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Tailoring Cytotoxicity of Antimicrobial Peptidomimetics with High Activity against Multidrug-Resistant *Escherichia coli*

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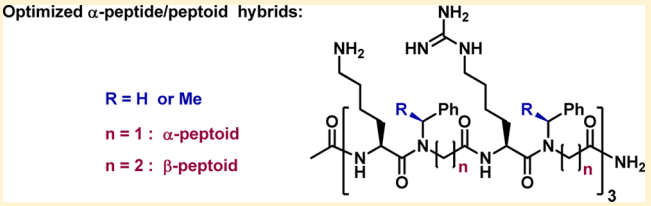
Supporting Information

ABSTRACT: Infections with multidrug-resistant pathogens are an increasing concern for public health. Recently, subtypes of peptide–peptoid hybrids were demonstrated to display potent activity against multidrug-resistant Gram-negative bacteria. Here, structural variation of these antibacterial peptidomimetics was investigated as a tool for optimizing cell selectivity. A protocol based on dimeric building blocks allowed for efficient synthesis of an array of peptide–peptoid oligomers representing length variation as well as different backbone designs displaying chiral or achiral peptoid residues. Lack of α-chirality in the side chains of the peptoid residues proved to be correlated to reduced cytotoxicity. Furthermore, optimization of the length of these peptidomimetics with an alternating cationic–hydrophobic design was a powerful tool to enhance the selectivity against Gram-negative pathogens over benign mammalian cells. Thus, lead compounds with a high selectivity toward killing of clinically important multidrug-resistant *E. coli* were identified.



INTRODUCTION

Infections with multidrug-resistant (MDR) pathogens are of growing concern, as these difficult-to-treat conditions are reported still more often and are linked to high morbidity and mortality.[1] Despite the urgent need for new treatment options, only limited effort is devoted to research and development of new anti-infective remedies from the big pharmaceutical companies, and therefore, medical organizations encourage stakeholders to act.[2,3]

Antimicrobial peptides (AMPs) are believed to constitute potential lead compounds for future antibiotics.[4,5] During development of potent representatives of this class of compounds, three major issues are often encountered: (i) Mode of action for these compounds is multifaceted and may comprise bacterial intracellular targets and/or (more typically) interactions with the bacterial membrane, and because of the latter, concomitant cytotoxicity toward mammalian cells is often observed. (ii) The amide bonds of the peptide backbone are readily cleaved by proteases, which significantly decreases bioavailability. (iii) Difficult and/or expensive synthesis is an issue.

Stabilization toward proteolytic degradation may readily be achieved by incorporation of a sufficient ratio of D-amino acids or other unnatural residues. Also, improvements in methods for large-scale peptide synthesis have led to reduced production costs which have facilitated that synthetic peptides and peptidomimetics account for an increasingly significant share of novel chemical entities in the pipelines in many pharmaceutical companies.[6,7] Thus, in recent years several peptide-based therapeutics are in clinical trials or have been marketed toward a variety of indications.[8−11]

Hybrid oligomers containing both natural amino acids and unnatural residues, including N-substituted glycines or N-substituted β-alanines (denoted α- and β-peptoids, respectively) in cationic–hydrophobic alternating sequences constitute promising scaffolds for potential lead compounds, as they confer both stability toward enzymatic degradation and activity against MDR bacteria. By contrast, the analogous all-D and all-L α-peptides proved not to exhibit selective antibacterial properties but were both less potent and highly cytotoxic.[12] Previous observations[13] have indicated that the length of such scaffolds is an important determinant for the level of cytotoxicity to human cells. Thus, we hypothesize that optimization of this property could improve the cell selectivity for prokaryotes over eukaryotes. In order to investigate the impact of length variation and α-chirality of side chains on the activity profile, an array representing different designs of peptidomimetics was prepared (Figure 1).

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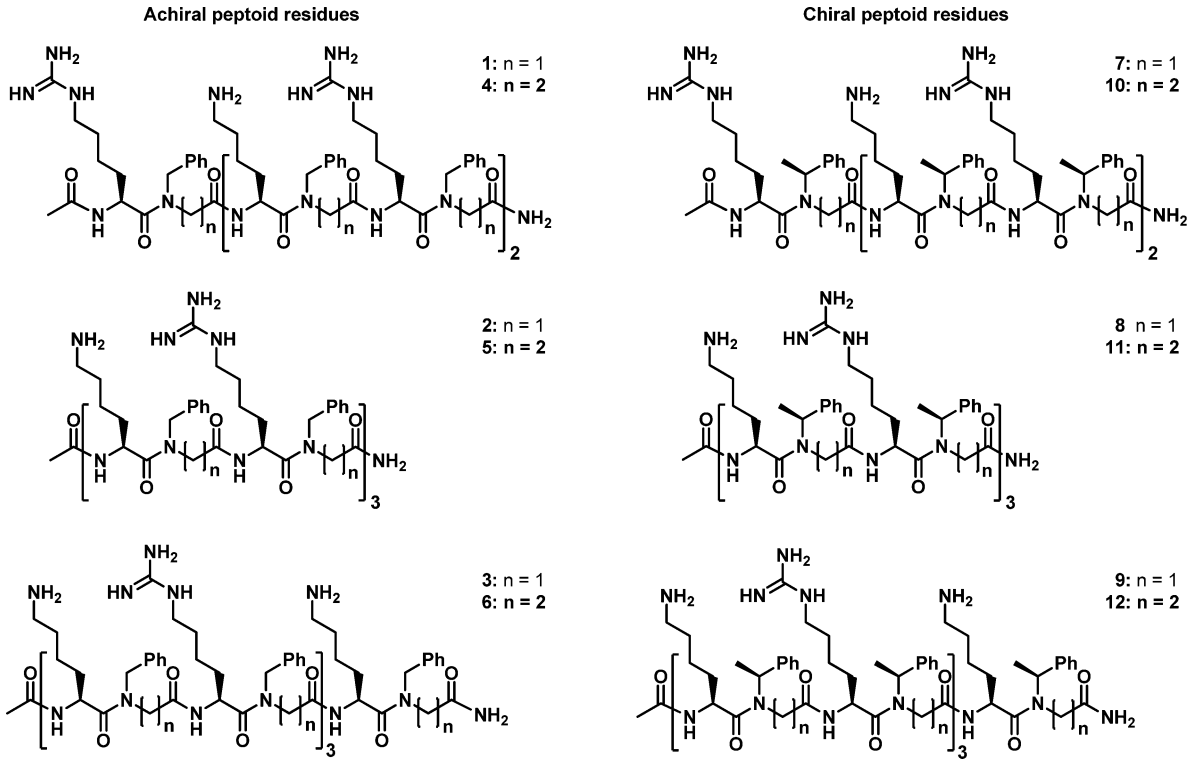


Figure 1. Peptidomimetic hybrids consisting of amino acids and either α- or β-peptoid residues with varying length and different degree of chirality.

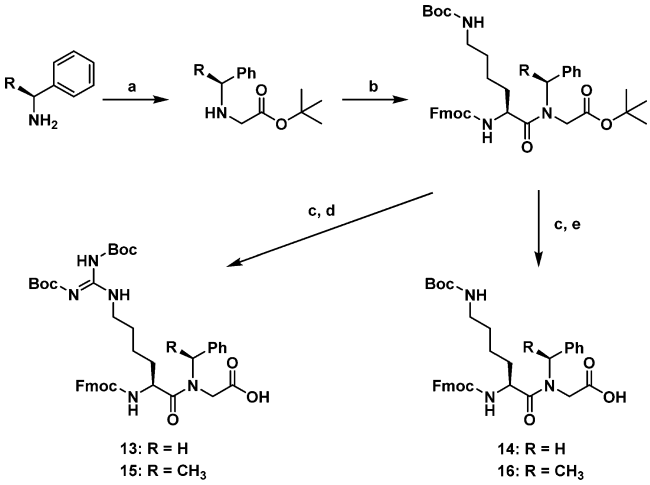
Four different subtypes of compounds displaying repetitive sequences alternating between a cationic moiety and a hydrophobic moiety have been synthesized in the lengths of 10, 12, and 14 residues. These peptidomimetics can be divided into two groups according to the α-peptoid (n = 1, i.e., **1**−**3** and **7**−**9**) or β-peptoid (n = 2, i.e., **4**−**6** and **10**−**12**) nature of the unnatural residues. The two groups each comprise two different series containing achiral (**1**−**6**) or chiral side chains (**7**−**12**). Previously, we have shown that AMP mimetics with a repetitive sequence containing homoarginine (hArg) as the cationic residue exhibit higher antibacterial activity compared to Lys-containing hybrids.[13] However, we anticipated that hArg residues also might confer higher cytotoxicity, and thus, a design containing both Lys and hArg as cationic moieties was chosen. In order to assess their cell selectivity, the compounds were screened against several MDR pathogens as well as toward an array of mammalian cell lines. The features of the CD spectra in the presence of membrane model systems mimicking bacteria or human cells, respectively, were correlated with cytotoxicity.

