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Electronic Supplementary Information

Amphiphile-mediated Enhanced Antibiotic Efficacy and Development of a Payload Nanocarrier for Effective Killing of Pathogenic Bacteria

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EXPERIMENTAL METHODS

1.0 Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of compound 1

MIC and MKC of **compound 1** was determined against *S. aureus* MTCC 96, *E. faecalis* MTCC 439, *E. coli* MTCC 433 and *P. aeruginosa* MTCC 2488. The bacterial strains were inoculated at 1% level in microtitre wells having the specific growth medium and grown overnight at 37^{o}C and 180 rpm in presence of varying concentrations of **compound 1**. The growth of the bacterial strains was determined by measuring absorbance at 600 nm in a microtitre plate reader (Perkin Elmer, USA). MIC of **compound 1** was recorded as the lowest amphiphile concentration which resulted in A_{600} reading of <0.1, indicating lack of cell growth. An aliquot (1% v/v) from all the culture wells which lacked cell growth (A_{600} = <0.1) was re-inoculated into fresh growth medium in microtitre wells and incubated overnight at 37^{o}C and 180 rpm in the absence of **compound 1**. MKC of the amphiphile was expressed as the lowest amphiphile concentration that prevented growth of the target bacterial cells following re-inoculation, as confirmed by A_{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).

2.0 Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of erythromycin, polymyxin B and tobramycin

MIC and MKC of erythromycin, polymyxin B and tobramycin was determined against *E. coli* MTCC 433, *S. aureus* MTCC 96 and *P. aeruginosa* MTCC 2488, respectively. Essentially the bacterial strains were grown in microtitre wells in the presence of varying concentrations of the antibiotics (5.0 µg/mL-400 µg/mL for erythromycin and polymyxin B and 0.06 µg/mL-10 µg/mL in case of tobramycin) and the MIC and MKC were determined by the absorbance method as described earlier.

3.0 *In vitro* resistance studies with compound 1

The protocol for determination of *in vitro* resistance of *S. aureus* MTCC 96 against **compound 1** in multiple step experiments was essentially based on a method described earlier. [1] The concentrations of **compound 1** used in these experiments corresponded to 2X-16X MIC against the target bacteria. Enumeration of *S. aureus* MTCC 96 colonies was accomplished on BHI-agar plates following an incubation of 48 h at 37°C.

4.0 Bactericidal efficacy of erythromycin and polymyxin B in combination with compound 1

The bactericidal activity of erythromycin in combination with **compound 1** was tested against representative Gram-negative bacteria *E. coli* MTCC 433 and *P. aeruginosa* MTCC 2488, while the antibacterial activity of polymyxin B in combination with **compound 1** was tested against *S. aureus* MTCC 96. Initially a 10 µL aliquot of bacterial cell suspension (10^6 CFU suspended in sterile PBS) of the respective target bacteria were inoculated onto sterile 96 well microtitre plate wells having specific growth media (100 µL) incorporated with a serial two fold dilution of erythromycin (5.0 µg/mL to 40 µg/mL in case of *E. coli* MTCC 433 and 10 µg/mL to 80 µg/mL in case of *P. aeruginosa* MTCC 2488) or various concentrations of polymyxin B (5.0 µg/ mL to 320 µg/ mL in case of *S. aureus* MTCC 433). For every concentration of erythromycin and polymyxin B, varying concentrations of **compound 1** was used in combination, depending upon the MIC of the amphiphile for the respective target bacterial strains. The cells were incubated at 37^{o}C and 180 rpm for 6h. Bacterial cell growth was estimated by measuring absorbance at 600 nm in a microtitre plate reader (Perkin Elmer, USA) and expressed as percentage growth inhibition compared to untreated cells. Fold decrease in the MIC of polymyxin B for amphiphile-treated cells compared to amphiphile-untreated cells was also determined. The ability of **compound 1** to sensitize *S. aureus* MTCC 96 to polymyxin B was quantified by determining the fractional inhibitory concentration (FIC) index. [2]

5.0 Anti-biofilm activity of compound 1

5.1. Estimation of biofilm biomass

Biofilm of *P. aeruginosa* MTCC 2488 was grown in sterile 96 well microtitre plate following a standard method. [3] To study the anti-biofilm activity of **compound 1**, the spent media in the microtitre well was decanted and fresh growth media incorporated with varying concentrations of **compound 1** (60 μM, 80 μM, 100 μM, 120 μM, 160 μM and 200 μM) was added to biofilms in the well and incubated for 24h. Subsequently the media was removed and wells were washed with sterile water (200 μL) to remove non-adherent bacteria. The wells were air dried for 45 min and 1% (v/v) crystal violet solution (150 μl) was added to each well and incubated for 45 min to stain the biofilm. The crystal violet stain incorporated by biofilms was solubilized with 95% ethanol (200 μl) and the biofilm biomass was estimated by transferring the ethanol-solubilized dye solution from each well into fresh well and measuring absorbance at 590 nm in a microtiter plate reader (Perkin Elmer, USA). The biomass obtained for untreated biofilm was also estimated for comparison. All experiments were performed in triplicates and a one way analysis of variance (ANOVA) was performed using Sigma Plot.

