrobial proteinaceous compounds for therapeutic purposes

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Abstract Gram-positive and -negative bacteria are dangerous pathogens that may cause human infection diseases, especially due to the increasingly high prevalence of antibiotic resistance, which is becoming one of the most alarming clinical problems. In the search for novel antimicrobial compounds, snake venoms represent a rich source for such compounds, which are produced by specialized glands in the snake's jawbone. Several venom compounds have been used for antimicrobial effects. Among them are phospholipases A_2, which hydrolyze phospholipids and could act on bacterial cell surfaces. Moreover, metalloproteinases and L-amino acid oxidases, which represent important enzyme classes with antimicrobial properties, are investigated in this study. Finally, antimicrobial peptides from multiple classes are also found in snake venoms and will be mentioned. All these molecules have demonstrated an interesting alternative for controlling microorganisms that are resistant to conventional antibiotics, contributing in medicine due to their differential mechanisms of action and versatility. In this review, snake venom antimicrobial compounds will be focused on, including their enormous biotechnological applications for drug development.

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Introduction

Gram-positive and -negative bacteria are dangerous pathogens that may cause human infection diseases, especially in view of antibiotic resistance, which is becoming an alarming clinical problem [1, 2]. In order to find novel antimicrobial compounds, multiple antibiotic sources have been explored in nature. Among them, animal venoms constitute one of the richest sources of pharmacologically active substances, since these compounds can be produced by self-defense, being useful for the development of unusual pharmaceuticals [1].

Snake venoms are produced in specialized venom glands located in the upper jawbone [2]. Venomous toxins, like other secreted proteins, are synthesized and metabolized in the secretory cells in the venom gland and are commonly transferred to the endomembrane system, where venom components are transported to the cell lumen by the secretory granules (Fig. 1) [3]. Snake venoms are an aqueous solution containing a mix of peptides and proteins, and also polyamides, histamines and alkaloids used in self-defense and predatory strategies [2]. However, mechanisms that regulate the concentration of these compounds, as well as for the regulation of pH and ionic strength in the lumen gland, are still unknown [2]. Venomous toxins may cause a variety of disturbances in an organism, including disarranging ion channels, nicotinic receptors, and enzymes, as well as disturbing neural and cardiovascular tissues and the neuromuscular system [4]. Furthermore, these molecules can also cause blood coagulation and homeostasis [4].

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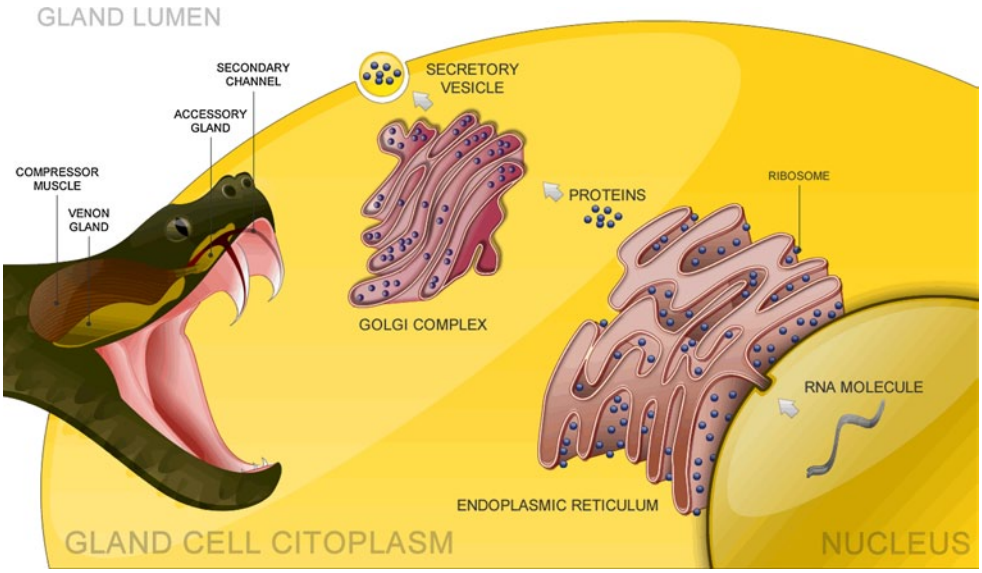

Fig. 1 Process of snake venom manufacturing showing the venom gland and a representation of venom production in gland cells

On the other hand, not just damaging properties have been obtained from venom. This secretion can be utilized as a pharmacological tool, and represents an enormous potential field for the discovery of new molecules that may cure diseases, even those which do not respond to current therapies [4]. To this end, this review will focus on antimicrobial compounds isolated from snake venoms that may be used as novel drugs against infectious diseases and as biotechnological tools that could help to solve health problems.

Antimicrobial enzymes from snake venom

Phospholipases A_2

Phospholipases A_2 (PLA_2) are members of an enzyme superfamily that has been divided into 11 groups according to source, molecular sizes, pathological effects, amino acid sequence similarities, structural homology, and disulfide bridge pattern [5]. Due to these characteristics, PLA_2s have also been classified as enzymes that hydrolyze phospholipids at the sn-2 position in a calcium-dependent manner, releasing fatty acids and lysophospholipids [6].

Snake venom PLA_2s are proteins with length of 120–130 amino acid residues that are cross-linked by seven disulfide bonds. They can be classified into the IA group, represented by Elapid snakes, and the IIA group, characterized by Viperid and Crotalid snakes [7, 8]. The latter group (IIA) can also be subdivided into two major subgroups. The first presents an aspartic acid at amino acid position 49 (Asp49), which has a negative charge that enhances Ca^{2+} binding and contributes to Asp49 hydrophobicity, and, consequently, its catalytic activity. The other subgroup is Lys49, which has a lysine at position 49 and shows a positive charge that prevents Ca^{2+} binding, showing low or no hydrophobicity, but also presenting catalytic activity [8]. In addition to their catalytic activity, IIA PLA_2s may also possess additional biological activities such as hemolytic [9], anticoagulant [10], platelet aggregation [11], neurotoxicity [12] and myotoxicity [13]. Furthermore, some studies have demonstrated that multiple pharmacological effects, such as antitumor [14] and antimicrobial activities, can also be correlated with PLA_2s snake venom activities.

