RESEARCH ARTICLE

[View Article Online](#)
[View Journal](#) | [View Issue](#) Cite this: *Med. Chem. Commun.*,
2019, 10, 1907Small antibacterial molecules highly active against
drug-resistant *Staphylococcus aureus*[†]Rajib Dey,^a Kathakali De,^a Riya Mukherjee,^a Sreyan Ghosh^a and Jayanta Halder ^{*ab}

The rapid growth of antibiotic resistance in *Staphylococcus aureus* coupled with their biofilm forming ability has made the infections difficult to treat with conventional antibiotics. This has created a massive threat towards public health and is a huge concern worldwide. Aiming to address this challenging issue, herein we report a new class of small antibacterial molecules (SAMs) with high antibacterial activity against multidrug-resistant *S. aureus*. The design principle of the molecules was based on the variation of hydrophobic/hydrophilic balance through incorporation of two quaternary ammonium groups, ethanol moieties, non-peptidic amide bonds and aliphatic chains. The lead compound, identified through a comprehensive analysis of structure–activity relationships, displayed high activity against clinical isolates of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) with MIC values in the range of 1–4 $\mu\text{g mL}^{-1}$. More importantly, this compound was capable of killing stationary phase bacteria and disrupting established biofilms of MRSA. Additionally, the compound revealed minimum toxicity towards human erythrocytes ($\text{HC}_{50} = 577 \mu\text{g mL}^{-1}$) and did not show significant toxicity towards mammalian cells (MDCK and A549) up to 128 $\mu\text{g mL}^{-1}$. Remarkably, the incorporation of non-peptidic amide bonds made the compounds less susceptible to degradation in human plasma, serum and mouse liver homogenate. Taken together, the results therefore indicate great promise for this class of molecules to be developed as potent antibacterial agents in treating infections caused by drug-resistant *S. aureus*.

Received 14th June 2019,
Accepted 26th July 2019

DOI: 10.1039/c9md00329k

rsc.li/medchemcomm

Introduction

Drug-resistant bacteria are responsible for a huge number of cases of mortality and morbidity and are shaping up to be an enormous threat towards public health globally. About 2 million patients are infected by different drug-resistant bacteria every year.^{1–4} Among various notorious pathogens, drug-resistant *Staphylococcus aureus* infections have created an alarming situation. *S. aureus* has already been reported to develop resistance against last resort antibiotics, which generally target bacterial cellular processes, as a result of mutation, production of drug-inactivating enzymes and elimination by bacterial efflux pumps.^{5–12} In this scenario, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) mediated infections are emerging rapidly, increasing the massive risk of community and hospital acquired infections. Moreover, MRSA and VRSA have been categorized as

high-priority pathogens by the WHO, requiring immediate attention.¹³ To add to the growing bacterial resistance against conventional antibiotics, the formation of impenetrable biofilms has further aggravated the scenario.^{14–17} The majority of hospital acquired (nosocomial) infections and chronic wound infections are caused by drug-resistant *S. aureus*-mediated biofilm formation. Additionally, drug-resistant *S. aureus* is also responsible for various serious diseases like endocarditis, osteomyelitis and sepsis.^{18–21} Unfortunately, there is a massive dearth of novel classes of antibacterial agents which can disrupt the biofilms and inactivate the resistant bacteria.

In this direction, antimicrobial peptides (AMPs), lipopeptides and their synthetic mimics have been developed that exhibit effective antibacterial activity.^{22–48} Due to the presence of positive charges, hydrophobic amino acids and peptidyl amide bonds, these classes of molecules can interact effectively with the negatively charged bacterial membrane. As a result of their membrane-targeting nature, AMPs and lipopeptides are known to possess a low propensity for triggering the development of bacterial resistance. Some of these membrane-active agents have also displayed potent anti-biofilm properties.^{49–51} However, their translation into clinical settings and applications is limited by the complex synthetic routes, instability in physiological fluids, low selectivity and low *in vivo* potency. Currently, a few synthetic

^a Antimicrobial Research Laboratory, New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bangalore 560064, India. E-mail: jayanta@jncasr.ac.in

^b School of Advanced Materials, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Jakkur, Bangalore 560064, India

[†] Electronic supplementary information (ESI) available: Materials and detailed experimental methods of small antibacterial molecule synthesis, characterization, biological assays and supplementary figures. See DOI: 10.1039/c9md00329k

membrane-active candidates (LTX-109 and brilacidin) are undergoing clinical trials to be developed as potent drugs.^{52,53} Therefore there is a pressing need for the development of a new class of antibacterial agents having potent activity along with the ability to disrupt the biofilms effectively to tackle the drug-resistant *S. aureus* infections.

To achieve this goal, herein, we have designed a new class of small antibacterial molecules (SAMs) with varying hydrophobic/hydrophilic balance *via* a simple three-step synthetic route. The hydrophilicity in the molecules was introduced through incorporation of two quaternary ammonium groups, two ethanol moieties and two nonpeptidic amide bonds. On the other hand, hydrophobicity was introduced by varying the backbone chain length and pendant lipophilicity. To construct a detailed structure–activity relationship (SAR), first their antibacterial activity against different bacteria and toxicity against human erythrocytes were evaluated. After analysing their selectivity indexes, two compounds were found to be optimum for their high activity against methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA). We have also tested the antibacterial activity of these compounds against different drug-resistant and clinically isolated strains of *S. aureus*. Bactericidal kinetics, activity against metabolically inactive bacteria and their membrane-active mechanism of action were explored. Furthermore, the potential of the compounds to destroy established biofilms of *S. aureus* was examined. The stability of the compounds in human plasma, serum and mouse liver homogenate was also investigated. To establish the efficacy of the compounds to be applicable in clinical settings, their cytotoxicity towards mammalian cells was evaluated.

