Supporting Information

Small Molecular Adjuvant Repurposes Antibiotics towards Gram-negative Bacterial Infections and Multispecies Bacterial Biofilm

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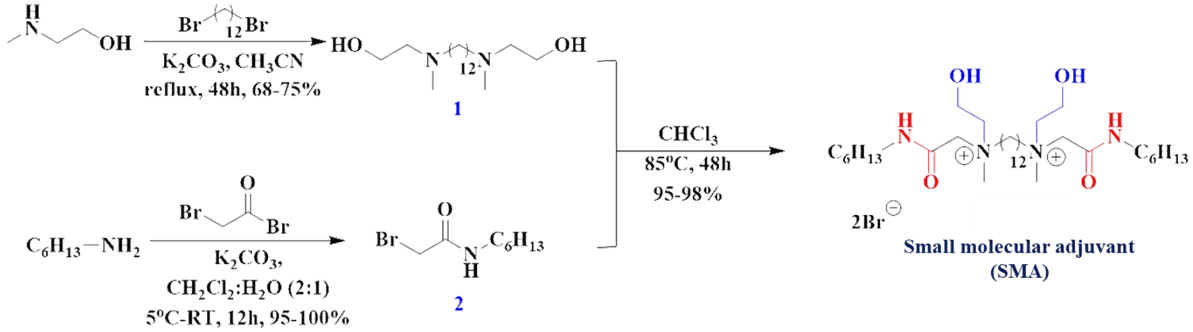
Synthesis of Small Molecular Adjuvants (SMA)

Concisely, 2-(methylamino) ethanol was reacted with dibromododecane to produce compound 1 with 68-75% yield. Activated hexyl amide 2 was synthesized by the reaction of 1-aminohexane with bromoacetyl bromide with 95-100% yield. Finally, SMA was synthesised by reacting intermediate 1 with the activated amide 2 with a good yield (95-98%). All the intermediates and SMA were characterized by ^{1}H NMR, ^{13}C NMR, HRMS (Figure S1, S2, S3).

Scheme S1. Synthesis of small molecular adjuvant (SMA)



Experimental section

Materials and Instrumentation

Reagent grade solvents were used after distilling and drying them. All reagents were bought from Sigma-Aldrich and Spectrochem, and were used directly deprived of purification. Nuclear Magnetic Resonance (NMR) spectra were recorded in deuterated solvent by using Brukar (AV-400) 400 MHz spectrometer. Optical density was measured by using Tecan Infinite Pro series M200 Microplate Reader. High resolution mass spectroscopy (HRMS) was measured by using Agilent Technologies 1290 infinity spectrometer. Bacterial strain, *K. pneumoniae* MTCC BAA2146 (NDM-1 producing strain) were obtained from MTCC (Chandigarh, India). *E. coli* R3336 (NDM-1 producing strain), *P. aeruginosa* R590, *A. baumannii* R674, and *K. pneumoniae* R3934 (NDM-1 producing strain) were acquired from

NIMHANS, Bangalore, India. Other NDM-1 producing bacterial strains *A. baumannii* A168, *A. baumannii* A157, and *K. pneumoniae* EN5136 were acquired from NICED, Kolkata, India. RAW 264.7 (ATCC® TIB-71™) cell lines were procured from ATCC (Rockville, MD, USA). Growth media and agar for bacteria, fungus, and mammalian cells were obtained from HIMEDIA, India. The *in-vivo* animal experiments were done by following the protocols permitted by the Institutional Animal Ethics Committee (IAEC) of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR) (201/Go/ReBi/S/2000/CPCSEA).

Synthesis of SMA

Synthesis of compound (N,N′-diethanol)-(N,N′-dimethyl)-1,12-diaminododecane (1)

Dibromododecane (4 mmol) was mixed with the 20 mL acetonitrile solution of (2-(methylamino)ethanol (10 mmol). Finally, solid K_2CO_3 (6 mmol) was mixed with this reaction mixture which then was stirred for 48 hours at 85° C. After completion of reaction K_2CO_3 was filtered out and the filtrate was further purified using column chromatography to get the pure product with 68-75 % yield.

Synthesis *N*-hexyl-1-bromoethanamide (2)

The 50 mL dichloromethane (DCM) solution of 1-aminohexane (30 mmol) was mixed with aqueous K_2CO_3 (45 mmol) solution and these two phased solution was stirred at 4-5 °C. Then, bromoacetyl bromide (45 mmol) solution in DCM (25 mL) was added to the reaction mixture drop wise for about 30 min by maintaining the temperature at 4-5 °C. Finally, the reaction mixture was stirred for 12 hours at room temperature. After that, the DCM layer was separated from the aqueous layer and the combined organic solution was finally concentrated to get the product with good yield (95-100%).

Characterization details:

***Compound (N,N'-diethanol)-(N,N'-dimethyl)-1,12-diaminododecane* (*1*)**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.585-3.557 (t, -NCH_2CH_2OH, 4H), 2.55-2.472 (t, -NCH_2CH_2OH, 4H), 2.404-2.362 (t, -NCH_2CH_2-, 4H), 2.233 (s, -$N(CH_3)_2$, 6H), 1.471-1.451 (m, -$NCH_2CH_2CH_2$-, 4H), 1.225-1.251 (m, -$NCH_2CH_2CH_2$-, 18H). HRMS: calculated m/z 317.3090 $[M+H]^+$; observed m/z 317.3064 $[M+H]^+$.