In addition to subgroups Lys49 and Asp49, some studies have suggested that other subgroups of IIA PLA_2 may be found in nature. Wei et al. [7] studied a novel subgroup known as promutoxin, containing an Arg at position 49 (R49 PLA_2) (Fig. 2a, b). This enzyme was first purified from *Protobothrops mucrosquamatus* snake venom by using liquid chromatography methods followed by SDS-PAGE and MALDI-TOF molecular mass determination. The enzyme was further challenged against Gram-positive and -negative bacteria [7]. Molecular mass analysis showed a single band of 15 kDa under reducing conditions and two bands of 15 and 24 kDa under non-reducing conditions. MALDI-TOF analysis showed a single peak with molecular mass of 13.656 kDa. Moreover, structural studies that involved a combination of different technologies such as MALDI-TOF, Edman N-terminal sequencing and molecular cloning were also performed to determine the primary structure of promutoxin [7]. Antimicrobial assays were performed against both Gram-positive and -negative bacteria showing that R49 PLA_2, at 250 $\mu g\ mL^{-1}$, was able to control *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Table 1) [7]. In studies performed with a homodimeric phospholipase A_2 from *Bungarus faciatus* snake venom (BFPA), Chunhua Xu et al. [15] purified and

characterized s-PLA_2-I (BFPA), showing 15 half-cysteines [15]. They also challenged BFPA against *Staphylococcus aureus* and *Escherichia coli,* showing a stronger activity against Gram-positive than Gram-negative bacteria [15]. These results may be explained by the overall cationic properties of BFPA, which makes it easy to penetrate the anionic Gram-positive bacterial cell wall [16]. It seems that reduced activity toward Gram-negative bacteria could be related to the absence of bactericidal/penetration-increasing protein (BPI), a protein able to bind and penetrate lipopolysaccharides produced by Gram-negative bacteria [17, 18].

Metalloproteinases

Snake venom metalloproteinases (SVMPs) comprise a complex sub-family of zinc-dependent enzymes that display a wide variety of biological activities such as hemorrhage [19], inhibition of platelet aggregation [19], coagulopathy [20], myonecrosis [21], and inflammatory responses [21]. All these activities are, however, closely related to each multi-complex SVMP domain function. These multi-complex domains are readily organized into three different classes called P-I, P-II, and P-III, which are classified according to the presence or absence of additional domains such as disintegrin and disintegrin-like domain, a high cysteine domain, and a lectin-binding domain [22, 23]. The first, P-I, is the smallest SVMP class, which shows molecular masses between 20 and 30 kDa, containing a pro-domain and the proteinase domain [24]. P-I typically displays fibrinogen or fibrinolytic activities [25]. Class P-II has intermediate molecular masses of 30–60 kDa and contains identical P-I domains in addition to a disintegrin domain [25]. The last one, P-III, is the largest SVMP class, which has molecular masses of 60–100 kDa and contains pro-, proteinase, disintegrin-like, and cysteine-rich domain structure [25]. Some authors suggest a fourth SVMP class, P-IV, which has an additional lectin-like domain linked by disulfide bounds to a P-III class SVMP. However, there are some doubts whether P-IV truly represents a novel class or if it is just a special P-III structure bonded by a single disulfide bridge with available C-type lectin-like proteins during venom synthesis [21].

Although SVMPs are known for their proteolytic, cell–matrix and cell–cell adhesion abilities, only a few studies have related these enzymes to direct antimicrobial activities. Samy et al. [26] reported the isolation and characterization of a metalloproteinase from *Agkistrodon halys* (AHM), aiming to understand how membrane permeability and antimicrobial functions could be related to such proteinases [26]. Isolation and purification methods based on gel filtration followed by reversed-phase HPLC, MALDI-TOF, and SDS-PAGE were applied, resulting in AHM purification that showed a molecular mass of 23 kDa. AHM showed inhibitory activities against *Bacillus pseudomallei*, *Proteus vulgaris*, and *S. aureus* (Table 1) [26]. Otherwise, AHM did not show any deleterious activity against *E. coli*, *P. aeruginosa*, or *Enterobacter aerogenis* (Table 1) [26]. Moreover, ultrastructure studies, using scanning electron microscopy (SEM) and transmission electron microscopy (TEM), were performed in order to demonstrate the cellular damages occasioned by AHM, such as cell perforation and cell wall roughening over Gram-positive and -negative bacteria.

In order to classify this enzyme, Samy et al. [26] also performed a comparative study of primary structure from AHM against other SVMPs such as contortrostatin, bothrostatin, and elegantin-2a, which revealed high levels of similarity due to a conserved domain constituted by disintegrin-like/cysteine-rich domains that are typical of the P-III SVMP class. This class seems to show bactericidal action by both bacterial anionic site recognition and also enzymatic degradation of phospholipid membranes, by its perforation followed by membrane disruption processes, events that preferentially occur in Gram-positive bacteria [26].

L-Amino oxidases

Another antimicrobial component of several snake venoms is L-amino acid oxidase (SV-LAAO), a classical flavoprotein commonly found in a wide variety of animal fluids such as milk [27], epithelial mucus [28], and venoms [29]. According to Du and Clemetson [16], SV-LAAOs are usually homodimerics, FAD-binding glycoproteins, with molecular masses around 110–150 kDa under non-denaturing conditions [16]. Each SV-LAAO monomer consists of three domains, with the whole structure being composed of a total of 14 α-helices and 18 β-strands. Moreover, Georgieva et al. [30] also propose that the stabilization of the L-amino acid oxidase quaternary structures by a pair of Zn^{2+} ions could be a relevant factor for biological activities [30], including the action on platelets, induction of apoptosis, hemorrhagic effects, cytotoxicity, and antimicrobial properties [31]. In addition, SV-LAAO can also show antimicrobial activities, which are related with the bacterial control by hydrogen peroxide H_2O_2 generated as a result of the oxidation of L-amino acids by LAAOs [18], as well as LAAO binding to bacterial membranes [32].

