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OVERVIEW

Harnessing snake venom phospholipases A_2 to novel approaches for overcoming antibiotic resistance

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Abstract

The emergence of antibiotic resistance drives an essential race against time to reveal new molecular structures capable of addressing this alarming global health problem. Snake venoms are natural catalogs of multifunctional toxins and privileged frameworks, which serve as potential templates for the inspiration of novel treatment strategies for combating antibiotic resistant bacteria. Phospholipases A_2 (PLA_2s) are one of the main classes of antibacterial biomolecules, with recognized therapeutic value, found in these valuable secretions. Recently, a number of biomimetic oligopeptides based on small fragments of primary structure from PLA_2 toxins has emerged as a meaningful opportunity to overcome multidrug-resistant clinical isolates. Thus, this review will highlight the biochemical and structural properties of antibacterial PLA_2s and peptides thereof, as well as their possible molecular mechanisms of action and key roles in development of effective therapeutic strategies. Chemical strategies possibly useful to convert antibacterial peptides from PLA_2s to efficient drugs will be equally addressed.

KEYWORDS

antibacterial peptides, antibiotic-resistant bacteria, peptidomimetics, phospholipase A_2, snake venom

1 | INTRODUCTION

In recent years, antibiotics have been responsible for saving thousands of lives, but their overuse has generated a dangerous public health problem of increasing significance (Ventola, 2015). It is predicted that, in the forthcoming 30 years, this health threat will become critical and cause more deaths than cancer (O'Neill, 2016). Several multidrug resistant bacterial strains have evolved, hindering and limiting the available therapeutics and cure of the patient (Chang et al., 2015). There are no boundaries for such microorganisms, whose spread to both developed and developing countries is dramatically calling for immediate solutions (da Costa, Loureiro, & Matos, 2013). Antibiotics are extremely valuable not only to treat infections, but also to prevent their possible onset in consequence of medical procedures, such as transplants, implants, surgery, and oncological treatment (Sommer, Munck, Toft-Kehler, & Andersson, 2017). However, pathogenic microorganisms have developed multiple strategies to escape conventional drugs (Ooi & O'Neill, 2017), making the antimicrobial resistance phenotype a huge challenge for researchers who face a race against time to identify new molecules and chemical structures with high potential to solve this issue (Buckland, 2017).

Natural products are a powerful window of opportunity to discover interesting compounds that can be used either per se or as models towards development of effective antibacterial drug candidates (Buss & Butler, 2004; Kingston, 2011). Snake venoms are a dynamic biochemical library of structurally and functionally diverse molecules, with a broad repertoire of activities ranging from lethal capacity to pharmaceutical and biotechnological applications (Almeida et al., 2017; Simoes-Silva et al., 2018). The biological and chemical diversities of venom compounds have play a crucial role in development of different medicines available in therapeutics and molecular tools used for research purposes (da Silva et al., 2011; Simoes-Silva et al., 2018). Since 1948, researchers have described the excellent antibacterial properties of snake venoms. Glaser (1948) reported the ability of venoms of two snake species of the genus *Crotalus*, to induce the death of human pathogens, such as *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. Several subsequent works have revealed and confirmed the antibiotic potential of secretions from

different families of snakes (Samy et al., 2007), thus constituting assorted biochemical libraries from which a variety of fascinating antibacterial biomolecules can be identified, isolated, and characterized (Figure 1). Among the numerous toxins from these natural collections, phospholipases A_2, L-amino acid oxidases, C-type lectin-like proteins, metalloproteases, cathelicidins, cardiotoxins, crotamine, cysteine-rich secretory proteins, disintegrins and serine proteases are the main biomolecules related to antibacterial activity (Chan et al., 2016; de Oliveira Junior, Silva Cardoso, & Franco, 2013). The snake venom proteomes are well diversified, varying in the classes and number of expressed proteins, as well as in the spectrum of biological effects (Fox & Serrano, 2008). In this way, these exciting cocktails of molecules represent one attractive open door to the search for novel antibiotic candidates capable to combat a wide range of Gram-positive and Gram-negative clinically relevant bacteria (Samy, Sethi, & Lim, 2016).

Phospholipases are amongst the best studied and characterized antibacterial venom proteins, which are not necessarily capable of hydrolyzing phospholipids (Samy et al., 2014). However, independent of their catalytic role they exhibit multiple biological activities of pharmacological interest (Lomonte, Angulo, & Moreno, 2010). Both isoforms found, Asp49 and Lys49 PLA_2s, have been efficient agents against multiple pathogenic bacteria, including drug-resistant strains (Páramo et al., 1998; Samy et al., 2014). The presence and quantity of PLA_2 isoforms vary according to each snake venom (Lomonte, Angulo, Sasa, & Gutierrez, 2009; Lomonte & Rangel, 2012), and their

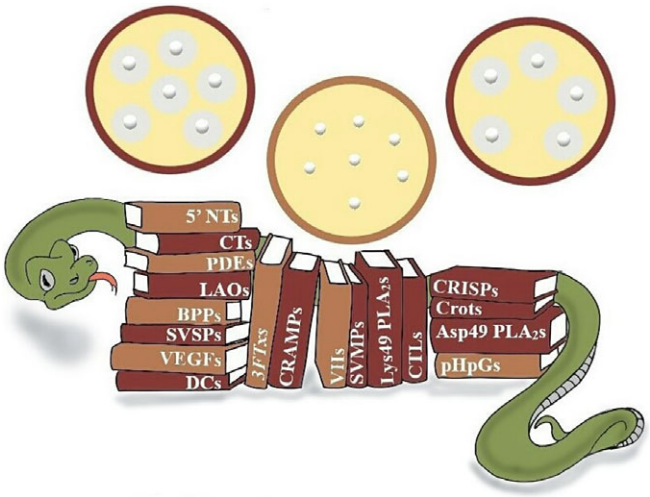

FIGURE 1 Snake venom: A complex library of valuable biomolecules, comprising diverse proteins and peptides with prominent antibacterial, including against multidrug-resistant clinical isolates. The main snake venom antibiotic components are highlighted in darker color. 5′ NTs: 5′-nucleotidases, CTs: Cardiotoxins, PDEs: Phosphodiesterases, LAOs: L-aminooxidases, BPPs: Bradykinin-potentiating peptides, SVSPs: Serine proteases, VEGFs: Snake venom vascular endothelial growth factors, DCs: Disintegrins, 3FTxs: Three-finger toxins, CRAMPs: Cathelin-related antimicrobial peptides, VHs: Venom hyaluronidases, SVMPS: Metalloproteinases, Lys49 PLA_2s: Catalytically inactive phospholipases, CTLs: C-type lectin-like proteins, CRISPs: Cysteine-rich secretory proteins, Crots: Crotamines, Asp49 PLA_2s: Catalytically active phospholipases, and pHpGs: Poly-Gly peptides

encouraging antibacterial activity has led to the synthesis and evaluation of small cationic oligopeptides inspired in PLA_2s (Costa et al., 2008; Lomonte et al., 2010). Such peptides are emerging as a new hope in these alarming times of world-spreading failure of antibacterial medicines currently available in the clinics.

In connection with the above, this review will focus on the prominent antibacterial activity of PLA_2s from snake venoms and their usefulness as a source of inspiration for the design of new antibacterial peptide-based molecules. Particular attention will be given to their structural and biochemical characteristics, mechanisms of action, limitations to their pharmaceutical use, and chemical strategies to overcome such limitations.

2 | OVERVIEW OF PLA_2S: BIOLOGICAL ROLES AND GENERAL ASPECTS

PLA_2s A_2 (EC 3.1.1.4) are a family of ubiquitous, multifunctional and pharmaceutically important toxins, generally responsible for Ca^{2+}-dependent catalytic hydrolysis of the *sn*-2 ester bond of membrane phospholipids, releasing fatty acids and lysophospholipids (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011; Fleer, Verheij, & de Haas, 1981; Ohno et al., 1997). One of the earliest enzyme of this superfamily that was studied in some detail was isolated from cobra venom at the end of the 19th century (De, 1944; Dennis et al., 2011; Harris & Scott-Davey, 2013; Wahlström, 1971), but due to their diversity and important toxicological, biotechnological, and pharmacological properties, snake venom PLA_2s (svPLA_2s) remain an active field of research, and a large number has been isolated and characterized until the present day. In fact, up to the time at which this review was prepared, and according to the National Center for Biotechnology Information (NCBI) and UniProt databases, over 450 different svPLA_2s had been characterized. Unfortunately, not all have their complete primary and tertiary structures elucidated, but this number represents a huge source of information to feed structure–activity relationships (SAR) studies towards the design of new functional molecules of pharmaceutical interest.

Most PLA_2s show a high conservation of protein structure and folding with an amino acid identity between 40% and 99% (Kini, 2003). They present an intriguing, wide range of biologically and pharmacologically relevant activities despite their high three-dimensional similarities (Lomonte & Rangel, 2012). Mammalian PLA_2s, for example, play a key role in cell proliferation and signaling (Hooks & Cummings, 2008), contraction of involuntary smooth muscles (Murthy & Makhlouf, 1998), and inflammatory processes (Nevalainen, 1993). On the other hand, most of the PLA_2s secreted from venomous animals are versatile proteins that do not always show enzymatic activity, but can induce myotoxicity (Almeida et al., 2016; Halpert & Eaker, 1976), inflammation (Pruzanski, Vadas, & Fornasier, 1986; Resende et al., 2017), as well as antibacterial (Forst et al., 1986; Sudarshan & Dhananjaya, 2014), antiviral (Cecilio et al., 2013), and antitumoral (Corin, Viskatis, Vidal, & Etcheverry, 1993; Gebrim et al., 2009) activities, among other biological effects (Rouault et al., 2006). Generally, svPLA_2s are among the main protein components of snake venoms (Bernardes et al., 2013; Boldrini-Franca et al., 2010; de Oliveira et al.,

2009). Proteomic and functional studies on snake venoms from *Agkistrodon contortrix contortrix* (Bocian et al., 2016), *Micrurus lemniscatus* (Casais et al., 2016), and *Agkistrodon piscivorus leucostoma* (Lomonte et al., 2014) have revealed that PLA_2s are the major component of these venom proteomes. In certain species of snakes, such as *Bungarus multicinctus*, svPLA_2s correspond to up 60% of the proteome, covering different isoforms (Bocian et al., 2016).

