Engineering of Marine-derived Antimicrobial Peptides (mAMPs) into Improved Anti-infective Drug Leads: A Mini-review

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Abstract. Marine-derived antimicrobial compounds possess chemical diversity varying from peptides, fatty acids to terpenes, alkaloids, and polyketides. These compounds, especially of peptide origin called antimicrobial peptides (AMPs), are present in the majority of marine organisms, including microbes (bacteria and fungi), invertebrates (molluscs, echinoderms, and sponges), vertebrates (fish and mammals), and plants (marine algae). They are defined by small molecular weight (less than 10 kDa), a net positive charge, and amphipathic structures. Moreover, due to their profound in vitro antimicrobial and cytotoxic activities and a low risk for resistance development, naturally occurring marine-derived AMPs (mAMPs) have been used as drug design templates for a large variety of semi-synthetic or synthetic AMPs, some of which have reached clinical trials. This mini-review aims to discuss AMPs from marine sources, mainly emphasizing the engineering of these peptides with improved pharmacological properties to develop drug candidates. Some selected recent examples of these engineered mAMPs as anti-infective drug leads are herein highlighted.

1. Introduction

Antimicrobial peptides (AMPs) belong to a group of peptide compounds exerting antimicrobial activity in vitro. These peptides are relatively small (comprising 5-60 amino acids or less than 10 kDa), have a net positive charge, and form amphipathic structures in a hydrophobic environment. In nature, AMPs are used as a vital tool for survival in many organisms as a part of the innate immune defense. Nowadays, AMPs are gaining increasing attention for promising drug leads because of their wide spectrum of activities against bacteria, fungi, parasites, viruses, and even cancer cells [1-7], and more importantly, they are less likely to develop resistance. These peptides mainly act on the lipid membrane structure of the incursive pathogens, creating a barrier in attaining resistance. Hence, AMPs may overcome antimicrobial resistance over conventional antibiotics [8-10].

The marine habitat has been regarded as one of the wealthiest reservoirs for AMPs. Its extreme and harsh conditions, such as a broad range of microbes (including the pathogenic microorganisms) as well as high salt concentration, drive marine organisms to produce various unique and potent bioactive molecules as their first line of defenses, in particular AMPs [11, 12]. Marine-derived AMPs (mAMPs) are produced from various marine species, including marine microorganisms (e.g., bacteria, fungi), invertebrates (e.g., molluscs, echinoderms, and sponges), plants (marine algae), and vertebrates (e.g., fish and mammals) [13]. Their chemical structure were found to vary from the terrestrial counterparts.
derivatives and often present novel and more diverse structures. Marine AMPs can be differed by their biochemical characteristics and structural features into linear α-helical (e.g., mixininidin), extended linear with the high content of one amino acid (glycine, histidine, proline, or tryptophan) (e.g., arasin), hairpin-like β-sheet or α-helical/β-sheet combined structure (e.g., hepcidins), and cyclic (primarily derived from sponges and tunicates; e.g., discodermin A) peptides. These mostly gene-encoded ribosomal peptides are extensively modified to adapt to a harsh marine environment, especially the high salinity. The salt-resistant feature of mAMPs allows peptides to retain their biological functions (particularly their anti-infective properties) in relatively high-salt environments, such as gastrointestinal fluid, saliva, serum, or other body fluids. Apparently, natural AMPs from marine organisms show tremendous prospects to be evolved into therapeutics, including anti-infective drug leads [14-16].

In spite of the propitious biological activity of the AMPs, they have some drawbacks towards the challenges for clinical applications, such as limited stability at certain pH; hemolytic side effect; elevated production cost; limited information on toxicity, pharmacodynamics, and pharmacokinetic properties; rapid degradation by protease; and cations-related reduced activity. However, a peptide can be altered in different approaches so that the expected properties, including effectiveness and stability, can be improved while the undesirable characteristics are attenuated. In addition, they can be optimized through the modification of their primary sequences. The rational design has received tremendous interest as well as depicts a significant revolution in the development of AMPs-based drug leads or drugs that have higher activity, less cytotoxicity, as well as feasible manufacturability [1, 17-19].

