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Non-hemolytic α-AApeptides as antimicrobial peptidomimetics†

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We report a new class of peptide mimetics, α-AApeptides, that display broad-spectrum activity against both Gram-negative and Gram-positive bacteria and fungi. With non-hemolytic activity, resistance to protease hydrolysis, and easy sequence programmability, α-AApeptides may emerge as a novel class of antibiotics.

Short cationic amphiphilic antimicrobial peptides (AMPs) are present in almost all organisms as a component of their innate defense against invading pathogens.[1] These peptides are emerging as a promising new generation of antibiotics because of their potent broad-spectrum antimicrobial activity, rapid action, ability to kill multidrug-resistant bacteria, and a low propensity for the development of resistance.[2–5] Many research findings support that AMPs kill bacteria through bacterial membrane disruption.[1,2] This is perhaps the major reason why acquiring resistance to AMPs is much more difficult, when compared to that of conventional antibiotic therapies.[6]

Despite being one of the most promising new generations of antimicrobial agents, AMPs face obstacles for therapeutic development. One of the biggest impediments is their intrinsic instability in the context of proteolytic degradation.[1,2] In contrast to conventional peptides, oligomeric peptidomimetics are protease-resistant, which can potentially circumvent the drawbacks of AMPs. In recent years, there has been significant interest in the development of antimicrobial peptidomimetics such as β-peptides,[7–11] peptoids,[12–15] aryl amides,[16,17] and oligoureas.[18] While the majority of these peptidomimetics were designed to mimic helical amphipathic antimicrobial peptides, for example magainin, the developments of antimicrobial peptide mimetics which imitate unstructured peptide antibiotics such as indolicidin are rare.[17,19–21] Some peptidomimetics are active against a variety of bacteria but are, however, hemolytic at higher concentrations, which sacrifices their selectivity.[11,12,18] Achieving potent, broad-spectrum antimicrobial activity, while retaining low hemolytic properties, is the immediate goal of antimicrobial peptidomimetic development.

We have recently proposed a new class of oligomeric peptidomimetics, termed "α-AApeptides", based on the α-chiral PNA backbone (Fig. 1).[22,23] α-AApeptides have many advantages over α-peptides, such as limitless diversification and resistance to protease degradation.[23] To continue to explore the potential application of α-AApeptides, herein we report for the first time the design, synthesis and evaluation of α-AApeptides as potential antimicrobial agents. We show that some α-AApeptides display potent, broad-spectrum activity against both Gram-positive and Gram-negative bacteria as well as fungus, and are highly selective (non-hemolytic). Coupled with straightforward solid phase synthesis, virtually limitless structural possibilities, low cost of production, simple tunability and programmability, and resistance to protease hydrolysis, α-AApeptides may lead to a new class of antimicrobial peptidomimetics.

Antimicrobial α-AApeptides were designed based on the notion that, while globally amphipathic structures are of importance, defined secondary structures are not necessary for the design of antimicrobial oligomers.[16,18,20,21] Gellman *et al.* have demonstrated that random copolymers can display different antibacterial activities by simply varying the ratio of hydrophobic and cationic groups, even the polymers are not structured at all.[21] Pre-organized helical oligomers can actually be more hemolytic and less selective.[12] α-AApeptides are very promising antimicrobial agents because they are likely to adopt an extended conformation[23] due to their conformational flexibility, which is believed to facilitate their penetration of bacterial cell walls.[16] The design of amphipathic α-AApeptide sequences is therefore very straightforward. They can be assembled by amphiphilic building blocks (containing both hydrophobic and cationic groups) and they would be potential antimicrobial agents, as the sequences can easily adjust their conformation to become globally amphipathic due to hydrophobic and

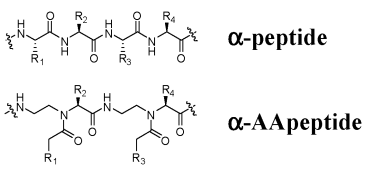

Fig. 1 Structures of an α-peptide and a corresponding AApeptide.

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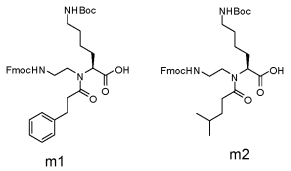

Fig. 2 α-AApeptide building blocks (**m1** and **m2**) used for the preparation of antimicrobial α-AApeptides.

electrostatic interactions when binding onto bacterial membranes, owing to their flexible backbone. We also predicted longer sequences would be more potent antimicrobial agents because more positive charges and hydrophobic groups can be involved in the interaction with bacterial membranes. To test our hypothesis, two amphiphilic α-AApeptide building blocks (Fig. 2) were synthesized, and a variety of α-AApeptide sequences with different lengths (**1–6**) were prepared (Fig. 3 and Fig. S1, ESI†) by assembling amphipathic building blocks on the solid phase *via* the reported protocol.[23] As controls, we prepared magainin II (a natural antimicrobial peptide) **7**, a 14-mer conventional peptide **8** with alternative phenylalanine and lysine residues and peptidomimetic **9**, the opposite α-AApeptide where the amino acid is Phe and the lysine like side chain comes off the amine (Fig. 3 and Fig. S1, ESI†).