Okubo et al. [29] demonstrated that LAAOs from *Bothrops mattogrosensis* venom (Bm-LAAO) (Fig. 2c, d) presented antimicrobial activity against multiple bacteria, showing a probable unusual protective function. Aiming to identify and isolate Bm-LAAO, gel filtration chromatography was utilized, resulting in three fractions with antimicrobial activities. The fraction with highest activity was applied onto a reversed-phase HPLC chromatograph,

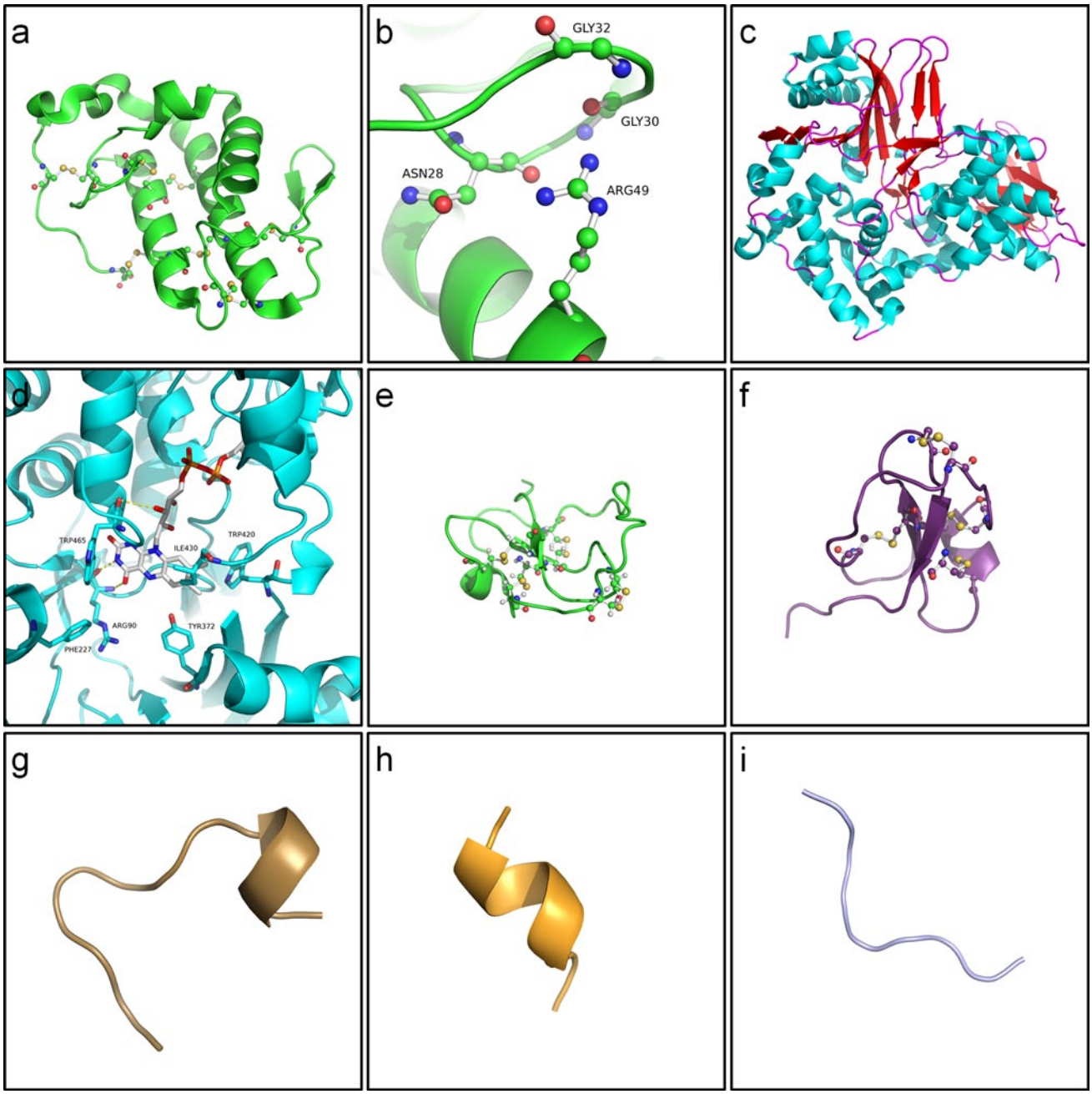

Fig. 2 Structure of proteinaceus compounds of snake venom. **a** Phospholipase A_2 protein structure and **b** related catalytic site showing the amino acids residues involved in enzyme reaction **c** L-Amino acid oxidase protein structure, and **d** related catalytic site showing the amino acids residues involved in enzyme reaction. Peptide structures of **e** nawaprin, **f** omwaprin, **g** Bm-LAOf1, **h** Bm-LAOf2 and **i** Bm-LAOf3. Structures were visualized by using PyMol

yielding a purified Bm-LAAO enzyme species. Bm-LAAO showed the highest antimicrobial activities, with MICs between 2 and 8 μg mL^{-1} when challenged against Gram-negative bacteria such as *Klebsiella pneumoniae*, *E. coli*, and *P. aeruginosa* and MICs among 8–32 μg mL^{-1} when challenged against Gram-positive bacteria such as *S. aureus* and *Streptococcus pyogenes* (Table 1) [29].

Other studies have shown that LAAO is an enzyme that is able to catalyze the oxidation of a wide number of amino acids [29], generating amino acids and hydrogen peroxide, H_2O_2. This ability seems to be essentially involved in antibacterial activity. Aiming to understand the H_2O_2 bactericidal mechanism of action, Zhang et al. [33] demonstrated that LAAO from *A. halys* snake venom

Table 1 Representative values about the MIC proteins and peptides against Bacteria

Microorganisms	MICs (μg mL^{-1})[a]									
	P. mucrosquamatus	A. halys	*Bothrops mattogrosensis*				B. fasciatus	C. durissus terrificus		*O. microlepidotus*
	PLA2	SVMP	LAAO	BmLAO-f1	BmLAO-f2	BmLAO-f3	BF-30	*Crotamine*	R-crotamine	Omwaprin
Gram-positive										
Bacillus magaterium	–	–	–	–	–	–	–	–	–	560
Bacillus subtilis	250	–	32	43	250	220	–	–	–	–
Staphylococcus aureus	–	7.5	32	43	250	220	4.7	–	–	5,600
Streptococcus pyogenes	–	–	8	43	125	110	–	–	–	–
Streptococcus warneri	–	–	–	–	–	–	–	–	–	1,700
Gram-negative										
Bacillus pseudomallei	–	30	–	–	–	–	–	–	–	–
Escherichia coli (ATCC8739)	–	–	4	22	250	220	–	–	–	–
Escherichia coli (O157:H7)	–	–	–	–	–	–	–	25	25	–
Escherichia coli (ML-35p)	–	–	–	–	–	–	–	50	25	–
Escherichia coli (ATCC25922)	–	–	–	–	–	–	2.3	100	50	–
Klebsiella pneumoniae	–	–	2	22	125	110	0.3	–	–	–
Proteus vulgaris	–	15	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa	250	–	8	22	250	220	2	–	–	–
Salmonella typhi	–	–	–	–	–	–	1,2	–	–	–
Salmonella typhimurium	250	–	8	43	250	220	–	–	–	–

