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Hydrophobicity Modulated Small Antibacterial Molecule Eradicates Biofilm with Potent Efficacy against Skin-infections

Mohini Mohan Konai,[1] Swagatam Barman,[1] Rahaf Issa,[2] Sheila MacNeil,[3] Utsarga Adhikary,[1] Kathakali De,[1] Peter N. Monk [2] and Jayanta Haldar*[1]

[1] Antimicrobial Research Laboratory, New Chemistry Unit and School of Advanced Materials, Jawaharlal Nehru Centre for Advanced Scientific Research, Jakkur, Bengaluru 560064, Karnataka, India.

[2] Department of Infection, Immunity and Cardiovascular Diseases, The University of Sheffield Medical School, Sheffield, UK.

[3] Department of Materials Science and Engineering, The University of Sheffield Medical School, Sheffield, UK.

*Corresponding author: jayanta@jncasr.ac.in, Telephone: +91-80-2208-2565, Fax: +91-80-2208-2627

The role of molecular arrangement of hydrophobic and hydrophilic groups for designing membrane-active molecules remains largely ambiguous. To explore this aspect, herein we report a series of membrane-active small molecules by varying the spatial distribution of hydrophobic groups. The two terminal amino groups of linear triamines such as diethylene triamine, bis(trimethylene)triamine and bis(hexamethylene)triamine were conjugated with cationic amino acids bearing variable side chain hydrophobicity (such as diaminobutyric acid, ornithine and lysine). The hydrophobicity was also modulated through conjugation of different long chain fatty acids with the central secondary amino group of the triamine. Molecules with constant backbone hydrophobicity displayed an enhanced antibacterial activity and decreased hemolytic activity upon increasing the side chain hydrophobicity of amino acids. On the other hand, increased hydrophobicity in the backbone introduced a slight hemolytic activity but a higher increment in antibacterial activity resulting in better selective antibacterial compounds. The optimized lead compound derived from structure-activity-relationship (SAR) studies was the dodecanoyl analogue of lysine series of compounds consisting of bis(hexamethylene)triamine as the backbone. This compound was active against various Gram-positive and Gram-negative bacteria at a low concentration (MIC ranged between 3.1-6.3 μg/mL) and displayed low toxicity towards mammalian cells (HC_{50} = 890 μg/mL and EC_{50} against HEK = 85 μg/mL). Additionally, it was able to kill metabolically inactive bacterial cells and eradicate preformed biofilms of MRSA. This compound showed excellent activity in a mouse model of skin-infection with reduction of ~4 log MRSA burden at 40 mg/kg dose without any sign of skin-toxicity even at 200 mg/kg. More importantly, it revealed potent efficacy in an *ex-vivo* model of human skin-infection (with

reduction of 85% MRSA burden at 50 μg/mL), which indicates great potential of the compound as an antibacterial agent to treat skin-infections.

KEYWORDS: Antibiotic-resistance, biofilms, skin-infections, membrane-active agents, molecular arrangement

Rampant emergence of antibacterial resistance and antibiotic tolerance due to biofilm formation has raised a challenging threat in the face of public health globally.[1-4] In this state of alarm, development of novel antibacterial molecules capable of displaying antibiofilm property is highly necessary. In the recent past, membrane-active molecules, specifically small molecular designs, have emerged as possible future antibacterial agents that bear enormous potential to solve the antibiotic crisis in the clinic. The design of this class of molecules derives its inspiration from naturally occurring antimicrobial peptides (AMPs).[5] Unlike the conventional antibiotics, AMPs act on bacteria primarily by targeting their membranes, which is advantageous because of slow to no propensity of resistance development.[5,6] AMPs are also reported to display antibiofilm properties, indicating their potential for developing as modern antibacterial agents.[7-9] However potent these AMPs as antibacterial agent may be, they suffer from several limitations such as complex synthetic design, expensive solid-phase synthesis, low selectivity, proteolytic degradation and so on.[6] Therefore, a plethora of synthetic mimics of AMPs have been developed to address the aforementioned limitations.[10-33] Despite the diversity in the structural design, the presence of net positive charge is a common feature of this class of molecules, which ensures selectivity towards negatively charged bacterial membrane through initial electrostatic interaction. Along with positive charge, the role of amphiphilicity has also been explored

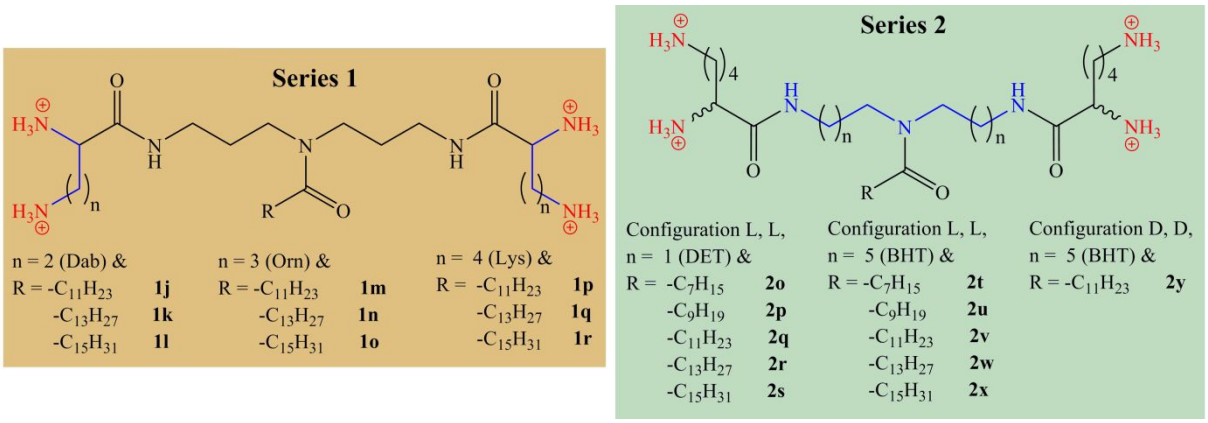


Figure 1. Chemical structure of small molecular antibacterial agents with hydrophobicity modulation between charges.

extensively in designing membrane-active molecules. The concept of facial amphiphilicity in designing membrane-active molecules was directly implemented into arylamide foldamers, urea, phenylene-ethylene oligomers, etc.[16,33] However, later on the concept of facial amphiphilicity was found to be very much design specific and is not applicable to all classes of membrane-active designs. For example, structurally flexible oligo-acyl-lysines that lack facial amphiphilic topology also displayed potent antibacterial activity.[34] Rather than amphiphilic topology, optimum hydrophobic/hydrophilic balance is recognized as a much more applicable concept in designing membrane-selective antibacterial molecules. It has even been seen that optimum hydrophobic/hydrophilic balance plays the major role over conformational stiffness to control antibacterial activity, when both the factors are incorporated in the same design.[18] However; the aspect of molecular arrangement of hydrophobic and hydrophilic groups could be another important concomitant parameter, which remains largely unknown. Here we investigate the role of molecular arrangement in a membrane-active design by modulating hydrophobicity between charges. The molecular design consists of a symmetric triamine in the backbone, where the

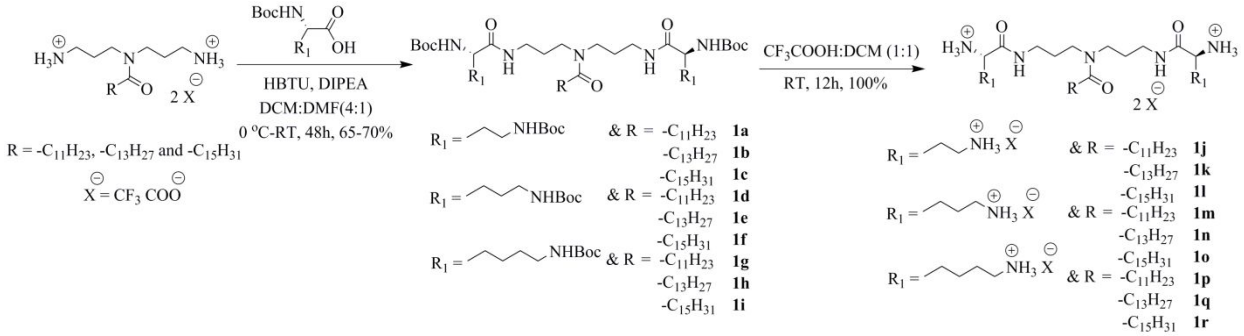
cationic amino acid is conjugated with the two terminal amino groups along with an aliphatic long chain which is attached with the middle secondary amino group. We have reported that molecules with norspermidine (bis(trimethylene)triamine) present in the backbone and consisting of various hydrophobic amino acids including lysine have displayed potent antibacterial efficacy.[22,35,40] Thus, we were interested to further explore this molecular design. In the first series of compounds, the modulation of hydrophobicity was achieved through varying hydrophobicity between α-amino group and the amino group present in side chain of positively charged amino acids (Series 1, Figure 1). In a second series of compounds, the backbone hydrophobicity of the design was varied by incorporating a triamine such as diethylene triamine and bis(hexamethylene)triamine instead of bis(trimethylene)triamine (Series 2, Figure 1). By varying the backbone triamine in the design, we could modulate hydrophobicity between the charges of two amino acids placed at either end of the design. In both the series of compounds, the aliphatic long chain was varied by incorporation of dodecanoyl, tetradecanoyl and hexadecanoyl groups to obtain the optimized compound. Additionally, for the second series of compounds, we also prepared octanoyl and decanoyl analogues to better understand the structure-activity relationship. The antibacterial activity of the compounds was evaluated against both classes of bacteria (Gram-positive and Gram-negative) and the toxicity was tested against human red blood cells (hRBCs). The optimized lead compound was then obtained by determining the selectivity index (HC_{50}/MIC). The antibacterial efficacy of the lead compound was evaluated further by performing additional experiments such as cytotoxicity against mammalian cells (HEK cell line), time-kill kinetics, activity against metabolically inactive bacteria (such as stationary and persister cells) and membrane-active mode of actions. The antibiofilm ability was investigated against established biofilms and resistance development

studies were performed to show the long-lasting antibacterial activity of this compound. The *in-vivo* antibacterial activity was examined in a mouse model of skin-infection and toxicity was evaluated in acute dermal model in mice. Finally, the potency of the compound was evaluated in an *ex-vivo* model of human skin-infection.

