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Short Antibacterial Peptides with Significantly Reduced Hemolytic Activity can be Identified by a Systematic L-to-D Exchange Scan of their Amino Acid Residues

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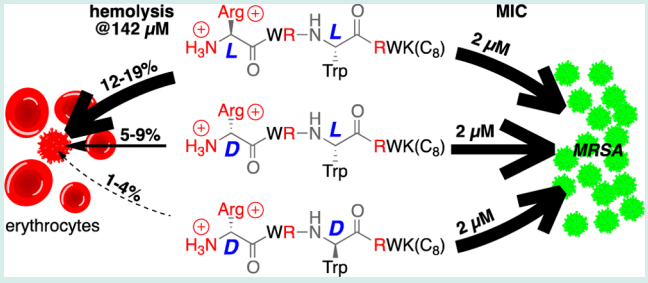
Supporting Information

ABSTRACT: High systemic toxicity of antimicrobial peptides (AMPs) limits their clinical application to the treatment of topical infections; in parenteral systemic application of AMPs the problem of hemolysis is one of the first to be tackled. We now show that the selectivity of lipidated short synthetic AMPs can be optimized substantially by reducing their hemolytic activity without affecting their activity against methicillin resistant *Staphylococcus aureus* (MRSA). In order to identify the optimized peptides, two sets of 32 diastereomeric H-^{D}Arg-WRWRW-^{L}Lys(C(O)C$_n$H$_{2n+1}$)-NH$_2$ (n = 7 or 9) peptides were prepared using a split−split procedure to perform a systematic L-to-D exchange scan on the central WRWRW-fragment. Compared to the all-L C$_8$-lipidated lead sequence, diastereomeric peptides had very similar antibacterial properties, but were over 30 times less hemolytic. We show that the observed hemolysis and antibacterial activity is affected by both differences in lipophilicity of the different peptides and specific combinations of L- and D-amino acid residues. This study identified several peptides that can be used as tools to precisely unravel the origin of hemolysis and thus help to design even further optimized nontoxic very active short antibacterial peptides.



KEYWORDS: *antibacterial peptides, lipidated peptide, hemolysis, L-to-D substitution scan, MRSA*

1. INTRODUCTION

In search for novel antibacterial agents, which need to be identified urgently in view of the rapid increase in occurrence of resistant pathogenic bacteria, antibacterial peptides are especially interesting.[1−3] As the chemical diversity that can be generated in even a relatively small peptide is already enormous, optimization of lead-sequences is a fundamental part of peptide-based drug-discovery. For example, more than 10^{13} decapeptides can be prepared using the 20 common proteinogenic amino acids, all of which have unique structural and functional properties and some of which should be antibacterial.[4] Multiplying this with a large set of readily available nonproteinogenic amino acids, many of which are available as both enantiomers, the number of peptides that can be prepared and analyzed is practically limitless. Fortunately, screening of the entire available peptidic chemical space for highly selective antibacterial peptides can be guided by optimization of previously identified active compounds using combinatorial methods. In view of the reported high systemic toxicity of antibacterial peptides, which often leads to unsuccessful clinical trials and limits their applications to topical infections,[2] the activity of the peptides has to be made more antibacterial specific.

Many AMPs are presently known, ranging from peptides that originate from natural sources[1] to artificially designed amide-bond containing synthetic compounds.[5] Much insight in the pharmacophore of AMPs has been gained over the years, showing that most active molecules are amphipathic, allowing them to interact with cell membranes. Within the family of synthetic peptides, short amphipathic AMPs containing lipophilic residues like tryptophan (Trp, W), phenylalanine (Phe, F), leucine (Leu, L) and cationic residues like arginine (Arg, R), lysine (Lys, K), and histidine (His, H) have provided active compounds. Beneficial from a pharmaceutical point of view is that small peptides can be produced on a large scale, are easily subjected to chemical tuning of their activity, and are generally only weakly immunogenic.[6] Particularly interesting AMPs are based on arginine and tryptophan residues: a sequence of only a few residues results in peptides with activity against Gram-positive bacteria.[7] Importantly, the intermediate activity of such short AMPs against Gram-negative bacteria was significantly increased by lipidation, even though this rendered them toxic against human cells.[8]

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Apart from enhancing the activity of AMPs by lipidation,[8,9] other methods comprise their conjugation with targeting[10] or organometallic moieties,[11] the preparation of multi/oligovalent constructs that simultaneously present several antibacterial peptides to the pathogen,[12] stabilization of the active conformation by biomimetic strategies,[13] or substituting single-atoms that have been shown to cause resistance.[14] Especially conjugation to organometallic fragments is a promising new strategy to modulate peptides.[15] Concerning ferrocenoyl-derivatized AMPs, it was shown that this activity was mostly caused by the lipophilicity of the organometallic fragment. Thus, lead-optimization studies can be performed using their cheaper lipidated counterparts. For this optimization, we used a combinatorial L-to-D substitution scan on the middle-section of the peptide (Figure 1), a strategy that has been successfully applied to improve the pharmacokinetic and -dynamic properties of therapeutic peptides. However, whereas an L-to-D substitution scan in the usual 15−50 amino acid long naturally occurring AMPs can rapidly become a laborious exercise, allowing only a few strategically placed rationally designed alterations, this is not the case for short AMPs. Nevertheless, inspiration for the current study is derived from longer structured peptides.