Results and discussion

Design and synthesis

A new class of small antibacterial molecules (SAMs) have been synthesized by incorporating the following features: (i) tunable hydrophobicity from lipophilic spacer chains and pendant lipophilic alkyl chains in the design, (ii) presence of two ethanol moieties for firm and persistent binding to the

hydrophilic lipid heads of the bacterial membrane bilayer,^{54,55} (iii) introduction of nonpeptidic amide bonds instead of peptide bonds formed by amino acids in conventional AMPs, and (iv) inclusion of two permanent positive charges from quaternary ammonium groups unlike the soft cationic charges found in AMPs or lipopeptides.

A total of twenty small antibacterial molecules (SAM 1–20) were synthesized in three steps following the reaction scheme as shown in Scheme 1. Briefly, 2-(methylamino) ethanol was reacted with dibromoalkanes to obtain the intermediate compounds **1a–1e** with 68–75% yield. Intermediates **2a–2d** were synthesized by the reaction of different 1-aminoalkanes with bromoacetyl with 100% yield.^{36,41} Finally, the intermediates **2a–2d** were attached to the diaminoalkanes to achieve the cationic SAMs with varied pendant hydrophobicity (SAM 1–20). Most of the compounds were purified and isolated by precipitation using diethyl ether to give 95–98% yield, whereas the compounds bearing the $-C_6H_{13}$ pendant long chain (SAM-1, SAM-5, SAM-9, SAM-13, SAM-17) were purified using reversed-phase HPLC with 61–68% yield. Different lipophilic pendant alkyl moieties ($-C_6H_{13}$, $-C_8H_{17}$, $-C_{10}H_{21}$, and $-C_{12}H_{25}$) and lipophilic spacer moieties ($m = 3, 6, 8, 10$ and 12) were used to generate a library of 20 cationic small molecules (SAM 1–20). All the final compounds were characterized (ESI⁺) by FT-IR, ¹H NMR, ¹³C NMR and high-resolution mass spectrometry (HRMS).

Antibacterial activity

The antibacterial activity of all the compounds was tested initially against growing planktonic cells of Gram-positive methicillin-resistant *S. aureus* (MRSA) and different Gram-negative bacteria (*Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) by determining their minimum inhibitory concentration (MIC) (Table 1). The glycopeptide antibiotic vancomycin and the lipopeptide antibiotic colistin were also used in this study. We have designed the compounds by varying both backbone hydrophobicity (in the spacer) and pendant hydrophobicity. The antibacterial activity of the compounds is a

Scheme 1 Synthesis of small antibacterial molecules (SAMs) bearing non-peptidic amide and ethanol groups.

Table 1 Antibacterial and hemolytic activities of small antibacterial molecules (SAMs)

Compounds	Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$)					$f_{\text{HC}_{50}}$ ($\mu\text{g mL}^{-1}$)
	^a MRSA	^b <i>E. coli</i>	^c <i>A. baumannii</i>	^d <i>K. pneumoniae</i>	^e <i>P. aeruginosa</i>	
SAM-1	256	>256	>256	>256	>256	>1024
SAM-2	2	16	32	32	8	178
SAM-3	1	1	2	4	2	34
SAM-4	2	16	8	128–256	64–128	37
SAM-5	256	>256	>256	>256	>256	>1024
SAM-6	1–2	4–8	16	32	16	222
SAM-7	0.5	1	2	2–4	4	31
SAM-8	1–2	8	4	128	128–256	31
SAM-9	128	256	>256	>256	>256	>1024
SAM-10	0.5–1	1–2	8	8	16	61
SAM-11	0.5	1	1	2	4	25
SAM-12	2	32	4	64	64–128	29
SAM-13	8	8	256	>256	256	>1024
SAM-14	0.25–0.5	0.5	4	4	4	28
SAM-15	0.5	2	1	4	8	18
SAM-16	4	32	8	64–128	64–128	32
SAM-17	1–2	2	64	128	128	577
SAM-18	0.25	0.5–1	2	2	4	12
SAM-19	2	4	2	16	32	16
SAM-20	2–4	32	32	128–256	64	22
Vancomycin	1	^g ND	ND	ND	ND	ND
Colistin	ND	1	0.5	1	1	ND

^a Methicillin-resistant *Staphylococcus aureus* ATCC33591. ^b *Escherichia coli* MTCC443. ^c *Acinetobacter baumannii* MTCC1425. ^d *Klebsiella pneumoniae* ATCC700603. ^e *Pseudomonas aeruginosa* MTCC424. ^f Concentration at which 50% hemolysis occurs against human erythrocytes.

^g Not determined.

manifestation of the positive charges and hydrophobicity present in the structures.

Effect of lipophilic spacer chain length on antibacterial activity. Among the various sets of compounds (SAM 1–4 with spacer $m = 3$ and $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$ lipophilic pendant chain; SAM 5–8 with spacer $m = 6$ and $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$ lipophilic pendant chain; SAM 9–12 with spacer $m = 8$ and $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$ lipophilic pendant chain; SAM 13–16 with spacer $m = 10$ and $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$ lipophilic pendant chain; and SAM 17–20 with spacer $m = 12$ and $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$ lipophilic pendant chain), those with a shorter spacer arm were found to be less active compared to the ones containing a longer spacer. Keeping the pendant hydrophobicity unchanged, the antibacterial activity was increased with increasing backbone hydrophobicity. For example, the MIC of compound SAM-1 ($m = 3$) was found to be $256 \mu\text{g mL}^{-1}$ against MRSA and $>256 \mu\text{g mL}^{-1}$ against all other tested bacteria. However, keeping the pendant hydrophobicity ($-\text{C}_6\text{H}_{13}$) the same, upon increasing the spacer length to $m = 6$ (SAM-5) and $m = 8$ (SAM-9), there was no significant improvement in the activities. However, when the spacer arm length was increased further to $m = 10$ (SAM-13) and $m = 12$ (SAM-17), both the compounds showed highly improved activity against MRSA (MIC = $8 \mu\text{g mL}^{-1}$ for SAM-13; MIC = $1-2 \mu\text{g mL}^{-1}$ for SAM-17) and *E. coli* (MIC = $8 \mu\text{g mL}^{-1}$ for SAM-13; MIC = $2 \mu\text{g mL}^{-1}$ for SAM-17). However, there was no significant improvement in the activities against other tested pathogens. Further, for compounds with $-\text{C}_8\text{H}_{17}$ pen-

