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Potent antibacterial lysine–peptoid hybrids identified from a positional scanning combinatorial library

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Abstract—In this paper, we describe the synthesis and screening of a biased positional scanning library made up of peptoids (*N*-alkylglycines) and lysines. The library consisted of 100 mixtures divided into four sub-libraries; OXXXKKK, XOXXKKK, XXOXKKK, and XXXOKKK, O being a defined peptoid building block and X a mixture of 25 peptoid building blocks. A theoretical number of 390,625 compounds were synthesized. The compound mixtures were screened against the American Type Culture Collection (ATCC) *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 bacterial strains, and the cytotoxic activities were assessed using a human blood hemolytic assay. The results from each sub-library were examined to identify the most potent amine at each position. On the basis of this knowledge eight new lysine–peptoid hybrids were synthesized and tested in the biological assays. One compound in particular, [*N*-(cyclohexylmethyl)glycyl]-[*N*-(1-methylhexyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(2-(3-chlorophenyl)ethyl)glycyl]-lysyl-lysyl-lysine amide, showed high antibacterial activity and low toxicity toward red blood cells.

1. Introduction

The dramatic increase in drug resistance among bacteria observed in the past decades has created an urgent need for rapid development of new antibacterial drugs.[1] In recent years, much work in this area has focused on natural antimicrobial peptides[2,3] which are ubiquitous in nature and are believed to be a major part of innate immunity.[4]

Antimicrobial peptides rapidly kill a wide range of pathogenic microorganisms,[5] including Gram-positive and Gram-negative bacteria, protozoa, yeasts, and fungi without exerting toxicity against the host. They target the bacterial membrane which is a fundamentally different mechanism as compared with traditional antibiotics. Therefore, this class of compounds holds promise and may be a part of the solution to the problem of antibiotic-resistant pathogens.[6] Unfortunately, antimicrobial peptides often exhibit deficiencies in one or more important pharmaceutical properties, such as absorption, metabolism, and excretion.[7] To overcome some of these problems, much work has focused on modification of the peptide backbone. By incorporation of D-amino acids, *N*-alkyl-α-amino acids or a variety of other non-natural amino acid derivatives, the biological lifetime and stabilization of secondary structures can be achieved.[8] N-Substituted glycines (peptoids), first reported by Simon et al.,[9] are a family of non-natural oligomers based on a polyglycine backbone, on which the side chains are appended to the amide nitrogen instead of the C^{α}-atom. The side-chain moiety is introduced by the reaction of primary amines, which yield an extremely diverse series of functionalized oligomers. Furthermore, peptoids have

Abbreviations: ACTH, adrenocorticotropic hormone; ATCC, American type culture collection; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumin; *C. albicans*, *Candida albicans*; CFU, colony-forming units; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DMSO, dimethylsulfoxide; *E. coli*, *Escherichia coli*; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; LC–MS, liquid chromatography–mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MHB, Mueller–Hinton broth; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMP, *N*-methyl-2-pyrrolidone; PBS, phosphate-buffered saline; PS-SCL, Positional scanning soluble combinatorial library; HPLC, reverse-phase high-performance liquid chromatography; *S. aureus*, *Staphylococcus aureus*; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

Keywords: Lysine–peptoid hybrids; Antibacterial activity; Solid-phase synthesis; Positional scanning soluble combinatorial library.

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been shown to be resistant to a wide range of pharmacologically relevant proteases.[10]

Combinatorial libraries have proven to be an efficient method for identifying peptide and peptoid compounds with potent biological activity,[11–16] including antimicrobial agents. Methods for generating combinatorial libraries may roughly be divided in five different categories:[17] (i) biological systems such as phages;[18] (ii) spatially addressable library approaches such as the multipin approach[19] or spot synthesis on cellulose;[20] (iii) synthetic solution library methods with affinity selection;[21] (iv) one-bead one-compound combinatorial method;[22,23] and (v) non-support-bound mixture-based combinatorial libraries which require deconvolution. These include the iterative and positional scanning approach (PS-SCLs).[24–26]

One of the major advantages of PS-SCLs is that libraries can be readily used in cell-based assay systems that require soluble interactants.[27] A PS-SCL consists of sub-libraries in which one position is occupied by a defined building block, the others by mixtures. For instance, the positional scanning of a trimer will consist of three sub-libraries; OXX, XOX, and XXO, where O represents a defined position, and X is occupied by a diverse set of building blocks. From these sub-libraries the most active building block for each position can be defined, and new more potent compounds can be synthesized.

