Tuning the Bactericidal Repertoire and Potency of Quinoline-based Amphiphiles for Enhanced Killing of Pathogenic Bacteria

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Electronic Supplementary Information

Materials

8-hydroxyquinoline, alkyl halides, valinomycin, 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), propidium iodide (PI), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3,3'-dipropylthiadicarbocyanine iodide (di$SC_3$5) were obtained from Sigma-Aldrich Chemicals, USA. DMSO and glutaraldehyde were purchased from Merck, Mumbai, India. HEPES buffer was procured from Sisco Research Laboratories (SRL), Mumbai, India.

Synthesis of amphiphiles

The amphiphiles synthesized in the present study included 8-alkoxy quinoline (neutral) and N-methyl 8-alkoxy quinolinium iodide (cationic) with varying alkyl chain from butyl, octyl and dodecyl. Neutral amphiphiles were prepared following the literature method.[1] For N-methylation, methyl iodide was added to a solution of 8-(alkoxy) quinoline in dry acetonitrile, and refluxed for 6 h to complete the reaction. The products obtained were re-

crystallized from methanol and characterized by NMR, IR and melting point. The neutral amphiphiles were designated as **compound 1** (R = C_4H_9), **compound 2** (R = C_8H_{17}) and **compound 3** (R = $C_{12}H_{25}$) whereas cationic amphiphiles consisted of **compound 4** (R = C_4H_9), **compound 5** (R = C_8H_{17}) and **compound 6** (R = $C_{12}H_{25}$), respectively. Synthesis of amphiphiles with even higher alkyl chain length ($C_{16}H_{33}$) resulted in the generation of highly insoluble products and hence their study was not pursued.

Characterization of amphiphiles

Melting points of the compounds were recorded using a Type *B-540 Buchi* melting point apparatus, maintaining the heating rate at 10 ^oC. The IR spectra of compounds were recorded at 4 per cm resolution with 10 scan using a *Perkin Elmer-Spectrum One FT-IR Spectrometer* from 4000 to 450 per cm using background spectrum of pure KBr, while ^1H and ^{13}C NMR spectra were recorded on a *Varian* 400 MHz spectrometer using tetramethylsilane (TMS) as internal standard.

Compound 4 [N-methyl-8-(butoxy) quinolinium iodide]

Yield: 65%, IR (KBr): 2921, 1597, 1533, 1467 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 1.014 (t, *J* = 7.2, 3H), 1.512 (m, 2H), 1.953 (m, 2H), 4.228 (t, 2H), 5.063 (s, 3H), 7.474 (d, ArH), 7.786 (m, ArH), 8.093 (m, ArH), 8.896 (m, ArH), 10.149 (d, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 13.847, 19.582, 30.961, 53.504, 70.893, 109.863, 116.681, 122.447, 122.729, 130.500, 130.973, 130.140, 147.507, 151.007 and 152.067. Light brown solid, Melting point 163 ^oC, Anal. Calcd $C_{14}H_{18}NOI$: C, 48.99; H, 5.29; N, 4.08. Found: C, 48.87; H, 5.21; N, 3.99.

Compound 5 [N-methyl-8-(octyloxy) quinolinium iodide]

Yield: 52%, IR (KBr): 2925, 1599, 1530, 1465 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.901 (t, *J* = 6.8, 3H), 1.311 (m, 8H), 1.525 (m, 2H), 1.986 (m, 2H), 4.248 (t, *J* = 6.8, 2H), 5.088 (s, 3H), 7.494 (d, ArH), 7.811 (m, ArH), 8.127 (m, ArH), 8.922 (m, ArH), 10.194 (d, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.229, 22.747, 26.393, 29.039, 29.275, 29.375, 31.891, 53.123, 71.213, 116.490, 122.439, 122.950, 130.912, 132.193, 147.362, 151.152 and 152.380. Light brown solid, Melting point 117 ^{o}C, Anal. Calcd $C_{18}H_{26}N_2$: C, 54.14; H, 6.56; N, 3.51. Found: C, 54.09; H, 6.52; N, 3.48.

