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Selective and broad spectrum amphiphilic small molecules to combat bacterial resistance and eradicate biofilms†

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Rationally designed amphiphilic small molecules selectively kill drug-sensitive and drug-resistant bacteria over mammalian cells. The small molecules disperse preformed biofilms and reduce viable bacterial count in the biofilms. Moreover, this class of membrane-active molecules disarms the development of bacterial resistance.

The continued emergence of drug-resistant pathogens along with the decline in the approval of new antimicrobial drugs pose unavoidable threats to human health.[1] Drug-resistant pathogenic bacteria are empowered with the abilities to either circumvent or thwart the action of commonly used therapeutic antibiotics. To tackle this problem, it is necessary to develop new antibacterial agents that act on bacterial cells selectively over the mammalian cells. The bacterial cell membrane, being mostly negatively charged (in sharp contrast to the zwitterionic mammalian cell membrane), is considered to be a major and inevitable target given its immense role in the survival of bacteria. Membrane-targeting antibacterial agents are thus considered to be an alternative to counter resistance development.[2]

Another major threat to public health is the formation of bacterial biofilms protected by extracellular polymeric substances (EPS). Biofilms are known to induce chronic infections and elevate bacterial resistance to antibiotics and host immune systems as a result of the diffusion barrier, genetic mutation, the presence of persistent cells with slow metabolism, and so on.[3] Notably, bacterial biofilms are known to cause more than 75% of microbial infections in humans.[3] Therefore, there is a pressing need for antibacterial agents that not only disarm bacterial resistance but also disperse established bacterial biofilms.

Membrane-targeting antimicrobial peptides (AMPs) and lipopeptides are known to overcome bacterial resistance and have exhibited the ability to disperse established bacterial biofilms.[4]

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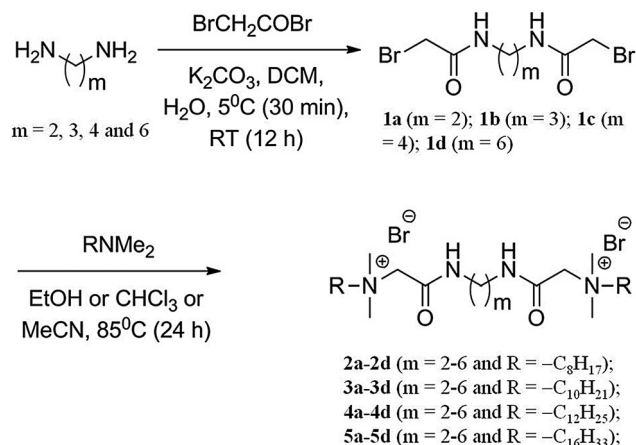
However, the high cost of manufacture, proteolytic degradation and low selectivity limit the use of these natural antimicrobials as therapeutic agents. Synthetic mimics of these natural antimicrobials, such as β-peptides, γ-AApeptides, aryl–amide foldamers, peptide dendrimers, oligoacyl lysines, oligoureas, small antibacterial peptides, antimicrobial polymers, alkylated peptoids, etc, have been widely demonstrated to overcome the aforementioned problems.[5] Though highly effective, the applications of peptidomimetics are still limited by the synthetic complexity, availability of frameworks and difficulty of introducing a variety of functional groups.[6]

Herein, we report the development of membrane-active amphiphilic small molecules in a facile and cost effective way using commercially available diaminoalkanes as scaffolds. The small molecules showed high selectivity towards bacterial cells over mammalian cells. These molecules dispersed established bacterial biofilms and reduced viable count within the biofilm. Furthermore, the molecules not only killed drug-resistant bacteria but also stalled the development of bacterial resistance. To mimic the structural features of antimicrobial peptides or lipopeptides composed of positive charges, lipophilic moieties either from hydrophobic amino acids or fatty acids, and peptide (amide) groups, we introduced two positive charges, two lipophilic moieties and two non-peptidic amide groups into the small molecules (Scheme 1). Furthermore, to fine-tune the structure–activity relationship of the molecules, the lipophilic moieties were varied between the amine groups of the diaminoalkane scaffold. To synthesize cationic small molecules in a simple two step process, various diamides obtained from diaminoalkanes by reacting with bromoacetyl bromide were quaternized with *N,N*-dimethylamino alkanes with quantitative yield (Scheme 1, see ESI† for experimental details and characterization).