N-hexyl-1-bromoethanamide (2): ^{1}H NMR: (400 MHz, $CDCl_3$): δ, 6.543 (br. s, amide –NHCO, 1H), 3.931 (s, -$COCH_2Br$, 2H), 3.312 (m, -$CONHCH_2$-, 2H), 1.574 (m, -$CH_2(CH_2)_3CH_3$, 2H), 1.336 (m, -$CH_2(CH_2)_3CH_3$, 6H), 0.886 (t, terminal –CH_3, 3H).

SMA: FT-IR (cm^{-1}): 3341 (OH str.), 3215 (amide NH str.), 2926 (CH_2 assym. str.), 2861 (CH_2 sym. str.), 1679 (amide I, C=O str.), 1551 (amide II, NH ben.), 1474 (CH_2 scissor); ^{1}H-NMR: (400 MHz, $CDCl_3$): δ8.51 (m, -$CONHCH_2$-, 2H), 4.57-4.46 (dd, -N^+CH_2CONH-, 4H), 4.15 (m, -$N^+CH_2CH_2OH$, 4H) , 3.77-3.74 (m, -$N^+CH_2CH_2OH$ and -$N^+CH_2CH_2CH_2$-, 8H), 3.41(s, -$N^+(CH_3)_2$, 6H) , 3.27-3.22 (m, -$NHCH_2CH_2$-, 4H) , 1.83 (bs, -$N^+CH_2CH_2CH_2$-, 4H), 1.58-1.53 (m, -$NHCH_2CH_2CH_2$-, 4H), 1.38-1.29 (m, -$N^+CH_2CH_2CH_2$ and -CH_2CH_3, 28H), 0.89-0.86 (t, terminal CH_3, 6H). 13C-NMR (600MHz, $CDCl_3$): 162.86, 64.71, 55.79, 50.72, 40.25, 39.83, 31.36, 29.39, 29.20, 28.92, 28.46, 28.35, 26.67, 26.44, 25.85, 22.52, 22.48, 14.03. HRMS: calculated m/z 300.28 $[M–2Br-]^{2+}$; observed m/z 300.278 $[M–2Br-]^{2+.}$

Synthesis of SMA

N-hexyl-1-bromoethanamide (2) (22.5 mmol) were mixed with the solution of compound 1 (7.5 mmol) in dry chloroform (7.5 mL), and the reaction mixtures were continuously stirred for 48 hours at 85°C. After the completion of reaction, the reaction mixtures were kept for cooling down at room temperature and finally relocated to a round bottom flask. Then the volume of the reaction mixtures was condensed to 1/10th of its original volume. Finally, the products were precipitated down by using of diethyl ether (75 mL). The precipitate was

continuously washed with diethyl ether for removing the unreacted reactants and the product was acquired by evaporating the excess organic solvent. Finally, compound SMA was further purified by using reversed phase-HPLC to get the yield in the range of 64-67 %.

Outer membrane permeabilization assay

Outer membrane permeabilization assay of SMA was performed against NDM-1 producing bacteria, *A. baumannii* A168. Briefly, the mid-log phase 6h grown bacterial cells was centrifuged down at 5000 rpm for 5 min. Then these bacterial cells were washed repeatedly with 5 mM HEPES buffer and finally re-suspended in 1:1 mixture of 5 mM HEPES buffer and 5 mM glucose. After that, 10 μM of NPN dye (N-phenyl-1-naphthylamine) was mixed with these bacterial suspensions and then incubated in dark condition for 45 minutes. After incubation, 190 μL of these cocktail bacterial suspensions was kept into the wells of black and clear-bottom 96-well plates and mixed with 10 μL of different concentrations of SMA. Finally, the fluorescence emission intensity was measured at 420 nm wavelength by exciting with 350 nm wavelength for 26 minutes. The experiment was performed in triplicate and the data was plotted by taking the average including standard deviation.

Membrane depolarization assay

The freshly 6 h grown bacterial cells (1×10^8 CFU/mL) were centrifuged down and repeatedly washed with HEPES buffer. Then, these washed cells were re-suspended in a mixture of 1:1:1 solution of 100 mM KCl, 5 mM HEPES, and 5 mM glucose solution. After that, the 2 μM final concentration of DisC3(5) dye was mixed with this bacterial suspension and incubated in dark condition for 1 h. Then, 190 μL of these dye mixed bacterial suspension was kept into the wells black well plate and subsequently measured the fluorescence intensity at 622 nm wavelength by exciting at the wavelength of 670 nm. After 4 minutes, 10 μL of different concentrations of SMA was mixed with it and the fluorescence

intensity was measured for 26 minutes. The experiment was performed in triplicate and the data was plotted by taking the average including standard deviation.

