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COMMUNICATION

Selectively Targeting Bacteria by Tuning the Molecular Design of Membrane-Active Peptidomimetic Amphiphiles

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Here we report the design of membrane-active peptidomimetic molecules with tunable arrangement of hydrophobic and polar groups. Inspite of the same chemical composition, the effective hydrophobicities of the compounds were different as a consequence of their chemical structure and conformational properties. The compound with a lower effective hydrophobicity revealed highly selective antibacterial activity over mammalian cells. This study, highlighting the role in membrane selectivity of the specific arrangement of the different moieties in the molecular structure, provides useful indications for developing non-toxic antibacterial agents.

The rapid emergence of drug-resistant bacteria, which are increasingly becoming difficult to treat with conventional antibiotic therapy, is a global health concern.[1] The alarm has already been set by various organizations, including the World Health Organization (WHO),[1b] calling for urgent development of new classes of antibacterial agents to combat such dangerous bacteria. To provide a possible solution to this challenging problem, membrane targeting antimicrobial peptidomimetics have been developed as promising class of agents.[2,3] These membrane targeting molecules are advantageous over conventional antibiotics, since development of resistance against them is slow, or even absent. However, in most cases, the toxicity towards mammalian cells limits their translation into the clinic.[3n] Therefore, highly selective antibacterial molecules showcasing specific targeting of bacterial membranes are yet to see the light of day. Various research groups, including ours, have seen that a threshold hydrophobicity is necessary for significant antibacterial activity.[3e] However, an increase in hydrophobic groups usually leads also to significant enhancement of toxicity towards mammalian cells, thereby resulting in toxic antibacterial molecules. On the other hand, efforts dedicated to improve the selectivity towards bacterial cells by reducing the total hydrophobicity usually resulted in a compromised antibacterial activity. Aiming to increase selectivity without impairing antibacterial activity, herein we designed two peptidomimetic compounds (bearing four positive charges), with the same total hydrophobicity, but with a different distribution of polar and apolar moieties (Figure 1). Both compounds contain an aliphatic chain (backbone), two arms, each ending with a lysine and a pendant long aliphatic moiety. While compound **1** has a greater backbone hydrophobicity, contributed by the bis(hexamethylene)triamine (total twelve methylene groups), compound **2** has only six methylene groups in the backbone contributed by bis(trimethylene)triamine. To obtain an equivalent hydrophobicity (and an equal total number of methylene groups) in these two compounds, pendant aliphatic moieties

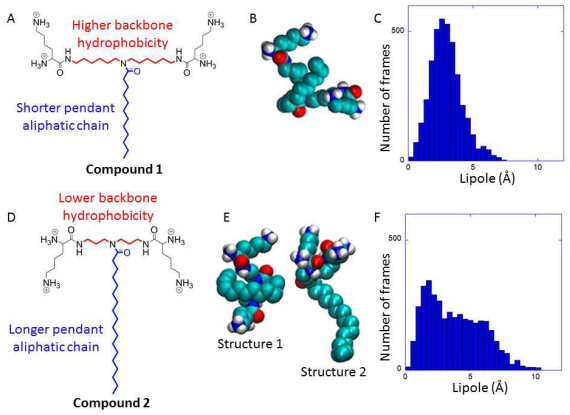

Fig. 1 Chemical structure of the compounds (A and D), the most representative structures of the compounds from MD simulation in water (B and E), the distribution of lipole values attained along the MD trajectories of the same compounds (C and F).

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†Electronic Supplementary Information (ESI) available: The details of synthesis and characterization of the compounds, experimental protocol for bioassays and supplementary figures. See DOI: 10.1039/x0xx00000x

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Table 1 Hydrophobicity, antibacterial and haemolytic activities of compounds.

Antibacterial Agents	logP[a]	RT[b] (min)	MIC (μg/mL) SA[c]	MRSA[d]	EC[e]	PA[f]	HC_{50} (μg/mL)
1	-0.1	12.5	1.6	3.1	3.1	3.1	860
2	-0.1	13.9	3.1	6.3	6.3	6.3	108
DTAB	ND	ND	6.3	50	50	>50	218
BAC-12	ND	ND	1.6	6.3	6.3	12.5	60