PLA2 phospholipase A_2, *SVMP* snake venom metalloproteinase, *LAAO* L-amino acid oxidase, *BmLAO-f1*, *BmLAO-f2*, *BmLAO-f3 B. mattogrosensis* L-amino acid oxidase fragments 1, 2 and 3, *R-crotamine* reduced crotamine, *BF-30* cathelicidin BF-30 from *Bungarus fasciatus*

[a] MIC (minimum inhibitory concentration) is defined as the lowest concentration that inhibited 100 % of bacterial growth

(AHP-LAAO) was able to inhibit both Gram-positive and -negative bacterial growth due to enhanced H_2O_2 production [33]. In order to relate the presence of H_2O_2 and antimicrobial activity, AHP-LAAO was challenged against *S. aureus* and *E. coli,* in the presence and absence of catalase, an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen. Interestingly, inhibitory effects ceased upon catalase addition. Catalase was able to destroy H_2O_2 yielded during AHP-LAAO enzymatic reactions, suggesting that the antimicrobial activity of AHP-LAAO could be directly attributed to the H_2O_2 produced [33].

Antimicrobial peptides

Antimicrobial peptides are defined as molecules that have a critical defense against all kinds of microorganisms, protecting the host from invasion of bacteria, fungi, and viruses [34]. Moreover, antimicrobial peptides have been focused as a new approach for drug development against infections [35]. They are part of the immune innate system and play a role in defending against microorganism infection. They are also capable of inactivating infectious agents and are a strong candidate for combating drug resistance [36]. Unfortunately, only a selected number of AMPs have been described to be suitable for pharmacological applications [37]. One of the greatest difficulties in designing useful AMPs is the unclear understanding of their structural determinants for membrane compound recognition [38]. Structure, hydrophobicity, folding, charges, and dynamic and AMP bond angles seem to be crucial factors that might influence their selectivity and activities [39, 40]. In this context, a wide variety of biochemical and biophysical studies have reported mechanisms of bioavailability, membrane disruption, and synergism activities of these molecules [41].

Antimicrobial peptides can be divided into four structural groups known as α-helical, β-sheet, α-hairpin, and extended peptides [37, 42]. Moreover, in addition to their diversity in amino acid sequences and structures, it is also very common for AMPs to share amphipathic properties [43, 44]. This amphipathic characteristic allows them to bind membranes of microorganisms and has been thought to cause cell lysis by interaction with lipids. Furthermore, AMPs can self-associate and form pores or can possibly act in disintegrating membranes in a detergent-like manner [37, 42]. Although a fold into the amphipathic structure seems to be a prerequisite for cell lysis, the exact mechanisms of action of most AMPs are still unclear [42]. Gram-positive and -negative bacterial cytoplasmic membranes predominantly present phospholipids with negative charges that give rise to an electrostatic attraction to the highly cationic AMPs [45]. In this context, phosphatidylglycerol (PG) and cardiolipin (CL) consists of anionic lipids that are most abundant in Gram-positive and -negative bacterial membranes. The interaction between AMPs and such lipids seems to be essential to understand the membrane disruption process [46]. Two recent studies developed by Epand et al. [47] show that activities of five AMPs (MSI-78, MSI-103, MSI-469, MSI-843, and MSI-1254) could promote lipid clustering in contrast to magainin 2, which is unable to do it [47]. Furthermore, all peptides demonstrated the formation of a crystalline phase in the presence of dimyristoyl phosphatidylglycerol (DMPG), a negatively-charged phospholipid [46]. Thus, cationic compounds such as most AMPs probably will cluster with lipid membranes [46].

In addition to their inner membrane, Gram-negative bacteria also have an outer membrane predominantly composed of the anionic lipopolysaccharide (LPS), which provides a strong barrier that must be overcome by AMP compounds [48–50]. Given this fact, studies have shown that, when some AMPs are evaluated against Gram-negative bacteria, higher peptide concentrations are required for the inhibitory process, when compared with Gram-positive ones [51]. The amphiphilic LPS is constituted of three distinct domains, which are known as a well-conserved lipid A moiety, a core oligosaccharide and a highly variable hydrophilic polysaccharide domain [52, 53]. Due to these characteristics, LPS is known to serve as a permeable barrier that can play an important role in the regulation of bacterial hydrophobic antibiotics and antimicrobial agents, modulating entry and insertion of AMPs in the inner plasma membrane [49, 54, 55]. In accordance with this fact, some studies have shown that insect cecropins, temporins, cathelicidins, protegrins, and some defensins are all active against Gram-positive bacteria, but not against Gram-negative microorganisms [49, 55]. Bearing this in mind, studies are needed into the role of cholesterol in different kinds of membrane bilayers with different AMPs, to help understand the exact role of cholesterol in selectivity of AMPs [56].

In recent studies about correlations in structure–activity of AMPs, it has been observed that LPS oligomerization may make European frog temporins inactive [49]. In studies examining ways to overcome AMP inactivation problems, it has been proposed that AMPs may cause structural disarrangements in Gram-negative bacteria's outer membrane by a self-promoted uptake mechanism based on the interaction of the peptide's cationic residues with LPS phosphate groups [55, 57]. Bhattacharjya et al. [58] showed that a hemolytic peptide from a bee venom named melittin was able to adopt a partial helical structure restricted to the cationic C-terminus in LPS micelles [58]. Otherwise, the melittin N-terminus was found to be unstructured and dynamic in LPS. These data suggest that C-termini could act as an anchoring region that disturbs LPS structure,

making hydrophobic N-terminus region insertion into the inner membrane possible. Furthermore, Matsuzaki et al.'s [59] studies showed that magainin and its analogues were able to induce leakage and create lesions at the outer membrane of Gram-negative bacteria by acquiring a helical fold when in contact with LPS, causing a disturbance of acyl chains of lipid A domain of LPS [55, 59].