RESULTS

Synthesis of Dimeric Building Blocks. On-resin oligomerization of amino acids and achiral peptoids to yield hybrid sequences may be carried out most conveniently by using monomer building blocks as previously reported.[12] In contrast, oligomerization of peptoid residues containing α-chiral side chains is strongly hampered because of steric hindrance, which makes on-resin assembly of dimeric building blocks, prepared via solution-phase couplings, more efficient.[14,15] Moreover, hArg building blocks are most readily obtained via the corresponding Lys building blocks by a guanidinylation step leading to bis-Boc-protected guanidino side chain functionalities. The array of compounds required for the present study consisted of both chiral[16] and achiral peptoid moieties in combination with both Lys and hArg. However, in order to employ a consistent solid-phase synthesis protocol, dimeric building blocks were utilized to obtain all oligomers as illustrated in Scheme 1.

The α-peptoid intermediates were prepared as described previously.[17] In brief, benzylamine or (*S*)-1-phenylethylamine was alkylated with *tert*-butyl 2-bromoacetate. Subsequently, the

Scheme 1[a]



[a]Reagents and conditions for preparation of dimeric hybrid building blocks: (a) 1.0 equiv of *tert*-butyl 2-bromoacetate, 2.0 equiv of Et_3N, THF, rt, 16 h; (b) 1.1 equiv of Fmoc-Lys(Boc)-OH, 1.5 equiv of TBTU, 2.5 equiv of DIPEA, dry DCM, rt, 16 h; (c) TFA−DCM 1:3, rt, 2 h; (d) 1.2 equiv of *N*,*N*′-bis-Boc-1*H*-pyrazole-1-carboxamidine, 5.0 equiv of DIPEA, DCM, rt, 16 h; (e) 1.3 equiv of Boc_2O, 5.0 equiv of DIPEA, DCM, rt, 16 h.

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Table 1. Minimal Inhibitory Concentration (μM) of the Peptidomimetics (Given as Median [Range], n = 3)[a]

		E. coli					
compd	no. of residues	susceptible strain[b]	ESBL-producing strain[c]	NDM-1-producing strain[d]	*A. baumannii*, MDR strain[e]	*S. aureus*, methicillin-resistant strain[f]	*E. faecium*, vancomycin-resistant strain[g]
1	10	8 [−]	16 [−]	8 [8−16]	>128 [−]	128 [−]	128 [64−128]
2	12	2 [−]	2 [2−4]	1 [−]	64 [−]	64 [−]	32 [−]
3	14	1 [1−2]	1 [1−2]	1 [−]	4 [−]	32 [32−64]	8 [−]
4	10	8 [−]	16 [−]	8 [−]	128 [−]	64 [−]	128 [−]
5	12	1 [−]	1 [−]	1 [−]	16 [−]	32 [32−64]	32 [−]
6	14	1 [−]	1 [−]	1 [−]	4 [2−4]	16 [16−32]	16 [−]
7	10	8 [−]	8 [−]	8 [−]	16 [−]	32 [32−64]	8 [−]
8	12	2 [−]	4 [−]	2 [−]	2 [−]	16 [−]	2 [1−2]
9	14	1 [−]	2 [−]	1 [−]	1 [−]	16 [−]	4 [2−4]
10	10	8 [−]	8 [−]	8 [−]	64 [−]	16 [−]	64 [−]
11	12	2 [−]	4 [−]	2 [−]	8 [−]	16 [−]	16 [−]
12	14	2 [−]	2 [−]	2 [−]	2 [1−2]	8 [4−8]	4 [4−8]
vancomycin		>128 [−]	>128 [−]	>128 [−]	>128 [−]	0.5 [−]	128 [−]
gentamicin		0.25 [0.125−0.25]	32 [−]	>128 [−]	>128 [−]	0.5 [0.5−1]	>128 [−]
cefotaxime		<0.125 [−]	>128 [−]	>128 [−]	>128 [−]	>128 [−]	>128 [−]
ciprofloxacin		<0.125 [−]	64 [−]	64 [−]	128 [−]	0.25 [−]	128 [−]

[a]MIC values for the compounds and control antibiotics were determined by using 2-fold dilution series in the range 0.125−128 μM. The MIC values are given as the median and range of three independent biological replicates in agreement with the CLSI standards using visual detection. No range, [−], indicates unambiguous results. [b]*E. coli* ATCC 25922. [c]Extended-spectrum β-lactamase (ESBL) producing *E. coli* (CTX-M-15), clinical isolate Aar 11, Statens Serum Institut, Copenhagen, Denmark; multidrug-resistant but colistin-susceptible. [d]New Delhi metallo-β-lactamase 1 (NDM-1) producing *E. coli*, clinical isolate no. 74859898, Hvidovre Hospital, Hvidovre, Denmark. [e]MDR strain only susceptible to colistin, clinical isolate no. E2-1228625, Hvidovre Hospital, Hvidovre, Denmark. [f]Methicillin-resistant *S. aureus* (MRSA) ATCC 33591. [g]Vancomycin-resistant *E. faecium* (VRE) ATCC 700221.

Table 2. Cytotoxicity of Peptidomimetics Given as the IC_{50} (μM)[a]

compd	no. of residues	HeLa[b]	Caco-2[c]	HepG2[d]	IEC-6[e]	NIH 3T3[f]	HUVEC[g]	erythrocytes[h]
1	10	506 ± 29	>1000	980 ± 104	972 ± 149	>1000	>1000	nd
2	12	316 ± 20	>1000	698 ± 77	636 ± 65	635 ± 90	>1000	>1024
3	14	99 ± 5	>1000	378 ± 33	319 ± 32	254 ± 11	418 ± 25	nd
4	10	508 ± 11	>1000	943 ± 49	>1000	745 ± 50	>1000	nd
5	12	228 ± 4	>1000	321 ± 23	457 ± 34	362 ± 28	>1000	nd
6	14	77 ± 3	353 ± 138	116 ± 5	266 ± 81	172 ± 11	458 ± 163	>1024
7	10	165 ± 6	583 ± 128	141 ± 31	146 ± 7	210 ± 16	398 ± 17	nd
8	12	52 ± 2	376 ± 37	77 ± 3	67 ± 3	121 ± 8	351 ± 30	nd
9	14	21 ± 0.5	182 ± 14	35 ± 1	30 ± 1	67 ± 10	145 ± 5	nd
10	10	136 ± 19	>1000	185 ± 19	186 ± 16	237 ± 14	nd	nd
11	12	39 ± 1	330 ± 26	100 ± 1	147 ± 8	135 ± 18	497 ± 23	nd
12	14	26 ± 1	197 ± 27	48 ± 2	80 ± 9	89 ± 5	236 ± 5	nd
melittin		1.9 ± 0.0	4.5 ± 0.1	2.5 ± 0.1	2.2 ± 0.1	6.4 ± 0.1	nd	nd