[a] water/octanol partition constant. [b] RP-HPLC retention time. [c] *S. aureus*. [d] methicillin-resistant *S. aureus*. [e] *E. coli*. [f] *P. aeruginosa*. ND stands for not determined and the results are compared with known membrane active compounds such as DTAB and BAC-12

with different chain lengths were attached to the middle nitrogen of the molecular design. Thus, a dodecanoyl chain was attached to **1**, whereas a longer octadecanoyl chain was included in **2**. The details of synthesis and characterization of the compounds are provided in the supporting information (SI) section. As a measure of total hydrophobicity of the

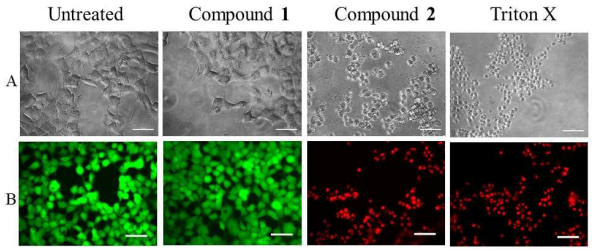

Fig. 2 Images of HEK cells treated with compound 1 and 2 (40 μg/mL each). (A) Bright field images. (B) Fluorescence microscopy images (merged). Scale bar: 50 μm.

compounds (based on the structural formula only), the water/octanol partitionconstant (logP) was determined, by using the ALOGPS server, and was found to be identical for both the compounds, with a value of -0.1 (Table 1). In spite of this similarity, the different RT values (12.5 min for compound **1** and 13.9 min for compound **2**) observed for the compounds suggested that the effective hydrophobicity differed significantly (Table 1). The different effective hydrophobicity of the two molecules was confirmed also by their aggregation properties: a cricical aggregation concentration of 44 μg/mL and 23 μg/mL was determined for compound **1** and **2**, respectively, by light scattering experiments. To explain this behavior, we performed molecular dynamics (MD) simulations by monitoring the conformations of the compounds in water. Figure 1C and 1F report the distribution of lipole values attained along the trajectories of the compounds. The lipole is a conformation-dependent measure of the spatial distribution of polar and apolar moieties, defined in analogy with the electrostatic dipole.[4] A narrow distribution of lipole values was observed for compound **1**, while compound **2** exhibited a bimodal-like distribution. A clustering analysis of the two trajectories, obtained a single conformational cluster for compound **1**, while two families were identified for compound **2**. The most representative structures of these clusters are shown in Figure 1B and 1E. Compound **1** populated a "folded" conformation, partially hiding the hydrophobic moieties from the water phase. A similar conformation was observed also in the most populated cluster of compound **2**; however, the additional cluster corresponded to structures where the octadecanoyl chain was exposed to water, resulting in higher lipole values. Overall, the simulations indicated that the long lipidated tail of compound **2** causes a higher exposure of the hydrophobic moieties to the water phase, as in this case compact conformations are unfavourable for entropic reasons. The conformational properties of compound **2** result in higher effective hydrophobicity as compared to compound **1**, in spite of the same total hydrophobicity in their chemical structures.

Next, the antibacterial activity of the compounds was investigated by determining minimum inhibitory concentration (MIC) against both Gram-positive and Gram-negative bacteria (Table 1 and Table S1). Compound **1** displayed a slightly superior antibacterial efficacy (MIC range: 1.6-3.1 μg/mL) over **2** (MIC range: 3.1-6.3 μg/mL). More importantly, this was less toxic, and displayed high selectivity towards bacterial over mammalian cells, with a very high HC_{50} value (concentration corresponding to 50% lysis of hRBCs) of 860 μg/mL, thereby significantly outperforming over compound **2**, which displayed a value of 108 μg/mL (similar to toxic antibacterial agents DTAB and BAC-12). Compound **1** also exhibited lower toxicity (higher EC_{50} values) against other mammalian cells (RAW and HEK cell lines) as well (Table S1). The live-dead assay with simultaneous staining using the dyes calcein-AM and propidium iodide (PI) further supported the less toxic nature of compound **1** over **2** (Figure 2 and Figure S7). Mammalian cells (HEK cell line) remained viable upon treatment with compound **1**, whereas the majority of cells were dead, upon exposure to compound **2**. Collectively these results suggest that the

