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The development of antimicrobial γ -AApeptides

Host-defense peptides (HDPs) are promising next generation of antibiotic agents, as they have the potential to circumvent emerging drug resistance, due to their mechanism of bacterial killing through disruption of their membranes. Nonetheless, HDPs have intrinsic drawbacks such as low-to-moderate activity, susceptibility to enzymatic degradation. In the past few years, we developed a new class of peptidomimetics named ' γ -AApeptides', which have superior resistance to proteolysis and a variety of diversification via straightforward synthesis. Our recent studies suggested that γ -AApeptides can mimic the bactericidal mechanism of HDPs and show potent and broad-spectrum activity against both Gram-positive and Gram-negative multidrug-resistant bacteria. In this review, we summarize our current studies of antimicrobial γ -AApeptides and discuss their potential future development as antimicrobial peptidomimetics.

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The emerging antibiotic resistance is viewed as one of the omnipresent threat in global public health field, due to the unrestrained abuse of antibiotics [1,2]. Multidrug-resistant pathogens including *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE) have been recognized as serious infections to life as they are no longer responsive to most conventional antibiotics [3,4]. In order to combat those bacterial pathogens, significant efforts are extended to developing the new generations of antibiotics with novel mechanisms. Antimicrobial peptides, also known as host-defense peptides (HDPs), have attracted considerable interest. HDPs are short cationic amphiphilic peptides present in almost every living organism, and are an excellent class of drug candidates [1,2]. Although the precise

mechanisms of HDPs are still under debate, there is consensus that HDPs are capable of adopting globally amphipathic structures, which have hydrophobic and cationic regions, and are critical for the disruption of bacterial membranes [5,6]. The cationic regions mainly contribute to the selectivity of HDPs by electrostatic interaction [7]. They preferentially associate with bacterial cell membranes that contain predominantly negatively charged phospholipids including cardiolipin and phosphatidylglycerol. On the contrary, the outer leaflet of the mammalian cell membranes has relatively weak electrostatic interaction with cationic HDPs, because it is composed of zwitterionic lipids such as phosphatidylcholine and sphingomyelin [8]. Upon association with bacterial membranes, HDPs utilize their hydrophobic side chains to penetrate membranes. Consequently, this hydrophobic interaction leads

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Figure 1. Structures of α-peptide, γ-AA peptide, α/γ-AA peptide and sulfono-γ-AApeptide.

to membrane thinning, disruption, depolarization and subsequent cell death [9–15]. By taking advantage of this mechanism of action, HDPs are bactericidal

rather than bacteriostatic found in conventional antibiotics. Meanwhile, the antimicrobial mechanism of HDPs generally lacks a specific cellular target. As such, although it is inevitable for resistance development in any therapeutic agents, HDPs are believed to have a lower risk of resistance. Therefore, HDPs are potential alternative approach for the development of antibiotic agent with novel antimicrobial mechanisms [3].

However, as the novel antibiotic agents, HDPs also possess some intrinsic drawbacks, such as susceptibility to proteolytic degradation and low-to-moderate activity [16]. To circumvent those drawbacks, over last decade, non-natural peptidomimetics that mimic the globally amphipathic structures and mechanism of action of HDPs have been developed and investigated, such as β-peptides [17–19], peptoids [20–24], aryl-amide oligomers [25,26], β-turn mimetics [27,28] and others [29,30]. Recently, we developed a new class of peptidomimetics termed ‘γ-AApeptides’ [31,32]. In this article, we summarize some of our results in the devel-

Figure 2. Synthesis of linear γ-AApeptides and alloc-protected γ-AApeptide building blocks. (A) Synthesis of linear γ-AApeptides. **(B)** Alloc-protected γ-AApeptide building blocks.