Compound 6 [N-methyl-8-(dodecyloxy) quinolinium iodide]

Yield: 45%, IR (KBr): 2930, 1595, 1535, 1470 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.882 (t, *J* = 7.2, 3H), 1.274 (m, 16H), 1.533 (m, 2H), 2.002 (m, 2H), 4.247 (t, *J* = 6.4, 2H), 5.089 (s, 3H), 7.501 (d, ArH), 7.817 (m, ArH), 8.128 (m, ArH), 8.958 (m, ArH), 10.185 (d, ArH). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.221, 22.778, 26.370, 29.024, 29.428, 29.520, 29.611, 29.718, 31.998, 53.077, 71.205, 116.567, 122.470, 122.874, 123.080, 130.950, 132.186, 147.469, 151.091 and 152.273. Light brown solid, Melting point 110 ^{o}C, Anal. Calcd $C_{22}H_{34}N_2$: C, 58.02; H, 7.52; N, 3.08. Found: C, 57.97; H, 7.48; N, 2.99.

Bacterial strains and growth conditions

The bacterial strains used in the present investigation were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The growth media used for propagating the bacterial strains were purchased from HiMedia, Mumbai, India. The bacterial strains comprised of Gram-positive strains of *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* MTCC 96 and MTCC 740, *Listeria monocytogenes* Scott A, and Gram-negative strains of *Escherichia coli* MTCC 433,

Pseudomonas aeruginosa MTCC 2488 and *Enterobacter aerogenes* MTCC 2822. *S. aureus* MTCC 96, *S. aureus* MTCC 740 and *L. monocytogenes* Scott A were propagated in Brain-Heart Infusion (BHI) broth at 37^{o}C and 180 rpm for 12 h, whereas *B. subtilis* MTCC 441, *E. coli* MTCC 433, *P. aeruginosa* MTCC 288 and *E. aerogenes* MTCC 2822 were grown in nutrient broth (NB) at 37^{o}C and 180 rpm for 12 h.

Screening of antibacterial activity of amphiphiles

Antibacterial activity of the amphiphiles was tested against Gram-positive *B. subtilis* MTCC 441, *L. monocytogenes* Scott A, *S. aureus* MTCC 96 and Gram-negative *E. coli* MTCC 433, *E. aerogenes* MTCC 2822 and *P. aeruginosa* MTCC 2488. Stock solution of the amphiphiles was prepared in DMSO. Target pathogens were grown in fresh media incorporated with varying concentrations of the amphiphile (50 and 100 µg/mL) for 24 h. The growth of amphiphile-treated cells was monitored periodically by measuring absorbance at 600 nm in a spectrophotometer (Cary 300, Varian) and was expressed as percentage growth compared to control (untreated cells).

Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC)

MIC and MKC of the synthetic amphiphiles were determined against *E. coli* MTCC 433 and *S. aureus* MTCC 96. Bacterial cultures were inoculated at 1% level in micro titre wells having the requisite growth medium and grown overnight at 37^{o}C and 130 rpm in presence of varying concentrations of the amphiphiles. The growth of the bacterial strains was determined by measuring absorbance at 600 nm in a micro titre plate reader (Infinite M200, TECAN, Switzerland). MIC of the amphiphile was recorded as the lowest amphiphile concentration which resulted in OD_{600} reading of <0.1, indicating lack of cell growth. An aliquot (1% v/v) from all the culture wells which lacked cell growth (OD_{600} = <0.1) was re-inoculated into fresh growth medium in micro titre wells devoid of amphiphile and incubated

overnight at 37°C and 130 rpm. MKC of the amphiphile was expressed as the lowest amphiphile concentration that prevented growth of the target bacterial cells following re-inoculation, as observed by OD_{600} reading of <0.1. The MIC and MKC values were calculated from three independent experiments, each having three replicas. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, USA).