Several studies on AMPs from marine species have been extensively reviewed so far [13, 14, 18, 20-24]. However, this mini-review focuses on the engineering of these marine peptides into improved desired properties, particularly anti-infective. Their chemical modifications are also highlighted. Some selected recent engineered mAMPs as anti-infective drug leads emphasizing on antibacterial and antifungal agents during 2015-2020 are documented and listed herein.

2. Modifications in naturally occurring marine-derived AMPs (mAMPs)
Post-translational modifications (PTMs) are prevalently found in naturally occurring AMPs as these alterations are critical to the diverse chemical structure and biological function of natural AMPs. As mentioned earlier, mAMPs undergo various PTMs (Table 1). Some of the PTMs are either specific to mAMPs or can be shared with terrestrial AMPs.

Most of these chemical modifications direct the proper peptides folding into various chemical scaffolds that are necessary to recognize the bacterial surfaces and membranes target. Moreover, to aid the design of mAMPs with improved stability as well as efficacy to be applied in therapeutics, PTMs can maintain protease activity and physiological salt concentration [33]. These features, therefore, become an advantage for peptide drug candidates.

Deciphering the relationship between PTMs and peptide properties has inspired peptide engineering. Some PTMs strategies have been found to apply in peptide engineering. A number of studies have corroborated substantial evidence that antibacterial potencies can be generated by modifying synthetic AMPs. Peptide engineering plays an essential role in developing naturally occurring mAMPs into an improved peptide as well as a new variation of therapeutic compounds. Herein, some of the recent engineered AMPs from marine sources against bacteria and fungi will be discussed below.
Table 1. Selected PTMs present in naturally occurring mAMPs

| Modifications | AMPs | Marine species origin | References |
|---------------|------|-----------------------|------------|
| Bromination   | cathelicidins | hagfish *Myxine glutinosa* | Shinnar et al., 2003 [25] |
| strongylocins | hagfish *Strongylocentrotus droebachiensis* | Li et al., 2008 [26] |
| jaspamide     | sponge *Jasps sp.* | Zabriskie et al., 1986 [27] |
| Unnatural amino acids | | | |
| (a) 6-bromotryptophan (bromination), dihydroxylysine, dihydroxyarginine, 3,4-dihydroxyphenylalanine | styelin | tunicate *Stylosia cava* | Taylor et al., 2000 [28] |
| (b) 3-methylisoleucine | polydiscamide A | sponge *Discodermia* spp. | Gulavita et al., 1992 [29] |
| N- or C-terminal modification | | | |
| (a) N-terminal blocking by formyl group and C-terminal lactonization with threonine | halicylindronide D | sponge *Halichondria cylindrata* | Li et al., 1995 [30] |
| (b) Oxidatively decarboxylated aromatic C-terminus | halocyamines | ascidian *Halocythina roretzi* | Azumi et al., 1990 [31] |
| (c) C-terminal amidation | clavanins | Hemocytes of sea squirt *Styela clava* | van Kan et al., 2002 [32] |

3. Engineered antibacterial mAMPs

It has been reported that high antimicrobial (antibacterial) activity is related to a free N-terminal amino group and amidated C-terminal on a peptide structure. The proper equilibrium of amphipathicity (cationic, e.g., arginine and aromatic, e.g., tryptophan residues), hydrophobicity (the hydrophobic interaction with bacterial membrane), cationicity (the number of positively-charged residues), and a free N-terminal amino group and amidated C-terminus constitute a favourable motif in AMPs [34-36].

