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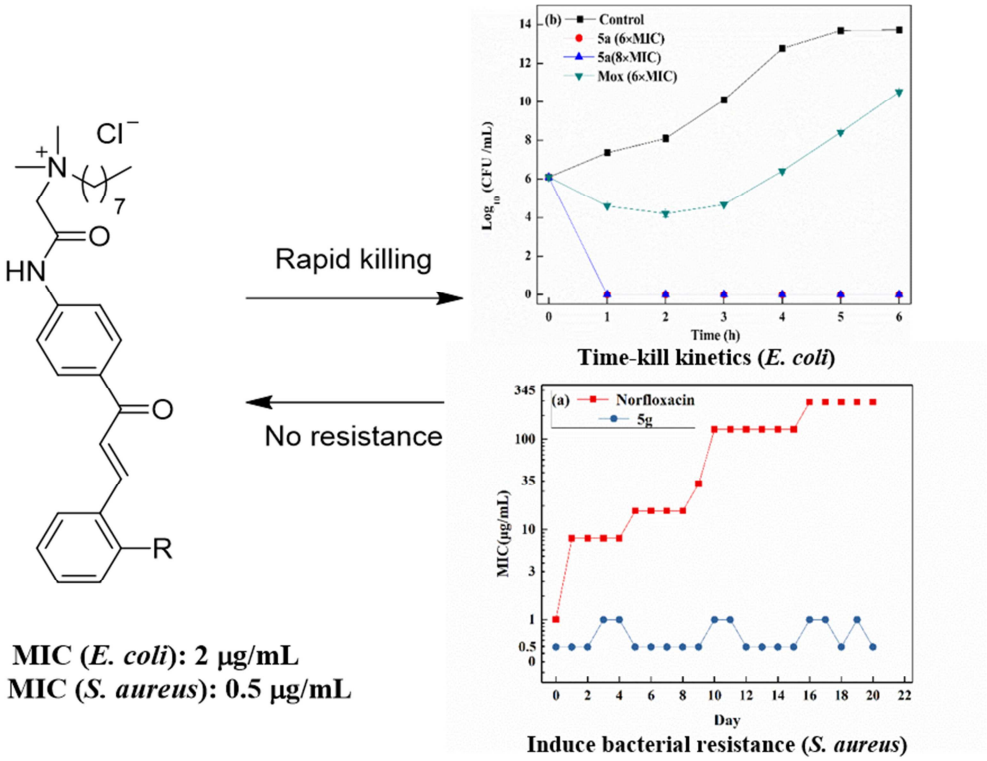
Graphical abstract



5a: R=H, MIC (*E. coli*): 2 μg/mL
5g: R=F, MIC (*S. aureus*): 0.5 μg/mL

Synthesis and Antibacterial Evaluation of Novel Cationic Chalcone Derivatives Possessing Broad Spectrum Antibacterial Activity

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ABSTRACT

There is an urgent need to identify new antibiotics with novel mechanisms that combat antibiotic resistant bacteria. Herein, a series of chalcone derivatives that mimic the essential properties of cationic antimicrobial peptides were designed and synthesized. Antibacterial activities against drug-sensitive bacteria, including *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Salmonella enterica*, as well as clinical multiple drug resistant isolates of methicillin-resistant *S. aureus* (MRSA), KPC-2-producing and NDM-1-producing Carbapenem-resistant *Enterobacteriaceae* were evaluated. Representative compounds **5a** (MIC: 1 μg/mL against *S. aureus*, 0.5 μg/mL against MRSA) and **5g** (MIC: 0.5 μg/mL against *S. aureus*, 0.25 μg/mL against MRSA) showed good bactericidal activity against both Gram-positive and Gram-negative bacteria, including the drug-resistant species MRSA, KPC and NDM. These membrane-active antibacterial compounds were demonstrated to reduce the viable cell counts in bacterial biofilms effectively and do not induce the development of resistance in bacteria. Additionally, these representative molecules exhibited negligible toxicity toward mammalian cells at a suitable concentration. The combined results indicate that this series of cationic chalcone derivatives have potential therapeutic effects against bacterial infections.

Keywords: Antimicrobial; Cationic chalcone derivatives; Broad spectrum antibacterial activity; Fluoride atom; Permeabilization; Depolarization

1. Introduction

The global threat of infectious diseases has been aggravated by the emergence of bacterial resistance to antibiotics. Such antibiotic resistance has stimulated the urgent need for the design of new antibiotics with different modes of action.[1-3] Methicillin-resistant *Staphylococcus aureus* (MRSA) was initially detected in Europe,[4] and is now responsible for the majority of community acquired and nosocomial infections.[5] KPC-2-producing *Klebsiella* pneumoniae (KPC) was initially identified in 1996 and has since spread to many countries.[6] New Delhi metallo-β-lactamase 1 (NDM-1)-producing *Enterobacteriaceae* hydrolyzes nearly all known β-lactam antibiotics. Moreover, the NDM-1 gene has quickly disseminated worldwide and this spread is uncontrollable.[7-9] Unfortunately, the development of new antibiotics has failed to keep pace with the development of drug-resistance over the past few decades.[3, 10] Thus, there is a critical global healthcare crisis, which requires the urgent development of more effective antibiotics.

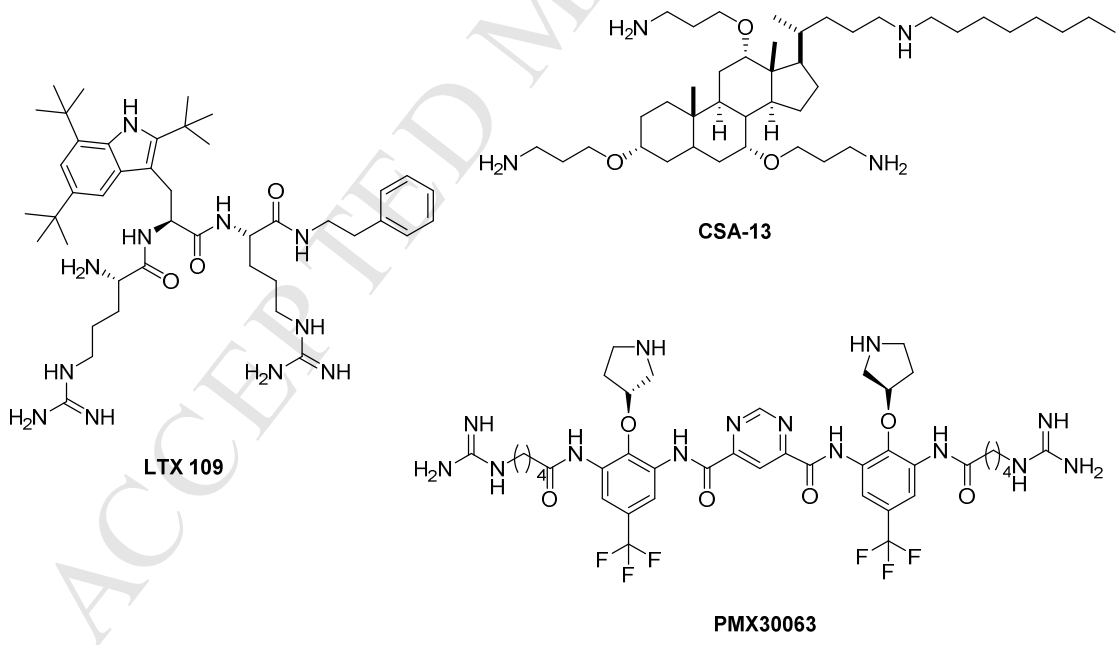


Figure 1. Three antibacterial peptide mimetic molecules in clinical trials.

Antimicrobial peptides (AMPs) provide broad-spectrum protection against bacterial infections for most organisms, [11, 12] and have been shown to overcome bacterial resistance. Cationic and amphiphilic AMPs attach to anionic microbial cell

surfaces and insert into the microbial membrane bilayers, which then leads to the disruption of the physical integrity of the cell membrane.[13] Despite the many advantages, AMPs still have some disadvantages, including easy degradation in vivo, toxicity in vivo and a high cost of manufacture, which impedes the use of AMPs as clinical antibacterial agents.[14, 15] Consequently, several groups have used amphipathic models of AMPs to mimic the action of AMPs and thus produce membrane-targeting antimicrobial agents.[16, 17] For example, α-peptides,[18] β-peptides,[19] antimicrobial polymers,[20] oligoacyl lysines,[21] oligoureas,[22] α-AA peptides[23] and cationic amphiphiles[24, 25] have showed effective activity against antibiotic-resistant bacteria. Additionally, membrane-active antibacterial molecules have been synthesized and reported to exhibit good activity and lower resistance tendencies.[16, 24, 26-28] There are some antibacterial peptide mimetic molecules including CSA-13[29], LTX-109[30] and PMX 30063[31] (Figure 1) that are currently undergoing clinical trials.

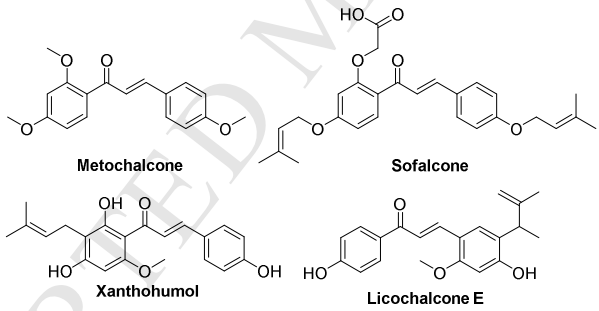


Figure 2. Representative chalcone compounds have been reported.

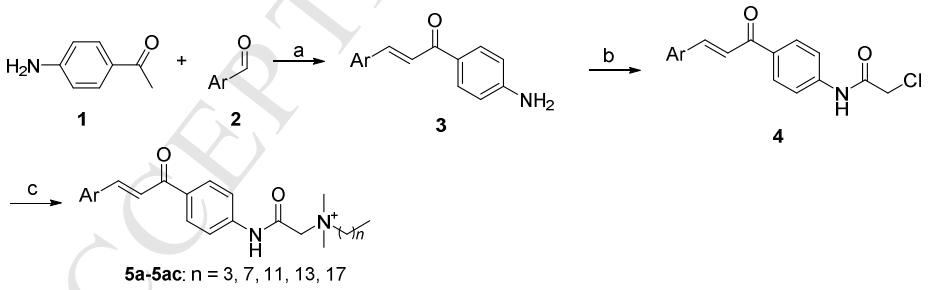
Natural products and their derivatives have historically been invaluable as a source of bioactive lead compounds for drug discovery.[32, 33] Chalcone (1, 3-diphenyl-2-propen-1-ones) is a key substructure of natural compounds that has been shown to be beneficial to human health.[34, 35] Chalcone and chalcone derivatives have a wide range of biological activities, including antibacterial,[36] antimalarial,[37, 38] and anti-inflammatory.[39] The unique α,β-unsaturated ketone structure of chalcone is responsible for the broad-spectrum of biological activities.[40] Furthermore, their interesting structural scaffolds and significant biological activities

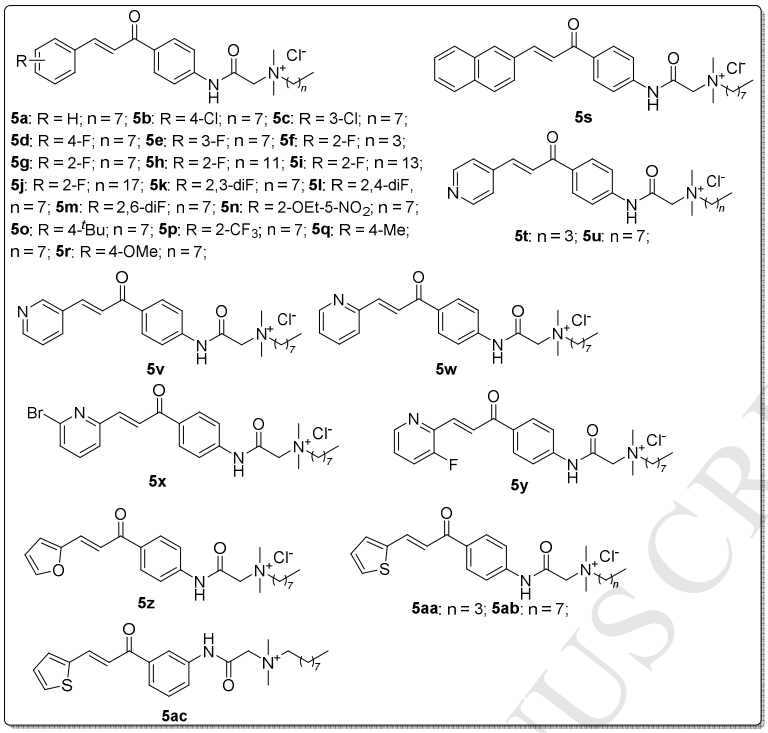
have prompted many groups to isolate and modify chalcone for the development of new drug candidates, and there are some existing drugs including metochalcone and antipeptic ulcer mucoprotective sofalcone.[41, 42] Natural chalcone derivatives such as xanthohumol[43] and licochalcone E[44] (Figure 2) show good antibacterial activity.

In our continuous endeavor for new structures with good biological activity against drug-resistant bacteria,[45, 46] antibacterial small molecular peptidomimetics were developed by taking advantage of the hydrophobic scaffold of chalcone.[45] These molecules contained cationic residues, which are positioned on the scaffold to recognize the outer membrane. Some compounds of this series exhibited potent antimicrobial properties against Gram-positive bacteria and Gram-negative bacteria, with rapid killing rates.. Importantly, the antibacterial mechanisms of our compounds involved depolarization and permeabilization of the bacterial cell membrane. These results provide new insights to the potential use of chalcone peptidomimetics as novel and efficient antimicrobial agents.

2.Results and discussion

2.1. Synthesis and characterization

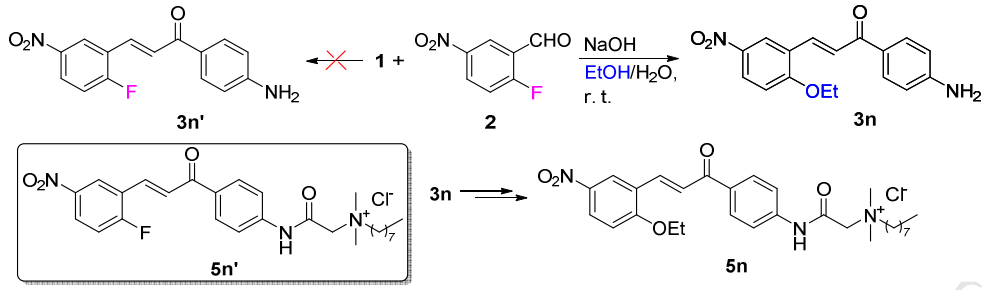




a): NaOH, EtOH, H_2O, r. t., overnight. b): Chloroacetyl chloride, K_2CO_3, acetone, r. t., 30 min. c): N,N-dimethyl alkylamine, acetonitrile, 85 °C, hydrothermal reaction vessel, 24 h.