Potentiation efficacy of SMA by performing the chequerboard assay

At first, all bacteria were grown for 6h in nutrient broth (0.5 μg/mL Meropenem-supplemented nutrient broths for *A. baumannii* A168, *A. baumannii* A157, and *K. pneumoniae* EN5136). Then the potentiation ability of SMA with various classes of antibiotics was investigated in MHB (Mueller Hinton broth) media by performing the renowned chequerboard assays. A mixture of 25 μL each of adjuvant (SMA) and antibiotics of different concentrations by 2-fold variations was kept into a 96-well plate followed by the addition of 150 μL of the 10^5 CFU/mL bacterial suspensions and then incubated for 18−24 h at 37 °C. This assay was repeated twice.

Time-kill kinetic against growing planktonic bacteria

The time-kill bactericidal activity of the leading combinations was examined against the growing planktonic NDM-1 producing bacteria *A. baumannii* A168 and *K. pneumoniae* R3934 by following the previously reported protocol. Briefly, the mid-log phase grown bacterial culture were diluted to 10^5 CFU/mL in MHB media and then was treated with the leading combinations. Same volume of Millipore water, individual treatment of SMA and antibiotics were taken as controls. Then the treated samples were incubated at 37 °C. At different time intervals like 0, 1, 2, 4, 6, 12, and 24 h, the bacterial viability was counted by serially 10-fold dilution in saline followed by drop-platted on Maconkey agar plate. Then the plates were incubated for 16-24 h at 37 °C, and finally bacterial colonies were counted visually. The duplicate data were plotted with standard deviation.

Cytotoxicity via LIVE/DEAD imaging

RAW 264.7 cells were seeded onto the wells (~10^4 cells/well) of a 96-well tissue culture plate. Then the grown cells were treated with the leading combinations. Triton-X (0.1 vol %) was taken as a positive control and for the negative control, the untreated cells were used. After 24 h of treatment, all cells were stained with a mixture of Calcein AM (2 μM final concentration) and propidium iodide (PI, 4.5 μM final concentration) and kept for incubation under 5% CO2 atmosphere for 15 min at 37 °C. After washing with PBS, the stained cells were then imaged in a Leica DM2500 fluorescence microscope where the band-pass filter for Calcein AM was at 500-550 nm wavelengths and a long-pass filter for PI was at 590-800 nm.

In-vivo systemic toxicity

In-vivo systemic toxicity of SMA was performed with female BALB/c mice (6-8 weeks; 18-22 g body weight). The systemic toxicity of SMA was executed through intra-peritoneal and sub-cutaneous injection. For each administration route, two groups of mice were taken. For both groups, 200 μL of saline was injected in control groups. As per the OECD guideline, different dosages of SMA utilized for the experiment, were 5.5, 17.5, 56, and 179 mg/kg. All the mice were kept under observation for 14 days. The mortality for each dosage of SMA was noted and finally, lethal dose for 50 % mortality (LD50) was calculated using the Spearman-Karber method.

Anti-biofilm assays against NDM-1 producing bacteria _A. baumannii_

The biofilm disruption ability of the leading combination was investigated against the preformed biofilm of NDM-1 producing *A. baumannii* A168 bacteria through the counting of viable biofilm-embedded bacteria, quantifying the biomass via crystal violet staining, and visualizing by the confocal laser microscopy (CLSM). Briefly, the 18 mm diameter glass coverslips were sterilized and kept in 6-wells plate. Then, the 2 mL of 10^5 CFU/mL bacterial suspension of *A. baumannii* A168 bacteria in BM2 biofilm media (supplemented with 0.5%

casamino acids, 0.5% glucose, and 200 μM $FeCl_3$) was poured onto the sterilized cover slip and incubated in stationary condition at 30 °C for 48 h. After that, the biofilm containing coverslips were washed with saline water and placed in another 6-well plate containing the 2 mL of different concentration of the examined compound concentrations followed by incubation for 24 h at 30 °C. Well without any compound was taken as negative control. After that, the treated coverslips were digested with 10 % (v/v) Trypsin-EDTA and finally 10-times serially diluted in saline to drop-platted on the Maconkey agar plate followed by the incubation at 37 °C for 24 h for counting the no of viable bacteria. For quantifying the percentage of biomass the treated coverslip was washed with saline and then stained with 0.1 % crystal violet followed by digesting with 95 % ethanol to take the optical density at 522 nm. For confocal microscopy, the treated biofilm were washed with saline, stained with SYTO-9 (green fluorescence) and PI (red fluorescence), and the images were visualized by utilizing confocal laser scanning microscopy (CLSM).

Minocycline uptake assay

The freshly mid-log phase grown bacterial culture of NDM-1 producing bacteria *K. pneumoniae* R3934 was harvested by centrifuging at 4500 rpm for 5 minutes and washed with the HEPES buffer. Then the washed cells were re-suspended in 5 mM HEPES buffer and mixed with 100 μg/mL of minocycline. Afterward, 190 μL of these minocycline-containing bacterial suspensions was placed into the black-well plates, and the fluorescence intensity was measured at emission wavelength of 405 nm by exciting at 535 nm for 4 minutes. Then, 10 μL of different concentrations of SMA was mixed with the wells, and the fluorescence intensity was measured for about 26 minutes. 10 μL of Millipore water was taken as a negative control. The results were plotted with the average of triplicate data.