[a]Values are given as the average ± SD (n = 3). nd: not determined. [b]HeLa: human cervical cancer cell line. [c]Caco-2: human epithelial carcinoma colon cell line. [d]HepG2: human hepatocellular carcinoma cell line. [e]IEC-6: normal rat small intestine epithelial cell line. [f]NIH 3T3: mouse fibroblast cell line. [g]HUVEC: human umbilical vein endothelial cells. [h]The IC_{50} value for erythrocytes is measured as the test compound concentration required for lysis of 50% red blood cells.

dimer was obtained via amide bond formation between the peptoid amine and Fmoc-Lys(Boc)-OH with *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) as coupling reagent. Simultaneous cleavage of the *tert*-butyl ester and the Boc protecting group with excess trifluoroacetic acid was followed either by N-protection with di-*tert*-butyl dicarbonate (to give the Lys building block) or by guanidinylation with the bis(Boc)-pyrazole reagent (to yield the hArg building block) as described previously.[14] Satisfactory purity of the crude oligomerization product was achieved by using a standard Fmoc protocol on a Rink amide resin with (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) as activator. Compound identity was verified with LC−HRMS, while purity was determined by using analytical HPLC. In addition to the α-peptoid−peptide dimeric building blocks, depicted in Scheme 1, the corresponding β-peptoid−peptide dimeric building blocks were prepared as described previously.[15]

Antibacterial Activity against Multidrug-Resistant Bacteria. The minimum inhibitory concentration (MIC) was determined by the microdilution broth method (according to CLSI recommendations[18]) for all test peptidomimetics and control antibiotics (Table 1). As expected, the clinically used antibiotics generally exhibited low activity against the MDR strains while the tested peptidomimetics with few exceptions demonstrated no to moderate activity against the Gram-positive

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bacteria methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VRE). Especially, peptidomimetics containing chiral peptoid residues (**7**−**12**) exhibited moderate activity (MIC of 4−16 μM), whereas the achiral peptoid residues (**1**−**6**) typically conferred low activity (MIC $\geq$ 16 μM) against the Gram-positive bacteria, with VRE being the most sensitive (MIC = 2 μM for compound **8**). By contrast, all backbone designs were demonstrated to confer promising activity (MIC of 1−4 μM) against the three *Escherichia coli* strains tested. Notably, elongation from 10 to 12 residues was essential for high activity (MIC $\leq$ 4 μM) against *E. coli*, whereas the shortest oligomers (**1**, **4**, **7**, and **10**) only demonstrated moderate potency (MIC of 8−16 μM). This trend was even more pronounced for *Acinetobacter baumannii*, for which a clear-cut length−activity relationship was observed with a typical 4-fold increase in potency for each added repetitive unit. A similar trend was also found for VRE. For both Gram-positive bacteria (MRSA and VRE) the highest activities were observed for longer compounds displaying α-chiral peptoid residues.

Cytotoxicity toward Mammalian Cells. In order to assess the impact of length variation on the cytotoxicity against mammalian cells, the IC_{50} values toward a range of different cell types were determined by the MTS/PMS assay[19] (Table 2). Of the three malignant cell lines (HeLa, Caco-2, and HepG2) in this study, the human cervical cancer cell line (HeLa) was significantly more sensitive to the test compounds than the other cell lines tested. Generally, a clear correlation between oligomer length and cytotoxicity was observed. For all oligomer series, an extended chain length resulted in increased cytotoxicity as reflected in an up to 3-fold decrease in the IC_{50} value for each additional dimeric unit (e.g., for **4** → **5** → **6** and **7** → **8** → **9** against HeLa cells). Notably, the presence of chirality in the peptoid residues conferred high cytotoxicity as compounds **7**−**12** typically exhibited 2- to 5-fold lower IC_{50} values compared to their achiral counterparts (**1**−**6**). Furthermore, the human epithelial carcinoma colon cell line (Caco-2) proved to be the least susceptible of the malignant cell lines, while human hepatocellular carcinoma (HepG2) cells were affected to a similar degree as the three benign cell lines: normal rat small intestine epithelial cells (IEC-6), mouse fibroblast cells (NIH 3T3), and human umbilical vein endothelial cells (HUVEC).

The nature of the peptoid residue proved to be less significant for cytotoxic activity, since oligomer subtypes containing α-peptoid residues were similar to their β-peptoid-containing counterparts. The hemolytic activity was investigated for two representative compounds (**2** and **6**), but despite strong cytotoxic activity of compound **6**, no hemolysis could be detected for either of these compounds.

Circular Dichroism Spectroscopy. A feasible approach for studying folding of oligomers involves circular dichroism (CD) spectroscopy.[20] By using this technique, we previously found that short peptoid−peptide hybrids in the presence of lipid micelles gave rise to characteristic CD spectra depending on their structural features.[12,21] The present test compounds were examined in the presence of appropriate lipid bilayer membrane models in the form of liposomes. Cholesterol is a highly abundant lipid in membranes of mammalian cells as opposed to bacterial cell membranes that are devoid of this component. In order to estimate the effect of this specific difference in membrane composition, the CD behavior of the test compounds was investigated in the presence of both cholesterol-containing liposomes and in a model lacking cholesterol. The lipid composition of the former was based on previous studies and consisted of the zwitterionic 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC), the anionic 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphoglycerol (POPG), and cholesterol in the molar ratio 5:3:2.[12] In the bacterial membrane model cholesterol was substituted with the zwitterionic phospholipid resulting in a lipid composition of POPC and POPG in the molar ratio 7:3. The CD spectra of compounds **1**−**3** containing achiral α-peptoid residues displayed features different from those of peptidomimetics containing achiral β-peptoid residues (i.e., **4**−**6**) (see Figure 2).

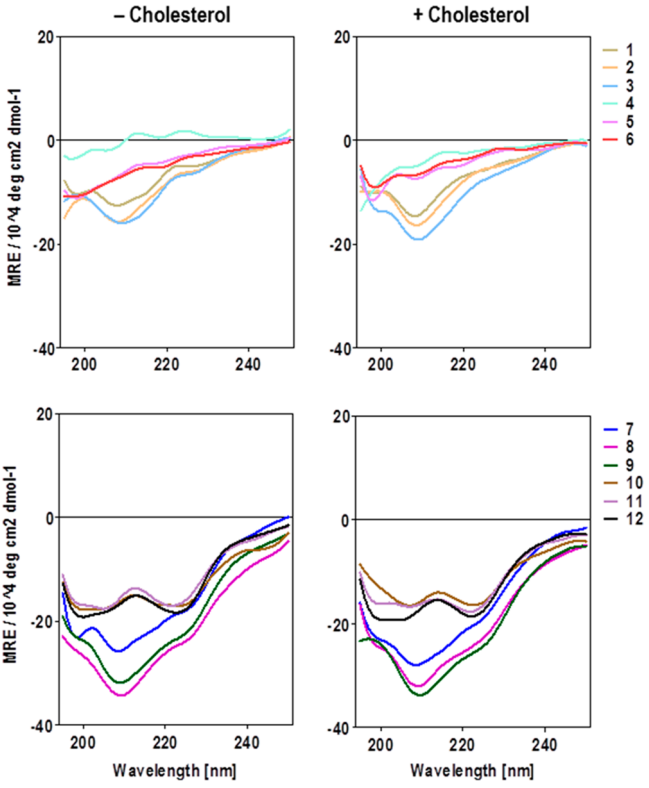

Figure 2. CD spectra of peptidomimetics (**1**−**12**) in the presence of model membranes. Spectra in the first column are obtained in the presence of liposomes without cholesterol, while spectra in the second column are obtained in the presence of cholesterol-containing liposomes.

For these two partially chiral subtypes only minor differences in CD behavior were observed between the two model membranes, suggesting that cholesterol is not contributing significantly to their interactions with human cell membranes. Likewise, the compounds containing chiral peptoid residues (i.e., **7**−**12**) gave rise to similar CD spectra in the presence of both models.