The antimicrobial activities of **1–9** were tested against the Gram negative *E. coli* and Gram positive *B. subtilis*, *S. epidermidis* and fungus *C. albicans* (Table 1). Hemolytic activities of the oligomers toward human red blood cells were

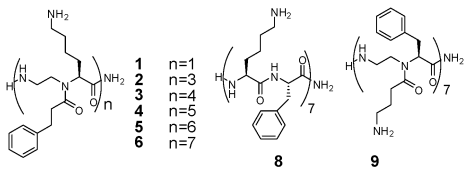

Fig. 3 Oligomers for the antimicrobial assay. **1–6** are α-AApeptides; **7** is magainin II; **8** is the 14-mer regular peptide with alternative phenylalanine and lysine residues. **9** is the opposite α-AApeptide where the amino acid is Phe and the lysine-like side chain comes off the amine.

also investigated to measure their selectivity (Table 1). As expected, the numbers of hydrophobic and cationic groups are important for antimicrobial activity, since longer α-AApeptides are found to be more potent (Fig. S2, ESI†). α-AApeptide **6** shows the best activity toward both Gram-positive and Gram-negative bacterial strains, and is amongst the most potent antimicrobial oligomeric peptidomimetics reported.[10,12] It also shows strong activity against the multi-drug resistant *S. epidermidis* strain RP62A, which is insensitive to a variety of conventional antibiotics. Two natural antimicrobial peptides, magainin **7** (helical)[24] and indolicidin (unstructured),[19,25–27] are both less active against all of the strains tested. Interestingly, the control peptide **8**, bearing exactly the same functional groups and backbone length as α-AApeptide **6**, did not show any activity at the concentrations tested. We hypothesize that such a distinct difference must result from the different backbones, and global amphipathic structures of the two peptides.[28] Surprisingly, control α-AApeptide **9** only shows very weak antibacterial activity, probably due to the existence of bulky chiral aromatic groups, which prevent the formation of an extended structure upon binding to bacterial cell membrane.

To test the selectivity of the oligomers, **1–9** were incubated with human red blood cells. None of them showed detectable hemolysis up to a concentration of 100 μg ml^{-1}. Then, the most active α-AApeptides **4**, **5** and **6**, along with control peptides magainin **7**, **8**, and **9**, were also tested at higher concentrations. Again, each of them effectively displayed almost 0% hemolysis at concentrations of up to 250 μg ml^{-1}, with the exception of peptide **8**, which showed 5% hemolysis at this concentration (Fig. S3, ESI†). It seems that the α-AApeptide backbone is slightly less hemolytic than that of a conventional peptide, as seen by comparing α-AApeptide **6** to peptide **8**, in which the same functional groups are present. Meanwhile, the evidence suggests that hemolytic activity is more likely related to functional groups present on the oligomer backbone than to the nature of the backbone, since indolicidin, containing multiple hydrophobic tryptophan residues, causes 10% hemolysis at 30 μg ml^{-1} and 100% hemolysis at approximately 200 μg ml^{-1}.[25]

Table 1 The antimicrobial and hemolytic activities of oligomers **1–8**. The microbial strains used were *E. coli* (JM109), *B. subtilis* (BR151), *S. epidermidis* (RP62A), and *C. albicans*. *S. epidermidis* is a multi-drug resistant isolate which is insensitive to β-lactam antibiotics, and a number of other antimicrobial agents. The minimum inhibitory concentration (MIC) is defined as the lowest inhibitor concentration that completely inhibits the growth of microbes during a 24 h incubation period at 37 °C. MHC (the minimum hemolytic concentration) is defined as the lowest concentration at which hemolysis is detected. H10 is defined as the concentration that causes 10% hemolysis of human red blood cells. Selectivity is defined as H10/MIC towards *B. subtilis*. Activities of the extended antimicrobial peptide indolicidin (ILPWKWPWWPWRR) were obtained previously by other research groups[19,25–27]

