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ARTICLE

Low-toxicity Amphiphilic Molecules linked by an Aromatic Nucleus Show Broad-spectrum Antibacterial Activity and Low Drug Resistance †

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Amphiphilic molecules linked by an aromatic nucleus were developed that showed high selectivity toward bacteria over mammalian cells, and low drug resistance. Promising compound 4g exhibited strong bactericidal activity against a panel of sensitive and resistant bacteria, low toxicity, the ability to reduce cell viability in biofilms, stability in mammalian fluids, rapid killing of pathogens, and high *in vivo* efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA).

Bacterial resistance and infection present a major challenge in the field of biomedicine[1, 2]. The rapidly increasing incidence of antibacterial resistance has caused escalating healthcare costs and high mortality rates[3]; while the development of new antibiotics has dramatically decreased[4]. Consequently, there is an immediate need for new agents with novel therapeutic activity against these resistant pathogens.

Bacteria have developed several different resistance mechanisms to respond to a single class of antibiotics such as modification of the drug target, cell wall permeability, molecular bypass and active efflux[5]. In addition, bacteria can synthesise a protective polysaccharide layer on their surface. This hydrated polymeric matrix, known as bacterial biofilm, is often the root of resistance to antimicrobial agents, contributing to chronic bacterial infections[6, 7]. Targeting the membrane of microorganisms is an effective and selective antimicrobial approach because bacterial biofilm has subtle differences to the mammalian cell membrane[8]. The role of antimicrobial peptides (AMPs) in innate immunity is to kill microorganisms and provide defence against infection[9-11]. AMPs interact with the target cell membrane via two steps, membrane binding (electrostatic interactions) and membrane insertion/permeation, eventually leading to cell death[12, 13]. Despite their antimicrobial properties, some disadvantages limit the use of AMPs including unknown toxicity, susceptibility to degradation by proteases, and the high cost of manufacture[14, 15]. These inherent limitations of AMPs have driven substantial efforts worldwide to develop synthetic mimics of AMPs. Many AMP-mimetics based on the inherent attributes of AMPs provide broad-spectrum antibacterial activity and have been shown to overcome bacterial resistance[16, 17].

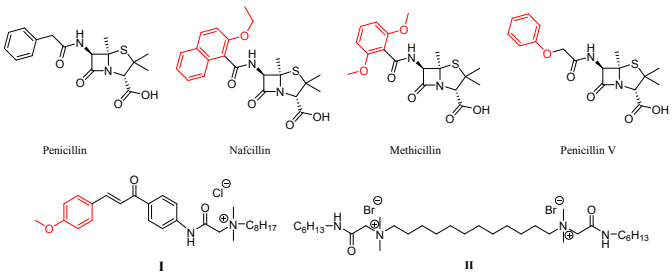

Fig 1 Antibiotics with a phenoxy group and cationic AMP-mimetics

In our continuing attempt to identify more potent compounds with great membrane selectivity and high antimicrobial activity against drug-resistance bacteria[18-20], chalcone derivatives I[19] (Fig. 1) with a 4-methoxy benzene substituent and moderate length alkane chain (eight carbon atoms), showed good antibacterial activity (in the range 1–4 μg/mL, Table 1) and moderate haemolytic activity. Inspired by the innovative discovery of active and low-toxicity cationic small molecules by Haldar's group (compound II[21]) and antibiotics with a phenoxy group (nafcillin, methicillin, and penicillin V showed better activity than penicillin, Fig. 1,[22]), we sought to find more effective antibacterial peptide mimics with low toxicity. The introduction of amide constructs could enhance the stability and the antibacterial activity of soft antimicrobial agents[23]. In view of the above considerations, amphiphilic molecules linked by an aromatic nucleus were designed and synthesized.

Compounds were designed to investigate the effects of an aromatic core, the length of the alkane chain, and the middle section on their membrane-selectivity. The general synthetic routes are shown in Schemes S1 and S2 (ESI†). Intermediates **1a**–**1f** reacted with **3a**–**3h** to obtain the final molecules **4a**–**4w**, using a previously reported

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method[19]. Compounds **5a–5d** were synthesized to verify the role of symmetry. Compounds **5e–5j** were obtained to investigate the influence of an aromatic core on antibacterial activity. The molecules