DISCUSSION AND CONCLUSIONS

In most earlier studies searching for novel AMP-derived lead compounds, investigation of cytotoxicity has often been confined to measurement of hemolytic activity that has been considered a reliable indicator for general cytotoxicity, and in several cases the ratio of antibacterial activity to hemolytic activity has been used as a selectivity index.[22−24] In recent years, a few investigations of AMPs have also included estimation of their potential anticancer and immunomodulatory

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properties,[25,26] and these studies indicated that evaluation of cytotoxicity should be expanded to comprise several other more relevant cell lines. Recently, we demonstrated that the ability of a peptidomimetic to lyse human erythrocytes may not be necessarily correlated with its cytotoxicity to HeLa cells.[12] This discrepancy has also recently been demonstrated in a study of cytotoxic properties of several members of distinct AMP families isolated from bee venom.[27] Here it was demonstrated that hemolytic activity and cytotoxicity against HUVEC and IEC-6 may deviate by 10-fold differences. Hence, it is implied that hemolysis preferably should not be applied as the only indicator for general cell toxicity.

Several in vitro cell viability assays for predicting acute cytotoxicity exist, and these are based on distinct end points such as morphology and metabolic activity. A careful choice of method is essential, as it has been shown that the sensitivity of the different assays may vary. In one study, three different approaches, the lactate dehydrogenase (LDH) assay, the water-soluble tetrazolium salt (WST-1) assay, and an assay measuring the intracellular level of ATPs, were all used to evaluate the cytotoxicity of the AMPs polymyxin B and gramicidin to different types of mammalian cells.[28] The results clearly indicated that the LDH assay lacked sensitivity compared to the other two assays. Another study reached a similar conclusion,[29] as it was demonstrated that the MTS/PMS assay[19] was more sensitive than the LDH assay. The MTS/PMS assay is based on the same principle as the WST-1 assay, namely, that the extracellular enzymatic conversion of a tetrazolium dye to a formazan product is directly correlated to the level of cell viability. The formazan product may readily be quantified by using simple spectrophotometric techniques. Therefore, an MTS/PMS assay was applied in the present work because of high sensitivity and ease of measurement.

The design of the present compounds was based on a number of earlier findings. First, it had been demonstrated that secondary structure could be adopted by α-peptoids as well as by β-peptoid/α-amino acid hybrids when at least 50% of the residues display α-chiral aromatic side chains.[14,30] Importantly, it had also been shown that a high degree of helicity of chiral α-peptoids may be linked to high hemolytic activity.[31] In addition, we had demonstrated that incorporation of peptoid modifications in every second position conferred enzymatic stability.[12] On the basis of these results, it was evident that oligomers alternating between a cationic α-amino acid and either an α- or a β-peptoid hydrophobic residue with or without α-chirality in the aromatic side chains were required in order to investigate a putative relationship between the degree of chirality and cell-selective activity. Second, it had been shown that for similar alternating oligomers guanidino functionalities in the cationic residues usually provide a higher antibacterial activity than amino groups.[13] In addition, another study on similar peptidomimetics reported that chain length rather than the type of cationic functionality was important for activity.[32] On the basis of these earlier observations, a detailed investigation of the influence of length variation on activity was warranted. All oligomers were prepared with alternating hArg and Lys residues in the cationic position in order to retain high antibacterial activity while attempting to keep cytotoxicity at a relatively low level.

Because of the simple repetitive nature of the oligomers, only two dimeric building blocks were required for each subtype of peptidomimetics, and these building blocks were readily obtained in multigram-scale syntheses from cheap starting materials, with Fmoc-Lys(Boc)-OH as the only α-amino acid component needed. Solid-phase assembly of oligomers gave in all cases crude products that were easily purified.

Expectedly, the design of alternating cationic–hydrophobic peptidomimetics displaying both amino and guanidino functionalities resulted in significantly higher activity of the shorter oligomers. As an example, the all-Lys 16-mers[12] corresponding to the present 12-mer peptidomimetics **2** and **5** exhibited 4- to 8-fold lower antibacterial activity against MDR *E. coli* strains and toward MRSA.[12]

The antibacterial activities of all α-peptoid-containing oligomers (**1**–**3** and **7**–**9**) toward *E. coli* strains were found to be very similar, while the peptidomimetics displaying achiral β-peptoid residues (i.e., **4**–**6**) were slightly more active than their chiral counterparts (**10**–**12**). This taken together with the observation that the α-chiral aromatic side chains appears to confer altered folding to these types of peptidomimetics in the presence of model membranes suggests that the propensity to adopt specific secondary structures may not be favorable for compounds with this alternating design when aiming at selective killing of *E. coli* strains. Interestingly, the opposite trend was seen for another Gram-negative bacterium, *A. baumannii*, that proved to be more susceptible to the fully chiral peptidomimetics (i.e., **7**–**12**), which were 2- to 32-fold more active than the corresponding oligomers with achiral peptoid units (i.e., **1**–**6**). Similar activity profiles were also found against the Gram-positive MRSA and VRE strains, as these were 2- to 4-fold and 2- to 16-fold, respectively, more susceptible to the fully chiral peptidomimetics. Thus, it may be concluded that antibacterial activity is enhanced by different structural features depending on the targeted bacterium: (i) high activity toward *E. coli* is promoted by incorporation of residues displaying guanidino groups but does not rely on the presence of α-chiral peptoid units, while dependence on oligomer length is moderate; (ii) potency against the Gram-positive MRSA and VRE as well as the Gram-negative *A. baumannii* is enhanced by α-chirality in the peptoid residues and strongly dependent on oligomer length and presence of guanidino functionalities in accordance with our previous findings.[12,13,32]

An array of peptidomimetics were tested toward six cell lines representing different tissue types including carcinogenic cells (i.e., human HeLa, Caco-2, and HepG2 cells), intestinal epithelium (rat IEC-6 cells), endothelium (HUVEC), and fibroblasts (NIH 3T3 cells). Of the cancer cell lines Caco-2 proved to be the least susceptible to the oligomers tested (IC_{50} > 300 μM for all 12-mers) while HUVEC was the most resistant benign cell line (IC_{50} > 350 μM for all 12-mers). In particular, for HeLa cells substantial cytotoxicity was seen at lower concentrations when the oligomer length was increased beyond 12 residues (IC_{50} < 100 μM for all 14-mers). Only a weak tendency toward increased cytotoxicity was observed for oligomers displaying β-peptoids (**4**–**6** and **10**–**12**) in comparison to the α-peptoid-containing counterparts (**1**–**3** and **7**–**9**), which is somewhat surprisingly as the extra methylene groups give rise to an elongation of the molecule. This infers that the number of side chains rather than the distance between the C- and N-termini is the most critical design parameter when attempting to limit cytotoxicity. Moreover, α-chirality of peptoid residues conferred at least 3-fold (and up to 10-fold) increased cytotoxicity toward all cell lines. In addition, dependence of cytotoxicity on oligomer length was more pronounced for fully chiral α-peptoid–peptide

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hybrids **7**–**9**, in agreement with the CD spectra obtained in the presence of the bacterial model that show a distinct increase in MRE when going from 10 to 12 residues.

However, in order to get a clear picture of structure–activity relationships with respect to the best overall antibiotic performance with the widest possible therapeutic window, three selectivity indices (SI = ratio of average IC_{50} to MIC values) were calculated from the activities determined for each member of the array of peptidomimetics (see Table 3). First, the average cytotoxicity against the three benign cell lines was compared to the mean MIC value toward the *E. coli* strains tested, which revealed that when targeting *E. coli* strains highest cell selectivity was reached for 12-mer peptidomimetics **2** and **5** incorporating achiral peptoid residues (SI > 450) but also the corresponding 14-mers **3** and **6** had favorable SIs of ∼300. By contrast, when *A. baumannii* was considered, the most favorable SIs (>65) were found for all 14-mer peptidomimetics (i.e., **3**, **6**, **9**, and **12**) as well as for the 12-mer **8** that has chiral α-peptoid units incorporated. No clear trend was seen for the SIs toward Gram-positive bacteria except that no compounds had SI > 25.