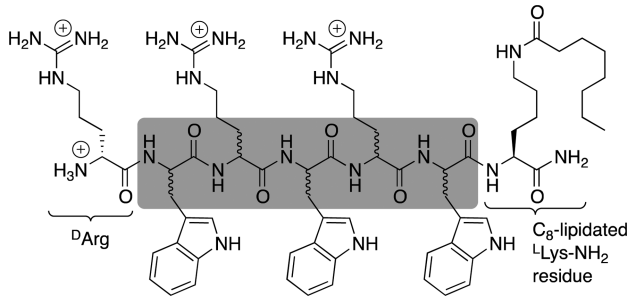

Figure 1. Chemical structure of the C_8-lipidated peptide for which an L-to-D exchange of the central five amino acid residues (highlighted by the gray box) was performed.

Specifically, for α-helical peptides it has been observed that the secondary structure depends on the environment,[16] which influences not only protein−protein interactions[17] but also the interaction between an α-helix and membrane.[16b,18] In case of membrane-targeting α-helical peptides an L-to-D substitution of only one or a few amino acid residues already disturbs the α-helical structure, resulting in a change in interface localization of the peptides.[19] Consequently, differences in activity against bacteria and erythrocytes can be measured.[20] For structurally ill-defined short AMPs,[21] such dependence has not been probed for and is much less likely. Despite this, we now show that it is possible to optimize the specificity of structurally ill-defined AMPs: an L-to-D exchange scan of amino acid residues of short lipidated AMPs can lead to peptides with an activity that is pronounced antibacterial and hardly hemolytic. Such findings are conveniently identified using combinatorial techniques.

The effect of an L-to-D exchange on the antibacterial and hemolytic properties of 64 diastereomeric lipidated peptides was determined. These peptides consist of two groups of 32 peptides each: one group was lipidated with octanoic acid (a C_8-tail, Table 1) and the other with decanoic acid (a C_{10}-tail, Table S1 in the Supporting Information). In the H-^{D}Arg-$^{L/D}$Trp-$^{L/D}$Arg-$^{L/D}$Trp-$^{L/D}$Arg-$^{L/D}$Trp-^{L}Lys(lipid)-NH_2 sequence, the five central amino acid residues are either L- or D-amino acid residues (Figure 1). Furthermore, an N-terminal D-arginine residue was incorporated to generate peptides that would resist proteolytic degradation of the peptides by means of aminopeptidases.[22]

2. RESULTS

2.1. General Considerations. In the discussion below the identity of the peptides is referred to using the length of the lipid-tail, that is, C_8 or C_{10}, and the enantiomer of the amino acid residues of the central WRWRW-sequence, that is, a row of five letters "L" or "D". It should be remembered, however, that the entire peptide has the sequence H-^{D}R-WRWRW-^{L}K(C(O)-C_nH_{2n+1})-NH_2. For example, C_8-LLDLD refers to the peptide with the sequence H-^{D}Arg-^{L}Trp-^{L}Arg-^{D}Trp-^{L}Arg-^{D}Trp-^{L}Lys(C-(O)C_7H_{15})-NH_2. The all-L peptides are composed of only L-amino acid residues and differ from the LLLLL peptides since the latter contains a D-arginine as N-terminal residue.

2.2. Antibacterial Activity. In this study, we set out to improve the specificity of lipidated short AMPs. For this, the previously determined high activity of the lead-sequence should not be altered to any significant extent. Fortunately, all peptides are comparable in activity against the Gram-positive bacteria, leaving room for an optimization of other pharmaceutically relevant properties. This was especially clear for activity against *B. subtilis*, where almost no differences were observed between the diastereomeric C_8- and C_{10}-lipidated peptides, but also for activity against the MRSA strain that was used in the panel. Concerning the activities of the diastereomeric C_8-lipidated peptides against Gram-negative pathogens, derivatives that were similarly or less active than the all-L lead peptide are almost exclusively obtained. One notable exception was a peptide that was twice as active against *P. aeruginosa* than any of the other peptides. Whereas the all-L C_8-lipidated peptide had an MIC-value of 18−36 μM the C_8-LLDDD peptide had an MIC-value of 4.6 μM; for comparison, the orally administered ciprofloxacin has a similar MIC-value of 2 μg/mL (5.4 μM) under the same conditions and using the same strain. Whereas the introduction of one N-terminal D-arginine residue as present in the C_8-LLLLL peptide is already beneficial for the activity, resulting in an MIC-value of 9−18 μM, an optimal activity is seen when the peptide has four subsequent D-amino acid residues at its N-terminus. Thus, this could be an improved sequence for the treatment of *P. aeruginosa* infections, which are often found in cystic fibrosis patients.[23] In combination with both the C_8-LLLDD and the C_8-LDDDD peptide, which have a lower activity, this peptide forms an interesting tool to unravel the underlying origin of this difference. Interestingly, the similar C_{10}-lipidated LLDDD-peptide had an MIC-value of 18 μM, which indicates that for this pathogen the length of the lipid-tail is not the main determinant for the activity.