RESULTS AND DISCUSSION

Design and Synthesis: In order to understand how molecular arrangement plays a role in dictating selective antibacterial activity, two series of compounds were synthesized by varying the positively charged amino acid (Series 1) and the backbone triamine of the design (Series 2). An aliphatic long chain in both the series was varied in order to tune the hydrophobicity in the molecules. In the first series, a total of nine compounds (**1j-1r**) were prepared by incorporating the amino acids diaminobutyric acid (Dab), ornithine (Orn) and lysine (Lys) with various aliphatic long chains (Series 1, Figure 1). In the second series of compounds, which were achieved by varying the backbone of the design, the amino acid was kept fixed as lysine (Series 2, Figure 1). In this series, the compounds (**2o-2s**) consisted of diethylene triamine in the

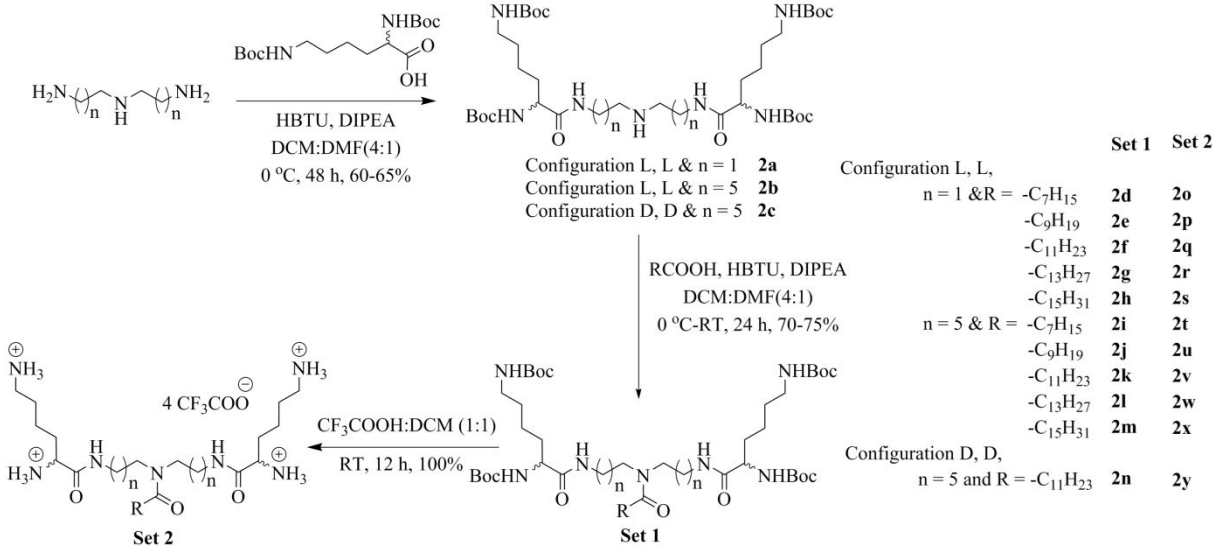
Scheme 1. Synthesis of small molecular antibacterial agents with hydrophobicity modulation in amino acid side chain.



backbone of the molecule, bearing aliphatic long chains varying from octanoyl to hexadecanoyl group. Compounds (**2t-2y**) also possessed varied long chains of octanoyl to hexadecanoyl with bis(hexamethylene)triamine present in the backbone of the molecules. All the compounds consisted of L-configuration of amino acid except the compound **2y**, which bears D-lysine in its structure.

Synthesis of the first series of compounds was achieved by two-step reactions starting

Scheme 2. Synthesis of small molecular antibacterial agents with hydrophobicity modulation in the backbone.



from the precursor amines (Scheme 1). The primary amino groups of the precursor amine were first coupled with carboxylic acid groups of N_α-Boc-N_γ-Boc-L-2,4-diaminobutyric acid, N_α-Boc-N_δ-Boc-L-ornithine or N_α-Boc-N_ε-Boc-L-lysine. In the next step, the Boc-groups were removed by using TFA (trifluoroacetic acid) to achieve the final compounds. The synthesis of the second series of compounds was achieved by three step reactions (Scheme 2). Two primary amino groups of the triamine (diethylene triamine or bis(hexamethylene)triamine) were first coupled

with carboxylic acid group of N_α-Boc-N_ε-Boc-L/D-lysines. In the second step, various aliphatic long chains were attached at the centrally placed secondary amino group by performing another coupling reaction (Scheme 2). Finally, the Boc groups were removed to achieve the final compounds (**2o-2y**). The details of synthesis, characterization, HPLC trace and HPLC purity percentage of the compounds are provided in the experimental section and supporting information (Supporting Information Figure S1-S33).

Antibacterial Activity: To investigate the role of molecular arrangement in guiding the antibacterial activity, the entire library of compounds was tested against various Gram-positive (*S. aureus*, MRSA) and Gram-negative (*E. coli, P. aeruginosa*) bacteria. The antibacterial activity is expressed in terms of MIC (minimum inhibitory concentration) values (Table 1).

Effect of Hydrophobicity Modulation in Amino Acid Side Chain. Analysing the antibacterial activity for the first series of compounds (**1j-1r**), where tunable hydrophobicity was borne out of the variation of amino acid side chains, suggested that decreasing the distance between charges leads to decrease in activity (Table 1). Dab series of compounds (**1j-1l**) were active with the MIC values mostly in the concentration range between 6.3-≥50 μg/mL (except the hexadecanoyl analogue, MIC = 3.1-6.3 μg/mL). Orn homologues (**1m-1o)**, with an additional methylene group in the side chain showed improvement in their antibacterial profile. The compounds exhibited the MIC values in the concentration range of 3.1-25 μg/mL except a value of >50 μg/mL was observed against *E. coli* for dodecanoyl analogue (**1m)**. Unsurprisingly, the Lys bearing compounds (**1p-1r**) that possess the highest hydrophobicity in their side chain showed the best antibacterial activity profile in this series. This becomes much clearer when comparing the MIC values of the lower long chain containing analogues of this series of compounds. While dodecanoyl analogue of Orn and Dab containing compound displayed MIC value of 25->50

μg/mL, corresponding Lys containing compounds displayed MIC values at concentration 12.5-50 μg/mL (except *E. coli*). Along the same line, the tetradecanoyl analogue of Lys containing compound displayed very good activity with the MIC values in the concentration range of 3.1-6.3 μg/mL, while Orn and Dab bearing analogues showed a concentration range of 3.1-25 μg/mL. Overall analysis suggested that Dab series of compounds are the least active, whereas Lys containing compounds, which possess the highest hydrophobicity in the side chain, are the most active. It was noteworthy that the above mentioned principle was not followed by

Table 1. Antibacterial and hemolytic activities of the compounds.

Compounds	Minimum Inhibitory Concentration (μg/mL)				HC_{50} (μg/mL)	SI[a] (HC_{50}/MIC)
	Gram-positive Bacteria		Gram-negative Bacteria			
	S. aureus	MRSA	*E. coli*	*P. aeruginosa*		
1j	25	25	>50	25	>1000	>40
1k	6.3	6.3	50	6.3	170	27
1l	3.1	3.1	6.3	3.1	60	19
1m	25	25	>50	25	>1000	>40
1n	6.3	6.3	25	6.3	305	48
1o	3.1	3.1	3.1	3.1	70	22.5
1p	12.5	12.5	>50	25	>1000	>80
1q	3.1	3.1	6.3	6.3	561	181
1r	3.1	3.1	3.1	3.1	133	43
2o	>50	>50	>50	>50	>1000	>20
2p	>50	>50	>50	>50	>1000	>20
2q	25	50	>50	50	>1000	>20
2r	6.3	6.3	25	12.5	765	121
2s	3.1	3.1	3.1	3.1	90	29
2t	>50	>50	50	50	>1000	>20
2u	12.5	12.5	25	12.5	>1000	>80
2v	3.1	3.1	6.3	3.1	860	277
2w	3.1	3.1	3.1	3.1	253	82
2x	3.1	3.1	3.1	3.1	87	28
2y	3.1	3.1	6.3	3.1	890	287

[a]SI stands for selectivity index that was determined considering MIC values against MRSA.

hexadecanoyl analogue compounds. This probably indicates that beyond an optimum length in lipidated chain, the effect of hydrophobicity variation between charges can no longer be seen due to greater hydrophobic effects from the long aliphatic chain in the design.

Effect of Hydrophobicity Modulation in the Backbone. Structure-activity analysis suggested that Lys containing compounds are the most active compounds in the series. We therefore moved forward with investigating the effect of backbone variation for this amino acid in particular. Overall, we saw that antibacterial activity is highly dependent on backbone hydrophobicity in the molecules. An improved antibacterial activity was found upon increasing the hydrophobicity in backbone from bis(trimethylene)triamine (six methylene groups) to bis(hexamethylene)triamine (twelve methylene groups) and it was compromised with decreased hydrophobic backbone of diethylene triamine (four methylene groups). The shortest long chain bearing compound (octanoyl analogue), **2t**, with highest backbone hydrophobicity (bis(hexamethylene)triamine) did not display much antibacterial activity. However, a moderate activity was observed for the next compound, **2u**, in this series that consisted of decanoyl long chain. Further increasing the long chain to dodecanoyl group, the resulting compound **2v** and its corresponding D-isomeric compound **2y** displayed excellent antibacterial activity. They were active with MIC values of 3.1-6.3 μg/mL. In comparison, the dodecanoyl analogue compounds with lesser backbone hydrophobicity, such as bis(trimethylene)triamine (**1p**) and diethylene triamine (**2q**) exhibited very limited antibacterial activity, which highlights the role of backbone hydrophobicity in dictating antibacterial activity. This role of backbone hydrophobicity was also reproducible in the tetradecanoyl derivatives. The tetradecanoyl compound with diethylene triamine and bis(trimethylene)triamine backbone showed MIC values in the concentration range of 6.3-25 μg/mL and 3.1-6.3 μg/mL respectively, whereas the analogue compound with

bis(hexamethylene)triamine backbone displayed the activity with MIC value of 3.1 μg/mL. Like the previous case, here too, we observed that the role of backbone hydrophobicity became unseen in case of the long lipidated derivatives. Table 1 illustrates that the hexadecanoyl analogue compounds of varied hydrophobic backbone **1r**, **2s**, **2x** displayed the MIC value of 3.1 μg/mL against all the bacteria tested. Collectively the results suggest that hydrophobicity between charges indeed plays an important role in membrane active design with shorter lipidated chain. However, this appears unseen for the higher lipidated analogues, where increased hydrophobic interaction brought about by long lipophilic moiety is guiding the antibacterial activity to a greater extent.