5.2. Fluorescence microscope analysis

Biofilm of *P. aeruginosa* MTCC 2488 was grown in sterile 96 well microtitre plate following a standard method. [3] The biofilm samples were then treated with 200 μM **compound 1** for 6 h. Subsequently, untreated biofilm samples as well as **compound 1**-treated biofilm samples were labeled separately with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) and propidium iodide (PI) by following the standard labeling procedures as described earlier. [4] The stained biofilms were observed under a 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 biofilms were recorded.

5.3. Field emission scanning electron microscope (FESEM) analysis

P. aeruginosa MTCC 2488 biofilm was grown on a cover slip by following the standard method as described earlier. [3] A representative biofilm sample was treated with 200 μM of **compound 1** for 6h. Subsequently, the amphiphile-treated as well as control biofilm samples

(untreated) were washed twice with filter-sterilized phosphate buffered saline (PBS) to remove spent media and finally with sterile MilliQ grade water. The samples were then air-dried in laminar hood and examined in a field emission scanning electron microscope (Zeiss Sigma, USA) and their images were recorded.

6.0 Synthesis of BSA nanoparticle (BNP)

BSA nanoparticle (BNP) was prepared by essentially following a desolvation technique described previously. [5] Initially, 100 mg BSA was dissolved in 2.0 ml of sterile MilliQ water (titrated to pH 8.2). To generate BSA nanoparticles (BNPs), this solution was subjected to desolvation by drop wise addition of 8.0 ml of ethanol under stirring conditions at room temperature. Following desolvation, 100 µL of 8% glutaraldehyde was added to the suspension and further stirred for 24h to facilitate cross-linking of the nanoparticles. Purification of BSA nanoparticles was achieved by five consecutive cycles of centrifugation (20,000 × g, 8 min for each cycle) with intermittent resuspension of the pellet in original volume of sterile MilliQ water (titrated to pH 8.2) between every centrifugation step. Each resuspension was performed in an ultrasonication bath for 5 min. The purified BSA nanoparticles were recovered from the final washing step and subjected to freeze-drying in a lyophilizer and the final freeze-dried preparation of BSA nanoparticle was stored in 4^{o}C till further use. The yield of BSA nanoparticles (Y_{np}) was calculated as follows:

$$Y_{np} = \frac{W_{Total\ BSA} - W_{Free\ BSA}}{W_{Total\ BSA}} X\ 100\%$$

where $W_{Total\ BSA}$ is the total amount of BSA and $W_{Free\ BSA}$ is the amount of non-desolvated free BSA. To ascertain the non-desolvated BSA, the nanoparticles were separated by centrifugation at 16,000 × g for 20 min at room temperature. An aliquot of the supernatant was diluted with sterile MilliQ water and the amount of BSA protein in the supernatant was determined by Bradford reagent (Sigma-Aldrich, USA) following manufacturer instructions.

7.0 Characterization of BSA nanoparticle (BNP) and BSA nanoparticle-compound 1 (BNP-C1) composite

7.1. Field emission scanning electron microscope (FESEM) analysis

BNP and BNP-C1 composite sample was added on separate clean sterile cover slip. The samples were then air-dried in laminar hood and examined in a field emission scanning electron microscope (Zeiss Sigma, USA). The images of BNPs and BP-C1 composite were recorded at various magnifications and the particle size was determined using ImageJ software (http://rsb.info.nih.gov/ij).

7.2. UV-visible and fluorescence spectroscopy

Samples of as prepared BNPs and BNP-C1 composite were subjected to UV-visible spectroscopy (Varian Cary 50, Varian Medical Systems, Inc., Palo Alto, CA) in scanning mode in a wavelength range of 200-800 nm. For fluorescence measurements of BNPs and BNP-C1 composite, the excitation and emission slit widths were set to 2.0 and 5.0 nm, respectively. For BNPs, a florescence emission spectrum was recorded at an excitation wavelength of 285 nm and an emission wavelength from 310-550 nm. For BNP-C1 composite, fluorescence measurements were acquired by setting the excitation at 370 nm while the emission wavelength was recorded between 385 and 650 nm. Both UV-visible as well as fluorescence spectra were recorded for multiple samples.

7.3. FT-IR analysis

FT-IR spectra of BSA, **compound 1**, BNP and BNP-C1 composite were recorded in KBr pellets at 4 cm^{-1} resolution in an infrared spectrometer (Spectrum One, Perkin-Elmer). Eight scans were performed for every sample in the range of 4000 to 450 cm^{-1}. A background spectrum for pure KBr was also measured.

8.0 Antibacterial activity of compound 1 following *in vitro* release from BNP-C1 composite

Initially, BNP-C1 composite (400 μM **compound 1** concentration) was dispersed in 500 μl of 10 mM HEPES buffer (pH 7.4) and incubated in an orbital shaker at 120 rpm at 37^{o}C for 24h to facilitate maximum *in vitro* release of **compound 1**. Target pathogenic bacteria, which included

Gram-positive *S. aureus* MTCC 96 and *E. faecalis* MTCC 439 and Gram-negative *E. coli* MTCC 433 and *E. aerogenes* MTCC 2822 were grown in fresh requisite growth media incorporated with varying concentrations of *in vitro* released **compound 1** (10.0 μM, 15.0 μM and 20.0 μM) at 37^{o}C and 130 rpm for 24h. The growth of **compound 1**-treated cells was monitored by measuring absorbance at 600 nm in a spectrophotometer (Cary 300, Varian) and was expressed as percentage killing compared to control (untreated cells).