In order to evaluate their potential as antibacterial yet non-toxic compounds, the antibacterial activity expressed as the minimum inhibitory concentration (MIC) and the hemolytic activity expressed as HC_{50} (concentration at which 50% hemolysis occurs) were determined. Among the four sets of molecules (**2a–2d** with $-C_8H_{17}$ lipophilic chain and m = 2–6; **3a–3d** with $-C_{10}H_{21}$ lipophilic chain and m = 2–6; **4a–4d** with $-C_{12}H_{25}$ lipophilic chain and m = 2–6; and **5a–5d** with $-C_{16}H_{33}$ lipophilic chain and

Scheme 1 Synthesis of the amphiphilic small molecules.

$m = 2–6$), **3a–3d** and **4a–4d** were found to be more active than **2a–2d** and **5a–5d** against both *S. aureus* and *E. coli*, thus showing a parabolic relationship between the activity and the lipophilic chain length (Table 1, and ESI,† Fig. S1). For example, the minimum inhibitory concentrations (MICs) for compounds **3a–3d** and **4a–4d** were 1.5–2 µg mL^{-1} and 1–2 µg mL^{-1} against *S. aureus* and 1.9–3.9 µg mL^{-1} for both sets against *E. coli*, whereas the MICs for molecules **2a–2d** and **5a–5d** were 1.9–22 µg mL^{-1} and 62.5–125 µg mL^{-1} against *S. aureus* and 3.9–125 µg mL^{-1} and >1000 µg mL^{-1} against *E. coli*, respectively. The toxicity towards human erythrocytes also increased as the lipophilic alkyl chain length increased from –C$_8$H$_{17}$ (HC$_{50}$ = 780 to >1000 µg mL^{-1} for **2a–2d**) to –C$_{10}$H$_{21}$ (HC$_{50}$ = 45–200 µg mL^{-1} for **3a–3d**) to –C$_{12}$H$_{25}$ (HC$_{50}$ = 31–53 µg mL^{-1} for **4a–4d**). A further increase in the length of the alkyl chain to –C$_{16}$H$_{33}$ showed a similar toxicity

(HC$_{50}$ = 32–49 µg mL^{-1} for **5a–5d**) as compounds **4a–4d** (Table 1, ESI,† Fig. S2). Thus, by varying the lipophilic alkyl chain, we could tune the selectivity (HC$_{50}$/MIC) of the small molecules. On the other hand, with the increase in the lipophilic spacer length, both the antibacterial and hemolytic activities were also found to vary. For example, the MICs of molecules **2a** ($m = 2$, R = –C$_8$H$_{17}$), **2b** ($m = 3$, R = –C$_8$H$_{17}$), **2c** ($m = 4$, R = –C$_8$H$_{17}$) and **2d** ($m = 6$, R = –C$_8$H$_{17}$) were 22 µg mL^{-1}, 18 µg mL^{-1}, 10 µg mL^{-1} and 2 µg mL^{-1} against *S. aureus* and 125 µg mL^{-1}, 62.5 µg mL^{-1}, 31.2 µg mL^{-1} and 3.9 µg mL^{-1} against *E. coli*, respectively, whereas the HC$_{50}$ values were >1000 µg mL^{-1} for **2a–2c** and 780 µg mL^{-1} for **2d** (Table 1, ESI,† Fig. S2). Thus, by varying both the lipophilic alkyl chain and spacer chain length, we were able to fine-tune the selectivity of the amphiphilic small molecules (Table 1).

Amongst all the tested molecules, molecules **2d** and **3a** were found to be the most potent in terms of their activity and selectivity. Molecules **2d** and **3a** showed selectivities of 410 and 100, respectively, against *S. aureus* and 200 and 50, respectively, against *E. coli* over human erythrocytes. These molecules also showed good activity (and selectivity) against *P. aeruginosa*, a difficult-to-treat Gram-negative pathogen (selectivities of **2d** and **3a** were 25 and 33, respectively) (Table 1). These molecules showed broad-spectrum activity not only against drug sensitive bacteria but also against various drug-resistant bacteria (Table 1). Against methicillin-resistant *S. aureus* (MRSA), molecules **2d** and **3a** displayed MIC values of 3 µg mL^{-1} and 1 µg mL^{-1}, respectively, thus showing selectivities of 260 and 200. The activities of molecules **2d** and **3a** against vancomycin-resistant *E. faecium* for VRE were also high as they exhibited MIC values of 6 µg mL^{-1} and 1 µg mL^{-1}, respectively (selectivity = 130 and 200, respectively). These molecules were also found to be active against β-lactam-resistant *Klebsiella pneumoniae*. The MICs of molecules **2d** and