dant hydrophobicity and varying spacer arms, the compounds exhibited much lower MIC values against all the tested bacteria. SAM-2 ($m = 3$, $-\text{C}_8\text{H}_{17}$) displayed MIC = $2 \mu\text{g mL}^{-1}$ against MRSA and MIC = $8-32 \mu\text{g mL}^{-1}$ against other Gram-negative pathogens. When the spacer arm was further increased while keeping the pendant length unchanged (SAM-6, $m = 6$; SAM-10, $m = 8$; SAM-14, $m = 10$; SAM-18, $m = 12$), the compounds exhibited higher activity against all the tested pathogens with activity showing an increasing trend with longer spacer length in the compound backbone. SAM-6 exhibited MIC = $1-32 \mu\text{g mL}^{-1}$ against the tested bacteria, whereas the MIC values of SAM-10, SAM-14, SAM-18 were $0.5-16 \mu\text{g mL}^{-1}$, $0.25-4 \mu\text{g mL}^{-1}$ and $0.25-4 \mu\text{g mL}^{-1}$, respectively. Most remarkably, besides MRSA and *E. coli*, SAM-14 and SAM-18 were also active against *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* with MIC = $4 \mu\text{g mL}^{-1}$ and MIC = $2-4 \mu\text{g mL}^{-1}$, respectively. On the other hand, when the spacer length is varied while keeping the pendant lipophilicity constant with $-\text{C}_{10}\text{H}_{21}$ (SAM-3, SAM-7, SAM-11, SAM-15 and SAM-19) and with $-\text{C}_{12}\text{H}_{25}$ (SAM-4, SAM-8, SAM-12, SAM-16 and SAM-20), the increasing trend of antibacterial activity with increasing spacer length was not followed. Compounds with $-\text{C}_{10}\text{H}_{21}$ pendant chains (SAM-3, SAM-7, SAM-11, SAM-15 and SAM-19) showed activity against all the tested pathogens where the activity of the compounds varied as follows: SAM-3: $1-4 \mu\text{g mL}^{-1}$, SAM-7: $0.5-4 \mu\text{g mL}^{-1}$, SAM-11: $0.5-4 \mu\text{g mL}^{-1}$, SAM-15: $0.5-8 \mu\text{g mL}^{-1}$ and SAM-19: $2-32 \mu\text{g mL}^{-1}$. Compounds with a $-\text{C}_{12}\text{H}_{25}$ pendant alkyl chain (SAM-4, SAM-8, SAM-12, SAM-16 and SAM-20) were not able to effect any activity against *K. pneumoniae* and *P. aeruginosa*.

(MIC $\geq 64 \mu\text{g mL}^{-1}$). Even though these compounds showed moderate activity on other bacteria, the activity was invariant irrespective of spacer arm length variation. Therefore, the effect of the spacer arm length is dominant when the pendant hydrophobicity is low, and with increasing spacer hydrophobicity, the activity increases. However, this tendency is not prevalent for compounds with longer pendant chains.

Effect of lipophilic pendant alkyl chain length on antibacterial activity. To examine how pendant hydrophobicity dictates the antibacterial activity, we have varied the hanging lipophilic chain while keeping the backbone spacer fixed. Keeping the spacer arm unchanged, pendant hydrophobicity was varied with $-\text{C}_6\text{H}_{13}$, $-\text{C}_8\text{H}_{17}$, $-\text{C}_{10}\text{H}_{21}$, and $-\text{C}_{12}\text{H}_{25}$. When the spacer arm was held at $m = 3$, compound with lowest pendant hydrophobicity SAM-1 ($m = 3$, $-\text{C}_6\text{H}_{12}$) was inactive against all the tested bacteria ($\geq 256 \mu\text{g mL}^{-1}$). Upon increasing the pendant chain length to $-\text{C}_8\text{H}_{17}$, compound SAM-2 showed moderate activity against all the bacteria with MIC values of $2\text{--}32 \mu\text{g mL}^{-1}$. Upon further increase in pendant length to $-\text{C}_{10}\text{H}_{21}$, there was a remarkable increase in activity against all the tested bacteria with MIC = $1\text{--}4 \mu\text{g mL}^{-1}$. SAM-4 ($m = 3$, $-\text{C}_{12}\text{H}_{25}$) showed moderate activity against MRSA (MIC = $2 \mu\text{g mL}^{-1}$), *E. coli* (MIC = $16 \mu\text{g mL}^{-1}$) and *A. baumannii* (MIC = $8 \mu\text{g mL}^{-1}$), but the compound did not show any activity against *K. pneumoniae* and *P. aeruginosa*. The same parabolic pattern was followed for compounds with longer spacer arms, where compounds with optimum hanging long chain length showed the highest activity whereas compounds with shorter or longer pendant chains showed low or moderate activity against the tested bacteria.