PS-SCLs may be generic or biased. The generic libraries are unrelated to any known structure, while the biased library is built around a known structure which previously has been characterized. Several small peptoids with potent biological activity have been identified using positional scanning combinatorial libraries. These include peptoids that neutralize bacterial endotoxins,[28] vanilloid receptor subunit 1 antagonists,[29] antifungal peptoids,[30] and antibacterial peptoids.[13–15]

In this paper, we describe the synthesis and screening of a biased lysine–peptoid PS-SCL. We synthesized four sub-libraries, each consisting of 25 peptoid mixtures (X), in the format $O_1X_2X_3X_4KKK$, $X_1O_2X_3X_4KKK$, $X_1X_2O_3X_4KKK$, and $X_1X_2X_3O_4KKK$. In each mixture the defined peptoid position was denoted O.

The 100 mixtures, a theoretical number of 390625 individual compounds, were screened against the *Staphylococcus aureus* and *Escherichia coli* bacterial strains. Furthermore, the cytotoxic activities were assessed in a human blood hemolytic assay. The results from each sub-library were examined to identify the most potent amine at each position. On the basis of this knowledge, eight new lysine–peptoid hybrids were synthesized and tested in the biological assays.

2. Results and discussion

In previous work, we have identified a series of novel lysine–peptoid hybrids with antimicrobial activity.[31,32] The hybrids were made up of the peptoid tetramer core [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl)glycyl]-[*N*-(butyl)glycine] amide and between one and six lysine residues. Since nearly all naturally occurring antimicrobial peptides are hydrophobic and cationic, we anticipated that conjugates made up of a peptoid tetracore and cationic lysines would result in derivatives with potent antibacterial activity and low cytotoxicity. Indeed, a number of lysine–peptoid hybrids, including [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl)glycyl]-*N*-(butyl)glycyl-lysyl-lysyl-lysine amide (Fig. 1) were very active toward *S. aureus* (3 μM) and *E. coli* (17 μM) and showed low cytotoxicity (5% at 50 μM) against human blood in a hemolytic assay. This prompted us to design a combinatorial library using lysyl-lysyl-lysine amide as lead structure.

A set of 37 primary amines were selected to comprise different functionalities and include hydrophobic and hydrophilic residues, aromatic residues, polar chains, and hydrogen-bond acceptors. Initially, we tested the coupling efficiency of the primary amines using the model system a_x-acetyl-lysyl-lysyl-lysine amide resin, where a_x was the primary amine evaluated. The 37 compounds were synthesized, cleaved, and subjected to HPLC and MALDI-TOF-MS. Of the 37 amines tested, 25 were chosen for use in the library. The criterion for successful incorporation was a purity of above 85%. Structures are shown in Figure 1.

Previously, we have reported on the synthesis of peptoids and lysine–peptoid hybrids, using 40 equiv of the amine to ensure high coupling efficiency. In the present library, where a mixture of 25 amines was used, a 40-fold excess would require a very large volume. However, Humet et al. reported the use of 20 equiv of the amine in a positional scanning combinatorial library of trimer peptoids with 22 different amines.[14] This prompted us to evaluate the effect of decreasing the molar ratio of amine to resin on the coupling efficiency. Thus, mixtures consisting of 1.1, 10, and 20 equiv of the tested amines were coupled to the bromoacetyl-KKK resin. A mixture of 37 lysine–peptoid hybrids, having the format $a_{(1-37)}$-acetyl-KKK-NH_2, was obtained.