Structure-function studies

Overnight grown cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were washed twice in phosphate buffer saline (PBS) and resuspended in the same buffer. Equimolar concentration of **compound 1**, **3** and **6** (4.5 and 7.5 µM) were added to 10^6 CFU/ mL target bacteria and incubated at 37°C and 180 rpm for 3 h. Membrane damage in cells were assessed by 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) leakage assay, propidium iodide (PI) uptake assay and fluorescence microscopy as follows:

cFDA-SE leakage assay

The fluorescent dye 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) was used to label cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96. Overnight grown cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were harvested by centrifugation at 3,000 x g for 10 min. The cell pellet was washed twice with sterile phosphate buffer and labeled with cFDA-SE (final concentration of 50 µM) at 37°C for 20 min. Termination of the labeling reaction was accomplished by pelleting the cells and washing of bacterial cells twice with phosphate buffer to remove excess cFDA-SE molecules. Equimolar concentration of **compound 1**, **3** and **6** (4.5 and 7.5 µM) were added to 10^6 CFU/mL cFDA-SE labeled target bacteria and incubated at 37°C and 180 rpm for 3 h. In case of control sample, only DMSO was added to labeled cells and incubated under the same conditions without amphiphile.

Leakage of carboxyfluorescein from cells was determined by measuring fluorescence of the cell free supernatant at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorimeter (FluoroMax-3, HORIBA). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control samples. Fluorescence measurements were taken for three independent samples.

PI uptake assay

The integrity of the membrane of amphiphile-treated target cells was determined by studying the uptake of the dye propidium iodide (PI). A 1.5 mM stock solution of PI was prepared in sterile MilliQ water and stored at 4°C. Equimolar concentration of **compound 1**, **3** and **6** (4.5 and 7.5 μM) were added to 10^6 CFU/mL target bacteria (in PBS) and incubated at 37°C and 180 rpm for 3 h. In case of control sample, only DMSO was added to cells and incubated under the same conditions without amphiphile. Following treatment with amphiphile, cells were washed with PBS to remove excess or unbound amphiphile and PI was added to both treated cells as well as control samples at a final concentration of 30 μM. After 30 min of incubation in a circulating water bath incubator (Amersham) set at 37°C, samples were centrifuged and washed in distilled water to remove excess dye. The cells were resuspended in PBS and fluorescence was measured in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 535 nm and emission wavelength of 617 nm. Fluorescence data for each sample was normalized with the optical density at 617 nm. The values obtained for untreated cells were subtracted from all experimental values. Fluorescence measurements were taken for three independent samples.

Fluorescence microscopy

Equimolar concentration of **compound 1**, **3** and **6** (4.5 and 7.5 µM) were added to 10^6 CFU/mL target bacteria (in PBS) and incubated at 37°C and 180 rpm for 3 h. In case of control sample, only DMSO was added to cells and incubated under the same conditions without amphiphile. Following treatment with amphiphile, cells were washed twice with PBS to remove excess amphiphile and both the treated and control cells were labeled with cFDA-SE and PI as mentioned previously. Stained samples were fixed in 2.5% glutaraldehyde and washed twice with phosphate buffer. A 10 µl aliquot of the fixed sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Eclipse Ti-U, Nikon) with a filter that allowed blue light excitation for cFDA-SE and green light excitation for PI stained cells. Images of the treated and control cells were recorded.