Scheme 1. Synthesis of cationic chalcone derivatives

Twenty-nine cationic chalcone analogs (**5a-5ac**), which differed in the length of the N,N-dimethyl alkylamine and had various substituted aryl rings, were designed and synthesized. The molecules were synthesized using a three-step method. The synthetic route is shown in Scheme 1. Intermediate compounds 4-aminochalcone **3** were synthesized via the aldol condensation reaction from various substituted aromatic formaldehydes and 4-aminoacetophenone (or 3-aminoacetophenone).[47] Compounds **4** were then obtained by the acylation of **3** using chloroacetyl chloride. Finally, cationic chalcone analogs **5** were obtained from N,N-dimethyl alkylamine and intermediates **4**.[24] The target molecules **5a-5ac** were characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectrometry (HRMS) as described for compound **5g** (Figure 3).



Scheme 2. The accidental synthesis of 5n'

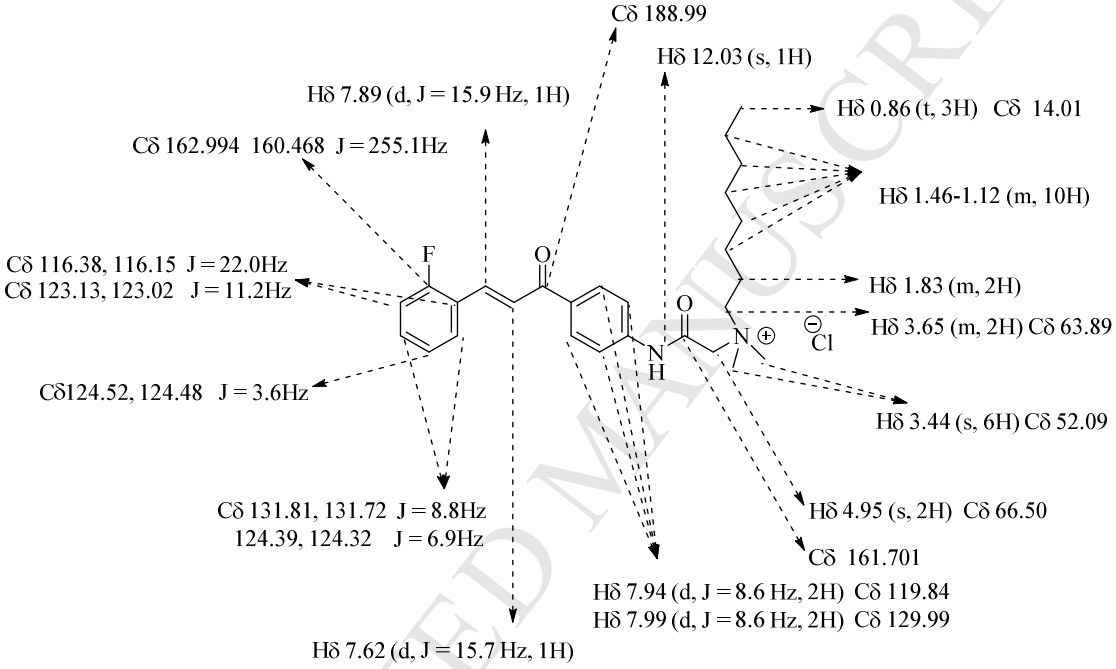


Figure 3. ^{1}H NMR and ^{13}C NMR chemical shifts of compound **5g**.

During the synthesis of compound **5n'**, unexpectedly **3n** was obtained rather than the production of **3n'** (Scheme 2). From the NMR and HRMS data of **3n**, we presumed that the fluorine of 2-fluoro-5-nitrobenzaldehyde (**2n**) or intermediate **3n'** was replaced by an ethoxyl during the aldol reaction in ethanol.[48] Compound **5n** was obtained from this accidental compound **3n** according the same method (Scheme 1).

Table 1. Minimal Inhibitory Concentration (MIC) and Hemolytic Activity (HC_{50}) of the Final Products

Compd	MIC(μg/mL)				HC_{50}(μg/mL)
	S. aureus ATCC29213	*E. faecalis* ATCC29212	*E. coli* ATCC25922	*S. enterica* ATCC13076	
5a	1	1	2	4	228
5b	2	2	4	8	＞500
5c	2	1	4	4	186
5d	2	2	2	4	83
5e	32	64	＞128	＞128	68
5f	16	16	64	128	262
5g	0.5	1	2	4	137
5h	4	8	16	32	293
5i	8	＞128	＞128	＞128	192
5j	＞128	＞128	＞128	＞128	444
5k	0.5	2	2	8	56
5l	2	2	4	4	54
5m	32	32	128	128	50
5n	8	2	＞128	＞128	102
5o	2	4	16	16	284
5p	2	4	8	16	136
5q	1	1	4	8	153
5r	1	2	4	4	124
5s	2	2	8	8	374
5t	128	128	>128	>128	＞500
5u	4	8	16	32	200
5v	4	8	16	32	127
5w	4	8	16	32	187
5x	4	8	16	32	363
5y	2	8	8	16	83
5z	1	4	4	16	137
5ab	0.5	1	4	8	133
5aa	32	32	64	128	360
5ac	2	4	16	16	249
VAN[a]	2	-[c]	-[c]	-[c]	-[c]
MEM[b]	-[c]	-[c]	<0.125	-[c]	-[c]

[a]VAN (vancomycin), [b]MEM (meropenem), [c]not determined.

2.2. Antibacterial activity

All compounds were initially screened for antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*), and against Gram-negative bacteria *Escherichia coli* (*E. coli*) and *Salmonella enterica* (*S. enterica*), and resistant bacteria including MRSA, KPC and NDM. The antibacterial efficacy was expressed as the minimum inhibitory concentration (MIC) that is required to inhibit the growth of the bacteria.[49] MIC values of the tested compounds are listed in Table 1.

In general, all compounds except **5e**, **5i**, **5j**, **5n** and **5t** showed good activity against all the bacteria tested. The cationic molecules were more effective toward Gram-positive bacteria than toward Gram-negative bacteria. For example, the range of MIC values for compounds **5a-5d** were 1–2 μg/mL against Gram-positive strains, whereas the range of MIC values were 2–8 μg/mL against Gram-negative strains. However, the antibacterial activities of the cationic molecules were found to vary significantly because of the different hydrophobicity of the alkyl chain. For example, compounds **5f-5j** with different alkyl chain lengths exhibited noticeably different antibacterial activity, with **5g** (medium hydrophobicity) showing the highest antibacterial activity. The antibacterial activity of compounds **5g-5j** decreased with increasing spacer length. This decrease in the antibacterial activity may be caused by the aggregation tendency because of the long hydrophobic chain.[50] This finding indicates the importance of an amphiphilic balance.

2.2.1. Effect of phenyl group and the fluoride atom on antibacterial activity

Different aryl groups of chalcone showed distinct activities. Compounds **5a-5s** with different substituted phenyl groups and compounds **5z-5ac** with thienyl or furanyl groups showed good activity, whereas compounds **5t-5y** with pyridyl groups showed poorer activity when compared with the activity of the other compounds. The MICs of mono-substituted phenyl compounds **5d**, **5e**, **5q**, **5r** and **5s** against Gram-positive bacteria ranged from 1 to 2 μg/mL, whereas the range of MIC values against Gram-negative bacteria were 4–8 μg/mL. P-methyl (**5q**) and p-methoxyl (**5r**)

achieved similar activity as compound **5a** against *S. aureus*. The presence of a fluoride atom was observed to be important for antibacterial activity. For example, compound **5g** with a fluoride atom substituted phenyl ring displayed the highest antibacterial activity. In addition, the position of the fluoride atom was found to affect the antibacterial activity. The different activity of **5d, 5e** and **5g** suggests that the position of the fluoride atom affects the antibacterial activity of these compounds. The compounds **5k-5m** with different positions of the second fluoride atom exhibited varying antibacterial activity. Compound **5k** displayed similar activity to that of **5g** ($MIC_{s.aureus}$ = 0.5 μg/mL), indicating the position of the fluoride atom modulates the antibacterial efficacy of the compounds.

2.2.2. Activity against drug-resistant bacteria

Table 2. MIC of Cationic Small Molecules against Drug-Resistant MRSA

Compd	MRSA								
	M-1	M-2	M-3	M-4	M-5	M-6	M-7	M-8	M-9
5a	1	0.5	1	8	4	0.5	1	4	2
5s	1	1	1	16	8	2	2	32	2
5b	1	0.5	1	4	2	0.5	2	1	2
5c	1	0.5	0.5	8	2	0.5	1	4	1
5d	1	0.5	0.5	8	2	0.5	1	4	2
5g	1	0.5	0.5	8	2	0.25	1	4	1
5l	1	1	0.5	8	4	0.5	1	4	2
5p	1	0.5	0.5	16	4	2	0.5	1	1
5q	1	0.5	0.5	4	2	0.5	1	4	2
5r	1	1	0.5	8	4	0.5	1	1	1
5z	4	2	2	32	8	2	4	16	2
5ab	1	1	0.5	16	4	1	2	1	2

The activity of compounds **5a-5d**, **5g**, **5l**, **5p-5s**, **5z** and **5ab** against drug-resistant bacteria MRSA (9 clinical isolated strains) was further tested. All selected molecules had MIC values in the range of 0.25–32 μg/mL (Table 2). Compound **5g** exhibited the highest activity with an MIC of 0.25 μg/mL against MRSA-6, and the MICs of the other compounds were primarily 0.5–8 μg/mL.

Table 3. MIC of Cationic Small Molecules against Drug-Resistant KPC

Compd	KPC												
	K-1	K-2	K-3	K-4	K-5	K-6	K-7	K-8	K-9	K-10	K-11	K-12	MIC_{90}
5s	16	16	16	＞128	32	16	＞128	8	16	＞128	＞128	＞128	＞128
5b	8	8	8	16	8	8	16	8	8	32	8	16	16
5c	4	4	4	8	4	4	8	4	4	8	8	8	8
5d	4	4	8	4	4	4	16	2	4	8	8	8	8
5g	4	4	8	8	4	4	16	4	4	16	8	16	16
5l	4	8	8	8	4	4	16	8	4	8	8	8	8
5p	8	8	8	＞128	8	4	＞128	4	8	16	8	16	＞128
5q	4	4	8	8	4	8	16	4	4	16	4	16	16
5r	8	8	8	8	4	8	2	8	8	16	16	16	16
5z	16	32	32	32	16	16	2	8	16	32	32	32	32
5ab	8	8	8	8	8	8	2	8	8	16	16	16	16

Antibacterial activity of selected compounds **5a-5d**, **5g**, **5l**, **5p-5s**, **5z** and **5ab** against the drug-resistant bacteria KPC (12 clinical isolated strains) was investigated. The MIC of compound **5c** ranged from 4 to 8 μg/mL (Table 3). Compounds **5r**, **5z** and **5ab** exhibited good activity against KPC-7 (MIC: 2 μg/mL).

Table 4. MIC of Cationic Small Molecules against Drug-Resistant NDM-1

Compd	NDM-1												
	N-1	N-2	N-3	N-4	N-5	N-6	N-7	N-8	N-9	N-10	N-11	N-12	MIC_{90}
5s	16	16	2	8	1	16	8	8	16	128	16	1	16
5b	16	4	4	8	16	8	4	4	8	16	8	8	16
5c	4	4	1	4	4	4	4	4	4	16	8	16	16
5d	4	4	1	4	16	4	4	16	8	16	4	16	16
5g	4	4	1	4	16	16	4	4	4	16	4	4	16
5l	4	4	2	4	8	4	4	4	4	16	4	2	8
5p	8	8	1	8	16	8	8	4	8	128	4	4	16
5q	4	4	2	8	8	4	4	4	8	1	4	32	8
5r	4	8	1	8	4	8	8	8	8	16	8	32	16
5z	16	16	4	8	32	16	16	16	16	32	16	64	32
5ab	4	8	2	8	4	8	8	8	2	4	8	8	8

The activity of compounds **5a-5d**, **5g**, **5l**, **5p-5s**, **5z** and **5ab** against the drug-resistant bacteria NDM (12 clinical isolated strains) was tested. The range of MIC values against NDM were primarily between 1 and 16 μg/mL (Table 4). Compound **5ab** showed the highest activity (MIC: 2–8 μg/mL). In addition, antibacterial activity toward NDM was better than the activity measured against KPC.

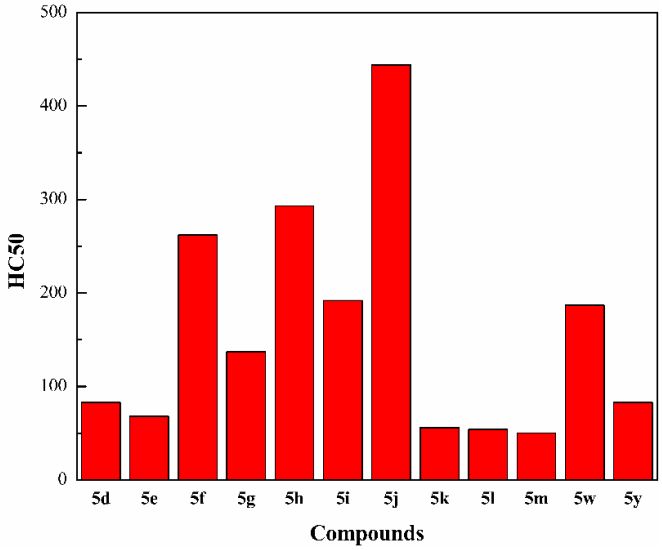


Figure 4. Hemolytic activity (HC_{50}) of part compounds.

2.3. Hemolytic activity

The ability of compounds to lyse red blood cells (RBCs), which were isolated from sheep blood, was used as a measure of the toxicity of the compounds toward mammalian cells. The hemolytic activity of all cationic molecules was represented as HC_{50} values (the concentration at which 50% hemolysis occurs). HC_{50} values of the cationic molecules ranged from 50 μg/mL to >500 μg/mL (Table 1). The HC_{50} values correlated with the presence or absence of a fluoride atom(s) and the length of the alkyl chain. The number of fluoride atoms on the phenyl group influenced the hemolytic activity. Here, the HC_{50} values of compounds **5d**, **5e** and **5g** with one fluorine atom were 83, 68 and 137 μg/mL, respectively, whereas the HC_{50} values of compounds **5k**, **5l** and **5m** that have two fluoride atoms were 56, 54 and 50 μg/mL, respectively. The HC_{50} value of **5g** was 2-fold higher than that of compound **5k** (Figure 4). The above results revealed that **5d**, **5e** and **5g** were more selective than compounds **5k**, **5l** and **5m** toward bacteria over mammalian cells. Similarly, the HC_{50} value of **5w** was 2-fold higher than that of compound **5y**. In addition, the selectivity values (HC_{50}/MIC) of the potential compounds **5a** and **5g** were 228 and 274. The HC_{50} values of compounds **5f-5j** did not systematically increase with an increase in the length of the alkyl chain. Thus, these results indicate that hydrophobicity did not

clearly affect hemolytic activity.

