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Novel lysine-peptoid hybrids with antibacterial properties[¶]

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Abstract: Here we report the design, synthesis and antibacterial activity of 20 lysine-peptoid hybrids. The hybrids are based on the peptoid lead structure [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl)glycyl]-*N*-(butyl)glycin amide (**1**) and contain between one and six lysine residues each. The compounds were tested for antibacterial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Furthermore, the hemolytic activity toward human erythrocytes was assessed. Several compounds with potent antibacterial activity and low hemolytic activity were identified. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; lysine-peptoid hybrids; solid-phase peptide synthesis

INTRODUCTION

The widespread use of antibiotics for treatment and prevention of bacterial infections has resulted in an alarming increase in multiresistant pathogenic bacteria [1]. It is therefore essential that research efforts focus on antimicrobial agents with alternative modes of action [2–9]. Small antimicrobial peptides (AMPs) play a key role in innate immunity [10–12] and are promising alternatives to traditional antibiotics. AMPs act very fast and are active against a wide range of pathogenic microorganisms [13], including Gram-positive and Gram-negative bacteria, viruses, protozoa and fungi. Currently, more than 1000 naturally occurring AMPs are known [14]. AMPs are typically from 10 to 40 amino acids in length, and may be roughly categorized according to their secondary structure as α-helices, β-sheets, extended backbone and loops. They carry a net cationic charge and approximately 50% of the total amino acid content consists of hydrophobic residues, yielding their amphipathic properties.

The majority of antimicrobial peptides target the negatively charged lipid bilayer of bacterial cells selectively, and not the outer membrane of mammalian cells, which are zwitterionic [15]. However, some cytotoxic peptides, such as melittin [16], are known. The exact mechanism by which antimicrobial peptides kill bacteria is not yet clearly understood [17–19], but is thought not to involve a receptor, although some exceptions have been reported [20,21].

In recent years, much research in this area has been focused on designing short analogues of naturally occurring antimicrobial peptides with increased antibacterial activity and low cytotoxity against mammalian cells [22–27]. One approach is to design positively charged, amphipathic peptides, consisting of cationic amino acid residues, e.g. lysine, and hydrophobic amino acid residues, e.g. leucine. Park *et al.* designed different Leu–Lys-rich peptides based on cecropin A (1–8)-magainin 2 (1–12) hybrids [28]. One peptide, in particular, H-KWKKLLKKPLLKKLLKKL-NH_2, showed strong antibacterial activity toward the tested Gram-positive and Gram-negative bacteria. The peptide also showed low hemolytic activity.

Diastereomers of short K_mL_n model peptides were reported by Shai and coworkers [29,30]. The peptides were composed of varying ratios of leucine and lysine, one-third of their sequence being composed of D-amino acids. One peptide, K_6L_9 (LKlLKkLlkKLLkLL-NH_2, lowercase amino acids are the D enantiomers), was found to cure neutropenic mice infected with gentamicin-sensitive *Pseudomonas aeruginosa* and gentamicin-resistant *Acinetobacter baumannii* bacteria, when injected intravenously. The all-L-amino acid parental peptide was ineffective.

Oligomeric N-substituted glycines [31] or 'peptoids' are structurally similar to α-amino peptides, but differ in that the sidechain is appended to the amide nitrogen instead of the α-carbon. Peptoids offer several advantages, including stability to proteolysis [32], biocompatibility [33] and facile synthesis by the submonomer approach. Furthermore, Goodson *et al.* have shown that some peptoids display antibacterial activity [34]. The tripeptoid [*N*-(dehydroabietyl)glycyl]-[*N*-(2-aminoethyl)glycyl]-*N*-(1,2,3,4-tetrahydro-naphthalen-1-yl)glycineamide, and some of its analogues, were active in the range of 3 to 12 μg/ml against a panel of Gram-positive and Gram-negative bacteria, which included isolates resistant to known antibiotics. The hemolytic activity of these compounds was modest. Potent lead structures have also been identified from combinatorial library

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approaches [35,36] or by designing peptoid analogues of antibacterial peptides [37–39].