Bactericidal activity of compound 6

Varying concentration of **compound 6** (1.64 – 4.4 µM) were added to 10^6 CFU/mL of *E. coli* MTCC 433 and *S. aureus* MTCC 96 (in PBS) and incubated at 37°C and 180 rpm. Viable cell numbers ($\log_{10}$ CFU/ mL) were determined at regular intervals by serial dilution and plating. For amphiphile-bacteria interactions studies, varying concentrations of **compound 6** (2.19, 3.29 and 4.4 µM) were added to 10^6 CFU/mL *E. coli* MTCC 433 and *S. aureus* MTCC 96 and incubated at 37°C and 180 rpm for 6 h. Samples were withdrawn periodically, centrifuged to separate cells and fluorescence intensity of unbound amphiphile was measured in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 370 nm and emission wavelength of 505 nm. Fluorescence micoscopic images of target bacteria treated with 4.4 µM **compound 6** were also recorded in a fluorescence microscope (Eclipse Ti-U, Nikon) with a filter that allowed blue light excitation.

Mode of action of compound 6

The experiments conducted to determine the mode of action of **compound 6** on *E. coli* MTCC 433 and *S. aureus* MTCC 96 included: (a) PI uptake assay, (b) cFDA-SE assay (c) Transmission electron microscope (TEM) analysis, (d) Effect of membrane potential and (e) 3,3'-dipropylthiadicarbocyanine iodide ($diSC_35$)-based membrane depolarization assay. A detailed description of the aforementioned assays is as follows:

PI uptake assay

Overnight grown cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were washed twice in phosphate buffer saline (PBS) and resuspended in the same buffer. Varying concentration of **compound 6** (1.64 – 4.4 µM) were added to 10^6 CFU/mL target bacteria and incubated at 37^{o}C and 180 rpm. Cells were withdrawn at periodic intervals and subjected to PI uptake assay as mentioned before.

cFDA-SE assay

Overnight grown cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were washed twice in phosphate buffer saline (PBS) and resuspended in the same buffer. Varying concentration of **compound 6** (1.64 – 4.4 µM) were added to 10^6 CFU/mL target bacteria and incubated at 37^{o}C and 180 rpm. Cells were withdrawn at periodic intervals, labeled with cFDA-SE as mentioned before and fluorescence measurements were conducted in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 488 nm and emission wavelength of 518 nm.

Transmission electron microscope (TEM) analysis

Overnight grown cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were washed twice in phosphate buffer saline (PBS) and resuspended in the same buffer. Approximately 10^6

CFU/mL were treated with 4.4 µM **compound 6** for 6 h at 37°C. Control samples consisted of untreated cells suspended in PBS. Treated as well as untreated cells were washed once with PBS and once with sterile MilliQ grade water and resuspended in MilliQ grade water. A 2.0 µl aliquot of each sample was spotted on carbon coated TEM grid (Pacific Grid, USA) and air-dried in laminar hood. The treated and untreated samples were examined in a transmission electron microscope (Jeol JEM 2100, Japan) operating at 200 kV and their images were recorded.

Effect of membane potential

Cells of *E. coli* MTCC 433 were pre-treated with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to collapse the transmembrane proton motive force as described earlier.[2] Approximately 10^6 CFU/mL CCCP treated and untreated cells (control) were taken in PBS and incubated with 4.4 µM **compound 6** for 6 h at 37°C. The bactericidal activity of the amphiphile on CCCP treated and control cells were compared by plating the samples at regular time intervals and determining the viable cell count.

Membrane depolarization assay

Cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were grown till mid-logarithmic phase (OD_{600} = 0.4-0.5). The cells were harvested by centrifugation and washed with a buffer solution (5 mM HEPES buffer, 5 mM glucose, pH 7.2). The pH of the buffer solution was adjusted to 7.2 using 1.0 N NaOH solution. Following the washing steps, cells were suspended in the same buffer to an OD_{600} of 0.05. The cell suspensions were incubated with 0.4 µM 3,3'-dipropylthiadicarbocyanine iodide ($diSC_35$) for 1h at 37°C followed by the addition of 100 mM KCl. Cell suspension (1.0 mL) was placed in a cuvette to which varying concentrations of **compound 6** (2.19 and 4.4 µM) was added and the fluorescence readings were monitored periodically in a spectrofluorimeter (FluoroMax-3, HORIBA) set to an

excitation wavelength of 622 nm and emission wavelength of 670 nm. Valinomycin (30 μM) was used as a positive control.

