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Lysine-Based α-peptide/β-peptoid peptidomimetics: Influence of hydrophobicity, fluorination and distribution of cationic charge on antimicrobial activity and cytotoxicity

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Abstract: Multidrug-resistant bacteria pose a serious threat to public health worldwide. Previously, α-peptide/β-peptoid hybrid oligomers were found to display activity against Gram-negative multidrug-resistant bacteria. In the present work, the influence of hydrophobicity, fluorination and distribution of cationic/hydrophobic residues on antimicrobial, hemolytic and cytotoxic properties of α-peptide/β-peptoid hybrids were investigated. An array of 22 peptidomimetics was tested. Analogues with enhanced hydrophobicity exhibited increased activity against Gram-positive bacteria. Incorporation of fluorinated residues into the peptidomimetics conferred increased potency against Gram-positive bacteria, while hemolytic properties and activity against Gram-negative bacteria depended on the degree and type of fluorination. Generally, shorter oligomers were less potent as compared to the corresponding longer analogues. However, some short analogues exhibited equal or higher antimicrobial activity. The hydrophobic/cationic alternating design proved superior to other distribution patterns of cationic side chains and hydrophobic moieties.

Introduction

Antimicrobial resistance (AMR) is a steadily growing threat to public health globally[1]. In particular, continuous inappropriate use of antibiotics in both animals and humans has accelerated the selection of multidrug-resistant (MDR) bacterial strains, which can be almost impossible to eradicate (*e.g.*, recently colistin-resistant *E. coli* strains have emerged)[2]. Although AMR now receives considerable attention, the drug development pipeline suffers from a lack of new compound classes capable of replacing the current drugs that rapidly are becoming ineffective[2c, 3]. In particular, MDR Gram-negative strains resistant to all currently used clinical antibiotics constitute a severe health problem[2b, 4]. Notably, only five entirely new classes of antibiotics have been launched in the period 2000-2012, and all of these are applicable to Gram-positive infections only[5].

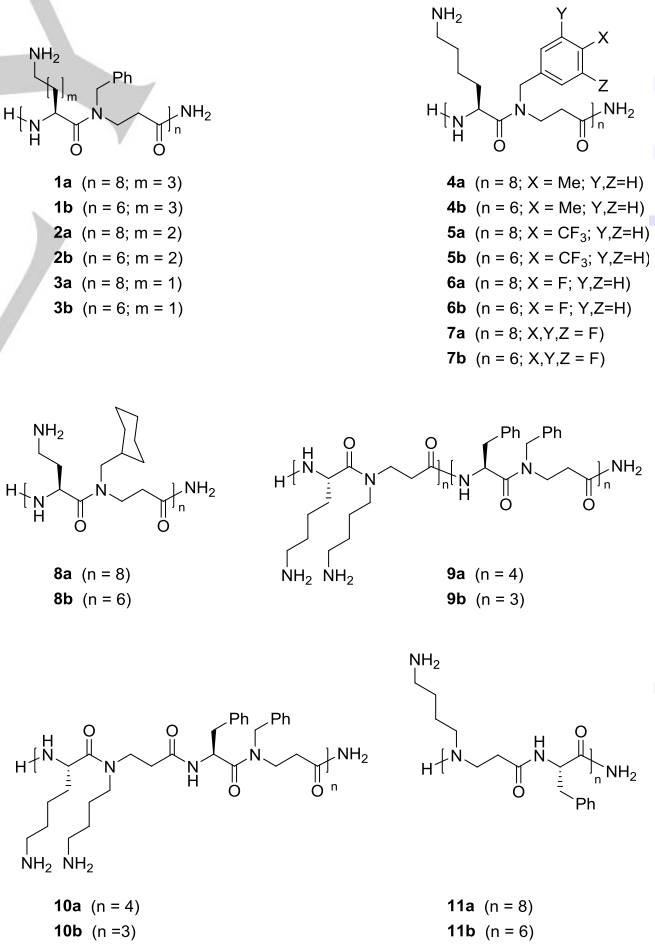

Figure 1. Structures of compounds belonging to the different subclasses of peptidomimetics investigated (suffixes a and b denote 16- and 12-mers, respectively).