By comparing the analytical HPLC traces of the three hybrid mixtures, we found that reducing the ratio of amine to resin to 1.1 equiv resulted in an unacceptable coupling efficiency for most amines. The two HPLC traces of the mixtures coupled with 10 and 20 equiv of amines were comparable and satisfactory. In two

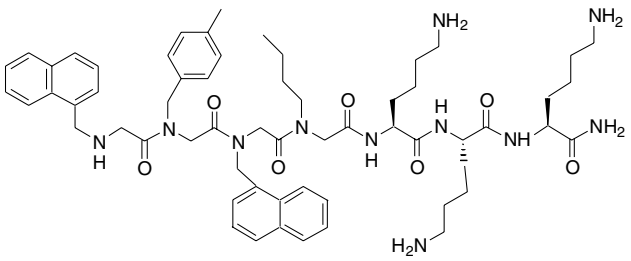

Figure 1. Lead structure identified in Ref. 31.

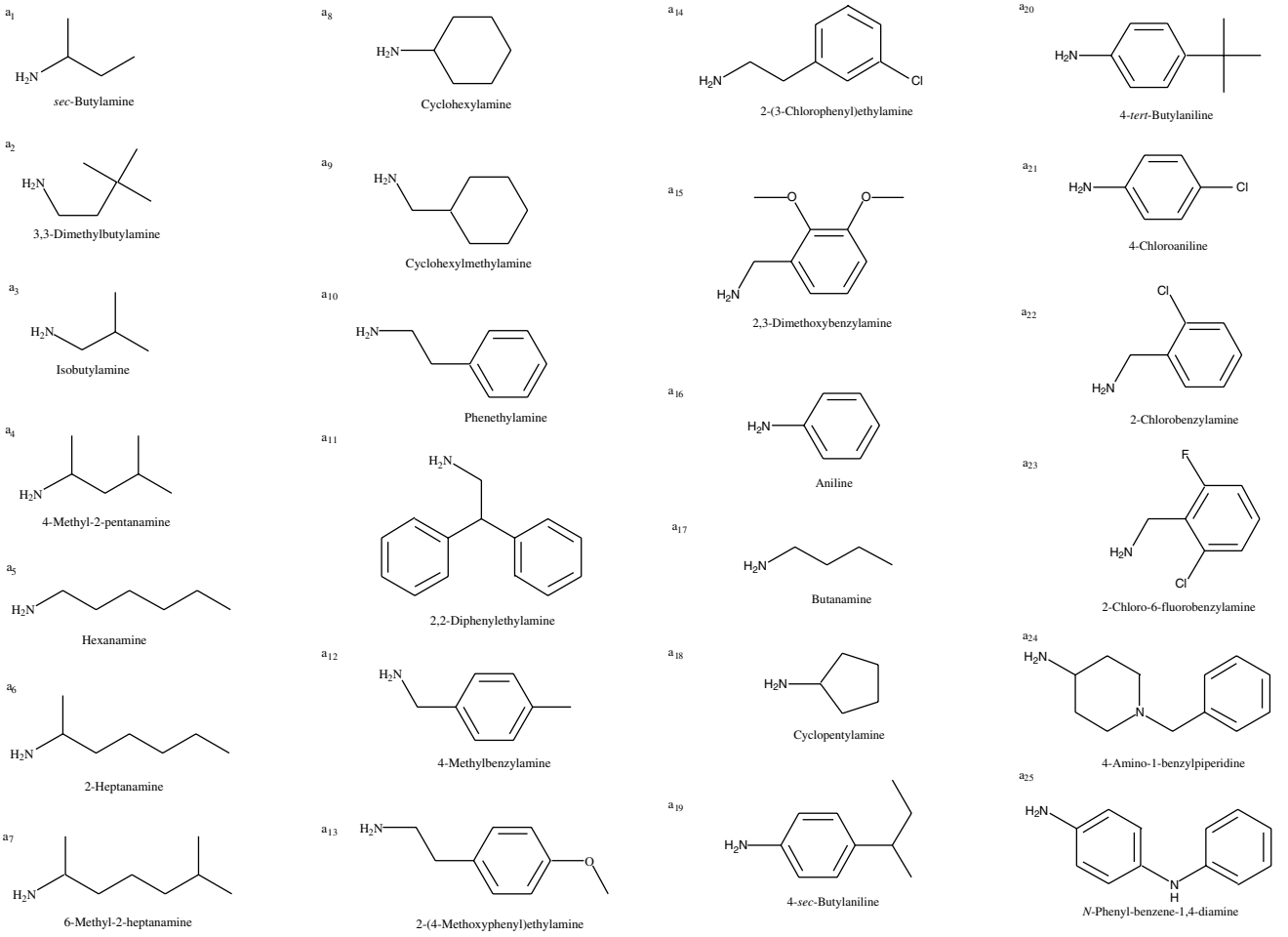

Figure 2. Structures of the amines used in the library.