	MIC[a] μM (μg ml^{-1})					
Oligomers	*E. coli*	*B. subtilis*	*S. epidermidis*	*C. albicans*	MHC[a]/μg ml^{-1}	Selectivity (H10/*B. subtilis* MIC)
1	293(90)	243(75)	>162(50)	—	>100	>1.3
2	64(60)	43(40)	>53(50)	—	>100	>2.5
3	16(20)	15(18)	>40(50)	—	>100	>5.6
4	13(20)	8(12.5)	20(30)	—	>250	>20
5	9.8(18)	6.8(12.5)	>27(50)	—	>250	>20
6	**2.1(4.5)**	**0.9(2)**	**4.6(10)**	**9.3(20)–14(30)**	**>250**	**>125**
7	16(40)	16(40)	>20(50)	>20(50)	>250	>6.3
8	>51(100)	>51(100)	>51(100)	>51(100)	~250	<2.5
9	38(75)	38(75)	>100	>51(100)	>250	>3.3
Indolicidin	13(25)	5(10)	10(20)	>33(64)	~30	~3

[a] For concentrations shown as "$>x$", x = 50, 100 or 250 μg ml^{-1}, which are the highest concentrations tested for that sequence.

Circular dichroism (CD) spectroscopy was employed to study the conformational structure of **1–8** in both aqueous buffer and in lipid vesicles that mimic bacterial membranes (Fig. S4, ESI†).[13] α-AApeptides have a different backbone compared to peptides; therefore CD cannot specifically elucidate a specific secondary structure. However, results suggest that α-AApeptides are probably unstructured in buffer solution, since their CD spectra are quite similar to monomer **1**, which is unlikely to have any secondary structure at all (Fig. S4a, ESI†). Magainin **7** is also randomly coiled in buffer, while peptide **8** displays a very weak minimum at 222 nm (Fig. S4a, ESI†), indicating the presence of some α-helical character. Upon binding to lipid vesicles, the CD of α-AApeptide **6** shows a minimum at 207 nm (Fig. S4b, ESI†), which is not observed in PBS. The reason is unclear, possibly because the amphipathic structure of α-AApeptide **6** is more defined on the lipid vesicles. Magainin **7** becomes highly helical (Fig. S4b, ESI†) under similar conditions, which is consistent with previous reports.[24] Interestingly, **8** completely lost its helical conformation when exposed to lipid vesicles, and turned into a random coil.

We believe α-AApeptides are not structured in aqueous solution due to their flexible backbone. However, upon binding to negatively charged bacterial membranes, they can easily adopt globally amphipathic structures to facilitate membrane disruption (Fig. S5, ESI†). Indeed, the flexibility of the AApeptide backbone leads to a stronger bacterial membrane-disruptive capability than conventional peptides,[16] which accounts for the potent antimicrobial activity of α-AApeptides. Control peptide **8**, containing alternative cationic and hydrophobic residues, displays positive charges all over the helical backbone in water solution. Forming an amphipathic structure for bacterial cell wall penetration is not favorable because regular peptide backbones have limited conformational freedoms, which lead to very weak antimicrobial activity. The same assumption can be used to explain the antibacterial activity of α-AApeptide **9**.

The antimicrobial mechanism of α-AApeptides in disrupting bacterial membranes was further assessed by fluorescence microscopy (Fig. S6, ESI†) using a double staining method with DAPI and PI. DAPI stains all bacterial cells irrespective of their viability, whereas PI only stains injured or dead cells with damaged membranes.[29,30] After incubation with α-AApeptide **6** for 2 h, strongly PI-stained red fluorescent *E. coli* and *B. subtilis* were observed, demonstrating that the membranes of those bacteria had been disrupted.

In conclusion, this is the first report of water-soluble α-AApeptides as antimicrobial peptidomimetics. Three α-AApeptides (**4**, **5** and **6**) exhibit high selectivity, broad-spectrum, and potent antibacterial activity against fungus, Gram-positive and Gram-negative bacteria, including clinically-relevant multi-drug resistant strains. α-AApeptide **6** was of significant interest as it displayed antimicrobial activities, activity that was equal to or better than the most antimicrobial oligomeric peptidomimetics,[10,12,14,18] yet displays no hemolytic activity at all, under tested experimental conditions. Such high selectivity is attributed to the intrinsic properties of the flexible α-AApeptide backbone in adopting amphipathic structures. Our results support recent findings that antimicrobial activity of peptides, or peptide mimics, is related to the presence of global amphipathicity upon interaction with bacterial membranes rather than to pre-defined secondary structures. The number (not just the ratio) and nature of cationic and hydrophobic groups also have profound impact on activity. The simple design and easy modular programmability should facilitate quick identification of more potent and selective antimicrobial α-AApeptides in the near future.

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