Connected to the multifunctional ability of AMPs, in membranes rich in LPS and anionic lipids, pardaxin is a peptide derived from the mucous glands of the *Pardichirus* fish, studies of which have demonstrated how these peptides interact with bilayer membranes [60, 61]. Hallock et al. [62] described the pardaxin (P1a) mechanism in bilayer membranes. In summary, these studies show pardaxin's behavior in lipidic bilayer components like 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), 1,2-dimyristoyl-phosphatidylcholine (DMPC), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (POPE), and 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG). Data indicated that bilayers constructed with POPC and POPE components reduced P1a's ability to cause membrane disruption. Furthermore, the POPG component alters the peptide/membrane interaction, suggesting that P1a has a preference and selectivity regarding bilayer composition, showing that the peptide mechanism is extremely complex and that bilayer disruption is composition-dependent.

In another study, Porcelli et al. [63] showed that synthetic pardaxin, named P4a, demonstrates a bend-helix-bend-helix in the presence of sodium dodecylphosphocholin. P4a could induce disorder in DMPC and in a hydrophobic bilayer core. Additionally, the pardaxin C-termini helix adopts a transmembrane conformation in DMPC bilayer. Otherwise, in POPC bilayers, pardaxin showed a lipid surface orientation and not a transmembrane orientation. These data suggest that P4a alters the head group dynamics. Bhunia et al. [39] developed a study explaining a pore-forming P4a in LPS micelles, demonstrating their efficacy in permeabilizing the outer in addition to the inner membrane. This study also demonstrates that P4a assumes random conformations in the aqueous buffer, but this conformation was modified to helical structures in the presence of an LPS micelle. NMR and infrared spectroscopy confirmed that P4a shows the presence of helices and loop/turn and extended conformations at the termini in an LPS micelle environment. Ramamoorthy et al. [38] showed that pardaxin dynamics became disordered in the presence of cholesterol. This same study also showed that a reduction in temperature could reduce pardaxin helix mobility. Furthermore, the presence of cholesterol decreases C-terminal helix disorder, since cholesterol increases the order of acyl chains in bilayers; consequently, the increase in acyl chains is one of the causes of reduction in motility of the C-terminal helix of pardaxin. These results imply that cholesterol may influence the barriel-stave formation performed by pardaxin.

A previous work developed by Wang et al. [36] isolated and characterized a cathelicidin antimicrobial peptide from *B. fasciatus* venom. The cathelicidin family is characterized by their cathelin domains, which are an anionic conserved domain with an N-terminal sequence conserved for about 100 residues [64, 65]. The cathelicidin precursor shows a highly conserved cathelin domain composed of 100 amino acid residues, which is flanked by a signal peptide fragment with approximately 30 residues in the N-terminus and also an antimicrobial peptide in the C-terminus cationic antimicrobial peptide [65]. The cathelicidin isolated by Wang et al. [36] was denominated cathelicidin-BF with a molecular mass of 3,637.5 Da and isoelectric point at 11.79. This cathelicidin is composed of 30 amino acid residues, including 12 basic residues (9 lysines and 2 arginines), 5 phenylalanines, and just one acidic residue (16 glutamic acid) [36]. The secondary structure elements were predicted by CD spectroscopy, indicating a highly α-helical conformation, although a three-dimensional structure was not elucidated [36]. However, CD data indicated that the N-terminal portion of cathelicidin-BF adopts an amphipathic α-helical conformation like that of any other cathelicidins [36]. The antimicrobial peptide cathelicidin-BF demonstrated strong antimicrobial activities against several microorganisms, being more efficient toward Gram-negative bacteria, and also against clinically isolated drug-resistant bacteria, including *K. pneumoniae*, *E. coli*, and *Salmonella typhi* clinical isolates (Table 1). Cathelicidin-BF was also effective against fungal pathogens *Candida albicans* ATCC2002 and *Pichia pastoris* (Table 2). Additionally, some saprophytic fungi were also affected by this peptide, including *Aspergillus terreus, A. niculans*, and *haetomium globosum* (Table 2) [36].

Table 2 Representative values about the MIC peptides against fungi

Microorganisms	MICs (μg mL^{-1})[a]	
	Cathelicidin (BF-30)	Pep5B
Aspergillus terreus	18.7	–
Aspergillus niculans	4.7	–
Chaetomium globosum	37.5	–
Candida albicans	4.7	8
Colletotrichum lindemuthinum	–	8
Fusarium oxysporum	–	8
Pichia pastoris	0.3	–
Saccharomyces cerevisiae	–	8

Pep5B peptide isolated from *Bothropos jararaca* snake venom

[a] MIC (minimum inhibitory concentration) is defined as the lowest concentration that inhibited 100 % of bacterial growth

Considering this information, the antimicrobial peptide cathelicidin BF-30 and two other commercial antibiotics (gentamicin and bacitracin) were evaluated against 23 clinical isolates of bacterial strains. Data indicate that cathelicidin shows higher efficiency in comparison to the other two antibiotics, especially against *E. coli*, *P. aeruginosa*, and *S. aureus*. [66]. Cathelicidin BF-30 was also evaluated in vivo for intraderrmal action in a burned rat model infected with *P. aeruginosa* strain [66]. The number of CFUs of rat's organs infected, including lungs and liver, showed a remarkable reduction with BF-30 treatment, suggesting that peptide could prevent the systemic effects of *P. aeruginosa* [66]. In summary, BF-30 demonstrates in vitro and in vivo bactericidal activity, including against resistant strains, representing a potent antibacterial activity, and is an important candidate for local treatment of infective burns [66].