Haemolytic activity

A preliminary toxicity study of all compounds was conducted against human erythrocytes (RBCs) which is represented as HC_{50} (concentration at which 50% haemolysis of RBCs occurs) in Table 1. Herein, it was observed that with increment in hydrophobicity either in the backbone or in the pendant lipophilicity, the toxicity against human red blood cells increased. Compounds with the same spacer arm length but varying pendant lipophilicity showed a huge variation in their HC_{50} values, where activity was observed to increase with increasing pendant chain length. For instance, in the case of compounds with spacer $m = 6$ and varying pendant chains, e.g. SAM-5 ($-\text{C}_6\text{H}_{13}$), SAM-6 ($-\text{C}_8\text{H}_{17}$), SAM-7 ($-\text{C}_{10}\text{H}_{21}$) and SAM-8 ($-\text{C}_{12}\text{H}_{25}$), the haemolytic activity values were $>1024 \mu\text{g mL}^{-1}$, $222 \mu\text{g mL}^{-1}$, $31 \mu\text{g mL}^{-1}$, and $31 \mu\text{g mL}^{-1}$, respectively. On the other hand, on maintaining the same pendant hydrophobicity ($-\text{C}_6\text{H}_{13}$) but varying the backbone spacer length (from $m = 3$ to $m = 12$), the HC_{50} values were found to be $>1024 \mu\text{g mL}^{-1}$ for SAM-1, SAM-5, SAM-9, and SAM-13 and $577 \mu\text{g mL}^{-1}$ for SAM-17. Similarly, when the pendant hydrophobicity was kept constant at $-\text{C}_8\text{H}_{17}$, upon varying the spacer length (from $m = 3$ to $m = 12$), the HC_{50} value varied from $222 \mu\text{g mL}^{-1}$ to $12 \mu\text{g mL}^{-1}$. This ascertains that the HC_{50} values vary with both pendant lipophilicity and spacer/backbone hydrophobicity.

Upon examination of the hemolytic activity and antibacterial activity of all the compounds, the selectivity indexes of the compounds were evaluated to identify which were more selective towards bacterial cells over mammalian cells. The selectivity indexes ($\text{HC}_{50}/\text{MIC}$) of the compounds against all the tested bacteria are presented in Fig. 1. All the SAMs were found to have very low selectivity towards the Gram-negative pathogens. However, SAM-13 and SAM-17 displayed moderate and high selectivity against *E. coli* with selectivity indexes of 125 and 288, respectively. The compounds SAM-6, SAM-10, SAM-13, SAM-14 and SAM-17 were shown to have high selectivity against MRSA, where SAM-6 and SAM-17 showed selectivity indexes of 222 and 577, respectively. Compound SAM-17 with spacer $m = 12$ and pendant chain $-\text{C}_6\text{H}_{13}$ was found to be the most selective compound against MRSA and *E. coli*.

Antibacterial activity against clinical isolates of methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA)

The selectivity index (Fig. 1) suggested that compounds SAM-6 and SAM-17 were most selective against methicillin-resistant *S. aureus*. To further explore the efficacy of these two compounds against clinically isolated pathogens, they were tested against three clinically isolated strains of methicillin-resistant *S. aureus* (MRSA) and two vancomycin-resistant *S. aureus* (VRSA) strains. To compare their efficacy with that of the conventional antibiotics methicillin and vancomycin, these were also included in the study. The antibacterial activity was represented in terms of MIC in Table 2. Both compounds exhibited potent antibacterial activity against all MRSA and VRSA strains. We observed that both SAM-6 and SAM-17 have MIC values between 1 and $4 \mu\text{g mL}^{-1}$ against all MRSA strains (Table 2). However, methicillin has shown MIC values between 16 and $32 \mu\text{g mL}^{-1}$ for MRSA R3545 and MRSA R3889, respectively, and $>32 \mu\text{g mL}^{-1}$ for MRSA ATCC33591 and MRSA R3890, confirming that these

Fig. 1 Selectivity index ($\text{HC}_{50}/\text{MIC}$) of the compounds against all tested bacteria. Compounds having MIC $\geq 128 \mu\text{g mL}^{-1}$ were not introduced in this figure.

Table 2 Antibacterial activity against drug-resistant *S. aureus* and cytotoxicity against mammalian cell lines

Compound	Minimum inhibitory concentration ($\mu\text{g mL}^{-1}$)						Cytotoxicity ($^{b}\text{EC}_{50}$; $\mu\text{g mL}^{-1}$)	
	MRSA ATCC33591	MRSA R3545	MRSA R3889	MRSA R3890	$^{a}\text{VRSA-1}$	VRSA-4	$^{c}\text{MDCK cell line}$	$^{d}\text{A549 cell line}$
SAM-6	1–2	1–2	1	4	2	2	45	44
SAM-17	1–2	1–2	1–2	2	2	2	188	194
Methicillin	>32	16–32	16–32	>32	ND	ND	^{e}ND	ND
Vancomycin	0.5	1	1	2	512	512	ND	ND

a Vancomycin-resistant *S. aureus*. b Concentration at which 50% cell viability occurs. c Madin–Darby canine kidney epithelial cells.

d Adenocarcinomic human alveolar basal epithelial cells. e Not determined.

strains have a high level of resistance to methicillin. On the other hand, vancomycin, a last-resort glycopeptide antibiotic, showed potent activity against all MRSA strains with MIC values of 0.5–2 $\mu\text{g mL}^{-1}$. However, it was found to be resisted by the VRSA strains (showing MIC 512 $\mu\text{g mL}^{-1}$). Remarkably, both the compounds SAM-6 and SAM-17 were found to be highly active against VRSA strains with MIC values of 2 $\mu\text{g mL}^{-1}$ (Table 2). This observation therefore suggests the potential of SAM-6 and SAM-17 to be developed as efficient therapeutic agents against drug-resistant clinical isolates of *S. aureus*.