PLA_2s can be classified into more than 15 different groups, but the main 4 are secreted PLA_2s (sPLA_2), cytosolic PLA_2s (cPLA_2), calcium-independent PLA_2 (iPLA_2), and platelet activation factor acetyl hydrolases (PAF-AHs) also known as lipoprotein-associated phospholipase A_2, (LpPLA_2s) (Dennis et al., 2011; Vasquez, Mouchlis, & Dennis, 2018). The svPLA_2s are part of sPLA_2s (Burke & Dennis, 2009). Generally, sPLA_2s are characterized by a low molecular weight of between 13 and 15 kDa, Ca^{2+} bound at the active site when catalytically active, and six conserved disulfide bonds plus one or two variable disulfide bonds (Dennis et al., 2011). Most sPLA_2 have highly conserved Ca^{2+}-binding loop (XCGXGG) and catalytic site (DXCCXXHD), and similar protein folding (Burke & Dennis, 2009). This structural uniformity was observed amongst sPLA_2s as diverse as the cobra venom Group IA (GIA) (Fremont, Anderson, Wilson, Dennis, & Xuong, 1993), human (Xu, Yi, Feng, Chen, & Liu, 2009), bovine (Steiner et al., 2001) and porcine pancreatic Groups IB (Dijkstra, Renetseder, Kalk, Hol, & Drenth, 1983; Sekar, 2007), plant Group XIB (GXIB) (Guy, Stahl, & Lindqvist, 2009), and procaryotic Group XIV (GXIV) (Dennis et al., 2011; Matoba, Katsube, & Sugiyama, 2002). Hence, it expected that novel sPLA_2s whose structure remains to be elucidated will share a similar three-dimensional structure, although the same does not apply to biological activities that, as mentioned before, cover a quite broad spectrum.

Snake venoms contain PLA_2s from groups IA and IIA (Dennis et al., 2011), respectively, found in the families *Elapidae* and *Viperidae* (Samy et al., 2012). The snakes of the *Elapidae* family are found in the tropics and subtropics worldwide, while *Viperidae* can be found throughout the world, with a wider climate distribution (Wallach, Williams, & Boundy, 2014). svPLA_2s belonging to group II can be subdivided into two main types, commonly referred to as isoforms Asp49 and Lys49, which display unique interactions and different molecular mechanisms of action (Dennis et al., 2011; Maraganore et al., 1984). Asp49 PLA_2s are toxins that show catalytic activity, while Lys49 PLA_2s are catalytically inactive (Gutiérrez & Lomonte, 2013). The catalytically active isoform can still be divided into acidic and basic subisoforms, according to their primary structure and pI, which impart distinct catalytic and biological properties. Normally, acidic Asp49 PLA_2 isoforms have higher catalytic activity and lower toxicity than the basic isoforms (Jimenez-Charris et al., 2016). The functional roles of acid isoforms remain unclear and continue a matter of discussion and research (Resende et al., 2017). In contrast, the Lys49 isoforms are invariably basic and lack the ability to hydrolyze membrane phospholipids (Lomonte & Rangel, 2012). Interestingly, all PLA_2 isoforms have showed an incredible functional diversity regardless of their ability to hydrolyze phospholipids. Due to this wide range of biological effects they can be used for therapeutic purposes such as anticoagulant, antiviral, antineoplastic, and antibacterial (Cecilio et al., 2013; Rodrigues et al., 2004; Sobrinho et al., 2018). These activities are not limited to svPLA_2s, and can be found in, for example, mammalian sPLA_2s (Nevalainen, Graham, & Scott, 2008). The most representative mammalian sPLA_2s belong to group IIA (PLA_2 GIIA), and are known to display bactericidal properties, and integrate the innate immune system (Dominiecki & Weiss, 1999; Nevalainen et al., 2008). In turn, svPLA_2 have attracted special interest due to the wide variety of isoforms and pharmacological properties, determined not only by the species but also by the breeding conditions of the snake (Calvete, 2011; Lomonte et al., 2009). This encloses a remarkable panoply of biomolecules whose therapeutic value can be exploited, while challenging researchers towards development of mimetic compounds aimed at pharmaceutical applications.

3 | STRUCTURE AND MODES OF ACTION OF ANTIBACTERIAL svPLA_2S

The antibacterial activity of svPLA_2s is one of their most promising therapeutic properties (Samy et al., 2012; Samy, Stiles, Franco, Sethi, & Lim, 2017) due to the increasing antibiotic resistance that has been detected in recent years (Norrby, Nord, & Finch, 2005). Various in vitro studies (Barbosa et al., 2005; Diz Filho et al., 2009; Sudarshan & Dhananjaya, 2014; Toyama et al., 2005) have established bactericidal and bacteriostatic activities of svPLA_2s against both Gram-negative bacteria such as *Xanthomonas axonopodis pv. passiflorae*, *E. coli*, *Vibrio cholerae*, *Klebsiella pneumoniae* and *Salmonella paratyphi*, and Gram-positive bacteria such as *S. aureus* and *B. subtilis*. The average inhibitory doses vary depending on the specific svPLA_2. For instance, svPLA_2 BFPA, from the banded krait (*Bungarus fasciatus*), presents a minimal inhibitory concentration (MIC) of 0.4 μM for *E. coli* (Xu et al., 2007), whereas its heterologous svPLA_2 OS2, from the coastal taipan (*Oxyuranus scutellatus scutellatus*), presents an impressive MIC of 3.1 nM for the same bacterial species (Rouault et al., 2006). Some svPLA_2s have a wide range of bactericidal action, as in the case of svPLA_2 VRV-PL-V from the Russel's viper (*Daboia russelii pulchella*), which significantly inhibits the growth of six bacterial species (Sudarshan & Dhananjaya, 2014). Yet, other svPLA_2s such as svPLA_2 MTX-I from the Brazil's lancehead pit viper (*Bothrops brazili*), have so far been tested against a single microorganism and their activity range remains unknown (Costa et al., 2008). The presence and abundance of antibacterial PLA_2s in venoms can confer important evolutionary advantages for snakes, such as decreased prey decomposition by bacteria specialized in exploiting organic matter and protection of the venom glands against bacterial infections (Lomonte & Rangel, 2012).

Generally, the antibacterial svPLA_2s are purified by one or two chromatographic methods, mainly reversed-phase high-performance liquid chromatography (RP-HPLC), or molecular exclusion and ion exchange (Stábeli, Simões-Silva, Kayano, & Calderon, 2012). The structural characteristics of these proteins are studied mainly by sequence analysis, molecular modeling, spectrometry, crystallography, circular dichroism (CD), and nuclear magnetic resonance (NMR) (Cox, Evans, Packman, Williams, & Woolfson, 1993; Vandermarliere, Stes, Gevaert, & Martens, 2014; Waudby, Launay, Cabrita, & Christodoulou, 2013). Enzymatic kinetics are investigated by potentiometric and

spectrophotometric techniques using natural and synthetic chromogenic substrates, such as 4-nitro-3-(octanoyloxy)benzoic acid (NOB) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol (β-Py-C10-HPG) (Dennis et al., 2011; Martins et al., 2014).

According to the NCBI database, there are approximately 35 svPLA$_2$s characterized and described in the literature regarding their antibacterial properties. This represents only 7.8% of all svPLA$_2$s studied so far, but it is important to consider that not all characterized phospholipases have been tested against bacteria. Antibacterial svPLA$_2$s have a molecular mass of approximately 14 kDa show a high similarity in their primary structure, sharing highly conserved regions, such as the active site and the calcium-binding region; they have variable pI, although most of them are basic, with many cationic regions in their structures (Nevalainen et al., 2008). These cationic domains are likely crucial for antimicrobial activity, as bioinformatic analysis tools such as AMPA (Antimicrobial Sequence Scanning System) revealed that regions with highest antibacterial potential in svPLA$_2$s are the positively charged C-terminal regions, which present considerable similitude with many host defense peptides. Table 1 summarizes the main properties of antibacterial svPLA$_2$s characterized thus far, and whose antibacterial effects have been studied through standard in vitro techniques, mainly agar well diffusion assay and Broth microdilution method.

As shown by data in Table 1, antibacterial properties are not exclusive to catalytically active Asp49 PLA$_2$s. Assays with Lys49 PLA$_2$s and Asp49 PLA$_2$s inactivated either chemically or by site-directed mutagenesis demonstrated conservation of antimicrobial activity independently of the catalytic hydrolysis of the glycerofospholipid *sn*-2-ester bond (Dhillon et al., 1987; Páramo et al., 1998). Examples of antibacterial catalytic and noncatalytic phospholipases are, respectively, Asp49 svPLA$_2$ BthA-I from the jararacussu pit viper (*Bothrops jararacussu*), and Lys49 PLA$_2$ BnpTX-I from the Neuwied's lancehead pit viper (*Bothrops pauloensis*).