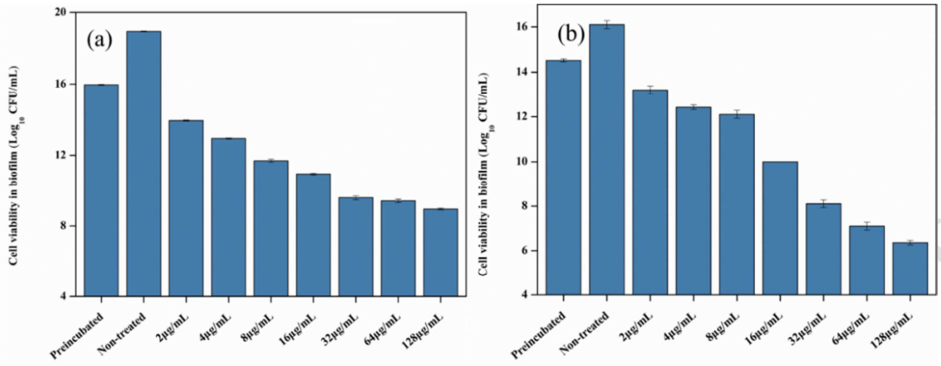


Figure 5. Biofilm disruption of chalcone derivatives at different concentrations. (a) Cell viability of *S. aureus* in biofilms treated with compound **5g**. (b) Cell viability of *E. coli* in biofilms treated with compound **5a**.

2.4. Disruption activity

The action of antimicrobial peptides involves the cationic moieties of the peptide interacting with the anionic moieties displayed on the surface of bacteria membranes. Subsequently, the hydrophobic moiety of the AMP disperses the established biofilms, leading to cell death.[51] The antibiofilm activity of the most promising cationic small molecules, **5g** and **5a**, was tested against established *S. aureus* and *E. coli* biofilms, respectively (Figure 5). *S. aureus* biofilms were cultured for 24 h and then treated with compound **5g** at different concentrations. As shown in Figure 5a, *S. aureus* cell viability in the biofilms decreased with increasing concentration of compound **5g**. The starting number of *S. aureus* in the biofilms was $10^{15.96}$ CFU/mL, which was used as a reference point. After 24 h, the cell viabilities in biofilms decreased to $10^{13.93}$, $10^{12.93}$, $10^{11.696}$, $10^{10.95}$, $10^{9.59}$, $10^{9.41}$, $10^{8.95}$ CFU/mL at **5g** concentrations of 2, 4, 8, 16, 32, 64 and 128 μg/mL, respectively. In contrast, the cell viability in not-treated biofilms increased to $10^{18.93}$ CFU/mL. The above results indicate that the cationic small molecule **5g** reduced bacteria viability in biofilms.

In addition, *E. coli* cell viability in biofilms was also observed to decrease with increasing concentrations of **5a** (Figure 5b). *E. coli* biofilms were cultured for 72 h and then treated with compound **5a** at different concentrations. The starting cell number of *E. coli* in the biofilms was $10^{14.51}$ CFU/mL. After 24 h, the cell viabilities

in the biofilms decreased to $10^{13.20}$, $10^{12.41}$, $10^{12.10}$, $10^{10.00}$, $10^{8.10}$, $10^{7.10}$, $10^{6.35}$ CFU/mL at **5a** concentrations of 2, 4, 8, 16, 32, 64 and 128 μg/mL, respectively. The cell viability in not-treated biofilms increased to $10^{16.1}$ CFU/mL. Thus, compound **5a** was able to reduce the number of viable bacteria even in mature (72 h) *E. coli* biofilms. These results are attributed to the properties of the compounds to eradicate preformed biofilms. Biofilm disruption activity of compounds **5g** and **5a** was visually observed by crystal violet staining (Figure S1).

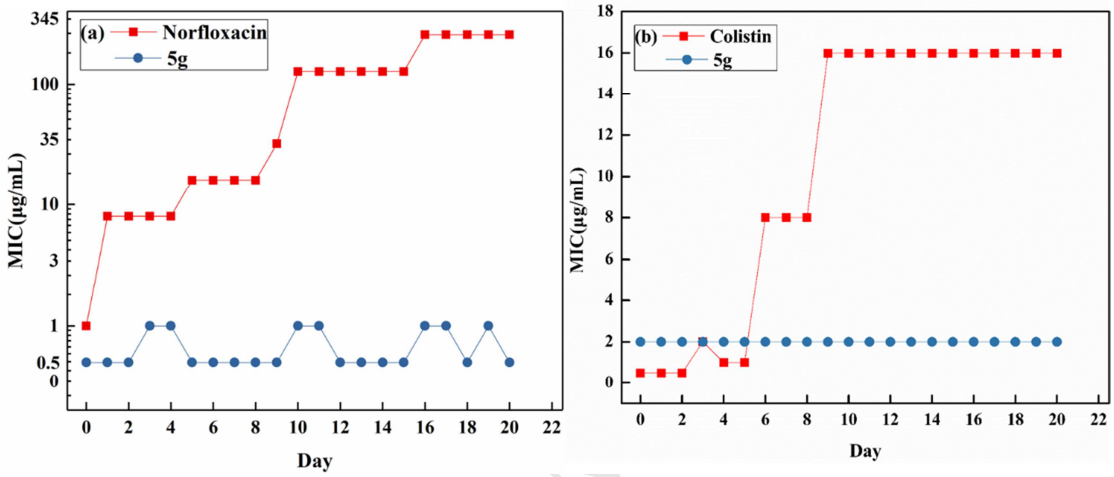


Figure 6. Propensity of the development of bacterial resistance against compound **5g**. (a) For *S. aureus*, where antibiotic norfloxacin was used as the control. (b) For *E. coli*, where the lipopeptide colistin was used as the control.

2.5. Propensity to induce bacterial resistance

Bacterial resistance against most antibiotics presents a serious problem.[52] Thus, the propensity of compounds to inhibit bacterial resistance is an important property. The ability of **5g** to suppress the development of resistance was tested. Norfloxacin and colistin were selected as positive controls for *S. aureus* and *E. coli*, respectively. Resistance is usually defined as a > 4-fold increase in the original MIC.[53] The results showed no change in the MIC of compound **5g**, indicating that exposure of the bacteria to this compound did not lead to the development of bacterial resistance over 20 generations. In contrast, norfloxacin and colistin showed 256-fold and 32-fold increases in the MIC values (Figure 6). The above results of the resistance study revealed that resistance by the tested bacteria toward **5g** is not easily developed.

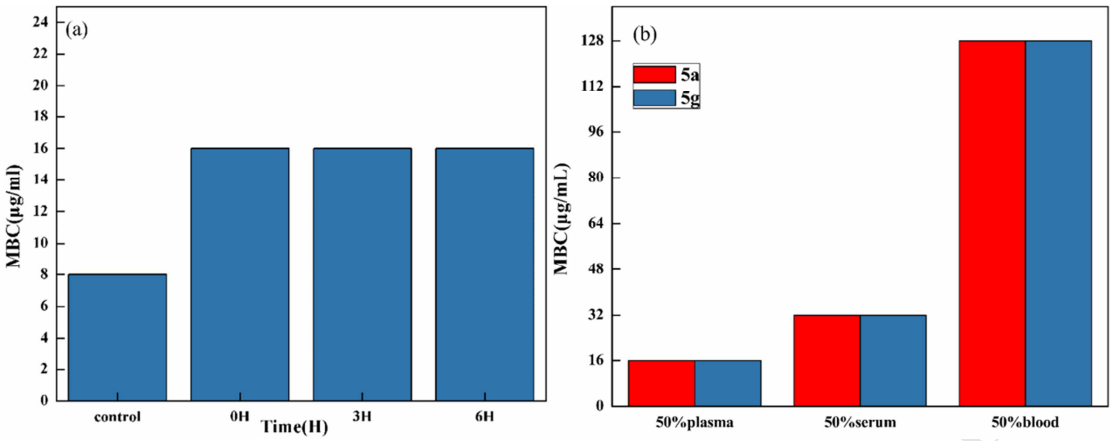


Figure 7. Plasma stability and bactericidal activity in complex mammalian fluids. (a) Compound **5g** against *S. aureus* in plasma treated at different times. (b) Compounds **5a** and **5g** against *S. aureus* in different culture mediums (50% serum, 50% plasma and 50% blood) against MRSA.

2.6. Plasma stability

Enzymes and proteins present in plasma will contribute to the degradation of natural antibacterial peptides. Plasma stability is an important factor that limits the application of antibacterial peptides.[21] The active compound **5g** was dissolved in 50% sterile Milli-Q water and 50% plasma. Three parallel tests were pre-incubated at 37 °C in 50% plasma for 0, 3 and 6 h. Bactericidal efficacy expressed as the minimum bactericidal concentration (MBC), which is the minimum drug concentration required to kill 99.9% of a tested microorganism, was examined. After the treatment, the MBC values of **5g** dissolved in 100% media as the positive control was 8 μg/mL. The MBC values of **5g** increased to 16 μg/mL after treatment in 50% plasma for 0, 3 and 6 h against *S. aureus* (Figure 7a). There was no influence of the incubation time on the observed activity. The above results indicate that compound **5g** is stable in plasma, even after a 6 h treatment.

2.7. Bactericidal activity in complex mammalian fluids

One of the major limitations of natural antimicrobial peptides is the loss of bactericidal efficacy in the presence of mammalian fluids due to enzymatic hydrolysis.

Activity in different culture mediums (50% serum, 50% plasma and 50% blood) was also determined to establish the efficacy of the compounds further. Bactericidal efficacy in different fluids was expressed as the MBC. It is interesting that **5a** and **5g** showed the same activity in different culture mediums. The MBC values of **5a** and **5g** in the 50% plasma were 16 μg/mL (Figure 7b). The MBC values of **5a** and **5g** in the 50% serum were 32 μg/mL, which is 2-fold higher than in 50% plasma. The MBC value was 128 μg/mL in 50% blood. These results indicate that compounds **5a** and **5g** were active in 50% plasma and 50% serum, whereas in 50% blood the compounds were not effective.

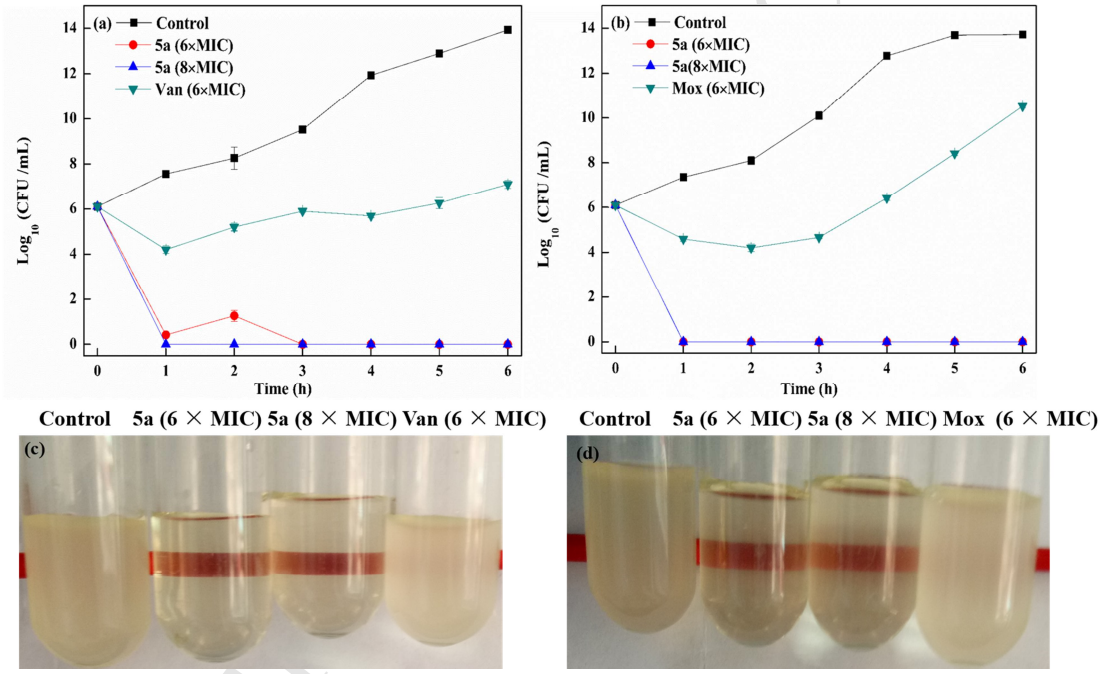


Figure 8. Time-dependent killing of pathogens by compound **5a**. (a) *S. aureus* was challenged with compound **5a** (at 6 × MIC and 8 × MIC) and vancomycin. (b) *E. coli* was challenged with compound **5a** (at 6 × MIC and 8 × MIC) and moxalactam. (c) Images show *S. aureus* after treatment for 6 h. (d) Images show *E. coli* after treatment for 6 h. The control was only treated with sterile water. Data are representative of 3 independent experiments.

2.8. Bactericidal time-kill kinetics

To study the bactericidal activity of the highest compound **5a**, a time-kill assay was carried out against *S. aureus* and *E. coli* at different concentrations (6 × MIC and

8 × MIC). Compound **5a** showed rapid bactericidal activity. The initial count of *S. aureus* was $10^{6.1}$ CFU/mL. The number of viable bacteria dropped to 10^{0} CFU/mL after 3 h at a concentration of 6 × MIC. In contrast, vancomycin (6 × MIC) did not kill the bacteria after 6 h. It is encouraging that compound **5a** also displayed the same rapid bactericidal activity against *E. coli* as observed for *S. aureus* (Figure 8). These results clearly demonstrate the superiority of compound **5a** over commonly used antibiotics vancomycin and moxalactam in killing both Gram-positive bacteria and Gram-negative bacteria.