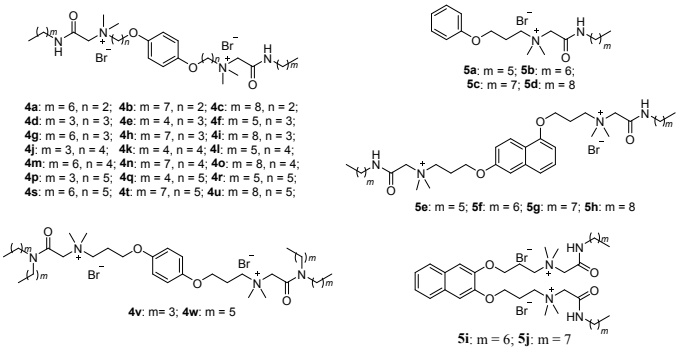

Fig 2 The structure of molecules **4a–4w** and **5a–5j**

were characterized by ^{1}H NMR, ^{13}C NMR, and HR-MS (Fig. 2). All of the compounds were confirmed to have ≥95% purity using HPLC. The antibacterial activity and haemolytic activity (HC_{50}) of amphiphilic molecules were evaluated against both sensitive bacteria (*Staphylococcus aureus*; *Enterococcus faecalis*; *Escherichia coli*; *Stenotrophomonas maltophilia*) and resistant bacterial strains. Some of these compounds showed excellent selectivity (HC_{50}/MIC $_{S.\ aureus}$) and activity against both negative and positive pathogens (Table 1). Compounds (**4d–4f**, **4j–4l**, **4p–4r**) with a short alkyl chain length (m = 3, 4, or 5) showed poor activity against the test strains (except **4r**), while the HC_{50} values for these compounds were more than 1280 μg/mL (except **4r**: 764 μg/mL). Compounds (**4a–4c**, **4g–4i**, **4m–4o**, **4s–4u**) with a moderate alkyl chain length (m = 6, 7, or 8) were effective against the four sensitive strains (MIC in the range of 0.25–2 μg/mL). For example, compounds **4s–4u** showed high activity against *S. aureus* (MIC in the range 0.25–0.5 μg/mL) and *E. coli* (MIC in the range 0.5–1 μg/mL). The MIC values of these compounds (**4a–4u**) increased with increasing chain length. Whereas the HC_{50} values of these compounds (**4a–4u**) decreased with increasing chain length. For example, the HC_{50} values of molecules **4d–4i** were >1280, >1280, >1280, 819, 287, and 45 μg/mL, respectively (Table 1). Compound **4g** (m=6; n=3) showed the best selectivity against *S. aureus* over erythrocytes.

The length of the middle section has a great impact on antibacterial or haemolytic activity. For example, compounds **4a**, **4g**, **4m**, and **4s** with the same alkane chain length (m = 6) showed similar antibacterial activity, while their haemolytic activities were different (>1280, 1219, 819, and 340 μg/mL, respectively), and compounds **4f**, **4l**, and **4r** with the same alkane chain length (m = 5) showed different antibacterial activity (8, 2, and 0.5 μg/mL against *S. aureus*, respectively). Compounds **4v** and **4w** with a branched chain were synthesized to increase hydrophobicity. The antibacterial activity of **4v** was slighter higher than that of **4d**. While the antibacterial activity of **4w** was higher than that of **4f**, suggesting that hydrophobicity has a positive influence on activity. The antibacterial activity of molecules **5a–5d** was impaired compared with that of amphipathic compounds **4f–4i**, indicating that symmetry plays an important role in the antibacterial activity. Molecules **5e–5j** were synthesized linked by naphthalene-1,6-diol or naphthalene-2,3-diol. In particular, compound **5e** ($MIC_{S.\ aureus}$: 0.5 μg/mL) showed better antibacterial activity than **4f** ($MIC_{S.\ aureus}$: 8 μg/mL), and higher selectivity ($HC_{50}/MIC_{S.\ aureus}$>2560) than **4g** ($HC_{50}/MIC_{S.\ aureus}$>2438), indicating that introduction of the naphthalene nucleus had promising results. Compounds **5e**, **5f**, **5g**, and **5i** showed excellent antibacterial activity ($MIC_{S.\ aureus}$: 0.5 μg/mL). The MICs of compounds **4a**, **4b**, **4g**, **4m**, **4r**, **4s**, **5e,** and **5f** were in the range of 0.5−4 μg/mL against 10 methicillin-resistant *S. aureus* isolates (Table S1, ESI†). The MIC values of these compounds against the NDM and KPC-producing strains were in the range of 1−8 μg/mL (Table S1, ESI†).