Cytotoxicity assay

Cytotoxicity of **compound 6** was assessed on human HT-29 colon adenocarcinoma cells by a standard XTT assay following the manufacturer instruction (Sigma-Aldrich, MO, USA). For cell culture experiments, Dulbecco's Modified Eagle Medium (DMEM) was procured from Sigma-Aldrich (USA) and fetal calf serum (FCS) was procured from PAA Laboratories, USA. HT-29 cells were grown in DMEM supplemented with 10% FCS. Cells were seeded into 96-well plates at a density of 1×10^6 cells per well and varying concentration of **compound 6** (4.4 and 44 μM) made in DMEM were added to the cells and incubated for a period of 24 and 48 h in a CO_2 incubator with 5% CO_2. Following incubation, the growth media was removed and fresh DMEM containing XTT solution was added. The plate was incubated for 2-3 h at 37^{o}C. Control cell (without amphiphile treatment) and solvent control were included in each assay. After incubation, the absorbance was measured at 450 nm. The assay was performed in six sets for each concentration of **compound 6**.

Reference

1 L. Andree, S. Jean and V. Z. Farchid, *Bull. Soc. Chim. Fr.*, 1987, **6**, 1027-1035.

2 M. L. Mangoni, N. Papo, G. Mignogna, D. Andreu, Y. Shai, D. Barra and M. Simmaco, *Biochemistry*, 2003, **42**, 14023-14035.

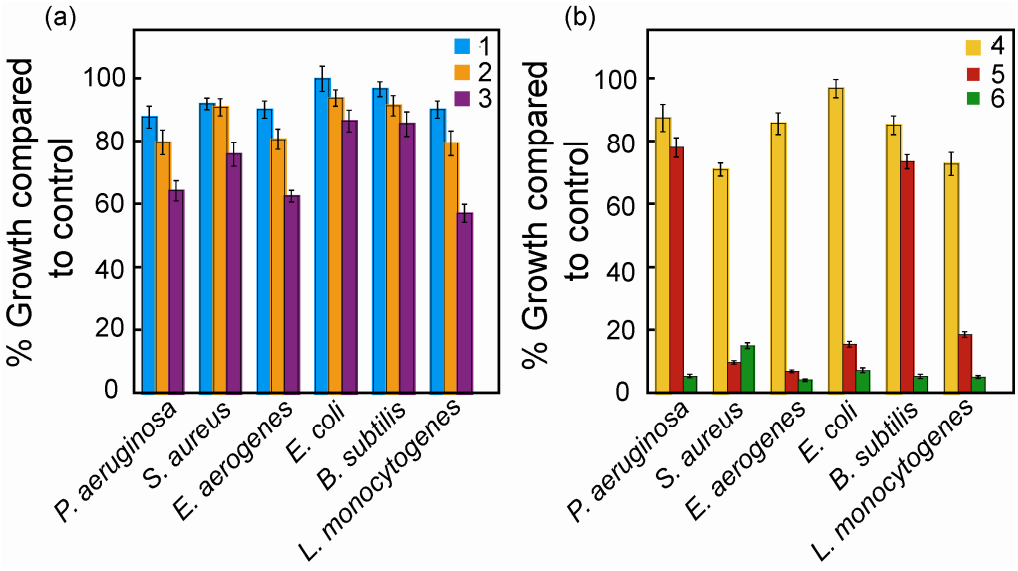

Fig. S1 Antimicrobial activity of **(a)** neutral (**compounds 1**, **2** and **3**) and **(b)** charged amphiphiles (**compounds 4**, **5** and **6**) against pathogenic bacterial strains. Amphiphiles were used at a concentration of 50 µg/ mL.