Figure 3. The design of antimicrobial AApeptides. (A) The basic structure of amphiphilic AApeptide building blocks. (B) The formation of amphipathic structure of AApeptides upon interaction with bacterial membranes. Reproduced with permission from [43] © 2013 Royal Society of Chemistry.

opment of antimicrobial γ -AApeptides that mimic the global structure, function and mechanism of HDPs.

Design & synthesis of γ -AApeptides

γ -AApeptides are a new class of peptide mimics designed in our lab in order to facilitate drug discovery and protein surface mimicry. They are termed ' γ -AApeptides' [31,32] because they contain γ -substituted-*N*-acylated-*N*-aminoethyl amino acid units (Figure 1) [33,34] derived from γ -chiral PNA backbones. Compared with natural peptides, the repeating unit of γ -AApeptides is comparable to a di- α -peptide residue, and γ -AApeptides are able to accommodate the same number of side functional groups on the backbone as peptides of the same lengths. Moreover, different side chains, which are introduced through acylation by carboxylic acids, contribute to the enormous diversity of γ -AApeptides, as well as their inherent resistance to biodegradation [31,32,35].

As a new class of peptidomimetics, γ -AApeptides start to show potential applications in drug discovery and chemical biology. For instance, they display inhibitory activity toward p53/MDM2 protein–protein interaction [31,32]. In addition, they also harness immune response and inhibit lipopolysaccharide (LPS)-activated Toll-like receptor 4 (TLR4) signaling [36]. In addition, γ -AApeptides have shown promise as potential antimicrobial agents [37–43]. Recently, γ -AApeptides exhibit comparable or enhanced performance compared with ciprofloxacin in the prevention of biofilm formation of both Gram-positive and Gram-negative bacteria [44].

Synthesis of γ -AApeptides

The synthesis of γ -AApeptides is carried out by using the strategy combining building block and submonomeric

approaches (Figure 2). In this strategy, alloc-protected γ -AApeptide building blocks (Figure 2B) are prepared and assembled on solid support to provide the desired γ -AApeptides sequences (Figure 2A) [31–32,35,36,44–47]. The derivatization of γ -AApeptides is virtually unlimited due to the freedom of using readily available carboxylic acids. Such features will give more space to expand their potential applications in drug discovery in the future.

Design of antimicrobial γ -AApeptides

We anticipated γ -AApeptides would be excellent candidates as the design of novel antimicrobial agents that mimic the structure, function and mechanism of HDPs, because of their limited conformational backbone flexibility [37,48], stability [31,32,35] and the unlimited chemodiversity [31,32]. Compared with canonical peptides, γ -AApeptides may be more flexible due to the existence of more dihedral angles. Therefore, antimicrobial AApeptides could be designed by joining amphiphilic γ -AApeptides' building blocks together, endowing their capability to adjust their conformation to adopt globally amphipathic structures upon interaction with bacterial cell membranes (Figure 3). Following this hypothesis, a number of γ -AApeptide sequences were developed and tested for their antimicrobial activity.

γ -AApeptides as antimicrobial agents

Linear antimicrobial γ -AApeptides

Based on our hypothesis, a focused library of linear γ -AApeptide sequences including γ 1 and γ 2 were synthesized and studied for their ability to kill bacteria (Figure 4 & Table 1) [37]. The results show that they can mimic HDPs and exhibit broad-spectrum activ-

Figure 4. Representative antimicrobial γ -AApeptides (cont. from facing page). A helical scaffold of γ_9 is also shown, in which red dots represent hydrophobic groups, and blue dots represent hydrophilic groups.

Data taken from [37–43].

ity against both Gram-positive and Gram-negative bacteria. A few mechanistic studies, such as drug resistance and fluorescence microscopy, were carried out. It shows that linear γ -AApeptide sequences kills bacteria by mimicking host-defense peptides through bacterial membrane disruption [37].

Lipo-linear antimicrobial γ -AApeptides

We postulated that lipo linear γ -AApeptides as a new class of antimicrobial peptidomimetics can mimic the bactericidal function of HDPs. Hence, lipo-linear γ -AApeptides were expected to display broad-spectrum activities against both Gram-positive

Table 1. The antimicrobial and hemolytic activities of γ -AApeptides.