Patch *et al.* [37] reported on the design and synthesis of several helical all-peptoid mimics of magainin-2 amide. All sequences included a lysine-like *N*-(4-aminobutyl)glycyl at every third position to provide a cationic, facially amphipathic helix and water solubility. The most active peptoid oligomer, [[*N*-(4-aminobutyl)glycyl]-[(*S*)-*N*-(1-phenylethyl)glycyl]-[(*S*)-*N*-(1-phenylethyl)glycyl]]$_3$-[*N*-(4-aminobutyl)glycyl]-[(*S*)-*N*-(1-phenylethyl)glycyl]-(*S*)-*N*-(1-phenylethyl)glycine-amide, showed good antibacterial activity against *Bacillus subtillis* and *Escherichia coli* and low hemolytic activity. In a recent excellent review of the many roles of peptoids in drug discovery, the same authors also reported [38] previously unpublished data on structurally related cationic peptoids with antibacterial activity. The peptoids ranged between 6 and 17 monomers in length. Macrocyclic peptidomimetics having a mixed peptide–peptoid backbone were described by Shankaramma *et al.* [39]. A peptide–peptoid protegrin I analogue displayed an activity of 24 and 8 µg/ml against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922, respectively. The hybrids were shown to possess antibacterial activity against Gram-positive and Gram-negative bacteria and a low hemolytic activity against human erythrocytes.

In previous work [40], we evaluated 50 primary amines for peptoid synthesis in a model system BB-[*N*-(4-methylbenzyl)glycyl]-BB-*N*-(butyl)glycin amide, BB being the monomer evaluated. A selection of products were screened for antibacterial activity. One particular peptoid sequence, [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl) glycyl]-*N*-(butyl)glycin amide (**1**), proved to be very interesting as lead structure. Since nearly all antimicrobial peptides are cationic and hydrophobic, we anticipated that synthesis of conjugates made up of **1** and cationic lysines would result in derivatives with potent antibacterial activity and low cytotoxicity. In this study, we report the design, synthesis and characterization of 20 such lysine-peptoid hybrids that have not been described previously. The compounds were synthesized by solid-phase synthesis, using a combination of Fmoc chemistry and the submonomer approach, purified by preparative high-performance liquid chromatography (HPLC) and tested for antibacterial activity against *S. aureus* ATCC 25 923 and *E. coli* ATCC 25 922. Finally, the cytotoxicity of the derivatives toward mammalian cells was assessed, using a hemolytic activity assay. Several lysine-peptoid hybrids with potent antibacterial activity and low hemolytic activity were identified. The results presented here suggest that lysine-peptoid hybrids are promising lead structures for developing future therapeutics.

MATERIAL AND METHODS

HPLC

Analytical HPLC was performed using a Waters C_{18}-reverse-phase column (Delta–Pak 100 Å 15 µm, Millipore, Billerica, MA, USA) on a Waters 600E system equipped with Millennium software. Samples were chromatographed at a flow-rate of 1.5 ml/min starting with 0.1% aqueous trifluoroacetic acid (TFA) (buffer A) for 10 min and increasing over 45 min to 0.1% TFA in CH_3CN/H_2O (9 : 1) (buffer B), detection at 220 nm. Preparative HPLC was done on a Waters C_{18}-reverse-phase column (SymmetryPrep™, 7 µm, Millipore). Samples were chromatographed at a flow-rate of 4 ml/min starting with buffer A/buffer B (9 : 1) for 10 min and increasing over 60 min to buffer A/buffer B (4 : 6) and finally to buffer B over an additional 8 min, detection at 220 nm.

MALDI-TOF-MS and LC-MS

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was done on a VG Tof Spec E Fisons instrument (Fisons Instruments, Beverly, USA), using α-cyano-*p*-hydroxycinnamic acid as matrix. Substance P and adrenocorticotropic hormone (ACTH) were used as calibrants.

Liquid chromatography-mass spectrometry (LC-MS) was done on a Bruker Esquire Mass Spectrometer (Bruker Daltonics, Billerica, USA). The LC part consisted of a HP 1100 equipped with a Vydac C_{18} column (cat # 238MS215) and a diode array detector. Samples were chromatographed at a flow-rate of 0.25 ml/min starting with 0.1% aqueous TFA (buffer A) and increasing over 25 min to 0.1% TFA in CH_3CN/H_2O (4 : 1) (buffer B), finally increasing to buffer B over 10 min, detection at 220 nm.