Table 3. Selectivity Indices (SIs) and Hydrophobicity (As Indicated by t_R)[a]

compd	no. of residues	SI (*E. coli*)[b]	SI (*A. baumannii*)[c]	SI (G^+)[d]	t_R (min)
1	10	>93	∼8	>8	18.20
2	12	>454	>12	>16	18.35
3	14	330	83	17	18.61
4	10	>86	>7	>10	18.50
5	12	>606	>38	>19	18.62
6	14	299	75	19	19.07
7	10	31	16	13	19.97
8	12	68	90	20	20.15
9	14	61	81	8	20.76
10	10	27	3	5	20.57
11	12	98	33	16	20.80
12	14	68	68	23	21.11

[a]Net hydrophobicity as indicated by the retention time (t_R) on analytical reversed-phase HPLC. [b]Selectivity index (SI) calculated as the ratio of the mean cytotoxicity against benign cell lines to the mean MIC value against the *E. coli* strains tested. [c]SI based on MIC for *A. baumannii*. [d]SI based on the mean MIC for the Gram-positive bacteria (MRSA and VRE).

Thus, identifying the optimal oligomer size was clearly the most important parameter in tailoring the activity profile as demonstrated by the present examination of its impact on both antibacterial and cytotoxic effects. Next, the lack of chirality in the peptoid residues proved to be crucial in keeping the cytotoxicity at an acceptable low level. This effect may be explained either by differences in secondary structure or by the increased hydrophobicity caused by the additional methyl groups introduced in the side chains of the α-chiral peptoid residues. Similar subtle contributions from higher hydrophobicity to increased hemolytic activity have been inferred for other types of peptidomimetics via their estimated net hydrophobicity as determined by the retention times (t_R) in reversed-phase analytical HPLC.[33] From Table 3 it is evident that there is a significant increase in hydrophobicity when comparing the t_R values of partially chiral oligomers **1**–**6** with those of the corresponding fully chiral oligomers **7**–**12**.

In conclusion, we find that the partially chiral subtypes of alternating peptoid–peptide hybrids appear to be particularly favorable sources of lead compounds against MDR *E. coli* strains, as a gradual increase of oligomer length reaches a sufficient antibacterial activity before the concomitant increase in cytotoxicity becomes detrimental to the cell selectivity. Noticeably, from this limited array of peptidomimetics explored we identified leads candidates (**2**, **3**, **5**, and **6**) that inhibited MDR *E. coli* growth at a concentration (i.e., 1–2 μM) 300- to 600-fold lower than the mean of the IC_{50} values for cytotoxicity against the benign mammalian cell lines.

EXPERIMENTAL SECTION

Starting materials and solvents were purchased from commercial suppliers (Alfa Aesar, CHEMsolute, Iris Biotech, Sigma Aldrich, VWR, AppliChem, Fluka, ABCR, LabScan, and Merck) and used without further purification. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired by using a 600 MHz Bruker Avance III HD equipped with a cryogenically cooled 5 mm dual probe optimized for ^{1}H and ^{13}C. Samples were dissolved in methanol-d_4 (Cambridge Isotope Laboratories) and analyzed at 300 K. The residual solvent peak was used as internal reference (CD_3OD: δ_H = 49.00; δ_H = 3.31). Coupling constants (J values) are given in hertz (Hz). Multiplicities are reported as follows: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). Additional peaks due to the presence of a minor rotamer are designated by an asterisk (*). Water used during analytical and preparative HPLC was filtered through a 0.22 μm membrane filter. Analytical HPLC was used to determine purity and was carried out on a Phenomenex Luna C18 (2) (3 μm) column (150 mm × 4.6 mm) using binary mixtures of eluent A (water–MeCN–TFA 95:5:0.1) and eluent B (water–MeCN–TFA 5:95:0.1) for elution with a flow rate of 0.8 mL/min. For elution of building blocks: a linear gradient of 0–80% B during 5 min followed by a linear rise to 100% B during 25 min; for peptidomimetics, a linear gradient of 10–60% B during 30 min. For UV detection, building blocks were monitored at λ = 267 nm and peptidomimetics at λ = 220 nm. All compounds had a purity of at least 95%. Retention times (t_R) are given for each compound. Preparative HPLC was performed by using a Luna C18 (2) (5 μm) column (250 mm × 21.2 mm) on an Agilent 1100 LC system with a multiple-wavelength UV detector. Elution was performed with a linear gradient of 10–40% B during 20 min at a flow rate of 20 mL/min; peptidomimetics were detected at λ = 220 nm. LC–HRMS was performed with a Phenomenex Luna C18 (2) (3 μm) column (150 mm × 4.6 mm) using binary mixtures of eluent C (water–MeCN–formic acid 95:5:0.1) and eluent D (water–MeCN–formic acid 5:95:0.1); elution was performed with a linear gradient of 10–60% D during 30 min at a flow rate of 0.5 mL/min. HRMS spectra were obtained by using a Bruker MicrOTOF-Q II Quadropol MS detector. The analyses were performed as ESI-MS (m/z): $[M + 5H]^{5+}$ for peptidomimetics and $[M + H]^+$ for building blocks.

Synthesis of Compound 13. Benzylamine (21.9 g) was dissolved in THF (75 mL). Et_3N (41.3 g, 2.0 equiv) and *tert*-butyl bromoacetate (39.9 g, 1.0 equiv) in THF (75 mL) were added. The mixture was stirred at room temperature for 16 h followed by filtration. The filtrate was concentrated in vacuo, and then the residue was dissolved in DCM (75 mL) and loaded onto a vacuum liquid chromatography (VLC) column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with DCM followed by DCM–MeOH 100:1 to 20:1. The appropriate fractions were concentrated in vacuo to yield an intermediate aza-Michael adduct (18.1 g, 41%).

Fmoc-Lys(Boc)-OH (42.3 g, 1.1 equiv) was dissolved in DCM (1000 mL). DIPEA (26.5 g, 2.5 equiv) and TBTU (39.5 g, 1.5 equiv) were added under stirring. After 15 min, the above intermediate aza-Michael adduct in DCM (200 mL) was added. The mixture was stirred at room temperature for 16 h and concentrated in vacuo until ∼100 mL of DCM remained. The solution was diluted with EtOAc (700 mL) and washed with 1 M HCl (3 × 300 mL), water (300 mL), 0.1 M NaOH (300 mL), saturated $NaHCO_3$ (300 mL), water (300 mL), and brine (300 mL). Subsequently, the solution was dried over Na_2SO_4

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and concentrated in vacuo. The crude product was dissolved in DCM (75 mL) and loaded onto a VLC column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by heptane−EtOAc 4:1 to 2:1. The appropriate fractions were concentrated in vacuo to yield an intermediate *tert*-butyl ester (34.1 g, 62%).

An aliquot of this intermediate *tert*-butyl ester (17.0 g) was treated with TFA−DCM 1:3 (230 mL) for 2 h. The mixture was concentrated in vacuo with DCM−toluene three times. The residue was redissolved in DCM (200 mL), and then DIPEA (16.4 g, 5.0 equiv) and pyrazol(Boc)$_2$ (9.4 g, 1.2 equiv) in DCM (200 mL) were added successively. The mixture was stirred at room temperature for 16 h and was then concentrated in vacuo, redissolved in DCM (50 mL), and loaded onto a VLC column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by 0.1% HOAc in heptane−EtOAc 2:1 to 1:1. The appropriate fractions were concentrated in vacuo to yield compound **13** (13.0 g, 68%; t_R = 14.30 min). HRMS: calcd for $C_{41}H_{51}N_5O_9$ $[M + H]^+$ 758.3764, found 758.3764; ΔM = 0.0 ppm. ^{1}H NMR (600 MHz, CD_3OD): δ = 1.46 (s, 9 H), 1.47 (s, 9 H), 1.28−1.85 (m, 6 H), 3.28 (t, J = 7 Hz, 2 H), 3.34* (m, 2 H), 3.80 (d, J = 17.2 Hz, 1 H), 3.90* (d, J = 18.7 Hz, 1 H), 4.14−4.50 (m, 5 H), 4.68 (m, 1 H), 4.85 (m, 1 H), 7.08−7.40 (m, 9 H), 7.64 (m, 2 H), 7.77 (m, 2 H). ^{13}C NMR (150 MHz, CD_3OD): δ = 23.9, 24.0*, 28.2, 26.6, 29.6, 26.7*, 32.5*, 32.8, 41.6, 41.7*, 48.4, 48.5*, 51.2*, 51.2*, 52.4, 53.3, 68.0, 80.5, 80.6*, 84.7, 120.9, 126.2*, 126.3, 128.2, 128.2, 128.5, 128.8, 128.9, 129.0, 129.2, 129.7, 129.9, 137.7, 137.8*, 142.6*, 142.6, 145.1, 145.3*, 145.3, 154.1*, 154.1, 157.4, 158.3, 158.4*, 164.2*, 164.3, 172.0, 172.4*, 175.2*, 175.3.