Table 1 MIC and HC$_{50}$ values of the amphiphilic small molecules

| Biocides	MIC (µg mL^{-1})						HC$_{50}$ (µg mL^{-1})
	Drug-sensitive bacteria			Drug-resistant bacteria			
	S. aureus	*E. coli*	*P. aeruginosa*	MRSA	VRE	*K. pneumoniae*	
2a	22	125	188	20	94	500	>1000
2b	18	62.5	188	17	125	500	>1000
2c	7.8	31.2	125	8	62	250	>1000
2d	1.9	3.9	31.2	3	6	31.2	780
3a	2	3.9	6	1.5	1.5	8	200
3b	1.9	3.9	6	1	1	6	140
3c	1.5	3.9	6	1	1	4	110
3d	1.5	1.9	3	1	1	3	45
4a	2	3.9	9	3.9	2	6	53
4b	1.9	2	10	3.9	1.5	6	40
4c	1.9	2	14	3.9	1.5	6	33
4d	1.0	1.9	28	8	1.5	8	31
5a	125	>1000	250	47	188	530	49
5b	94	>1000	186	47	147	517	41
5c	62.5	>1000	186	47	137	500	37
5d	62.5	>1000	250	31	94	500	32
Colistin	ND	0.4	0.4	ND	ND	1.2	>798
Vancomycin	0.63	ND	ND	0.63	750	ND	>1000

P. aeruginosa = *Pseudomonas aeruginosa*, MRSA = methicillin-resistant *S. aureus*, VRE = vancomycin-resistant *E. faecium*, *K. pneumoniae* = beta-lactam-resistant *Klebsiella pneumoniae*, ND = not determined, MIC = minimum inhibitory concentration after 24 h of treatment with the biocides (at 2-fold serial dilution) in growth media (nutrient medium/Luria–Bertani medium/brain–heart infusion medium), HC$_{50}$ = concentration at which 50% hemolysis (human red blood cells) occurs after 1 h of treatment with the biocides. Variation in values ≤5%.

3a were 31.2 µg mL^{-1} and 8 µg mL^{-1}, respectively, thus showing a selectivity of 25 each against this bacterium. The antibacterial activities of these molecules were also compared with a Gram-positive antibiotic, vancomycin, and a Gram-negative antibiotic, colistin (Table 1). Vancomycin showed activity only against the Gram-positive bacteria (MIC = 0.63 µg mL^{-1} against *S. aureus* and MRSA) except against VRE (MIC = 750 µg mL^{-1}), whereas colistin showed activity only against the Gram-negative bacteria (MIC = 0.4 µg mL^{-1} against *E. coli* and *P. aeruginosa* and 1.2 µg mL^{-1} against *K. pneumoniae*).

One of the major limitations of the natural antimicrobial peptides is the loss of antibacterial efficacy in the presence of blood plasma due to enzymatic hydrolysis.[7] However, the most potent molecule **2d** did not reveal any loss in activity when incubated with 50% plasma for 0 h, 3 h and 6 h prior to determining its activity against *S. aureus*. The MIC was determined to be 2 µg mL^{-1} after 0 h, 3 h and 6 h incubation in 50% plasma (ESI,† Fig. S3a). The cationic molecule **2d** was also found to be active in 50% serum (MBC, minimum bactericidal concentration = 3 µg mL^{-1}), 50% plasma (MBC = 6 µg mL^{-1}) and 50% blood (MBC = 12 µg mL^{-1}) against MRSA. The cationic molecule **2d** was also found to be active in 50% serum (MBC, minimum bactericidal concentration = 3 µg mL^{-1}), 50% plasma (MBC = 6 µg mL^{-1}) and 50% blood (MBC = 12 µg mL^{-1}) against MRSA (ESI,† Fig. S3b). The above results thus indicated that the molecule retained its antibacterial efficacy (only 2–4 fold increase in MBC), even in very complex mammalian fluids like human serum, plasma and blood. These molecules were found to show rapid bactericidal activity against both types of bacteria. Molecule **2d** killed *S. aureus* (~5 log reduction) at 240 min in growth medium and in <10 min in a relatively less complex medium (HEPES–glucose buffer (1:1)) at 11.4 µg mL^{-1} (ESI,† Fig. S4a and b). On the other hand, **2d** killed *E. coli* (~5 log reduction) at 360 min in growth medium and 20 min in HEPES–glucose buffer (1:1) at 23.4 µg mL^{-1} (ESI,† Fig. S4c and d).