Hemolytic Activity and Selectivity: Preliminary toxicity study was performed by evaluating the hemolytic activity of the compounds. In order to quantify the hemolytic activity, the HC_{50} (defined as compound concentration corresponding to 50% lysis of the hRBCs) values were determined (presented in table 1).

Effect of Hydrophobicity Modulation in Amino Acid Side Chain. An overall observation suggested that hemolytic activity was highly dependent on hydrophobicity variation in the amino acid side chain. Analysing the results for the compounds (**1j-1r**), it was found that dodecanoyl analogues of Dab, Orn and Lys bearing compounds were completely non-toxic even at 1000 μg/mL. However, the effect of side chain hydrophobicity on hemolytic activity was observed for tetradecanoyl and hexadecanoyl derivatives. While the tetradecanoyl derivative of Dab and Orn containing compounds displayed HC_{50} values 170 μg/mL and 305 μg/mL, respectively, the Lys bearing compound showed a higher value of 561 μg/mL. Similarly, the hexadecanoyl derivative of Lys containing compound displayed a higher HC_{50} value of 133 μg/mL compared to Dab and

Orn bearing compounds that showed the values of 60 μg/mL and 70 μg/mL, respectively. Taken together the results suggest that the Lys series of compounds which possess higher side chain hydrophobicity are the least toxic compared to Dab and Orn bearing compounds that possess lesser hydrophobicity in amino acid side chain. This was reflected markedly in the selectivity index as determined by the ratio of HC_{50} and MIC values. The tetradecanoyl derivative of Lys consisting compound displayed a selectivity index of 181, whereas the corresponding Orn and Dab derivatives showed much lesser values of 48 and 27 respectively (Table 1). Thus, Lys series of compounds were highly selective compared to Dab and Orn comprising compounds.

Effect of Hydrophobicity Modulation in the Backbone. Hemolytic activity was also found to be dependent on backbone hydrophobicity. Overall analysis suggested that shorter long chain containing compounds (such as octanoyl and decanoyl derivatives) were completely non-toxic (HC_{50} value >1000 μg/mL), whereas hexadecanoyl derivatives, **2s** and **2x** were relatively toxic and displayed HC_{50} values of 87 and 90 μg/mL, respectively. The dodecanoyl and tetradecanoyl derivatives with decreased hydrophobic backbone (diethylene triamine) displayed HC_{50} values of >1000 μg/mL and 765 μg/mL, respectively. In comparison, the tetradecanoyl derivative with increased hydrophobic backbone (bis(hexamethylene)triamine), **2w**, showed a HC_{50} value of 253 μg/mL, and the dodecanoyl analogue compound (**2v**) displayed a value of 860 μg/mL. Therefore, a general analysis suggested that an increase in backbone hydrophobicity resulted in a slight increase in hemolytic activity. However, the simultaneous increasing effect on antibacterial activity leads to high selectivity index as compared to the analogue compounds with decreased backbone hydrophobicity. The dodecanoyl derivative (**2v)** and its corresponding D-isomeric compound, **2y**, displayed the highest selectivity index of 277 and 287 respectively, when

calculated for MRSA (Table 1). Combined together, the results therefore suggested that an increase in hydrophobicity between charges leads to selective antibacterial compounds.

Cytotoxicity against HEK Cells: To further investigate the selectivity of the lead compound **2y**, toxicity was determined against HEK cells by both MTT assay and live-dead assay through fluorescence microscopy. Both the experiments suggested that the compound was not toxic even at a concentration of 40 µg/mL (6-13 folds higher as compared to MIC values). In Figure 2, the

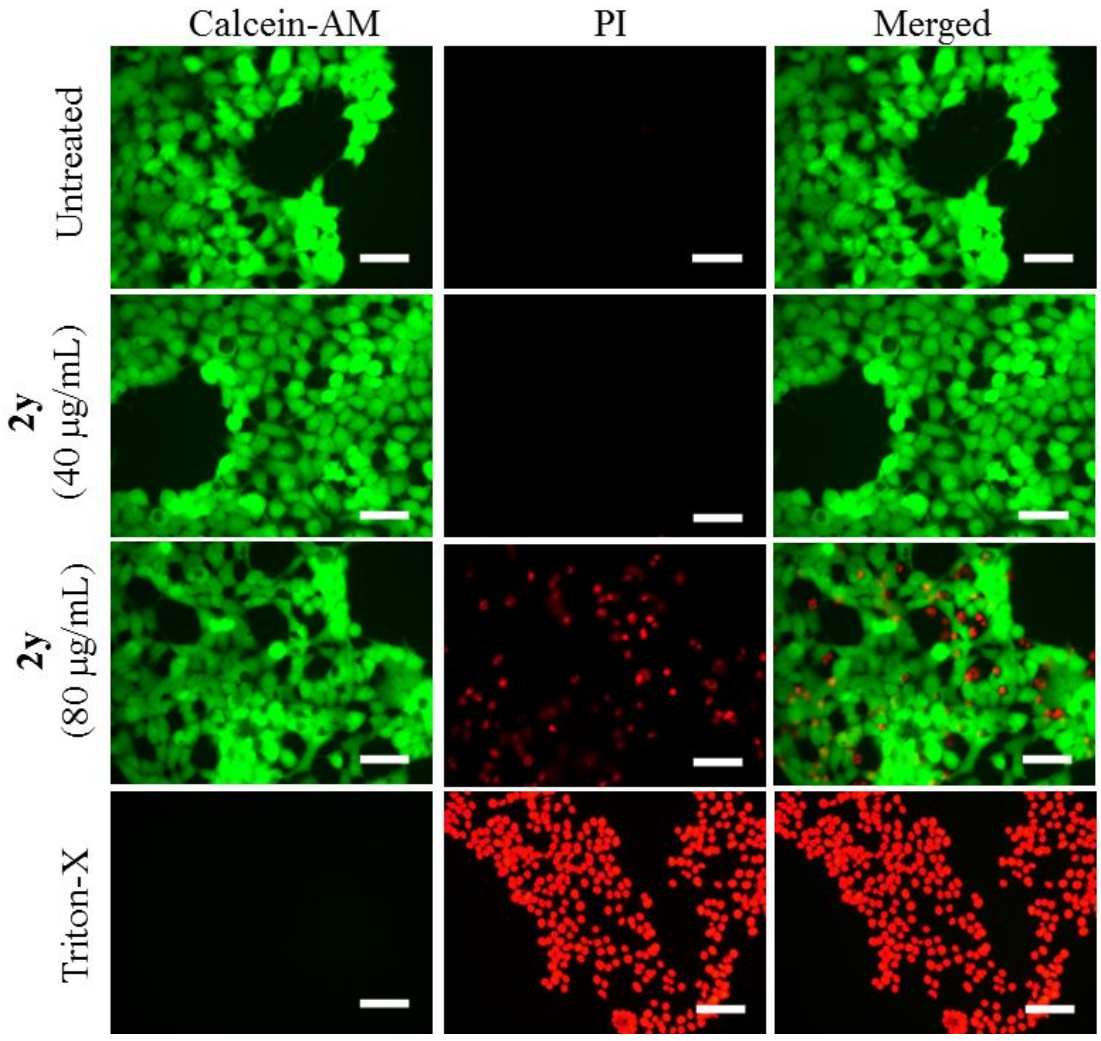


Figure 2. Fluorescence microscopy images of HEK cells under different conditions. Scale bar: 50 µm.

live-dead assay suggested that there were no dead cells (corresponding to red fluorescence), in complete agreement with the MTT assay which showed 100 % cell viability at this concentration (Supporting Information Figure S34a). However, at the next higher concentration of 80 μg/mL, the compound displayed slight toxicity. To have a quantitative readout, the EC_{50} value (concentration of the compound at which 50% of the treated cells remain viable) was determined. The EC_{50} value was found to be 85 μg/mL (Supporting Information Figure S34b), which was significantly higher (13-27 fold) compared to the concentration at which it displayed antibacterial activity.