Antimicrobial peptides (AMPs) play a key role in the innate immune system of all higher organisms[6]. Since their discovery more than three decades ago, AMPs and their synthetic analogues have received considerable attention from academia, and recently also by the pharmaceutical industry. Thus, in 2016 at least six synthetic AMPs were undergoing clinical trials[7]. Moreover, several AMPs have proved effective *in vitro* against a wide range of pathogens[8] while exhibiting a low tendency of resistance development[9], albeit they possess some unresolved cytotoxicity issues[10]. The detailed antimicrobial mechanisms of AMPs remain to be elucidated although multiple studies have established that most cationic AMPs initially interact with the negatively charged bacterial membrane. When a threshold concentration or a critical degree of surface adsorption is reached, transient or lasting pores are formed with ensuing bacterial killing due to concomitant disturbance of the membrane potential and/or loss of cytosolic content[11]. Yet, some AMPs have demonstrated alternative bactericidal mechanisms, *e.g.*, inhibition of cell wall synthesis as well as of nucleic acid or protein synthesis[12]. Nevertheless, AMPs inherently possess a number of disadvantages: (i) susceptibility to proteolytic degradation, (ii) pH- and/or salinity-dependent activity, or (iii) loss of activity due to binding to serum proteins[13]. However, incorporation of unnatural amino acids or mimetic residues may improve the bioavailability profile and metabolic stability of AMPs[13a]. A number of such peptidomimetics have been investigated, *e.g.*, α-peptoids[14], β-peptoids[15], β-peptides[16], α-peptide/β-peptide hybrids[17], lipo-AA-peptides[18], and peptide/peptoid hybrids[19]. Previously, α-peptide/β-peptoid hybrids were compared with peptidomimetics having other backbone designs (but also displaying alternating cationic-hydrophobic sequences) and were found to possess superior cell selectivity[19b]. Furthermore, potency against food-related bacteria[20], anti-biofilm activity[21], and immunomodulatory properties[22] has been reported for this compound class. Interestingly, in contrast to most AMPs these peptidomimetics display increased potency when tested in media containing up to 25% plasma[23].

Structure-activity studies have revealed that antimicrobial activity may be correlated to enhanced hydrophobicity for both AMPs and peptidomimetics, albeit with a resulting loss of cell selectivity and augmented hemolytic properties[17, 24]. Thus, it remains a challenge to design peptidomimetics possessing a fine-tuned balance between positive net charge and overall hydrophobicity that confers a pharmacological activity profile that allows for systemic use. A number of research groups have examined several ways to adjust the hydrophobicity of AMPs and antimicrobial peptidomimetics, *e.g.*, via end-group modification[19b, 24b], lipidation with fatty acids[25], or via introduction of bulky[26] or fluorinated amino acids in order to achieve a higher membrane affinity. The last approach was investigated by introducing hexafluoro-leucine residues into magainin or buforin resulting in enhanced antimicrobial activity and increased resistance to proteolytic degradation, while retaining low hemolytic activity[27]. By contrast, replacement of valine by hexafluoro-leucine in protegrin analogues led to reduced potency, while some analogues even lacked activity[28]. Incorporation of fluorine atoms[29], trifluoromethyl groups[30] or longer fluorinated tails[31] have also been found to enhance the antimicrobial activity, but often with concomitant increased hemolytic activity. Due to these diverging findings of the effect of fluorination further exploration of peptidomimetics was clearly warranted. In the present work we subject the class of α-peptide/β-peptoid hybrids to an investigation of structure-activity relationships (SARs) with focus on the influence of hydrophobicity (methylation or fluorination of phenyl rings and incorporation of cyclohexane moieties), sequential distribution of cationic residues, and side chain length on antimicrobial and hemolytic properties.

Results and Discussion

Six different subsets of α-peptide/β-peptoid hybrids containing cationic and hydrophobic residues in a 1:1 ratio were designed and obtained in oligomer lengths of 12 and 16 residues (Figure 1). As guanidinium-functionalized analogues previously were found to exhibit lower cell selectivity[19a], lysine was chosen as the typical cationic component. The β-peptoid analogue of phenylalanine (βNphe) was used as the typical hydrophobic component. To investigate whether the distance between the amino functionalities and the backbone is of importance for the antimicrobial properties, a subset of compounds comprising analogues with ornithine and (S)-2,4-diaminobutyric acid (Dab) as cationic residues in addition to lysine (i.e. **1**-**3**) was included. In another compound series hydrophobicity was increased by introduction of methyl, fluoro and trifluoromethyl substituents onto the phenyl rings (i.e. **4**-**7**) as well as by replacing the phenyl rings with cyclohexane moieties (to give **8**). In previous studies, we have exclusively explored the subclass of α-peptide/β-peptoid peptidomimetics with an alternating cationic/hydrophobic design. Therefore, we also examined analogues with alternative distributions of charged and hydrophobic side chains (i.e. **9** and **10**), but with similar backbone in order to elucidate whether the original design in fact is optimal. For comparison, also the inversed analogues (i.e. **11**) of **1** were included.

The required hydrophobic peptoid building blocks were prepared by an aza-Michael addition approach[19b, 32], while the cationic peptoid building block with a lysine-like side chain was obtained via alkylation of the appropriate amine with ethyl 3-bromopropanoate, followed by saponification and Fmoc protection[19b, 33]. Oligomers were prepared by Fmoc-based solid-phase synthesis on Rink amide resins using either MW-assisted or manual protocols[19b, 32]. Acidic cleavage from the resin and subsequent purification by preparative HPLC afforded the target compounds.