Organism	MIC ($\mu\text{g ml}^{-1}$)								
	γ^1	γ^2	γ^3	γ^4	γ^5	γ^6	γ^7	γ^8	γ^9
Gram-negative									
<i>Escherichia coli</i>	5	5	5	5	–	–	2	3	4
<i>Klebsiella pneumoniae</i>	>50	5	5	3	>50	8	3	–	–
<i>Pseudomonas aeruginosa</i>	>50	>50	5	3	18	8	5	2	4
Gram-positive									
<i>Bacillus subtilis</i>	3	2	3	3	5	1	–	–	–
<i>Staphylococcus epidermidis</i> (MRSE)	8	5	4	3	8	2	1	2	2
<i>Enterobacter faecalis</i> (VRE)	15	5	5	4	20	5	3	4	2
<i>Staphylococcus aureus</i> (MRSA)	15	5	4	3	30	1	2	4	2
Hemolysis (H_{50})	>500	300	>500	>500	>500	100	100	>500	100
The microbial organisms used are <i>E. coli</i> (JM 109), <i>B. subtilis</i> (BR151), methicillin-resistant <i>S. epidermidis</i> (RP62A), vancomycin-resistant <i>E. faecalis</i> (ATCC 700802), methicillin-resistant <i>S. aureus</i> (ATCC 33592), <i>K. pneumoniae</i> (ATCC13383) and <i>P. aeruginosa</i> (ATCC 27853). The MIC is the lowest concentration that completely inhibits growth after 24 h. H_{50} is the concentration causing 50% hemolysis. “–” indicates no test was carried out [37–41,49].									
MIC: Minimum inhibitory concentration; MRSA: Methicillin-resistant <i>Staphylococcus aureus</i> ; MRSE: Methicillin-resistant <i>Staphylococcus epidermidis</i> ; VRE: Vancomycin-resistant <i>Enterococci</i> .									

and Gram-negative bacteria. To test the hypothesis, lipo-linear γ -AApeptides γ^3 and γ^4 were designed and investigated for their antimicrobial mechanism (Figure 4 & Table 1) [38]. It is remarkable that both γ^3 and γ^4 are even more potent and broad-spectrum than the previously reported linear sequence γ^2 [37] toward the Gram-negative bacterium *P. aeruginosa*. In addition, lipo-linear γ -AApeptides exert bactericidal ability through its membrane disruption properties like HDPs, as evidenced by fluorescence microscopy, membrane depolarization and drug resistance studies [38].

Cyclic antimicrobial γ -AApeptides

In the effort of our continuous exploration of antimicrobial γ -AApeptides, cyclic antimicrobial γ -AApeptides were also developed to mimic the structure and function of HDPs [39]. Cyclic antimicrobial peptides are common in nature, such as gramicidin S, tyrocidine, polymyxin B and protegrin I, which generally are semi-rigid in their backbone conformations with substituents positioned in the well-defined space [27] compared with the linear γ -AApeptides, cyclic γ -AApeptides may have enhanced antimicrobial activity due to their more rigid conformation. Based on this rationale, cyclic antimicrobial γ -AApeptides were designed by joining amphiphilic building blocks together, so as to form a globally amphipathic conformation upon the interaction with bacterial membranes [39]. As expected,

those cyclic γ -AApeptides including γ^5 and γ^6 are among the most potent antimicrobial peptidomimetics with broad-spectrum activity, especially toward the most clinically relevant strain *S. aureus* (MRSA).