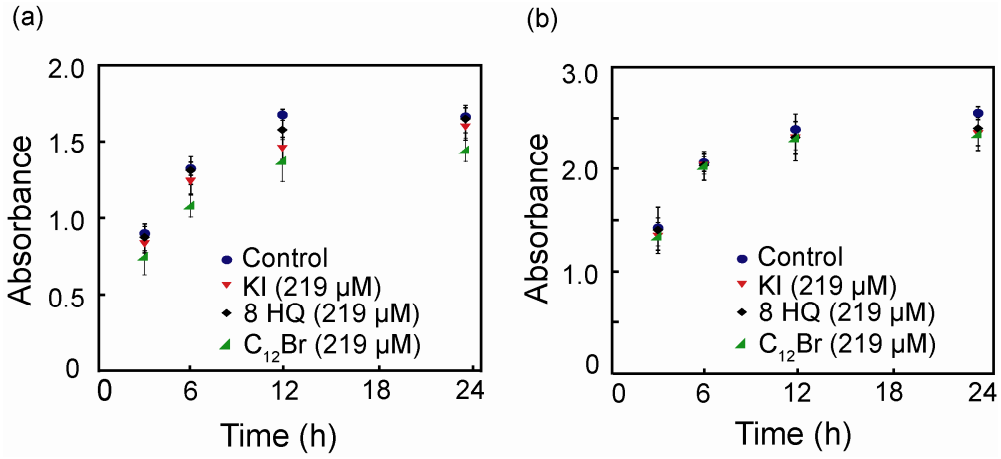

Fig. S2 Control experiments to ascertain the effect of potassium iodide (KI), 8-hydroxy quinoline (8HQ) and 1-bromo dodecane ($C_{12}Br$) on the growth of **(a)** *E. coli* MTCC 433, **(b)** *S. aureus* MTCC 96. The growth of the bacterial strains was monitored by measuring absorbance at 600 nm at regular intervals.

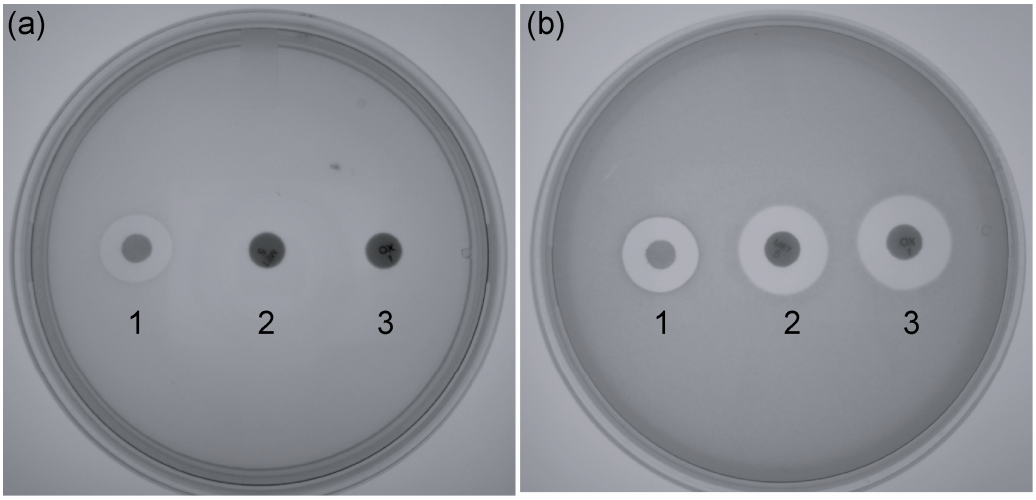

Fig. S3 Disc diffusion assay to ascertain the susceptibility of **(a)** *S. aureus* MTCC 96 and **(b)** *S. aureus* MTCC 740. Disc 1 was impregnated with 4.4 µM of **compound 6**. Discs 2 and 3 are ready to use discs of methicillin (5.0 µg) and oxacillin (1.0 µg) respectively.