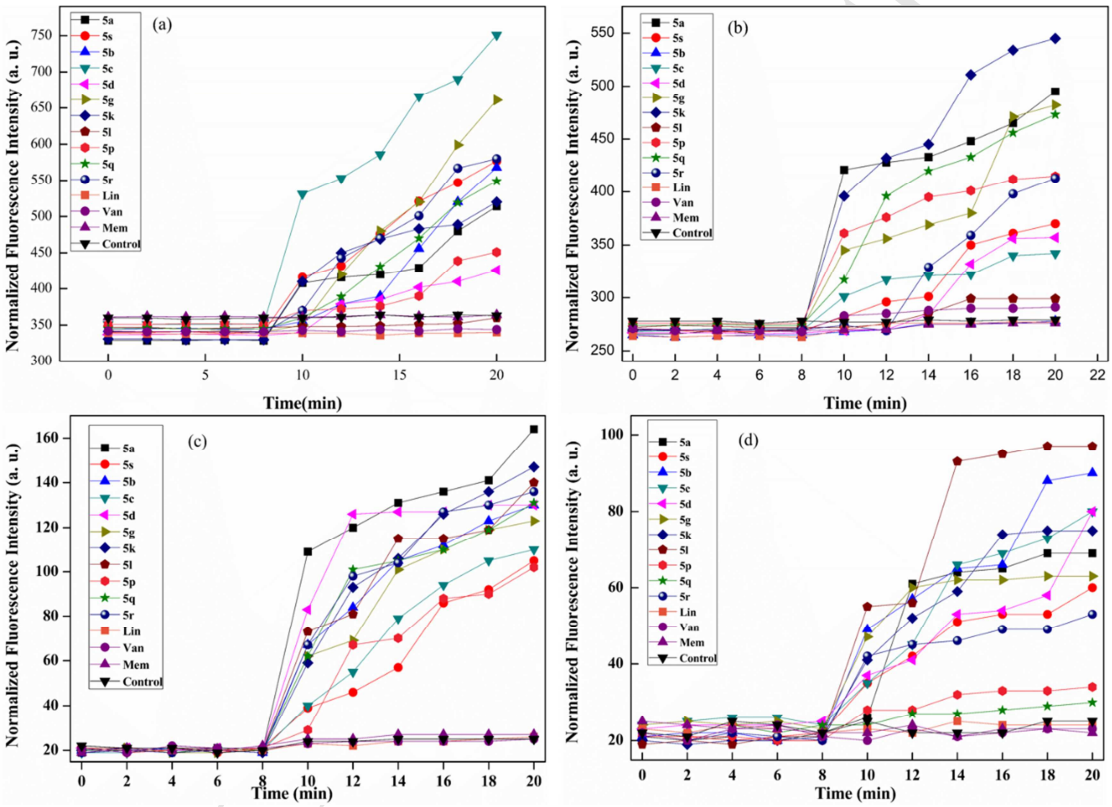


Figure 9. Mechanism of antibacterial action of potential cationic chalcone derivatives. (a) and (b) cytoplasmic membrane depolarization of *S. aureus* and *E. coli*, respectively, at 10 μg/mL. (c) and (d) inner membrane permeabilization of *S. aureus* and *E. coli*, respectively, at 10 μg/mL.

2.9. Mechanism of antibacterial action

To confirm the mechanism of action, 11 selected compounds (**5a**, **5s**, **5b**, **5c**, **5d**, **5g**, **5k**, **5l**, **5p**, **5q**, **5r**) with good antibacterial activity were used in the cytoplasmic

membrane depolarization assay and the inner membrane permeabilization assay.

2.9.1. Cytoplasmic membrane depolarization

In the cytoplasmic membrane depolarization assay, the membrane potential-sensitive cyanine dye $DiSC_{35}$ was used indirectly to reflect the integrity of the cytoplasmic membrane. If depolarization of the cell membrane is enhanced, the dye embeds into the hydrophobic environment of the bilayer of the membrane and the fluorescence intensity enhances significantly.[54] Depolarization of the cytoplasmic membrane was determined using *S. aureus* and *E. coli*. The results are shown in Figure 9a, 9b, which showed that all compounds were able to depolarize the membranes of *S. aureus* and *E. coli*. However, the membrane depolarization ability by the compounds varied. Compounds **5c**, **5g** and **5r** showed the highest depolarization activity of the cytoplasmic membrane of *S. aureus*, and compounds **5k**, **5a** and **5g** showed the highest depolarization activity of the cytoplasmic membrane of *E. coli*. The other compounds showed lower activity against both *S. aureus* and *E. coli*. The above results agree with the MIC data.

2.9.2. Inner membrane permeabilization

Permeabilization of the cytoplasmic inner membranes of bacteria *S. aureus* and *E. coli* was studied using propidium iodide (PI). The PI dye fluoresces red after embedding in double-stranded DNA. Although PI cannot pass through live cell membranes, it can traverse damaged cell membranes and stain the nucleus. Thus, using the unique attributes of PI, the permeabilization of the inner membrane was monitored. The results are shown in Figure 9c, 9d. Fluorescence intensity enhancement was observed following treatment of the cells with the aforementioned compounds. Thus, the chalcone derivatives were efficient in permeabilizing the inner membranes of both *S. aureus* and *E. coli*. Nonetheless, there were differences in the ability of the compounds to permeabilize the inner membranes. Compounds **5a**, **5k** and **5l** showed the highest permeabilization activity towards the membrane of *S. aureus*, and compounds **5l**, **5b** and **5c** showed the highest permeabilization activity

towards the membrane of *E. coli*. The above results agree with the MIC data.

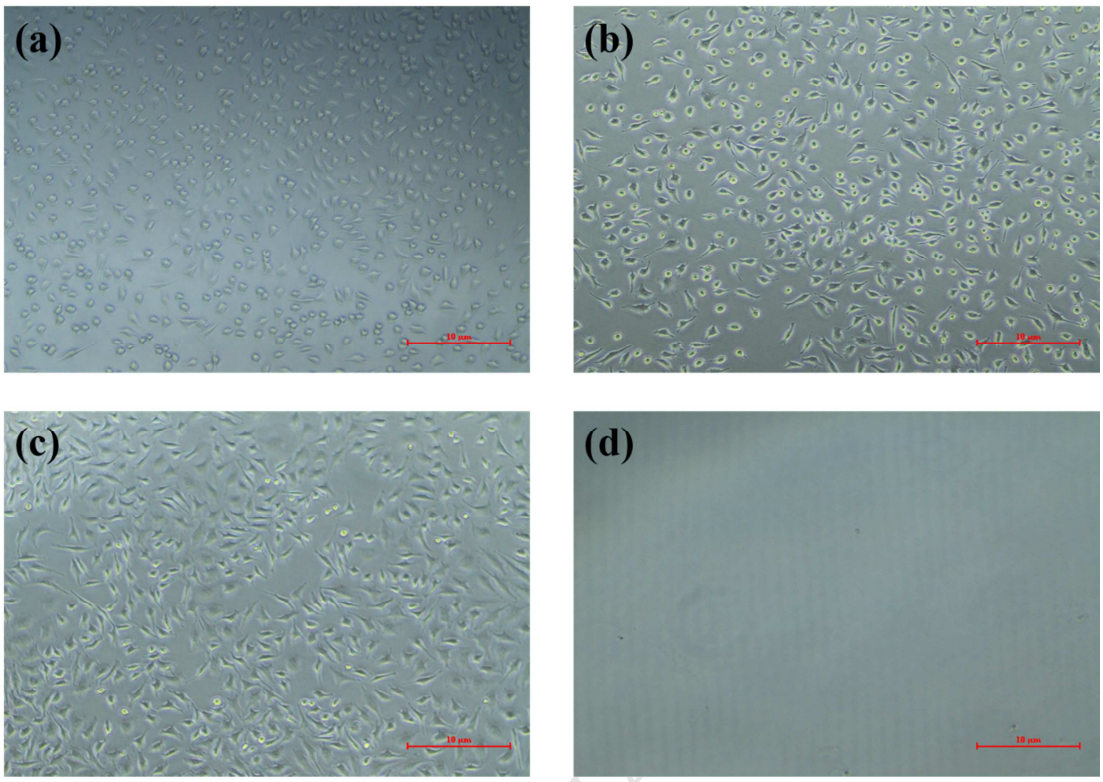


Figure 10. Electron scanning microscopy images of HeLa cells after treated with small molecule **5a** for 24 h. (a) Non-treated cells (negative control). (b) Cells treated with **5a** (2 × MIC). (c) Cells treated with **5a** (4 × MIC) and (d) cells treated with 0.1% Triton X (positive control). Scale bar is 10 μm.

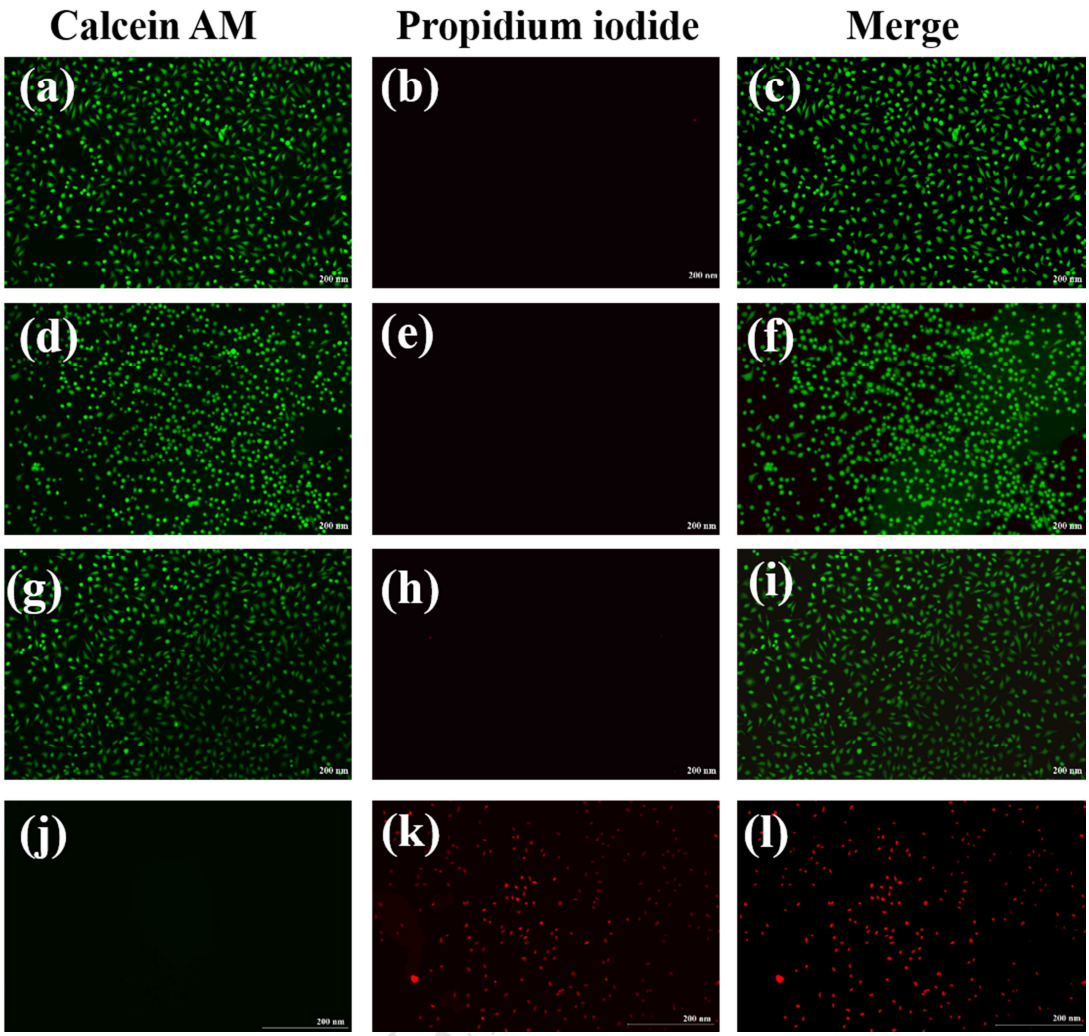


Figure 11. Fluorescence microscopy images of HeLa cells after treatment with **5a** for 24 h and staining with calcein AM and propidium iodide (PI). (a - c) Non-treated cells (negative control). (d - f) Cells treated with **5a** (2 × MIC). (g - i) Cells treated with **5a** (4 × MIC), and (j-l) cells treated with 0.1% Triton-X (positive control). Scale bar is 200 nm.

2.10. Electron scanning and fluorescence microscopy

The toxicity of compound **5a** against HeLa cells was studied. Cells were seeded into the wells and then treated with compound **5a** at different concentrations (2 × MIC and 4 × MIC). The treated cell lines were imaged by optical microscopy to visualize the cell morphology (Figure 10). The images presented in Figure 10b, 10c show that the treated cells had spindle shapes with intact morphology at **5a** concentrations of 2

× MIC and 4 × MIC, and the results were similar to the untreated cell line (Figure 10a). In contrast, cells treated with Triton-X had shapes that indicated extensive cell damage (Figure 10d). Using the different attributes of the dyes calcein-AM and PI, fluorescent microscopic studies were carried out to visualize live and dead cells. Cells treated with compound **5a** fluoresced green even at 2 × MIC and 4 × MIC concentrations (Figure 11d-f, g-i) and were similar to untreated cells (Figure 11a-c). In contrast, cells treated with 0.1% Triton-X fluoresced red (Figure 11j-l). These results thus indicate that compound **5a** is non-toxic toward mammalian cells at 4 × MIC value.

3. Conclusions

A new series of cationic molecules with the chalcone skeleton and a hydrophobic alkyl chain were designed and synthesized. These compounds displayed potent broad-spectrum and membrane-active antibacterial activity against various drug-sensitive and drug-resistant bacteria. Molecules with different substituted aryl rings showed different antibacterial and hemolytic activities. Moreover, a fluoride atom was found to influence positively the activities of the compounds. In addition, the cationic chalcone derivatives were shown to be stable under plasma conditions and even in complex mammalian fluids such as serum and plasma. The potential compounds can depolarize and permeabilize bacterial membranes, leading to the rapid death of bacteria. Importantly, the compounds did not allow bacteria to develop resistance and exhibited negligible toxicity toward mammalian cells at a given concentration. The results indicate that these cationic chalcone derivatives could be potential therapeutic agents to treat bacterial infections in the future.

4. Experimental section

4.1. Materials and instrumentation: Reagents and solvents were purchased from commercial sources (shanghai darui finechemical, Energy Chemical, Aladdin, Macklin) and were used without further purification. All the solvents were of reagent grade. All the chemicals were used as supplied. Drug-sensitive bacterial strains, *E. coli, S. aureus, E. faecalis, S. enterica* were obtained from American type culture

collection. Drug-resistant bacteria, Methicillin-resistant *S. aureus* (MRSA), Carbapenemase-producing *K. pneumonia* (KPC) and NDM-1-producing *Enterobacteriaceae* (NDM-1) were collected from a teaching hospital located in Zhengzhou, Henan Province. All isolates were identified by VITEK2 compact (bioMerieux, France) and 16S rRNA gene sequencing. PCR and nucleotide sequencing were employed to screen for the presence of bla_{NDM} gene, the primers and PCR program conditions were described previously [55]. Sheep RBCs were used for hemolytic assay.