Cytotoxicity

To establish the potential of the compounds to be developed as a therapeutic regimen, the toxicity of SAM-6 and SAM-17 was further determined against two different mammalian cell lines (MDCK and A549) using the Alamar Blue assay (Fig. 2A and S61†). The EC_{50} value (concentration at which 50% of cells were viable) was determined by analysing the sigmoidal plot of cell viability against the tested concentration of the compounds (Table 2). The EC_{50} value of SAM-6 was 45 $\mu\text{g mL}^{-1}$ against both the cell lines, which was 22-fold more compared to its MIC value against VRSA, whereas SAM-17 was found to have an EC_{50} value of 188 $\mu\text{g mL}^{-1}$ against MDCK and 194 $\mu\text{g mL}^{-1}$ against A549, which was 94–97 fold higher compared to its MIC against VRSA. SAM-17 did not

display any significant toxicity even up to 128 $\mu\text{g mL}^{-1}$ against both the cell lines (Fig. 2A).

Further, the toxicity of compound SAM-17 was determined against MDCK cells through a live/dead assay upon staining with calcein-AM and propidium iodide (PI) dyes. The results suggested that after 24 h of exposure to SAM-17 (32 $\mu\text{g mL}^{-1}$), all the cells were alive, similar to the untreated ones (Fig. 2B), which confirmed the nontoxic nature of the optimized compound.

Time-kill kinetics against MRSA

Having investigated the potency of these compounds against a number of drug-resistant *S. aureus* strains followed by exploring their cytotoxicity, we were then interested in finding out the rate at which the compounds could effect the killing. To this end, we performed a time-kill kinetics assay of SAM-6 and SAM-17 against MRSA ATCC33591 and MRSA R3545 (Fig. 3). As evident from the experimental observation, SAM-6 showed complete killing of MRSA ATCC33591 (5.4 log CFU mL^{-1} reduction) within 2 h and MRSA R3545 (5.6 log CFU mL^{-1}) within 4 h at 16 $\mu\text{g mL}^{-1}$. At a higher concentration of 32 $\mu\text{g mL}^{-1}$, SAM-6 showed complete killing within 1 h for both the bacteria (Fig. 3A and B). Compound SAM-17 revealed more than 2 log CFU mL^{-1} reduction in bacterial count within 6 h for both MRSA ATCC33591 and MRSA R3545 at 16 $\mu\text{g mL}^{-1}$. Upon increasing the concentration to 32 $\mu\text{g mL}^{-1}$,

Fig. 2 (A) Cell viability of MDCK and A549 cells against SAM-17 at different concentrations through the Alamar Blue assay. (B) Fluorescence microscopy images of MDCK cells stained with calcein-AM and propidium iodide (PI). (i) Nontreated cells, (ii) cells treated with SAM-17 (32 $\mu\text{g mL}^{-1}$) and (iii) cells treated with Triton-X. Scale bars: 50 μm . All cells were obtained from ATCC (Rockville, MD, USA).

Fig. 3 Time-kill kinetics against planktonic bacteria. For SAM-6 against (A) MRSA ATCC33591 and (B) MRSA R3545 and for SAM-17 against (C) MRSA ATCC33591 and (D) MRSA R3545. Asterisks (*) indicate complete killing of bacteria. The detection limit of this experiment is 50 CFU mL⁻¹.

compound SAM-17 exhibited complete killing (5 log CFU mL⁻¹ reduction) of MRSA ATCC33591 within 8 h, whereas at the same concentration, SAM-17 displayed complete killing (6 log CFU mL⁻¹ reduction) of MRSA R3545 within 12 h (Fig. 3C and D).

Activity against metabolically inactive stationary phase MRSA

In the case of biofilm-mediated infection, different phases of bacteria including metabolically inactive and planktonic phases are present. Therefore, as we have already established that our compound has potent bactericidal activity against planktonic cells, next we tested the bactericidal activity of SAM-17 against metabolically inactive stationary phase MRSA cells (MRSA ATCC33591 and MRSA R3545). The bactericidal activity at different concentrations (8 µg mL⁻¹, 16 µg mL⁻¹,

and 32 µg mL⁻¹) of SAM-17 was investigated in comparison with vancomycin at 32 µg mL⁻¹ (Fig. 4). According to the result, vancomycin was totally inactive against stationary phase bacteria of both MRSA strains, while compound SAM-17 at 32 µg mL⁻¹ killed more than 99% (2.05 log CFU mL⁻¹ reduction) of metabolically inactive MRSA ATCC33591 (Fig. 4A) and for MRSA R3545, SAM-17 killed >99% (2.8 log CFU mL⁻¹ reduction) stationary phase bacterial cells (Fig. 4B). Therefore, our compound not only exhibited activity against different drug-resistant planktonic phase bacteria but also showed significant ability to kill the stationary phase MRSA, whereas the known antibiotic vancomycin did not show any activity against stationary phase cells.

Antibiofilm activity

Most bacterial infections are biofilm-mediated and conventional antibiotics fail to penetrate the biofilms which renders them ineffective for the treatment of biofilm-related infections. Therefore, we investigated the ability of SAM-17 to eradicate established biofilms of MRSA ATCC33591 and MRSA R3545 by employing crystal violet staining and also by estimation of the viable cells (Fig. 5). According to the result, SAM-17 was found to reduce more than 4 log(CFU mL⁻¹) bacterial burden at a concentration of 16 µg mL⁻¹ against both the bacteria (Fig. 5A and B), whereas vancomycin was found to reduce 1 log(CFU mL⁻¹) bacterial count. Crystal violet staining suggested that SAM-17 was able to reduce 60% of the biomass of MRSA ATCC33591 biofilms and 80% of the biomass of those of MRSA R3545 (Fig. 5C and D).