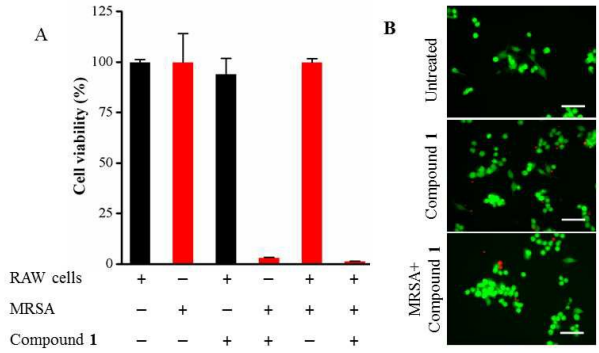

Fig. 3 Antibacterial activity of compound **1** (40 μg/mL) in co-culture with mammalian cells. (A) Cell viability, RAW cells were quantified by MTT assay (black) and MRSA cells were quantified by colony counting (red). (B) Merged fluorescence microscopy images of RAW cells at different conditions showing the effect of compound exposure. Scale bar: 50 μm.

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molecular design with a lower effective hydrophobicity leads to decreased affinity towards mammalian cells without compromising interaction with bacterial cells. The highly selective antibacterial activity of compound **1** was then investigated in co-culture with mammalian cells (RAW cells). The cell viability data clearly demonstrated that compound **1** drastically reduced MRSA cell viability. The MRSA viability was reduced to below 5%, both in the absence and in the presence of RAW cells (red bar, Figure 3A). In contrast, the RAW cell viability of the compound **1** treated sample was similar (94±7.8 % cell viability) to untreated control (black bar, Figure 3A), which was further confirmed by fluorescence microscopy (Figure 3B and Figure S8). Investigation of the mechanism of action of compound **1** suggested that it depolarized the

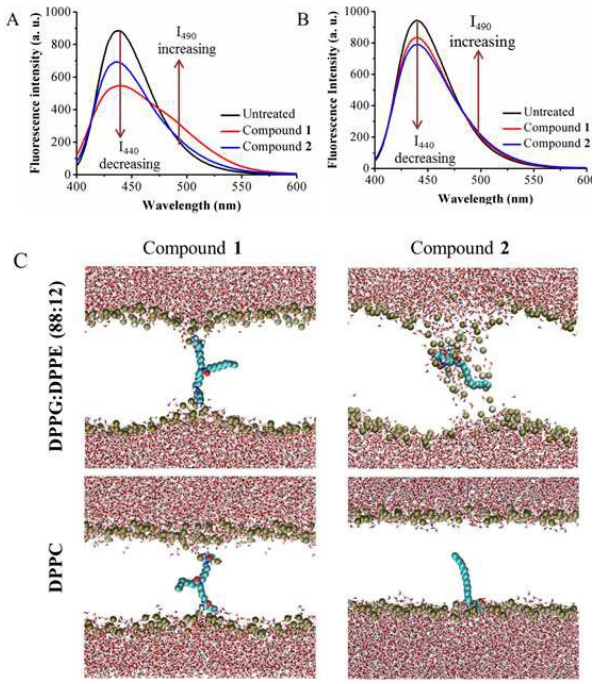

Fig. 4 Biophysical and MD simulation studies. (A) Laurdan fluorescence of DPPG:DPPE (88:12) and (B) DPPC liposome at compound concentration of 100 µg/mL. (C) Final frames of the simulations.

membrane of MRSA even at 2.5 µg/mL and the effect was concentration-dependant (Figure S9).