Comparison of the activity of the peptides that differ only in the length of their lipid tail (Table 1 vs Table S1 in the Supporting Information) shows that for almost all bacteria the MIC-values are equal to 4 times lower for the C_{10}-lipidated peptides when compared to the C_8-lipidated versions. The overall higher activity of the C_{10}-lipidated peptides could be associated with a more efficient uptake, but could similarly indicate that the more lipophilic C_{10}-tail is a more suitable anchor for the bacterial membrane.

The depth of tuning that can be obtained by performing an L-to-D substitution scan is further illustrated by the activity of the diastereomeric C_{10}-lipidated peptides against Gram-

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Table 1. Antibacterial Activity (MIC-Values, Given in μM), Hemolytic Activity (As %-Hemolysis of Samples Treated with 142 μM (250 μg/mL) of Peptide), and Retention Times of the C_8-Lipidated L-to-D-Substituted H-^{D}R-WRWRW-^{L}K(C_8)-NH_2 Peptides[a]

		Gram-negative			Gram-positive				
		E. coli	*A. baumannii*	*P. aeruginosa*	*S. aureus*	*S. aureus* (MRSA)	*B. subtilis*		
entry	chirality of the amino acid residues in the ^{N}WRWRW^{C}-section	MIC	MIC	MIC	MIC	MIC	MIC	hemolysis (in %) with 142 μM peptide	retention time (min)[c]
0	*all-L*[b]	*2–5*	*5–9*	*18–37*	*2–5*	*2*	*0.6–1.1*	*15.6 ± 3.2*	*19.1*
1	LLLLL	4.6–9.1	18–36	9.1–18	4.6	2.3	1.1–2.3	6.9 ± 1.8	19.1
2	LLLLD	9.1–18	18–36	9.1	9.1	2.3	1.1	6.0 ± 0.5	19.2
3	LLLDL	36	9.1–18	18–36	4.6	2.3	1.1	2.7 ± 0.1	18.8
4	LLDLL	18	9.1–18	18	9.1	2.3	1.1	2.7 ± 1.4	18.6
5	LDLLL	9.1–18	9.1–18	18–36	4.6	2.3–4.6	1.1	4.2 ± 0.6	18.8
6	DLLLL	9.1–18	9.1–18	18–36	4.6	2.3	1.1	4.2 ± 0.8	18.8
7	LLLDD	4.6–9.1	9.1–18	18	4.6	2.3	0.6–1.1	5.1 ± 2.3	19.1
8	LLDLD	36	18–36	36	4.6	2.3	0.6–1.1	1.3 ± 0.2	18.7
9	LDLLD	18–36	18–36	36–73	9.1	2.3	1.1	0 – 2.9	19.0
10	DLLLD	18	36	18	9.1	2.3	1.1	2.4 ± 0.8	19.2
11	LLDDL	9.1	18	18	4.6	2.3	1.1–2.3	3.7 ± 1.7	18.9
12	LDLDL	36	18–36	36–73	9.1	4.6–9.1	1.1	0 – 4.1	18.8
13	DLLDL	n.a.	18	36	9.1	4.6–9.1	1.1	3.7 ± 0.3	18.8
14	LDDLL	36	18–36	73	9.1	2.3–4.6	1.1	0.5 ± 0.5	18.6
15	DLDLL	36	18	36–73	9.1	4.6	1.1	1.6 ± 0.6	18.4
16	DDLLL	9.1–18	18	18	4.6	2.3	0.6–1.1	4.1 ± 0.9	19.1
17	LLDDD	9.1	18–36	4.6	4.6	1.1–2.3	1.1–2.3	6.2 ± 0.4	19.4
18	LDLDD	36	18	73	4.6	2.3	1.1	0–0.9[d]	18.6
19	DLLDD	18–36	36	73	4.6	2.3–4.6	1.1	0.5 ± 0.5	18.8
20	LDDLD	73	18–36	73	9.1	2.3–4.6	1.1	0.8 ± 0.1	18.8
21	DLDLD	n.a.	n.a.	n.a.	9.1	4.6	1.1	0–0.6[d]	18.9
22	DDLLD	18	18	18	9.1	2.3	0.6–1.1	8.0[e]	19.3
23	LDDDL	36	9.1–18	73	9.1	2.3	1.1	3.3 ± 0.3	18.8
24	DLDDL	36	18–36	36–73	4.6	2.3	1.1–2.3	1.0 ± 0.7	18.7
25	DDLDL	36	18	36	4.6	2.3	1.1–2.3	3.5 ± 1.2	18.8
26	DDDLL	18–36	18	18	4.6	2.3	1.1	5.3 ± 0.3	19.2
27	LDDDD	18	18–36	18–36	9.1	2.3	1.1	1.3 ± 0.2	18.7
28	DLDDD	9.1–18	36	36–73	4.6	2.3	1.1	0–1.4[d]	19.1
29	DDLDD	18	18	36–73	2.3	2.3	1.1	3.1 ± 1.1	18.9
30	DDDLD	36	18	36–73	9.1	2.3	1.1	0–3.1[d]	18.8
31	DDDDL	9.1	18	9.1–18	9.1	2.3	1.1	8.5 ± 3.8	19.1
32	DDDDD	9.1	18–36	9.1	4.6	2.3	1.1	15 ± 2.2	19.5