Time-kill Kinetics: The time kill-kinetics of **2y** was performed against both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Complete killing (>5 log CFU/mL reduction) of *S. aureus* was found within 180 and 360 min at 25 μg/mL and 12.5 μg/mL, respectively (Supporting Information Figure 35a). Although the compound displayed comparatively slower killing kinetics against *E. coli*, around 3 log reduction (corresponds to bactericidal activity) in cell viability was observed within 360 min of treatment with 50 μg/mL of compound (Supporting Information Figure 35b). Taken together, the results illustrated the bactericidal nature of the compound against both of the bacteria. Moreover, the faster rate of killing of *S. aureus* than *E. coli* probably indicated the better interaction of the compound with Gram-positive bacteria, which are known to possess a much simpler cell envelope structure compared to Gram-negative ones.[40]

Antibacterial Activity against Non-dividing Dormant Bacteria: Although conventional antibiotics are capable of killing growing bacterial cells, they remain ineffective against non-

dividing dormant bacteria. Therefore, new antibacterial agents that would also be active against these slow or non-growing bacteria are highly sought after. To this end, we have investigated the killing efficacy of optimized compound, **2y**, against metabolically inactive stationary and persister cells of *S. aureus* as well as *E. coli*. The dormant cells were created by following a reported protocol and the details are provided in the experimental section.[35] At first, the midlog phase dividing bacterial cells were allowed to grow to non-dividing stationary phase cells. The persister bacterial cells were then prepared from stationary phase culture by treating with ampicillin sodium. The efficacy of the test compound was compared with conventional antibiotics ampicillin (for both *S. aureus* and *E. coli*), vancomycin (for *S aureus*) and

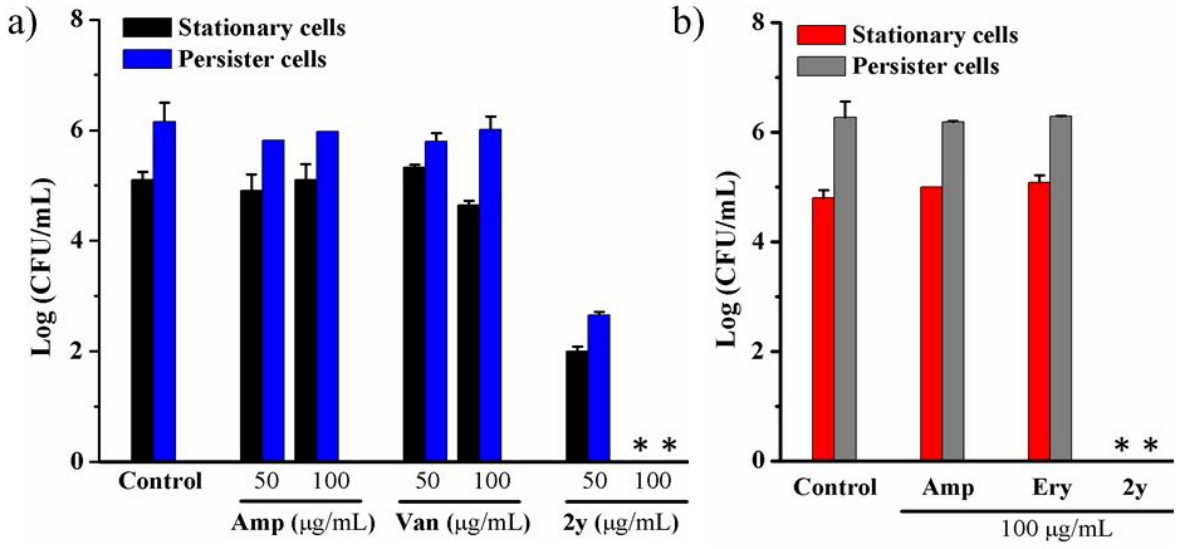


Figure 3. Antibacterial efficacy against stationary and persister cells: (a) antibacterial activity against *S. aureus*, (b) antibacterial activity against *E. coli*. Potency of the compound **2y** was compared with the antibiotics Amp (for both *S. aureus* and *E. coli*), Van (for *S. aureus*) and Ery (for *E. coli*). In the figure Amp, Van and Ery represents Ampicillin, Vancomycin and Erythromycin respectively and the symbol asterisks (*) correspond to <50 CFU/mL.

erythromycin (for *E. coli*). The results suggested that the test compound, **2y**, was capable of killing dormant cells of both *S. aureus* and *E. coli*, whereas all the control antibiotics remained ineffective against such dormant bacterial cells. This clearly illustrated the effectiveness of this new antibacterial compound over conventional antibiotics. Figure 3a demonstrates that compound **2y** not only reduced bacterial viability (~ 3 log reduction at concentration of 50 μg/mL), but was also capable of completely killing the stationary (~ 5 log reduction) and persister (~ 6 log reduction) *S. aureus* within 2 h at a concentration of 100 μg/mL. In comparison, ampicillin and vancomycin (a last-resort antibiotic against Gram-positive infections) remained completely ineffective even at the concentration of 100 μg/mL. Furthermore, compound **2y** also displayed high efficacy against the stationary and persister cells of *E. coli*. Complete killing of bacterial cells (~ 5 log reductions in stationary and ~ 6 log reductions in persister viability) was observed upon treatment with 100 μg/mL of test compound (Figure 3b). Here too, the control antibiotics ampicillin and erythromycin remained completely ineffective at this concentration. Taken together the results therefore suggested enormous potential of **2y** to treat metabolically inactive dormant bacteria against which conventional antibiotics fail to show any activity.

Membrane Depolarization of Growing Planktonic and Non-dividing Dormant Bacteria: An optimum membrane potential is crucial for the functioning of various biological processes in bacteria. Therefore, a perturbation in bacterial membrane potential can lead to death of bacterial cells. Membrane targeting mode of action against both growing planktonic as well as metabolically inactive stationary and persister cells was hence investigated by performing membrane depolarization assay. Compound **2y** was assayed against all such metabolically

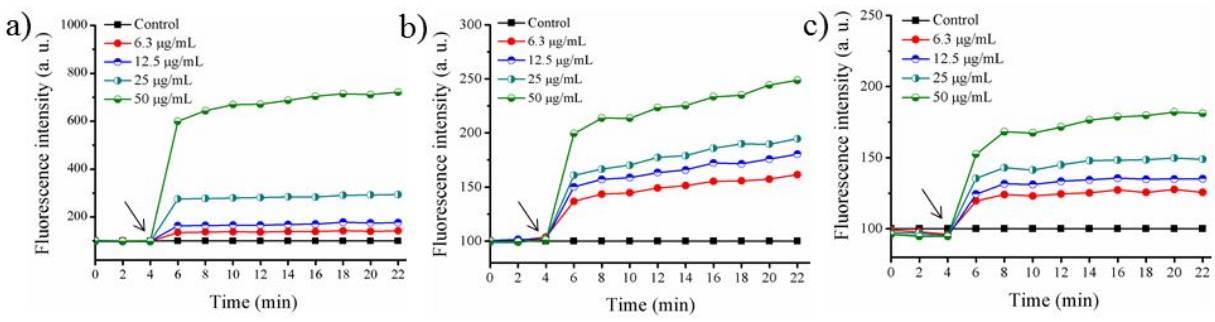


Figure 4. Membrane depolarization of metabolically distinct *S. aureus* cells; (a) Growing planktonic cells, (b) Stationary phase cells, (c) Persister cells. Studies were performed by using the membrane potential sensitive dye $DiSC_3$ (5) and the arrow in the figures corresponds to addition of compound.

distinct cells. The dye $DiSC_3$ (5) was used to monitor the effect of compound on bacterial membrane potential. $DiSC_3$ (5) distributes inside bacterial cells based on the normal bacterial membrane potential, but any disruption of this normal potential results in release of dye into the external medium that leads to an increase in fluorescence intensity. Our results suggested that test compound **2y** showed appreciable destabilization in membrane potential irrespective of the metabolic state of both *S. aureus* and *E. coli* cells (Figure 4 and Supporting Information Figure S36). An increase in fluorescence was observed even at a concentration of 6.3 μg/mL, and importantly a dose-dependent enhancement in intensity was seen with increase in the compound concentration (Figure 4). Furthermore, the propidium iodide (PI) assay revealed that the test compound, **2y**, does not show a significant membrane permeabilization effect (Figure S37). Taken together, the results therefore suggested that this class of compounds act on metabolically distinct bacterial cells primarily by destabilizing their membrane potential.

Resistance Studies: In this current state of affairs where there is a rapid emergence of antibiotic resistance, it is imperative to develop new antibacterial agents possessing long-lasting efficacy. Therefore, propensity of resistance development against **2y** was investigated for both *S. aureus*

(Gram-positive representative) as well as *E. coli* (Gram-negative representative). Results clearly suggested that bacteria were unable to develop resistance against this new compound even after 14 subsequent passages. The MIC values of the test compound remained almost unchanged over the experimental time span (Supporting Information Figure S38). On the contrary, *S. aureus* and *E. coli* were observed to develop high level of resistance against the antibiotics norfloxacin and colistin, respectively.[22,35] Taken together, these results firmly suggested that both classes of bacteria find difficulties in developing resistance against this new compound, thereby highlighting the long-lasting antibacterial efficacy over conventional antibiotics.

Anti-biofilm Activity: Biofilm associated infection is one of the major threats faced by public health today. According to the current scenario, 70-80% of infections are in association with biofilm formation, and are usually difficult to treat with conventional antibiotic therapy.[36] MRSA is one such dangerous bacterium, which shows a high propensity for biofilm formation. A major percentage of community as well as hospital acquired infections are caused by MRSA.[37,38] The World Health Organization (WHO) has unsurprisingly categorised MRSA as one of the critical bacteria, against which new antibacterial agents need to be developed urgently.[39] Hence, we investigated the efficacy of the optimized compound **2y** as an anti-biofilm agent to tackle MRSA infections. The biofilm disruption ability of the compound was measured by crystal-violet (CV) staining and the efficacy was compared with the anti-MRSA antibiotics fusidic acid and vancomycin (a last resort antibiotic for MRSA). The results suggested that the compound **2y** could eradicate a significant amount of the established biofilm, whereas fusidic acid and vancomycin hardly displayed any efficacy (Figure 5a). Compound **2y** displayed about 40% and 80% reduction in biofilm biomass at 20μg/mL and 40μg/mL respectively, whereas control