9.0 Anti-biofilm activity of compound 1 following *in vitro* release from BNP-C1 composite

Initially, BNP-C1 composite (400 μM **compound 1** concentration) was dispersed in 500 μl of 10 mM HEPES buffer (pH 7.4) and incubated in an orbital shaker at 120 rpm at 37^{o}C for 24h to facilitate maximum *in vitro* release of **compound 1**. Biofilm of *P. aeruginosa* MTCC 2488 was grown in sterile 96 well microtitre plate following a standard method [2] and then treated with varying concentrations of *in vitro* released **compound 1** (60 μM, 80 μM and 100 μM) at 37^{o}C for 24h. The anti-biofilm activity of *in vitro* released **compound 1** was ascertained by measuring biofilm biomass through crystal violet staining as described previously.

10.0. Antibacterial activity of BNP-C1 composite

10.1. cFDA-SE leakage assay

A stock solution of cFDA-SE (500 μM) was prepared in ethanol and stored at -20^{o}C. 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, resuspended in the same and labelled with cFDA-SE (final concentration of 50 μM) at 37°C for 20 min. Subsequently, cells were pelleted by centrifugation and washed twice with sterile phosphate buffer to remove excess dye molecules. Varying concentrations of BNP-C1 composite (2.0 μM, 3.0 μM and 4.0 μM **compound 1** concentration) were added separately to cFDA-SE labelled-target bacteria (10^6 CFU/mL) and incubated at 37^{o}C and 180 rpm for 3h. In case of control experiments, only PBS and BSA nanoparticles suspended in PBS were also added to labelled cells and incubated under the same conditions. Following incubation, cells were pelleted by centrifugation and leakage of carboxyfluorescein from the 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. For every BNP-C1 composite concentration and control sample, fluorescence measurements were acquired from three independent experimental samples.

10.2. Membrane depolarization assay

The ability of BNP-C1 composite to depolarize the transmembrane potential of target bacteria was tested by $DiSC_35$-based membrane depolarization assay. A stock solution of $DiSC_35$ (3.0 mM) was made in ethanol and stored at -20^{o}C. Cells of *E. coli* MTCC 433 and *S. aureus* MTCC 96 were grown till mid-logarithmic phase (A_{600} = 0.4-0.5). The cells were harvested by centrifugation, washed with a buffer solution (5.0 mM HEPES buffer, 5.0 mM glucose, pH 7.2) and resuspended in the same buffer to an A_{600} of 0.05. The cell suspensions were incubated with $DiSC_35$ (0.4 μM) for 1 h at 37^{o}C followed by the addition of KCl (100 mM). Subsequently cell suspensions (1.0 mL) were placed in a cuvette to which varying concentrations of BNP-C1 composite (2.0, 3.0 and 4.0 μM **compound 1** concentration) was added and fluorescence readings were monitored intermittently in a spectrofluorimeter (FluoroMax-3, HORIBA) set to an excitation wavelength of 622 nm and emission wavelength of 670 nm. Cells treated with valinomycin (30 μM) and BSA nanoparticles alone were used as positive and negative control samples, respectively. Fluorescence measurements were taken for three independent experimental samples.

11.0. Reference

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2. A. Giacometti, O. Cirioni, M. S. Del Prete, A. M. Paggi, M. M. D'Errico and G. Scalise, *Peptides* 2000, **21**, 1155.

3. M. Musken, S. D. Fiore, U. Romling and S. Haussler, *Nat*. *Protocols*, 2010, **5**, 1460.

4. M. D. Adhikari, S. Goswami, B. R. Panda, A. Chattopadhyay and A. Ramesh, *Adv*. *Healthcare Mater*., 2013, **2**, 599.

5. K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. von Briesen and D. Schubert, *Int*. *J*. *Pharmaceutics*, 2003, **257**, 169.

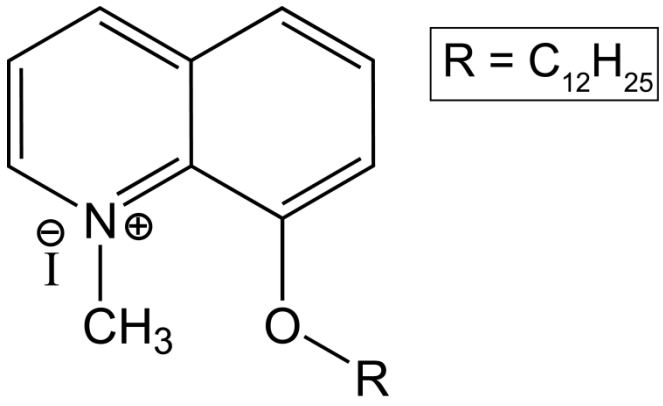

Fig. S1 General structure of **compound 1**.