Membrane permeabilization and disruption resulting in a loss of membrane integrity of the bacteria is the main mechanism of action for cationic antimicrobials.[8] The amphiphilic small molecules were similarly found to dissipate the membrane potential of both Gram-positive and Gram-negative bacteria when a bacterial suspension in HEPES–glucose (1:1) buffer was treated with a particular concentration of all the small molecules (40 µg mL^{-1}) (ESI,† Fig. S5a and b). The molecules also showed membrane permeabilization against both types of bacteria (ESI,† Fig. S5c–e). Interestingly, even the least active compounds **5a–5d** showed membrane permeabilization in the buffer. This could be due to lesser interactions of the amphiphilic molecules with the less-complex buffer, which was verified by determining the MBCs in the above buffer where a cationic and hydrophobic molecule **5d** showed good activity (MBC = 3.9–7.8 µg mL^{-1} against *E. coli*) (ESI,† Table S1).

To establish the ability of this class of small molecules in dispersing preformed bacterial biofilms, matured *S. aureus* biofilms at the solid–liquid interface (developed for 24 h in a flat bottom 96-well plate) with an initial count of 11.8 log$_{10}$ CFU per well of bacteria were treated with **2d** at five different
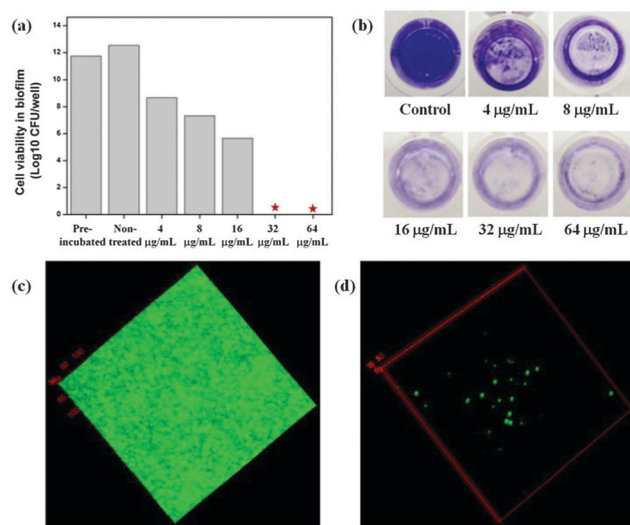

Fig. 1 Antibiofilm activity of small molecule **2d**. (a) Cell viability in biofilms, indicating the presence of live bacteria in the biofilm after plating and counting the colonies of *S. aureus* (star represents <50 CFU per mL); (b) images of the treated and non-treated biofilms of *S. aureus* after staining with crystal violet; (c) and (d) CLSM images of non-treated and treated (32 µg mL^{-1} of **2d**) *S. aureus* biofilms after staining with SYTO 9.

concentrations (4 µg mL^{-1}, 8 µg mL^{-1}, 16 µg mL^{-1}, 32 µg mL^{-1} and 64 µg mL^{-1}). The molecule was found to reduce the cell viabilities in the biofilms (8.77, 7.45 and 5.9 log$_{10}$ CFU per well at 4 µg mL^{-1}, 8 µg mL^{-1}, and 16 µg mL^{-1}, respectively, and 0 CFU per well at 32 µg mL^{-1} and 64 µg mL^{-1}), whereas the cell viability in the non-treated biofilm increased to 12.6 log$_{10}$ CFU per well (Fig. 1). The compound **2d** was also able to reduce the cell viabilities in matured *E. coli* biofilms at both the solid–liquid and liquid–air interfaces (developed for 72 h in a flat bottom 96-well plate) from an initial count of 21.5 log$_{10}$ CFU per well to 19.6, 19.3, 15.3, 12.1 and 9.4 log$_{10}$ CFU per well at 4 µg mL^{-1}, 8 µg mL^{-1}, 16 µg mL^{-1}, 32 µg mL^{-1} and 64 µg mL^{-1}, respectively, whereas the cell viability in the non-treated biofilm increased to 23.3 log$_{10}$ CFU per well, which was evident from crystal violet staining. The antibiofilm activity of compound **2d** was also evaluated against matured biofilms formed on a cover slip (18 mm diameter) at the solid–liquid interface for both *S. aureus* and *E. coli*. Biocide **2d** at 32 µg mL^{-1} was found to reduce the viable count from 17.7 log$_{10}$ CFU per mL to 5.6 log$_{10}$ CFU per mL against the *S. aureus* biofilm and completely eradicated the *E. coli* biofilm with an initial bacterial count of 7.2 log$_{10}$ CFU per mL (ESI,† Fig. S6). The ability of the compound to disperse a preformed biofilm was further visualized by confocal laser scanning microscopy (CLSM) images of both treated (**2d** at 32 µg mL^{-1}) and non-treated bacterial biofilms grown on cover slips (Fig. 1, ESI,† Fig. S7).