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 400 MHz and 101 MHz spectrometer, respectively, and TMS as internal standard reference. Coupling constants (J) are given in hertz (Hz). High resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer. Analytical thin layer chromatography (TLC) was performed on glass plates pre-coated with silica gel, (Qingdao Marine Chemical Factory (China)) to monitor the reactions. Visualization was accomplished using UV light. Column chromatography was performed on silica gel (300-400 meshes). For optical density and fluorescence measurements, Tecan Infinite Pro series M200 Microplate Reader was used. The biological experiments were performed with a 1300 series A_2 Biological Safety Cabinet. TDL-5M Desktop Low-speed Refrigerated Centrifuge was used in antibacterial studies.

4.2. General procedure for the synthesis of Chalcone analogs (3a-3w)

NaOH (9.25 mmol, 1.25 eq.) in H_2O (50 mL) was stirred at room temperature. Compound 1-(4-aminophenyl) ethan-1-one or 1-(3-aminophenyl) ethan-1-one (7.40 mmol, 1.00 eq.) and aromatic aldehyde (7.58 mmol, 1.02 eq.) were dissolved in ethanol (50 mL). Then ethanol solution was added into H_2O solution. The reaction mixture was stirred at room temperature. After the reaction was completed (monitored by TLC light petroleum / EtOAc, 2/1, v/v), the mixture was poured into ice water (50 mL). The precipitate formed was filtered and washed with water. The products were dried under vacuum and purified via silica gel column chromatography (light petroleum /EtOAc, 3/1, v/v) to obtain intermediate products **3a-3w** in 10%-86% yield. The purified compounds were subsequently characterized using ^{1}H NMR, ^{13}C NMR. The NMR

data of compounds **(3a-3w)** were listed in supplementary material.

4.3. General procedure for the synthesis of amide coupling compounds (4a-4w)

Chloroacetyl chloride (0.6 mmol, 1.20 eq.) was dropped to the mixture of intermediates **3a-3w** (0.5 mmol, 1.00 eq.) and K_2CO_3 (0.6 mmol, 1.20 eq.) in acetone (1.5 mL). The reaction mixture was stirred at room temperature for 0.5-1 h. After the reaction finished (monitored by TLC), the mixture was poured into ice water (3 mL). The precipitate formed was filtered and washed with water. The products were dried under vacuum to obtain target compounds **4a-4w** without any further purification. The purified compounds were in 41%-91% yield and subsequently characterized using ^{1}H NMR, ^{13}C NMR. The NMR data of compounds **(4a-4w)** were listed in supplementary material.

4.4. General procedure for the synthesis of amphiphilic small molecules (5a-5ac)

Compounds **(4a-4w)** (528.73 μmol, 1.0eq.) were reacted with N, N- dimethyl tertiary amine (1.58 mmol, 3 eq.) in anhydrous acetonitrile (6 mL) in a screw-top pressure tube at 85 °C for about 24 h. After the reaction, the solvent was evaporated and the products were precipitated using excess ether. Finally, the products were filtered and washed with ether for 3-5 times. The products were dried under vacuum to obtain target compounds **5a-5ac** without any further purification. The purified compounds were in 50%-95% yield and subsequently characterized using ^{1}H NMR, ^{13}C NMR, IR and HR-MS.

4.4.1. ***(E)N-(2-((4-cinnamoylphenyl)amino)-2-oxoethyl)-N,N-dimethyloctan-1-aminium chloride (5a).*** Light yellow solid, yield: 78%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.21 (s, 1H), 8.24 (d, J = 8.0 Hz, 2H), 8.04 – 7.88 (m, 5H), 7.77 (d, J = 15.5 Hz, 1H), 7.48 (s, 3H), 4.64 (s, 2H), 3.58 (s, 2H), 3.34 (s, 6H), 1.79 (s, 2H), 1.24-1.23 (m, 6H), 1.23-1.13 (m, 4H), 0.83 (d, J = 6.1 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.61, 162.71, 143.60, 142.37, 134.70, 133.16, 130.54, 129.84, 128.88, 121.89, 119.09, 64.88, 62.32, 51.24, 31.12, 28.37, 25.67, 22.00, 21.83, 13.91. IR υ (cm −1): 764, 975, 1447, 1601, 1661, 1696, 3025, 3166, 3245; HR-MS (ESI) Calculated for $C_{27}H_{37}ClN_2O_2$ [M-Cl] $^{+}$: 421.2850, found: 421.2856.

4.4.2. ***(E)-N-(2-((4-(3-(3-chlorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N dimethyloctan-1-aminium chloride (5b).*** White solid, yield: 93%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.87 (s, 1H), 8.19 (s, 2H), 7.90 (d, *J* = 18.9 Hz, 5H), 7.71 (d, *J* = 14.1 Hz, 1H), 7.52 (s, 2H), 4.50 (s, 2H), 3.51 (s, 2H), 3.26 (s, 6H), 1.74 (s, 2H), 1.42-0.98 (m, 10H), 0.82 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.51, 162.69, 142.37, 142.12, 135.00, 133.69, 133.10, 130.52, 129.89, 128.92, 122.65, 119.12, 64.93, 62.33, 51.26, 31.11, 28.35, 25.66, 21.98, 21.83, 13.89. IR υ (cm $^{-1}$): 723, 984, 1465, 1596, 1658, 1698, 3085, 3170, 3245; HR-MS (ESI) Calculated for $C_{27}H_{36}Cl_2N_2O_2$ [M-Cl] $^{+}$: 455.2460, found: 455.2464.

4.4.3. ***(E)-N-(2-((4-(3-(3-chlorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim ethyloctan-1-aminium chloride (5c).*** Light yellow solid, yield: 67%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.99 (s, 1H), 8.24 (d, *J* = 8.6 Hz, 2H), 8.11 – 8.02 (m, 2H), 7.92 (d, *J* = 8.6 Hz, 2H), 7.84 (d, *J* = 6.1 Hz, 1H), 7.72 (d, *J* = 15.6 Hz, 1H), 7.53 – 7.46 (m, 2H), 4.56 (s, 2H), 3.60 – 3.50 (m, 2H), 3.30 (s, 6H), 1.78 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.18 (m, 4H), 0.84 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.94, 163.23, 143.00, 142.37, 137.47, 134.27, 133.46, 131.16, 130.48, 128.38, 123.89, 119.56, 65.38, 62.80, 51.73, 31.62, 28.87, 26.17, 22.50, 22.33, 14.41. IR υ (cm −1): 727, 971, 1447, 1600, 1665, 1699, 3027, 3163, 3245; HR-MS (ESI) Calculated for $C_{27}H_{36}Cl_2N_2O_2$ [M-Cl] $^{+}$: 455.2460, found: 455.2463.

4.4.4. ***(E)-N-(2-((4-(3-(4-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim ethyloctan-1-aminium chloride (5d).*** White solid, yield: 83%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.04 (s, 1H), 8.22 (d, *J* = 8.5 Hz, 2H), 8.05 – 7.88 (m, 5H), 7.75 (d, *J* = 15.6 Hz, 1H), 7.32 (t, *J* = 8.6 Hz, 2H), 4.57 (s, 2H), 3.65 – 3.48 (m, 2H), 3.31 (s, 6H), 1.78 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.18 (m, 4H), 0.84 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.50, 163.13 (d, *J* = 250.07 Hz), 162.70, 142.36, 133.13, 131.39 (d, *J* = 3.0 Hz), 131.19 (d, *J* = 8.6 Hz), 129.84, 121.78, 119.07, 115.89 (d, *J* = 21.7 Hz), 64.88, 62.31, 51.23, 31.11, 28.36, 25.66, 22.00, 21.83, 13.90. IR υ (cm −1): 739, 983, 1465, 1597, 1656, 1697, 3085, 3172, 3241; HR-MS (ESI) Calculated for $C_{27}H_{36}ClFN_2O_2$ [M-Cl] $^{+}$: 439.2755, found: 439.2762.

4.4.5. ***(E)-N-(2-((4-(3-(3-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim***

ethyloctan-1-aminium chloride (5e). Light yellow solid, yield: 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 8.24 (d, J = 8.7 Hz, 2H), 8.05 (d, J = 15.6 Hz, 1H), 7.91 (dd, J = 23.1, 9.5 Hz, 3H), 7.80 – 7.65 (m, 2H), 7.52 (dd, J = 14.2, 7.9 Hz, 1H), 7.30 (td, J = 8.6, 2.3 Hz, 1H), 4.59 (s, 2H), 3.56 (dd, J = 10.2, 6.5 Hz, 2H), 3.31 (s, 6H), 1.79 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.18 (m, 4H), 0.84 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.49, 162.73, 162.56 (d, *J* = 244.62), 142.51, 142.09 (d, *J* = 2.7 Hz), 137.29 (d, *J* = 8.1 Hz), 132.97, 130.80 (d, *J* = 8.4 Hz), 129.94, 125.53 (d, *J* = 2.4 Hz), 123.33, 119.07, 117.16 (d, *J* = 21.4 Hz), 114.62 (d, *J* = 22.0 Hz), 64.87, 62.32, 51.23, 31.11, 28.36, 25.67, 22.00, 21.84, 13.89. IR υ (cm −1): 733, 971, 1446, 1601, 1665, 1697, 3028, 3165, 3239; HR-MS (ESI) Calculated for $C_{27}H_{36}ClFN_2O_2$ [M-Cl] $^{+}$: 439.2755, found: 439.2763.

4.4.6. ***(E)-N-(2-((4-(3-(2-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim ethylbutan-1-aminium chloride (5f).*** Light yellow solid, yield: 76%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (s, 1H), 8.32 – 8.09 (m, 3H), 8.02 (d, *J* = 15.7 Hz, 1H), 7.93 (d, *J* = 8.5 Hz, 2H), 7.84 (d, *J* = 15.7 Hz, 1H), 7.54 (dd, *J* = 13.2, 6.7 Hz, 1H), 7.34 (dd, *J* = 13.0, 6.2 Hz, 2H), 4.58 (s, 2H), 3.66 – 3.50 (m, 2H), 3.31 (s, 6H), 1.77 (d, *J* = 7.4 Hz, 2H), 1.33 (dd, *J* = 14.4, 7.2 Hz, 2H), 0.94 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.44, 162.73, 160.87 (d, *J* = 251.3 Hz), 142.53, 134.71 (d, *J* = 4.2 Hz), 132.89, 132.59 (d, *J* = 8.6 Hz), 129.92, 129.09, 124.93 (d, *J* = 3.4 Hz), 124.01 (d, *J* = 4.0 Hz), 122.32 (d, *J* = 11.2 Hz), 119.14, 116.05 (d, *J* = 21.6 Hz), 64.77, 62.35, 51.18, 23.87, 19.13, 13.43. IR υ (cm −1): 743, 963, 1456, 1598, 1659, 1691, 3034, 3163, 3235; HR-MS (ESI) Calculated for $C_{23}H_{28}ClFN_2O_2$ [M-Cl] $^{+}$: 383.2129, found: 383.2135.

4.4.7. ***(E)-N-(2-((4-(3-(2-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim ethyloctan-1-aminium chloride (5g).*** Light yellow solid, yield: 84%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.03 (s, 1H), 7.97 (dd, *J* = 19.1, 8.6 Hz, 4H), 7.89 (d, *J* = 15.9 Hz, 1H), 7.66 (d, *J* = 7.4 Hz, 1H), 7.62 (d, *J* = 15.7 Hz, 1H), 7.44 – 7.32 (m, 1H), 7.20 (t, *J* = 7.5 Hz, 1H), 7.16 – 7.05 (m, 1H), 4.95 (s, 2H), 3.75 – 3.57 (m, 2H), 3.44 (s, , 6H), 1.83 (s, 2H), 1.46-1.12 (m, 10H), 0.86 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 188.99, 161.73 (d, J = 255 Hz), 161.71, 141.79, 137.19, 134.27, 131.77 (d, *J*

= 8.8 Hz), 129.74, 124. 43 (d, J = 6.9 Hz), 123.08 (d, *J* = 11.2 Hz), 119.84, 116.27 (d, *J* = 21.9 Hz), 66.50, 63.90, 52.10, 31.54, 28.96, 26.17, 22.91, 22.52, 14.01. IR υ (cm −1): 723, 980, 1444, 1600, 1662, 1696, 3026, 3165, 3235; HR-MS (ESI) Calculated for $C_{27}H_{36}ClFN_2O_2$ [M-Cl] $^+$: 439.2755, found: 439.2764.

4.4.8. ***(E)-N-(2-((4-(3-(2-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyldodecan-1-aminium chloride (5h).*** Light yellow solid, yield: 77%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.05 (d, *J* = 21.5 Hz, 1H), 8.20 (d, *J* = 8.6 Hz, 2H), 8.15 (t, *J* = 7.7 Hz, 1H), 8.01 (d, *J* = 15.7 Hz, 1H), 7.93 (d, *J* = 8.0 Hz, 2H), 7.84 (d, *J* = 15.7 Hz, 1H), 7.53 (dd, *J* = 13.5, 6.9 Hz, 1H), 7.34 (dd, *J* = 13.0, 5.5 Hz, 2H), 4.57 (s, 2H), 3.66 – 3.48 (m, 2H), 3.30 (s, 6H), 1.78 (s, 2H), 1.30 – 1.17 (m, 18H), 0.83 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.29, 162.73, 160.87 (d, *J* = 251.4 Hz), 142.58, 134.63 (d, *J* = 4.1 Hz), 132.86, 132.46 (d, *J* = 8.7 Hz), 129.81, 129.06, 124.83 (d, *J* = 3.1 Hz), 123.86 (d, *J* = 3.9 Hz), 122.32 (d, *J* = 11.2 Hz), 119.09, 115.95 (d, *J* = 21.7 Hz), 64.72, 62.33, 51.29, 31.27, 29.03, 28.94, 28.76, 28.72, 28.39, 25.68, 22.05, 21.86, 13.83. IR υ (cm −1): 753, 981, 1467, 1601, 1659, 1704, 3091, 3175, 3246; HR-MS (ESI) Calculated for $C_{31}H_{44}ClFN_2O_2$ [M-Cl] $^+$: 495.3381, found: 495.3388.