additional experiments, a mixture of 25 amines was coupled to bromoacetyl-KKK-resin, using 10 and 20 equiv of amine, respectively. Following cleavage and work-up, the lysine–peptoid mixtures showed similar HPLC traces (data not shown).

On the basis of these results, we decided to synthesize the lysine–peptoid library using an excess of 10 equiv of amines instead of 20 or 40 equiv. The library was designed as a positional scanning combinatorial library divided into four sub-libraries; OXXX-KKK-NH_2, XOXX-KKK-NH_2, XXOX-KKK-NH_2, and XXXO-KKK-NH_2 where O represents a defined diversity position and X a mixture of the 25 amines.

This resulted in 100 mixtures, containing nearly 4000 compounds each (25^4/100). Following synthesis, which was carried out in a 100-well Teflon block,[33] the compounds were cleaved from the resin, lyophilized, and a stock peptide solution in 1% DMSO was prepared. The compound concentration was determined by amino acid analysis, and the solutions were diluted to 200 μM for use in the biological assays.

The minimum inhibitory concentration (MIC) was determined for each compound mixture against the Gram-positive bacterium *S. aureus* and the Gram-negative bacterium *E. coli*.

The ability of the mixtures to lyse human red blood cells was also determined, as a measure of the hybrids' cytotoxicity. The activity of the 100 mixtures was examined and the most potent amine in each sub-library was chosen for use in a set of second generation lysine–peptoid hybrids. Finally, a second generation of eight lysine–peptoid hybrids was synthesized and their biological activity are evaluated. The antibacterial- and the hemolytic activity of the compound mixtures are shown in Figures 3a–d.

The antibacterial activity is displayed as columns representing the inverse MIC value, thus the most active mixture displays the highest column. This is also true for the hemolytic activity, hence what is preferred is two high columns for the bacterial activity, and as low as possible a column for the hemolytic activity.

Figure 3a represents compound mixtures with specific amines in position 4, XXXO-KKK-NH_2. The most potent amine at this position was a_{14}; 2-(3-chlorophenyl)ethylamine, displaying MIC values of 1.8 and 6.25 μM against *S. aureus* and *E. coli*, respectively,

