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Effect of side chain hydrophobicity and cationic charge on antimicrobial activity and cytotoxicity of helical peptoids

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ABSTRACT

Peptoids are peptidomimetic polymers that are resistant to proteolysis and less prone to immune responses; thus, they can provide a practical alternative to peptides. Among the various therapeutic applications that have been explored, cationic amphipathic peptoids have demonstrated broad-spectrum antibacterial activity, including activity towards drug-resistant bacterial strains. While their potency and activity spectrum can be manipulated by sequence variations, bacterial selectivity and systemic toxicity need to be improved for further clinical development. To this aim, we incorporated various hydrophobic or cationic residues to improve the selectivity of the previously developed antibacterial peptoid **1**. The analogs with hydrophobic residues demonstrated non-specific cytotoxicity, while those with an additional cationic residue showed improved selectivity and comparable antibacterial activity. Specifically, compared to **1**, peptoid **7** showed much lower hemolysis and cytotoxicity, while maintaining the antibacterial activity. Therefore, we believe that peptoid **7** has the potential to serve as a promising alternative to current antimicrobial therapies.

Antimicrobial peptides (AMPs) are naturally occurring antibiotic agents, found in most living organisms, that exhibit broad-spectrum activity.[1] AMPs participate in the innate immune response against various pathogens and encompass a diverse group of compounds, with varied sequences and sizes that generally consist of several clusters of cationic or anionic residues, together with hydrophobic residues. Because AMPs are ubiquitous in nature, their mode of actions cannot be defined by a single mechanism; however, the general consensus is that the amphipathic scaffold of AMPs allows formation of transmembrane pores upon contact with microorganisms to induce membrane rupture.[2] Once they have penetrated inside the cells, some AMPs participate in additional intracellular interactions, such as nucleic acid binding[3,4] and enzyme inhibition,[5] to further promote antimicrobial activity. The antimicrobial selectivity and potency of AMPs depend on their physicochemical properties, such as the net charge, secondary structure, hydrophobicity, size, and balance between hydrophobic and hydrophilic residues.[6–8]

Among the many variants of AMPs, cationic AMPs with antibacterial activity have been studied most extensively, because of their highly selective and potent cytotoxicity. Cationic AMPs bind to negatively charged lipopolysaccharides or phospholipids on the bacterial membrane and exert rapid cytotoxicity.[9,10] This unique mode of action is highly specific toward many strains of bacteria, including drug-resistant strains.[11–15] Given the systemic toxicity and prevalent drug resistance with the currently available antimicrobial treatments, AMPs hold promise for novel broad-spectrum therapeutic agents with low toxicity.[16] However, AMPs have several inherent limitations, such as the possibility of proteolytic degradation and their potential immunogenicity;[17,18] therefore, various alternative approaches have been explored to counter these limitations, including combination therapy,[19] specific delivery mechanisms,[20] and non-natural amino acid analogs.[21]

Peptoids are peptidomimetic polymers, consisting of a versatile molecular scaffold, based on a poly-*N*-substituted glycine backbone, which generally limits proteolysis and immune responses, while maintaining similar physicochemical properties to peptides.[22] In addition, peptoids can be readily synthesized through conventional solid-phase peptide synthesis techniques, and any chemical moiety in the form of a primary amine can be incorporated into peptoids to

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provide structural diversity. As a practical alternative to peptides, peptoid analogs have been developed for various therapeutic applications, including antimicrobial[23,24] and anticancer agents[25–28] and cellular delivery vehicles.[29–31] In particular, several recent studies reported amphipathic peptoids that were highly effective against a broad spectrum of bacteria[32–35] and fungi.[36,37]

Peptoid **1** (Fig. 1) is a cationic amphipathic compound with potent antibacterial activity against drug-resistant bacterial strains, such as *P. aeruginosa*[38] and *M. tuberculosis*.[39] Previously, we studied various factors affecting the antibacterial potency and selectivity of **1** by differing the chain length, amphipathicity, and net charge.[24,25,38–40] We observed that the ratio of cationic and hydrophobic residues and the presence of aromatic side chains appear to be critical for the antibacterial activity. More importantly, the antibacterial potency and activity spectrum can be manipulated by sequence variations; however, bacterial selectivity and systemic toxicity need to be improved for further clinical development. To this end, we designed a series of cationic, amphipathic peptoids containing hydrophobic substituents in their aromatic side chains, as shown in Fig. 1. We incorporated one cationic monomer, *N*lys, per two hydrophobic monomers to create a group of three monomers, defined as one repeating unit (n = 1). Peptoids can form a polyproline type-I-like helix with three monomers per turn.[41] Consequently, with one cationic helical face and two hydrophobic faces, the peptoid would mimic both the amphipathic and helical attributes of the AMP. In our previous study, compared to shorter peptoids, peptoids with twelve monomers (n = 4) generally showed higher cytotoxicity; however, in this work, we included peptoids with nine (n = 3), ten, and thirteen (n = 3 and 4, with an additional *N*lys group) monomers to diversify the net charge and helicity. We also included a known AMP, pexiganan,[42] for comparison. All peptoids were synthesized on an automated peptide synthesizer by following the previously described submonomer pathway[40,43] and purified using high performance liquid chromatography (HPLC). The complete sequences, molecular weight, net charge, HPLC elution percentages, and charge to length ratio (CTLR) of the synthesized peptoids are described in Table 1. We found that the elution order of each of the peptoids from reversed-phase HPLC corresponds to the clogP value of each residue. For example, peptoid **3**, which contains the most hydrophobic substituent (*N*spe(pCl), clogP = 0.481), eluted at the highest percentage of acetonitrile (64.1%); whereas peptoid **7**, which contains the least hydrophobic substituent (*N*spe, clogP = -0.197) with an additional *N*lys, eluted at the lowest percentage of acetonitrile (48.0%), indicating that the hydrophobicity of the aromatic monomers can be directly translated to the overall hydrophobicity of each peptoid.[44]