Six bacterial strains representing important Gram-positive and Gram-negative human pathogens were selected to explore the spectrum of antimicrobial activity: *Staphylococcus aureus*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Acinetobacter baumannii*, and *Salmonella* Typhimurium. In addition, a canine clinical isolate of *Staphylococcus pseudintermedius* was included as an example of an important veterinary pathogen. *S. pseudintermedius* is the main cause of integumentary infections in dogs, and these infections represent the main cause for antibiotic treatment of dogs[34].

Also, *S. pseudintermedius* poses a problem for pet owners and veterinarians due to potential zoonosis[35]. The antimicrobial activity of the peptidomimetics was evaluated by determination of minimal inhibitory concentrations (MIC values; Table 1) using broth microdilution according to recommendations

Table 1. Antimicrobial activity, hemolytic properties, and hydrophobicity of peptidomimetics.

No.	Modification	MIC[a] (μg/ml; also stated as μM in brackets)							%B[b]	HD_{10} (μM[c] also stated as hemolysis observed at 150 μM in brackets)
		S. aureus[d]	*S. pseudintermedius*[e]	*E. faecium*[f]	*P. aeruginosa*[g]	*E. coli*[h]	*A. baumannii*[i]	*S.* Typhimurium[j]		
1a	Length of cationic side chain	32 (13.7)	2 (0.9)	32 (13.7)	8 (3.4)	4 (1.7)	8 (3.4)	4 (1.7)	34.4	>150 (5 %)
1b		>64 (>36.5)	4 (2.3)	>64 (>36.5)	>64 (>36.5)	8 (4.6)	64 (36.5)	64 (36.5)	33.0	>150 (5 %)
2a		16 (7.2)	4 (1.8)	16 (7.2)	4 (1.8)	4 (1.8)	8 (3.6)	2 (0.9)	33.4	>150 (11 %)
2b		>64 (>38.3)	4 (2.4)	>64 (>38.3)	64 (38.3)	16 (9.6)	32 (19.2)	32 (19.2)	30.6	>150 (9 %)
3a		8 (3.8)	8 (3.8)	8 (3.8)	4 (1.9)	8 (3.8)	8 (3.8)	4 (1.9)	35.3	18.8 (14 %)
3b		>64 (>41.9)	2 (1.4)	64 (41.9)	8 (5.2)	8 (5.2)	>64 (>41.9)	4 (2.6)	33.2	>150 (5%)
4a	Phenyl ring substitution	8 (3.3)	4 (1.6)	4 (1.6)	8 (3.3)	16 (6.5)	8 (3.3)	8 (3.3)	40.4	2.6 (21 %)
4b		8 (4.3)	2 (1.1)	8 (4.3)	8 (4.3)	8 (4.3)	8 (4.3)	16 (8.7)	38.8	37.5 (15 %)
5a		16 (5.6)	8 (2.8)	8 (2.8)	16 (5.6)	8 (2.8)	16 (5.6)	8 (2.8)	46.9	<2.3 (100 %)
5b		4 (1.8)	4 (1.8)	2 (0.9)	8 (3.7)	8 (3.7)	8 (3.7)	2 (0.9)	45.9	<2.3 (100 %)
6a		2 (0.8)	2 (0.8)	4 (1.6)	4 (1.6)	8 (3.2)	4 (1.6)	4 (1.6)	36.9	>150 (9 %)
6b		8 (4.3)	2 (1.1)	16 (8.6)	4 (2.1)	8 (4.3)	8 (4.3)	8 (4.3)	36.1	>150 (7 %)
7a		4 (1.4)	2 (0.7)	2 (0.7)	4 (1.4)	8 (2.8)	4 (1.4)	2 (0.7)	43.7	4.7 (68%)
7b		2 (0.9)	2 (0.9)	2 (0.9)	4 (1.9)	4 (1.9)	4 (1.9)	2 (0.9)	41.9	37.5 (22%)
8a	Aliphatic β-peptoid	4 (1.7)	4 (1.7)	2 (0.8)	4 (1.7)	4 (1.7)	4 (1.7)	2 (0.8)	43.5	>150 (47%)
8b		4 (2.2)	2 (1.1)	4 (2.2)	2 (1.1)	8 (4.4)	2 (1.1)	2 (1.1)	41.5	>150 (20%)
9a	Alternative distribution of cationic residues	16 (6.9)	8 (3.4)	64 (>27.4)	16 (6.9)	16 (6.9)	16 (6.9)	16 (6.9)	49.0	38.5 (88 %)
9b		16 (9.1)	8 (4.6)	32 (18.2)	16 (9.1)	16 (9.1)	32 (18.2)	32 (18.2)	45.0	9.4 (52 %)
10a		16 (6.9)	8 (3.4)	16 (6.9)	8 (3.4)	16 (6.9)	8 (3.4)	8 (3.4)	37.3	>150 (6 %)
10b		64 (36.5)	2 (1.1)	64 (36.5)	8 (4.5)	16 (9.1)	64 (36.5)	4 (2.3)	35.7	>150 (10%)
11a	Inverse design	32 (13.7)	4 (1.7)	32 (13.7)	4 (1.7)	4 (1.7)	4 (1.7)	4 (1.7)	35.8	<2.3 (5 %)
11b		>64 (>36.5)	4 (2.3)	>64 (>36.5)	16 (9.1)	4 (2.3)	16 (9.1)	>64 (>36.5)	34.6	18.8 (9 %)
Colistin		>64	16	>64	0.25	<0.25	0.5	<0.25	-	>150 (2%)
Gentamicin		<0.25	16	8	1	1	8	1	-	>150 (1%)