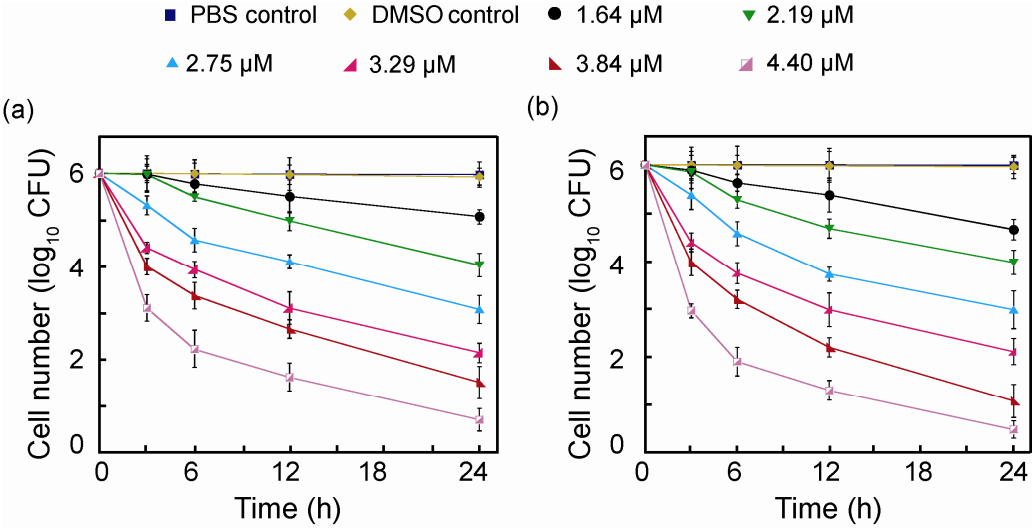

Fig. S4 Effect of varying concentrations of **compound 6** on the viability of (a) *E. coli* MTCC 433 and (b) *S. aureus* MTCC 96.