The molecular mechanisms underpinning the antibacterial effect of svPLA$_2$s are varied and, unlike antibacterial PLA$_2$ from mammals, svPLA$_2$s do not require a complement protein to promote Gram-negative cell lysis (Koduri et al., 2002; Samy et al., 2012). Mechanisms of bacterial cell wall destabilization by svPLA$_2$s have been put forward in several literature reports. Samy, Gopalakrishnakone, Ho, and Chow (2008) have shown that the walls of *S. aureus* treated with an Asp49 PLA$_2$ were severely damaged promoting the release of cellular content compared to the untreated control. This basic toxin promoted morphological alterations such as permeabilization and damage to the bacterial membrane in *S. aureus*. Páramo et al. (1998) proposed a catalytically independent bactericidal mechanism exerted by an isoform Lys49 PLA$_2$. The cationic antimicrobial proteins exert their bacterial inhibitory effect through a process of thinning and destabilization of the membrane lipid bilayer, resulting in the permeabilization and expulsion of cellular content (Glukhov, Stark, Burrows, & Deber, 2005; Heller et al., 2000). On the other hand, in the case of anionic antibacterial proteins, a protein-lipid head group interaction is proposed that would have a similar effect: bacterial lysis (Dennison et al., 2006). Furthermore, apart from protein-mediated lysis, it has been proposed that PLA$_2$s also inhibit the biosynthesis of macromolecules and promote the expression of autolytic enzymes (Otvos Jr. et al., 2000; Samy et al., 2012).

Other studies have proposed that C-terminal derived from svPLA$_2$ has the bactericidal effect and that it explains its therapeutic activity (Lomonte et al., 2010). This molecular region is composed by cationic and hydrophobic amino acids, with a length that varies from 12 to 16 amino acids and an elevated affinity for anionic phospholipids (Costa et al., 2008; Diaz et al., 2001). The dose requirements for this bactericidal mechanism are greater than for the process driven by mammalian antibacterial sPLA$_2$ enzymes (Santamaria, Larios, Quiros, et al., 2005). However, due to its short length and versatility, (Lomonte, Pizarro-Cerda, Angulo, Gorvel, & Moreno, 1999) small biomimetic peptides inspired have been synthetized, which have shown a hopeful activity not only antibacterial, but also anticancer (Nevalainen et al., 2008; Páramo et al., 1998).

4 | svPLA$_2$S-INSPIRED ANTIMICROBIAL PEPTIDES

It is now established that both Asp49 and Lys49 svPLA$_2$s are interesting macromolecules with multiple activities impelling clinical and therapeutic treatments (Cecilio et al., 2013; Lomonte et al., 2010). Though some studies have validated the antibacterial potential of C-terminal region of Asp49 PLA$_2$s (Costa et al., 2008), multiple studies have been carried out on Lys49 PLA$_2$s, due to the independence of catalytic activity suggesting the presence of noncatalytic molecular regions capable of exerting different bioactivities. In this connection, Lomonte, Moreno, Tarkowski, Hanson, and Maccarana (1994) demonstrated for the first time that biomimetic peptides replicate the effects of their parent svPLA$_2$s. Since this pioneering study, many synthetic peptides based on the same molecular regions were evaluated for a better understanding of the mechanisms of myotoxic, antibacterial, antiviral, and antitumoral actions of Lys49 svPLA$_2$s (Almeida et al., 2018; Gebrim et al., 2009; Lomonte et al., 2010). Therefore, these catalytically inactive toxins are taken as valuable templates for creation and innovation in antibiotherapy, building on smaller peptide segments with antibacterial potential (Figure 2). However, antibacterial oligopeptides reproducing the C-terminal sequences of Lys49 svPLA$_2$s have also other types of bioactivity, from toxic effects such as neurotoxicity and myotoxicity, to therapeutically relevant effects, such as antitumoral properties (Lomonte et al., 2010). This molecular region, usually with an extension of 13 amino acids, is characterized by the presence of basic cationic residues, similarly to antimicrobial peptides (AMPs) (Shagaghi, Palombo, Clayton, & Bhave, 2016), and seems to be fundamental for the antibacterial properties of Lys49 svPLA$_2$s. One of the main advantages of AMPs, like svPLA$_2$s-inspired antibacterial peptides, is their very low probability of generating resistance to antibiotics (Memariani et al., 2017).

To date, more than 15 C-terminal peptides derived from Lys49 svPLA$_2$s have been synthetized, notwithstanding different biological activities were evaluated, from toxic to pharmacological effects. Biomimetic antimicrobial peptides inspired in this region have been reported (Table 2). They have an average molecular mass of between 1,500 and 1,800 Da, which both avoid immune allergic reactions

TABLE 1 Antibacterial PLA_2s from snake venoms

Isoforms PLA_2	PLA_2 toxin	Snake species	pI	MW	Active against	References
Lys49	Basic PLA_2	*Agkistrodon halys blomhoffii*	10.2	13,982	*Escherichia coli*	Forst et al. (1986)
	mt-I	*Bothrops nummifer*	nd	16,000	*Salmonella typhimurium*	Gutiérrez, Lomonte, and Cerdas (1986); Santamaria et al. (2005)
	mt-II	*Bothrops godmani*	8.9	13,400	*S. typhimurium*	Arni et al. (1999); Diaz, Gutierrez, and Lomonte (1992); Santamaria et al. (2005)
	mt-I	*Bothrops schlegelii*	9.3	15,000	*S. typhimurium*	Angulo et al. (1997); Santamaria, Larios, Angulo, et al. (2005)
	mt-II	*Bothrops asper*	9.1	13,341	*S. typhimurium*	Lomonte and Gutiérrez (1989); Santamaria, Larios, Angulo, et al. (2005)
	mt-IV	*Bothrops asper*	nd	15,500	*S. typhimurium*	Diaz, Lomonte, Zamudio, and Gutierrez (1995); Santamaria, Larios, Angulo, et al. (2005)
	BthTX-I	*Bothrops jararacussu*	9.0	14,124	*E. coli* and *Staphylococcus aureus*	Barbosa et al. (2005)
	MjTX-II	*Bothrops moojeni*	8.2	13,887	*E. coli*	Stabeli et al. (2006)
	BFPA	*Bungarus fasciatus*	7.5	13,288	*E. coli* and *S. aureus*	Xu et al. (2007)
	MTX-II	*Bothrops brazili*	8.2	13,965	*E. coli*	Costa et al. (2008)
	CoaTx-II	*Crotalus oreganus abyssus*	8.7	13,868	*Pseudomonas aeruginosa* 31NM, *E. coli* ATCC 25922, *S. aureus* BEC9393 and *Rib1*	Almeida et al. (2016)
	BnuTX-I	*Bothrops neuwiedi urutu*	nd	13,373	*E. coli* (ATCC 25922), *S. aureus* (ATCC 29213), *P. aeruginosa* (ATCC 27853) *and Klebsiella pneumoniae* (ATCC 13883)	Correa et al. (2016)
	LmutTX	*Lachesis muta muta*	≈8.6	13,890	*S. aureus*, methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*	Diniz-Sousa et al. (2018)
Asp49 basic	mt-I	*Bothrops godmani*	8.2	14,300	*S. typhimurium*	Diaz et al. (1992), Santamaria, Larios, Angulo, et al. (2005)
	mt-I	*B. asper*	8.72	10,700	*S. typhimurium*	Gutiérrez, Ownby, and Odell (1984), Santamaria, Larios, Angulo, et al. (2005)
	mt-III	*B. asper*	9.5	nd	*S. typhimurium*	Kaiser, Gutierrez, Plummer, Aird, and Odell (1990), Santamaria, Larios, Angulo, et al. (2005)
	F17	*Crotalus durissus terrificus*	8.2	14,664	*Xanthomonas axonopodis pv. passiflorae*	Oliveira, Toyama, Novello, Beriam, and Marangoni (2002)
	F15	*Crotalus durissus terrificus*	8.9	14,500	*Xanthomonas axonopodis pv. passiflorae* and *Clavibacter michiganensis michiganensis*	Toyama et al. (2003)
	BnpTX-I	*Bothrops pauloensis*	7.8	14,000	*E. coli* and *S. aureus*	Rodrigues et al. (2004)
	BnpTX-II	*B. pauloensis*	nd	14,000	*E. coli* and *S. aureus*	Rodrigues et al. (2004)
	BthTX-II	*B. jararacussu*	8.5	14,056	*E. coli* and *S. aureus*	Barbosa et al. (2005)
	Crototoxin	*Crotalus durissus collilineatus*	8.3	14,000	*Xanthomonas axonopodis pv. passiflorae*	Toyama et al. (2005)
	OS2	*Oxyuranus scutellatus scutellatus*	8.4	13,317	*E. coli*	Rouault et al. (2006)
	MTX-I	*Bothrops brazili*	8.0	13,870	*E. coli*	Costa et al. (2008)
	PLA_2A	*Crotalus durissus ruruima*	8.7	14,299	*X. axonopodis*	Diz Filho et al. (2009)
	VRV-PL-V	*Daboia russellii pulchella*	7.3	13,587	*S. aureus*, *Bacillus subtilis*, *E. coli*, *Vibrio cholerae*, *K. pneumoniae*, and *Salmonella paratyphi*	Sudarshan and Dhananjaya (2014)
	VRV-PL-VIIIa	*Daboia russelii pulchella*	8.4	13,611	*S. aureus*, *B. subtilis*, *E. coli*, *V. cholerae*, *K. pneumoniae* and *S. paratyphi*	Sudharshan and Dhananjaya (2015)
Asp49 acidic	BthA-1	*B. jararacussu*	5.3	13,685	*E. coli* and *S. aureus*	Roberto et al. (2004)
	Daboiatoxin (DbTx)	*Daboia russelli siamensis*	4.6	15,000	*Burkholderia pseudomallei* (Strains KHW and TES) and *S. aureus*	Samy et al. (2006); Samy et al. (2007)