Amino Acid Analysis

Amino acid analysis was performed on a Waters PicoTag analyzer (Waters, Milford, USA), after samples were hydrolyzed with 6 M aqueous HCl and 0.1% phenol at 110 °C. The concentration of each antibacterial solution was determined by including a standard, L-α-amino butanoic acid.

Materials

Sterile 96-well polypropylene plates were from COSTAR, Corning Incorporated, (Corning, USA); Sterile 96-well polystyrene microtiter plates were from Nunc (Roskilde, Denmark); Mueller–Hinton broth was from Fluka (Buchs, Switzerland). Fresh human blood in citrate-phosphate-dextrose buffer was obtained from Copenhagen University Hospital (Copenhagen, Denmark).

Chemicals

TentaGel S RAM resin (loading 0.23 meq/g) was from RAPP Polymers (Tübingen, Germany); 1-hydroxybenzotriazole (HOBt) and L-Fmoc-Lys(Boc)-OH were purchased from PerSeptive Biosystems (Hamburg, Germany) or Novabiochem (Läufelfingen, Switzerland); *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-*N*-methyl methanaminium hexafluorophosphate *N*-oxide (HATU) and *N*-[(1*H*-benzotriazol-1-yl)(dimethylaminomethylamino)methylene]-*N*-methyl

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methanaminium tetrafluoroborate *N*-oxide (TBTU) were from AnaSpec Inc. (San Jose USA); piperidine, anhydrous ampicillin, diisopropylamine, triisopropylsilane (TIS), *N*,*N*′-diisopropylcarbodiimide (DIPCDI), 1-naphthalenemethylamine, 4-methylbenzylamine, and butylamine were obtained from Fluka (Buchs, Switzerland); L-α-amino butanoic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Aldrich (Steinheim, Germany); bovine serum albumin (BSA), Triton X-100, ACTH, Substance P and Melittin were obtained from Sigma–Aldrich (St Louis, USA); TFA was from Merck (Darmstadt, Germany); DCM was from Riedel de Haën (Seelze, Germany); phenylisothiocyanate was obtained from Pierce (Rockford, USA). All starting chemicals were used without further purification.

Antibacterial Activity

Strains used for determining antibacterial activity included the two American Type Culture Collection (ATCC) strains *E. coli* ATCC 25 922 and *S. aureus* ATCC 25 923. The minimum inhibitory concentration (MIC) of each peptide was determined using a broth microdilution assay modified from the method of Hancock [41]. Dilutions of the stock solution to the required test concentration were made to reach a final concentration of 0.2% BSA and 0.01% acetic acid. Serial twofold dilutions of the peptides were made in 0.2% BSA and 0.01% acetic acid in sterile 96-well polypropylene microtiter plates. To each well was added 100 µl of the peptide and 100 µl of the test bacteria in Mueller–Hinton broth to reach a final concentration of 2×10^5 CFU/ml. Following the addition of MTT [42], (10 µl, 3 mM), the MIC of each peptide was read as the lowest concentration of peptide that inhibited visible growth of the bacteria after 24 h incubation at 37 °C. All MIC determinations were performed in duplicate and are the average of three independent determinations, using the same stock solution. Ampicillin was used as control.

Hemolytic Activity Study

Human blood was washed three times (3000 rpm) with a cold solution of 0.15 M, pH 7.2 phosphate buffered saline (PBS). The erythrocytes were diluted to a final concentration of 0.5% in PBS. To each well of a v-shaped polypropylene microtiter plate 75 µl of the diluted erythrocytes and 75 µl of peptide solution (100 µM) were added. The microtiter plates were allowed to incubate at 37 °C for 1 h, and centrifuged for 10 min at 4000 rpm. The supernatant (60 µl) of each well was then transferred to a flat-bottomed polystyrene microtiter plate and the absorbance was read at 414 nm. PBS and 0.1% Triton X-100 were used as references. Melittin was used as positive control. The hemolysis percentage was calculated as follows [28]: $[(A_{peptide} - A_{PBS})/(A_{Triton} - A_{PBS})] \times 100$.