Lipo-cyclic antimicrobial γ -AApeptides

In consideration of the effect of cyclization, which reduces the structure motility and facilitates bacterial membrane disruption, and lipidation that encourages antimicrobial agent interactions with membranes, we hypothesized that γ -AApeptides with both cyclization and lipidation could be more potent in killing bacteria. To test the hypothesis, a series of lipidated cyclic γ -AApeptides including different numbers of amphiphilic γ -AApeptide building blocks were designed, synthesized and then measured for their activity against a range of multidrug-resistant bacterial pathogens (Figure 4 & Table 1) [40]. Particularly, γ^7 , with a small amphipathic ring and a C_{16} lipid tail outside, shows very potent activity against all tested drug-resistant stains. More intriguingly, γ^7 is demonstrated to be more effective to prevent the formation of biofilms and to reduce biofilm biomass in both Gram-positive and Gram-negative bacteria [44]. The similar structures, which have lipid tail inside ring with amphipathic structure, possess weak activity against bacteria. The possible reason is that the lipid tail on the ring has limited orientation and fails to interact with bacterial membranes. In addition, among the same amphipathic structures,

the precise distribution of functional groups in molecules will also affect the strength of interaction with bacterial membranes. Furthermore, the ability of lipidated cyclic γ -AApeptides to damage bacteria was evaluated by fluorescence microscopy, suggesting a mechanism analogous to HDPs. Interestingly, lipo-cyclic γ -AApeptides were also shown to harness the immune response and inhibit LPS-activated TLR4 signaling, endowing lipo-cyclic γ -AApeptides dual-functional roles as novel antimicrobial and anti-inflammatory agents [40].

Hybrid antimicrobial lipo- α / γ -AApeptides

Most γ -AApeptides that display potent and broad-spectrum antimicrobial activity against an array of antibiotic resistant Gram-positive and Gram-negative bacteria are long sequences and require a number of steps to synthesize. We hypothesized that short antimicrobial γ -AApeptides may also have potential application in antimicrobial field. As such, a focused library was synthesized by utilizing a hybrid backbone of canonical α -amino acid residues fused with γ -AApeptides (Figure 4 & Table 1) [41]. As anticipated, γ 8 [41] exerts bactericidal activity rapidly by mimicking HDPs without showing any hemolytic activity at concentrations up to 350 μgml^{-1} . Although alkylation was supposed to increase the overall hydrophobicity of molecules and enhance the interactions between hybrid peptides and bacterial membranes, the sequences containing two alkyl tails failed to show any antimicrobial activity. It indicates that specific orientations of the peptide backbones are required to maximize the interactions with bacterial membranes [50,51]. Again, studies including time kill and fluorescence microscopy suggested that lipo- α / γ -AApeptides can mimic the mode of action of HDPs and kill bacterial pathogens via membrane disintegration. In addition, lipo- α / γ -AApeptides also benefit from a more economical synthetic process when compared with previous γ -AApeptides [41].

Helical antimicrobial sulfono- γ -AApeptides

In order to explore the impact of different subclasses of γ -AApeptides on their antimicrobial activity, we prepared a series of sulfono- γ -AApeptides [49], which were believed to form helical structures, and have the ability to mimic the action of helical HDPs such as magainin 2 [10]. Based on the above-mentioned hypothesis and structural model, these sulfono- γ -AApeptides were designed with distinct amphipathicity, length and hydrophobicity, in order to understand the structure–function relationship existing in this class of foldamer for antimicrobial application (Figure 4 & Table 1) [42]. X-ray crystallography and 2D-NMR [49] analysis suggested that

sulfono- γ -AApeptides form stable helical structures in solution. As expected, sequence γ 9 shows significant antimicrobial activity. Although some sequences have same cationic charges and hydrophobic groups, their different location on the helical scaffold affects antimicrobial activity, as the distances between two adjacent side chains are not always the same on the sulfono- γ -AApeptides helical scaffold compared with side chains in α -helix [42]. In addition, small-angle x-ray scattering (SAXS) studies [52,53] shows the acetylation of N-terminus of the sequences significantly promote the folding of the sequences, and highly dense positive charges destabilize the helical folding [37,54]. Interestingly, the sequences with slightly less helical propensity in solution are more potent and selective than the stronger helix-forming sequences. Furthermore, time-kill and fluorescence microscopy studying suggest that similar to HDPs, the sequence γ 9 kill both Gram-positive and Gram-negative bacteria via compromising their membranes. Finally, it should also be noted that sulfono- γ -AApeptides are highly resistant to enzyme degradation [42], augmenting their potential antimicrobial applications.