Crotamine consists of a myotoxin from rattlesnake venom peptide with 42 amino-acid residues, a positive net charge of 8 and pI 9.5, with high similarity to mammal's β-defensins formed by 2–3 antiparallel β-sheets, and six conserved cysteine residues involved in disulphide bond formation [67]. Crotamine shows antibacterial activity against *E. coli* strains O157:H7, ML-35p, and ATCC 25922 (Table 1). Some analysis of mechanism action demonstrates that crotamine kills the bacteria by first penetrating the membrane [67]. In order to evaluate the importance of disulfide bonds in antibacterial crotamine activity, the molecule was reduced and further tested against *E. coli* strains, demonstrating a tenuous increase in activity against all strains in comparison to native crotamine (Table 1). Furthermore, reduced crotamine also showed an increase in salt resistance when compared to non-reduced, suggesting that the disulfide bond is dispensable for these properties [42]. It was therefore also suggested that disulfides are important in snake venom, providing stability in vitro; indeed, the myotoxic lethality effect showed a decrease of about 50 % [67, 68].

Moreover, Gomes et al. [69] reported the isolation and characterization of a novel peptide (PepBj) from *Bothropos jararaca* snake venom. PeBj was further challenged against different fungi. The purification methods were based, firstly, on gel filtration chromatography, which resulted in the separation of the crude venom into eight fractions. Several fractions were tested against *Colletotrichum lindemuthianum*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, and *C. albicans*, but only a single fraction showed significant antifungal activity (Table 2) [69]. This fraction was further applied onto a reverse-phase chromatograph, yielding the purified PepBj. Mass spectrometry analysis showed a major peak with molecular mass of 1,370 Da. In addition to antifungal assays, Gomes et al. [69] performed optical and scanning electron microscopy, showing that PepBj induced various hyphal morphological alterations coupled with pseudo-hyphae formation in evaluated fungi. Moreover, using SYTOX green assay, it was observed that PepBj was also able to induce hyphal membrane permeability, showing strong SYTOX green fluorescence in the cytosol and nucleus [69], suggesting that the peptide's actions are based on intercellular damages.

Another important class of antimicrobial peptides isolated from snake venoms consists of waprin (WAP) [70] The first member of a waprin family, called nawaprin, was isolated from *Naja nigricollis* snake venom (Fig. 2e) [70]. In this study, a three-dimensional structure of nawaprin was determined by nuclear magnetic resonance spectroscopy. Structural data showed that nawaprin is relatively flat and disc-like in shape, characterized by a spiral backbone configuration, composed of circular segments projected to the molecule outside, containing a short antiparallel β-strand, as well as circular segments projected to the molecule inside, connected by four disulfide bounds (Fig. 2e) [70]. Although the cysteine residues are conserved, different structural arrays can be observed among waprin family members. Furthermore, after new studies on waprin (WAP), it became known that the WAP domain is found in proteins with different functions, such as elafin and secretory leukocyte proteinase inhibitor (SLPI), which are proteinase inhibitors with high antimicrobial activities [71, 72], ps20 with growth-inhibitory activity [73], and single WAP motif protein 1 and 2 (SWAM1 and SWAM2), which presented antimicrobial activities when evaluated against *E. coli* and *S. aureus* (Table 1) [74].

Another member of the waprin family, a small protein of 50 amino acid residues, was isolated from *Oxyuranus microlepidotus* and named omwaprin (Fig. 2f). First, the peptide shows antimicrobial activity against Gram-positive bacteria, such as *Bacillus magaterium* and *Streptococcus warneri* (Table 1). Furthermore, a three-dimensional homology model of omwaprin was constructed (Fig. 2f). This model revealed that the omwaprin structure consists of a spiral backbone conformation with two circular segments connected by four disulfide bonds, as well as three β-strands in a loop-structure composition with both N-terminal and C-terminal projected to the molecule outside (Fig. 2f) [31]. The structural model of omwaprin showed that its N-terminal part has four positive charge residues, which seems essential for omwaprin's antimicrobial activities [31]. In order to confirm this hypothesis, deletion mutagenesis was carried out, resulting in a protein with six amino acid deletions, as well as a secondary structure, which was submitted to circular dichroism spectroscopy analysis (CD analysis), showing similar structures to omwaprin. The mutant cationic segment without N-terminus showed no antimicrobial activity against *B. magaterium* and *S. warneri* (Table 1), even at a high concentration, clearly suggesting that the positive charges of N-terminal

residues are important in omwaprin's antimicrobial activities [31].

Peptide fragments from higher venom proteins have also been demonstrated to be effective to control microorganisms. Okudo et al. [29] demonstrated that Bm-LAAO fragments could also show antimicrobial activity against multiple bacteria. Three fragments, named Bm-LAOf1 (Fig. 2g), Bm-LAOf2 (Fig. 2h), and Bm-LAOf3 (Fig. 2i), were identified, and further synthesized and evaluated against Gram-positive and -negative bacteria, against which they showed activity (Table 1). Molecular modeling analyses suggested that Bm-LAOf1 presented an α-helix region with a net charge of +3 as well as a coil conformation with hydrophobic and cationic C-terminal sequences (Fig. 2g) [29]. Bm-LAOf2 structurally presented a hydrophobic helix conformation with a negative charge of −1 and a definition of a dipole helix (Fig. 2h) [29]. Moreover, Bm-LAOf3 structurally presented a coil conformation due to the presence of two proline residues, which make helix formation difficult (Fig. 2i) [29]. Hydrophobic residues at the N-terminal and C-terminal were also observed, which could contribute to peptide–lipid interaction in the three peptides evaluated here. However, when compared with natural protein Bm-LAAO (Fig. 2c, d), the three peptide fragments showed lower microbial development inhibitions [29], despite the clear benefits of lower molecular masses.