Synthesis of Compound 14. An intermediate *tert*-butyl ester was prepared as described for compound **13**. The intermediate *tert*-butyl ester (17.0 g) was treated with TFA−DCM 1:3 (230 mL) for 2 h. The mixture was concentrated in vacuo with DCM−toluene three times. The residue was redissolved in DCM (200 mL), and then DIPEA (16.4 g, 5.0 equiv) and Boc_2O (7.2 g, 1.3 equiv) in DCM (200 mL) were added successively. The mixture was stirred at room temperature for 16 h. The mixture was concentrated in vacuo, redissolved in DCM (50 mL), and loaded onto a VLC column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by 0.1% HOAc in heptane−EtOAc 2:1 to 1:1. The appropriate fractions were concentrated in vacuo to yield compound **13** (10.7 g, 68%; t_R = 15.52 min). HRMS: calcd for $C_{35}H_{41}N_3O_7$ $[M + H]^+$ 616.3017, found 616.3022; ΔM = 0.8 ppm. ^{1}H NMR (600 MHz, CD_3OD): δ = 1.40 (s, 9 H), 1.41* (s, 9 H), 1.26−1.82 (m, 6 H), 2.93−3.05 (m, 2 H), 3.74 (d, J = 17.2 Hz, 1 H), 3.91* (d, J = 18.7 Hz, 1 H), 4.14−4.52 (m, 5 H), 4.60−4.70 (m, 1 H), 4.85 (m, 1 H), 7.08−7.39 (m, 9 H), 7.65 (m, 2 H), 7.77 (m, 2 H). ^{13}C NMR (150 MHz, CD_3OD): δ = 23.9, 24.0*, 28.8, 30.6, 32.6*, 32.8, 41.0, 41.1*, 48.4*, 48.4, 51.2, 52.3*, 52.5*, 53.2, 68.0, 79.8, 120.9, 126.2*, 126.3, 126.3, 128.2, 128.2, 128.5, 128.6*, 128.8, 128.9, 129.0, 129.2, 129.7, 129.9, 137.6, 137.8*, 138.9, 142.6*, 142.6, 145.1, 145.3*, 145.3*, 158.4, 158.5*, 158.5, 172.0, 172.3*, 175.3*, 175.3.

Synthesis of Compound 15. (*S*)-1-Phenylethylamine (11.5 g) was dissolved in THF (40 mL), and then Et_3N (19.2 g, 2.0 equiv) and *tert*-butyl bromoacetate (18.5 g, 1.0 equiv) in THF (40 mL) were added successively. The mixture was stirred at room temperature for 16 h followed by filtration. The filtrate was concentrated in vacuo, and then the residue was dissolved in DCM (50 mL) and loaded onto a VLC column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with DCM followed by DCM−MeOH 100:1 to 50:1. The appropriate fractions were concentrated in vacuo to yield an intermediate amine (12.9 g, 58%).

Fmoc-Lys(Boc)-OH (28.3 g, 1.1 equiv) was dissolved in DCM (650 mL), and then DIPEA (17.7 g, 2.5 equiv) and TBTU (26.4 g, 1.5 equiv) were added under stirring. After 15 min the intermediate amine in DCM (50 mL) was added. The mixture was stirred at room temperature for 16 h and concentrated in vacuo. The crude product was dissolved in EtOAc (500 mL) and then washed with 1 M HCl (3 × 200 mL), water (200 mL), 0.1 M NaOH (200 mL), saturated $NaHCO_3$ (200 mL), water (200 mL), and brine (200 mL). Subsequently, the solution was dried over Na_2SO_4 and concentrated in vacuo. The crude product was dissolved in DCM (50 mL) and loaded onto a VLC column (height, 8.0 cm; diameter, 9.5 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by heptane−EtOAc 7:1 to 2:1. The appropriate fractions were concentrated in vacuo to yield an intermediate *tert*-butyl ester (20.4 g, 54%).

An aliquot of this intermediate *tert*-butyl ester (10.4 g) was treated with TFA−DCM 1:2 (100 mL) for 2 h. The mixture was concentrated in vacuo with DCM−toluene three times. The residue was redissolved in DCM (100 mL), and then DIPEA (9.8 g, 5.0 equiv) and pyrazol(Boc)$_2$ (5.7 g, 1.2 equiv) in DCM (200 mL) were added successively. The mixture was stirred at room temperature for 16 h and was then concentrated in vacuo and redissolved in EtOAc (200 mL). The solution was washed with 1 M HCl (2 × 100 mL), 10% citric acid (2 × 100 mL), water (2 × 100 mL), and brine (100 mL). The mixture was dried over Na_2SO_4, concentrated in vacuo, redissolved in DCM (40 mL), and loaded onto a VLC column (height, 8.0 cm; diameter, 8.0 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by heptane−EtOAc 4:1, 0.1% HOAc in heptane−EtOAc 4:1 to 1:1. The appropriate fractions were concentrated in vacuo to yield compound **15** (9.9 g, 84%; t_R = 14.56 min). HRMS: calcd for $C_{42}H_{53}N_5O_9$ $[M + H]^+$ 772.3901, found 772.3905; ΔM = 0.5 ppm. ^{1}H NMR (600 MHz, CD_3OD): δ = 1.45−1.48 (m, 18 H), 1.25−1.65 (m, 4 H), 1.66 (d, J = 6.9 Hz, 3H), 1.70−1.88 (m, 2 H), 3.32−3.41 (m, 2 H), 3.85* (d, J = 18.9 Hz, 1 H), 3.96 (d, J = 17.3 Hz, 1 H), 4.10−4.38 (m, 5H), 5.48 (q, J = 6.7 Hz, 1 H), 5.85* (q, J = 6.9 Hz, 1 H), 7.21−7.40 (m, 9H), 7.68 (m, 2 H), 7.78 (m, 2 H). ^{13}C NMR (150 MHz, CD_3OD): δ = 16.3*, 17.9, 24.0, 28.2, 28.6, 29.7*, 29.8, 41.7, 45.4, 45.9*, 48.4*, 48.5, 52.5, 53.0*, 53.8*, 56.2, 67.9*, 68.0, 80.6, 84.5, 120.9, 126.2, 126.3, 126.3, 128.2, 128.2, 128.2, 128.8, 128.8*, 129.0*, 129.5, 129.8, 140.8*, 141.1, 142.6, 142.6*, 145.1, 145.2*, 145.3, 154.1*, 154.2, 157.4*, 157.5, 158.3*, 158.4, 164.3, 172.2, 172.6*, 174.5, 175.3*.