Future perspective

The findings regarding the development of antimicrobial γ -AApeptides demonstrate that γ -AApeptides have great potential to become a new class of antibiotic agents to combat drug-resistant bacterial pathogens, because they not only are able to mimic mechanism of HDPs, but also have enhanced stability, bioavailability and limitless derivatization compared with HDPs. To further develop more potent γ -AApeptides for antimicrobial applications, a few aspects will be explored in the future. First of all, more diverse functional groups need to be investigated by taking advantage of the unique and novel backbone of γ -AApeptides, since the functional groups we studied are limited to date [43]. It is anticipated that their biological activity can be improved by introducing a wide variety of functional side chains, such as hydrophobic, bulky groups and cationic groups. In addition, their antimicrobial mechanisms will be carried out more systematically, which could gain insight into the general mechanism of action in this class of peptidomimetics, so as to assess their potential to induce drug resistance in bacteria. Moreover, it would be very helpful if crystal structures [49,55] of certain antimicrobial γ -AApeptides can be obtained, which will assist in understanding their structure–function relationship and thus contributing to the future rational design. In addition, it is also critical to investigate the *in vivo* activity of antimicrobial γ -AApeptides, so as to better determine

the potential of γ -AApeptides as a new generation of antibiotics combating drug resistance.

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Executive summary

Background

- The emerging antibiotic resistance is viewed as one of the omnipresent threat in global public health field, due to the unrestrained abuse of antibiotics.
- Host-defense peptides (HDPs) are promised to be next generation of antibiotic agents, as they have the potential to circumvent emerging drug resistance. However, their low stability and low-to-moderate activity hamper their further development.

Design & synthesis of γ -AApeptides

- γ -AApeptides is designed to be a new class of peptide mimics and start to show potential applications in drug discovery and chemical biology by taking advantage of their special backbone.

Synthesis of γ -AApeptides

- Alloc-protected γ -AApeptide building blocks are used to prepare γ -AApeptides with diverse functional groups on solid phase, which facilitates derivatization and optimization.

Design of antimicrobial γ -AApeptides

- Antimicrobial AApeptides are designed by joining amphiphilic γ -AApeptides building blocks together, and they could adjust their conformation to adopt globally amphipathic structures upon interaction with bacterial cell membranes.

Linear antimicrobial γ -AApeptides

- Linear γ -AApeptide sequences with multiple amphiphilic and hydrophobic building blocks were synthesized and show ability to inhibit the growth of all tested Gram-negative and Gram-positive bacteria.

Lipo-linear antimicrobial γ -AApeptides

- Lipo-linear γ -AApeptides are also broad-spectrum antimicrobial agents.

Cyclic antimicrobial γ -AApeptides

- Cyclic antimicrobial γ -AApeptides could also form globally amphipathic conformations upon the interaction with bacterial membranes and show good antimicrobial activity.

Lipo-cyclic antimicrobial γ -AApeptides

- Lipidated cyclic γ -AApeptides with both amphipathic ring and lipid tail also show potent activity drug-resistant stains.

Hybrid antimicrobial Lipo- α/γ -AApeptides

- Lipo- α/γ -AApeptides can mimic the mode of action of HDPs and kill bacterial pathogens via membrane disintegration.

Helical antimicrobial Sulfonyl- γ -AApeptides

- Sulfonyl- γ -AApeptides can form helical structures and show antimicrobial activity by killing both Gram-positive and Gram-negative bacteria via compromising their membranes.

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