Perspectives for possible therapeutics and industrial applications

Increasing bacterial resistance caused by the indiscriminate use of antibiotics is growing every year. Therefore, research focusing on organisms resistant to antibiotics is also underway in order to find new alternatives sources to combat them [75]. The cationic proteinaceous compounds, for example, have emerged during recent years for therapeutics against parasites and microorganisms [76]. These compounds, in addition to direct antimicrobial activity, could mediate a series of immunomodulation activities, providing opportunities to develop novel therapeutic products [76]. They are called host-defense proteins since they are related to immune system activities, including modulation of pro-inflammatory cytokine and chemokine production and also the stimulation of macrophages, neutrophils and T lymphocyte activation proliferation [77]. Nevertheless, some obstacles are commonly found in the development of viable antimicrobial components. One of them is the determination of drug pharmacokinetics, which relates how the doses and compounds concentrations may be administered in a patient. However, the greatest challenge is to discover the best way of administering the drug [76, 78], as previously observed for some antibiotics, such as amino glycosides and capreomycin, which is administered intravenously [79, 80]. It is important to note that a lower dose could be best for treatment, since allergic reactions to certain types of drugs and antibiotics could occur as a side effect. Furthermore, a short treatment time or a lower number of doses would be more appropriate since long treatments and high dosages may improve pressure selection and lead to higher microorganism resistance [81]. Oral administration is clearly preferential for antibiotics [82]. Nevertheless, proteins and peptides can be easily degraded by digestive proteolytic enzymes, and gastrointestinal tracts may not be able to adsorb the protein and peptides [76]. This digestion process could affect therapeutic procedures and thus hinder the use of proteinaceous compounds for systematic applications. Moreover, there is a dependence on ideal physiologic conditions such as pH and salt to reach high antimicrobial activities [76]. Hence, the solution for administering drugs is by direct administration in blood vessels. This type of administration makes the compound absorbed more quickly, thus avoiding loss by degradation or excretion, leading to rapid drug distribution throughout the body. However, it is important to remember that some antibiotics should undergo metabolism in the body to become active.

Although no proteinaceous antimicrobial compound from snake venom has been licensed for clinical use until now, current studies continue bringing new expectations about their viability as a new class of drugs. One alternative to circumvent these problems may be the use of nanotechnology, sending nano-coated compounds that could move directly to the proper infection location. Several nano-encapsulated proteins and peptides have been investigated for drug application [83]. This technique would offer protection and improve the pharmacokinetics from easy degradation as well reducing tissue rejection and damage [78].

An enormous challenge of protein and peptide use as a biotechnological tool involves the production itself, which is generally very expensive and time consuming, needing new technologies for development and production of these molecules. Some techniques have been used to obtain proteinaceous compounds, including natural isolation, recombinant expression, and chemical synthesis [77]. The first is a common technique in academia, but not at the industrial level, due to higher costs, lower reproducibility, and time needed [77]. The chemical synthesis technique is commonly used to produce proteinaceous compounds derived from natural sources like snake venom, but it is extremely expensive for synthesis involving proteins higher than 30 amino acid residues in length. Another problem is the fact that most molecules from snake venom have disulfide bonds and post-translational modifications, which increases the costs and difficulties of synthesis [84–86]. With the limitation of these techniques described above, the heterologous expression system has been widely used in the

last few decades, because it involves the fusion of carrier proteins which contain a chemical and enzymatic cleavage site, allowing the peptide target to be released [77]. Some of these proteins act in stabilization, neutralizing the charge of the peptide and generating a nontoxic expression and soluble peptide, being a clear solution in the production of the proteins and peptides described here [87].

Nevertheless, some precautions have to be taken when choosing a heterologous system from proteins to peptides, since bacteria cannot produce very extensive proteins, with proper folding and glycosylation patterns [88]. For AMP production, the most widely used heterologous system is bacteria (*E. coli*) and yeasts (*S. cerevisiae*), representing about 97 % [89]. However, plants and transgenic plants have been receiving more attention in this area for their ability to produce proteins larger then bacterial systems [88]. The *E. coli* heterologous system is the most utilized microorganism because of its rapid growth, wide availability of commercial vector expressions, and the extensive knowledge in genetics, biochemistry, and physiology [90]. Notwithstanding this, there are some challenges to be overcome. The first consists of preventing AMPs from destroying the host's bacterial heterologous system in use. The second is the AMPs' chemical properties and size, because some studies demonstrate that AMPs are a target of proteases synthesized by bacteria, and also the bacterial system sometimes cannot carry out post-translational modification [91, 92]. Thus, for expression success, the AMPs have to be fused with a carrier harboring anionic properties [91].

Otherwise, yeasts such as *S. cerevisiae* and *P. pastoris* for heterologous expression systems are the most widely used [92]. Some advantages are clearly observed in the yeast system over the prokaryotic system, including post-translational and transcriptional modifications such as glycosylation [93]. Both these yeast systems allow recombinant protein secretion, resulting in fewer purification steps and facilitating the scale-up process [94]. Regarding AMP production, several cases do not require carrier proteins, as described in the *E. coli* system, to make scale-up processes easier [92]. Plants modified genetically have long been used as expression platforms for peptides [88]. These systems are developed mostly for crop improvement, but plants could also be utilized as bioindustries, demonstrating their enormous potential as a platform for proteins and peptide expression, even if the host has to be used primarily for crop improvement [92].

In order to complete the gaps in knowledge about proteins and peptides where no experimental derived structure exists, the methodologies focusing computational-driven three-dimensional structure prediction have been successfully used. However, when there an experimentally determined structure exists, in silico methods can also be useful in predicting binding location surfaces for other molecules, estimating the binding energy and predicting the movements and flexibility that are required for some events, including the mechanism of action [95]. Many modeling strategies have used ab initio to predict some protein structures. Ab initio consists of characterizing a 3D protein structure using only the primary sequence of the protein as an input [95]. Otherwise, in addition to ab initio, homology modeling has been performed, utilizing structural and evolutionary similarities by means of a template, which is a protein structure, previously elucidated, with identity to the protein target. In contrast, threading methods can be used by models which are not evolutionarily related because they use proteins which have the same fold but do not necessarily have homologous proteins [95]. Modeling building involves first of all identifying the best template for the alignment. The templates are chosen according to the greatest identity/homology between the target and template [95]. Some approaches to sequence conservations can also be used, and for a refinement, loop structures and side chains can be refined by molecular dynamics [20, 95]. In summary, despite some limitations, like an elevated computational cost for frisking the energy space and also the complication of accurately selecting the native structure from a broad range of alternative conformations, these in silico analyses are also promising for use in predictions of proteins and peptides derived from snake venoms.