Fig. 4 Bactericidal activity of SAM-17 against stationary phase cells of (A) MRSA ATCC33591 and (B) MRSA R3545. The data plotted are the average of two independent experiments with their standard deviation.

Fig. 5 Biofilm disruption ability of SAM-17. Quantification of viable cells in biofilms after treatment for (A) MRSA ATCC33591 and (B) MRSA R3545. Quantification of reduction of biofilm biomass after treatment by crystal violet staining for (C) MRSA ATCC33591 and (D) MRSA R3545.

Vancomycin was found to reduce 10% of the biomass of both biofilms. Therefore, the results strongly suggest that the optimized compound disrupts the biofilms of clinically relevant MRSA and kills the pathogens, proving its efficacy as an excellent antibacterial agent.

Mechanism of action

Next, we explored the membrane targeted mechanism of action (membrane permeabilization and depolarization) of

compounds SAM-6 and SAM-17 through a spectroscopic technique against MRSA R3545 (Fig. 6A–D) and MRSA ATCC33591 (Fig. S62A–D†) at different concentrations (4–32 µg mL^{−1}). First, the ability of the compounds to permeabilize the bacterial membrane was investigated by employing the dye propidium iodide (PI) (Fig. 6A and B and S62A and B†). The increase in fluorescence intensity upon exposure of the compounds substantiated the fact that PI passed through the membrane of the dead bacterial cells. The results ascertained that with increasing concentration of the compounds, the extent of permeabilization increased. Further, to understand whether the compounds also act by depolarising the bacterial cell membrane, DiSC3(5), a dye sensitive to membrane potential, was employed in the experiment (Fig. 6C and D and S62C and D†). Upon addition of the compounds, the fluorescence intensity increased gradually, indicating a change in the potential across the bacterial membrane caused by treatment with the compounds. The result highlighted the fact that both the compounds caused significant perturbation in the membrane potential of both the bacteria. With increasing concentration of SAM-6 and SAM-17, this effect of depolarization was found to increase. It was also found that the extent of both membrane permeabilization and depolarization was more pronounced for SAM-6 compared to SAM-17 at the same concentration. The above results therefore collectively confirmed that the compounds act on bacteria by targeting their membrane.

Live/dead assay of MRSA

We have also performed live/dead assay by staining with the dyes SYTO-9 and propidium iodide (PI) in order to visualize the live and dead cells of MRSA ATCC33591 (Fig. 6E). In the

Fig. 6 Membrane-active mechanism of action. Membrane permeabilization of MRSA R3545 by (A) SAM-6 and (B) SAM-17. Membrane depolarization of MRSA R3545 by (C) SAM-6 and (D) SAM-17. The red arrow indicates compound addition. (E) Live/dead assay against MRSA ATCC33591 through fluorescence microscopy. (i) Untreated cells and (ii) cells treated with SAM-17 (32 µg mL^{−1}). Scale bars: 20 µm.

case of untreated cells, we observed green fluorescence upon SYTO-9 staining which indicated that all the cells were alive. In comparison, images of the cells treated with compound SAM-17 ($32 \mu\text{g mL}^{-1}$) suggested only the presence of dead cells which was confirmed from the bright red fluorescence in the PI channel with very few live cells (Fig. 6E).

Antibacterial activity upon incubation with mammalian fluid and mouse liver homogenate

Antimicrobial peptides suffer from instability under physiological conditions which limits their application in clinical settings.⁵⁶ Therefore, we rationally designed our molecules by incorporation of non-peptidic amide bonds which are less susceptible to degradation in a physiological environment. We were interested to check the stability of SAM-17 in plasma and serum by investigating its antibacterial activity in such mammalian fluids. After incubation with plasma and serum for 3 h, the antibacterial activity of SAM-17 was investigated. SAM-17 exhibited a MIC value of $4 \mu\text{g mL}^{-1}$ against MRSA ATCC33591 in both cases, showing minimum alteration from their MIC values in bacterial growth medium (Fig. S63[†]). Furthermore, the antibacterial activity of SAM-17 was investigated in the presence of mouse liver homogenate. After incubation with extracted liver homogenate, the MIC of the compound was determined against MRSA ATCC33591. At different concentrations of liver homogenate, MIC values of SAM-17 were altered only by two-fold ($4 \mu\text{g mL}^{-1}$) (Fig. S63[†]). Altogether the results therefore suggested that the SAMs are less susceptible to degradation under physiological conditions unlike antimicrobial peptides, proving their excellent potential to be engineered as potent antibacterial agents.

Conclusion

In order to address the ever-rising threats of notorious infections caused by drug-resistant *S. aureus*, we have developed a new class of small antibacterial molecules (SAMs) displaying high activity against clinical isolates of MRSA and VRSA. Additionally, this class of membrane-active molecules have significant bactericidal activity against difficult to treat metabolically inactive cells of MRSA and could disrupt established biofilms, whereas vancomycin showed a negligible effect. More importantly, the optimized compounds show minimal toxicity against mammalian cells. Collectively, all the results therefore emphasize that this class of small antibacterial molecules bear potential to be developed as new antibacterial agents against multidrug-resistant *S. aureus*.