Selective bacterial membrane targeting was investigated by performing biophysical studies. To mimic the membrane of bacterial cells and mammalian cells, liposomes were prepared using DPPG:DPPE (88:12) and DPPC lipids, respectively. The dye, laurdan (6-Dodecanoyl-2-dimethylaminonaphthalene) was inserted in the lipid bilayers to monitor the effects of the compounds. The fluorescence property of laurdan dye is sensitive towards the dipole moment of its surroundings.[2d] In untreated liposomes, the laurdan emission spectrum was peaked at 440 nm. After compound treatment, the intensity at this wavelength decreases, followed by an increase at 490 nm. This finding indicates penetration of water molecules below the lipid head groups, where the probe is located, due to bilayer perturbation by the compounds. Laurdan fluorescence spectra showed that both compounds (**1** and **2**) exerted greater effects in charged liposomes (mimicking bacterial membranes) than in neutral liposomes. For anionic liposomes, a more pronounced effect was observed for compound **1** than **2**, supporting our earlier results on their comparative antibacterial profiles (Figure 4A, Figure S10). MD simulations were also performed to characterize the interaction of the compounds with lipid membranes. The compounds were simulated in duplicate (Figures S11-S13). Like the biophysical experiment, this study was performed for both lipid compositions, mimicking bacterial and mammalian membranes. By following our established "minimum bias" approach,[5] (see supporting information) simulations were started from a random mixture of lipids, water and the compounds. During the trajectories, water and lipid phases separated into two distinct phases after a few ns, and then the phospholipids self-organized into a bilayer. Membrane perturbation was stronger for the DPPG:DPPE bilayer, where insertion of phosphate groups (and the associated water molecules) caused by the compounds was more marked compared to DPPC membranes (Figure 4C). For the DPPG:DPPE bilayer, the charged groups of compound **1** were situated at the head groups region of the bilayer, whereas the molecular backbone comprising of larger hydrophobicity was inserted into the hydrophobic core of the bilayer along with the shorter dodecanoyl aliphatic tail (simulation 1, Figures S11 and S13). Additionally, one of the simulations showed that a transmembrane configuration is possible, with a local membrane thinning effect (Figure 4C). For compound **2** however, no such transmembrane configuration was observed (Figure S11). Rather, in one of the simulations, a distinct water-filled channel was found, lined by several lipid head groups, which indicates strong membrane perturbation when compound **2** is inserted into the bilayer (Figures 4C and S11). On the other hand, no significant membrane perturbations were caused by the compounds in DPPC membranes. Here too, for compound **1** transmembrane configuration is possible, definitely with a lesser extent of membrane thinning effect (Figure 4C). In case of compound **2**, the charged groups, and the molecular backbone along with the polar secondary amide group were always located in the head groups region of the bilayer, whereas only the octadecanoyl aliphatic tail inserted in the hydrophobic core of the membrane, parallel to the lipid chains (Figures S11 and S12). Taken together, these results provide a clear atomic-level picture of the position and orientation of compounds **1** and **2** in the membrane. The strong interactions with the charged head groups of DPPG:DPPE (88:12) lipids explain the selectivity observed experimentally for the compounds. In addition, the two molecules behave differently when bound to the membrane. Due to its shorter aliphatic chain, compound **1** is forced to insert the hydrophobic molecular backbone in the lipid core, to optimize its membrane interaction. In this way, the polar amide bonds and secondary amide group are located in the tail region of the bilayer lipids. We hypothesize that this topology

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leads to a reduction in the hydrophobic driving force for binding to neutral membranes, and thus reduces the toxicity of the compound. This interpretation is reminiscent of the finding that interruption of the hydrophobic sector of helical AMPs by introduction of polar groups, or of helix breaking residues, often leads to an increase in selectivity.[6] A further reduction in this driving force is provided by the lower effective hydrophobicity observed for compound **1** in water, which is similar to the observation that helix breaking residues cause an increase in selectivity of helical AMPs by allowing compact conformations with a reduced effective hydrophobicity.[5b] In contrast, the ability of compound **1** to attain a transmembrane orientation with greater extent of membrane thinning effect, allowed by the hydrophobic molecular backbone, could be related to the higher antimicrobial activity of this compound.

In conclusion, a new strategy towards selective targeting of bacteria with membrane-targeting peptidomimetic is introduced. Our studies revealed that even by keeping the chemical composition constant in a molecular design, effective hydrophobicity of the molecules can be tuned due altering the positional arrangement of hydrophobic moieties in the chemical structure. The molecule with a lower effective hydrophobicity was non-toxic and displayed potent antibacterial activity against various pathogenic bacteria. In contrast, the molecule with greater effective hydrophobicity was toxic towards mammalian cells. The selective antibacterial activity was further confirmed in the more realistic conditions of a co-culture with mammalian cells. Biophysical studies using model membranes also supported our findings. Moreover, the molecular dynamics simulation studies revealed that the two compounds behave differently both in solution and when bound to membranes, and clarified our experimental results with an atomic level picture. Altogether, these findings highlighting the role of molecular structure and conformation in cell selectivity, might lead the way for the rational design of non-toxic antibacterial agents.

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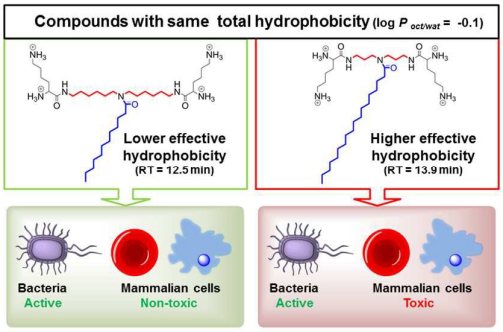

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