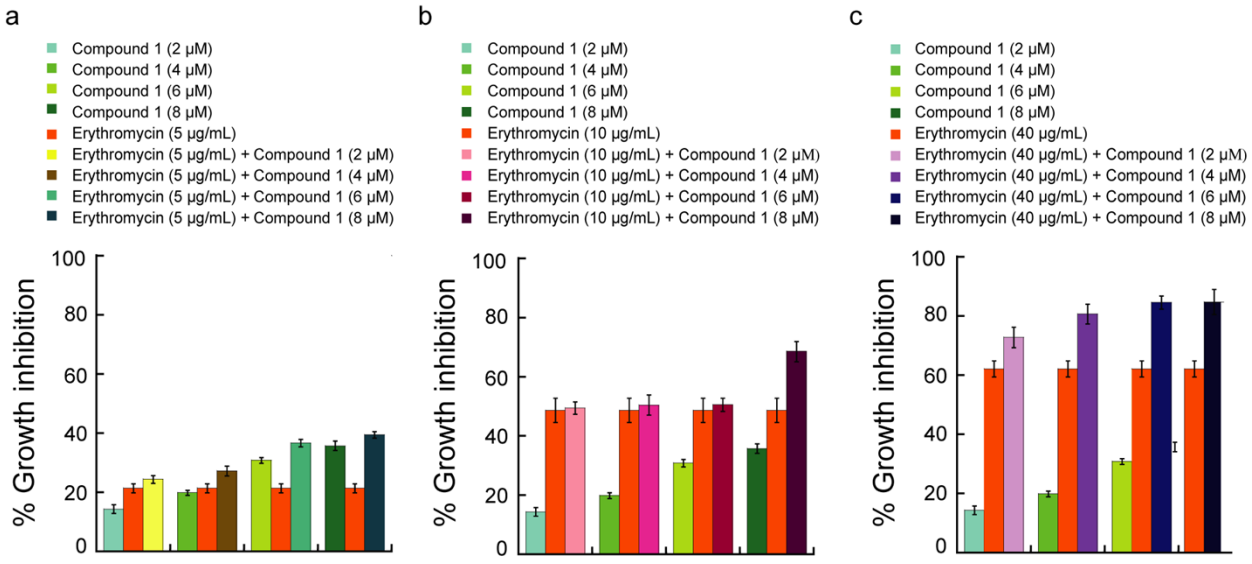

Fig. S2 Effect of combined treatment of **compound 1** and erythromycin on the growth of *E. coli* MTCC 433.

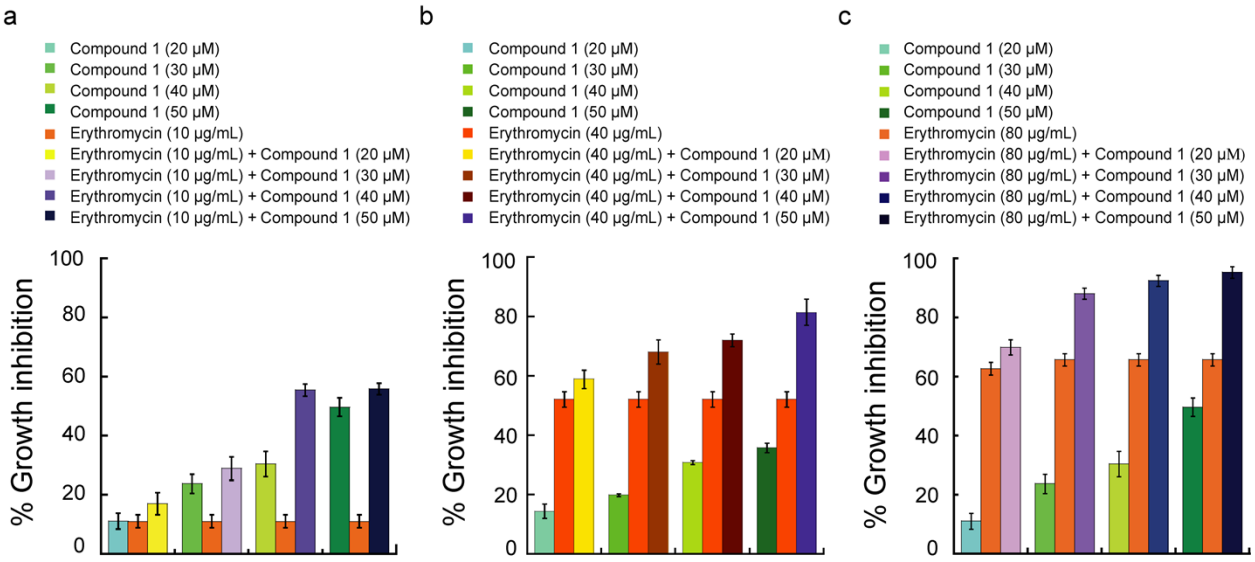

Fig. S3 Effect of combined treatment of **compound 1** and erythromycin on the growth of *P. aeruginosa* MTCC 2488.