All hemolysis determinations were performed in duplicate and are the average of three independent determinations using the same stock solution.

Synthesis of Lysine–peptide Hybrids

Compounds **1**–**21** were synthesized manually on a TentaGel S RAM resin (200 mg, 0.23 meq/g), using a combination of Fmoc SPPS [43] and the submonomer approach [31].

Coupling of Peptoid Monomers

The resin or resin-bound peptoid/peptide chain was bromoacetylated by adding 0.6 M bromoacetic acid (10 eq) in NMP (*N*-methylpyrrolidone) and 3.2 M DIPCOI (12.8 eq). The support was agitated for 30 min and drained; then another portion of bromoacetic acid/DIPCDI was added for yet another 30 min. After washing with NMP, the sidechain was introduced by nucleophilic substitution of the halide with a primary amine (40 eq) in NMP and agitated for 2 h.

Coupling of Lysine Residues

Two different acylation procedures were used, depending on the nature of the resin-bound *N*-terminal residue. When coupling Fmoc–Lys(Boc)-OH to a peptoid residue, a fivefold excess of HATU/DIEA (1 : 1.5) in NMP was used. When coupling Fmoc–Lys(Boc)-OH to another lysine residue, or as the first amino acid, TBTU/HOBt/DIEA (1 : 1 : 1.5) was employed. Acylation reactions were performed for 2 h, followed by a 2 h recoupling. Deprotection of the Fmoc group was effected with 20% piperidine in NMP. Following synthesis, the peptide-resin was washed with NMP, EtOH and ether and dried *in vacuo*. The resin was treated with TFA/H_2O/TIS (95 : 2.5 : 2.5) for 2 h, filtered and then washed with TFA/H_2O (95 : 5). TFA was removed by evaporation and the product precipitated in ether. The product was washed twice with ether, dried and lyophilized from 50% aqueous CH_3CN.

RESULTS AND DISCUSSION

Design and Synthesis

We have previously reported on the evaluation of primary amines for peptoid synthesis in the model system BB-[*N*-(4-methylbenzyl)glycyl]-BB-*N*-(butyl)glycin amide, BB being the monomer evaluated. A selection of the model compounds were tested for antibacterial activity toward *S. aureus* and *E. coli* where especially [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl) glycyl]-*N*-(butyl)glycin amide **1** proved to be very active. On the basis of this sequence, we designed 20 lysine-peptoid hybrids, differing in length, number of lysine residues and hydrophobicity. The peptoid building blocks are abbreviated X, Y, Z (X: *N*-(butyl)glycyl, Y: *N*-(1-naphthalenemethyl)glycyl, Z: *N*-(4-methylbenzyl)glycyl) (Figure 1). The compounds reported in this study are shown in Table 1A and 1B. In order to get a full overview of the results, we found it necessary to display the same data in two different ways. In Table 1A, the lysine-peptoid hybrids are shown in sequence series. The hybrids are divided into two main groups. Group 1 (compounds **2**–**12**) contains between one and six lysine residues outside the YZYX-core. Group 2 (compounds **13**–**21**) contains between two and five lysine residues, located both inside and outside the YZYX-core. In Table 1B, the hybrids are