[a]K = lysine; W = L- or D-tryptophan; R = L- or D-arginine; n.a. indicates inactive peptides, which means MIC-values exceeded 73 μM. MIC stands for minimal inhibitory concentration, which is the lowest concentration of peptide at which bacterial growth is inhibited; these values are given with one digit when lower than 10 and as an integer when higher than 10. [b]These values were taken from ref 8, values in that paper were obtained using identical standard protocols and hRBCs from the same person. The all-L peptide is composed of only L-amino acid residues and differs from the LLLLL peptide since the latter contains a D-arginine as N-terminal residue. [c]The retention time of the peptides is determined using a reversed phase C_{18} column and benzyl alcohol as internal standard. [d]A range is given since the standard deviation was larger than the average value, which could suggest that less than 0% hemolysis would be possible. [e]Single data point.

negative bacteria (Table S1, Supporting Information). Whereas the all-L-C_{10} peptide published previously, with the sequence H-RWRWRW-K(C(O)C_9H_{19})-NH_2, had an MIC of 4–9 μM against *A. baumannii*, the C_{10}-DDDDD peptide is significantly less active with an MIC of 36–72 μM. Fortunately, at least six peptides with different combinations of L- and D-residues have MIC-values that were similar to that of the all-L peptide, that is, peptides with C_{10}-DLLDL, C_{10}-LDDLL, C_{10}-DLDLL, C_{10}-DLLDD, C_{10}-DLDLD, C_{10}-DLDDL, and C_{10}-DDDLD residues in the WRWRW-sequence (entries 13, 14, 15, 19, 21, 24, and 30 in Table S1 of the Supporting Information).

2.3. Hemolytic Activity. The effect of an L-to-D substitution scan on the hemolytic activity was determined next, using a concentration of the peptides that was significantly higher than the MIC-values against Gram-positive bacteria, that is, 142 μM for the C_8-lipidated peptides or 140 μM for the C_{10}-lipidated peptides *vs* 0.6–9.1 μM, which corresponds to approximately 230–15 times higher amounts than are needed to inhibit bacterial growth. What immediately became clear is a pronounced effect of different combinations of L- and D-amino acid residues on the hemolytic activity (Chart 1). The difference between the most and least hemolytic diastereomeric peptides is around 30-fold for the C_8-lipidated peptides, that is, DDDDD versus DLDLD (entries 32 and 21, Table 1), and about a factor of 10 for the C_{10}-lipidated peptides, that is, DDDDD versus DLDLD (entries 32 and 21, Table S1, Supporting Information). As could be expected, the hemolytic activities of the C_8- and C_{10}-lipidated peptides roughly follow