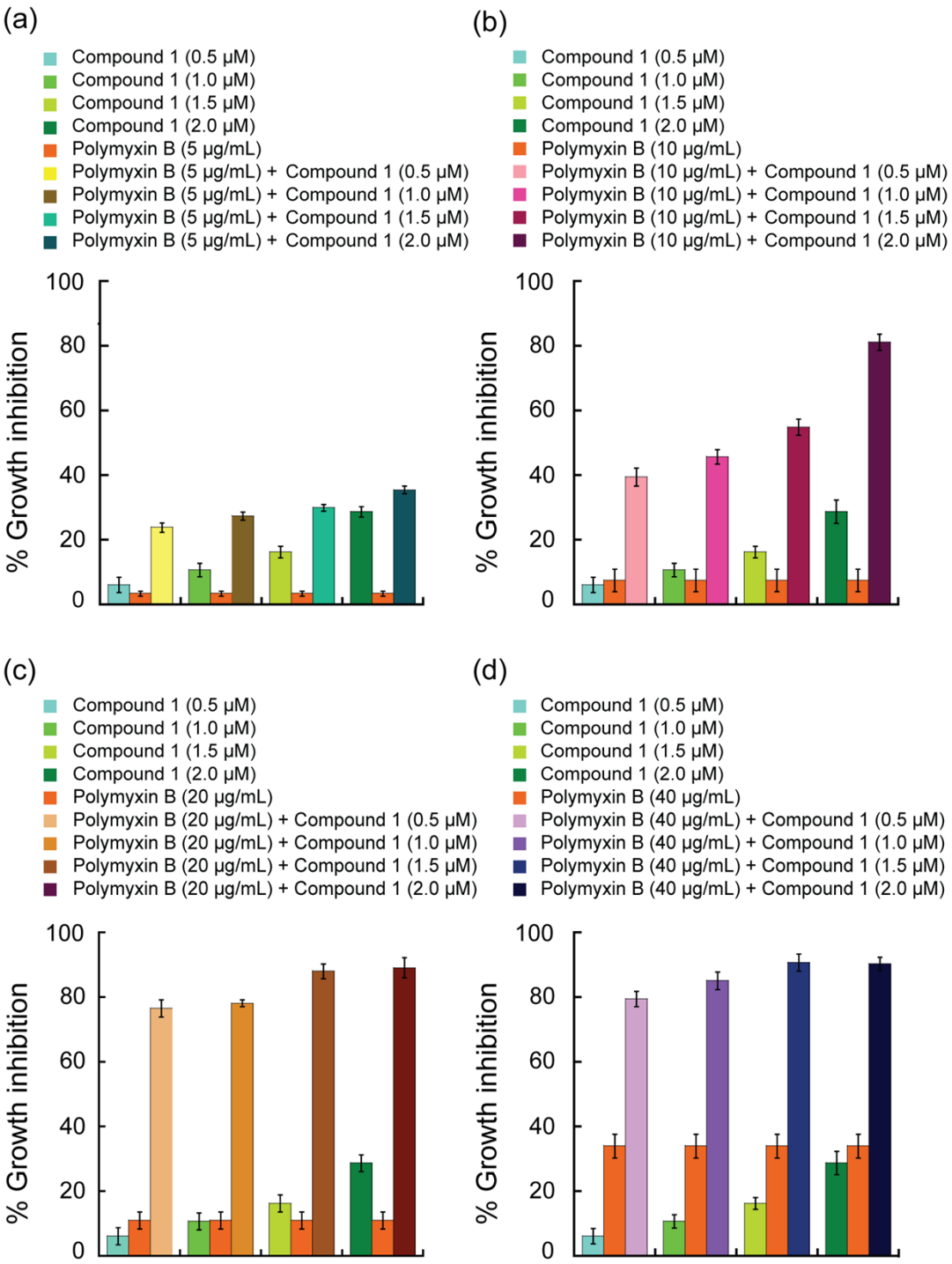

Fig. S4 Effect of combined treatment of **compound 1** and polymyxin B on the growth of *S. aureus* MTCC 96.

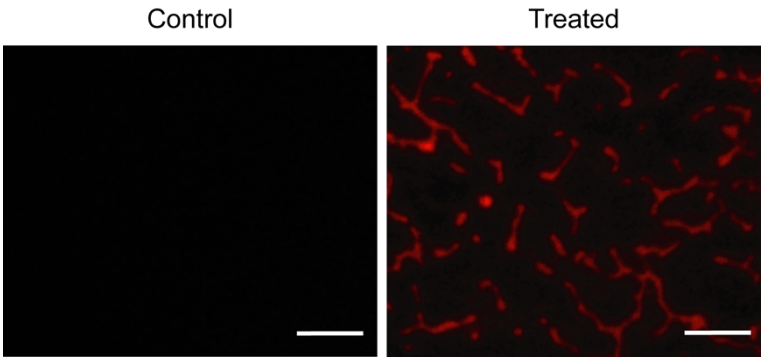

Fig. S5 Fluorescence microscope analysis to study the effect of **compound 1** (200 µM) on *P. aeruginosa* MTCC 2488 biofilm. Control and treated biofilm were stained with propidium iodide. Scale bar for the images is 50 µm.