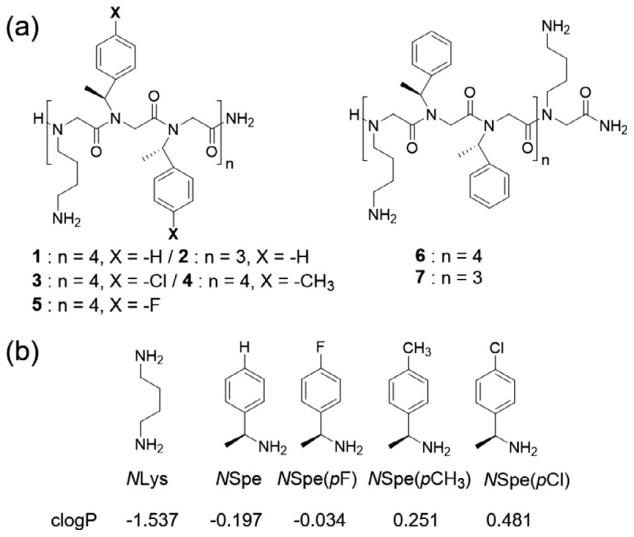

Fig. 1. (a) Structures of cationic, amphipathic peptoids. (b) Structures of the peptoid side chains and their clogP values. The clogP calculation was performed using the Molinspiration free log *P* calculator.[47]

For peptoids **1**–**7**, circular dichroism (CD) spectra were measured at 190–260 nm to evaluate the peptoid backbone conformations (Fig. 2). As expected, peptoids exhibited the typical polyproline type-I (PPI)-like α-helical peptoid CD signatures, with two negative Cotton effects at 202 nm and 220 nm.[41] Red-shifted peaks were observed for **3** and **4** because of the decreased energy gap in the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions (Fig. 2(a)). The degree of the peak shift roughly correlates with the Woodward–Fieser rule;[45] para-methyl and para-chloro substituents exhibited +5 and +10 nm red-shifts, respectively. For the para-fluorine-containing **5**, no distinct peak shift was observed, likely because of the unavailability of non-bonding electrons on the fluorine. Among the 12mer peptoids (**6** is a 13mer), CD of peptoid **1** indicated a helical fold with the greatest intensity. Although the molar absorptivities of chlorobenzene and toluene are larger than those for benzene, decreased intensities of CD minima were observed in **3** and **4**, suggesting that the helical integrity of the two peptoids is weaker than **1**. Additional *N*lys incorporation at the C-terminus of peptoid **1** resulted in a slightly weakened helical fold for **6**, the CD signature of which was similar to that of the para-fluorine-containing **5**. The length-dependent CD change is provided in Fig. 2(b). Peptoid 12mers and 9mers are shown in solid lines and dotted lines, respectively (**6** and **7** are a 13mer and a 10mer, respectively). In general, longer peptoids are expected to have greater helical integrity, as was observed for **1** and **2**; however, a similar CD signature was observed **6** and **7**.