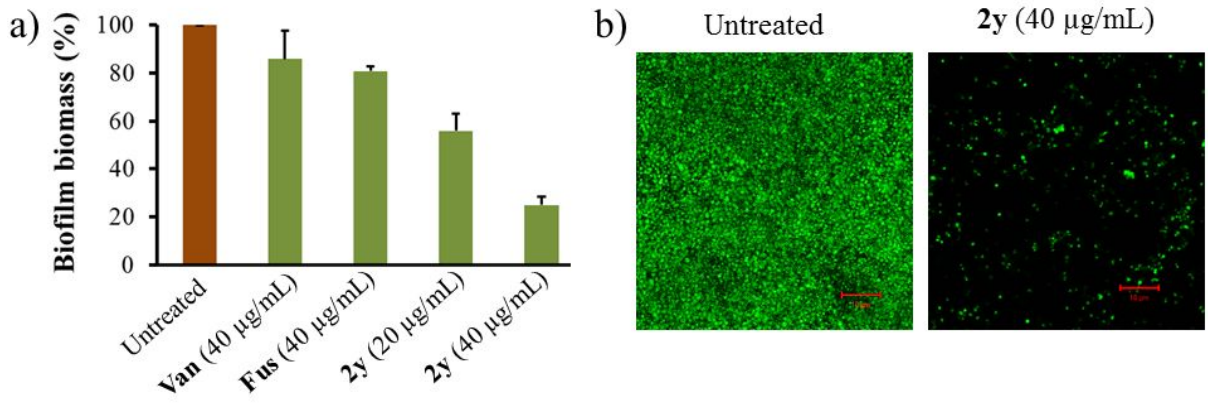


Figure 5. (a) Quantification of biofilms disruption through CV staining. In figure Van and Fus represent the antibiotics Vancomycin and Fusidic acid, respectively, (b) Confocal laser scanning microscopy (CLSM) images of MRSA biofilms after staining with SYTO-9. The scale bar is 10 µm in the figure.

antibiotics showed around 10-20% reduction at 40µg/mL. Further, the extent of biofilm disruption was visualized through confocal laser scanning microscopy (CLSM), where the biofilms were stained with the dye Syto-9, a fluorescent nucleic acid stain. Compared to the untreated control, the compound treated sample possesses lesser green fluorescence, which confirmed that the compound has significant biofilm eradication ability.

***In-vivo* Efficacy in Mice Model:** In order to evaluate the efficacy of **2y** as a topical antibacterial agent, toxicity was evaluated in mice by performing acute dermal toxicity studies. A dose of 200 mg/kg was used for this study. After exposure of the test compound, the treated mice group was kept under careful observation for 14 consecutive days. It was found that the compound did not induce any kind of adverse effects such as irritation, tremors, convulsions, salivation and so

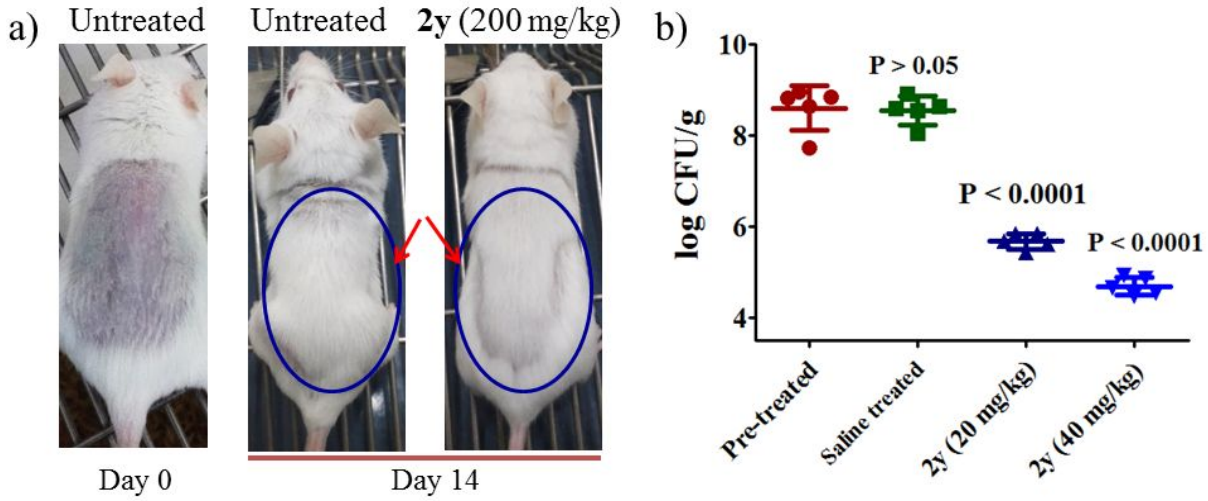


Figure 6. (a) Photographic images of the mice after 14 days of compound exposure. Blue circle indicated normal fur growth in mice for both untreated as well as compound treated case, (b) Bacterial titer in mice model of superficial skin-infection.

on. Additionally, normal fur growth was observed, similar to untreated group of mice further indicating nontoxic nature of the compound (Figure 6a). Thus, the results suggest that this compound has potential for development as an agent for treatment of topical infections.

The *in-vivo* activity of **2y** was then evaluated in a mouse model of MRSA skin infection. Two groups of infected mice received either 20 mg/kg or 40 mg/kg of the compound treatment (40 µL, applied topically), whereas saline was administered to the control group of mice. After four days of continuous treatment (four doses: 24 h interval), the lead compound reduced bacterial burden by 2.8 log and 3.9 log at 20 mg/kg and 40 mg/kg, respectively (Figure 6b). Encouragingly, the test compound not only demonstrated activity under *in-vitro* scenario, but was also found to show excellent *in-vivo* activity. These results suggest that compound **2y** shows significant promise as an agent for treating MRSA skin-infections.

Toxicity in *Ex-vivo* Model of Human Skin: To elucidate the real possibility of using compound **2y** as a topical antibacterial agent, efficacy was tested in human skin. The toxicity of compound was first evaluated towards human skin *ex-vivo*. At first, models of human skin was engineered *ex-vivo* by following established protocol.[43] The models were cut into 4 equal pieces and then fully submerged in presence of compound **2y** for 24 h at concentrations from 20 µg/mL to 5000

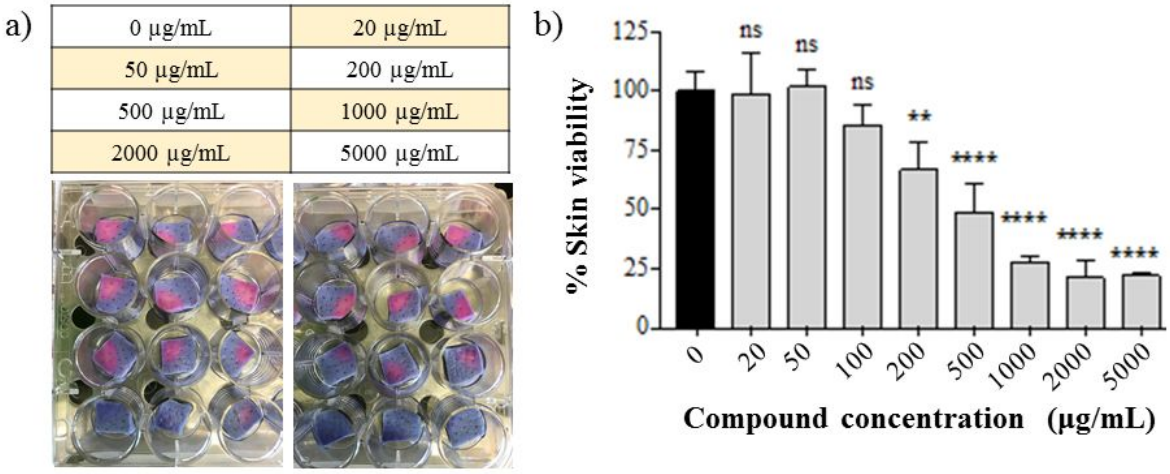


Figure 7. Toxicity of compound **2y** in *ex-vivo* model of human skin after 24 h exposure. (a) Photographic images showing skin post-staining with alamar blue. Blue, dead tissue or decellularised dermis. Pink, viable tissue. (b) Quantification of skin viability, where data is expressed as % viability to untreated control (0 µg/ml). *n*=3. One-way ANOVA (*vs.* untreated control).

µg/mL. Treatment with compound **2y** revealed a dose-dependent toxicity response (Figure 7). Concentrations of up to 100 µg/mL did not cause any significant damage or reduce the tissue viability, as quantified by alamar blue assay. Concentrations between 20-100 µg/mL were therefore selected for testing against MRSA-infected *ex-vivo* skin.

Antibacterial Activity in *Ex-vivo* Model of Human Skin Infection: Antibacterial activity of compound **2y** was evaluated in *ex-vivo* model of MRSA-infected human skin at non-toxic concentrations of ≤100 μg/mL. Skin was treated with single or multiple doses (up to 3) of compound **2y** or vancomycin at 24 h intervals (Figure 8a-c). Although, vancomycin was found to be highly effective at higher concentration such as 8 μg/mL and 16 μg/mL (Figure 8a), no significant activity was observed at 4 μg/mL, regardless of the number of doses/length of

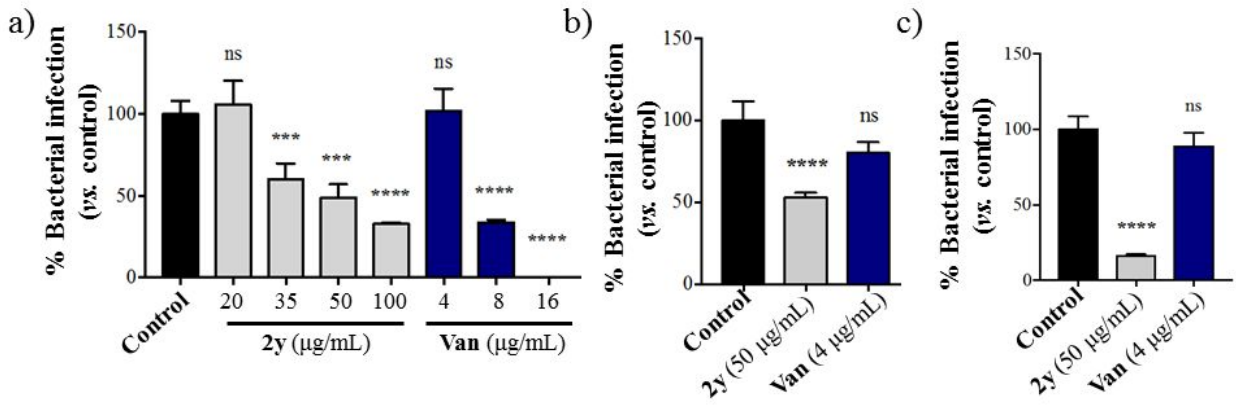


Figure 8. Antibacterial activity of **2y** in *ex-vivo* model of human skin infection. MRSA-infected skin was treated with 1 dose (a), 2 doses (b), or 3 doses (c) of **2y** (MIC = 12.4 μg/mL) or Van (vancomycin, MIC = 0.8 μg/mL) at 24 h intervals. Data (CFU per mg skin) is expressed as % bacterial infection to untreated control, $n = 3$, one-way ANOVA (*vs.* control).

treatment. Our test compound, **2y**, also revealed significant efficacy, with a single dose of 100 μg/mL showing comparable reduction to vancomycin at 8 μg/mL. Treatment with compound **2y** demonstrated a dose-dependent reduction in bacterial infection (Figure 8a). After 3 doses, the compound was able to reduce bacterial infection by 85% at 50 μg/mL (Figure 8c). Taken together the results therefore indicated real possibility of this newly developed compound to be developed as antibacterial agent to treat skin infections.