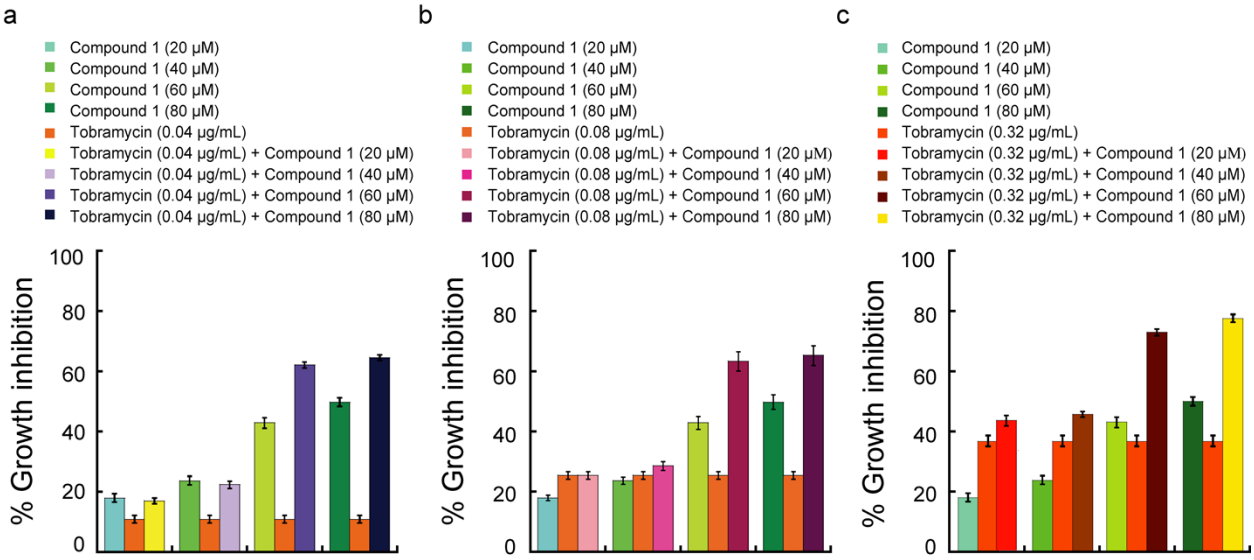

Fig. S6. Effect of combined treatment of **compound 1** and tobramycin on the growth of *P. aeruginosa* MTCC 2488 biofilm.

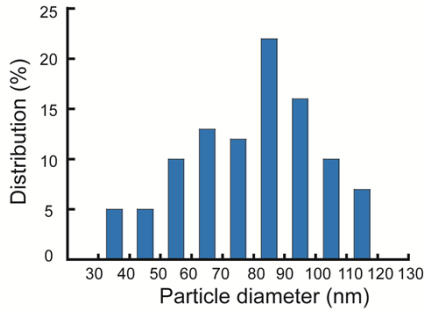

Fig. S7 Particle size distribution of BSA nanoparticles (BNPs) determined by ImageJ analysis software (http://rsb.info.nih.gov/ij).

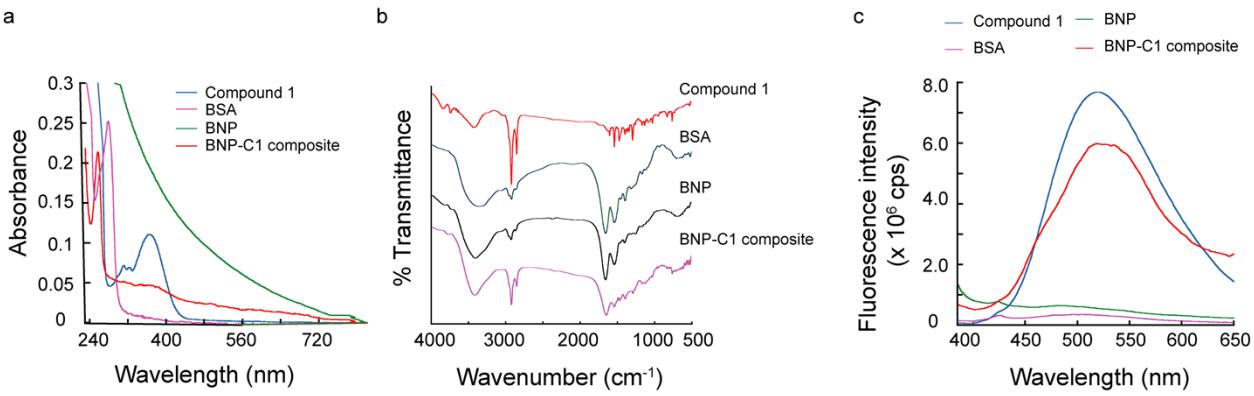

Fig. S8 (a) UV-visible absorption spectra of **compound 1**, bovine serum albumin (BSA), BSA nanoparticle (BNP) and BSA-nanoparticle-**compound 1** composite (BNP-C1 composite). (b) FTIR analysis of **compound 1**, bovine serum albumin (BSA), BSA nanoparticle (BNP) and BSA-nanoparticle-**compound 1** composite (BNP-C1 composite). (c) Fluorescence emission spectra of **compound 1**, bovine serum albumin (BSA), BSA nanoparticle (BNP) and BSA-nanoparticle-**compound 1** composite (BNP-C1 composite).