As previously described [29], in silico analysis of three fragments of L-amino acid oxidase from *B. mattogrosensis* was performed by homology modeling. Okubo et al. [29] utilized a template of *Vipera ammodytes* LAO which were characterized by X-ray diffraction (PDB 3kve) [27]. The three fragments isolated in the study showed 100 % of identity with the sequence acquired. BmLAO-f1 showed a coil conformation with a C-terminus with hydrophilic and cationic characteristics composed of Lys^{10}, Lys^{11}, and His^{13}. Moreover, APD prediction described the structure of α-helical region containing a +3 charge. The central region was characterized by a proline-rich region with hydrophobic residues (Phe^{3} and Leu^{7}) conferring 30 % of hydrophobic ratio, which probably favors interaction with cell membrane phospholipids. In addition, BmLAO-f2 presented hydrophobic helical conformation with a negative charge (−1), which is characterized by Lys^{1}, Lys^{2}, Glu^{5}, Asp^{6}, and Asp^{7} residues, and also a dipole helix formation. In this peptide, the Phe^{3} and Trp^{4} residues (25 % hydrophobic ratio) provide a possible interaction with lipid membranes. Finally, BmLAO-f3 presented Pro^{4} and Pro^{5} residues that probably make helix formation difficult, thus presenting a coil conformation. This peptide presents 22 % of ratio with an Ile1 and Phe^{8} at the N- and C termini, respectively, which may contribute to peptide–lipid interaction. As demonstrated by Okubo et al. [29], in silico analysis can

provide a wide range of information about AMPs and their structures, suggesting which residues could be important for bacterial membrane interactions.

Another tool that is promising in a study of AMPs from snake venom is the nuclear magnetic resonance (NMR) spectrometry technique. Nuclear magnetic resonance (NMR) consists of another technology that is much used for AMP study. The multidimensional solution NMR technique is well established to elucidate globular proteins and to investigate membrane-associated polypeptides and their relationship with micellar and bicellar complexes [92, 93]. Solid-state NMR is a remarkable technique that could be used to study AMP structures in the presence of liquid-disordered phospholipid bilayers [94]. In order to clearly comprehend solid-state NMR, it needs to be recognized that most NMR interactions could be closely dependent on molecule alignment relative to magnetic field direction [94]. In NMR spectra of solid and semi-solid samples, and also of large molecular complexes, dipolar and quadrupolar interactions are anisotropic. In order to reestablish well-resolved spectra, two approaches are well established, fast spinning around the magic angle (MAS) which unchangeably averages the orientation-dependent NMR interactions, or sample uniaxial alignment, which is relative to the magnetic field direction [94]. Both of them result in a relatively narrow line protein shape in the solid state or also when associated with lipid bilayers [94]. The valorization of narrow lines has been used to simplify the interpretation of data, because high resolution spectra are important in understanding the systems which have so far been investigated [43]. In order to determine AMP backbone conformation in multilamellar vesicles (MLVs), solid-state NMR techniques are used to measure the dipolar couplings under MAS [43]. Solid-state NMR experiments showed clear membrane orientation modifications with variable composition when they react with AMPs, including MSI-78 (also known as pexiganan, a magainin 2 analogue isolate from *Xenopus leavis*) and MSI-594 (hybrid of MSI-78, a magainin 2 analog) [43]. Several researchers have demonstrated how different AMPs act on membranes, using the NMR technique, such as pardaxin (from the Red Sea Moses sole, *Pardachirus marmoratus*), MSI-594, and MSI-367 [$(KFAKKFA)_3$-NH2] (Synthetic peptide previously formulated in silico) [38, 39, 46, 48, 55, 61–63, 96, 97], and also several review works have been published demonstrating the importance of NMR to AMP study [45, 98].

In order to examine how AMPs interact with LPS, Bhunia et al. [48] used NMR to evaluate how the MSI-594 peptide interacts with LPS micelles. The study determined the MSI-594 three-dimensional structure in a complex with LPS and also in a free form. Firstly, MSI-594 in free solution is completely unstructured. However, in the presence of *E. coli* and *S. typhimurium,* LPS of the two-dimensional Tr-NOESY spectra showed a large amount of NOE connectivity, indicating a peptide-folding structure. Strikingly, the MSI-594 LPS induced short helix (I2-K10) and longer C-termini helix (I13-L24) structures that were connected by a short loop (K11-G12). This new structure seems to help the MSI-594 cross the outer membrane LPS barrier [48]. By using a similar strategy, Bhunia et al. [39] elucidated the interactions of LPS and pardaxin (Pa4). This study was used for Pa4 three-dimensional structure elucidation in complex with LPS, showing the ability of Pa4 to disrupt the outer membrane. During this process, Pa4 adopts a clear helical conformation. The structure provided in LPS micelles showed two cationic residues (Lys^8 and Lys^{16}) in the middle of the N-terminus helix and at the beginning of the C-terminus, which can interact with the lipid A bisphosphate group in LPS. These interactions make it easier for hydrophobic residues to contact acyl LPS chains, facilitating membrane disruption [39]. Furthermore, aiming to evaluate AMP selectivity, a synthetic peptide called MSI-367 [$(KFAKKFA)_3$-NH_2], which was formulated in silico, was synthesized by Thennarasu et al. [96]. MSI-367 has ~48 % of helical propensity which folds a maximum of five helical turns under favorable conditions. Moreover, MSI-367 has nine positively charged lysines that possibly bind in negatively charged lipids on bacterial membranes. ^{31}P NMR data indicated modifications in the lipid orientation and bilayers caused by MSI-367 peptide binding. NMR data, also obtained in the presence of *E. coli* lipids, showed the presence of lipid–peptide electrostatic interactions, embracing the peptide at the lipid–water interface and providing the basis for bacterial cell selectivity [96].

In summary, the NMR technique could shed some light on how peptides behave in bacterial and eukaryote membranes, displaying what kind of interactions AMPs can develop with these lipidic bilayers. By using animal venom prototypes, studies with NMR have shown a real improvement in the characterization of AMPs derived from snake venoms. This technology could also demonstrate the types of modifications that snake venom peptides can cause in mammalian cell membranes, providing novel insights into peptide specificity. Nevertheless, at the moment, peptides derived from snake venoms have not been widely evaluated by solid-phase NMR, which also makes this technique promising for ophidian peptide studies.

It looks increasingly likely that many bacterial infection problems may be solved with the use of therapeutic properties which snake proteinaceous compounds can give us, and production may be solved by using novel technologies of chemical synthesis and heterologous expression. Therefore, further studies must be done to understand more about the mechanisms of action of these components and how they really act in the human body, so that they can be improved as new drugs to combat microorganism-resistant infections.

As previously described in this review, proteinaceous snake venom compounds could be extremely valuable in solving the problems of hospital infections, due to their incredible versatility and modes of action that provide a wide spectrum of activities, functions and low concentrations to be active.

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