[a] Minimal inhibitory concentrations (*i.e.* MIC values) for the peptidomimetics and control antibiotics were determined by using 2-fold dilution series in the range 0-64 μg/mL. The MIC values are given as the median of two independent biological replicates each with three technical replicates in agreement with the CLSI standards by using visual detection. [b] Hydrophobicity as indicated by the percentage of acetonitrile (%B) at the peak elution time on analytical reversed-phase HPLC. [c] The hemolytic dose (HD_{10}) is given as the concentration derived from a 2-fold dilution series that resulted in lysis of 10% human erythrocytes. [d] *Staphylococcus aureus* [ATCC 29213], [e] Canine clinical methicillin-resistant isolate of *Staphylococcus pseudintermedius* [29063], [f] vancomycin-resistant *Enterococcus faecium* [BM4147], [g] *Pseudomonas aeruginosa* [ATCC 27853], [h] *Escherichia coli* [ATCC 25922], [i] *Acinetobacter baumannii* [ATCC 19606], [j] *Salmonella* Typhimurium [U1].

by the Clinical Laboratory Standards Institute (CLSI)[36].Hemolytic activity was estimated as the peptidomimetic concentration that gives rise to 10% lysis of human red blood cells after incubation for 1 hour at 37 °C[37]. Effect on mammalian cell viability was determined by the MTS/PMS assay[38], and is reported as the IC_{50} values in both hepatocytes and fibroblasts.

Development of a potent and cell-selective antimicrobial peptidomimetic is challenging since SAR studies, performed by us and others[17, 24], on AMPs and antimicrobial peptidomimetics infer that improved antimicrobial activity often is accompanied by a similar rise in cytotoxicity. Hence, proper design of stable and non-toxic lead compounds requires a detailed understanding of

how factors like length, cationicity, amphipathicity and hydrophobicity define antimicrobial potency and cell selectivity for a specific class of compounds[39]. The relative hydrophobicity of peptides and peptidomimetics can be correlated to their retention in reverse-phase HPLC, and it is usually expressed as the percentage of acetonitrile at peak elution[39a]. Peptidomimetics were synthesized as 12- and 16-mers (denoted by suffixes a and b in Figure 1) to examine whether increased hydrophobicity might compensate for the general observation that shorter analogues exhibit diminished antimicrobial activity, whereas cytotoxicity appears to be correlated more distinctly to oligomer length[20, 22, 40]. Expectedly, a 2- to 16-fold decrease in antimicrobial activity was generally observed for the shorter oligomers, but for each bacterium certain shorter peptidomimetics exhibited equal or even slightly enhanced potency. Thus, for the Gram-positive *S. aureus* and VRE only two short analogues (*i.e.* **5b** and **7b**) were equally or slightly more potent, while this trend was more pronounced for MRSP as several short analogues (**5b**-**8b**) displayed similar or even 4-fold increased potency. Besides two compounds with slightly higher activity (**5b** and **8b**), the shorter analogues displayed retained activity or an up to 16-fold reduction in potency toward the Gram-negative *P. aeruginosa* and *A. baumannii*. For *E. coli* and *S.T*yphimurium the common trend was that the activity of the shorter analogues ranged from slightly enhanced to significantly reduced (up to 16-fold) activity (Table 1). Notably, for most bacteria the highly hydrophobic 12-mers **5b**, **7b** and **8b** exhibited similar potency (ranging from 2-fold decrease to 2-fold increase) as the corresponding 16-mers.

Interestingly, peptidomimetics with shorter cationic side chains displayed improved antimicrobial activity against *S. aureus* and VRE, whereas the opposite tendency was found for MRSP. Thus, replacement of Lys with Dab improved the activity against *S. aureus* from a MIC of 32 µg/mL to 8 µg/mL (*i.e.* **1a** versus **3a**), while the same modification led to decreased activity against MRSP (MIC of 2 µg/mL versus 8 µg/mL for **1a** and **3a,** respectively). By contrast, no general trend for the potency against Gram-negative bacteria was observed for this structural variation. Replacement of Lys (in **1a**) with Dab (to give **3a**) confers a slight increase in hydrophobicity as estimated by the increased retention in reverse phase HPLC (elution at 34.4% B and 35.3% B, respectively), and these findings are thus in accordance with earlier observations on the activity of end group-modified peptidomimetics against *S. aureus*[24b].