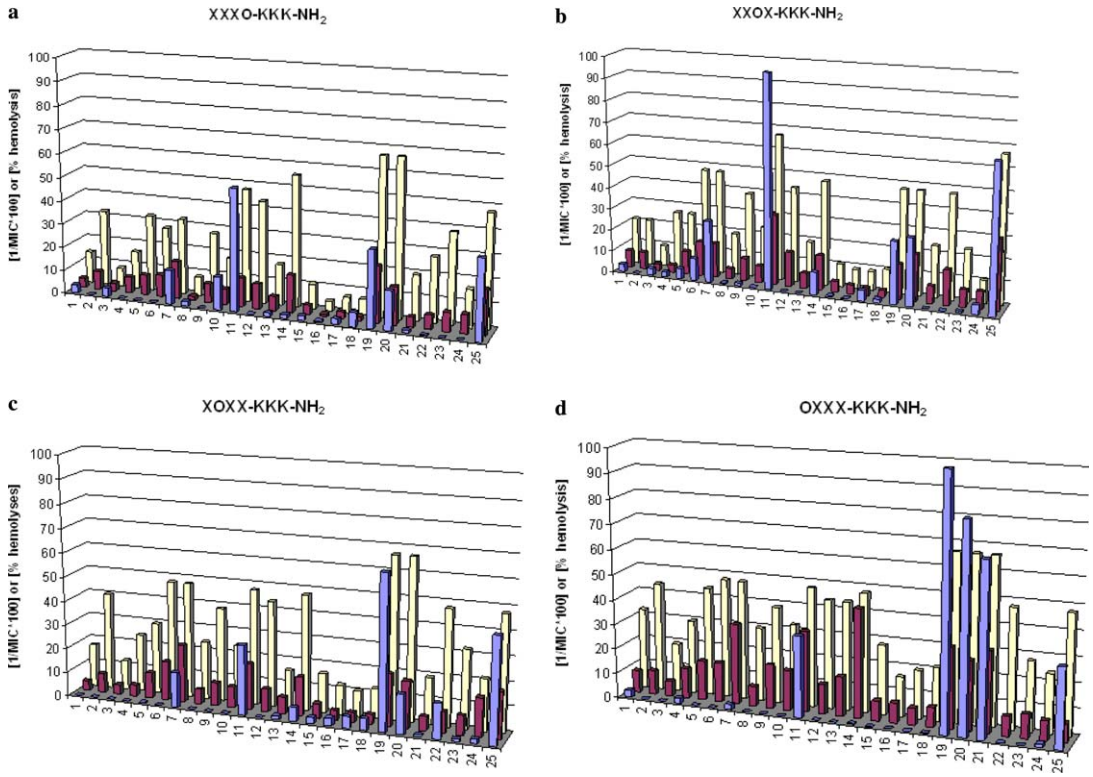

Figure 3. (a–d) Biological activities of the compound mixtures in the four sub-libraries. O represents the defined diversity position, and the *x*-axis refers to the amine occupying this position for the particular compound mixture in accordance with the numbers given in Figure 2. The blue columns represent the percentage hemolysis of the compound mixtures. The red and the yellow columns represent the activity of the library mixtures against *Escherichia coli* and *Staphylococcus aureus*, respectively. The antibacterial activities are shown as (1/MIC) * 100. It should be mentioned that MIC values above 47 at the *x*-axis only show the lowest possible activity.

and a hemolytic activity against human red blood cells of only 1%. Thus, a_{14} was chosen to be the amine at this position in the second generation compounds. Other amines which showed antibacterial activity at position 4 included: a_{19} (4-*sec*-butylaniline), MIC values of <1.5 and 4.2 μM against *S. aureus* and *E. coli*, respectively, and a_{20} (4-*tert*-butylaniline), MIC values of <1.5 and 6.25 μM against *S. aureus* and *E. coli*, respectively. However, they also showed high hemolytic activity of 33% for a_{19} and 17% for the a_{20}-mixture. The highest hemolytical activity (52%) was observed for the compound mixture with a_{11} (2,2-diphenylethylamine).

The amine selected for position 3, XXOX-KKK-NH_2 (Fig. 3b), was a_{12} (4-methylbenzylamine), displaying MIC values of <2.1 and 6.25 μM against *S. aureus* and *E. coli*, respectively, and 0% hemolytic activity. The same values were observed for the mixture containing a_{22} (2-chlorobenzylamine). However, we selected 4-methylbenzylamine over 2-chlorobenzylamine, since a chlorine containing building block was chosen for position 4. As was the case for position XXXO-KKK-NH_2, a_{11} turned out to be the most hemolytic amine. The most active amine at position 2, XOXX-KKK-NH_2 (Fig. 3c), was a_6 (2-heptanamine). This amine displayed MIC values of <2.1 and 6.25 μM against *S. aureus* and *E. coli*, respectively, and 0% hemolytic activity. Mixtures with a_7, a_{11}, a_{14}, a_{19}, a_{20}, and a_{25} all showed higher antibacterial activity than a_6, but also higher hemolytic activity. The most potent amines at the N-terminal position (Fig. 2d) were a_5 (hexanamine), a_6 (2-heptanamine), a_7 (6-methyl-2-heptanamine), a_9 (cyclohexylmethylamine), a_{10} (phenethylamine), a_{12} (4-methylbenzylamine), a_{13} (2-(4-methoxyphenyl)-ethylamine), and a_{14} (2-(3-chlorophenyl)ethylamine). They all showed very low MIC values, <2.3–6 and 6.25–16 μM, against *S. aureus* and *E. coli*, respectively, and 0–2% hemolytic activity. Furthermore, we noticed that the hemolytic activity for the compound with a_{19-21} increased drastically with values reaching 100%, 82%, and 69% hemolysis, respectively.