Table 1 MIC and HC_{50} values of compounds **I** and **II**, molecules **4a–4w** and **5a–5j**, and commercial antibiotics

Com.	MIC(μg/mL)[a]				HC_{50}[f]	SI[g]
	S. a[b]	*E. f*[c]	*E. c*[d]	*S. m*[e]		
I[19]	1	-[f]	4	-[f]	124	124
II[21]	1	- f	2	- f	805	805
4a	1	2	1	1	>1280	>1280
4b	0.5	0.25	1	1	617	1234
4c	0.5	0.5	1	1	78	156
4d	4	32	8	16	>1280	>320
4e	16	32	32	64	>1280	>80
4f	8	2	8	16	>1280	>160
4g	0.5	2	1	1	1219	2438
4h	0.5	1	0.5	1	287	574
4i	0.5	0.5	1	0.5	45	90
4j	4	16	8	4	>1280	>320
4k	8	32	16	32	>1280	>160
4l	2	16	4	16	>1280	>640
4m	0.5	1	1	2	819	1638
4n	0.5	2	1	0.5	132	264
4o	1	0.5	1	2	20	20
4p	4	16	8	16	>1280	>320
4q	2	32	8	8	>1280	>640
4r	0.5	2	1	4	764	1528
4s	0.25	0.5	0.5	1	340	1360
4t	0.5	0.25	1	1	34	68
4u	0.5	1	1	2	14	28
4v	4	4	64	32	>1280	>320
4w	0.5	1	2	4	132	264
5a	64	128	128	128	>1280	>20
5b	16	32	64	128	>1280	>80
5c	8	8	32	64	616	77
5d	4	4	16	16	438	109.5
5e	0.5	4	2	2	>1280	>2560
5f	0.5	1	1	1	406	812
5g	0.5	0.5	1	2	50	100
5h	1	0.5	2	2	20	20
5i	0.5	1	2	2	103	206
5j	1	1	2	4	21	21
V[h]	1	-[j]	-[j]	-[j]	-[j]	-[j]
M[i]	-[f]	-[j]	0.0625	-[j]	-[j]	-[j]

[a] Minimum inhibitory concentration; [b] *S. aureus*; [c] *E. faecalis*; [d] *E. coli*; [e] *S. maltophilia*; [f] the concentration that causes 50% haemolysis of red blood cells (RBCs); [g] Selectivity index, $HC_{50}/MIC_{S.\ aureus}$; [h] vancomycin; [i] meropenem; [j] not determined.

Stability in complex mammalian fluids is an important property that limits the application of AMPs. Compound **4g** was pre-incubated in 50% plasma for 2, 4, and 6 h at 37 °C. The minimum bactericidal concentration (MBC) values of **4g** were 2 μg/mL in control media and 8 μg/mL in plasma after different treatment times (Fig. S1a, ESI†). The MBC value of compound **4g** against MRSA was 32 μg/mL both in 50% serum and 50% blood (Fig. S1b, ESI†). Compound **4g** showed better bactericidal activity in 50% plasma (8 μg/mL) than in other solutions. The MBC and MIC values of **4g** at different concentrations of trypsin were evaluated and showed good activity (MBC: 8 μg/mL;

MIC: 1 μg/mL at a trypsin concentration of 80 μg/mL) (Fig. S1c, ESI†). The above results indicated that **4g** is stable in complex mammalian fluids and that incubation time has no effect on the bactericidal activity in plasma.

The analysis of time-kill kinetics of optimized compound **4g** against *S. aureus* was carried out at different concentrations. For the exponential phase strains, *S. aureus* could be removed completely by treatment with compound **4g** for 6 h at 3 or 4 μg/mL (Fig. 3a). For the stationary phase strains, compound **4g** removed *S. aureus* irreversibly after 16 h treatment at 12 μg/mL (Fig. 3b). While the number of viable bacteria treated with vancomycin was $10^{6.7}$ CFU/mL after 24 h. These results suggested the superiority of compound **4g** over commonly used antibiotics in killing bacteria. Photographs of the test strains treated for 6 or 24 h are shown in Fig. S2 (ESI†).