EtBr efflux assay

A freshly 6 h grown bacterial culture of NDM-1 producing *A. baumannii* A168 bacteria was harvested at 4500 rpm for 5 min and re-suspended in fresh MHB media. Then, these fresh bacterial suspensions were mixed with 5 μM ethidium bromide (EtBr) and incubated in dark condition at 140 rpm at 37 °C for 2 h. Next, the EtBr-stained cells were harvested by centrifuging down, washed with media to amputate the extracellular EtBr, and replaced with fresh MHB. Then these freshly prepared EtBr-stained bacterial suspensions were treated with different concentrations of SMA and then no of stained cells were analysed by Flow cytometry ($\lambda_{emission}$ = 530 nm and $\lambda_{exitation}$ = 600 nm). Without any compound treatment was taken as positive control which showed the efflux of EtBr. 10 μg/mL concentration of CCCP was taken as a negative control which stopped the EtBr efflux.

Resistance frequency study

The frequency of resistance grown by *A. baumannii* A168 bacteria against the leading combination consisting of SMA and Fusidic acid was evaluated. Firstly, the nutrient agar plates were prepared by supplementing with the combination of SMA (16 μg/mL) and Fusidic acid (8 μg/mL), only Fusidic acid (512 μg/mL; 8× MIC), and Colistin (8× MIC). Then, the mid-log phase grown bacteria were re-suspended in saline at different concentrations (started from 10^8 CFU/ml to 10^5 CFU/mL). Then, the 100 μL of these various bacterial suspensions were spread-platted on different compounds-supplemented nutrient agar plates and incubated for 24 h at 37 ˚C. The frequency of resistance was calculated through dividing the number of obtained resistant mutants by the number of cells challenged.

Bactericidal activity against multispecies bacterial co-culture

The bactericidal activity of the leading combination of SMA and Fusidic acid was investigated against the multispecies bacterial co-culture of MRSA ATCC 33591 and *A. baumannii* A168. Mid-log phase grown cultures of the both bacteria were diluted to 10^5

CFU/mL in MHB media and mixed. Then, 150 µL of this co-mixture of both bacteria-MRSA ATCC33591 and *A. baumannii* A168, was treated with 50 µL of the combination therapy, SMA, Fusidic acid, Vancomycin, and Colistin for 12 h. After incubation, 20 µL of the treated co-culture was serially diluted by 10-times in saline and finally, it was drop-platted on the manitol salt agar plate (for MRSA specific colony) and the Maconkey agar plate (for *A. baumannii* specific colony) simultaneously. Then these two types' plates were incubated for 24 h at 37 °C and post-incubation the no of viable bacteria for MRSA and *A. baumannii* was visually counted. The data for duplicate experiment was represented with standard deviation.

Anti-biofilm efficacy against multispecies bacterial biofilm

The biofilm disruption ability of the leading combination consisting of SMA and Fusidic acid was investigated against multispecies bacterial biofilm of MRSA ATCC33591 and *A. baumannii* A168 through the counting of viable biofilm-embedded bacteria and quantifying the biomass via crystal violet staining. Briefly, the 18 mm diameter glass coverslips were sterilized and kept in 6-wells plate. Then, the 2 mL co-mixture of 10^5 CFU/mL MRSA ATCC33591 bacteria and 10^6 CFU/mL *A. baumannii* A168 bacteria in tryptic soya broth (TSB) biofilm media supplemented with 0.5 % glucose was poured onto the sterilized cover slip and incubated in stationary condition at 37 °C for 48 h. After that, the biofilm containing coverslips were washed with saline water and placed in another 6-well plate containing the 2 mL of different concentrations of the examined compound followed by incubation for 24 h at 37 °C. Well without any compound was taken as negative control. After incubation, for quantifying the percentage of biomass, the treated coverslips were washed with saline and then stained with 0.1 % crystal violet followed by digesting with 95 % ethanol to take the optical density at 522 nm. For the counting of viable biofilm-embedded bacteria, the treated coverslips were digested with 10 % (v/v) Tripsin-EDTA and consequently it was 10-times

serially diluted in saline. Finally, it was drop-platted on the manitol salt agar plate (for MRSA specific colony) and the Maconkey agar plate (for *A. baumannii* specific colony) simultaneously. Then these two types' plates were incubated for 24 h at 37 °C and post-incubation the no of viable bacteria for MRSA and *A. baumannii* was visually counted. The data for duplicate experiment was represented with standard deviation.

Mice skin infection model

6 to 8 weeks old female BALB/c mice (18−22g) were used for the *in-vivo* model. Briefly, all mice were condensed neutropenic by inoculating two dosages of cyclophosphamide (i.p.) (150 mg/kg first dose and 100 mg/kg after 3 days of the first dose). After 24 h of the second dose, mice were sedated with the cocktail of ketamine-xylazine (ketamine = 40 mg/kg**;** xylazine = 2 mg/kg) intraperitoneal injection. Then the dorsal area of each mouse was shaved clearly. Next, a wound (inflaming and glittering of the skin without haemorrhage) was formed at the shaved dorsal area. After that, 20 µL saline suspension of 10^7 CFU/mL NDM-1 producing *A. baumannii* A168 bacteria was contaminated at the wound site. After 4 h of infection, for the pre-treatment count, one mouse was sacrificed and the infected skin was regimented in saline to get the count of viable bacterial per gram of skin. Then, mice (n = 4 per group) were treated with saline (50 µL; untreated), SMA (40 mg/kg), Fusidic acid (40 mg/kg), Colistin (40 mg/kg), and the combination of SMA (40 mg/kg) and Fusidic acid (40 mg/kg) daily at the infected site. The compound solution was thoroughly spread on the whole infected surface. Total five dosages (one dose per day) were given topically. At five days, all mice were sacrificed; the infected skin was collected and homogenized in saline, serially diluted, and drop-plated on Maconkey agar plate for cell counting. The bacterial count was presented as log CFU/g of the tissue with mean standard error.