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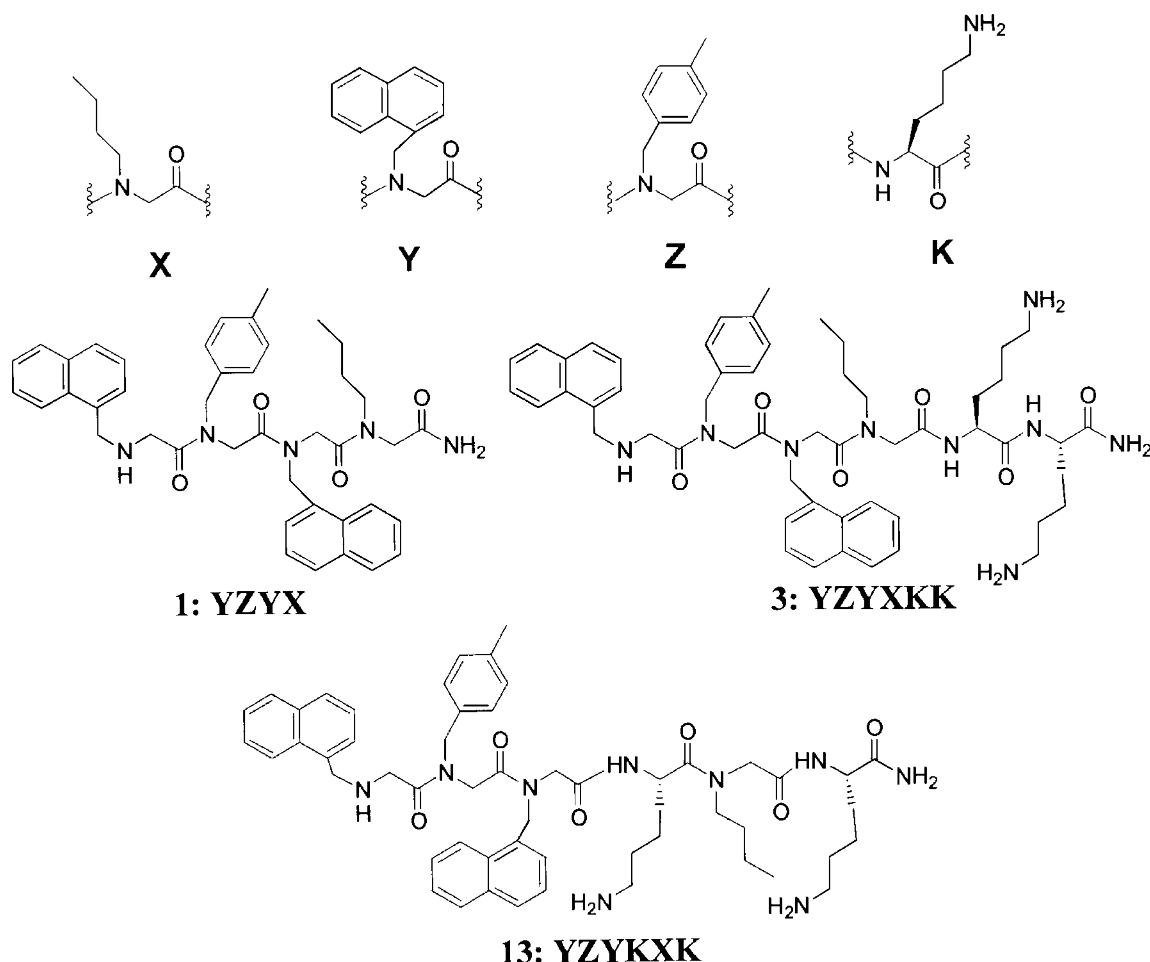

Figure 1 Representative structures of monomers and compounds used in this study. The peptoid building blocks are abbreviated X, Y, Z; (X: *N*-(butyl)glycyl, Y: *N*-(1-naphthalenemethyl)glycyl, Z: *N*-(4-methylbenzyl)glycyl).

arranged according to the number of lysine residues, allowing comparison of positional isomers.

Compounds **1**–**21** were purified by preparative RP-HPLC to more than 95% purity, and the molecular mass of each compound was verified by MALDI-TOF-MS. A stock solution of each lysine-peptoid hybrid was prepared and the exact concentration was determined by amino acid analysis, except for compound **1**, which was determined by weight. The stock solution was used for MIC determination and hemolytic activity experiments. The hybrids were tested for antibacterial activity against *E. coli* ATCC 25 922 and *S. aureus* ATCC 25 923. Furthermore, the hemolytic activity toward human erythrocytes was assessed. The results are shown in Table 1A and 1B.