To obtain SARs to establish a putative correlation between increased hydrophobicity and activity, the phenyl rings of the β-peptoid residues were replaced with substituted aryl moieties displaying methylation in the 4-position (to give **4a/b**) as well as fluorination in position 4 (*i.e.* **6a/b**) and simultaneously in positions 3, 4 and 5 (*i.e.* **7a/b**), or trifluoromethylation in position 4 (*i.e.* **5a/b**). Moreover, to extend the hydrophobicity range further, analogues containing cyclohexane-based peptoid residues were included. Addition of methyl and trifluoromethyl groups enhanced the activity against *S. aureus* and VRE 2- to 8-fold, *e.g.*, MIC against *S. aureus* was improved from 32 µg/mL (**1a**) to 8 µg/mL (**4a**), while MIC against VRE was lowered significantly for **5b** as compared to that of **1b** (more than 64-fold). By contrast, a 2- to 4-fold decrease in activity against Gram-negative bacteria was typically observed. Fluorination directly on the aromatic rings (as in **6a** and **7a**) led to significantly increased activity against *S. aureus* and VRE (8- to 16-fold), while potency toward MRSP and Gram-negative bacteria remained unchanged. Remarkably, the trisfluorinated 12-mer **7b** exhibited high activity (MIC values of 2-4 µg/mL) against all pathogens included in the test panel. Likewise, incorporation of cyclohexane-based peptoid units (to give **8a/b**) conferred substantially increased activity against both Gram-positive and Gram-negative bacteria. Analogues with an inverse design having lysine-like β-peptoid residues, but with similar backbone structure (*i.e*. **11a/b**), were somewhat more hydrophobic than the parent compounds **1a/b**, but still exhibited similar activity profiles to all tested bacteria as previously found for *E. coli*[19b].

In addition, analogues displaying alternative arrangements of the cationic and hydrophobic residues, but with identical backbone, were investigated since all previous work on α-peptide/β-peptoid hybrids has concerned representatives with alternating cationic and hydrophobic residues[19b, 22, 40]. Hence, compounds **10a/b** and **9a/b** in which all cationic residues were placed pairwise or completely segregated in each end of the sequence, respectively, were examined as well. Analogues **10a/b** exhibited similar antimicrobial activity as the parent peptidomimetics **1a/b**, while possessing slightly increased hydrophobicity. By contrast, compounds **9a/b** having a longitudinal amphipathic structure consistently showed unchanged or decreased antimicrobial activity albeit being among the most hydrophobic compounds tested in the present study.

Traditionally, hemolytic activity has been used as a general indicator of cytotoxicity, and oligomer length and hydrophobicity appear to be the major determinants of the hemolytic properties of peptidomimetics[22, 40]. For the pairs **1a**/**b**, **2a/b**, and **6a/b** no substantial differences in hemolytic properties were observed between 16-mers and 12-mers. A slight shortening of the side chain length of cationic residues (*i.e.* Lys → Orn) only had a minor effect on the hemolytic properties (HD_{10} remained above 150 µM), whereas incorporation of Dab into a 16-mer led to significantly increased hemolysis (HD_{10} of 19 µM). Expectedly, incorporation of more hydrophobic β-peptoid residues displaying methyl (*i.e.* **4a/b**) or trifluoromethyl (*i.e.* **5a/b**) substituents on the phenyl rings increased the hemolytic activity substantially as HD_{10} typically was close to or lower than the MIC value. Trisfluorinated analogues **7a/b** also followed this pattern and exhibited high hemolysis that may be correlated to high hydrophobicity (~ elution at 46.0% B and 44.1% B) as compared to **1a/b** (35.2% B and 34.7% B). However, compounds **6a/b** containing monofluorinated phenyl rings remained almost non-hemolytic in accordance with an only slightly increased hydrophobicity as compared to that of **1a/b**.

Besides an influence on the antimicrobial activity, the distribution of cationic residues within the sequence also had an impact on the hemolytic properties of the peptidomimetics. Thus, location of all cationic residues in one end significantly boosted the hydrophobicity of **9a** (~51.6% B), which conferred increased hemolytic properties. Notably, compound **10a** with a pairwise arrangement of cationic residues remained almost non-hemolytic despite a slightly increased hydrophobicity. Collectively, these results infer that the original design with alternating cationic and hydrophobic residues indeed is the most favorable as it allows for potent antimicrobial activity while keeping hemolysis low. As anticipated from their quite hydrophobic nature (45.8% B; 43.7% B) compounds **8a/b**

Table 2. Effect of selected compounds on mammalian cells given as cell viability (IC_{50} values given in µg/mL [a] and selectivity indices (SI)).