To evaluate the antibacterial activity of the synthesized peptoids, we determined the minimal inhibitory concentration (MIC) against a Gram-negative strain with *E. coli* and a Gram-positive strain with *B. subtilis*. Because our library was composed of slight variants of peptoid **1**, the assay was performed in a narrow concentration range of 0.4–6 μM using 2-fold serial dilutions. As shown in Table 2, two control compounds, peptoid **1** and pexiganan demonstrated potent antibacterial activity against both strains. Peptoid **2**, which contains nine monomers (n = 3), with the same repeating sequence as peptoid **1**, was slightly less active (3.1 μM for *E. coli*, 1.6 μM for *B. subtilis*) than peptoid **1** (1.6 μM for *E. coli*, 0.8 μM for *B. subtilis*). Peptoids with hydrophobic substituents (**3**–**5**) displayed generally poor to moderate activity against *E. coli*, but they maintained comparable activity against *B. subtilis*, except for peptoid **3**. The most hydrophobic peptoid, **3**, which contains para-chlorine substituents, appeared to be the least effective compound against both strains, exhibiting MIC values greater than 6.1 μM. The poor antimicrobial activity of para-chlorobenzyl-containing peptoids was also observed by Cobb and coworkers.[46] In contrast, the para-fluorine-containing **5** showed comparable MIC values to **1**, which is not surprising given that fluorine is often used as an isostere of hydrogen; however, the fluorine substitution resulted in the most hemolytic peptoid in the library. Interestingly, peptoids containing an additional *N*lys residue, **6** and **7**, demonstrated slightly enhanced antibacterial activities compared to their parent peptoids **1** and **2**. Specifically, peptoid **6**, which contains an additional *N*lys group at the *N*-terminus of peptoid **1**, demonstrated the most potent MIC (1.6 μM for *E. coli*, 0.4 μM for *B. subtilis*) among the series of peptoids. In addition, peptoid **7** exhibited comparable antibacterial activity to **1**, although **7** had a shorter chain of ten monomers (n = 3).

To assess toxicity of these peptoids toward mammalian cells, we determined the concentration of each compound that will cause hemolysis of rat erythrocytes (HD_{10}/HD_{50}). Peptoids with

Table 1
Sequences of peptoids and pexiganan.

Compounds	Sequence	MW (Da)[a]	Net charge[b]	CTLR[c]	HPLC elution[d](% CH_3CN)
1	H-(*N*lys-*N*spe-*N*spe)$_4$-NH_2	1819.36	+4	0.33	53.6
2	H-(*N*lys-*N*spe-*N*spe)$_3$-NH_2	1368.78	+3	0.33	51.6
3	H-(*N*lys-*N*spe(*p*Cl)-*N*spe(*p*Cl))$_4$-NH_2	2094.90	+4	0.33	64.1
4	H-(*N*lys-*N*spe(*p*CH_3)-*N*spe(*p*CH_3))$_4$-NH_2	1931.58	+4	0.33	61.1
5	H-(*N*lys-*N*spe(*p*F)-*N*spe(*p*F))$_4$-NH_2	1963.29	+4	0.33	56.2
6	H-(*N*lys-*N*spe-*N*spe)$_4$-*N*lys-NH_2	1947.54	+5	0.38	50.3
7	H-(*N*lys-*N*spe-*N*spe)$_3$-*N*lys-NH_2	1496.96	+4	0.40	48.0
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2	2477.22	+9	0.41	43.7

[a] Molecular weight is calculated for the free base form, and not as a TFA salt.
[b] Net charge is counted based on the number of cationic side chains at neutral pH.
[c] CTLR stands for charge-to-length ratio.
[d] Percentage of acetonitrile in water, 0.1% (v/v) TFA at HPLC elution.

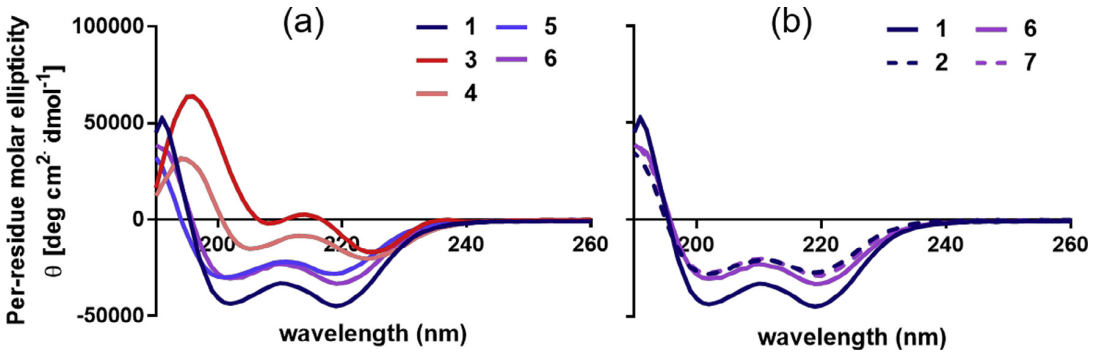

Fig. 2. Circular dichroism (CD) spectra of (a) 12mers and (b) comparison between 12mers and 9mers. **6** and **7** are a 13mer and a 10mer, respectively. Spectra were recorded as the per-residue molar ellipticity at 190–260 nm. A peptoid concentration of 50 μM in Tris buffer (10 mM, pH 7.4) was used. Data were acquired at 25 °C.

Table 2
Antibacterial and hemolytic activities of peptoids and pexiganan.