Synthesis of Compound 16. An intermediate *tert*-butyl ester was prepared as described for compound **15**. The intermediate *tert*-butyl ester (10.0 g) was treated with TFA−DCM 1:2 (100 mL) for 1 h. The mixture was concentrated in vacuo with DCM−toluene three times. The residue was redissolved in DCM (100 mL), and then DIPEA (9.4 g, 5.0 equiv) and Boc_2O (4.14 g, 1.3 equiv) in DCM (100 mL) were added successively. The mixture was stirred at room temperature for 16 h. The mixture was concentrated in vacuo, redissolved in DCM (40 mL), and loaded onto a VLC column (height, 8.0 cm; diameter, 8.0 cm; column material, 60H silica gel; column pretreated with heptane). Gradient elution was carried out with heptane followed by heptane−EtOAc 4:1, 0.1% HOAc in heptane−EtOAc 4:1 to 1:1. The appropriate fractions were concentrated in vacuo to yield compound **16** (2.46 g, 27%; t_R = 15.93 min). HRMS: calcd for $C_{36}H_{43}N_3O_7$ $[M + H]^+$ 630.3170, found 630.3171; ΔM = 0.2 ppm. ^{1}H NMR (600 MHz, CD_3OD): δ = 1.41 (s, 9 H), 1.24−1.54 (m, 4 H), 1.66 (d, J = 6.9 Hz, 3H), 1.78−1.89 (m, 2 H), 2.95−3.11 (m, 2 H), 3.86* (d, J = 18.8 Hz, 1 H), 3.96 (d, J = 17.3 Hz, 1 H), 4.11−4.38 (m, 5H), 5.49 (q, J = 6.8 Hz, 1 H), 5.84* (q, J = 6.9 Hz, 1 H), 7.20−7.39 (m, 9H), 7.65 (m, 2 H), 7.78 (d, J = 7.6 Hz, 2 H). ^{13}C NMR (150 MHz, CD_3OD): δ = 16.3*, 17.9, 23.9, 24.0*, 28.8, 30.5*, 30.7, 41.0, 41.1*, 45.3, 45.9*, 48.4*, 48.4, 52.6*, 53.0*, 53.8, 56.1, 67.9*, 68.0, 79.8, 120.9, 126.2, 126.3, 126.3*, 128.2*, 128.2*, 128.2, 128.8, 129.0, 129.6*, 129.7, 141.1, 142.6*, 142.6, 145.1, 145.2*, 145.3, 158.3*, 158.5, 158.5, 172.3, 172.6*, 174.5, 175.4*.

General Protocol for Synthesis of Peptidomimetics. Peptidomimetics were prepared as previously described.[15] In brief, Rink amide resin (loading, 0.70 mmol/g) and Teflon reactors (10 mL) were used for all compounds. Fmoc deprotection conditions were the following: 20% piperidine in DMF (2 × 10 min, each time with 5 mL under shaking at room temperature). Washing conditions were with DMF, MeOH, and DCM (each 3 × 3 min with 5 mL). Coupling conditions were the following: building block, PyBOP, and DIPEA; 2.0

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equiv for loading, 2.5 equiv for the first two couplings, and 3.0 equiv for all subsequent couplings (≥2 h under shaking at room temperature). Capping was applied after coupling no. 4: Ac_2O−DIPEA−NMP 1:2:3 (5 mL for 10 min at room temperature). Final Fmoc deprotection of the N-terminus was followed by acetylation as described above for capping, and then the resin was washed. Cleavage and simultaneous side chain deprotection involved TFA−water 95:5 (5 mL, 1 h under shaking at room temperature). The filtrate was collected, and the resin was eluted with DCM (2 mL), MeOH (2 mL), TFA−water 95:5 (2 mL), and DCM (2 mL). The combined filtrates were concentrated in vacuo and then coevaporated with MeOH−toluene (3 × 5 mL). The crude product was purified by using preparative HPLC and concentrated in vacuo as described in the above section for general procedures. Finally, the product was dissolved in water (1 mL) and lyophilized.

Peptidomimetic ***1***. Analytical HPLC: t_R = 18.20 min. HRMS: calcd for $[M + 5H]^{4+}$ 391.2374, found 391.2376; ΔM = 0.5 ppm.

Peptidomimetic ***2***. Analytical HPLC: t_R = 18.35 min. HRMS: calcd for $[M + 5H]^{5+}$ 368.4251, found 368.4254; ΔM = 0.8 ppm.

Peptidomimetic ***3***. Analytical HPLC: t_R = 18.61 min. HRMS: calcd for $[M + 5H]^{5+}$ 423.4573, found 423.45774; ΔM = 0.2 ppm.

Peptidomimetic ***4***. Analytical HPLC: t_R = 18.50 min. HRMS: calcd for $[M + 5H]^{5+}$ 327.4063, found 327.4070; ΔM = 2.1 ppm.

Peptidomimetic ***5***. Analytical HPLC: t_R = 18.62 min. HRMS: calcd for $[M + 5H]^{5+}$ 385.2438, found 385.2440; ΔM = 0.5 ppm.

Peptidomimetic ***6***. Analytical HPLC: t_R = 19.07 min. HRMS: calcd for $[M + 5H]^{5+}$ 443.0796, found 443.0800; ΔM = 0.9 ppm.

Peptidomimetic ***7***. Analytical HPLC: t_R = 19.97 min. HRMS: calcd for $[M + 5H]^{4+}$ 409.0078, found 409.0086; ΔM = 2.0 ppm.

Peptidomimetic ***8***. Analytical HPLC: t_R = 20.15 min. HRMS: calcd for $[M + 5H]^{5+}$ 385.2440, found 385.2456; ΔM = 4.2 ppm.

Peptidomimetic ***9***. Analytical HPLC: t_R = 20.76 min. HRMS: calcd for $[M + 5H]^{5+}$ 443.0787, found 443.0791; ΔM = 0.9 ppm.

Peptidomimetic ***10***. Analytical HPLC: t_R = 20.57 min. HRMS: calcd for $[M + 5H]^{4+}$ 426.5276, found 426.5273; ΔM = 0.7 ppm.

Peptidomimetic ***11***. Analytical HPLC: t_R = 20.80 min. HRMS: calcd for $[M + 5H]^{5+}$ 402.0617, found 402.0621; ΔM = 1.0 ppm.

Peptidomimetic ***12***. Analytical HPLC: t_R = 21.11 min. HRMS: calcd for $[M + 5H]^{5+}$ 462.7186, found 462.7180; ΔM = 1.3 ppm.

Bacterial Strains and Antimicrobial Agents. Three standard laboratory test strains (*E. coli* ATCC 25922, *S. aureus* ATCC 33591 [MRSA], *E. faecium* ATCC 70022 [VRE], all purchased from the ATCC, Manassas, VA, USA) and three clinical isolates (*E. coli* Aar 11 [ESBL], Statens Serum Institut, Copenhagen, Denmark; *E. coli* 74859898 [NDM-1]; and *A. baumannii* E2-1228625, Hvidovre Hospital, Hvidovre, Denmark) were used for antimicrobial activity experiments. Antimicrobial control substances were purchased as the commercial products for parenteral use (gentamicin as hexamycin, Sandoz Pharmaceutical, Holzkirchen, Germany; vancomycin as vancomycin "Fresenius Kabi", Bad Homburg, Germany, ciprofloxacin and cefotaxime as ciprofloxacin "Villerton" and cefotaxim "Villerton", respectively, FarmaPlus, Oslo, Norway).

In Vitro Antimicrobial Activity Experiments. Minimum inhibitory concentrations (MIC) were determined by the microdilution broth method according to the standards[11] of the Clinical and Laboratory Standards Institute (CLSI) as previously described.[34] In brief, fresh overnight colonies were suspended to a turbidity of 0.5 McFarland units and were then further diluted in Mueller−Hinton BLII broth (MHB; Becton Dickinson, Albertslund, Denmark). These bacterial suspensions were added to wells in polypropylene trays containing 2-fold serial dilutions of oligomers. The polypropylene trays (Nunc, Roskilde, Denmark) were incubated at 35 °C in ambient air for 18 h. The MIC value was determined as the lowest concentration showing no visible growth compared to the control without antibacterial compound added to the MHB-diluted 0.5 McFarland standard. The concentration test range was 0.5−128 μM test compound, and the MIC values are specified as the median and range of three biological independent replicates performed on separate days.