Ethical statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Jawaharlal Neheru Center for Advanced Scientific Research (JNCASR) and approved by the Animal Ethics Committee of JNCASR.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

We thank Prof. C. N. R. Rao, FRS (JNCASR), for his constant support and encouragement. R. D. thanks CSIR for a research fellowship. We acknowledge JNCASR and DAE-BRNS/JH/4432 for financial support. We thank Swagatam Barman (JNCASR) for helping with the live/dead assay experiment. J. H. acknowledges a Sheikh Saqr Career Award Fellowship. We acknowledge Dr. Sidharth Chopra, CSIR-Central Drug Research Institute, Lucknow, India, for providing VRSA strains.

References

- 1 V. M. D'Costa, C. E. King, L. Kalan, M. Morar, W. W. Sung, C. Schwarz, D. Froese, G. Zazula, F. Calmels, R. Debruyne, G. B. Golding, H. N. Poinar and G. D. Wright, *Nature*, 2011, 477, 457–461.
- 2 E. D. Brown and G. D. Wright, *Nature*, 2016, 529, 336–343.
- 3 G. Taubes, *Science*, 2008, 321, 356–361.
- 4 J. Davies and D. Davies, *Microbiol. Mol. Biol. Rev.*, 2010, 74, 417–433.
- 5 G. J. Moran, A. Krishnadasan, R. J. Gorwitz, G. E. Fosheim, L. K. McDougal, R. B. Carey and D. A. Talan, *N. Engl. J. Med.*, 2006, 355, 666–674.
- 6 L. B. Rice, *Am. J. Med.*, 2006, 119, S11–S19.
- 7 G. Cornaglia and G. M. Rossolini, *Clin. Microbiol. Infect.*, 2009, 15, 218–223.
- 8 H. W. Boucher and G. R. Corey, *Clin. Infect. Dis.*, 2008, 46, S344–S349.
- 9 S. Stefani, D. R. Chung, J. A. Lindsay, A. W. Friedrich, A. M. Kearns, H. Westh and F. M. MacKenzie, *Int. J. Antimicrob. Agents*, 2012, 39, 273–282.
- 10 G. Dhanda, P. Sarkar, S. Samaddar and J. Haldar, *J. Med. Chem.*, 2019, 62(9), 3184–3205.
- 11 G. W. Kaatz, F. McAleese and S. M. Seo, *Antimicrob. Agents Chemother.*, 2005, 49(5), 1857–1864.
- 12 B. D. Schindler, P. Jacinto and G. W. Kaatz, *Future Microbiol.*, 2013, 8(4), 491–507.
- 13 *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*, WHO, 2017.
- 14 D. Davies, *Nat. Rev. Drug Discovery*, 2003, 2, 114–122.
- 15 C. R. Arciola, D. Campoccia, P. Speziale, L. Montanaro and J. W. Costerton, *Biomaterials*, 2012, 33, 5967–5982.
- 16 H. McCarthy, J. K. Rudkin, N. S. Black, L. Gallagher, E. O'Neill and J. P. O'Gara, *Front. Cell. Infect. Microbiol.*, 2015, 5, 1–9.
- 17 M. Bhattacharya, D. J. Wozniak, P. Stoodley and L. Hall-Stoodley, *Expert Rev. Anti-infect. Ther.*, 2015, 13, 1499–1516.
- 18 J. W. Costerton, P. S. Stewart and E. P. Greenberg, *Science*, 1999, 284, 1318–1322.
- 19 J. Knox, A. C. Uhlemann and F. D. Lowy, *Trends Microbiol.*, 2015, 23(7), 437–444.