Spectra of SMA:

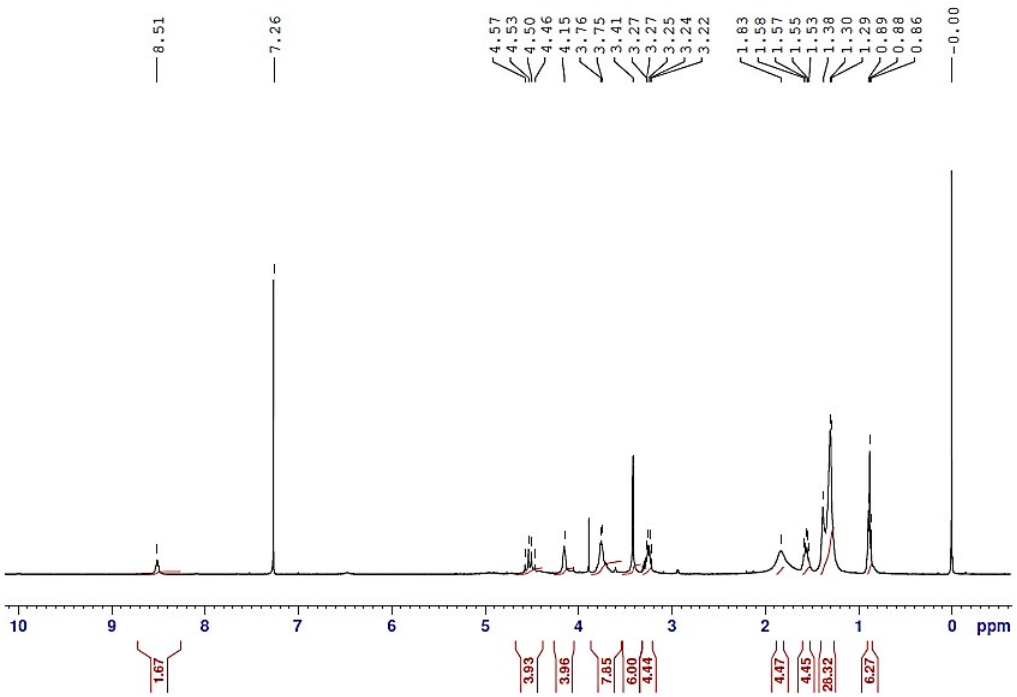


Figure S1. [1]HNMR spectrum of **SMA** was recorded in $CDCl_3$ at 400MHz.

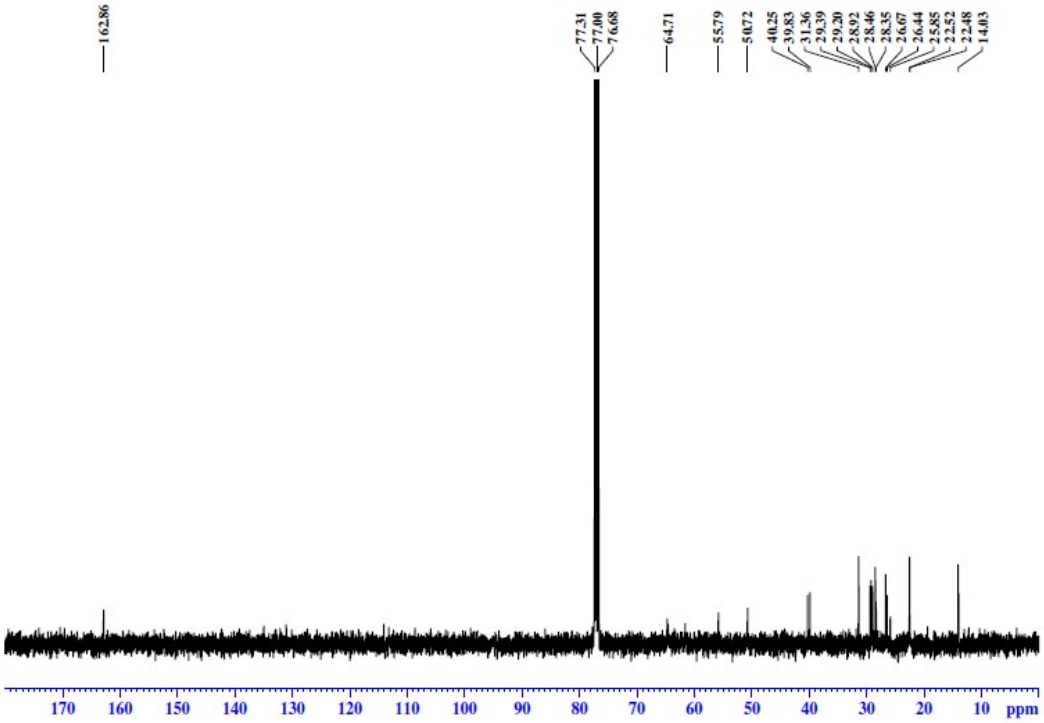


Figure S2. [13]CNMR spectrum of **SMA** was recorded in $CDCl_3$ at 600MHz.