4.4.9. ***(E)-N-(2-((4-(3-(2-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyltetradecan-1-aminium chloride (5i).*** Light yellow solid, yield: 79%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.85 (s, 1H), 8.19 (d, *J* = 8.8 Hz, 2H), 8.13 (t, *J* = 7.5 Hz, 1H), 8.00 (d, *J* = 15.7 Hz, 1H), 7.89 (d, *J* = 8.7 Hz, 2H), 7.83 (d, *J* = 15.7 Hz, 1H), 7.53 (dd, *J* = 13.1, 6.4 Hz, 1H), 7.33 (dd, *J* = 13.2, 5.7 Hz, 2H), 4.51 (s, 2H), 3.53 (dd, *J* = 10.1, 6.6 Hz, 2H), 3.28 (s, 6H), 1.76 (s, 2H), 1.29 – 1.16 (m, 22H), 0.83 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.24, 162.72, 160.88 (d, *J* = 251.5 Hz), 142.57, 134.63 (d, *J* = 4.1 Hz), 132.86, 132.42 (d, *J* = 8.7 Hz), 129.80, 129.03, 124.80 (d, *J* = 3.1 Hz), 123.82 (d, *J* = 3.8 Hz), 122.33 (d, *J* = 11.2 Hz), 119.07, 115.92 (d, *J* = 21.7 Hz), 64.67, 62.31, 51.30, 31.28, 29.10, 29.05, 28.96, 28.78, 28.73, 28.40, 25.68, 22.06, 21.86, 13.82. IR υ (cm −1): 758, 985, 1468, 1597, 1661, 1696, 3084, 3161, 3245; HR-MS (ESI) Calculated for $C_{33}H_{48}ClFN_2O_2$ [M-Cl] $^+$: 523.3694, found: 523.3697.

4.4.10. ***(E)-N-(2-((4-(3-(2-fluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-di***

methyloctadecan-1-aminium chloride (5j). Light yellow solid, yield: 78%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.81 (s, 1H), 8.19 (d, *J* = 8.8 Hz, 2H), 8.13 (t, *J* = 7.5 Hz, 1H), 8.00 (d, *J* = 15.7 Hz, 1H), 7.89 (d, *J* = 8.7 Hz, 2H), 7.84 (d, *J* = 15.8 Hz, 1H), 7.53 (dd, *J* = 13.1, 6.5 Hz, 1H), 7.33 (dd, *J* = 13.2, 5.5 Hz, 2H), 4.51 (s, 2H), 3.53 (dd, *J* = 10.1, 6.6 Hz, 2H), 3.29 (s, 6H), 1.76 (s, 2H), 1.35 – 1.12 (m, 32H), 0.84 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 186.21, 161.60, 159.75 (d, *J* = 251.3 Hz), 141.38, 133.57 (d, *J* = 4.2 Hz), 131.76, 131.43 (d, *J* = 8.8 Hz), 128.76, 127.94, 123.77 (d, *J* = 3.2 Hz), 122.79 (d, *J* = 3.8 Hz), 121.19 (d, *J* = 11.2 Hz), 117.97, 114.90 (d, *J* = 21.5 Hz), 63.53, 61.10, 50.23, 30.13, 27.92, 27.90, 27.85, 27.80, 27.55, 27.19, 24.46, 20.93, 20.63, 12.77. IR υ (cm −1): 757, 985, 1467, 1597, 1661, 1696, 3084, 3161, 3249; HR-MS (ESI) Calculated for $C_{37}H_{56}ClFN_2O_2$ [M-Cl] $^{+}$: 579.4320, found: 579.4323.

4.4.11. ***(E)-N-(2-((4-(3-(2,3-difluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyloctan-1-aminium chloride (5k).*** White solid, yield: 73%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.10 (s, 1H), 8.21 (d, J = 8.8 Hz, 2H), 8.05 (d, J = 15.7 Hz, 1H), 7.98 (d, J = 7.6 Hz, 1H), 7.94 (d, J = 8.8 Hz, 2H), 7.80 (d, J = 15.7 Hz, 1H), 7.54 (dd, J = 17.2, 8.3 Hz, 1H), 7.33 (dd, J = 12.8, 7.7 Hz, 1H), 4.59 (s, 2H), 3.56 (dd, J = 10.2, 6.5 Hz, 2H), 3.31 (s, 6H), 1.79 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.20 (m, 4H), 0.84 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.52, 168.00, 155.21 (dd, *J* = 246.9, *J* = 12.7 Hz), 153.79 (dd, *J* = 253.6, 13.4 Hz), 147.92, 138.64 (t, *J* = 3.6 Hz), 137.95, 135.21, 130.64 (d, *J* = 4.1 Hz), 130.36 (d, *J* = 5.3 Hz), 130.30 (d, *J* = 3.0 Hz), 129.93 (d, *J* = 8.2 Hz), 129.49 (d, *J* = 2.6 Hz), 124.39, 124.19, 70.17, 67.61, 56.49, 36.36, 33.60, 30.93, 27.24, 27.10, 19.12. IR υ (cm −1): 743, 963, 1467, 1602, 1661, 1700, 3081, 3165, 3250; HR-MS (ESI) Calculated for $C_{27}H_{35}ClF_2N_2O_2$ [M-Cl] $^{+}$: 457.2661, found: 457.2666.

4.4.12. ***(E)-N-(2-((4-(3-(2,4-difluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyloctan-1-aminium chloride (5l).*** Light yellow solid, yield: 75%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.11 (s, 1H), 8.38 – 8.10 (m, 3H), 8.01 (d, *J* = 15.7 Hz, 1H), 7.93 (d, *J* = 8.4 Hz, 2H), 7.78 (d, *J* = 15.7 Hz, 1H), 7.33 (dt, *J* = 16.5, 9.1 Hz, 2H), 4.59 (s, 2H), 3.66 – 3.51 (m, 2H), 3.31 (s, 6H), 1.79 (s, 2H), 1.31-1.25 (m, 6H),

1.25-1.16 (m, 4H), 0.83 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.34, 163.51 (dd, *J* = 252.4, 12.7 Hz), 162.73, 161.25 (dd, *J* = 254.9, 12.7 Hz), 142.54, 133.75 (d, *J* = 1.9 Hz), 132.87, 130.71 (dd, *J* = 10.0, 3.6 Hz), 129.87, 123.76, 119.26 (d, *J* = 3.8 Hz), 119.13, 112.58 (d, *J* = 3.4 Hz), 112.37 (d, *J* = 3.5 Hz), 104.55 (t, *J* = 26.1 Hz), 64.90, 62.35, 51.25, 31.10, 28.35 (d, *J* = 1.9 Hz), 25.67, 21.98, 21.84, 13.87. IR υ (cm −1): 749, 974, 1466, 1600, 1661, 1695, 3093, 3173, 3240; HR-MS (ESI) Calculated for $C_{27}H_{35}ClF_2N_2O_2$ [M-Cl] $^+$: 457.2661, found: 457.2668.

4.4.13. ***(E)-N-(2-((4-(3-(2,6-difluorophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyloctan-1-aminium chloride (5m).*** Brown solid, yield: 95%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (s, 1H), 8.09 (d, *J* = 8.7 Hz, 2H), 7.90 (dd, *J* = 17.4, 12.4 Hz, 3H), 7.69 (d, *J* = 16.0 Hz, 1H), 7.63 – 7.50 (m, 1H), 7.27 (t, *J* = 8.8 Hz, 2H), 4.55 (s, 2H), 3.60 – 3.49 (m, 2H), 3.30 (s, 6H), 1.78 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.19 (m, 4H), 0.84 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.53, 162.76, 160.77 (dd, J = 254.6, 6.8 Hz), 142.62, 132.63, 132.47 (d, *J* = 11.1 Hz), 129.80, 128.91, 127.31 (t, *J* = 7.5 Hz), 119.25, 112.47 (d, *J* = 5.0 Hz), 112.20 (d, *J* = 9.4 Hz), 111.93 (d, *J* = 15.3 Hz), 64.87, 62.27, 51.24, 31.12, 28.36, 25.66, 22.00, 21.83, 13.89. IR υ (cm −1): 749, 962, 1469, 1597, 1661, 1696, 3084, 3161, 3249; HR-MS (ESI) Calculated for $C_{27}H_{35}ClF_2N_2O_2$ [M-Cl] $^+$: 457.2661, found: 457.2668.

4.4.14. ***(E)-N-(2-((4-(3-(2-ethoxy-5-nitrophenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimethyloctan-1-aminium chloride (5n)***. Light yellow solid, yield: 58%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (s, 1H), 8.82 (s, 1H), 8.29 (d, J = 7.3 Hz, 1H), 8.21 (d, J = 8.3 Hz, 2H), 8.13 (d, J = 15.8 Hz, 1H), 7.94 (dd, J = 23.0, 12.0 Hz, 3H), 7.32 (d, J = 9.2 Hz, 1H), 4.56 (s, 2H), 3.55 (s, 2H), 3.30 (s, 6H), 1.78 (s, 2H), 1.31-1.26 (m, 6H), 1.26-1.18 (m, 4H), 0.84 (d, J = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.48, 162.73, 162.04, 142.49, 140.85, 135.86, 132.99, 129.92, 127.17, 124.31, 123.97, 123.73, 119.10, 113.01, 65.26, 64.91, 62.33, 51.25, 31.11, 28.36, 25.67, 21.99, 21.83, 14.31, 13.89. IR υ (cm −1): 747, 956, 1467, 1589, 1660, 1699, 3045, 3191, 3249; HR-MS (ESI) Calculated for $C_{29}H_{40}ClN_3O_5$ [M-Cl] $^+$: 510.2962, found: 510.2963.

4.4.15. ***(E)-N-(2-((4-(3-(4-(tert-butyl)phenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,***

N-dimethyloctan-1-aminium chloride (5o). Brown solid, yield: 87%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.84 (s, 1H), 8.19 (d, *J* = 8.5 Hz, 2H), 8.00 – 7.85 (m, 3H), 7.81 (d, *J* = 8.2 Hz, 2H), 7.72 (d, *J* = 15.5 Hz, 1H), 7.48 (d, *J* = 8.2 Hz, 2H), 4.52 (s, 2H), 3.73 – 3.42 (m, 2H), 3.28 (s, 6H), 1.77 (s, 2H), 1.35 – 1.17 (m, 19H), 0.85 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.65, 162.67, 153.54, 143.57, 142.22, 133.32, 132.01, 129.77, 128.70, 125.70, 121.11, 119.12, 64.93, 62.33, 51.26, 34.63, 31.11, 30.88, 28.35, 25.66, 21.99, 21.83, 13.90. IR υ (cm −1): 754, 982, 1467, 1599, 1661, 1699, 3053, 3187, 3250; HR-MS (ESI) Calculated for $C_{31}H_{45}ClN_2O_2$ [M-Cl] $^{+}$: 477.3476, found: 477.3482.

4.4.16. ***(E)-N,N-dimethyl-N-(2-oxo-2((4(3(2(trifluoromethyl)phenyl)acryloyl)phenyl)amino)ethyl)octan-1-aminium chloride (5p).*** Light yellow solid, yield: 83%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.13 (s, 1H), 8.38 (d, *J* = 7.7 Hz, 1H), 8.25 (d, *J* = 8.0 Hz, 2H), 8.08 (d, *J* = 15.3 Hz, 1H), 7.97 (t, *J* = 11.2 Hz, 3H), 7.83 (dd, *J* = 16.5, 7.8 Hz, 2H), 7.69 (t, *J* = 7.4 Hz, 1H), 4.61 (s, 2H), 3.56 (d, *J* = 7.3 Hz, 2H), 3.33 (s, 6H), 1.79 (s, 2H), 1.40-1.25 (m, 6H), 1.25-1.02 (m, 4H), 0.83 (d, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 192.40, 167.64, 147.64, 143.05, 138.29, 138.01, 137.67, 135.13, 134.98, 134.71 (d, *J* = 10.7 Hz), 133.65, 133.26 (d, *J* = 8.2 Hz), 132.92, 131.07 (d, *J* = 5.3 Hz), 130.99, 130.52, 127.80, 124.33, 70.29, 67.63, 56.38, 36.37, 33.68, 31.00, 27.27, 27.23, 19.05. IR υ (cm −1): 757, 978, 1459, 1597, 1665, 1713, 3049, 3169, 3245; HR-MS (ESI) Calculated for $C_{28}H_{36}ClF_3N_2O_2$ [M-Cl] $^{+}$: 489.2723, found: 489.2728.

4.4.17. ***(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(p-tolyl)acryloyl)phenyl)amino)ethyl)octan-1-aminium chloride (5q).*** Light yellow solid, yield: 66%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.08 (s, 1H), 8.21 (d, *J* = 8.7 Hz, 2H), 7.99 – 7.84 (m, 3H), 7.80 (d, *J* = 8.0 Hz, 2H), 7.72 (d, *J* = 15.6 Hz, 1H), 7.29 (d, *J* = 8.0 Hz, 2H), 4.59 (s, 2H), 3.56 (dd, *J* = 10.0, 6.6 Hz, 2H), 3.31 (s, 6H), 2.36 (s, 3H), 1.78 (s, 2H), 1.32-1.25 (m, 6H), 1.25-1.16 (m, 4H), 0.84 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.55, 162.69, 143.65, 142.30, 140.58, 133.26, 131.98, 129.75, 129.50, 128.87, 120.80, 119.07, 64.86, 62.32, 51.23, 31.12, 28.37, 25.68, 22.00, 21.84, 21.06, 13.90. IR υ (cm −1): 735, 984, 1465, 1597, 1658, 1698, 3086, 3170, 3238; HR-MS (ESI) Calculated

for $C_{28}H_{39}ClN_2O_2$ [M-Cl] $^+$: 435.3006, found: 435.3013.

4.4.18.

(E)-N-(2-((4-(3-(4-methoxyphenyl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dimeth yloctan-1-aminium chloride (5r). Light yellow solid, yield: 85%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H), 8.20 (d, *J* = 8.7 Hz, 2H), 7.97 – 7.79 (m, 5H), 7.72 (d, *J* = 15.5 Hz, 1H), 7.03 (d, *J* = 8.7 Hz, 2H), 4.57 (s, 2H), 3.83 (s, 3H), 3.55 (dd, *J* = 10.2, 6.5 Hz, 2H), 3.30 (s, 6H), 1.78 (s, 2H), 1.31-1.25 (m, 6H), 1.25-1.19 (m, 4H), 0.84 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.46, 162.66, 161.29, 143.58, 142.15, 133.44, 130.73, 129.67, 127.34, 119.34, 119.05, 114.37, 64.87, 62.31, 55.35, 51.23, 31.12, 28.36, 25.67, 22.00, 21.83, 13.90. IR υ (cm −1): 748, 977, 1444, 1596, 1659, 1699, 3086, 3169, 3229; HR-MS (ESI) Calculated for $C_{28}H_{39}ClN_2O_3$ [M-Cl] $^+$: 451.2955, found: 451.2963.