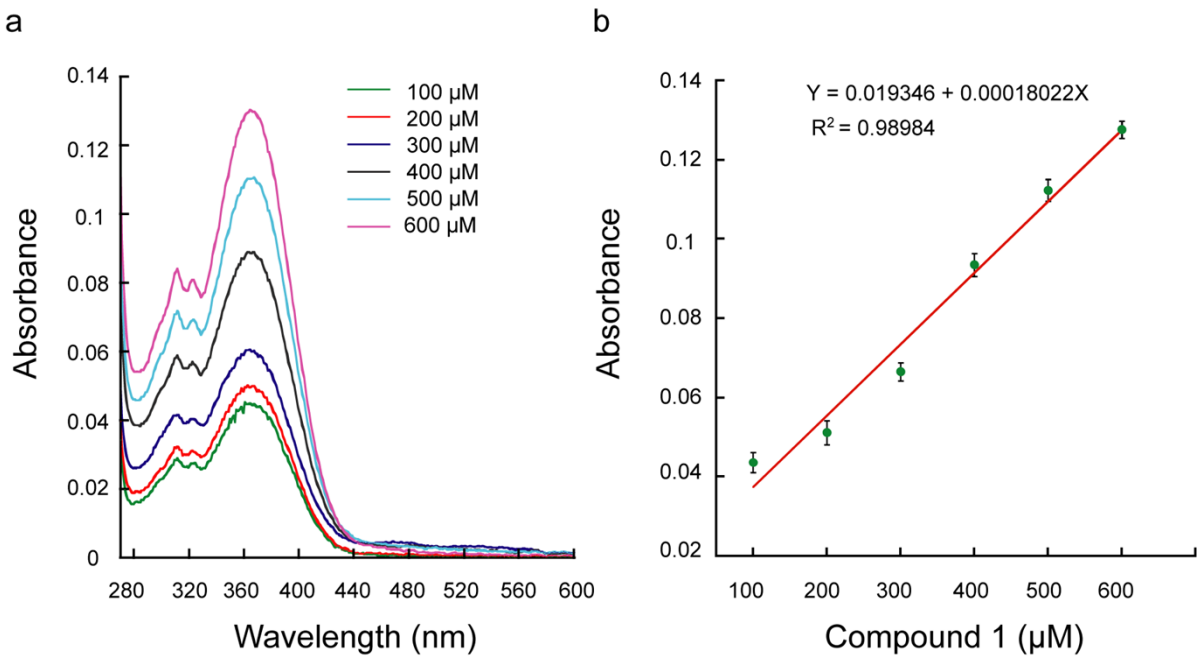

Fig. S9 (a) UV-visible absorption spectra of varying concentrations of **compound 1**. (b) Calibration plot of absorbance versus concentration for **compound 1**.