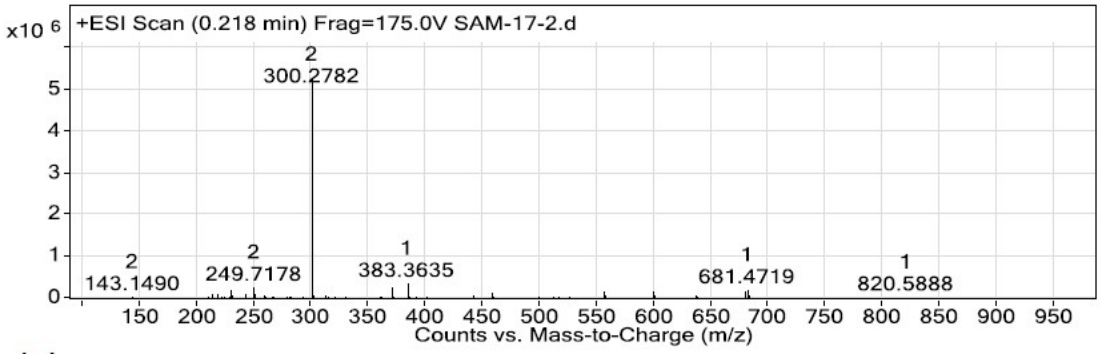


Figure S3. HRMS spectrum of **SMA**

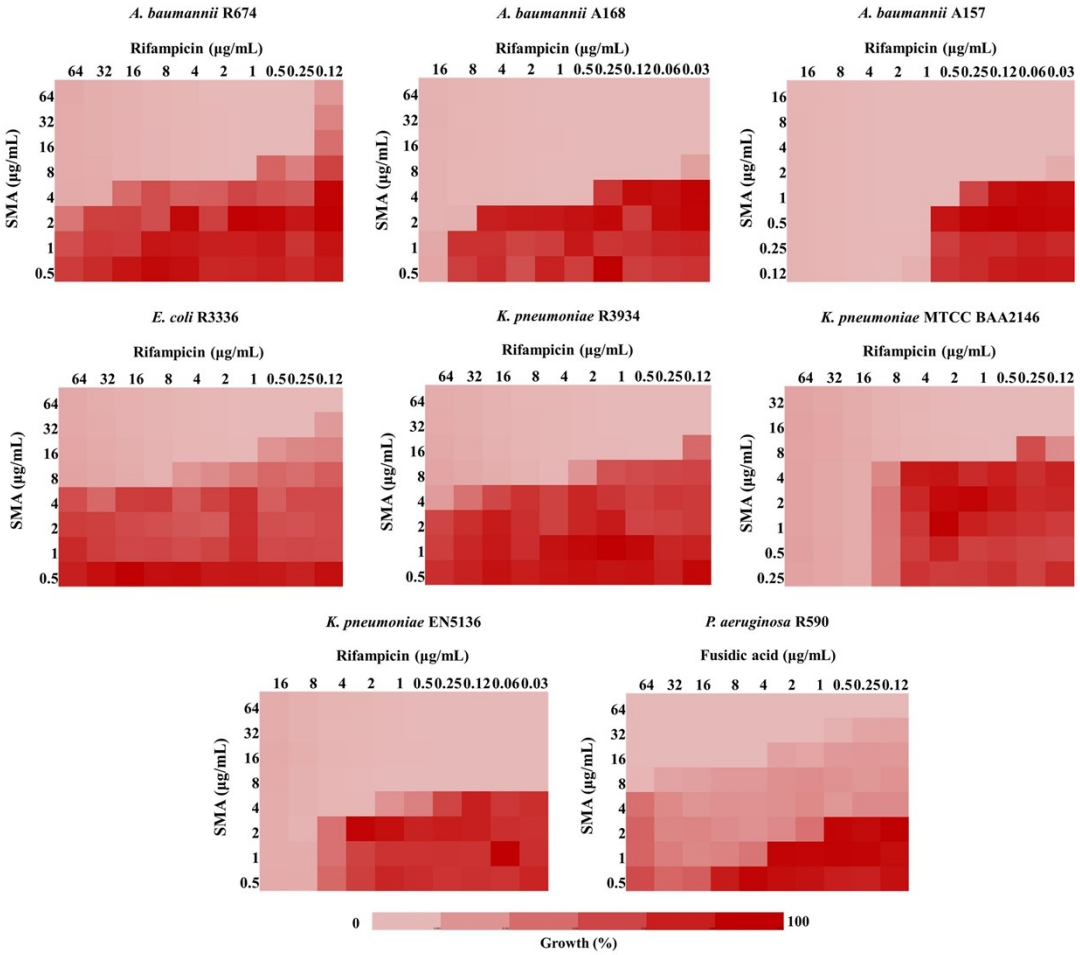


Figure S4. Chequerboard assays of SMA with Rifampicin against multi-drug resistant Gram-negative pathogens