Assay for in Vitro Hemolytic Activity. The lysis of human red blood cells was measured as previously described[33] with modifications. In brief, freshly drawn human red blood cells (hRBCs) were washed twice with PBS buffer (made from buffer tablets; Sigma Aldrich, DK, 0.01 M phosphate, 2.7 mM KCl, 0.137 M NaCl, and pH 7.4) and centrifuged two times for 8 min at 3000 rpm/116*g* and 4000 rpm/206*g* (Sigma 204, Sigma Laborzentrifugen, Osterode am Harz, Germany), respectively. A 2-fold serial dilution of peptidomimetics in PBS buffer was added to each well in a sterile polypropylene V-bottom 96-well plate (Whatman, U.K.), to the total volume of 75 μL. A 0.5% v/v hRBC suspension (75 μL in PBS buffer) was added to each well to reach a final volume of 150 μL in each well. The plate was incubated (37 °C) for 1 h, and the cells were subsequently pelleted by centrifugation at 4000 rpm/206*g* (B4i multifunctional centrifuge, Thermo Fisher Scientific, U.S.) for 10 min. The supernatants (75 μL) were transferred to clear, flat-bottomed plastic 96-well plates (Nunc, Roskilde, Denmark). The concentration of hemoglobin was detected by measuring the OD at 414 nm ($VERSA_{max}$ microplate reader, Molecular Devices, Sunnyvale, CA, U.S.). The OD of cells incubated with melittin (400 μg/mL) defined 100% hemolysis, and the OD of cells incubated with PBS buffer defined 0% hemolysis. The concentration test range was 8−1024 μM.

Cell Culturing. The effect of the peptidomimetic compounds on cell viability was assessed on cell monolayers grown to ∼90% confluence after 21−25 h of culturing under standard conditions (5% CO_2/95% O_2 at 37 °C). Cell lines of different origin were used. WT HeLa, HepG2, and NIH 3T3 were from ATCC (Manassas, VA, U.S.), IEC-6 and HUVEC were from ECACC (Salisbury, U.K.), and the Caco-2 cells were from Deutsche Sammlung von Mikroorganismen and Zellkulturen (DSMZ, Braunschweig, Germany). The WT HeLa and HepG2 cells were cultured in Eagle's minimal essential medium (EMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) of nonessential amino acid (NEAA) mixture and 1 mM sodium pyruvate. IEC-6 cells were cultured in DMEM with 5% (v/v) FCS and 5 μg/mL insulin. NIH 3T3 cells were cultured in DMEM supplemented with 10% (v/v) newborn calf serum (NCS) (Gibco, Paisly, U.K.). Caco-2 cells were cultured in DMEM with 1% (v/v) NEAA and 10% (v/v) FCS; and HUVEC cells were cultured in Ham's F-12K (Kaighn's) medium supplemented with 10% (v/v) FCS, 0.1 mg/mL heparin, and 0.03 mg/mL endothelial cell growth supplement. All culturing media were supplemented with penicillin (10 000 IU/mL), streptomycin (10 mg/L), and L-glutamine (2 mM). All cell media and supplements were from Sigma-Aldrich (St. Louis, MO, U.S.) except the serum, which was purchased from Gibco (Paisly, U.K.). The number of cells seeded in the 96-well plates (Corning Costar, Sigma-Aldrich, Brøndby, Denmark) was adjusted to obtain a similar level of confluence; i.e., the seeding densities were the following: WT HeLa (8000 cells/well), HepG2 (23 000 cells/well), IEC (16 000 cells/well), NIH 3T3 (22 000 cells/well), Caco-2 (25 000 cells/well), and HUVEC (15 000 cells/well). Prior to seeding the HUVEC cells, the wells were surface-coated with 0.6 μg/cm^2 collagen (Calfskin, Sigma-Aldrich, Brøndby, Denmark). A cell passage number of 4−10 were used.

Cell Viability Assessment. For the viability assessment, the MTS/PMS assay[19] was applied. First, the adhered cells were washed with 37 °C Hanks' balanced salt solution (HBSS from Sigma-Aldrich, St. Louis, MO, U.S.) containing 10 mM Hepes (AppliChem, Darmstadt, Germany), pH 7.4, and exposed to 100 μL of test compound dissolved in the appropriate culturing medium for each cell line at concentrations in the range 0−2000 μM for 1 h at 37 °C and 50 rpm. After the cells were washed with HBSS containing 10 mM Hepes (pH 7.4), 100 μL of an MTS−PMS solution consisting of 240 μg/mL MTS (Promega, Madison, WI, U.S.) and 2.4 μg/mL PMS (Sigma-Aldrich, Buchs, Switzerland) in HBSS was added to the cells and incubated for 1.5−4 h at 37 °C with horizontal shaking and under light protection. For the HUVEC cells, the PMS concentration was 9.6 μg/mL. The absorbance was measured at 492 nm on a POLARstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) and the relative viability calculated by using 0.2% (w/v) sodium dodecyl sulfate as a positive control and untreated cells as a negative control. Controls

were included on all 96-well plates and did not change significantly between individual experiments. Data were confirmed in two independent biological replicates performed on separate passages of cells and separate days with a total number of at least four replicates. For simplicity, data from one biological replicate with three technical replicates are reported.

Preparation of Liposomes. The liposomes were prepared from POPC and POPG in a 7:3 molar ratio or POPC, POPG, and cholesterol in a 5:3:2 molar ratio as previously described.[35,36] The particle size distribution was determined as previously described.[12] All liposome preparations had a narrow size distribution with a mean diameter of approximately 100 nm. ζ potentials were determined by laser Doppler electrophoresis by using a Zetasizer (Malvern Instruments, Worcestershire, U.K.) in 10 mM Tris buffer (pH 7.5). The ζ potential of the cholesterol-containing liposomes were in the same range as the liposomes without cholesterol, i.e., -63.6 ± 2.5 vs -61.3 ± 1.3 ($n = 3$). In order to provide standardized lipid concentrations during CD measurements, determination of the lipid concentration was performed by using the phospholipid B enzymatic colorimetric method. For this purpose an mti-diagnostics phospholipids kit (mti-diagnostics, Idstein, Germany) was acquired. In brief, the phospholipids were hydrolyzed to free choline by phospholipase D. The liberated choline was oxidized to betaine by choline oxidase with simultaneous production of hydrogen peroxide. The latter reacted quantitatively with 4-aminoantipyrine and phenol to form a compound with an absorption maximum at 505 nm.

Circular Dichroism Measurements. The CD measurements were carried out as previously described with minor modifications.[12] In brief, solutions of 20 μM test compound and 2 mM model membranes in 10 mM Tris buffer (pH 7.5) were used. Measurements were performed by using a quartz cell with a path length of 1 mm on a DSM 1000 CD spectrophotometer (Olis, U.S.), and data were collected at 1.0 nm intervals. All spectra were obtained at far-UV wavelengths (195–250 nm) as the average of five scans followed by background subtraction. Measurements were monitored to ensure that the HV values did not exceed 700. All spectra were smoothed by using a 13-point second-order Savitzky–Golay routine. The raw data, $\theta_{degrees}$, were converted by using the equation for mean residue ellipticity: MRE = $\theta_{degrees}$/(path length in millimeters × the molar concentration × the number of residues).[37]

■ ASSOCIATED CONTENT

Supporting Information

Analytical HPLC data on target compounds **1**–**12** and building blocks **13**–**17**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

AMP, antimicrobial peptide; Boc_2O, di-*tert*-butyl dicarbonate; DIPEA, diisopropylethylamine; EMEM, Eagle's minimal essential medium; ESBL, extended-spectrum β-lactamase; FBS, fetal bovine serum; hArg, homoarginine; HBSS, Hanks' balanced salt solution; hRBC, human red blood cell; HUVEC, human umbilical vein endothelial cell; LDH, lactate dehydrogenase; MRE, mean residue ellipticity; MTS/PMS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium)/phenazinemethosulfate; NDM-1, New Delhi metallo-β-lactamase 1; NEAA, nonessential amino acid; NCS, newborn calf serum; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphoglycerol; PyBOP, (benzotriazol-1-yloxy)tris-(pyrrolidino)phosphonium hexafluorophosphate; SI, selectivity index; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; t_R, retention time in analytical HPLC; VLC, vacuum liquid chromatography; WST, water-soluble tetrazolium salt

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