Comp´d	Effect on HepG2[b] viability	Eukaryote/prokaryote selectivity index		Effect on NIH 3T3[c] viability	Eukaryote/prokaryote selectivity index		Erythrocyte/prokaryote selectivity index	
	IC_{50} (µg/mL)	IC_{50}/MIC		IC_{50} (µg/mL)	IC_{50}/MIC		HD_{10}/MIC	
		G+	G-		G+	G-	G+	G-
1a	318.1 (280.8 – 359.9)	15	53	832.3 (723.8 – 959.8)	38	139	>16	>60
3a	151.2 (133.3 – 173.1)	19	25	399.2 (344.6 – 461.6)	50	67	4.9	6.6
4a	65.2 (49.3 – 86.6)	12	7	527.2 (451-0 – 863.0)	100	53	1.2	0.6
6a	44.9 (41.2 – 49.4)	17	9	132.4 (113.9 – 154.6)	49	27	>140	>75

[a] Values are given as the average (n = 6 in two different cell passages), also stated as 95% confidence interval in brackets. [b] HepG2: human hepatocellular carcinoma cell line. [c] NIH 3T3: mouse fibroblast cell line.

exhibited intermediate hemolytic activity as compared to that of **1a/b** and **5a/b**.

Recent results show that viability testing on different appropriate cell lines may give a more comprehensive picture of the selectivity towards bacteria, and thus complement results from hemolysis studies[40]. Hence, four representative peptidomimetics **1a**, **3a**, **4a** and **6a** were selected for testing in viability assays with hepatocytes (HepG2) and fibroblasts (NIH 3T3) (Table 2). Generally, a correlation between antimicrobial activity and cytotoxicity was observed. Despite displaying lower hemolysis than analogues **3a** and **4a**, peptidomimetic **6a** demonstrated the highest impact on viability of mammalian cells, indicating a critical effect of fluorination. Surprisingly, compound **4a** demonstrated lower cytotoxicity toward fibroblasts than analogues **3a** and **6a**, while exhibiting the highest hemolytic activity. The ranking of compounds in relation to their cytotoxic effects followed the same trend in both cell lines. Notably, comparison of the two cell lines infers that HepG2 hepatocytes appear more sensitive toward all tested compounds than the NIH 3T3 fibroblasts. In an attempt to reveal a potential direct correlation between cytotoxicity, hemolytic activity and antimicrobial activity, selectivity indices (SI) were calculated as ratios between IC_{50} or HD_{10} and an average potency against Gram-positive or Gram-negative bacteria (Table 2). Interestingly, all tested analogues displayed similar selectivity indices when comparing their effect on HepG2 and Gram-positive species, whereas **1a** showed higher selectivity between HepG2 cells and Gram-negative species. Peptidomimetics **4a** and **1a** demonstrated the best cell selectivity between NIH 3T3 cells and Gram-positive and Gram-negative bacteria, respectively. Also, **6a** displayed the highest cell selectivity toward mammalian erythrocytes and both Gram-positive and Gram-negative species, while in particular **3a** and **4a** proved to be considerably less selective. Overall, when targeting Gram-positive bacteria it appears that fluorination (*i.e.* **6a** versus **1a**) may be beneficial, but only to a very limited degree as the absolute IC_{50} values were lowered considerably, while the increase in antibacterial potency was even more pronounced. On the other hand, the performance of the starting peptidomimetic **1a** was superior against Gram-negative species.

Conclusions

In the present SAR study, we have explored the effects of hydrophobicity (via methylation and fluorination), rearrangement of cationic residues, length of cationic side chains, and oligomer length on antibacterial activity and cytotoxicity. The results show that even though hydrophobicity is often considered a key element in optimization of antibacterial activity, it constitutes a two-bladed sword as the hydrophobicity must be kept below a certain threshold level in order to avoid an ensuing loss of selectivity for bacteria over mammalian erythrocytes and other relevant cell types. Somewhat surprisingly, modification towards very high hydrophobicity may in fact even confer diminished antimicrobial activity, *e.g.*, as seen for the trifluoromethylated analogues. Nevertheless, limited fluorination at a single position in the aromatic β-peptoid residues proved to be sufficient to dramatically increase activity against both Gram-positive and Gram-negative bacteria without affecting the hemolytic properties (*e.g.*, as seen for **6a**), albeit it resulted in acquisition of some cytotoxicity. By contrast, incorporation of three fluorine atoms directly on each phenyl ring or as a trifluoromethyl substituent both led to decreased cell selectivity. Rearrangement of cationic residues induced increased hydrophobicity without

affecting the antimicrobial properties of the compounds, whereas the hemolytic activity as well as cytotoxicity was increased as compared to the simple cationic/hydrophobic alternating design. An interesting trend was seen for MRSP. In this case shorter peptidomimetics consistently displayed activity comparable to the corresponding longer analogues despite a clear opposite trend for other bacteria. Also, the present results further corroborate our previous observations for this subclass of peptidomimetics: namely that a slightly increased level of hydrophobicity is beneficial for both potency and cell selectivity when Gram-positive bacteria is targeted, whereas such modifications seem to be of very limited advantage when combating Gram-negative species.