CONCLUSIONS

To summarize, a series of small molecules were developed by varying spatial distribution of hydrophobic groups in a membrane-active design. Antibacterial and toxicity studies revealed that some of the compounds were selective towards bacterial killing over mammalian cells, with insights into the role of molecular arrangement of hydrophobicity. This finding highlighting the role of molecular arrangement of hydrophobicity will have significant insight to design non-toxic membrane-active molecules with high antibacterial potency. The lead compound was rapidly bactericidal against growing planktonic bacteria and also completely eradicates their stationary and persister cells. The compound also displayed potent antibiofilm activity and bacteria were unable to develop resistance against this compound even after 14 continuous passages. More importantly, it displayed *in-vivo* activity with significant reduction of bacterial burden without any sign of toxicity in mice and revealed good antibacterial efficacy in *ex-vivo* model of human skin-infections. Thus, the lead molecule bears potential to be developed as an antibacterial agent to treat skin infections.

EXPERIMENTAL SECTION

Antibacterial Assay: The assay was performed by following our reported protocol.[40-42] Detailed experimental protocol is provided in supporting information.

Hemolytic Assay: The details of experiment is provided in supporting information.

Toxicity study against HEK cells:

Live-dead assay and cell morphology analysis through bright filed images.[42] Briefly, human embryo kidney (HEK 293) cells were seeded into 96-well plate in complete DMEM media

(supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin) and grown to 70-80% confluency. After that, the cells were treated with 40 μg/mL and 80 μg/mL of compound **2y**. Two control experiments were also performed, while in one case cells were treated with media containing no compound (untreated) and for the other case cells were treated with 0.1 vol % Triton X-100 solution. The plate was then incubated for 24 h at 37 ºC under 5% CO_2 atmosphere. At the end, the media was discarded and cells were washed once with 1X PBS and stained with calcein AM (2 μM, Fluka) and propidium iodide (PI, 4.5 μM) (Sigma-Aldrich) for 15 min. After that, the dye containing solution was discarded and the cells were washed with 1X PBS to remove the excess dyes. The images of the cells were then captured with a 40X objective in a Leica DM2500 fluorescence microscope. A band-pass filter for calcein AM at 500-550 nm and a long-pass filter for PI at 590-800 nm were used while imaging. For bright field images another experiment was performed by following the same protocol as mentioned above, where the images of the cells directly were captured after 24 h of compound treatment instead of staining with dyes.

MTT assay. The experiment was performed by following established protocol with slight modification.[40] Briefly, the HEK cells seeded into 96-well plate in complete DMEM media (supplemented with 10% fetal bovine serum and 5% penicillin-streptomycin) and grown to 70-80% confluency. After that the medium was diacarded and various concentration of test compound **2y** diluted in DMEM media was added to the cells. Both the negative and positive controls were included in the experiment. As the negative control, same volume of media was added and for the positive control 0.1 vol % of Triton X-100 was added instead of compound. The treated plate was then incubated for 24 h at 37 °C under 5% CO_2 atmosphere. At the end, the 10 μL of MTT solution (5 mg/mL concentration) was added to wells and incubated for another 4

h. After that, it was centrifuged at 1100 rpm for 5 min and the supernatant was discarded and washed with 1X PBS. 100 µL of DMSO was then added to solubilize formazan crystals formed due to reduction of MTT. Finally, the optical density (OD) of the plate was then recorded at 570 nm and the percentage of cell viability was calculated using the following equation: Cell viability (%) = $(A_t\text{-}A_{TXt})/(A_{nt}\text{-}A_{TXt})\times100$; where A_t corresponds to OD of treated sample, A_{nt} for non-treated negative control and A_{TXt} OD of Triton X-100 treated positive control. Each concentration had triplicate values and the average data was plotted against concentration followed by fitted with sigmoidal plot. From the curve the concentration corresponds to 50% cell viability was determined to be EC_{50} value.

Time-Kill Kinetics: Time kill kinetics of optimized compound **2y** was investigated against both *S. aureus* as well as *E. coli*. Briefly, the test compound of required concentartion was prepared in autoclaved milipore water and 50 µL was added to the wells of 96-well plate. Then, 6 h grown mid-log phase bacterial culture was diluted to ~10^6 CFU/mL in nutrient broth and 150 µL of these suspension were added to the compound containing wells. In case of control experiment same volume of autoclaved milipore water was added instead of compound solution. After that, 20 µL of aliquots from that solution were 10-fold serially diluted in 0.9 % saline at different time intervals (0, 30, 60, 180 and 360 min). 20 µL of these serially diluted dilutions were then spot plated on agar plates and incubated for 24 h at 37 ºC. Colonies were then counted and bacterial cell viability was presented in logarithmic scale.

Antibacterial activity against dormant bacterial cells: Preparation of stationary and persister cells was achieved by established protocol.[35] Briefly, 6 h grown midlog phase culture of *S. aureus* and *E. coli* were diluted to 1:1000 in nutrient and LB broth and allowed to grow for 16 h

to atain the stationary phase. To prepare persister cells, stationary phase culture was treated with ampicillin sodium (100 μg/mL for *S. aureus* and 300 μg/mL for *E. coli*) of for 3h at 37 °C. These stationary and persister bacterial culture were centrifused at 9000 rpm for 2 min, and washed with MEM (*S. aureus*) or M9 media (*E. coli*). It was then resuspended and diluted to ~$10^{5\text{-}6}$ CFU/mL in MEM (*S. aureus*) or M9 media (*E. coli*). 150 μL of these bacterial suspension were then added to the 96-well plate containing 50 μL of test compound **2y** and control antibiotics in respective medium. In case of the control experiment, same volume of respective media was added instead of any antibacterial agents. The plate was then incubated at 37 ºC for 2 h under shaking. At the end, the bacterial suspension was serially diluted as 10-fold in saline and 20 μL of the diluted solutions were spot-plated on agar plates and incubated for 48 h at 37 ºC. Finally, to determine cell viability bacterial colonies were counted and result was represented as Log CFU/mL.

Membrane depolarization and permeabilization assay: Reported experimental protocol was followed with little modification.[41] Detailes are provided in the supporting information.

Resistance studies: Resistance study was performed by following reported experimental protocol.[35] At first, the MIC values of **2y** was determined against both the *S. aureus* and *E. coli* by following the experimental protocol as described in antibacterial assay. For the next day, the second MIC experiment was performed for the compound where the bacterial suspension was prepared from the grown bacteria at sub-MIC (MIC/2) concentration of first MIC experiment. Next day then third MIC experiment was performed same as the previous experiment by taking bacterial suspension from sub-MIC concentration of second MIC experiment. This process was repeated for total of 14 contineous day. At the end of the experiment, the fold of increase in MIC values were then determined and plotted it against the number of days.

Quantification and visualization of biofilm disruption: At first, MRSA biofilms were grown on glass coverslip by following reported protocol.[41] Briefly, midlog phase MRSA culture (~10^8 CFU/mL) was diluted to ~10^5 CFU/mL in nutrient broth supplemented with 1% glucose and 1% NaCl. 2 mL of the bacterial suspension was then added into 6 well plate containing a sterile 18 mm of sterile glass coverslip. Biofilm was the allowed to grow on coverslip for 24 h under stationary condition at 37 ºC. After that, the coverslips consisting of biofilms were carefully washed with 1X PBS and placed into the wells of the 6-well plate containing 2 mL of the various concentration of **2y** and control antibiotics (diluted in the complete biofilm media). In case of untreated control experiment the same volume of respective medium was added instead of any antibacterial agents. The plate was incubated at 37 ºC for 24 h under stationary condition. At the end, the coverslips were washed with 1X PBS and placed into wells of another 6-well plate and allowed to dry. After that, the disrupted biofilms contained coverslips were stained with crystal-violet (2 mL of 0.1% w/v solution prepared in sterile milipore water) for 5-10 min. The stained biofilms were washed with 1X PBS and placed into the wells of another 6-well plate and 2 mL of 95% ethanol in water was added to solubilize the stained CV. Finally, the OD of the solution at 520 nm was recorded by using plate reader and percentage of biofilm biomass was calculated by considering 100 percentage for untreated control. To visualize the extent of biofilm disruption by the test compound **2y**, experiment was performed by following the same protocol. Here the only difference was that the disrupted biofilms were stained with the dye syto-9 instead of crystal-violet and images were captured by using confocal laser scanning microscopy.

Acute dermal toxicity:[41] *In-vivo* toxicity was evaluated by performing acute dermal toxicity experiment. Briefly, female BALB/c mice (6-8 weeks-old) of body weight 18-22g were randomly divided in two groups having five mice in each. The mice were anesthetized with

intraperitoneal injection of 150 μL of xylazine-ketamine cocktail. In the anesthetic condition, the fur from the back of the mice (~1/10th of total body surface) was removed without any injury on the skin. After 24 h, the compound 2y (200 mg/kg) was applied in the shaved area on skin of one group of mice. The remaining group was left untreated as the control experiment. The treated group mice were kept under continuous observation for 2 h to monitor if compound exposure results any abnormal behavior of mice. After that, both treated as well as untreated group of mice were kept under observed for a period of 14 days with specific attention towards furs regrowth in the shaved area.