(*Continues*)

TABLE 1 (Continued)

Isoforms PLA_2	PLA_2 toxin	Snake species	pI	MW	Active against	References
	$PnPLA_2$	*Porthidium nasutum*	4.6	15,803	*S. aureus* ATCC 25923 and ATCC 29213	Vargas et al. (2012)
	$BmooPLA_2$	*Bothrops moojeni*	5.2	13,601	*S. aureus* and *E. coli*	Silveira et al. (2013)
	NN-XIb-PLA_2	*Naja naja*	nd	nd	*S. aureus*, *B. subtilis*, *E. coli*, *V. cholerae*, *K. pneumoniae* and *S. paratyphi*	Sudarshan and Dhananjaya (2016)
Not defined	Mulgatoxin	*Pseudechis australis*	nd	13,200	*B. pseudomallei* (Strains KHW and TES)	Samy et al. (2006)
	Mb-PLA_2	*Montivipera bornmuelleri*	nd	13,650	*E. coli*, *P. aeruginosa*, and *S. aureus*	Accary, Mantash, Mallem, Fajloun, and Elkak (2015)
	$WaPLA_2$	*Walterinnesia aegyptia*		14,000	*B. cereus*, *B. subtilis*, *Enterococcus faecalis*, *Staphylococcus epidermidis*, *S. aureus*, *E. coli*, *K. pneumonia*, *P. aeruginosa*, and *S. enteric*	Bacha, Alonazi, Elshikh, and Karray (2018)

when administered in vivo and promotes rapid diffusion (Lomonte et al., 2010). Moreover, given their short linear sequences, their chemical synthesis is much simpler than those of much longer or structurally complex (e.g., containing several disulfide bridges) AMPs (Lomonte et al., 2010). The net charge of AMP from the C-terminal region of Lys49 $svPLA_2$s is +5 or +6 at neutral pH, reflecting their cationic nature that is important for the initial interaction of the peptides with the anionic bacterial membranes (Memariani et al., 2017). These small AMPs have a hydrophobicity between +18.33 kcal mol^{-1} and +22.24 kcal mol^{-1}, and their average pI ranges from 9.90 to 10.14. Interestingly, despite the 20 standard amino acids encoded by eukaryotic DNA, the C-terminal region of Lys49 $svPLA_2$s make use of only 10 such amino acids and exclude negatively charged ones. Actually, this region, and AMPs thereof, show high degree of conservation at certain amino acid residues, namely, at positions 1, 2, 8, 12 and 13, which are exclusively occupied by Lys residues. Arg and His also are highly conserved (Figure 3a,b), which altogether reinforces the importance of these cationic amino acids for the antibacterial properties displayed by the C-terminal region of Lys49 $svPLA_2$s. These biochemical and structural characteristics play a fundamental role for the mechanism of antibacterial action, as discussed further below.

In addition, several variants of antibacterial Lys49 $svPLA_2$s C-terminal peptides have been synthesized, with the intention of improving their therapeutic profile, by (a) increasing activity and/or stability against degradation by proteases, (b) reducing toxicity, and (c) establish relevant SAR towards a better understanding of their mechanism of action in order to guide rational design of more potent and safer mimetics (Lomonte et al., 1999; Santamaria, Larios, Angulo, et al., 2005; Santamaria, Larios, Quiros, et al., 2005) (Table 3). In several cases, sequence modifications have consisted in inserting hydrophobic residues known as relevant in many AMPs, especially Trp (Diniz-Sousa et al., 2018; Santamaria, Larios, Angulo, et al., 2005). Trp and other hydrophobic residues are larger than other amino acids usually found in Lys49 $svPLA_2$s C-terminal peptides, hence their modified

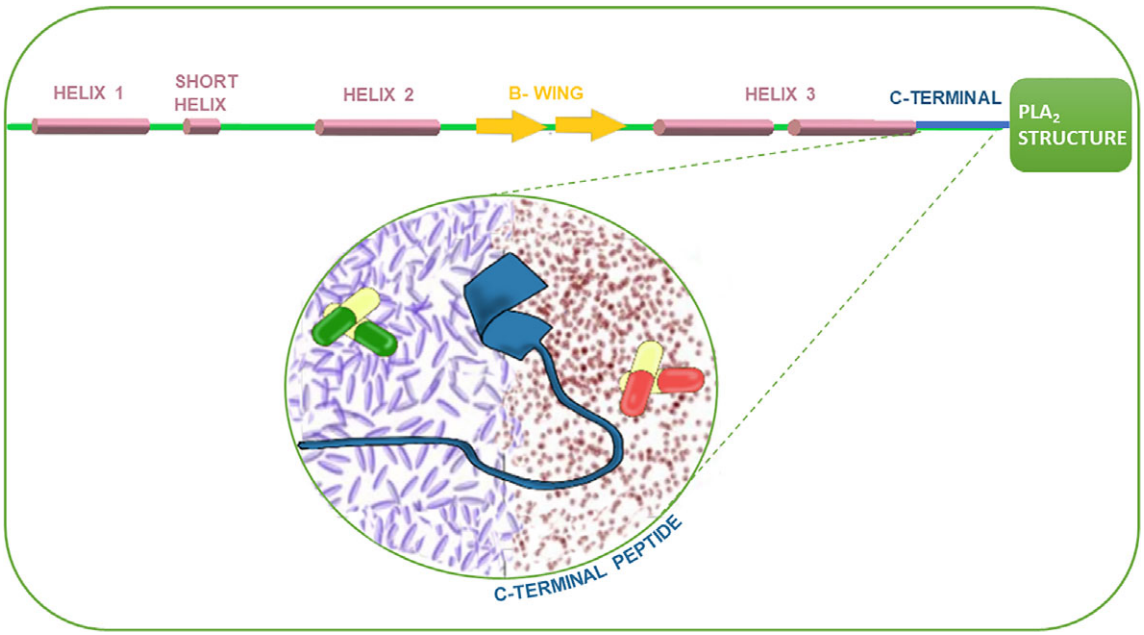

FIGURE 2 C-terminal antibacterial region of $svPLA_2$s. Cationic peptides mimicking this PLA_2 are equally capable to kill both Gram-positive and Gram-negative bacteria

TABLE 2 Physicochemical properties of antibacterial peptides inspired in Lys49 PLA_2 toxins from snake venoms. Physicochemical descriptors were determined in silico using tools available on the ExPASy server

Peptides	PLA_2 toxin template	Snake species	pI	Net charge[a]	Average molecular weight	Hydrophobicity (kcal mol^{-1})[b]	Bioactive against	References
p115–129	Myotoxin II	*Bothrops asper*	10.02	+6	1,731.18	+19.20	*Vibrio cholerae* O1 Ogawa S-LPS, *V. cholerae* O1 Inaba R-LPS, *Brucella abortus* 45/20 R-LPS attenuated, *Escherichia coli* 29,648 S-LPS, *E. coli* K-12 W-1485 serotype Ra, *Salmonella typhi* 6,539 S-LPS, *Shigella sonnei* 25,931 S-LPS, *Staphylococcus aureus* Cowan-1	Páramo et al. (1998)
pepMTX-II	MTX-II	*Bothrops brazili*	10.14	+6	1,705.14	+22.24	*E. coli* ATCC 29648	Costa et al. (2008)
p-BthTX-1 $(p\text{-BthTX-1})_2$	Bothtropsin-1	*Bothrops jararacussu*	10.14	+6	1,739.16	+21.78	*E. coli* ATCC 25922, *Staphylococcus epidermidis* ATCC 35984, *S. aureus* ATCC 25923, *S. aureus* SA16, *S. aureus* SA33, *S. aureus* SA88, *S. aureus* SA90, *Enterococcus faecium* VRE16, *E. faecium* HSJRP8, *Klebsiella pneumoniae* ATCC 700603, *K. pneumoniae* ATCC BAA1705, *E. coli* ATCC 35218, *E. coli* CA4	Santos-Filho et al. (2015); Santos-Filho et al. (2017)
PepC	LmutTX	*Lachesis muta muta*	9.90	+5	1,559.93	+18.49	Methicillin-resistant *S. aureus* (MRSA)	Diniz-Sousa et al. (2018)
pC-CoaTxII	CoaTx-II	*Crotalus oreganus abyssus*	10.14	+6	1,715.18	+18.33	*Pseudomonas aeruginosa* 31NM, *P. aeruginosa* ATTC 27853, *E. coli* ATCC 25922, *S. aureus* BEC9393, *S. aureus* rib1	Almeida et al. (2018)

[a] Net charge corresponds to the sum of basic and acidic amino acid residues at neutral pH.
[b] Hydrophobicities were expressed based on Wimley-White scale, assuming neutral pH.

versions have not only variable hydrophobicity (ranging from +8.93 kcal mol^{-1} to +20.97 kcal mol^{-1}) but also higher mass, typically between 1,600 and 2,000 Da (Shagaghi et al., 2016). A more detailed description of the most relevant AMPs developed upon modification of Lys49 $svPLA_2$s C-terminal peptides follows, including some considerations on their mechanisms of action.