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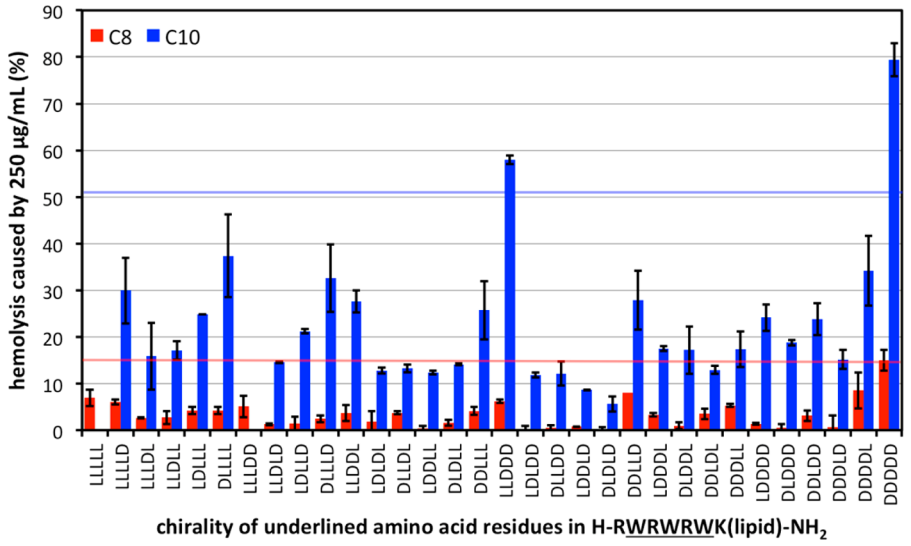
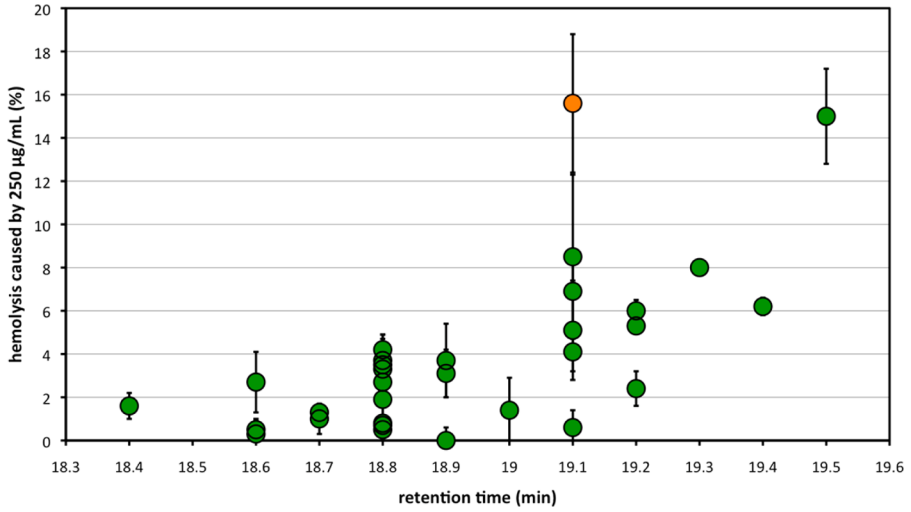
Chart 1. Hemolytic Activities Observed for the Different Diastereomeric Lipidated Peptides[a]



[a]Percentage hemolysis is obtained from duplicate experiments and the average is given for samples treated with 250 μg/mL (142 μM for C_8 and 140 μM for C_{10}) of lipidated peptide. Values for C_{10}-LLLLL and C_{10}-LLLDD could not be obtained due to insufficient amounts of compound. The red and blue horizontal lines indicate the percentage of hemolysis caused by the all-L C_8 or all-L C_{10} reference peptides (see section 3.1 for details), respectively.[8]

Chart 2. Retention Time of the 32 C_8-Lipidated Peptides versus the Percentage of Hemolysis of Human Red Blood Cells That Is Caused by 142 μM of the Peptide[a]



[a]The orange circle at 19.1 min with ~16% hemolysis represents the all-L-C_8 peptide.[8].

each other in a parallel fashion and the same combination of L- and D-amino acid residues results in the two peptides that are most and least hemolytic (Chart 1). Clearly, a screening of hemolytic activity of diastereomeric antibacterial lipopeptides can lead to multiple peptides with significantly reduced hemolytic activity.

2.4. Hemolytic Activity versus Lipophilicity of the Different Diastereomeric C_8-Lipidated Peptides. Previously, a higher lipophilicity, as inferred from longer retention times on a reversed-phase C_{18} column, was related to increased hemolysis.[8] In the present case, different diastereomeric H-(Arg-Trp)$_3$-Lys(C_8)-NH_2 peptides displayed different retention times (Table 1). To be more specific, a difference of 1.1 min was observed between the least and most strongly retained peptide, i.e. between the C_8-DDDDD peptide with t_R = 19.5 min and the C_8-LDLDL peptide with a t_R = 18.4 min; for the C_{10}-lipidated peptides, a similar trend was observed (Table S1, Supporting Information). This retention time of the peptides on a C_{18}-column was taken as a measure for the apparent lipophilicity and plotted against the hemolytic activity (Chart 2).

A plot of the retention time of the peptides against the observed hemolysis clearly shows the general trend that a higher lipophilicity causes more hemolysis is maintained. However, some notable exceptions are also identified. Specifically, most of the peptides cause less than 5% hemolysis even though their retention times vary within a range of 18.4–19.2 min. Alternatively, a set of six peptides with t_R = 19.1 min is found to have a hemolytic potential that ranges from 1% to 15% hemolysis.