***In-vivo* activity in mice model of MRSA skin-infections:** *In-vivo* anti-MRSA activity of compound **2y** was evaluated by following a reported experimental protocol.[41] Briefly, BALB/c mice (female, 6-8 weeks-old) in the range of 18-22 g of body weight were randomly divided in 4 groups with five mice in each. Mice were then rendered neutropenic by intraperitoneally injection of two doses of cyclophosphamide, the doses of 150 mg/kg and 100 mg/kg were administrated four days and one day before the experiment. The mice were then anesthetized by intraperitoneal injection of 150 μL of xylazine-ketamine cocktail solution. In the anesthetized condition, the fur on the back of the mice was removed by using a trimmer followed by they were shaving using a razor. Mice were shaved such that a wound could be created, which was clearly observed as ruptured and damaged area on the skin. The mice were then infected at the wound by placing 20 μL of freshly prepared MRSA suspension in sterile saline (~10^7 CFU of bacteria per mice). The infected mice were then left untreated for 24 h to allow MRSA biofilm formation on mice skin. The bacteria in the biofilm of infection site was quantifyed by sacrificing one group of mice. The tissue sample at the infection site was collected in 10 mL of sterile saline and homogenized. The suspension was then serially diluted by 10-fold in sterile

saline. After that, each dilution was spot plated on agar plate and incubated at 37 °C. At the end of 24 h, bacterial colonies were counted and results were expressed as log CFU/g of weight of the tissue collected from mice. However, the two groups of mice out of three were treated with either 20 mg/kg or 40 mg/kg of compound **2y** (40 µL, applied topically) and the remaining group was treated with same volume of saline. All the groups of mice were treated with 4 doses of compounds or saline at 24 h interval. 24 h after the last dose (at day 5) all the mice were sacrificed and tissue sample was collected and bacterial cell viability was determined as mentioned for the pre-treatment sample.

Ethics statement for *ex-vivo* studies: All work using human keratinocytes and fibroblasts was performed on samples from abdominoplasty or breast reduction surgeries. Participants provided written informed consent to donate the skin, which was stored in the patient's clinical notes. The protocol and consent form were approved by the Sheffield NHS Trust, ethics committee (reference number 15/YH/0177). Tissues and cells were stored for a maximum of 1 week, and used on an anonymous basis.

Generation of *ex-vivo* skin models: Skin constructs were produced as previously described.[43] Briefly, human dermal fibroblasts and keratinocytes were isolated from split-thickness skin biopsies. Sterile de-epidermised dermis (DED), measuring 2 cm^2, was used as a scaffold. DED was placed in tissue culture wells and a sterile steel ring (with a diameter of 27 mm) was placed on top. Gentle pressure was then applied to obtain a seal. Green's media was added to cover the dermis around, but not submerge, the ring. Keratinocytes (between P1-P2) and fibroblasts (between P5-P8) were seeded in the centre of the ring at a density of 300,000 and 100,000 cells per model, respectively. Models were incubated for 2 days as a submerged culture. Models were then cultured at an air-liquid interface for 14-18 days. Media was replaced every 2-3 days.

Toxicity in *ex-vivo* skin models: Each human skin model was cut into 4 pieces measuring ~1cm^2 and washed once with PBS. Skin was then transferred to a 24-well plate and exposed to compound, **2y**, at a concentration of 0 to 5 mg/mL for 24h. After incubation, skin was washed twice with PBS and stained with 50 μg/mL Alamar blue for 3h. Aliquots from each well were transferred to a black opaque 96-well plate. Fluorescence was measured at excitation 530nm and emission 590nm.

***Ex vivo* activity in human skin models:** Antimicrobial activity of compound, **2y**, was assessed in the skin models using established protocols.[43] Briefly, human skin models were cut into 4 pieces measuring ~1cm^2. Skin was wounded by burning with a soldering iron at 150ºC for 2 seconds. Skin was then infected with 1x10^7 mid-log phase MRSA (clinical skin isolate obtained from Sheffield Northern General Hospital) for 5h. Infected skin was then washed in PBS and incubated for a further 18h in the presence of compound **(2y)** at concentration of 0-100 μg/mL or vancomycin at 5x-40x MIC (Observed MIC: 0.8μg/mL). After incubation, skin was washed, weighed, cut into 16 pieces, lysed in 10% saponin and the number of CFU was determined. For multiple dose studies, infected skin was treated with 3 doses of compound **2y** (50 μg/mL), vancomycin (5xMIC) or media only at 24h intervals. Finally, 24h after the last dose (day 4 of infection), skin was processed as detailed previously.

ASSOCIATED CONTENT

Supporting Information. Details of synthesis and characterization of the compounds, spectral data, HPLC trace of the compounds, table corresponding to HPLC purity, molecular weight,

retention time, figures for cytotoxicity assay, time-kill kinetics and resistance studies. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+91) 80-2208-2565. Fax: (+91) 80-2208-2627. E-mail: jayanta@jncasr.ac.in

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

Dab, diaminobutyric acid; Orn, Ornithine; Lys, Lysine; HBTU, *N,N,N′,N′*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate; $(Boc)_2O$, Di-tert-butyldicarbonate; TFA, trifluoroacetic acid; MRSA, Methicillin-resistant *S. aureus;* MIC, minimum inhibitory concentration; HC_{50}, 50% hemolytic concentration; $DiSC_3(5)$, 3, 3'-dipropylthiadicarbocyanine iodide.

REFERENCES

1. Bush, K.; Courvalin, P.; Dantas, G.; Davies, J.; Eisenstein, B.; Huovinen, P.; Jacoby, G. A.; Kishony, R.; Kreiswirth, B. N.; Kutter, E.; Lerner, S. A.; Levy, S.; Lewis, K.; Lomovskaya, O.; Miller, J. H.; Mobashery, S.; Piddock, L. J. V.; Projan, S.; Thomas, C. M.; Tomasz, A. ; Tulkens, P. M.; Walsh, T. R.; Watson, J. D.; Witkowski, J.; Witte, W.; Wright, G. D; Yeh, P.; Zgurskaya, H. I. Tackling antibiotic resistance. *Nat. Rev. Microbiol.* **2011**, *9*, 894-896.
2. Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. Bad Bugs, No Drugs: No ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **2009**, *48*, 1-12.
3. Stewart, P. S.; Costerton, J. W. Antibiotic resistance of bacteria in biofilms. Lancet 2001, 358, 135-138.
4. Parsek, M, R.; Singh, P. K. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol.* **2003**, 57, 677-701.
5. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389-395.
6. Fjell1, C.D.; Hiss, J. A.; Hancock, R. E. W.; Schneider, G. Designing antimicrobial peptides: form follows function. *Nature Rev. Drug Discov.* **2011**, *11***,** 37-51.
7. Kapoor, R.; Wadman, M. W.; Dohm, M. T.; Czyzewski, A. M.; Spormann, A. M.; Barron, A. E. Antimicrobial peptoids are effective against *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **2011**, *55*, 3054-3057.

8. De Zoysa, G. H.; Cameron, A. J.; Hegde, V. V.; Raghothama, S.; Sarojini, V. Antimicrobial peptides with potential for biofilm eradication: synthesis and structure activity relationship studies of battacin peptides. *J. Med. Chem.* **2015**, *58*, 625-639.

9. Dou, X.; Zhu, X.; Wang, J.; Dong, N.; Shan, A. Novel design of heptad amphiphiles to enhance cell selectivity, salt resistance, antibiofilm properties and their membrane-disruptive mechanism. *J. Med. Chem.* **2017**, *60*, 2257-2270.

10. Haug, B. E.; Stensen, W.; Kalaaji, M.; Rekdal, O.; Svendsen, J. S. Synthetic antimicrobial peptidomimetics with therapeutic potential. *J. Med. Chem.* **2008**, *51*, 4306-4314.

11. Patch, J. A.; Barron, A. E. Helical peptoid mimics of magainin-2 amide. *J. Am. Chem. Soc.* **2003**, *125*, 12092-12093.

12. Porter, E. A.; Wang, X.; Lee, H. S.; Weisblum, B.; Gellman, S. H. Non-haemolytic beta-amino-acid oligomers. *Nature* **2000**, *405*, 298-298.

13. Violette, A.; Averlant-Petit, M. C.; Semetey, V.; Hemmerlin, C.; Casimir, R.; Graff, R.; Marraud, M.; Briand, J. P.; Rognan, D.; Guichard, G. *N,N′*-Linked oligoureas as foldamers: chain length requirements for helix formation in protic solvent investigated by circular dichroism, NMR spectroscopy, and molecular dynamics. *J. Am. Chem. Soc.* **2005**, *127*, 2156-2164.

14. Radzishevsky, I. S.; Rotem, S.; Bourdetsky, D.; Navon-Venezia, S.; Carmeli, Y.; Mor, A. Improved antimicrobial peptides based on acyl-lysine oligomers. *Nat. Biotechnol.* **2007**, *25*, 657-659.

15. Barman, S.; Konai, M. M.; Samaddar, S.; Haldar, J. Amino acid conjugated polymers: Antibacterial agents effective against drug-resistant *Acinetobacter baumannii* with no detectable resistance. *ACS Appl. Mater. Interfaces* **2019**, *11*, 33559-33572.

16. Choi, S.; Isaacs, A.; Clements, D.; Liu, D. H.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 6968-6973.

17. Thaker, H. D.; Cankaya, A.; Scott, R, W.; Tew, G. N. Role of amphiphilicity in the design of synthetic mimics of antimicrobial peptides with gram-negative activity. *ACS Med. Chem. Lett.* **2013**, *4*, 481-485.

18. Thaker, H. D.; Sgolastra, F.; Clements, D.; Scott, R. W.; Tew, G. N. Synthetic mimics of antimicrobial peptides from triaryl scaffolds. *J. Med. Chem.* **2011**, *54*, 2241-2254.