Experimental Section

General procedure

Starting materials and solvents were purchased from commercial suppliers (Iris Biotech, Sigma-Aldrich, and Merck) and used without further purification. Water used for analytical and preparative HPLC was filtered through a 0.22 µm Millipore membrane filter. Purity was determined by analytical HPLC using a Phenomenex Luna C18(2) column (150 mm × 4.6 mm; 3 µm particle size) on a Shimadzu Prominence and Shimadzu Nexera system using an aqueous MeCN gradient with 0.1% TFA added (eluent A: 5:95 MeCN–H_2O + 0.1% TFA, eluent B: 95:5 MeCN–H_2O + 0.1% TFA); a flow rate of 0.5 mL/min was used. For elution of peptidomimetics, a linear gradient of 10→60% B during 10 min was used with UV detection at λ = 220 nm. All tested compounds had a purity of at least 95%. Preparative HPLC was performed by using a Phenomenex Luna C18(2) column (250 × 21.2 mm; 5 µm particle size) on a Shimadzu Prominence system by using the same eluents as for analytical HPLC. Elution was performed with a linear gradient of 10→50% B during 20 min at a flow rate of 40 mL/min with UV detection at λ = 220 nm. HRMS spectra were obtained by using a Bruker MicrOTOF-Q II Quadropol MS detector.

General protocol for automated MW-assisted synthesis of peptidomimetics (1a, 4a, 5a, 5b, 6a, 6b, 10b)

Peptidomimetics were prepared by automated microwave (MW)-assisted Fmoc-based solid-phase peptide synthesis (SPPS) on a CEM™ Liberty microwave peptide synthesizer. H-Rink-Amide ChemMatrix® resin (PCAS BioMatrix Inc., Quebec, Canada) (loading 0.52 mmol/g, 0.1 mmol) was used. Fmoc (9-fluorenylmethyloxycarbonyl) deprotection conditions: excess 20% piperidine in DMF, initially 75 °C (MW), 30 s, subsequently 75 °C (MW), 180 s. Coupling conditions: 5.0 equiv of building block, 5.0 equiv N,N,N′,N′-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), DIPEA, DMF, 75 °C (MW), 15 min. Resin was transferred to a Teflon vessel fitted with a polypropylene filter by using DMF and DCM. Upon draining, the resin was washed with DCM. Cleavage and side chain deprotection was performed with TFA–H_2O (95:5; 2 × 1 h, each with 5 mL under shaking at room temperature). The filtrates were collected, and the resin was further eluted with TFA (2 mL), MeOH (2 mL), and DCM (2 mL). The combined filtrates were concentrated *in vacuo* and co-evaporated with MeOH-toluene (3 × 5 mL). The crude product was purified by preparative HPLC, and the appropriate fractions were concentrated *in vacuo* and lyophilized; identity was verified by HRMS and purity (>95%) was determined by analytical UHPLC.

General protocol for manual synthesis of peptidomimetics (1b, 2a, 2b, 3a, 3b, 4b, 7a, 7b, 8a, 8b, 9a, 9b, 10a, 11a, 11b)

Several peptidomimetics were prepared manually as previously described[16]. In brief, H-Rink-Amide ChemMatrix® resin (PCAS BioMatrix Inc., Quebec, Canada) (loading 0.52 mmol/g, 0.05 mmol) and Teflon vessels (10 mL; fitted with a polypropylene filter) were used. Coupling conditions used for peptoid building blocks: 3.0 equiv building block, 3.0 equiv benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP), and 6.0 equiv DIPEA (>2 h under shaking at room temperature). Coupling conditions for Fmoc-protected amino acid building blocks: 5.0 equiv building block, 5.0 equiv PyBOP and 10.0 equiv DIPEA (>2 h under shaking at room temperature). Fmoc deprotection conditions: 20% piperidine in DMF (2 × 10 min, each time with 5 mL under shaking at room temperature). Washing conditions: DMF, MeOH, and DCM (3 × 3 min, each time with 5 mL under shaking at room temperature). Capping was applied after loading, and after couplings number 6, 9, and 12: Ac_2O–DIPEA–NMP 1:2:3 (5 mL for 10 min under shaking at room temperature). Cleavage and side chain deprotection followed by purification were performed as described above for MW-assisted synthesis of peptidomimetics.

Determination of antibacterial activity *in vitro*

Six strains were used in MIC determinations: *Staphylococcus aureus* [ATCC 29213], a canine clinical methicillin-resistant isolate of *Staphylococcus pseudintermedius* [29063], vancomycin-resistant *Enterococcus faecium* [BM4147], *Pseudomonas aeruginosa* [ATCC 27853], *Escherichia coli* [ATCC 25922], *Acinetobacter baumannii* [ATCC 19606], *Salmonella* Typhimurium [U1]. All strains were cryo-preserved in Brain Heart Infusion (BHI) broth supplemented with glycerol 15% (v/v) at –80°C, and cultivated aerobically at 37 °C in nutrient agar base (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) calf blood. Minimal inhibitory concentrations (MICs) were determined by the broth microdilution method according to the Clinical Laboratory Standards Institute (CLSI)[36]. In brief, colonies from fresh overnight cultures were suspended to a turbidity of 0.5 McFarland, and the resulting suspensions were further diluted 1:1000 in Mueller−Hinton Broth II. These bacterial suspensions were added to microtiter plates 96 U-well (Almeco A/S) containing 2-fold serial dilutions of peptidomimetics in saline followed by incubation at 37 °C in ambient air for 18 h. The MIC values were determined as the lowest concentration showing no visible growth as compared to the control without peptidomimetic added. Experiments were performed twice as triplicates on two different days.