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Within this particular set of peptides with t_R = 19.1 min two diastereomeric peptides likely form useful tools to study the underlying mechanism of hemolysis and determine how differences in primary structure are related to hemolysis. Both C_8-DDDDL and C_8-DLDDD have one particular L-amino acid residue in the central WRWRW-sequence, only at different positions. Apparently, shifting the L-configuration from the tryptophan residue that is closest to the N-terminus to the arginine residue that is closest to the lipidated lysine residue does not affect lipophilicity but decreases hemolysis from 4.7−12.3% to 0−1.4%, respectively. Clearly, certain combinations of L- and D-amino acid residues can suppress hemolysis while their antibacterial activity is hardly affected. This indicates that hemolysis can be reduced by taking advantage of the differences in membrane architecture and components between erythrocytes and bacteria.

3. EXPERIMENTAL PROCEDURES

3.1. Synthesis of the L-to-D Substituted Peptides. All peptides were prepared manually by means of a split-couple-split strategy. For this, Fmoc-^{L}Lys(Mtt)-OH was coupled to 1.2 g of ChemMatrix-Rink resin (loading = 0.6 mmol/g) using TBTU, HOBt, and D*i*PEA in DMF (15 mL for 3 h). After removal of the Fmoc-group using 20% piperidine in DMF (2 × 20 mL, each 10 min), followed by washing with DMF (5 × 20 mL, each 2 min), the amount of resin was split in two portions. Using the above-mentioned coupling reagents in half the volume of DMF (7.5 mL), Fmoc-^{L}Trp(Boc)-OH was coupled to one batch and Fmoc-^{D}Trp(Boc)-OH to the other batch. Again, after removal of the Fmoc-group, and washing of the resin, each batch was split in two equal portions to which either Fmoc-^{D}Arg(Pbf)−OH or Fmoc-^{L}Arg(Pbf)-OH were coupled. This process was repeated until the last tryptophan residue was attached. After coupling of Fmoc-^{D}Arg(Pbf)-OH to the terminal amino-group of the tryptophan residues of all the 32 batches, each of them was washed with DMF and DCM. Removal of the Mtt-group from the amino-group on the side-chain of the C-terminal lysine residue was achieved by repeated treatments of each batch with 2% TFA and 5% TIS in DCM. After washing with DCM and Et_2O the resin was dried under vacuum and the dry pellet was split into two portions. Of all peptides one portion was reacted with octanoic acid (C_8) and the other portion with decanoic acid (C_{10}) using TBTU, HOBt, and D*i*PEA in NMP as coupling reagents. Finally, after removal of the N-terminal Fmoc-group on the arginine residue, each of the 64 peptides was cleaved from the resin using TFA/TIS/water −92.5/5/2.5 (%, v/v/v). Precipitation of the cleaved peptides in cold (−20 °C) Et_2O/*n*-hexane −1/1 (%, v/v) and purification by semipreparative HPLC on a C_{18}-column afforded 64 peptides in high purity (>95%). A representative set of C_8-lipidated and C_{10}-lipidated peptides has been analyzed by HPLC and HR-MS (see Supporting Information).

3.2. Minimal Inhibitory Concentration. Corresponding MICs were tested against *Escherichia coli* DSM 30083, *Acinetobacter baumannii* DSM 30007, *Pseudomonas aeruginosa* DSM 50071, *Bacillus subtilis* DSM 402, *Staphylococcus aureus* DSM 20231 (type strain), and *Staphylococcus aereus* ATCC 43300 (MRSA) in a microtiter plate assay according to CLSI guidelines. *E. coli*, *A. baumannii*, *S. aureus*, and *B. subtilis* were grown in Mueller Hinton broth, *P. aeruginosa* in cation-adjusted Mueller Hinton II. Peptides were applied as dissolved in DMSO. Serial dilution in culture media was carried out automatically with the Tecan Freedom Evo 75 liquid handling workstation (Tecan, Männedorf, Switzerland), and typically covered a range from 128 or 64 μg/mL to 0.5, 0.125, or 0.0625 μg/mL, depending on the availability of the peptide. Dilutions were inoculated with 10^5 bacteria/mL taken from late exponential cultures grown in the same media in a total volume of 200 μL per well. Cells were incubated for 16 h at 37 °C, lowest peptide concentration inhibiting visible bacterial growth was taken as MIC.

3.3. Hemolysis. Was performed as described previously.[8]

3.4. Apparent Lipophilicity of the C_8-Lipidated Peptides. A reversed-phase C_{18}-column was used in combination with HPLC-buffers consisting of buffer A: water/acetonitrile/TFA − 95/5/0.1 (%, v/v/v), and buffer B: acetonitrile/water/TFA − 95/5/0.1 (%, v/v/v). After 5 min of buffer A (1 mL/min), a gradient was applied that replaces 100% A with 100% B in 20 min (correlating to 5%/min). Samples were dissolved in DMSO and eluted compounds were detected by the absorption of two wavelengths, that is, 214 and 254 nm. Analysis of the lipidated peptides was performed in one consecutive session; retention times were determined in the presence of benzyl alcohol as internal standard.