Compounds	MIC[a] (μM)		HD_{10}/HD_{50}[b]	H_{max}[c] (200 μM)
	E. coli	*B. subtilis*		
1	1.6	0.8	9.1/63.4	100
2	3.1	1.6	119.5/>200	38.7 ± 5.4
3	>6.1	6.1	<6.25/10.4	100
4	>6.1	0.8	<6.25/8.3	100
5	3.1	0.4	<6.25/<6.25	100
6	1.6	0.4	19.5/>200	48.1 ± 3
7	1.6	0.8	>200/>200	9.8 ± 0.8
Pexiganan	0.8	0.4	113.4/>200	21.5 ± 2.9

[a] MIC: minimal inhibitory concentration. These concentrations represent the mean values of three independent experiments performed in duplicate.
[b] HC_{10} and HC_{50} are the concentrations of the compounds that cause 10% and 50% hemolysis of rat erythrocytes, respectively.
[c] H_{max} is the percent (%) hemolysis at the highest concentration tested (200 μM).

hydrophobic substituents (**3**–**5**) generally appeared to be toxic, inducing lysis of 10% (HD_{10}) and 50% (HD_{50}) of rat erythrocytes at concentrations in the low micromolar range (less than 10.4 μM), while peptoid **1** was moderately toxic (HD_{10} = 9.1 μM and HD_{50} = 63.4 μM). However, peptoids **2**, **6**, **7**, and pexiganan induced the same extent of hemolysis at much higher concentrations, greater than 200 μM. The differences in hemolytic toxicity were more clearly observed, when the percent hemolysis induced by each compound at 200 μM (H_{max}) was measured. Hydrophobic peptoids **3**–**5**, as well as peptoid **1**, demonstrated complete hemolysis of erythrocytes, whereas peptoids **2**, **6**, **7** and pexiganan showed a relatively low percent hemolysis. In particular, peptoid **7** demonstrated the lowest percentage of hemolysis (9.8%), indicating that the antibacterial activity of **7** is highly selective. The addition of a *N*lys group at the C-terminus noticeably reduced hemolytic toxicity, while maintaining comparable antibacterial activity. Overall, peptoids with greater charge-to-length ratio (CTLR) appear to exhibit improved selectivity; in contrast, peptoids with greater hydrophobicity and helical fold show decreased selectivity.

Next, we wanted to examine whether these cationic amphipathic peptoids also exerted cytotoxicity against cancer cells, as we have previously observed.[25,26] Therefore, we determined the cytotoxicity of the peptoids against human leukemia Jurkat T-cells and human prostate cancer LNCaP cells, in parallel with normal human lung fibroblasts (MRC5) for comparison. As shown in Table 3, LC_{50} values of all the compounds were generally in agreement with the percent hemolysis of erythrocytes. Peptoids **3**–**5**, which induced hemolysis at low concentrations, showed relatively low LC_{50} values (1.58–12.0 M) against cancer cells and normal cells; whereas, the least hemolytic peptoid **7** showed low cytotoxicity against the tested eukaryotic cells (28.9–80.5 M). Although we were unable to identify compounds with cancer-specific cyto-

Table 3
LC_{50} values (μM) against cancer cells (Jurkat and LNCaP) and normal cells (MRC5).[a]

	Jurkat	LNCaP	MRC5
1	2.21	5.0	8.0
2	18.6	27	40.0
3	1.58	10.0	3.0
4	ND[b]	12.0	8.0
5	2.47	9.5	9.5
6	5.04	ND[b]	10.0
7	28.9	ND[b]	80.5
Pexiganan	ND[b]	ND[b]	21.2

[a] These concentrations represent mean values of three independent experiments performed in duplicate.
[b] ND: Not Determined.

toxicity in this series, these results again confirmed that peptoids with hydrophobic substituents (**3**–**5**) are toxic to mammalian cells and demonstrated that the antibacterial activity of peptoid **7** is highly specific toward bacteria. It is interesting to note that this remarkable specificity of **7** was achieved by adding only one additional *N*lys group to peptoid **2**.

In conclusion, we designed and evaluated the antibacterial activity of cationic, amphipathic peptoids. We incorporated hydrophobic or cationic residues to improve the selectivity of the previously developed antibacterial peptoid, peptoid **1**. The hydrophobic analogs (**3**–**5**) demonstrated a non-selective cytotoxicity against mammalian cells; however, compared to their parent peptoids (**1** and **2**), the analogs with an additional *N*lys group (**6** and **7**) exhibited an improved selectivity and comparable activity. In particular, compared to peptoid **1**, a shorter sequence analogue, peptoid **7**, demonstrated the same extent of antibacterial activity but showed much lower hemolysis and cytotoxicity; therefore, we believe that peptoid **7** is a promising antimicrobial candidate with a good therapeutic window.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2017.11.034.

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