Historically, the first biomimetic antimicrobial peptide derived from a PLA_2 toxin to be reported was p115–129, a peptide inspired in myotoxin II from the highly venomous pit viper *Bothrops asper* (Lomonte et al., 1994). This peptide was evaluated against a large panel of microbes, including Gram-positive and Gram-negative bacteria, showing higher antibacterial activity than the parent myotoxin, while being equally nonhemolytic. Transmission electron microscopy (TEM) analysis showed p115–129 to induce alterations in the morphology of the bacterial membranes, as well as the internal formation of granules in *V. cholerae* and *S. aureus* (Páramo et al., 1998). These findings led to the chemical synthesis of the first modified peptide based on the sequence of peptide segments derived from PLA_2s: peptide p115-W3, which is a variant of p115–129 possessing an increased content in hydrophobic amino acids. Peptide p115-W3 has

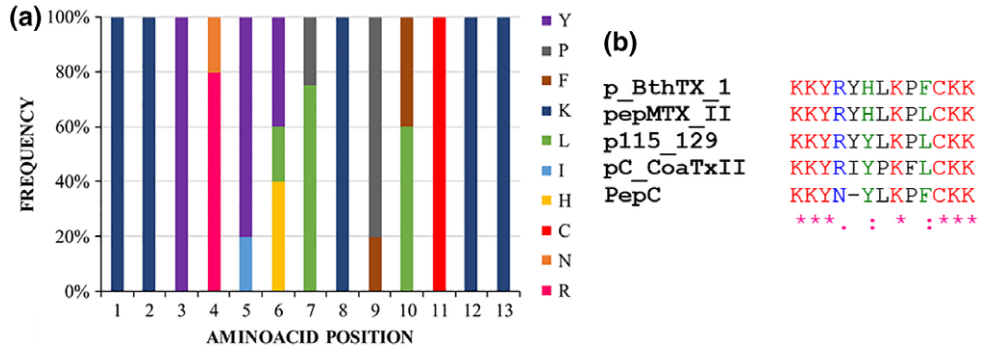

FIGURE 3 Primary structure of antibacterial peptides mimicking Lys49 PLA_2s C-terminus. (a) Frequency of amino acid residues per position. (b) Multiple sequence alignment

TABLE 3 Physicochemical properties of antibacterial variant peptides inspired by biomimetic peptides derived from Lys49 PLA_2 toxins. Physicochemical parameters were determined in silico using tools available on the ExPASy server

Modified peptides	Parent biomimetic peptide	Snake species	pI	Net charge[a]	Average molecular weight	Hydrophobicity (kcal mol^{-1})[b]	Bioactive against	References
p115-W3	p115–129	*Bothrops asper*	10.60	+6	1,800.29	+15.06	*Escherichia coli* ATCC 29648, *Salmonella enterica* serovar *Typhimurium* D984, *Staphylococcus aureus* ATCC 23923, *Brucella abortus* 45/20	Lomonte et al. (1999), Santamaria, Larios, Quiros, et al. (2005)
pEM-1			11.39	+6	1,768.23	+15.58	*S. typhimurium* ATCC 14028, *S. aureus* ATCC 23923	Santamaria, Larios, Angulo, et al. (2005)
pEM-2			11.39	+6	1,742.19	+15.94	*S. typhimurium* ATCC 14028, *S. aureus* ATCC 23923, *S. enterica* serovar *Typhimurium* D984, *Pseudomonas aeruginosa* ATCC 27853, *S. sonnei* ATCC 25931, *V. cholerae* Owaga IMS 124, *E. coli* 25,922, *Enterococcus faecalis* ATCC 29212, *Klebsiella pneumoniae* ATCC 13883, *B. abortus* 45/20, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 19636, *P. aeruginosa* ATCC 9027, *P. aeruginosa* (CI 05), *P. aeruginosa* (CI 11), *Acinetobacter baumannii* (CI 02), *A. baumannii* (CI 06), *S. aureus* (CI 07), *S. aureus* (CI 12), *E. faecalis* (CI 02), *E. faecalis* (CI 04)	Santamaria, Larios, Angulo, et al. (2005), Santamaria, Larios, Quiros, et al. (2005)
pEM-3			10.14	+6	1,754.21	+17.82	*S. typhimurium* ATCC 14028, *S. aureus* ATCC 23923	Santamaria, Larios, Angulo, et al. (2005)
pEM-4			10.14	+6	1,754.21	+17.82		
pEM-5			10.14	+6	1,754.21	+17.82		
pEM-6			10.19	+5	1,649.08	+13.64		
pEM-7			10.31	+6	1,777.25	+16.44		
pEM-8			10.31	+6	1,777.25	+16.44		
pEM-9			10.70	+6	2,073.53	+8.93		
PepC-W	PepC	*Lachesis muta muta*	10.20	+5	1,606.01	+17.11	Methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* ATCC 27853	Diniz-Sousa et al. (2018)
pEM-2-W5K	pEM-2	*B. asper*	11.43	+7	1,684.15	+20.83^{-}	*E. coli* ATCC 25922, *S. aureus* ATCC 25923, *S. aureus* ATCC 29213, *S. aureus* ATCC 19636, *P. aeruginosa* ATCC 27853, *P. aeruginosa* ATCC 9027	Yu et al. (2010)
pEM-2-W5A/A9W			11.39	+6	1,742.19	+15.94		
pEM-2-W5K/A9W			11.43	+7	1,799.28	+18.24^{-}		
PV	pEM-2 MP-VT1[c]	*B. asper and peptides from other venoms*	11.39	+6	1,968.51	+13.44^{-}	*P. aeruginosa* (CI 05), *P. aeruginosa* (CI 11), *A. baumannii* (CI 02), *A. baumannii* (CI 06), *S. aureus* (CI 07),*S. aureus* (CI 12), *E. faecalis* (CI 02), *E. faecalis* (CI 04)	Memariani et al. (2017)
BVP	pEM-2 MP-B[c] MP-VT1[c]		10.60	+5	1,582.05	+16.94^{-}		
PVP	pEM-2 MP-VT1[c]		11.43	+7	1,726.23	+19.08^{-}		
PV3	pEM-2 MP-VT1[c]		11.47	+8	1,823.43	+20.97		

[a] Net charge corresponds to the sum of basic and acidic amino acid residues at neutral pH.
[b] Hydrophobicities were expressed based on Wimley-White scale, assuming neutral pH.
[c] Antibacterial hybrid peptides, which combined molecular regions of pEM-2 and peptides from other venoms, such as mastoparan-VT1 and mastoparan-B.

three Trp residues replacing three Tyr residues of the parent sequence, which led to increased activity against *E. coli*, possibly owing to an improved interaction with the cell membrane of this Gram-negative bacterial species (Lomonte et al., 1999).

In a subsequent study, a series of 10 substituted peptides inspired in p115–129 were synthesized and tested for their antimicrobial activity. One particular sequence, pEM-2, stood out for its significantly higher antibacterial activity as compared to all the others, and overall results suggested that higher activity was observed for sequences possessing three Trp residues (Santamaria, Larios, Angulo, et al., 2005). Later, the D-enantiomer of pEM-2 was also produced and tested, equally showing antibacterial effects against Gram-negative bacteria such as *Pseudomonas aeruginosa* and *V. cholerae*; interestingly, both enantiomers displayed higher antibacterial activity than the well-known antimicrobial protein lactoferricin B. Moreover, an endotoxin neutralization assay was also carried out, comparing the same pair of enantiomers with polymyxin B, a clinically relevant AMP: the L-enantiomer was equipotent to polymyxin B, whereas the D-enantiomer was slightly less active. In addition, a low cytolytic activity of pEM-2 on muscle cells was determined, indicating feeble interaction with eukaryotic cells (Santamaria, Larios, Quiros, et al., 2005).

Myotoxin II from the pit viper *B. brazili* (Brazilian lancehead) equally possesses an antibacterial C-terminal sequence, on which peptide pepMTX-II was inspired and evaluated against *E. coli* ATCC 29648. Although the peptide showed promising activity, it was lower than that of the parent protein, MTX-II. Moreover, further cytotoxicity assays using *Candida albicans* and parasite cell lines indicated that pepMTX-II lacks selectivity between eukaryotic and prokaryotic cells (Costa et al., 2008).

In addition to their antimicrobial properties, the toxicity of svPLA$_2$s-inspired peptides is a critical issue that must be considered. For this reason, in 2010, pEM-2 was used as a structural template to generate antibacterial analogues with more attenuated hemolytic activity (Yu et al., 2010). In one such analog, pEM-2-W5K, Trp5 was replaced by Lys, which promoted higher selectivity for bacterial cells, that is, the antibacterial properties were preserved, whereas lytic activity on human red blood cells (RBC) was significantly reduced. Another analog, pEM-2-W5K/A9W, consisted in the substitution of residues Trp5 and Ala9 by Lys and Trp, respectively. This led to a more potent antibacterial action, but also increased the hemolytic effect (Yu et al., 2010).

Gebrim et al. (2009) worked with peptide p-BthTX-I, inspired in the C-terminal region of Bothropstoxin-1, from the *B. jararacussu* snake venom. This peptide was initially highlighted for its antitumor activity, but 6 years later, both the peptide monomer and its disulfide bridged dimer, (p-BthTX-I)$_2$, were evaluated against Gram-positive and Gram-negative bacteria (Santos-Filho et al., 2015) with promising results, whereby the dimer revealed stronger activity against *E. coli* and *S. aureus* than the monomer. In addition, upon incubation of the p-BthTX-I monomer in the assay medium, formation of the (p-BthTX-I)$_2$ dimer was observed in a few minutes and reached completion (i.e., full monomer-to-dimer conversion) within 6 h. Considering this fact, the authors suggested that the antibacterial effect displayed by p-BthTX-I was probably and mainly due to its dimeric form. Noticeably, the peptide showed no effect against *C. albicans*, epithelial cells, erythrocytes and macrophages, demonstrating in this way that it is selective against prokaryotic cells (Santos-Filho et al., 2015).