4.4.19. ***(E)-N,N-dimethyl-N-(2-((4-(3-(naphthalen-2-yl)acryloyl)phenyl)amino)-2-ox oethyl)octan-1-aminium chloride (5s).*** Brown solid, yield: 56%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.13 (s, 1H), 8.59 (d, *J* = 15.3 Hz, 1H), 8.28 (dd, *J* = 12.4, 6.3 Hz, 4H), 8.06 (dd, *J* = 21.3, 6.5 Hz, 3H), 7.97 (d, *J* = 8.5 Hz, 2H), 7.70 – 7.59 (m, 3H), 4.61 (s, 2H), 3.61 – 3.51 (m, 2H), 3.32 (s, 6H), 1.79 (s, 2H), 1.30-1.24 (m, 6H), 1.24-1.19 (m, 4H), 0.84 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.54, 162.73, 142.46, 139.47, 133.34, 133.12, 131.32, 131.17, 130.77, 129.92, 128.78, 127.20, 126.27, 125.66, 124.38, 122.93, 119.14, 64.87, 62.34, 51.23, 31.11, 28.37, 25.67, 22.00, 21.85, 13.90. IR υ (cm −1): 738, 969, 1488, 1598, 1656, 1694, 3087, 3165, 3225; HR-MS (ESI) Calculated for $C_{31}H_{39}ClN_2O_2$ [M-Cl] $^+$: 471.3006, found: 471.3013.

4.4.20. ***(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(pyridin4yl)acryloyl)phenyl)amino) ethyl)butan-1-aminium chloride (5t)***. Light brown solid, yield: 50%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.97 (s, 1H), 8.68 (d, *J* = 6.0 Hz, 2H), 8.23 (d, *J* = 8.8 Hz, 2H), 8.18 (d, *J* = 15.7 Hz, 1H), 7.92 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 6.0 Hz, 2H), 7.68 (d, *J* = 15.7 Hz, 1H), 4.55 (s, 2H), 3.62 – 3.48 (m, 2H), 3.30 (s, 6H), 1.84 – 1.70 (m, 2H), 1.40 – 1.26 (m, 2H), 0.94 (t, *J* = 7.4 Hz, 3H).^{13}C NMR (101 MHz, DMSO-d_6) δ 187.56, 162.75, 150.31, 142.66, 141.83, 140.64, 132.71, 130.09, 126.28, 122.48,

119.16, 64.82, 62.39, 51.21, 23.87, 19.13, 13.42. IR υ (cm −1): 747, 981, 1482, 1600, 1660, 1699, 3087, 3165, 3225; HR-MS (ESI) Calculated for $C_{22}H_{28}ClN_3O_2$ [M-Cl] $^+$: 366.2176, found: 366.2180.

4.4.21. ***(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(pyridin-4yl)acryloyl)phenyl)amino) ethyl)octan-1-aminium chloride (5u)***. Light brown solid, yield: 90%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.42 (s, 1H), 8.68 (d, *J* = 4.6 Hz, 2H), 8.23 (d, *J* = 8.7 Hz, 2H), 8.16 (d, *J* = 15.7 Hz, 1H), 7.85 (d, *J* = 8.3 Hz, 4H), 7.68 (d, *J* = 15.6 Hz, 1H), 4.42 (s, 2H), 3.57 – 3.49 (m, 2H), 3.27 (s, 6H), 1.75 (s, 2H), 1.32 – 1.23 (m, 10H), 0.85 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.50, 162.77, 150.31, 142.72, 141.81, 140.64, 132.66, 130.08, 126.22, 122.49, 119.11, 64.88, 62.32, 51.23, 31.11, 28.37, 25.67, 21.92, 13.90. IR υ (cm −1): 748, 988, 1444, 1620, 1660, 1697, 3090, 3171, 3240; HR-MS (ESI) Calculated for $C_{26}H_{36}ClN_3O_2$ [M-Cl] $^+$: 422.2802, found: 422.2809.

4.4.22. ***(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(pyridin-3-yl)acryloyl)phenyl)amino) ethyl)octan-1-aminium chloride (5v)***. Light brown solid, yield: 52%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.77 (d, *J* = 16.4 Hz, 1H), 9.04 (s, 1H), 8.69 – 8.56 (m, 1H), 8.37 (d, *J* = 8.0 Hz, 1H), 8.23 (d, *J* = 8.7 Hz, 2H), 8.10 (d, *J* = 15.7 Hz, 1H), 7.89 (d, *J* = 8.5 Hz, 2H), 7.77 (d, *J* = 15.7 Hz, 1H), 7.51 (dd, *J* = 7.8, 4.8 Hz, 1H), 4.50 (s, 2H), 3.63 – 3.45 (m, 2H), 3.28 (s, 6H), 1.77 (s, 2H), 1.45 – 1.16 (m, 10H), 0.84 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.36, 162.73, 150.93, 150.32, 142.52, 140.15, 135.09, 132.90, 130.54, 129.97, 123.88, 123.74 119.09, 64.89, 62.31, 51.23, 31.11, 28.37, 25.66, 21.92, 13.90. IR υ (cm −1): 748, 976, 1467, 1599, 1664, 1697, 3086, 3166, 3240; HR-MS (ESI) Calculated for $C_{26}H_{36}ClN_3O_2$ [M-Cl] $^+$: 422.2802, found: 422.2805.

4.4.23. ***(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(pyridin-2yl)acryloyl)phenyl)amino) ethyl)octan-1-aminium chloride (5w)***. Light brown solid, yield: 86%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.11 (s, 1H), 8.69 (d, *J* = 2.8 Hz, 1H), 8.35 – 8.02 (m, 3H), 7.92 (d, *J* = 11.9 Hz, 4H), 7.71 (d, *J* = 15.4 Hz, 1H), 7.43 (s, 1H), 4.59 (s, 2H), 3.54 (s, 2H), 3.30 (s, 6H), 1.77 (s, 2H), 1.33-1.22 (m, 6H), 1.22-1.14 (m, 4H), 0.81 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 188.33, 163.25, 153.29, 150.48, 143.13 (d, *J* = 11.8

Hz), 137.66, 133.39, 130.33, 125.43(d, *J* = 3.9Hz) 125.26, 119.70, 65.36, 62.81, 51.73, 31.61, 28.87 (d, *J* = 2.2 Hz), 26.18, 22.42 (d, *J* = 15.1 Hz), 14.39. IR υ (cm $^{-1}$): 748, 973, 1466, 1600, 1661, 1697, 3086, 3171, 3229; HR-MS (ESI) Calculated for $C_{26}H_{36}ClN_3O_2$ [M-Cl] $^{+}$: 422.2802, found: 422.2806.

4.4.24. ***(E)-N-(2-((4-(3-(6-bromopyridin-2-yl)acryloyl)phenyl)amino)-2-oxoethyl)-N, N-dimethyloctan-1-aminium chloride (5x)***. Light yellow solid, yield: 81%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.76 (s, 1H), 8.14 (d, *J* = 8.8 Hz, 2H), 8.08 (d, *J* = 15.4 Hz, 1H), 7.97 (d, *J* = 7.4 Hz, 1H), 7.91 – 7.88 (m, 2H), 7.86 (d, *J* = 7.7 Hz, 1H), 7.70 (d, *J* = 7.8 Hz, 1H), 7.64 (d, *J* = 15.4 Hz, 1H), 4.49 (s, 2H), 3.53 (dd, *J* = 10.2, 6.6 Hz, 2H), 3.28 (s, 6H), 1.76 (s, 2H), 1.31-1.26 (m, 6H), 1.26-1.20 (m, 4H), 0.84 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.65, 162.76, 154.23, 142.63, 141.64, 140.74, 140.45, 132.71, 129.96, 128.98, 126.25, 124.35, 119.23, 64.90, 62.28, 51.24, 31.12, 28.37, 25.66, 21.92 (d, *J* = 17.3 Hz), 13.91. IR υ (cm $^{-1}$): 724, 966, 1465, 1610, 1662, 1712, 3036, 3176, 3235; HR-MS (ESI) Calculated for $C_{26}H_{35}BrClN_3O_2$ [M-Cl] $^{+}$: 500.1907, found: 500.1914.

4.4.25. ***(E)-((2-((4-(3-(3-fluoropyridin-2-yl)acryloyl)phenyl)amino)-2-oxoethyl) (methyl)(octyl)-l4-azanyl)methylium chloride (5y)***. Light brown solid, yield: 85%. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.86 (s, 1H), 8.49 (d, *J* = 3.8 Hz, 1H), 8.16 (d, *J* = 15.3 Hz, 1H), 8.02 (dd, *J* = 20.4, 11.9 Hz, 3H), 7.93 (d, *J* = 8.3 Hz, 2H), 7.46 (t, *J* = 9.0 Hz, 1H), 7.37 – 7.28 (m, 1H), 4.93 (s, 2H), 3.75 – 3.63 (m, 2H), 3.48 (s, 6H), 1.81 (s, 2H), 1.36-1.28 (m, 4H), 1.27-1.18 (m, 6H)), 0.84 (t, *J* = 6.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 191.28 – 188.76 (m), 191.28 – 142.26 (m), 134.44, 133.81, 129.92, 126.55 (d, *J* = 4.0 Hz), 125.87 (d, *J* = 4.1 Hz), 123.84 (d, *J* = 19.4 Hz), 119.76, 65.90, 63.58, 52.12, 31.52, 28.95 (d, *J* = 1.7 Hz), 26.15, 22.87, 22.49, 14.00. IR υ (cm $^{-1}$): 745, 943, 1470, 1600, 1663, 1694, 3045, 3161, 3225; HR-MS (ESI) Calculated for $C_{26}H_{35}ClFN_3O_2$ [M-Cl] $^{+}$: 440.2713, found: 440.2715.

4.4.26. ***(E)-N-(2-((4-(3-(furan-2-yl)acryloyl)phenyl)amino)-2-oxoethyl)-N,N-dim ethyloctan-1-aminium chloride (5z)***. Dark brown solid, yield: 70%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.99 (s, 1H), 8.11 (s, 2H), 7.92 (d, *J* = 9.9 Hz, 3H), 7.57 (s, 2H), 7.12 (s, 1H), 6.71 (s, 1H), 4.55 (s, 2H), 3.54 (s, 2H), 3.29 (s, 6H), 1.77 (s, 2H),

1.37-1.14 (m, 10H), 0.84 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.03, 162.69, 151.15, 146.14, 142.27, 133.10, 130.13, 129.59, 119.16, 118.50, 116.96, 113.10, 64.88, 62.29, 51.23, 31.11, 28.36, 25.66, 21.91 (d, *J* = 17.1 Hz), 13.90. IR υ (cm $^{-1}$): 767, 965, 1472, 1600, 1659, 1697, 3083, 3164, 3224; HR-MS (ESI) Calculated for $C_{25}H_{35}ClN_2O_3$ [M-Cl] $^+$: 411.2642, found: 411.2649.

4.4.27. *(E)-N,N-dimethyl-N-(2-oxo-2-((4-(3-(thiophen-2-yl)acryloyl)phenyl)amino)ethyl)butan-1-aminium chloride (5aa)*. Light brown solid, yield: 65%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (d, *J* = 8.8 Hz, 2H), 7.90 (t, *J* = 11.9 Hz, 3H), 7.80 (d, *J* = 5.0 Hz, 1H), 7.70 (d, *J* = 3.5 Hz, 1H), 7.57 (d, *J* = 15.3 Hz, 1H), 7.20 (dd, *J* = 5.0, 3.7 Hz, 1H), 4.52 (s, 2H), 3.61 – 3.49 (m, 2H), 3.29 (s, 6H), 1.81 – 1.70 (m, 2H), 1.40 – 1.27 (m, 2H), 0.94 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.12, 162.66, 142.23, 139.74, 136.38, 133.12, 132.75, 130.36, 129.69, 128.69, 120.19, 119.16, 64.81, 62.35, 51.20, 23.87, 19.13, 13.43. IR υ (cm $^{-1}$): 736, 973, 1462, 1597, 1651, 1698, 3041, 3162, 3245; HR-MS (ESI) Calculated for $C_{21}H_{27}ClN_2O_2S$ [M-Cl] $^+$: 371.1788, found: 371.1790.

4.4.28. *(E)-N, N-dimethyl-N-(2-oxo-2-((4-(3-(thiophen-2-yl)acryloyl)phenyl)amino)ethyl)octan-1-aminium chloride (5ab)*. Light yellow solid, yield: 88%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.75 (s, 1H), 8.13 (d, *J* = 8.6 Hz, 2H), 7.91 (d, *J* = 15.3 Hz, 1H), 7.87 (d, *J* = 8.6 Hz, 2H), 7.80 (d, *J* = 5.0 Hz, 1H), 7.70 (d, *J* = 3.4 Hz, 1H), 7.57 (d, *J* = 15.3 Hz, 1H), 7.23 – 7.17 (m, 1H), 4.49 (s, 2H), 3.58 – 3.49 (m, 2H), 3.28 (s, 6H), 1.76 (s, 2H), 1.31 – 1.21 (m, 10H), 0.84 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 187.09, 162.67, 142.20, 139.73, 136.40, 133.12, 132.78, 130.38，129.69, 128.70, 120.14, 119.14, 64.90, 62.2, 51.25, 31.11, 28.35, 25.6, 21.91, 13.91. IR υ (cm $^{-1}$): 744, 965, 1472, 1600, 1666, 1699, 3047, 3160, 3225; HR-MS (ESI) Calculated for $C_{25}H_{35}ClN_2O_2S$ [M-Cl] $^+$: 427.2414, found: 427.2417.

4.4.29. *(E)-N,N-dimethyl-N-(2-oxo-2-((3-(3-(thiophen2-yl)acryloyl)phenyl)amino)ethyl) octan-1-aminium chloride (5ac)*. Dark brown solid, yield: 57%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.79 (s, 1H), 8.38 (s, 1H), 8.01 – 7.92 (m, 2H), 7.90 (d, J = 7.8 Hz, 1H), 7.82 (d, J = 5.0 Hz, 1H), 7.71 (d, J = 3.4 Hz, 1H), 7.56 (t, J = 7.9 Hz, 1H), 7.48 (d, J = 15.4 Hz, 1H), 7.21 (dd, J = 4.9, 3.8 Hz, 1H), 4.51 (s, 2H), 3.54 (dd, J =

10.2, 6.6 Hz, 2H), 3.29 (s, 6H), 1.78 (s, 2H), 1.32 – 1.19 (m, 10H), 0.84 (t, J = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 188.28, 162.34, 139.59, 138.50, 138.09, 137.04, 133.14, 130.59, 129.37, 128.77, 124.02, 120.15, 118.97, 64.84, 62.31, 51.29, 31.11, 28.36, 25.67, 21.91 (d, J = 15.8 Hz), 13.90. IR υ (cm $^{-1}$): 748, 966, 1479, 1601, 1664, 1701, 3043, 3159, 3230; HR-MS (ESI) Calculated for $C_{25}H_{35}ClN_2O_2S$ [M-Cl] $^{+}$: 427.2419, found: 427.2418.