Antibacterial Activity

The lead structure **1** was very active against both strains, displaying MIC values of <2 μM and 3–6 μM against *S. aureus* and *E. coli*, respectively. The majority of the analogues also showed potent MIC values. Thus, all of the lysine-peptoid hybrids displayed an antibacterial activity of 12 μM or lower against *S. aureus*; the most active were compounds **5**–**12** with a MIC value of 2 μM or better. The activity against the Gram-negative bacterium *E. coli* was less profound than that of *S. aureus*. Seventeen out of twenty hybrids showed an activity below 26 μM against *E. coli*. The Group 1 compounds were generally more active than the Group 2 compounds. The former group showed an activity between <2–4 μM against *S. aureus*, compound **2** being the only exception with a MIC value of 10 μM. In contrast, the most active of the Group 2 compounds (**18**, **20** and **21**), showed a two- to threefold better activity toward *E. coli*, when compared with Group 1 compounds. For the series **YZYXK**, **YZYXKK**, **YZYXKKK**, **YZYXKKKK** and **YZYXKKKKK**, the MIC values toward *S. aureus* indicated that there is a correlation between the number of lysine residues at the *C*-terminus and activity, MIC values going from 10 to <2 μM. The same tendency was shown in the activity against *E. coli*, MIC values going from 23 to 15 μM. A different trend was observed for the series **KYZYX**, **KKYZYX**, **KKKYZYX**, **KKKKYZYX**, and **KKKKKYZYX**. The MIC values toward *E. coli* indicated that adding a lysine residue at the *N*-terminus

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Table 1 (A) lists the lysine-peptoid hybrids in sequence series. (B) lists the same data according to the number of lysine residues. Lysine residues are in grey (A) and (B)

A						B						
	Compound[a,b]	RT[c]	S. aureus[d]	E. coli[d]	% H[e]		Compound[a,b]	#K	RT[c]	S. aureus[d]	E. coli[d]	% H[e]
1	YZYX	22.3	<2[f]	3–6[f]	100	**1**	YZYX	0	22.3	<2[f]	3–6[f]	100
2	YZYXK	19.5	10	23	100	**2**	YZYXK	1	19.5	10	23	100
3	YZYXKK	18.1	4	21	51	**7**	KYZYX	1	19.8	2	15	93
4	YZYXKKK	17.2	3	17	5	**3**	YZYXKK	2	18.1	4	21	51
5	YZYXKKKK	16.8	<2	15	2	**8**	KKYZYX	2	18.3	2	25	31
6	YZYXKKKKK	16.5	<2	15	3	**13**	YZYKXK	2	17.6	9	25	13
7	KYZYX	19.8	2	15	93	**4**	YZYXKKK	3	17.2	3	17	5
8	KKYZYX	18.3	2	25	31	**9**	KKKYZYX	3	17.5	2	25	23
9	KKKYZYX	17.5	2	25	23	**14**	YZKYKXK	3	15.9	12	75	6
10	KKKKYZYX	17.1	<2	25	6	**16**	YZYKXKK	3	16.8	7	38	0
11	KKKKKYZYX	16.8	<2	50	6	**19**	KKYZYKX	3	16.4	5	15	15
12	KKKYZYXKKK	14.9	<2	25	4	**5**	YZYXKKKK	4	16.8	<2	15	2
13	YZYKXK	17.6	9	25	13	**10**	KKKKYZYX	4	17.1	<2	25	6
14	YZKYKXK	15.9	12	75	6	**15**	YKZKYKXK	4	15.0	10	10	5
15	YKZKYKXK	15.0	10	10	5	**17**	YZKYKXKK	4	15.4	5	23	4
16	YZYKXKK	16.8	7	38	0	**20**	KKYZKYKX	4	15.0	8	7	6
17	YZKYKXKK	15.4	5	23	4	**6**	YZYXKKKKK	5	16.5	<2	15	3
18	YKZKYKXKK	14.5	5	4	6	**11**	KKKKKYZYX	5	16.8	<2	50	6
19	KKYZYKX	16.4	5	15	15	**18**	YKZKYKXKK	5	14.5	5	4	6
20	KKYZKYKX	15.0	8	7	6	**21**	KKYKZKYKX	5	14.4	7	6	7
21	KKYKZKYKX	14.4	7	6	7	**12**	KKKYZYXKKK	6	14.9	<2	25	4
22	Ampillicin[g]	n.d.	1.25	62.5	n.d							
23	Melittin	n.d.	n.d	n.d	100							

[a] The peptoid building blocks are abbreviated X, Y, Z; (X: *N*-(butyl)glycyl, Y: *N*-(1-naphthalenemethyl)glycyl, Z: *N*-(4-methylbenzyl)glycyl).
[b] All compounds were synthesized as *C*-terminal amides.
[c] RT: RP-HPLC retention time in minutes.
[d] MIC values are reported in μM.
[e] Percent hemolysis against human erythrocytes at 50 μM compound concentration.
[f] Concentration of the peptoid tetramer was determined by weight.
[g] MIC values of ampicillin in μg/ml.

results in decreased activity, MIC values increasing from 15 to 50 μM. On the contrary, all five compounds displayed the same potent activity against *S. aureus*, independent of charge.