4. DISCUSSION

In this paper, we give a detailed account of the effect of the inversion of α-carbon atom of amino acid residues in two closely related groups of lipidated arginine and tryptophan containing short AMPs on their antibacterial and hemolytic properties. By preparing and analyzing two sets of 32 diastereomeric lipidated peptides we determined the extent in which the spectrum of activity of these peptides could be optimized. Although such a tuning of antibacterial versus hemolytic properties has been carried out for longer α-helical AMPs,[18] a detailed and systematic study on a comprehensive series of diastereomeric short lipidated AMPs was not yet performed. Even more, the effect of differences in the spatial orientation by which the amino acid side chains "branch off" from the peptide backbone was expected to be much less drastic for such short AMPs than known for AMPs that have a defined secondary structure under biologically relevant conditions. That the specificity of structurally ill-defined lipidated short AMPs could be optimized to the same level as was shown for α-helical antibacterial peptides was unexpected.

Compared to the all-L containing AMP lead-sequence, an L-to-D substitution scan leads to many peptides that have antibacterial activities in the same range as the lead-sequence. This is especially clear when the pathogenic Gram-positive bacteria are considered, but also emerges from the activity against Gram-negative bacteria, although to a lesser extent. Interestingly, a peptide with the sequence H-^{D}Arg-^{L}Trp-^{L}Arg-^{D}Trp-^{D}Arg-^{D}Trp-^{L}Lys(C(O)C_7H_{15})-NH_2 was identified to be very active against *P. aeruginosa*, having an MIC-value of 4.6 μM; it was even more active than its C_{10}-lipidated version. This was especially surprising in view of the usually higher activity of C_{10}-lipidated peptides over their C_8-lipidated counterparts. Since new antibacterial agents against *P. aeruginosa* are urgently needed in view of the infections this pathogen causes in the later stages of cystic fibrosis, this is a promising lead-sequence for further studies.

Whereas antibacterial activity was mostly maintained over the set of L-to-D substituted peptides, almost all diastereomeric AMPs had significantly reduced hemolytic activity. Even though the C_{10}-lipidated all-L peptide was 3.5 times more hemolytic than its C_8-lipidated counterpart, about one-fourth of the C_{10}-

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lipidated diastereomeric peptides was equally or less hemolytic than the most hemolytic C_8-lipidated peptides (Chart 1). Within the set of C_8-lipidated peptides, a 30-fold difference between most and least hemolytic was determined; for the C_{10}-lipidated peptides a 10-fold improvement was obtained. To assess if the origin of the observed differences in hemolytic properties was related to differences in lipophilicity between the diastereomeric AMPs, we compared the apparent lipophilicity of the C_8-lipidated peptides using their retention times on a reversed-phase (C_{18}) HPLC column (Table 1). This comparison confirmed the general trend that more lipophilic peptides are also more hemolytic, but also showed that underlying combinations of L- and D-amino acid residues are at least equally important (Chart 2). Two areas underscore this point: several peptides (i.e., DDDLD, DLDDD, DDLLL, LLLDD, and LLLLL) with similar retention times (t_R = 19.1 min) have very different hemolytic potential (1−15%) and several peptides with very similar hemolytic potential (∼2%) have very different retention times (t_R = 18.4−19.0 min). Also, at least five peptides have similar retention times and cause a similar level of hemolysis, even though they have several amino acid residues with different chirality, that is, LDDDL, LDLLL, DLLLL, DLLDL, and DDLDL.

To assess if the reduced hemolysis is due to a generally weaker affinity for membranes, we compared the percentage of hemolysis caused by 142 μM with the activity against the three Gram-negative bacteria of the test-panel (see Supporting Information for the graphical representations). It should be noted that even though the retention times of this closely related set of compounds on a C_{18}-column could be taken as a rough estimate of the general affinity of these compounds for a membrane, such a suggestion is best validated by correlating two activity parameters. If the differences in activity would be due to generally weaker affinity for a membrane, there should be a linear correlation between hemolysis and MIC-values against several bacteria. Analysis of our data set at best shows a rough correlation between the antibacterial activity and hemolysis; notable exceptions to a linear correlation are clearly present. Specifically, the correlation chart for hemolysis and *P. aeruginosa* identifies 11 peptides with MIC-values of 18.2 μM but that cover a range of hemolysis percentage of 2.4 ± 0.8 to 8.5 ± 3.8. Against *E. coli* a similar pattern can be seen, with 8 peptides that have MIC-values of 9.1 μM and cause a percentage of hemolysis that ranges from 3.7 ± 1.7 to 15.0 ± 2.2, and also 8 peptides with MIC-values of 18.2 μM and hemolysis that ranges from 0−1.4% to 8.0%. Against *A. baumannii* the absence of a direct correlation between hemolysis and MIC-values is even less clear, with 15 peptides that have an MIC-value of 18.2 μM and cause 0−0.9 to 8.5 ± 3.8 percentage hemolysis. Thus, even though the correlation between high hemolysis and high antibacterial activity roughly applies, clear exceptions are found, strongly suggesting that factors other than general weaker membrane binding are also at play. This finding will be useful for lead-optimization strategies for short structurally ill-defined (lipidated) AMPs.