19. Chongsiriwatana, N. P.; Miller, T. M.; Wetzler, M.; Vakulenko, S.; Karlsson, A. J.; Palecek, S. P.; Mobashery. S.; Barron, A. E. Short alkylated peptoid mimics of antimicrobial lipopeptides. *Antimicrob. Agents Chemother.* **2011**, *55*, 417-420.

20. Niu, Y.; Padhee, S.; Wu, H.; Bai, G.; Qiao, Q.; Hu, Y.; Harrington, L.; Burda, W. N.; Shaw, L. N.; Cao, C.; Cai, J. Lipo-γ-AApeptides as a new class of potent and broad-spectrum antimicrobial agents. *J. Med. Chem.* **2012**, *55*, 4003-4009.

21. Ghosh, C.; Manjunath, G. B.; Akkapeddi, P.; Yarlagadda, V.; Hoque, J.; Uppu, D. S. S. M.; Konai, M. M.; Haldar, J. Small molecular antibacterial peptoid mimics: The simpler the better! *J. Med. Chem.* **2014**, 57, 1428-1436.

22. Konai, M. M.; Ghosh, C.; Yarlagadda, V.; Samaddar, S.; Haldar, J. Membrane active phenylalanine conjugated lipophilic norspermidine derivatives with selective antibacterial activity. *J. Med. Chem.* **2014**, *57*, 9409 -9423.

23. Hoque, J.; Konai, M. M.; Sequeira, S. S.; Samaddar, S.; Haldar, J. Antibacterial and antibiofilm activity of cationic small molecules with spatial positioning of hydrophobicity: an in vitro and in vivo evaluation. *J. Med. Chem.* **2016**, 59, 10750-10762.

24. Lin, S.; Koh, J. J.; Aung, T. T.; Lim, F.; Li, J.; Zou, H.; Wang, L.; Lakshminarayanan, R.; Verma, C.; Wang, Y.; Tan, D. T.; Cao, D.; Beuerman, R. W.; Ren, L.; Liu, S. Symmetrically substituted xanthone amphiphiles combat gram-positive bacterial resistance with enhanced membrane selectivity. *J. Med. Chem.* **2017**, *60*,1362-1378.

25. Su, M.; Xia, D.; Teng, P.; Nimmagadda, A.; Zhang, C.; Odom, T.; Cao, A.; Hu, Y.; Cai, J. Membrane-active hydantoin derivatives as antibiotic agents. *J. Med. Chem.* **2017**, *60*, 8456-8465.

26. Ilker, M. F.; Nusslein, K.; Tew, G. N.; Coughlin, E. B. Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives. *J. Am. Chem. Soc.* **2004**, *126*, 15870-15875.

27. Mowery, B. P.; Lindner, A. H.; Weisblum, B.; Stahl, S. S.; Gellman, S. H. Structure-activity relationships among random nylon-3 copolymers that mimic antibacterial host-defense peptides. *J. Am. Chem. Soc.* **2009**, 131, 9735-9745.

28. Nederberg, F.; Zhang, Y.; Tan, J. P. K.; Xu, K. J.; Wang, H. Y.; Yang, C.; Gao, S. J.; Guo, X. D.; Fukushima, K.; Li, L. J.; Hedrick, J. L.; Yang, Y. Y. Biodegradable nanostructures with selective lysis of microbial membranes. *Nat. Chem.* **2011**, *3*, 409-414.

29. Wang, J.; Chen, Y. P.; Yao, K.; Wilbon, P. A.; Zhang, W.; Ren, L.; Zhou, J.; Nagarkatti, M.; Wang, C.; Chu, F.; He, X.; Decho, A. W.; Tang, C**.** *Chem. Commun.* **2012**, *48*, 916-918.

30. Uppu, D. S. S. M.; Akkapeddi, P.; Manjunath, G. B.; Yarlagadda, V.; Hoque, J.; Haldar, J. Polymers with tunable side-chain amphiphilicity as non-hemolytic antibacterial agents. *Chem. Commun.* **2013**, *49*, 9389-9391.

31. Thoma, L. M.; Boles, B. R.; Kuroda, K. Cationic methacrylate polymers as topical antimicrobial agents against *Staphylococcus aureus* nasal colonization. *Biomacromolecules* **2014**, *15*, 2933-2943.

32. Yadav, K.; Kumar, S.; Mishra, D.; Asad, M.; Mitra, M.; Yavvari, P. S.; Gupta, S.; Vedantham, M.; Ranga, P.; Komalla, V.; Pal, S.; Sharma, P.; Kapil, A.; Singh, A.; Singh, N.; Srivastava, A.; Thukral, L.; Bajaj, A. Deciphering the Role of Intramolecular Networking in Cholic Acid-Peptide Conjugates on the Lipopolysaccharide Surface in Combating Gram-Negative Bacterial Infections. *J. Med. Chem.* **2019,** *62,* 1875-1886.

33. Sgolastra, F.; Deronde, B. M.; Sarapas, J. M.; Som, A.; Tew, G. N. Designing mimics of membrane active proteins. *Acc. Chem. Res.* **2013**, *46*, 2977-2987.

34. Ivankin, A.; Livne, L.; Mor, A.; Caputo, G. A.; DeGrado, W. F.; Meron, M.; Lin, B.; Gidalevitz, D. Role of the conformational rigidity in the design of biomimetic antimicrobial compounds. *Angew. Chem. Int. Ed.* **2010**, *49*, 8462 -8465.

35. Konai, M. M.; Adhikary, U.; Samaddar, S.; Ghosh, C.; Haldar, J. Structure-activity relationship of amino acid tunable lipidated norspermidine conjugates: disrupting biofilms with potent activity against bacterial persisters. *Bioconjug. Chem.* **2015**, *26*, 2442-2453.

36. Conlon, B. P.; Nakayasu, E. S.; Fleck, L. E.; LaFleur, M. D.; Isabella, V. M.; Coleman, K.; Leonard, S. N.; Smith, R. D.; Adkins, J. N.; Lewis, K. Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature* **2013**, *503*, 365-370.

37. Boucher, H. W.; Corey, G. R. Epidemiology of methicillin-resistant *Staphylococcus aureus*. *Clin. Infect. Dis.* **2008**, *46*, S344-349.

38. Elston, J. W.; Barlow, G. D. Community-associated MRSA in the United Kingdom. *J. Infect.* **2009**, *59*, 149-155.

39. Willyard, C. The drug-resistant bacteria that pose the greatest health threats. *Nature* **2017**, *543*, 15.

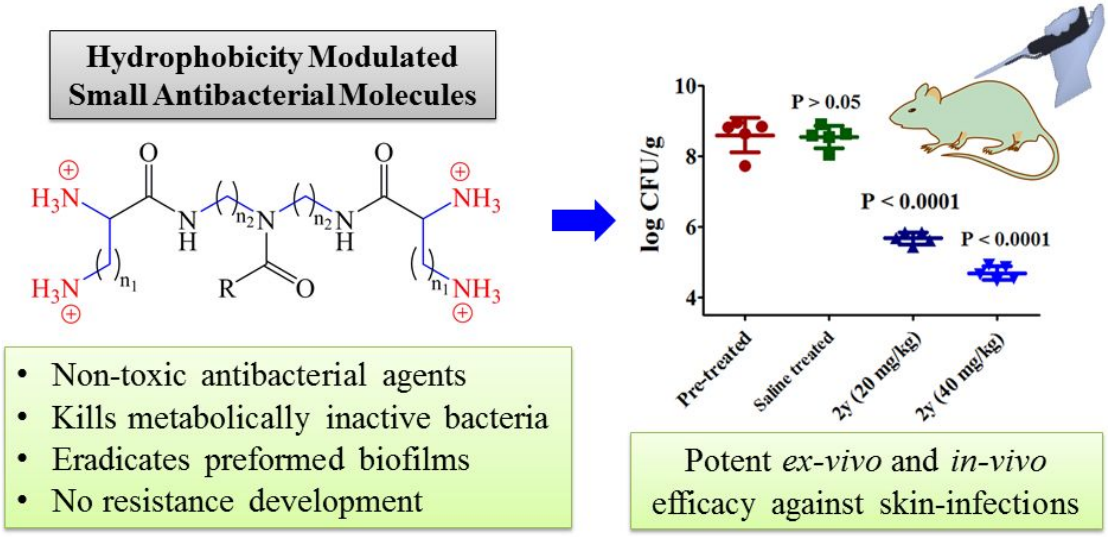
40. Konai, M. M. Haldar, J. Lysine-based small molecules that disrupt biofilms and kill both actively growing planktonic and nondividing stationary phase bacteria *ACS Infect. Dis.* **2015**, *1*, 469-478.

41. Konai, M. M.; Haldar, J. Fatty acid comprising lysine conjugates: anti-MRSA agents that display in-vivo efficacy by disrupting biofilms with no resistance development. *Bioconjug. Chem.* **2017**, *28*, 1194-1204.

42. Konai, M. M.; Adhikary, U.; Haldar, J. Design and Solution-Phase Synthesis of Membrane-Targeting Lipopeptides with Selective Antibacterial Activity. *Chem. Eur. J.* **2017**, 23, 12853-12860.

43. Ventress, J. K.; Partridge, L. J.; Read, R. C.; Cozens, D.; MacNeil, S.; Monk, P. N. Peptides from Tetraspanin CD9 Are Potent Inhibitors of Staphylococcus Aureus Adherence to Keratinocytes. *PLoS One* **2016**, *11*, e0160387.

TOC graphic


Hydrophobicity Modulated Small Antibacterial Molecules
• Non-toxic antibacterial agents
• Kills metabolically inactive bacteria
• Eradicates preformed biofilms
• No resistance development
log CFU/g
10
8
6
4
P > 0.05
P < 0.0001
P < 0.0001
Pre-treated
Saline treated
2y (20 mg/kg)
2y (40 mg/kg)
Potent ex-vivo and in-vivo efficacy against skin-infections