- 20 H. F. Wertheim, D. C. Melles, M. C. Vos, W. V. Leeuwen, A. V. Belkum, H. A. Verbrugh and J. L. Nouwen, *Lancet Infect. Dis.*, 2005, **5**, 751–762.
- 21 S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland and V. G. Fowler, *Clin. Microbiol. Rev.*, 2015, **28**(3), 603–661.
- 22 M. Zasloff, *Nature*, 2002, **415**, 389–395.
- 23 Y. X. Chen, C. T. Mant, S. W. Farmer, R. E. W. Hancock, M. L. Vasil and R. S. Hodges, *J. Biol. Chem.*, 2005, **280**, 12316–12329.
- 24 C. Ghosh, P. Sarkar, R. Issa and J. Haldar, *Trends Microbiol.*, 2019, **27**(4), 323–338.
- 25 E. A. Porter, X. Wang, H. S. Lee, B. Weisblum and S. H. Gellman, *Nature*, 2000, **404**, 298.
- 26 S. Choi, A. Isaacs, D. Clements, D. H. Liu, H. Kim, R. W. Scott, J. D. Winkler and W. F. DeGrado, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 6968–6973.
- 27 M. F. Ilker, K. Nusslein, G. N. Tew and E. B. Coughlin, *J. Am. Chem. Soc.*, 2004, **126**, 15870–15875.
- 28 A. Colomer, A. Pinazo, M. A. Manresa, M. P. Vinardell, M. Mitjans, M. R. Infante and L. Pérez, *J. Med. Chem.*, 2011, **54**, 989–1002.
- 29 I. S. Radzishhevsky, S. Rotem, D. Bourdetsky, S. Navon-Venezia, Y. Carmeli and A. Mor, *Nat. Biotechnol.*, 2007, **25**, 657–659.
- 30 F. Nederberg, Y. Zhang, J. P. K. Tan, K. J. Xu, H. Y. Wang, C. Yang, S. J. Gao, X. D. Guo, K. Fukushima, L. J. Li, J. L. Hedrick and Y. Y. Yang, *Nat. Chem.*, 2011, **3**, 409–414.
- 31 A. Makovitzki, D. Avrahami and Y. Shai, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 15997–16002.
- 32 J. Hoque, P. Akkapeddi, V. Yarlagadda, D. S. S. M. Uppu, P. Kumar and J. Haldar, *Langmuir*, 2012, **28**, 12225–12234.
- 33 D. S. S. M. Uppu, P. Akkapeddi, G. B. Manjunath, V. Yarlagadda, J. Hoque and J. Haldar, *Chem. Commun.*, 2013, **49**, 9389–9391.
- 34 C. Ghosh, G. B. Manjunath, P. Akkapeddi, V. Yarlagadda, J. Hoque, D. S. S. M. Uppu, M. M. Konai and J. Haldar, *J. Med. Chem.*, 2014, **57**, 1428–1436.
- 35 M. M. Konai, C. Ghosh, V. Yarlagadda, S. Samaddar and J. Haldar, *J. Med. Chem.*, 2014, **57**, 9409–9423.
- 36 J. Hoque, M. M. Konai, S. Samaddar, S. Gonuguntla, G. B. Manjunath, C. Ghosh and J. Haldar, *Chem. Commun.*, 2015, **51**, 13670–13673.
- 37 M. M. Konai, U. Adhikary, S. Samaddar, C. Ghosh and J. Haldar, *Bioconjugate Chem.*, 2015, **26**, 2442–2453.
- 38 C. Ghosh, G. B. Manjunath, M. M. Konai, D. S. S. M. Uppu, J. Hoque, K. Paramanandham, B. R. Shome and J. Haldar, *PLoS One*, 2015, **10**, e0144094.
- 39 C. Ghosh, M. M. Konai, P. Sarkar, S. Samaddar and J. Haldar, *ChemMedChem*, 2016, **11**, 2367–2371.
- 40 D. S. S. M. Uppu, M. M. Konai, U. Baul, P. Singh, T. K. Siersma, S. Samaddar, S. Vemparala, L. W. Hamoen, C. Narayana and J. Haldar, *Chem. Sci.*, 2016, **7**, 4613–4623.
- 41 J. Hoque, M. M. Konai, S. S. Sequeira, S. Samaddar and J. Haldar, *J. Med. Chem.*, 2016, **59**, 10750–10762.
- 42 M. M. Konai and J. Haldar, *Bioconjugate Chem.*, 2017, **28**, 1194–1204.
- 43 M. M. Konai, B. Bhattacharjee, S. Ghosh and J. Haldar, *Biomacromolecules*, 2018, **19**(6), 1888–1917.
- 44 K. Yadav, S. Kumar, D. Mishra, M. Asad, M. Mitra, P. S. Yavvari, S. Gupta, M. Vedantham, P. Ranga, V. Komalla, S. Pal, P. Sharma, A. Kapil, A. Singh, N. Singh, A. Srivastava, L. Thukral and A. Bajaj, *J. Med. Chem.*, 2019, **62**, 1875–1886.
- 45 M. Schmidt, S. Harmuth, E. R. Barth, E. Wurm, R. Fobbe, A. Sickmann, C. Krumm and J. C. Tiller, *Bioconjugate Chem.*, 2015, **26**, 1950–1962.
- 46 Y. Niu, S. Padhee, H. Wu, G. Bai, Q. Qiao, Y. Hu, L. Harrington, W. N. Burda, L. N. Shaw, C. Cao and J. Cai, *J. Med. Chem.*, 2012, **55**(8), 4003–4009.
- 47 Y. Hu, M. N. Amin, S. Padhee, R. E. Wang, Q. Qiao, G. Bai, Y. Li, A. Mathew, C. Cao and J. Cai, *ACS Med. Chem. Lett.*, 2012, **3**, 683–686.
- 48 Q. Zhang, P. Ma, J. Xie, S. Zhang, X. Xiao, Z. Qiao, N. Shao, M. Zhou, W. Zhang, C. Dai, Y. Qian, F. Qi and R. Liu, *Biomater. Sci.*, 2019, **7**, 2144–2151.
- 49 B. E. Haug, W. Stensen, M. Kalaaji, Q. Rekdal and J. S. Svendsen, *J. Med. Chem.*, 2008, **51**, 4306–4314.
- 50 B. Mensa, G. L. Howell, R. Scott and W. F. DeGrado, *Antimicrob. Agents Chemother.*, 2014, **58**, 5136–5145.
- 51 C. Nagant, B. Pitts, P. S. Stewart, Y. Feng, P. B. Savage and J. P. Dehaye, *MicrobiologyOpen*, 2013, **2**, 318–325.
- 52 R. Kapoor, M. W. Wadman, M. T. Dohm, A. M. Czyzewski, A. M. Spormann and A. E. Barron, *Antimicrob. Agents Chemother.*, 2011, **55**, 3054–3057.
- 53 G. H. De Zoysa, A. J. Cameron, V. V. Hegde, S. Raghothama and V. Sarojini, *J. Med. Chem.*, 2015, **58**, 625–639.
- 54 W. Kim, W. Zhu, G. L. Hendricks, D. V. Tyne, A. D. Steele, C. E. Keohane, N. Fricke, A. L. Conery, S. Shen, W. Pan, K. Lee, R. Rajamuthiah, B. B. Fuchs, P. M. Vlahovska, W. M. Wuest, M. S. Gilmore, H. Gao, F. M. Ausubel and E. Mylonakis, *Nature*, 2018, **556**, 103–107.
- 55 T. J. Cuthbert, B. Hisey, T. D. Harrison, J. F. Trant, E. R. Gillies and P. J. Ragogna, *Angew. Chem., Int. Ed.*, 2018, **57**, 12707–12710.
- 56 A. Breijl, M. Riool, P. H. S. Kwakman, L. Boer, R. A. Cordfunke, J. W. Drijfhout, O. Cohen, N. Emanuel, S. A. J. Zaat, P. H. Nibbering and T. F. Moriarty, *J. Controlled Release*, 2016, **222**, 1–8.