Lastly, one of the most striking findings of this study is that the all-L peptides are among the most hemolytic compounds. Since these all-L peptides usually form the starting point in a study that attempts to uncover new antibacterial peptides, it is very likely that the specificity of a short lipidated AMP with promising antibacterial activity can be further optimized by reducing its hemolytic properties substantially. Although it is too early to explain the findings of this study in great detail, the following established facts might be involved: tryptophan indole rings play an important role in carbohydrate recognition,[24] they are known to function as membrane-anchors for transmembrane proteins,[25] and that the guanidine-group of the arginine residue favorably interacts with phosphate-esters.[26] Both eukaryotic cells and erythrocytes are composed of phospholipid-based membranes that are richly decorated with functional groups that can interact with the functional groups that are present in the peptides. Different combinations of L- and D-amino acid residues result in different spatial arrangements of the functionalities that can participate in such interactions.[27]

5. CONCLUSIONS

A detailed study on the effect of an L-to-D exchange on the antibacterial and hemolytic activity of short lipidated antibacterial peptides is presented. This study clearly shows that a systematic L-to-D substitution scan of amino acid residues in a short lipidated AMP leads to diastereomeric peptides that have an optimized spectrum of activity. A convenient and reliable solid-phase based split-and-split procedure was applied to two sets of 32 stereoisomeric peptides with the sequence H-^{D}Arg-$^{L/D}$Trp-$^{L/D}$Arg-$^{L/D}$Trp-$^{L/D}$Arg-$^{L/D}$Trp-^{L}Lys(lipid)-NH_2 (lipid = caprylic (C_8) or capric acid (C_{10})). Antibacterial properties against Gram-positive bacteria were fully retained, against Gram-negative bacteria the activity was mostly retained. For the C_{10}-lipidated peptides a trend very similar to the C_8-lipidated peptides was observed (see Table 1 in the Supporting Information). Fortunately, a significant effect was observed with respect to the hemolytic properties of these peptides. In fact, under similar conditions the all-L C_8-lipidated peptide (i.e., H-$(RW)_3K(C(O)C_7H_{15})$ displayed 15.6 ± 3.2% hemolysis whereas all-but-one stereoisomers of this peptide were significantly less hemolytic. At least seven C_8-lipidated peptides, that is, LLDLD, LDDLL, LDLDD, DLDDL, LDDDD, DLDDD, and DDDLD, were almost not hemolytic and still possessed strong activity against Gram-positive bacteria. This significantly increases the potential therapeutic window of these compounds. In view of the mentioned two-dimensional optimization of activity we were able to significantly reduce their activity in one dimension, i.e. against erythrocytes, and were able to maintain their activity in the other dimension, that is toward prokaryotic organisms. We show that both the apparent lipophilicity of the peptide and a specific combination of L- and D-amino acid residues are responsible for the observed hemolysis, indicating that spatial arrangements of functional groups help to orchestrate the interaction with erythrocytes on an atomic level and contribute to the hemolytic potential of lipidated short AMPs. For example, the C_8-DDLDD and C_8-DDDDL peptide both have one L-tryptophan residue in their sequence have quite similar retention times and antibacterial activities, but still cause a significantly different percentage of hemolysis, that is, 2−4.2% and 4.7−12.3%, respectively. This paper clearly shows that a relatively convenient and labor-friendly strategy can help to identify short lipidated antibacterial peptides that are very active against many pathogenic bacteria and are hardly hemolytic, suggesting these and similar peptides can ultimately be used in systemic applications in a clinical setting.

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■ ASSOCIATED CONTENT

Supporting Information

Antibacterial and hemolytic activity of the 32 C_{10}-lipidated diastereomeric peptides, three charts that compare MIC-values against the three Gram-negative pathogens in the panel with the percentage hemolysis caused by 142 μM of the peptides, and HPLC and HR-MS analysis of a representative sample of the peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AMP, antimicrobial peptide; Arg (or R), arginine residue; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; D*i*PEA, *N*,*N*-di-*iso*-propylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI-MS, electrospray ionization mass spectrometry; Et_2O, diethylether; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; hRBCs, human red blood cells; HR-MS, high-resolution mass spectrometry; Lys (or K), lysine residue; MIC, minimal inhibitory concentration; MRSA, methicillin resistant *S. aureus*; Mtt, 4-methyltrityl; NMP, *N*-methyl-2-pyrrolidone; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PBS, phosphate buffered saline; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TIS, triisopropylsilane; Trp (or W), tryptophan residue

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