The antibacterial activities were increased for both bacterial strains tested. Generally, we found that the lysine–peptoid hybrids were more active against *S. aureus* than *E. coli*, which is in agreement with other studies on antimicrobial peptides, for example, indolicidin,[34] anoplin,[35] and peptoids.[36] This is probably due to the fact that Gram-positive bacterial membranes consist of a single bilayer membrane and a peptidoglycan–teichoic acid network, which furnishes the bacteria with an overall negatively charged surface. Contrary, Gram-negative bacteria are made up of an inner and outer membrane with an intervening layer of peptidoglycan in the periplasmic space. The outer membrane consists mainly of lipopolysaccharides, resulting in a negatively charged surface.[37] The most hemolytic mixtures at 50 μM compound concentration were the a_{11} (2,2-diphenylethyl-

amine), a_{19} (4-*sec*-butylaniline), a_{20} (4-*tert*-butylaniline), a_{21} (4-chloroaniline), a_{25} (*N*-phenylbenzene-1,4-diamine). Generally, we observed that the hemolytic activity correlated to some extent with the hydrophobicity of the lysine–peptoid hybrid mixtures.

On the basis of the above findings, we synthesized eight new lysine–peptoid compounds, the only variable being the amine at the N-terminal position; structures are shown in Figures 4a and b. The eight peptide–peptoid hybrids all showed the same very low antibacterial activity, ranging from 0.78 to 1.56 μM against *S. aureus* and 1.56 to 3.13 μM against *E. coli*. The lysine–peptoid hybrids presented in this paper, display among the lowest MIC values of any antibacterial peptoid or lysine–peptoid hybrid reported in the literature. The hemolytic activity of the eight second generation lysine–peptoid differed as can be seen in Table 1. The compounds may be divided into three structural classes, depending on the N-terminal amine; aliphatic amines, hybrids **1**–**3**, cyclic amine, compound **4**, and the aromatic amine hybrids **5**–**8**.

Several antimicrobial peptides and peptoids have been identified from combinatorial libraries. Four series of Fmoc N^a-derivatized antimicrobial tetrapeptides, which were active against Gram-positive and Gram-negative bacteria, were reported by Houghten and co-workers.[38,39] The MIC values were in the range of 4–8 and 32–62 μg/mL for *S. aureus* and *E. coli*, respectively. Furthermore, the tetrapeptides also displayed activity against methicillin-resistant *S. aureus*, in the range 32–124 μg/mL. These authors have also identified several hexapeptides, with MIC values ranging from 10 to 21 μg/mL against *S. aureus* and *E. coli*, respectively. Hong and Oh reported a decapeptide, KKVVFKVKFK, which inhibited the growth of *Candida albicans* and showed a broad range of antibacterial activity but no hemolytic activity.[40] The most active peptoid identified from a combinatorial library of trimeric peptoids displayed MIC values of 5 and 10 μM against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), respectively.[15] Humet et al. reported on an antibacterial peptoid from a positional scanning

Table 1. MIC values and hemolytical data

Compound	*S. aureus* (μM)	*E. coli* (μM)	Hemolysis (%)
1	0.78–1.56	1.56–3.13	13
2	0.78–1.56	1.56–3.13	16
3	0.78–1.56	1.56–3.13	26
4	0.78–1.56	1.56–3.13	7
5	0.78–1.56	1.56–3.13	19
6	0.78–1.56	1.56–3.13	16
7	0.78–1.56	1.56–3.13	10
8	0.78–1.56	1.56–3.13	46