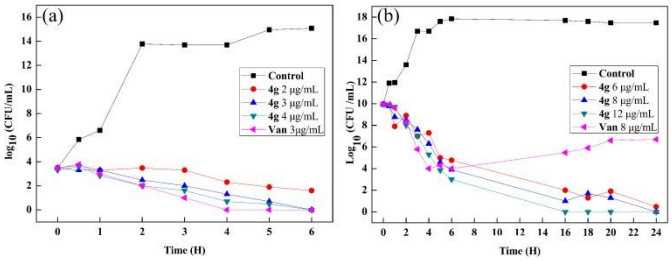

Fig 3 Time-dependent killing of pathogens by compound **4g**. *S. aureus* was grown to (a) exponential phase, and (b) stationary phase, challenged with compound **4g** or Van (vancomycin). The control was treated with sterile water.

The propensity of bacteria to develop drug resistance was assessed through resistance selection studies after prolonged passage at sub-inhibitory concentrations (0.5 × MIC). Only a small change was detected in the MIC of compound **4g** against both *S. aureus* and *E. coli* after 16 passages. Whereas, in the case of norfloxacin and colistin, the MICs started to increase after 1 and 7 days, increasing by a factor of 256 and 32, respectively (Fig. 4). Thus, compound **4g** had major advantages over conventional antibiotics norfloxacin and colistin and induced less bacterial resistance.

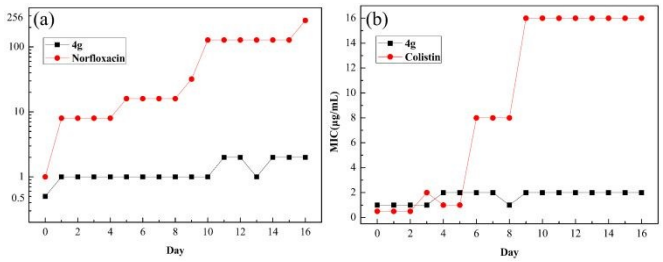

Fig 4 Studies on the emergence of resistance in **4g** toward (a) *S. aureus* (norfloxacin as control) and (b) *E. coli* (colistin as control).

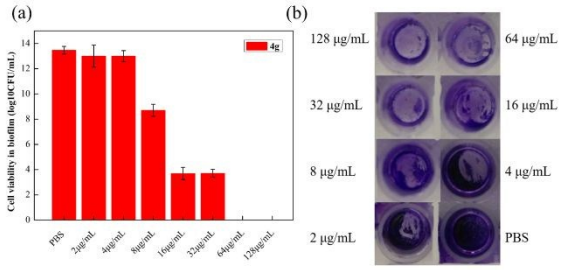

Fig 5 Effect of **4g** on bacterial biofilms. (a) Cell viability of *S. aureus* in biofilms treated with compound **4g**; (b) Images of *S. aureus* biofilms treated with **4g** after staining with crystal violet.

Bacteria can form a protective biofilm that acts as a barrier against the host immune response and provides resistance against antibiotics[23]. The cell viability in biofilms decreased with an increasing concentration of compound **4g** (Fig. 5a). Cell viability decreased to 13.00, 8.70, and 3.70 $\log_{10}$ CFU/mL at 4, 8, and 16 mg/mL, respectively, and 0 $\log_{10}$ CFU/mL at 64 and 128 mg/mL. Whereas the cell viability treated with buffer solution was 13.47 $\log_{10}$ CFU/mL. The destructive activity of compound **4g** on biofilm was visually observed by crystal violet staining (Fig. 5b). The minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) were also evaluated (Table S2, ESI†). The 70% minimal biofilm inhibit concentrations ($MBIC_{70}$) of compound **4g** were 10.1 and 22.9 μg/mL against *S. aureus* and *E. coli* biofilms, respectively. The MBECs of compound **4g** were 8 μg/mL against *S. aureus* and 16 μg/mL against *E. coli*. The MBIC and MBEC values were in agreement with the results of the biofilm disruption test (Fig. 5).

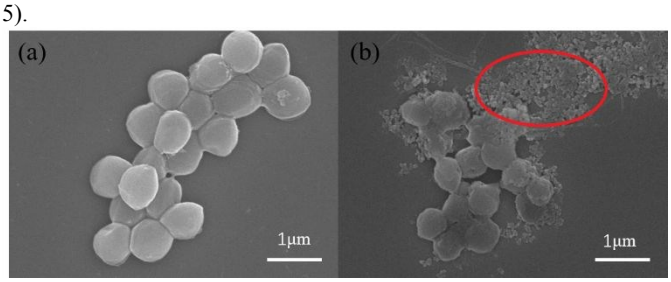

Fig 6 FESEM images of cell membrane damage. (a) Untreated *S. aureus*; (b) *S. aureus* treated with compound **4g** at 16 μg/mL. Scale bar 1 μm.