One of the main problems of peptide-based drugs in clinical applications is their low oral bioavailability, due to, among other factors, degradation by serum proteases (Arias, Piga, Hyndman, & Vogel, 2018). With this in mind, the stability of (p-BthTX-I)$_2$ in human serum was analyzed. The most abundant serum degradation product identified, peptide KKYRYHLKPFC, was able to retain the antibacterial activity of the (p-BthTX-I)$_2$. Actually, the degradation peptide product exhibited an increased antibacterial effect against strains of *S. aureus*, *Enterococcus faecium*, multidrug-resistant *K. pneunomniae* and *E. coli*. The ability of (pBthTX-I)$_2$ and KKYRYHLKPFC to eliminate *Staphylococcus epidermidis* biofilms was also tested, with both peptides revealing identical anti-biofilm activity, as well as activity against the planktonic forms of that bacterial species (Santos-Filho et al., 2017).

The biochemical study of the Lys49PLA$_2$ LmutTX, from the venomous pit viper *Lachesis muta muta*, revealed a new C-terminal antimicrobial peptide sequence, PepC, which prompted the synthesis of a PepC-W variant, where Tyr residues were replaced by Trp. PepC-W showed an overall better performance than PepC against *S. aureus*, *S. aureus* resistant to methicillin (MRSA) and *P. aeruginosa*. Moreover, PepC-W cytotoxicity assays against the C2C12 line of muscle myoblasts of mouse C3H resulted in low activity, validating its selectivity against prokaryotic cells (Diniz-Sousa et al., 2018).

The most recently characterized antibacterial peptide from Lys49 svPLA$_2$s is pC-CoaTxII. This peptide was tested on five bacterial strains, including clinical isolates resistant to antibiotics. Peptide pC-CoaTxII showed greater activity against Gram-negative bacteria, although its effects against Gram-positive bacteria were significant. Experimental results show pC-CoaTx-II to be more active against both ATCC and clinical drug-resistant isolates, as compared to the parent protein (Almeida et al., 2018).

The use of antibacterial peptides has a great potential, however, there are a few drawbacks that need to be addressed. In this connection, short hybrid antimicrobial peptides (SHAMPs) have been generated based on peptides from svPLA$_2$s, like pEM-2. This chemical strategy consists of the joining together the sequences of different AMPs. The most promising hybrid was synthesized from pEM-2 and three fragments of MP-VT1, an AMP from the venom of the greater banded hornet, *Vespa tropica* (Memariani et al., 2017; Yang, Wang, Lee, & Zhang, 2013). This amphipathic peptide hybrid, named PV3 and whose amino acid sequence is KKWRKLLKKLKKLL, was active against several bacterial species, including ATTC strains and drug-resistant bacterial clinical isolates. In addition, PV3 displayed low activity against human RBC and hepatic embryonic cells, which highlights its selectivity against prokaryotic cells. In turn, another SHAMP, termed PV and mainly constituted by the pEM-2 sequence, displayed the highest antibacterial effect, but along with strong hemolytic activity (Memariani et al., 2017).

Several experimental and bioinformatics studies have addressed the unraveling of molecular mechanisms underpinning the antibacterial activity of small cationic peptides inspired in Lys49 svPLA$_2$s (Almeida et al., 2018; Lomonte et al., 2010; Santos-Filho et al., 2015). Interesting findings were made, although much more remain to be unveiled. A better understanding of the mechanisms of antibacterial

action and their sequence-dependent differences are essential to explore the potential and applications of these AMPs. The mechanism of action of synthetic Lys49 svPLA$_2$ peptides has been initially and mainly associated with the presence of positive and hydrophobic amino acid residues in their primary structures, enabling a stronger interaction with anionic bacterial membranes (Páramo et al., 1998). As such, many assays and studies have been carried out in order to dissect the possible modes of interaction between these AMP and bacterial cell membranes, raising many hypotheses that have been thoroughly discussed. Assays using p115–129 have evidenced that this peptide does interact with components of bacterial cell walls and membranes, namely, lipopolysaccharides of the outer membrane and the lipoteic acids of the cell wall, with apparently no internalization. This mode of action has been also proposed for several AMPs (Guani-Guerra, Santos-Mendoza, Lugo-Reyes, & Teran, 2010). Interestingly, bacteria belonging to the *Brucella* genus showed resistance to p115–129 and its variants, p115-W3 and pEM-2, while *E. coli* bacteria were susceptible to these peptides (Lomonte et al., 2010; Páramo et al., 1998). When generating *Brucella abortus* and *E. coli* chimeras, damage caused by those peptides was comparable to that observed in susceptible strains of *E. coli* and *V. cholerae* (Páramo et al., 1998). Although bacterial membranes are mostly formed by negatively charged lipids, there is considerable variability in membrane composition between different bacterial species and even between strains of the same species (Samy et al., 2017), which upholds the distinct effects induced by a given peptide or peptide family against different bacteria.

Cell membranes are not the only possible targets of AMPs. Peptide interactions with specific membrane receptors or even intracellular targets have to be considered (Santamaria, Larios, Quiros, et al., 2005). This said, synthesis and evaluation of enantiomers of AMPs found or inspired in nature may shed some light on whether membrane receptors or other specific targets, which are typically enantioselective proteins, participate in the mechanism of peptide antibacterial action. For instance, finding that both D and L enantiomers of pEM-2 have identical antibacterial activities suggests that this specific peptide interaction with cell membranes does not imply recognition by receptors (Araya & Lomonte, 2007; Santamaria, Larios, Quiros, et al., 2005). In such cases, a new avenue of opportunity is opened, since D enantiomers are more resistant to proteolytic degradation than their L counterparts (Santamaria, Larios, Quiros, et al., 2005). Like pEM-2, many other AMPs have been found to act through molecular mechanisms not involving recognition by cell membrane receptors (Reddy, Yedery, & Aranha, 2004).

The effects of pEM-2 on monolayers and lipid bilayers were later studied by atomic force microscopy (AFM), Langmuir–Blodgett (LB) monolayer technique and calcein fluorescence (CF) assays. These studies were performed using bacterial cell liposome models that emulate the membrane composition of *E. coli*, *S. aureus* and *B. subtilis*. In addition, pure DPPC (zwitterionic) and DPPG (anionic) lipid membrane models were used for comparison. Lipid membrane interaction assays indicated a higher peptide surface concentration on anionic membranes than on zwitterionic membranes, at high surface pressures. In the CF assays, stronger fluorescence was observed with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) membranes, *E. coli*, *S. aureus* and *B. subtilis* models, while only a weak signal was observed with 1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol (DPPG) membranes. Based on these observations, the authors considered that electrostatic interactions are important for the initial peptide binding to the membrane, while hydrophobic interactions and membrane composition seem crucial to the subsequent peptide-mediated lysis of the cell membranes (Won & Ianoul, 2009).

In another study on pEM-2, researchers have demonstrated the peptide disruptive action on lipid membranes. Electrically neutral 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) and negatively charged 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) large unilamellar vesicles (LUVs) were used in CF assays that showed that pEM-2 had stronger interactions with POPG than with POPC vesicles. At a peptide/membrane ratio of 0.1, 50% of the calcein was released from POPG whereas only 10% form POPC (Yu et al., 2010). Similar results were obtained in studies using SHAMPs that comprise the pEM-2 sequence, such as the aforementioned PV and PV3. In membrane permeability assays using these SHAMPS, *S. aureus* was susceptible to their permeation activity, with detection of β-galactosidase release, reflecting rapid disruption of the *S. aureus* membrane (Memariani et al., 2017).

Other spectroscopic techniques have been used to unravel the modes of antimicrobial action of synthetic Lys49 svPLA$_2$ peptides. An UV-Raman resonance study of pEM-2 indicated that electrostatic interactions of this peptide with lipid membranes play an important role in its antibacterial function, which is common amongst many other AMPs (Reddy et al., 2004). Another important observation was that the pEM-2 tends to adopt an α-helical structure upon interaction with the membranes (Quan & Ianoul, 2009), a finding that was later confirmed by CD and NMR (Yu et al., 2010).

CD spectroscopy assays with p-BthTX-I showed that this peptide adopts a random coil conformation when interacting with trifluoroethanol (TFE), as well as with lipopolysaccharide (LPS) micelles and LUVs. According to the authors, p-BthTX-I exerts a mechanism of action different from the peptide–membrane interaction models most frequently associated to α-helical AMPs (Santos-Filho et al., 2015). This was supported by CF assays also performed, as measurement of fluorescence emission upon calcein release demonstrated that p-BthTX-I had a low interaction with membrane models (POPC and POPC:POPG). Hence, it appears that p-BthTX-I mode of antimicrobial action does not involve bacterial membrane permeabilization (Santos-Filho et al., 2015).

Subsequently, the molecular basis for the action of pC-CoaTx-I was investigated in silico. From this study, it was suggested that the peptide is inserted into the cell membrane, which then promotes the opening of water channels, with may either (a) cause an osmotic imbalance to bacterial cells leading to their disruption, or (b) interfere with the nonpolar interactions of the phospholipid bilayer, causing severe membrane destabilization and consequent bacterial death (Almeida et al., 2018).