4.5. Microorganisms and culture conditions: The antibacterial activity of all the cationic chalcone derivatives was evaluated against both Gram-positive bacteria (*S. aureus*, *E. faecalis*, MRSA) and Gram-negative bacteria (*E. coli*, *S. enterica*, KPC, NDM-1). All the bacteria were cultured in Muller-Hinton broth (5.0 g of beef extract, 17.5 g of casein hydrolysate, and 1.5 g of starch in 1000 mL of distilled water) while *E. coli* was grown in Luria-Bertani broth (10.0 g of tryptone, 5.0 g of yeast extract, and 10 .0 g of NaCl in 1000 mL of distilled water). Brain-heart infusion broth (5.0 g beef heart infusion form, 12.5 g of calf brains infusion form, 2.5 g Na_2HPO_4, 2.0 g D-glucose, 10 g of peptone and 5.0 g NaCl in 100 mL of sterile distilled water) was used for stock samples of bacteria, the freeze dried stock samples of bacteria in 33.3% glycerol were stored at -80 ºC. For solid media, Mueller-Hinton agar (5.0 g of beef extract, 17.5 g of casein hydrolysate, 1.5 g of starch and 12.5 g of agar in 1000 mL of distilled water) was used as growth medium.

4.6. Cell culture: Human cervical carcinoma cell line (HeLa cell), maintained in complete DMEM media (Bioind) supplemented with 10% FBS (Zeta Life), at 37 ºC in a humidified atmosphere of 5% CO_2 in air. All the cells were mycoplasma free. The cells were trypsinized, counted and seeded in 96-well plates for viability studies or in 12-well plates for other studies. The cells were allowed to adhere overnight before they were used for experiments.

4.7. Antibacterial assay (minimum inhibitory concentration): Minimum inhibitory concentration (MIC) of all the small molecular compounds (**5a-5ac**) were determined by broth microdilution method according to CLSI guidelines. The test medium for most species was cation-adjusted Muller-Hinton broth (MHB). The 4-6 h grown culture as described in the microorganism and culture condition section gives about 10^8 CFU/mL

of bacteria. The bacterial cultures were then diluted to give approximately 10^6 CFU/mL in different media (Muller-Hinton broth media for *S. aureus*, *E. faecalis*, MRSA, *S. enterica*, KPC, NDM-1 and Luria-Bertani media for *E. coli*) which were then used for determining antibacterial efficacy. All the final compounds were water soluble at room temperature. Stock solutions of the final compounds were prepared with sterile Milli-Q water. Then the stock solutions were serially diluted to different concentration (256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL, 2 μg/mL, 1 μg/mL, 0.5 μg/mL) by using different media (Muller-Hinton broth media for *S. aureus*, *E. faecalis*, MRSA, *S. enterica*, KPC, NDM-1 and Luria-Bertani media for *E. coli*). These dilutions (100 μL) were added to the wells of 96 well plate followed by the addition of 100 μL of bacterial suspension (10^6 CFU/mL). Two controls were made: one containing 200 μL of media (negative contrast) and the other containing 200 μL of bacterial solution (10^6 CFU/mL, positive contrast). The plates were then incubated at 37 ºC for 16-20 h. After the incubation, read the results. Each concentration was determined in twice and the whole experiment was repeated at least twice. The antibacterial activity was thus expressed as minimum inhibitory concentration (MIC). A glycopeptides antibiotic vancomycin and a β-lactams antibiotic meropenem were used to compare the antibacterial efficacy in this study. To determine the minimum bactericidal concentration (MBC), the bacterial suspension that appeared to have less/little turbidity in the MIC experiment was plated (20 μL) and the agar plates were incubated for 20-24 h at 37 °C. Concentration at which no bacterial growth (no bacterial colony) was observed was taken as the MBC of the respective compounds.

4.8. Antibacterial activity in plasma (Plasma stability): Bacteria (*S. aureus*) was grown in a similar way as mentioned in the microorganism and culture condition and finally diluted in the respective media to give 10^6 CFU/mL. The fresh sterile defiber sheep blood (from commercial resource) was centrifuged at 3500 rpm for 10 min. The plasma, separated from the blood cells after centrifugation, was carefully collected. The test compound (**5g**) was dissolved in 50% sterile Milli-Q water and 50% plasma at a concentration of 512 μg/mL. Three such test samples were preincubated at 37 °C in 50% plasma for 0 h, 3 h and 6 h respectively. Then the three samples were serially

diluted to several concentration (256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL). After that, 50 μL of the above solutions was added to wells of a 96-well plate and 150 μL of the bacterial suspension (10^5 CFU/mL) was added to wells. The plate was then incubated for 20-24 h at 37 °C and antibacterial efficacy (minimum bactericidal concentration) of the test compounds was determined as described in the previous section (antibacterial assay).

4.9. Bactericidal activity in complex mammalian fluids: The fresh sterile defiber sheep blood was bought from a biochemical reagent company (China). Plasma was obtained as mentioned above. Serum was isolated by using serum tube containing sheep blood and then centrifuging the blood at 3,500 rpm for 10 min. Methicillin-resistant *S. aureus* (MRSA) was grown in way as mentioned in the microorganism and culture conditions. Finally, MRSA was diluted with 50% Muller-Hinton broth (MHB) medium and 50% mammalian media (serum, plasma, blood), individually in a way to give 10^5 CFU/mL of MRSA in 50% serum, 50% plasma, and 50% blood (having 50% MHB medium). The tested molecules (**5a**, **5g**) was dissolved in sterile water with the serial dilution method at the concentration of (512 μg/mL, 256 μg/mL, 128 μg/mL, 64 μg/mL, 32 μg/mL, 16 μg/mL, 8 μg/mL, 4 μg/mL). Then 50 μL of the dilutions was added to the wells of a 96-well plate and 150 μL of the bacterial suspension (10^5 CFU/mL) was added separately to the wells containing the dilutions of the compounds (**5a**, **5g**). The plate was then incubated for 20-24 h at 37 °C and minimum bactericidal concentration of the test compounds was determined by plating the bacterial suspension (20 μL) directly from the wells onto Muller-Hinton agar (MHA) plate. The agar plates were incubated at 37 °C for 20-24 h and colonies were observed to determine the MBC.

4.10. Time-dependent killing: An overnight culture of bateria *S. aureus* and *E.coli* was diluted 1:10,000 in MHB medium and incubated at 37 °C with aeration at 225 rpm for 2 h (early exponential) or 5 h (late exponential). Bacteria were then challenged with compound **5a** at 6×MIC and 8×MIC (a desirable concentration at the site of infection) and two antibiotics vancomycin (6 μg/mL, *S.aureus*) or moxalactam (12 μg/mL, *E.coli*) in culture tubes at 37 °C and 225 rpm. At different intervals, 100

μL bacteria solution were removed to 96-well plate, centrifuged at 4,000 rpm for 3 min (TDL 5M centrifuge) and resuspended in 100 μL of sterile phosphate buffered saline (1×PBS). Ten-fold serially diluted suspensions were plated on MHA plates and incubated at 37 °C overnight. Colonies were counted and CFU per mL was calculated. Experiments were performed with biological replicates.

4.11. Biofilm disruption assay (determination of viable count and imaging): The bacteria *S. aureus* and *E. coli* (4-6 h grown, mid-log phase) were diluted to ~10^5 CFU/mL into suitable media (MHB for *S. aureus* and M9 media supplemented with 0.02% casamino acid and 0.5% glycerol for *E. coli* respectively). The 96-well plates containing 100 μL of these suspensions were incubated under stationary conditions (for about 24 h for *S. aureus* and 72 h for *E. coli*). After incubation, the bacteria suspensions were centrifuged at 3,500 rpm for 5 min, the medium was removed and the wells were washed with 1×PBS once. Compound **5g** against *S. aureus* and **5a** against *E. coli* (100 μL at 2, 4, 8, 16, 32, 64, and 128 μg/mL) was then added to the wells containing preformed bacterial biofilms and allowed to incubate for 24 h at 37°C. A control was made where 100 μL of the above medium was added. After 24 h, medium was discarded and the planktonic cells were removed by washing with 1×PBS. Then 100 μL of trypsin-EDTA solution was added to the treated biofilm to make the suspension of bacterial cells present within the biofilm. Cell suspension was then assessed by plating the 10-fold serial dilutions of the suspension on suitable agar plates. After 24 h of incubation, bacterial colonies were counted and cell viability was expressed as log10 (CFU/well) along with the control. For visualizing the disruption of biofilm by the small molecules, 100 μL of 0.1% of crystal violet (CV) was added into the wells and incubated for 10 min. Then the crystal violet solution was discarded and the plates were washed twice with 1×PBS. Finally, imaging of the stained wells was taken using a digital camera.

4.12. Cytoplasmic membrane depolarization assay: The 4-6 h grown bacteria (mid-log phase) were harvested (3,500 rpm, 5 min), washed and resuspended with 1×PBS (*S. aureus*) and 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution at 1:1:1 ratio (*E. coli*). Then the bacterial suspension (~10^8 CFU/mL, 150 μL) was

added to the wells of a 96-well plate (Black plate, clear bottom with lid). Then 3, 3′-dipropylthiadicarbocyanine iodide (diSC35) (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli* (additional 50 μL of 200μM of EDTA was also added in case of *E. coli*). After the incubation, fluorescence was measured for the next 8 min at every 2 min interval at an excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 5 nm). Bacterial suspensions were then transferred to another well-plate containing 10 μL of 420 μg/mL of small molecules **5a, 5s, 5b, 5c, 5d, 5g, 5k, 5l, 5p, 5q, 5r** and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval, the final concentration of small molecules**5a, 5s, 5b, 5c, 5d, 5g, 5k, 5l, 5p, 5q, 5r** was 20 μg/mL. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

4.13. Inner membrane permeabilization assay: The 4-6 h grown bacteria (mid-log phase) were harvested (3500 rpm, 5 min), washed and resuspended similarly as the previous method. Then the bacterial suspension (~10^8 CFU/mL, 150 μL) was added to the wells of a 96-well plate (Black plate, clear bottom with lid). Then propidium iodide (PI) (10 μM, 50 μL) was added to the wells containing bacterial suspension and pre-incubated for about 30 min for *S. aureus* and 40 min for *E. coli*. After the incubation, fluorescence was measured for the next 8 min at every 2 min interval at an excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). Bacterial suspensions were then transferred to another well-plate containing 10 μL of 420 μg/mL of small molecules and fluorescence intensity was monitored immediately for another 12 min at every 2 min interval, the final concentration of small molecules **5a, 5s, 5b, 5c, 5d, 5g, 5k, 5l, 5p, 5q, 5r** was 20 μg/mL. A control experiment was performed by treating the preincubated bacterial suspension and dye solution only with sterile Milli-Q water (50 μL).

4.14. Propensity of bacterial resistance development: In order to evaluate the propensity of developing bacterial resistance towards the compounds, one of the potent compound **(5g)** was used in the study. First, MIC of compound **(5g)** was determined

against *S. aureus* and *E. coli*, and subsequently the compounds was challenged repeatedly at the 1/2 MIC level. Two control antibiotics norfloxacin and colistin were chosen for *S. aureus* and for *E. coli*, respectively. In case of norfloxacin and colistin, the initial MIC values were determined against respective bacteria. After the initial MIC experiment, serial passaging was initiated by transferring bacterial suspension grown at the sub-MIC of the compound/antibiotics (at MIC/2) and was subjected to another MIC assay. After 24 h incubation period, cells grown at the sub-MIC of the test compound/antibiotics were once again transferred and assayed for MIC experiment. The process was repeated for 20 passages for both *S. aureus* and *E. coli* respectively. The MIC for test compound to the control antibiotics was plotted against days to determine the propensity of bacterial resistance development.

4.15. Hemolytic activity: Red blood cells (RBCs) were isolated from sheep blood and resuspended in 1×PBS (5%). RBC suspension (150 μL) was then added to solutions of serially diluted small molecules **(5a-5ac)** at the concentration of (5120, 2560, 1280, 640, 320, 160, 80, 40, 20, 10 μg/mL) in a 96-well plate (50 μL). Two controls were prepared, one 50 μL RBC suspension (5%) and the other with 50 μL of 0.1% solution of Triton X-100. The plate was then incubated for 1 h at 37 °C. After the incubation, the plate was centrifuged at 3,500 rpm for 5 minutes. Supernatant (100 μL) from each well was then transferred to a fresh 96-well plate and absorbance at 540 nm was measured. Percentage of hemolysis was determined as $(A–A_0) / (A_{total}–A_0) \times 100$, where A is the absorbance of the test well, A_0 is the absorbance of the negative control (5% RBC suspensions), and A_{total} the absorbance of wells with 0.1% Triton X-100).

4.16. Cytotoxicity study: Cytotoxicities of the small molecules were evaluated by the Cell Counting Kit-8 (CCK-8). Briefly, 5×103 cells in 100 μL medium were seeded to each of 96-well plates. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium (100 μL) containing the candidate compound (**5a**) in different concentration. And only media was used as negative control. At the end of the treatment (24 h), the medium was discarded and washed twice with the new culture medium, then added 100 μL new medium (with 5% CCK-8) to each well. Cells were incubated at 37 °C for a further 4 h and then the absorbance at 450 nm was measured

using a Microplate Reader. Results were expressed as percent viability = [A-A_0 / A_{nc}-A_0] × 100%, where A is the absorbance of the treated cells, A_{nc} is the absorbance of the negative control and A_0 is absorbance of the background (new medium containing 5% CCK-8). The average 50% inhibitory concentration (IC_{50}) was determined from the dose-response curves according to the inhibition ratio for each concentration. Each concentration was analyzed in triplicate and the experiment was repeated three times.

4.17. Fluorescence and electron scanning microscopy: As mentioned above for the cytotoxicity study, cells were seeded into the wells of a 12-well plate and then treated with compound (**5a**) at various concentrations (1, 2, 4, 8 and 16μg/mL). For positive control 0.1% Triton X-100 was used. All the treated and untreated cells (as negative control) were washed once with 1×PBS (the images were captured with a 10× objective in electron microscope) and stained with 2 μM calcein AM (Fluka) and 4.5 μM propidium iodide (PI) (Sigma-Aldrich) (50 μL of 1:1 calcein AM:PI) for 15 min at 37 °C under 5% CO_2-95% air atmosphere. Finally, the images were captured with a 10× objective in fluorescence microscope using a band-pass filter for calcein AM at 500-550 nm and a long-pass filter for PI at 590-800 nm.

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1. Twenty-nine cationic chalcone analogs (**5a**-**5ac**) were designed and synthesized. Representative compounds **5a** (MIC: 1 μg/mL against S. aureus, 0.5 μg/mL against MRSA) and **5g** (MIC: 0.5 μg/mL against S. aureus, 0.25 μg/mL against MRSA) showed good bactericidal activity against both G+ and G- bacteria, including the drug-resistant species MRSA, KPC and NDM.

2. The phenyl group and the fluoride atom was found to play an important influence in the antibacterial activity and hemolytic Activity.

3. The cationic chalcone derivatives were shown to be stable under plasma conditions. The potential compounds can depolarize and permeabilize bacterial membranes. Importantly, the compounds did not allow bacteria to develop resistance and exhibited negligible toxicity toward mammalian cells.