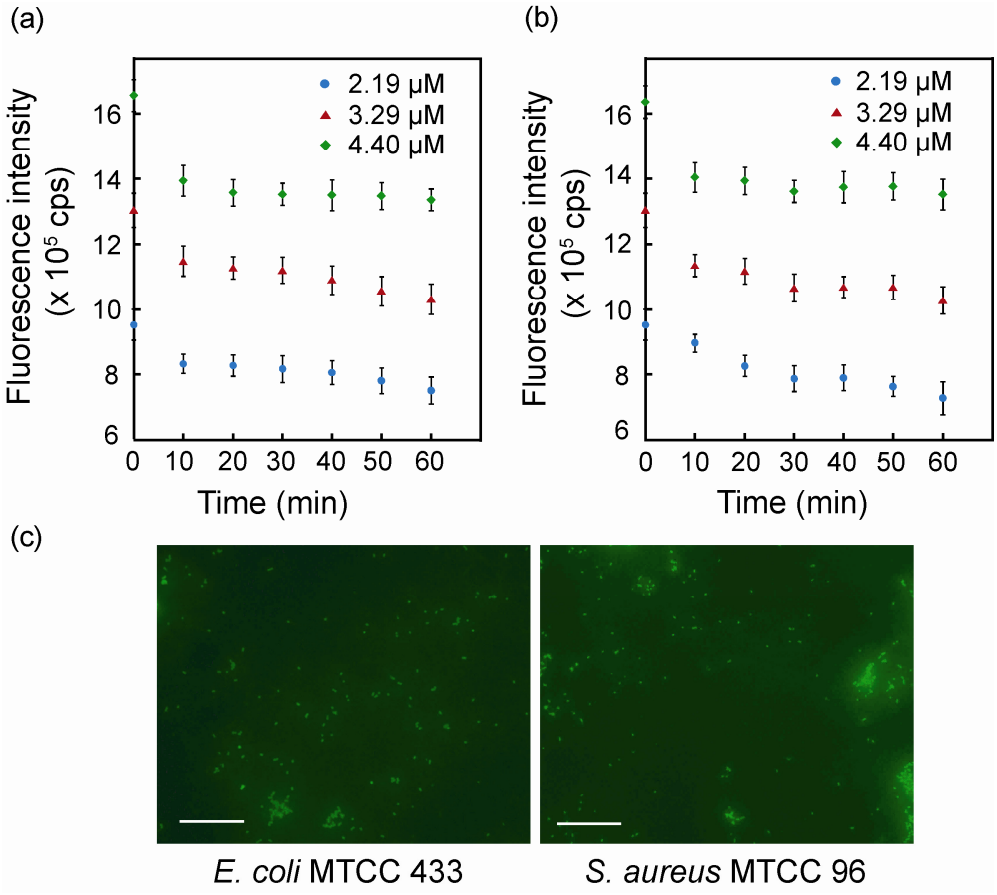

Fig. S5 Fluorescence intensity curves of unbound amphiphile following sequestration of varying concentrations of **compound 6** by 10^6 CFU of (a) *E. coli* MTCC 433 and (b) *S. aureus* MTCC 96. (c) Fluorescent images of bacterial cells after 1h interaction with **compound 6**. Scale bar for the images was 50 μ.

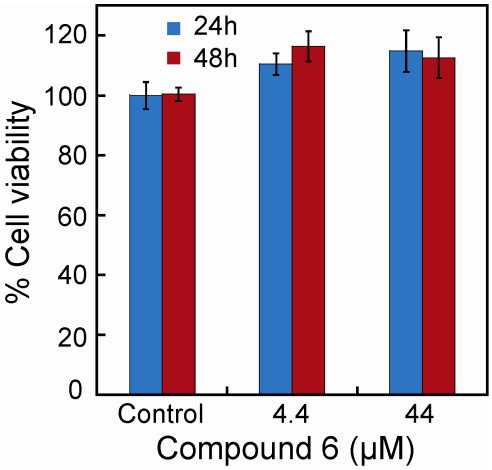

Fig. S6 XTT-based cytotoxicity assay to determine % viability of HT-29 cells following exposure to varying concentrations of **compound 6**. Each data point represents mean ± SD from six samples.

Table S1. Calculated hydrophobicity (LogP value) of neutral amphiphiles

Synthetic Amphiphile	**Hydrophobicity* (LogP value)**
Compound 1	3.17
Compound 2	4.61
Compound 3	6.04

* Hydrophobicity of the amphiphiles was calculated using the software jlopP Version 1.1 (http://www.vls3d.com/JME_EditorOK.dir/run_jlogp.html)

Table S2. MIC and MKC of synthetic amphiphiles and corresponding absorbance values for bacterial strains at MIC and MKC values of amphiphiles

Synthetic Amphiphile	***E.coli* MTCC 433**		***S. aureus* MTCC 96**	
	MIC (µM)/ OD_{600} ± standard deviation	**MKC(µM)/ OD_{600} ± standard deviation**	**MIC (µM)/ OD_{600} ± standard deviation**	**MKC (µM)/ OD_{600} ± standard deviation**
Compound 1	3000/0.046 ± 0.005	3000/0.085 ± 0.004	2000/0.053 ± 0.008	3000/0.073 ± 0.006
Compound 2	2000/0.063 ± 0.004	3000/0.029 ± 0.004	1500/0.065 ± 0.002	3000/0.019 ± 0.005
Compound 3	2000/0.055 ± 0.008	4000/0.039 ± 0.004	500/0.037 ± 0.005	1000/0.066 ± 0.005
Compound 4	2000/0.058 ± 0.004	2000/0.036 ± 0.002	2000/0.071 ± 0.002	4000/0.04 ± 0.005
Compound 5	80/0.032 ± 0.005	120/0.057 ± 0.002	60/0.03 ± 0.007	120/0.031 ± 0.009
Compound 6	15/0.069 ± 0.004	20/0.067 ± 0.004	5/0.02 ± 0.007	15/0.047 ± 0.002