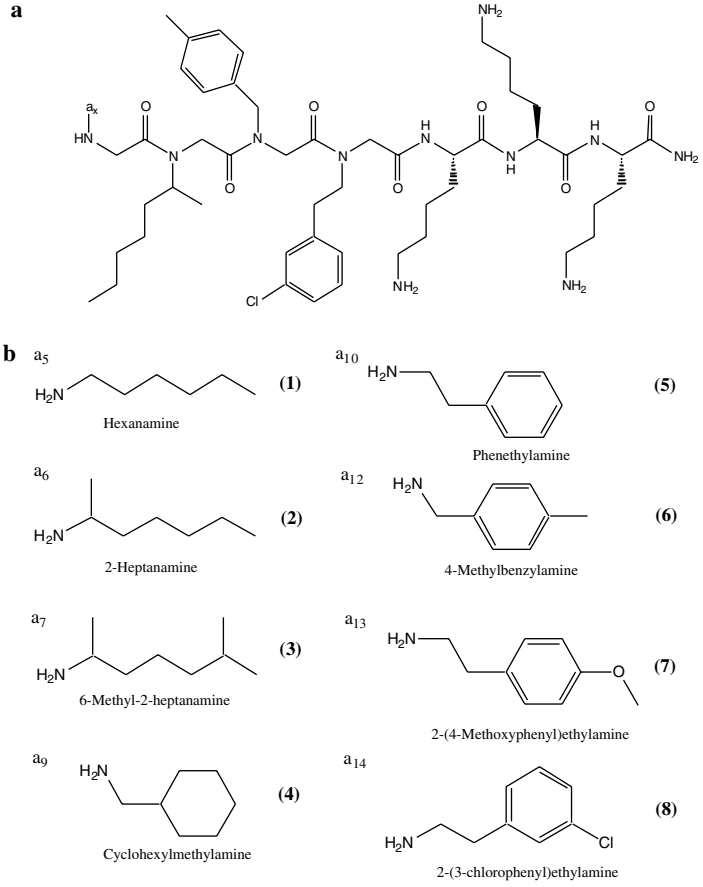

Figure 4. (a) General structure of the eight second generation lysine–peptoid hybrids. Each compound carries a different amine at the N-terminal position. (b) Amines used for second generation lysine–peptoid hybrids. Numbers in parentheses refer to the number given for the compound having the particular amine at the N-terminal position.

combinatorial library also composed of trimeric peptoids. However, the most active compound displayed MIC values of 45 and 90 μM against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922), respectively.[14]

3. Conclusion

We have identified eight new antibacterial lysine–peptoid hybrids, using a positional scanning combinatorial library approach. The antibacterial compounds all display an improved antibacterial activity profile as compared with the lead compound [*N*-(1-naphthalenemethyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(1-naphthalenemethyl)glycyl]-[*N*-(butyl)glycyl]-lysyl-lysyl-lysine amide. Furthermore, the MIC values are fully comparable with previous literature reports on naturally occurring antibacterial peptides, antibacterial peptoids, and lysine–peptoid hybrids. One compound in particular, [*N*-(cyclohexylmethyl)glycyl]-[*N*-(1-methylhexyl)glycyl]-[*N*-(4-methylbenzyl)glycyl]-[*N*-(2-(3-chlorophenyl)ethyl)-glycyl]-lysyl-lysyl-lysine amide, showed high antibacterial activity and low toxicity toward red blood cells. The lysine–peptoid hybrid displayed MIC values of 0.78–1.56 and 1.56–3.13 μM against *S. aureus* and *E. coli*, respectively, and a hemolytic activity of 7%. This compound may be an excellent lead structure for developing future therapeutics.

4. Experimental

4.1. Library synthesis

The peptide–peptoid hybrids were prepared by a combination of Fmoc solid-phase peptide synthesis[41] and the sub-monomer approach.[9]

TentaGel S Ram, 10× 500 mg, (0.23 meq/g) was allowed to swell in NMP (*N*-methylpyrrolidone) in 20 mL polypropylene syringes overnight. Following Fmoc deprotection with 20% piperidine in NMP and thoroughly washing, the first lysine monomer (L-Fmoc-Lys(Boc)-OH) was introduced together with 1-hydroxybenzotriazole (HOBt) and *N*,*N*′-diisopropylcarbodiimide (DIPCDI) as couplings agents (4:4:4). The deprotection and coupling steps were performed twice. After coupling the three lysines a small amount of the resin was cleaved and the product was subjected to HPLC and MALDI-TOF-MS to check for impurities and proper couplings. The first bromoacylation was done in the syringes by adding 0.6 M bromoacetic acid (10 equiv) in NMP and 3.2 M DIPCDI (12.8 equiv), this step performed twice for 1/2 h. The resin was subsequently distributed into a 100-well Teflon block, in which the further synthesis was done. Next, the side chain was introduced by nucleophilic substitution of the halide with a primary amine (40 equiv) or a mixture of 25 primary amines (10 equiv) in NMP for 2 h. The next three peptoid residues were synthesized in the same stepwise manner.