A novel cationic peptide with high polyalanine content, namely Pa-MAP 1.9, has been rationally designed based on a synthetic multifunctional peptide analogue Pa-MAP. The Pa-MAP is derived from HPLC-8, a peptide obtained originally from the polar fish *Pleuronectes americanus* [37]. This newly analogue synthetic mAMPs seems to be a promising anti-infective drug leads against the pathogenic Gram-negative infection. It showed greater antibacterial properties primarily against Gram-negative strains, including *E. coli*, *Klebsiella pneumoniae*, and *Enterococcus faecalis*. It has been suggested that adopting the conformation of α-helical, which may favour the insertion into the lipid bilayer as well as the orientation mimicking the bacterial membrane, can facilitate a bacterial membrane interaction [34].
A bioengineering approach has been applied to develop β-hairpin mAMPs arenicin-1 derived from marine lugworm *Arenicola marina* as a template for antimicrobials with potent activity and less toxicity [38]. The shortened 17-residue analogue of arenicin-1, namely ALP1 [39] (Figure 1), was obtained by heterologous expression in the *Escherichia coli* strain. It has been reported that the ALP1 analogue demonstrates potent antibacterial activity towards both Gram-positive and Gram-negative pathogens, including multidrug resistance strains, no cytotoxicity at high peptide concentration (up to 50 µM), as well as good tolerance to physiological or high salt concentrations. These features perform the peptide a favourable chemical temp for further designing antibacterial agents with more potent and nontoxic properties [38, 39]. In addition, recently, Orlov et al. (2019) [40] redesigned arenicin-1 through branching or strand rearrangement, specific amino acids substitution, as well as structure cyclization or linearization. Those generated analogues make both the outer and inner *E. coli* membrane cells permeable, indicating the membranolytic mechanism of action. Their findings also suggest that the arenicin scaffold could be acceptable as a model for refining new antibacterial compounds.

Moreover, the other member of the arenicin family, NZ17074 (N1), which is a variant of arenicin-3 (GFCWNVCVYRNGVRVCHRRCN; changes vs. arenicin-3 in bold; Tyr5Asn, Tyr17His), also exhibits potent antimicrobial activity towards Gram-negative bacteria and fungi, and is currently entering preclinical evaluations [41]. However, due to its significant degree of cytotoxicity in normal eukaryotic cells, N1 has been modified by replacing multiple residues with hydrophobic/cationic residues (alanine/lysine) and by removing disulfide bond, generating several analogues to reduce cell toxicity and generate more therapeutically molecules. Two chemically synthetic analogues, N2 (Gly1Ala, Gly12Ala; a ‘rocket’ analogue bearing two disulfide bonds) and N6 (Cys3Ala, Cys20Ala; a ‘kite’ analogue bearing a Cys7-Cys16 disulfide bond), demonstrated potent activity against *E. coli* and *Salmonella enteritidis*, with N2 showing higher activity than N1. The increased flexibility in the N- and C- terminus of both newly synthesized analogues led to lower haemolytic and cytotoxic effects than N1 [42, 43].

Furthermore, the improved antibacterial activity of the synthetic N6 analogues against intracellular *S. typhimurium* has demonstrated a novel platform of cell-penetrating peptides (CPPs)-cleavable linker-AMP as an efficient AMPs delivery and a C-terminal modification as an effective strategy to largely improve peptides stability. Those analogues, the CPP-linker-N6, the C-amidated N6, and CPP-linker-C-amidated N6, had almost no hemolysis and cytotoxicity but with increased antibacterial activity. However, further investigations are required to be applied clinically [44].

**Figure 1.** Chemical structures of antibacterial peptide arenicin-1 and its ALP1 analogue.
A very recently promising lead arenicin-3 derivative for further clinical evaluation, named AA139 analogue, has also been successfully synthesized by Cooper and his coworkers [45]. They developed the synthetic peptide AA139 (Val8Ala, Tyr9Arg, Val13Ala) by structure-guided optimization by combining the structure-activity/toxicity relationship evaluation with the membrane models and NMR solution structures. The peptide analogue showed reduced mammalian toxicity and propitious efficacy in various pathogenic Gram-negative in vivo models.