***In vitro* hemolytic activity**

The lysis of human red blood cells (hRBCs) was measured as previously described[37]. In brief, hRBCs from a freshly drawn type 0^+ blood sample were washed with PBS buffer (pH 7.2, 10 mM Tris, 150 mM NaCl), and then centrifuged once at 700 × g for 8 min and twice at 1000 × g for 8 min each time. Two-fold serial dilutions of the peptidomimetics in PBS buffer were added to each well in a sterile round-bottom 96-well plate for a total volume of 20 µL in each well. A 1% v/v hRBC suspension (80 µL in PBS buffer) was added to reach a total volume of 100 µL in each well. The plate was incubated (37 °C) for 1 h, and then the cells were pelleted by centrifugation at 1000 × g for 10 min. The supernatant (60 µL) was transferred to a new 96-well plate, and the hemoglobin content was detected by measuring the OD with a Molecular Devices VersaMax Microplate Reader at 414 nm. OD of cells incubated with melittin (400 µg/mL) defined 100% hemolysis, while OD of cells incubated with PBS buffer defined 0% hemolysis. Tests were performed twice in triplicates on two different days.

Cell culturing

Two different cell lines were used: HepG2 and NIH 3T3 (from ATCC, Manassas, VA, USA), obtained from a similar level of confluence after 21−25 h of culturing under standard conditions (5% CO_2/95% O_2 at 37 °C); *i.e.* the seeding densities were the following: HepG2 (6.97×10^4 cells/cm^2), NIH 3T3 (6.67×10^4 cells/cm^2). 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. NIH 3T3 cells were cultured in DMEM supplemented with 10% (v/v) newborn calf serum (NCS) (Gibco, Paisly, UK). 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 obtained from Sigma-Aldrich (St. Louis, MO, USA), except serum (Gibco, Paisly, UK). 96-well plates were from Corning Costar (Sigma-Aldrich, Brøndby, Denmark).

Cell viability

Cell viability assessment was performed on cell monolayers grown to ~90% confluence in 96-well plates by using the MTS/PMS assay as previously described[40]. Briefly, the adhered cells were washed with 37 °C Hanks' balanced salt solution (HBSS from Sigma-Aldrich, St. Louis, MO, USA) containing 10 mM HEPES (AppliChem, Darmstadt, Germany), pH 7.4, and exposed at 37°C to 100 µL of peptidomimetic dissolved in the medium used for culturing of each cell line (at concentrations in the range 0−1000 µM) for 1 h. After exposure the cells were washed twice with 37 °C HBSS containing 10 mM HEPES (pH 7.4), and then 100 µL of an MTS/PMS solution, consisting of 240 µg/mL MTS (Promega, Madison, WI, USA) and 2.4 µg/mL PMS (Sigma Aldrich, Buchs, Switzerland) in HBSS, were added to the cells, which then were incubated for 1 h at 37 °C with horizontal shaking (50 rpm) protected from light. A POLARstar OPTIMA plate reader (BMG Labtech, Offenburg, Germany) was used to measure the absorbance at 492 nm. The relative viability was calculated by using 0.2% (w/v) sodium dodecyl sulfate (SDS) as a positive control, while cells exposed to medium without test compound were used as a negative control. Data were obtained in two independent biological replicates performed on separate passages of cells and on separate days with a total number of six replicates.

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Keywords: Antimicrobial, peptidomimetics, structure-activity relationships, fluorination, hydrophobicity

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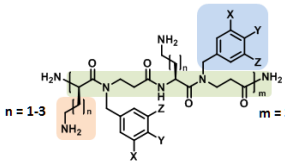
Entry for the Table of Contents

Library design:

- Length
- Hydrophobicity
- Fluorination
- Lys homologues
- Charge distribution



X,Y,Z = F

X,Z = H and Y = F, CH_3, or CF_3

βNPhe (*i.e.* X,Y,Z = H) → βNCyHex

From a structure-activity relationship study comprising an array of α-peptide/β-peptoid hybrids with an alternating cationic-hydrophobic design, displaying variation in length and side chains, it was found that fluorination of the hydrophobic residues led to increased antibacterial activity. For the longer oligomers a concomitantly increased cytoxicity toward human cells were observed, while some 12-mers exhibited an acceptable activity profile.