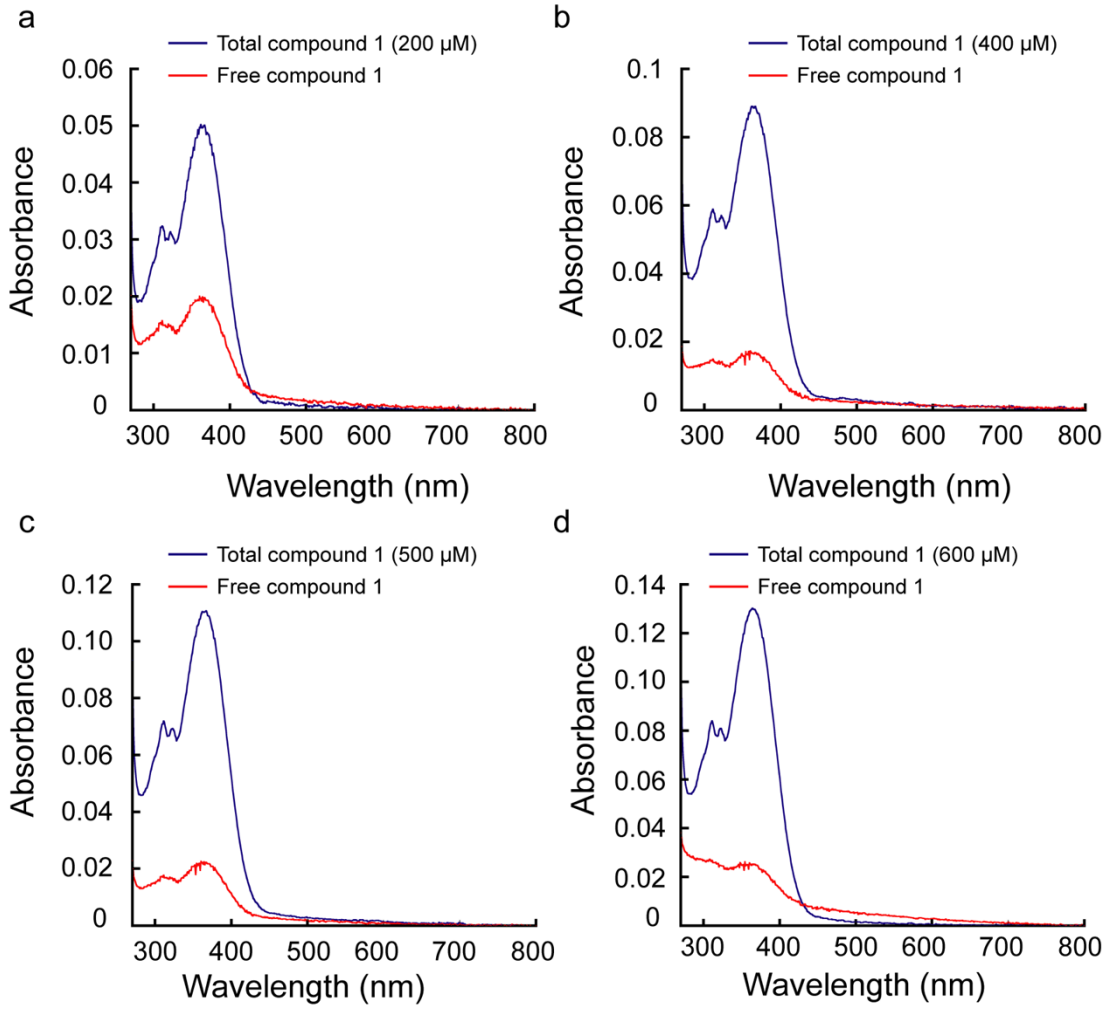

Fig. S10 UV-visible absorption spectra of **compound 1** for estimation of loading capacity (LC) and encapsulation efficiency (EE) of BSA nanoparticles. Total **compound 1** indicates the initial concentration of **compound 1** used for amphiphile-loading experiments and free **compound 1** is the compound recovered in the supernatant following generation of BSA nanoparticle-**compound 1** composite.

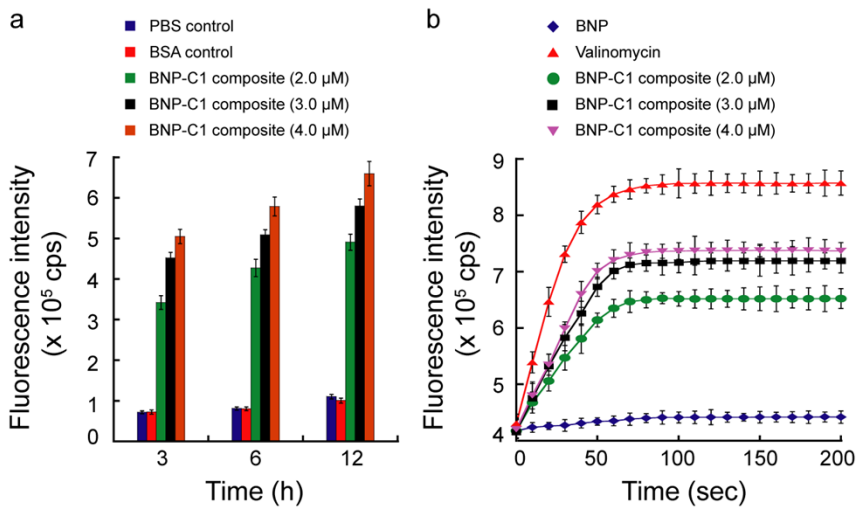

Fig. S11 (a) cFDA-SE leakage assay to ascertain membrane-directed activity of BNP-C1 composite on *E. coli* MTCC 433. (b) $DiSC_3$5-based membrane depolarization assay in *E. coli* MTCC 433 cells treated with BNP-C1 composite. The concentrations of **compound 1** present in BNP-C1 composite are indicated in parenthesis.

Table S1. Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of **compound 1** against representative pathogenic bacteria.

Pathogenic Bacteria	MIC of compound 1 (μM)	MKC of compound 1 (μM)
S. aureus MTCC 96	5.0	15
E. faecalis MTCC 439	20	40
E. coli MTCC 433	15	20
P. aeruginosa MTCC 2488	120	150

Table S2. MIC of **compound 1** against *S. aureus* MTCC 96 in multistep resistance development experiment.

Test compound	Selection step/Medium	MIC of compound 1 (μM) / OD_{600} ± standard deviation
Compound 1	Step 1/ Agar	5.0/0.02 ± 0.007
Compound 1	Step 2/ Agar	5.0/0.03 ± 0.006
Compound 1	Step 3/ Agar	5.0/0.05 ± 0.006

Table S3. Minimum inhibitory concentration (MIC) and minimum killing concentration (MKC) of erythromycin and polymyxin B against representative target bacteria.

Target Pathogen	Antibiotic	
	Erythromycin	
Gram-negative Bacteria		
	MIC (µg/mL)	**MKC (µg/mL)**
E.coli MTCC 433	80	160
P. aeruginosa MTCC 2488	160	320
	Polymyxin B	
	MIC (µg/mL)	**MKC (µg/mL)**
S. aureus MTCC 96	160	180

Table S4. Fold reduction in MIC of polymyxin B in combination with **compound 1** and determination of fractional inhibitory concentration (FIC) index.

Indicator strain	Compound 1 (µM)	Folds reduction in MIC of Polymyxin B	FIC index[a]	Effect[b]
S. aureus MTCC 96	0.5	2.0	0.60	ADD
	1.0	4.0	0.45	SYN
	1.5	8.0	0.42	SYN
	2.0	16.0	0.36	SYN

[a] Fractional inhibitory concentration (FIC) index was assessed as described in the experimental section. [b] An FIC index of ≤ 0.5 is considered to demonstrate synergism, FIC index of > 0.5-1.0 indicates additive effect, FIC index >1.0 - <4.0 indifference and FIC index of ≥ 4.0 indicates antagonism. [2] ADD: Additive; SYN: Synergism; IND: Indifference; ANT: Antagonism.