Field emission scanning electron microscopy (FESEM) images showed that untreated bacteria retained well-defined morphology and the smooth surface characteristic of bacteria (Fig. 6a). However, after treatment with **4g** for 3 h, the morphology of the cell membrane became irregular (Fig. 6b). Red fragments indicated that cells had been completely destroyed. Thus, the above results indicated that compound **4g** killed bacteria by disrupting their cell membranes.

The ability of compounds to depolarize the bacterial cell membrane was investigated using $Disc_3(5)$[24]. The fluorescence intensity was significantly enhanced after treatment with selected compounds (Fig. S3a, b, ESI†), indicating that these molecules were efficient in depolarizing the cytoplasmic membranes of *S. aureus* and *E. coli*. The membrane permeabilization of Gram-positive and Gram-negative bacteria was monitored using the nuclear fluorescence dye PI (propidium iodide). Compounds **4b**, **4r**, **4s**, and **5f** exhibited high permeabilization of the *S. aureus* inner membrane (Fig. S3c, ESI†), and compounds **4a**, **4b**, **4m**, **5e**, and **5f** exhibited high permeabilization of *E. coli* (Fig. S3d, ESI†). Thus, the results suggested that molecules permeabilize the inner membrane of both Gram-positive and Gram-negative bacteria. An outer membrane permeabilization assay was performed using *E. coli* (Fig. S3e, ESI†). All compounds tested enhanced the permeability of *E. coli*. Compounds **4b**, **4s**, and **5f** showed the highest activity with respect to permeabilizing the outer membrane of *E. coli*.

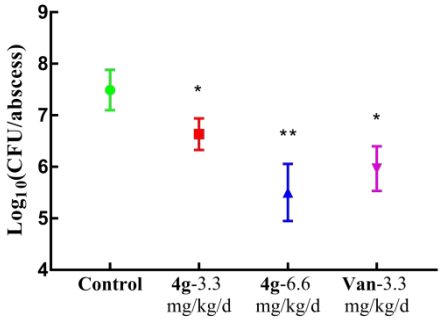

Fig 7 *In vivo* efficacy of compound **4g** in a mouse model of MRSA-1115041 skin infection. (a) Bacterial counts of skin samples. P-values (*) were 0.041, 0.0072, and 0.0106 for samples treated with 3.3 mg/kg/d **4g**, 6.6 mg/kg/d **4g**, and 3.3 mg/kg/d vancomycin (Van), compared with the control.

The toxicity of the most promising compound, **4g**, toward Hela cells was evaluated. The electron scanning microscopy images of HeLa cell morphology (Fig. S4b, c, ESI†) were unaltered after treatment with molecule **4g** compared with the control (Fig. S4a, ESI†). HeLa cells were evaluated by staining with calcein AM and PI to visualize LIVE and DEAD cells by fluorescence microscopy. The cells treated with **4g** fluoresced green (Fig. S5d-I, ESI†), and the same phenomenon was observed with the negative control (Fig. S5a–c, ESI†). The results confirmed the less-toxic nature of the bacterial membrane-disrupted compound **4g** (concentration 64 × $MIC_{S.\ aureus}$).

A mouse model of MRSA skin infection was established to evaluate the antibacterial activity *in vivo* (Fig. S6)[25]. Kunming mice were purchased from the Animal Center of Zhengzhou University (Zhengzhou, China), and all animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Zhengzhou University and approved by the Animal Ethics Committee of Zhengzhou University. Compound **4g** showed similar activity at a dose of 6.6 mg/kg/d with vancomycin (3.3 mg/kg/d) (Fig. 7). The antibacterial activity of compound **4g** was improved with an increased dose. The skin tissue samples were imaged by haematoxylin and eosin staining. In normal (healthy, uninfected) skin samples, the layers could be seen clearly (Fig. S7a, ESI†); while, MRSA-infected skin showed a serious inflammatory reaction. The epidermis separated from the dermis, and multifocal areas of inflammatory cell infiltration of the dermis as well as the subcutaneous tissue were observed (Fig. S7b, ESI†). The width of the skin was narrower with **4g**-treated sections (Fig. S7c, ESI†) than with the infected samples. Vancomycin-treated infected skin also showed minimal inflammation (Fig. S7e, ESI†). These data indicated that **4g** was efficient at reducing MRSA infection *in vivo* in a mouse model.