The ability of the small molecules to suppress bacterial resistance development was evaluated by challenging one of the potent molecules (**2d**) at sub-MICs repeatedly against both *S. aureus* and *E. coli*. To evaluate the propensity of developing resistance, bacteria grown at the sub-MIC level (MIC/2) were used for successive MIC assays and the process was repeated for 20 passages. The cationic biocide showed no change in the
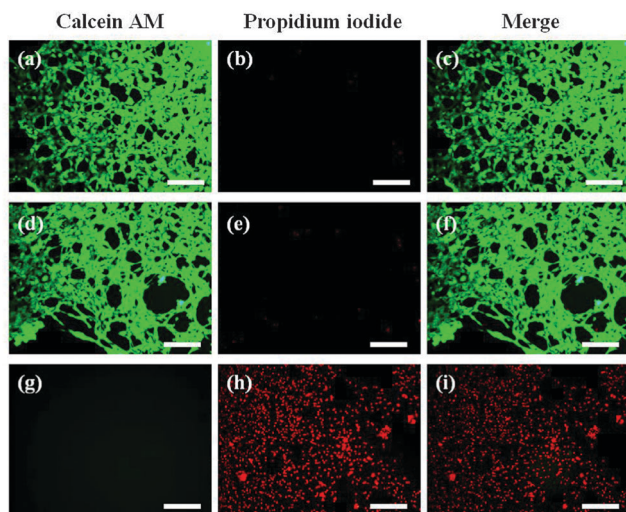
Fig. 2 Fluorescence microscopy images of HEK cells after treatment with small molecule **2d** for 24 h and staining with calcein AM and propidium iodide (PI). (a–c) Non-treated cells (negative control); (d–f) cells treated with **2d** (128 µg mL^{-1}); and (g–i) cells treated with 0.1% Triton X (positive control). Scale bars are 20 µm.

MIC against both the bacteria even after 20 passages, whereas around 805-fold and 250-fold increases in the MIC were observed for antibiotic norfloxacin against *S. aureus* and lipopeptide colistin against *E. coli*, respectively (ESI,† Fig. S8). The above results thus indicated that bacteria were less prone to developing resistance against this type of molecule.

In order to further evaluate the toxicity of the small molecules, human embryo kidney cells (HEK 293) were treated with the most potent molecule (**2d**) for 24 h. The half-maximal inhibitory concentration (IC$_{50}$) was found to be 220 µg mL^{-1} in the lactate dehydrogenase (LDH) assay, following the manufacturer protocol (lactate dehydrogenase activity assay kit, Sigma-Aldrich, catalog number MAK066). Also, the treated cells, visualized by the LIVE/DEAD staining method, showed green fluorescence even at 128 µg mL^{-1} (64 times the MIC) and were similar to the untreated cells, whereas cells treated with Triton-X were found to have completely red fluorescence (Fig. 2, ESI,† Fig. S9). These results thus indicated that these biocides are indeed non-toxic towards mammalian cells.

In summary, novel membrane-active amphiphilic small molecules, developed in a facile and cost-effective way, were highly active towards drug-sensitive and drug-resistance pathogenic bacteria but were less or non-toxic to human erythrocytes and human kidney cells. The molecules killed bacteria mainly by disrupting the membrane integrity and hindered the propensity of developing bacterial resistance. Furthermore, the small molecules dispersed the preformed Gram-positive and Gram-negative bacterial biofilms and reduced the viable bacteria in biofilms.

The structure–activity relationship, demonstrated by varying the nature of the lipophilic alkyl chain and spacer chain length, emphasized the role of the optimum amphiphilicity in developing non-toxic yet potent membrane-active antibacterial agents.

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