When analyzing the sequences of peptides in Figure 3a,b, a predominance of basic peptides is observed, including highly conserved Lys residues, which is a common trait in membrane-active AMPs (Chan, Prenner, & Vogel, 2006). This denotes the importance of such residues for the initial, electrostatically driven, steps of the interaction between Lys49 svPLA$_2$ peptides and bacterial cell membranes (Arias

et al., 2018; Won & Ianoul, 2009). Hydrophobic aliphatic and aromatic amino acids such as Pro, Ile, Asn, Leu, Phe, and Tyr are equally important for the antimicrobial activity of these peptides (Kini & Evans, 1989; Páramo et al., 1998; Reddy et al., 2004; Saravanan et al., 2014). In fact, while the electrostatic interactions promoted by basic residues are related to selective recognition and binding to bacterial membranes, hydrophobic residues promote subsequent steps that are crucial for antibacterial activity (Páramo et al., 1998; Won & Ianoul, 2009). Cysteine has been found relevant for the activity of some AMP (Brouwer et al., 2018), and this is also observed for Lys49 svPLA$_2$s C-terminal peptides as those depicted in Figure 3. These invariably possess a Cys residue at position 11, and assays with p-BthTX-I and its analogue where Cys was replaced by Ser showed a decrease in antimicrobial activity, probably due to the fact that the substituted peptide was no longer able to form a dimer resembling (p-BthTX-I)$_2$ (Santos-Filho et al., 2015). In turn, substituted analogues in Table 3 have increased activity as compared to native sequences on which they were inspired (Diniz-Sousa et al., 2018; Santamaria, Larios, Angulo, et al., 2005). This stems from the fact that replacements were deliberately done in order to increase antimicrobial activity; in some variants, one or more specific residues, mainly Tyr, were replaced by Trp, as this amino acid is known to promote strong interactions with bacterial membranes (Arias et al., 2018; Lomonte et al., 2010). In other variants, as pEM-1 and pEM-2, Ala was inserted as this residue is known to possess higher α-helix propensity (Pace & Scholtz, 1998), which is in turn relevant for the antimicrobial activity of α-helical cationic and amphipathic AMPs (Santos-Filho et al., 2015). In other cases, such as pEM-9, Arg was introduced to improve antimicrobial activity (Arias et al., 2018), as this residue conserves the cationic nature of Lys, while enabling establishment of additional hydrogen bonds strengthening peptide-cell interactions (Silva et al., 2014).

Altogether, data on Lys49 PLA$_2$s C-terminal peptides and their variants suggest that a common model of interaction with membranes cannot be assumed a priori. Establishment of specific mechanisms of action and of relevant sequence-activity relationships is of chief importance to improve characteristics such as specificity, stability and broad spectrum activity, if one intends to take advantage of the promising biological properties of svPLA$_2$-inspired antimicrobial peptides for clinical applications (Memariani et al., 2017; Saravanan et al., 2014).

5 | PEPTIDES AS DRUGS: CHALLENGES AND PERSPECTIVES

Clinical application of small antimicrobial peptides from or inspired by nature has well identified pros and cons, as briefly compiled in Figure 4. Considering the difficulties that were earlier experienced when addressing the development of peptide-based drugs, peptides were a "no go area" for medicinal chemists and pharma companies until very recently.

However, the paradigm is rapidly changing as, despite their limitations, peptides have several important advantages over small therapeutic molecules, starting with their natural origin, which generally implies higher safety, selectivity, and specificity. Peptides are the main effectors of all signaling transduction processes, being involved in most endocrine pathways; thus, in principle, they can be used to regulate virtually all such biochemical events, constituting an almost unlimited source for potential drugs. Moreover, their greatest disadvantage may turn out to be an advantage: peptides have short half-lives in vivo, mainly due to proteolytic degradation, but this also means that their degradation products are smaller peptides and amino acids, and conjugates thereof, all of which endogenous-like and easily eliminated; in other words, peptide drugs are less prone to accumulate in the body and cause toxicity, reducing the risks of their therapeutic use. Finally, as compared to large proteins and antibodies, which have conquered a niche in pharmaceutical development earlier and easier than peptides, the latter can penetrate further into tissue, have considerably higher stability, and are generally less immunogenic/allergenic, while being produced at considerably lower costs. Hence, the recent revival of peptide-based drugs both in academia and in the pharmaceutical industry comes with no surprise, as peptide therapeutics can address many unmet medical needs, by complementing or even replacing small molecules or large protein-based therapeutics currently in the clinics (Craik, Fairlie, Liros, & Price, 2013; Henninot, Collins, & Nuss, 2018; Otvos Jr. & Wade, 2014). Accordingly, peptide drug sales have been steadily increasing, at present having an expected volume of sales likely over US$ 70 billion in 2019 (insulins included) (Henninot et al., 2018), and US$ 47 billion (insulins excluded) by 2024 (Al Musaimi, Al Shaer, de la Torre, & Albericio, 2018).

Since 2000, the number of peptide-based drugs being placed in the market has been increasing, and many more have entered clinical trials, in order to address diverse medical conditions (Figure 5). Although many of the peptides currently in clinical use are hormones or hormone-stimulating peptides, a significant number of peptide-based drugs are now under clinical development to address infectious diseases (Fernandes & Martens, 2017; Greber & Dawgul, 2017; Henninot et al., 2018). If any doubts would remain on the relevance of peptides against multidrug-resistant (MDR) bacteria, one just has to notice that, as of today, the last resort against nosocomial MDR infections are glycopeptides like dalbavancin, oritavancin, telavancin, and colistin, or the lipopeptide daptomycin (Chin et al., 2018; Chung & Khanum, 2017; Giamarellou, 2006). Moreover, the natural AMPs known as defensins have inspired development of one of the newest hopes against MDR infections, brilacidin, an investigational new drug (IND) active even against nonreplicating bacteria (Fernandes & Martens, 2017).

All the above explains why about 10% of peptide drugs presently in development target microbes, with particular emphasis on ESKAPE pathogens, that is, MDR strains of *E. faecium*, *S. aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species that are the leading cause of severe nosocomial infections worldwide (Santajit & Indawattana, 2016). As such, any knowledge gained on new AMPs, like the aforementioned Lys49 PLA$_2$s C-terminal peptides, regarding structure, SAR, activity and modes of action, is of invaluable relevance towards the development of peptide-based drugs targeted at MDR infections, which are amongst the greatest public health menaces of our times.

Still, one cannot disregard that conversion of naturally occurring/inspired bioactive peptides into therapeutically useful drugs is a difficult and laborious challenge, starting right from the preclinical stage.

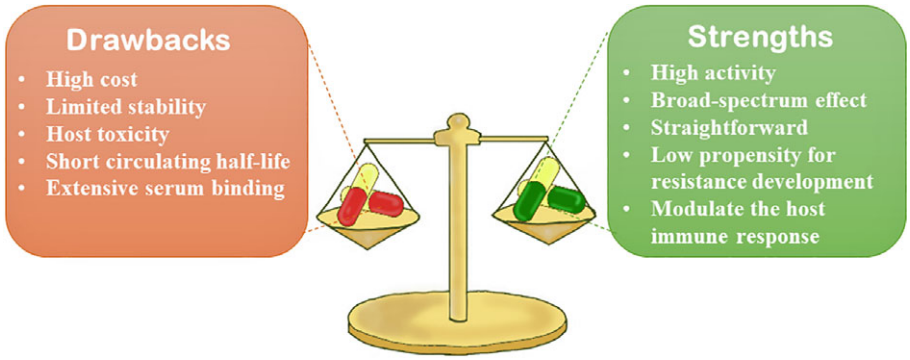

FIGURE 4 Pros and cons of antimicrobial peptides as drugs

For instance, it has already been mentioned that an increase in Trp content can improve antibacterial activity of Lys49 PLA_2s-derived AMPs; however, that was also found to potentiate toxicity against eukaryotic cells, meaning that a fine sequence tuning is required to ensure a favorable balance between activity and safety (Diniz-Sousa et al., 2018; Santamaria, Larios, Angulo, et al., 2005). Insertion of positively charged residues as Lys to promote peptide accumulation onto the negatively charged membranes should be equally used with caution: while Lys residues promote formation of a hydrophilic patch eventually allowing water entrance and diffusion through the membrane, causing its destabilization (Almeida et al., 2018; Yu et al., 2010), they also make peptides more prone to proteolytic degradation (Arias et al., 2018).

Even when structure–activity issues are conveniently dealt with, one needs to address the greatest challenge of all in developing a peptide-based drug: the generally poor absorption, distribution, metabolism, and excretion (ADME) properties of natural peptides, the vast majority of which display an oral bioavailability below 1% (Di, 2015). Setting aside peptide encapsulation strategies, which lay beyond scope of this review, a few classical strategies to tackle this situation include (a) N-terminal acylation and C-terminal amidation, to avoid proteolytic degradation by amino and carboxypeptidases, respectively; (b) identification of sites amenable to undergo proteolysis by endopeptidases, in order to perform suitable backbone modifications, like replacement of the labile amide bond by a bioisostere, or of L-amino acids by their N-methylated and/or D-enantiomer counterparts, or by β-amino acid analogues; (c) increase stability and/or lock conformation upon cyclization; (d) coupling to a small serum protein-ligand molecule to increase blood circulation time, or (e) production of peptidomimetics, including peptoids, peptaibols, azapeptides and other mimetics, though this approach is the least attractive, as it usually joins additional synthetic effort with relevant drop in activity (Di, 2015; Henninot et al., 2018; Molchanova, Hansen, & Franzyk, 2017; Qvit, Rubin, Urban, Mochly-Rosen, & Gross, 2017; Raza et al., 2018; Sierra, Fusté, Rabanal, Vinuesa, & Viñas, 2017).