4. Engineered antifungal mAMPs

Pathogen resistance and drug toxicity have restrained the treatments for fungal infections. From previous studies, the antifungal mAMP Cm-p1 (SRSELIVHQR) has been isolated from a marine mollusc Chenchritis muricatus. Moreover, to improve its antifungal activity, Cm-p5 (SRSE-LIVHQRLF) has been engineered by adding two more residues, leucine and phenylalanine to the C-terminus of Cm-p1. Besides antifungal property towards human fungal pathogens such as Trichophyton mentagrophytes, T. rubrum, Candida parapsilosis, and Cryptococcus neoformans, this modification has considerably increased fungistatic effect towards pathogenic Candida albicans (EC = 146 μg mL⁻¹; MIC = 10 μg mL⁻¹). Moreover, this newly engineered analogue exhibited low toxicity towards mammalian cell cultures and low haemolytic. In silico study and isothermal titration calorimetry showed Cm-p5 binds to a bilayer membrane of phosphatidyserine and phosphatidylserine and possesses affinity from the least to the highest level to ergosterol, mammalian phospholipid, and fungal phospholipid, respectively, but no affinity to chitin. However, this analogue was unfortunately incapable of regulating the kidney fungal burden in a systemic candidiasis mice model [46, 47].

Cm-p5 analogue, however, is also not suitable for therapeutic application because the analogue is readily degraded by proteases. Two arginine residues in the peptide design seems to lead to trypsin cleavage, and the free N-terminal can be prone to be attacked by aminopeptidase. As the structure-activity relationship is determined, the covalent modification is expected to enhance stability. Therefore, based on the following rational design study of this peptide, glutamic acid and histidine residues are essential in the helix stabilization, allowing Vicente et al. (2019) to synthesize a cyclic helical-stabilized analogue of Cm-p5, named CysCysCm-p5, by changing those residues for cysteine [48] (Figure 2). The new cyclic CysCysCm-p5 has shown greater fungal control capacity (than conventional antibiotic fluconazole), low cytotoxicity, and a stabilized α-helix and disulfide bridges that may promote metabolic stability and activity in vivo. Thus, this monomer shows a favourable systemic antifungal drug lead.

**Figure 2.** Helical projections of antifungal peptide Cm-p1 (a) and its analogues Cm-p5 (b) and CysCys-Cm-p5 (c). Adapted from Vicente et al., [48].

In addition, further modified monomers of CysCys-Cm-p5, parallel and anti-parallel dimers were generated by intramolecular disulfide bond formation. Recently, Rosenau and his coworkers [49] have
demonstrated that these new derivates initially exhibited semi-inhibitory concentration (10-21 μg mL⁻¹) toward C. aurius biofilm in vitro that did not observe in the previous cyclic Cm-p5.

Another recent engineered antifungal mAMPs is the N-methylated analogue of a marine bacteria-derived high proline content cyclotetrapeptide [50]. The non-methylated naturally occurring cyclotetrapeptide was previously obtained from the seaweed Digenea sp.-associated Pseudomonas sp. and Pseudovalteromonas sp. [51]. The newly synthesized methylated analogue exhibited increased antifungal activity towards Microsporum audouinii and T. mentagrophytes (dermatophyte pathogens) compared to its non-methylated natural peptide and considerable activity against C. albicans. Besides modulating the biological properties or activities, the N-methylation of the cyclopeptides has shown several advantages. These advantages include increasing the hydrophobic interaction (via minimizing the hydrogen-bond donors), improving the efficacy (via improving receptor selectivity), and improving the oral bioavailability (via avoiding inter-and intra-molecular hydrogen bonds formation) [52].

5. Concluding remarks

AMPs from marine organisms are a promising treatment for antibiotic resistance. These peptides show advantageous post-translational modifications that enable coping with harsh marine environmental conditions such as high salinity. The modifications aim to increase the half-life of mAMPs in life systems, endowing improved stability and bioavailability to protease resistance in vivo. These characteristics lead to mAMPs as fascinating chemical models for the design of drugs or pharmaceuticals. Elucidation of the modification-peptide property correlation prompts peptide manipulation. Further continued structural alterations of the naturally occurring mAMPs are needed to obtain (semi)synthetic peptides with increased pharmacokinetics as well as bioactivity, which will be a tremendous battle against microbial infections.

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