A series of amphiphilic molecules linked by an aromatic nucleus were designed and synthesized as AMP-mimetics. Some compounds showed high membrane selectivity ($HC_{50}/MIC_{S.\ aureus}$ of **4g** and **5e** were 2438 and >2560, respectively) and excellent antibacterial activity against a panel of bacteria including various multidrug-resistant isolates. Mechanistic studies and SEM images indicated that these compounds depolarized and permeabilized bacterial membranes, leading to irregular cell morphology and the rapid death of bacteria. Lead compound **4g** possesses optimal attributes, such as stability in plasma and trypsin, the ability to disrupt established *S. aureus* biofilms, lower drug resistance, high *in vivo* efficacy against MRSA, and no detectable toxicity. Overall, these results suggest that amphiphilic molecules linked by an aromatic nucleus are highly promising compounds for development into pharmaceuticals.

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Conflicts of interest

There are no conflicts of interest to declare.

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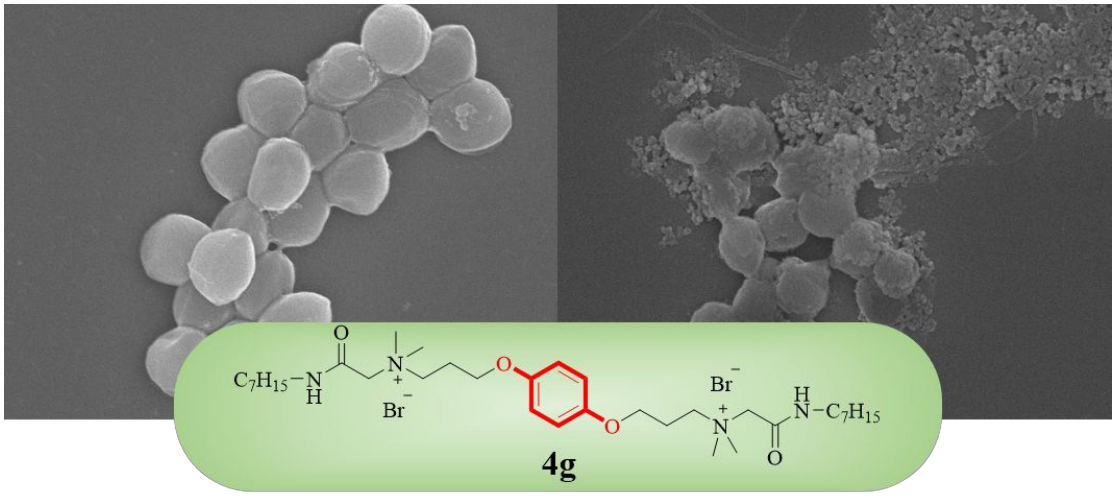
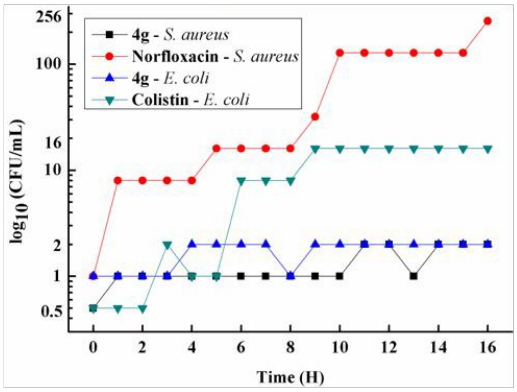
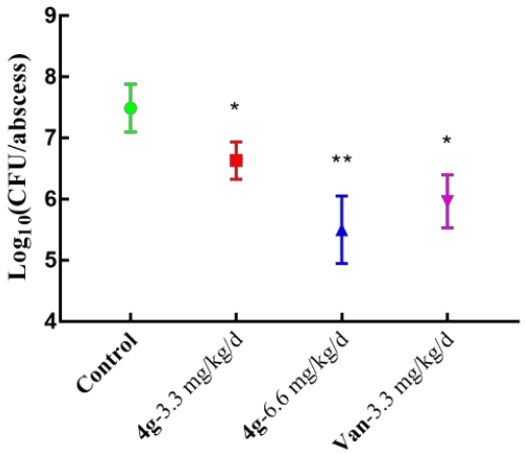
Amphiphilic molecules linked by aromatic nucleus, possessing strong bactericidal activity, high selectivity, less drug resistance, high in vivo efficacy against MRSA, were developed.







Resistance studies