Furthermore, we noticed that the activity of **13**–**21** toward *E. coli* increased significantly for analogues containing four or five cationic lysine residues. This tendency is in contrast to the one found for compounds **7**–**11**, where the activity toward *E. coli* decreased while adding additional lysine residues to the *N*-terminus.

Table 1B lists the hybrids according to the number of lysine residues present.

When comparing positional isomers $K = 1$–3, we observed that analogues having one or more lysine residues at the *N*-terminus, or at least two adjacent lysine residues at the *C*-terminus, were generally more active against *S. aureus* than their positional isomers. This difference was most distinct for $K = 1$ and $K = 2$. Thus, for $K = 1$, **KYZYX** showed a fivefold better activity against *S. aureus* compared to **YZYXK**. For $K = 2$, **YZYXKK** (**3**) and **KKYZYX** (**8**) showed better activity against *S. aureus* than **YZYKXK** (**13**). For $K = 3$, **YZYXKKK** (**4**) and **KKKYZYX** (**9**) were more active against *S. aureus* than **YZKYKXK** (**14**), **YZYKXKK** (**16**) and **KKYZYKX** (**19**). However, there was no distinct difference in activity against *E. coli* for the $K = 1$ and $K = 2$ series. For $K = 3$, **KKYZYKX** (**19**) was more active toward *E. coli* than **YZYXKKK** (**4**), **KKKYZYX** (**9**), **YZKYKXK** (**14**) and **YZYKXKK** (**16**).

Furthermore, we noticed that hybrids containing four or five lysine residues, of which at least two were located inside the YZYX-core, did not show selectivity between *S. aureus* and *E. coli*, the only exception being compound **17**. Thus, the compounds **15**, **18**, **20** and **21**, all had a MIC ratio *S. aureus*/*E. coli* from 1 to 1.25. These findings are similar to the results reported by Dathe *et al.* [44], who investigated different analogues of magainin II amide with cationic charges ranging between +3 and +7. They reported that a charge increase beyond +5 with retention of other structural

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motifs led to loss of antimicrobial selectivity. However, contrary to our results, a dramatic increase of hemolytic activity was observed.

Goodson *et al.* [34] identified several peptoid trimers with antibacterial activity against a panel of Gram-positive and Gram-negative bacteria. The MIC values ranged from 5 to 20 μM against Gram-positive bacteria and from 10 to >40 μM against Gram-negative bacteria. The compounds presented in this paper have significantly improved antibacterial activity compared to the aforementioned work. Thus, 14 of 21 lysine-peptoid hybrids showed a MIC value of 5 μM or below against *S. aureus*. Similarly, 19 out of 21 displayed a MIC value below 40 μM against *E. coli*. Furthermore, the antibacterial activity of positional isomers of the trimer [*N*-(dehydroabietyl)glycyl]-[*N*-(2-aminoethyl)glycyl]-*N*-(4-chlorobenzyl)glycine amide was also evaluated. The authors found similar Gram-positive activities of the six positional isomers (10–20 μM). This finding is in agreement with our work, where we observed a similar activity of the lysine-peptoid hybrids against *S. aureus* (2–10 μM).