In the particular case of development of AMPs into drugs, a popular approach has been cyclization, mainly due to the fact that many of the orally active peptides known are cyclic, including peptide antibiotics like actinomycin D, bacitracin A, beauvericin, colistin, cyclopeptolide 1, cyclotides, dalbavancin, daptomycin, echinocandins, gramicidins, griselimycin and derivatives, micafungin, mycoplanecins, oritavancin, permetin A, polymyxins B1 and B2, pristinamycin and related antibiotics, surotomycin, telavancin, valinomycin, and vancomycin, among others (Falanga et al., 2017; Joo, 2012; Nielsen et al., 2017; Tapeinou, Matsoukas, Simal, & Tselios, 2015). As such, chemical approaches to cyclic homodetic (cyclization through an amide bond) or heterodetic (cyclization through a non-amide bond) head-to-tail, head/tail-to-side chain, side chain-to-side chain peptides (Figure 6) have deserved special attention from peptide chemists (Davies, 2003; Lambert, Mitchell, & Roberts, 2001; Martí-Centelles, Pandey, Burguete, & Luis, 2015; Tam &

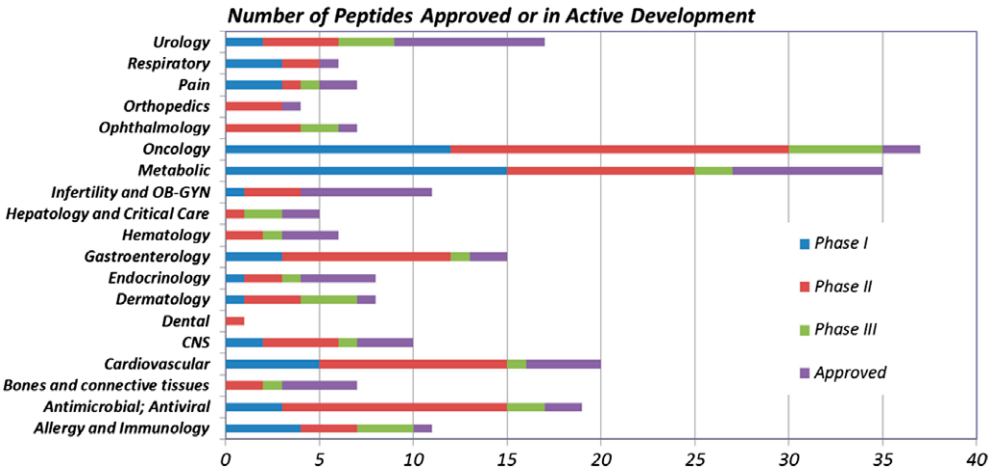

FIGURE 5 Peptide drugs approved or currently in development, sorted by therapeutic area Reproduced with permission from Henninot et al. (2018)

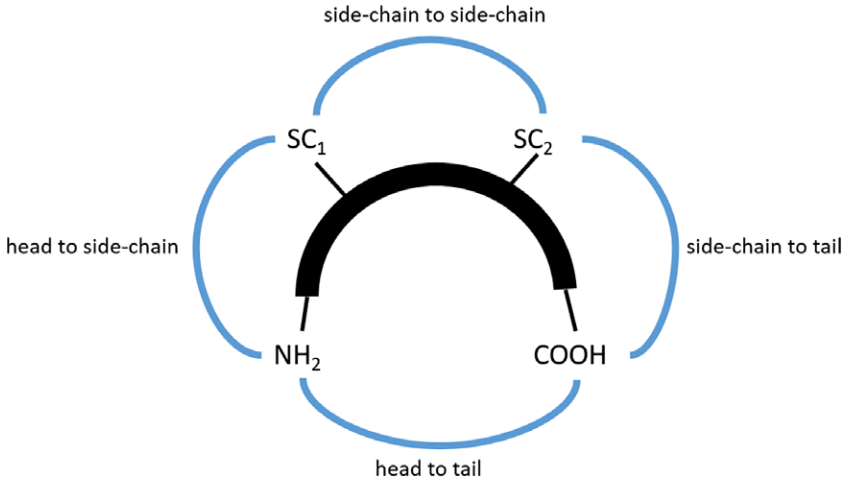

FIGURE 6 Peptide cyclization orientations depending on use of N-terminal (NH_2), C-terminal (COOH) and/or side-chain (SC) functional groups

Wong, 2012; White & Yudin, 2011). One classical route is oxidation of thiol groups to produce a disulfide bridge between the side chains of Cys residues, which may either belong or be added to the original sequence; this is relatively simple from a synthetic viewpoint if only one disulfide bridge is to be formed (Ahn et al., 2008), but can be laborious if multiple disulfide bridges are targeted, although improved methods have been emerging over the past 15 years (e.g., Cheneval et al., 2014; Vila-Perelló, Sánchez-Vallet, García-Olmedo, Molina, & Andreu, 2003). One limitation of disulfide-bridged peptides is their lability to bioreducing conditions (Yang, Chen, Vlahov, Cheng, & Low, 2006), which has often led researchers to invest on homodetic cyclic peptides, given the higher chemical and biochemical stability of amide bonds (Monaim et al., 2018; Rohrbacher, Deniau, Luther, & Bode, 2015; Valldosera et al., 2008).

With the fast-growing portfolio of commercially available non-natural amino acids whose side chains are tailored for specific modifications, other chemoselective strategies for peptide cyclization have been developed over the past few decades, which include, among many others, (a) Michael-type (thiol-ene) additions, (b) Diels–Alder reactions, (c) Huisgen's 1,3-dipolar (azide-alkyne) cycloadditions, (d) (native) chemical ligation, and (e) Grubbs reactions (olefin metathesis), the latter leading to the so-called hydrocarbon-stapled peptides (Figure 7) (Davies, 2003; Lambert et al., 2001; Lau, de Andrade, Wu, & Spring, 2015; Li, Aneja, & Chaiken, 2003; Martí-Centelles et al., 2015; Turner, Oliver, & Lokey, 2007; Verdine & Hilinski, 2012; Wallensky & Bird, 2014; White & Yudin, 2011).

Stapled peptides are particularly interesting for the development of novel peptide-inspired antibiotics, by offering enzymatically stable locked bioactive conformations like, for example, amphipathic α-helices adopted by many AMPs when interacting with bacterial membranes (Cromm, Spiegel, & Grossman, 2015; Klein, 2017; Lau

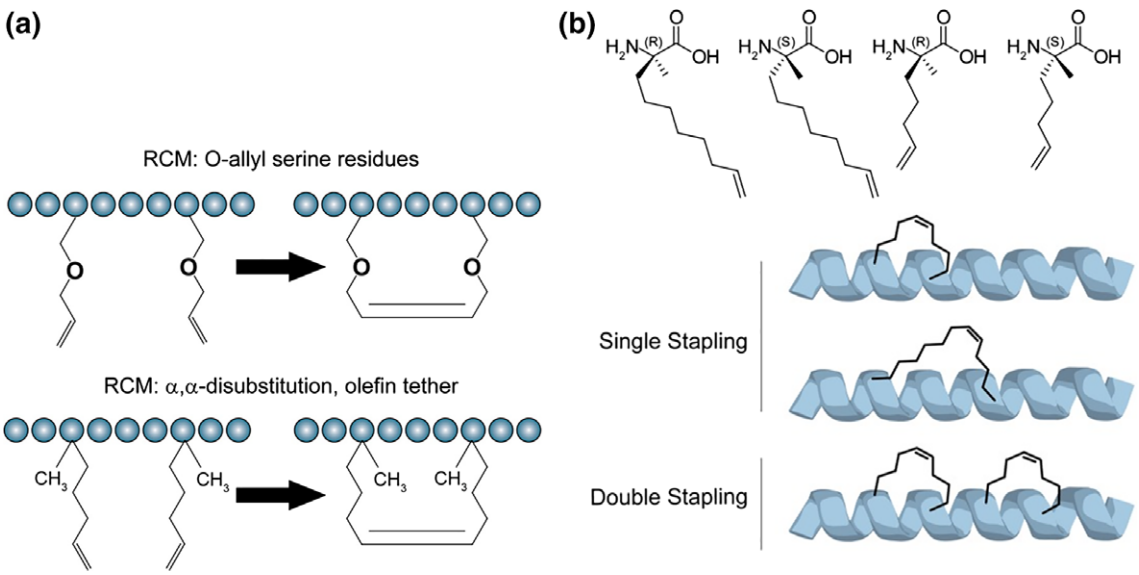

FIGURE 7 Stapled peptides via ruthenium-catalyzed olefin metathesis. (a) Ring closure metathesis (RCM) pioneer strategies towards hydrocarbon (top, Blackwell and Grubbs approach) and all-hydrocarbon (bottom, Schafmeister and Verdine approach) tethers; (b) all-hydrocarbon peptide stapling to produce stabilized α-helices: Chiral non-natural amino acids are inserted at i, i + 4 or i, i + 7 positions to produce tethers that span one or two helical turns, respectively; double-stapled peptides can be produced by modification at both i, i + 4 and i, i + 7 Adapted from Wallensky and Bird (2014)

et al., 2015; Migon, Neubauer, & Kamysz, 2018; Wallensky & Bird, 2014; Xie, Gao, Shull, & Teng, 2016).

6 | CONCLUDING REMARKS

Collective efforts from multidisciplinary teams are mandatory when targeting translation of relevant findings from basic research into the clinics. The remarkable biological properties of many natural or synthetic AMPs reported thus far should not be taken as a mere academic exercise unworthy of further development to produce valuable drug candidates. Fortunately, both academia and industry are changing gears regarding peptide-based drugs, and market projections accompany this trend. Recent development of anti-thrombotic agents inspired in snake venom proteins (Huang, Hsu, & Kuo, 2016) presage a bright future for bioactive peptides derived from such toxins. In particular, identification of new antimicrobial molecules from natural sources and hit-to-lead-to-candidate efforts thereof are crucial to keep pace in the fight against resistant pathogens.

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