Following synthesis the product was cleaved from the resin with trifluoroacetic acid/triisopropylsilane/H_2O (95:2.5:2.5), precipitated in ether, and lyophilized from 50% acetonitrile. Stock solutions were prepared in 1% DMSO, and the concentrations determined by amino acid analysis.

4.2. Synthesis of individual peptide–peptoid hybrids

The eight single peptide–peptoid hybrids were synthesized as described above, although the first six residues (three lysines and three N-substituted glycines) were synthesized in four syringes, whereupon the resin was distributed into eight 5 mL syringes and the last peptoid monomer was coupled. The products were cleaved from the resin, precipitated in ether, and lyophilized twice from 50% acetonitrile. The compounds were characterized by reverse-phase HPLC and MALDI-TOF, and purified by preparative HPLC. Stock solutions were prepared in 1% DMSO, and the concentrations determined by amino acid analysis.

4.3. HPLC

Analytical high-performance liquid chromatography was performed using a Waters C_{18}-reverse-phase column (Symmetry® C_{18} 5 μM, 4.6 × 250 mM, Part No. WAT054275, Waters Corp., Milford, MA, USA) on a Waters 600E system equipped with Millennium software. Preparative HPLC was done on a Vydac C_{18}-reverse-phase column (10–15 μM, 22 × 250 mM, Part No. 218TP101522, VYDAC, Hesperia, CA, USA).

4.4. MALDI-TOF-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.

4.5. Amino acid analysis

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

4.6. Bacterial assay

The minimal inhibitory concentration (MIC) of each peptoid–peptide hybrid was determined using a broth microdilution assay modified from the method of Hancock.[42] Briefly, serial twofold dilutions of the peptides were made in solutions of 0.2% bovine serum albumin and 0.01% acetic acid in 96-well polypropylene microtiter plates (Costar, Corning Inc., Corning, NY) in volumes of 100 μL. To each well was added 100 μL of the test organism in Mueller–Hinton broth to a final concentration of approximately 2×10^5 colony-forming units/mL. The plates were incubated overnight at 37 °C and the MIC of each lysine–peptoid hybrid was

read as the lowest concentration of lysine–peptoid hybrid that inhibited visible growth of the organism 100%. All MIC determinations were performed in duplicate and are the average of three independent determinations. Ampicillin was used as a positive control.

4.7. Hemolytic assay

Human blood was received in citrate–dextrose–phosphate buffer from Copenhagen University Hospital (Copenhagen, Denmark). The erythrocytes were spun at 1.000*g* and washed three times with 10 volumes of cold 0.15 M phosphate-buffered saline (PBS). The erythrocytes were diluted to a final concentration of 0.5% in PBS. To each well of a Costar v-shaped 96-well polypropylene microtiter plate were added 75 μL of human erythrocytes in PBS and 75 μL of peptide–peptoid solution in PBS (100 μM). The microtiter plates were allowed to incubate at 37 °C for 1 h and subsequently centrifuged for 10 min at 1.000*g*. Sixty microliters of the supernatant was then transferred to a Nunc 96-well flat-bottomed polystyrene microtiter plate and read at 414 nm in a spectrophotometer to evaluate heme release. PBS and 0.1% Triton X-100 were used as references. Melittin was used as positive control. The hemolysis percentage was calculated as follows:[43] $[(A_{\text{peptide}} - A_{\text{PBS}})/(A_{\text{Triton}} - A_{\text{PBS}})] \times 100\%$. All hemolysis determinations were performed in duplicates and are the average of three independent determinations.

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