The fact that the lysine-peptoid derivatives were more active against *S. aureus* than *E. coli* is in agreement with numerous reports on antibacterial peptides, e.g. melittin [45], ranalexin [46] and anoplin [47]. The selectivity may be accounted for in terms of the membrane architecture and composition of Gram-negative and Gram-positive bacteria as described by Lohner [15]. Antibacterial peptides have a balance between hydrophobicity and cationic charge, which is also the case for lysine-peptoid hybrids. One hypothesis which explains bacterial killing is the carpet model [48]. This model does not require a specific peptide structure, only a net positive charge, and enough hydrophobicity to allow membrane interaction. The cationic peptides bind onto the negatively charged phospholipid bilayer surface. When a threshold concentration is reached, the membrane is permeated in a detergent-like manner. The permeation pathway varies for different peptides. However, in order to elucidate the bacterial killing mechanism of the lysine-peptoid compounds, further investigations are needed, such as interaction with model membranes [49], and ATR-FTIR [50] to determine lysine-peptoid hybrid orientation in contact with membranes.

Hemolytic Activity Study

The hemolysis percentage against human erythrocytes at 50 μM compound concentration was assessed. The results are shown in Table 1A and 1B. Generally, we observed that the hemolytic activity correlated very well with the number of lysine residues in the hybrid. The series **2–6** showed hemolysis percentage values decreasing from 100 to 2–3%. A similar trend was observed for compounds **7–11**, which displayed hemolysis percentage values decreasing from 93 to 6%. Compound **12**, **KKKYZYXKKK**, which carries three lysine residues at both termini, showed virtually no hemolytic activity. Furthermore, the hybrids containing one or more lysine residues inside the YZYX-core, compounds **13–21**, displayed a hemolytic activity below 15%.

Our finding that the hemolytic activity of the hybrids correlates with the number of lysine residues is in agreement with previous reports on antibacterial peptides, e.g. indolicidin. This tridecamer peptide, ILPWKWPWWPWRR-NH_2, is active against a wide range of microorganisms and has also been reported to be strongly hemolytic toward red blood cells. Kolodkin and coworkers [51] showed that indolicidin analogues, in which 3–5 hydrophobic amino acids were replaced with lysine residues, displayed very low hemolytic activity, while antibacterial activity was retained. RP-HPLC retention times are generally thought to correlate with hydrophobicity and, to some extent, hemolytic activity [52,53]. In agreement with this, we noticed that the most hydrophobic compounds in this study were also among the most hemolytic compounds. Thus, **YZYX** (22.3 min), **YZYXK** (RT 19.5 min) and **KYZYX** (19.8 min) showed a hemolysis percentage between 93–100%. Thirteen out of fourteen hybrids with a retention time below 17.5 min showed a hemolytic activity of less than 10%, **KKYZYKX** (16.4 min) being the only exception. In addition, we found a good correlation between RP-HPLC retention times and hemolytic activity (correlation coefficient $r = 0.84$). This is in agreement with the work of Blondelle *et al.* on omission analogues of melittin [54]. They reported a correlation coefficient $r = 0.82$. For the series **2–6** and **7–11**, we found the correlation between hydrophobicity and hemolytic activity was even better, $r = 0.98$ and 0.97, respectively.

CONCLUSIONS

In this paper, we present a novel class of antibacterial compounds, lysine-peptoid hybrids. The hybrids are based on the peptoid lead structure [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl)glycyl]-*N*-(butyl)glycin amide. Our study revealed some interesting results. Firstly, we identified several lysine-containing analogues which displayed antibacterial activity against *S. aureus* and *E. coli*; MIC values for the most active ranging from <2–8 μM and 4–20 μM, respectively. Our best candidates have an activity profile that is fully comparable with previous literature reports on naturally occurring antibacterial peptides [55] (such as buforin II, cecropin P1, magainin II and nisin), antibacterial peptoids [34] and peptide–peptoid hybrids [39]. Furthermore, we identified two analogues which showed a high selectivity toward *S. aureus* but not *E. coli*, and four analogues

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which were very active, but did not show any selectivity toward either of the bacterial strains tested. Secondly, we were able to show that the hemolytic activity of the analogues correlated well with the number of lysine residues present in the hybrids, and that hemolytic activity may be reduced very significantly, while retaining antibacterial activity. All of the analogues containing four or more lysine residues, showed a very low hemolytic activity and good antibacterial activity. We are currently conducting related studies, in order to further characterize the lysine-peptoid hybrids, including antibacterial activity toward medically relevant strains, enzymatic stability and CD spectroscopy. In conclusion, our results indicate that lysine-peptoid hybrids are promising lead structures for developing new antibacterial agents.

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