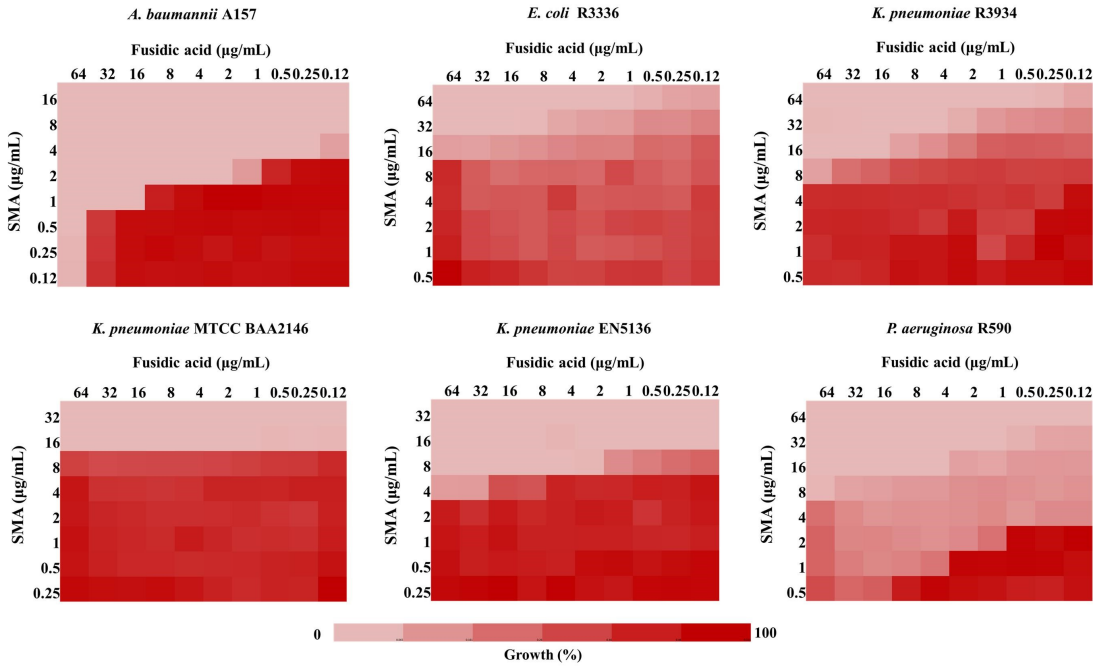


Figure S5. Chequerboard assays of SMA with Fusidic acid against multi-drug resistant Gram-negative pathogens

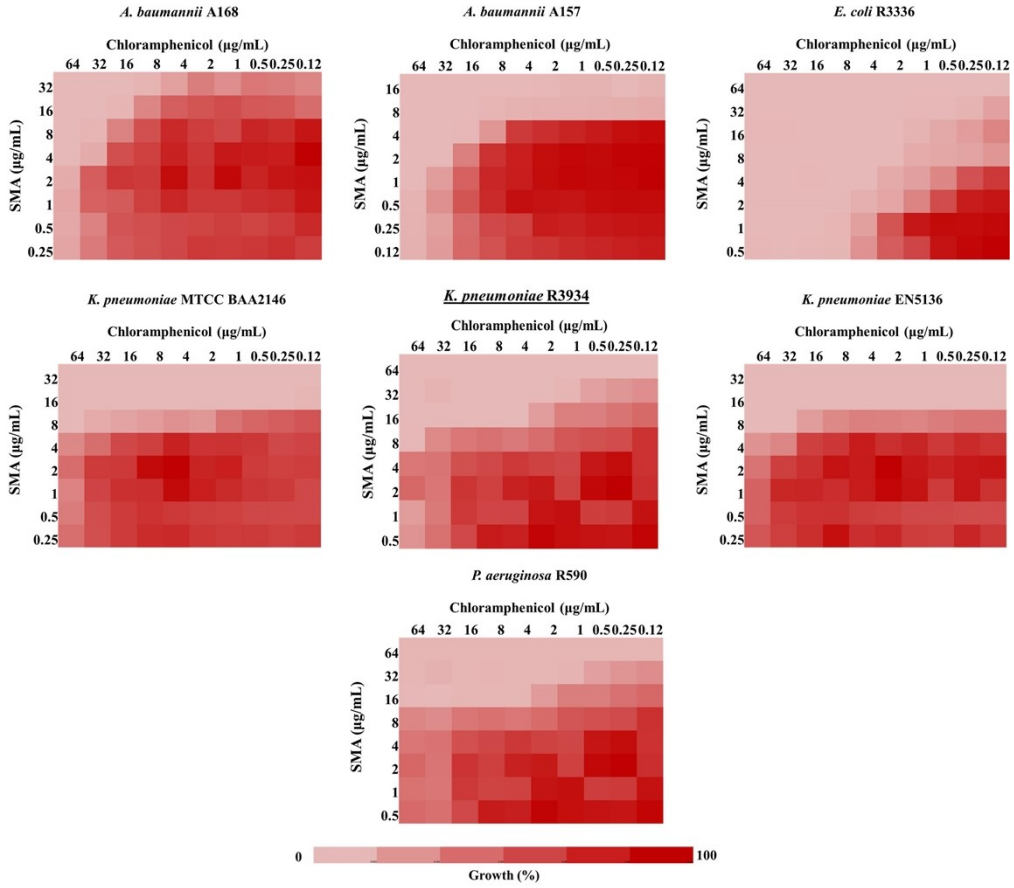


Figure S6. Chequerboard assays of SMA with Chloramphenicol against multi-drug resistant Gram-negative pathogens

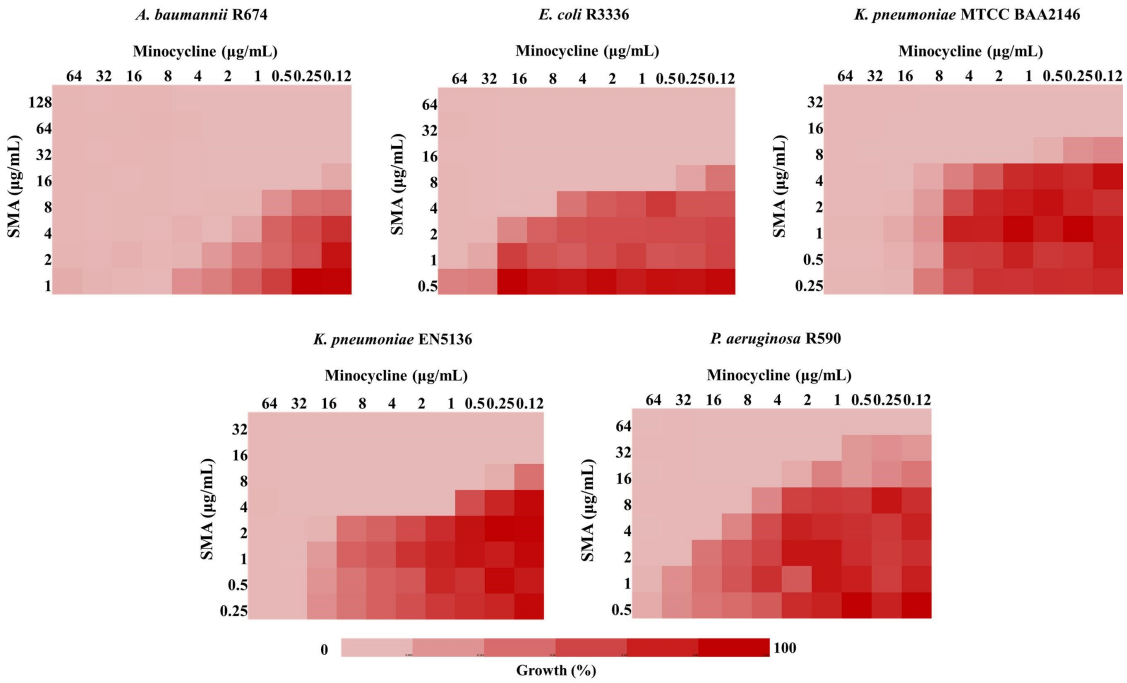


Figure S7. Chequerboard assays of SMA with Minocycline against multi-drug resistant Gram-negative pathogens

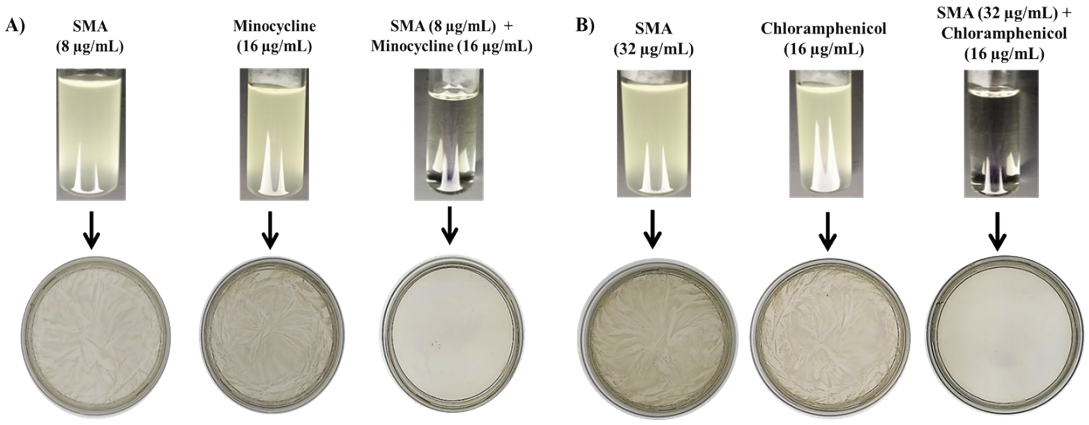


Figure S8. Visual turbidity test of the combination of **A)** SMA and Minocyline against *K. pneumoniae* R3934; **B)** SMA and Chloramphenicol against *K. pneumoniae* R3934.

Compound	Minimum Inhibitory Concentration (µg/mL)			
	[a]MRSA ATCC33591	MRSA R3889	[b]VRSA-1	VRSA-4
SMA	1-2	1-2	2	2
Vanocomycin	0.5	1	512	512

Table S1. Antibacterial activity of SMA against *Staphylococcus aureus.* [a]Methicillin-resistant *S. aureus*; [b]Vancomycin-resistant *S. aureus*.