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ARTICLE

Identification of benzochromene derivatives as a highly specific NorA efflux pump inhibitor to mitigate drug resistant strains of *S. aureus*.

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Increased expression of efflux transport proteins confer Multi Drug Resistant (MDR) phenotype to drug resistant bacteria, which can be mitigated by Efflux pump Inhibitors (EPI). EPI's have a dual advantage of restoring efficacy of antibiotic and retarding evolution of drug resistant mutants. In this study, 17 heterocyclic derivatives synthesized by Polyethylenimine (PEI) catalyzed one-pot protocol were evaluated for its EPI potential. Based on *in silico* studies and *in vitro* studies, 5 benzochromene(BC) based compounds (among 17 heterocyclic derivatives), were observed to significantly potentiate the effect of ciprofloxacin (CPX) and displayed a best modulation factor 32, against NorA overexpressed mutant SA-1199B. Toxicity analyses using zebrafish model showed that, of the benzochromene compounds evaluated, BC9 exhibited low toxicity. BC9 inhibited NorA efflux pump at a Minimum effective concentration (MEC) of 2µg/ml (4.03 µM) and even at subinhibitory concentrations, it was effective in reversing CPX MIC of both ATCC strain and clinical isolate of MRSA by 128 and 4 fold respectively. Our results show that benzochromene derivative BC9 is a highly specific NorA inhibitor that can be employed to mitigate MDR strains of *S.aureus* that overexpresses NorA efflux pump.

A Introduction

Multi drug resistant (MDR) bacterial strains render antimicrobial agents ineffective and severely hamper effective treatment which increases mortality and co morbidity. Hence, World Health Organization (WHO) announced that MDR strains are a great threat to public health. Among infections caused by MDR strains, MRSA accounts for 80,461 invasive infections annually in USA alone.[1] Increased prevalence of multi-drug resistance pathogens catalyzed by horizontal gene transfer[2] and fuelled by indiscriminate antibiotic use, cannot be contained by the limited number of available antibiotics. Above all due to evolutionary selection pressures, microbes quickly develop resistance to even drugs like vancomycin[3] and lipopeptide daptomycin[4] which were once considered to abrogate evolution of resistant mutants. In such a scenario, it is imperative to urgently resort to alternate strategies to curtail MDR bacteria. Hence of late, research focus has shifted from anti-bacterial agents to compounds that target drug resistance mechanisms like beta lactamase inhibitors,[5] efflux pump inhibitors[6] quorum sensing inhibitors, peptidomimetics, phage therapies etc.[7] Very recently both lytic and lysogenic phages have been engineered with CRISPR Cas system to selectively target antibiotic resistant bacteria.[8]

Drug resistance in microbial pathogens arises due to various mechanisms like enzymatic inactivation, mutation of the target site, decreased penetration or increased efflux,[9] among these, extrusion of diverse classes of structurally unrelated drugs through the efflux pumps seems to be one of the favoured mechanisms adopted by many pathogens which is evident from the analysis of clinical isolates.[6,10,11] A recent study has shown that efflux serves as a primary mechanism to withstand antimicrobial stress and confers resistance towards fluoroquinolones and biocides in *Staphylococcus aureus.*[12] The ability of gram positive bacterium like *S. aureus* to thrive in gut was recently attributed to the presence of MnhF efflux pump that expels bile salts.[13] Various reports have shown that efflux pumps play an important role in virulence and lack of or defect in efflux pump is closely associated with attenuated virulence.[14,15] By targeting efflux, EPI's impart new lease of life

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to the existing array of antibiotic molecules that were initially rendered ineffective by MDR bacteria. By virtue of its ability to increase intracellular drug concentration, EPI can resensitize MDR bacteria to existing antibiotics and are likely to decrease the propensity of emergence of drug resistant mutants.[16]

Methicillin resistant *Staphylococcus aureus* (MRSA) in the form of Health Care Associated Community Onset MRSA (HACO-MRSA), Heath care onset MRSA (HA-MRSA) and Community acquired MRSA (CA-MRSA) continues to be leading cause of nosocomial infections in USA despite a substantial decrease in HACO-MRSA and HA-MRSA infections in 2011 relative to 2005.[1] Estimates show that invasive MRSA infections account for 18,650 deaths annually.[17] MDR phenotype due to efflux in gram positive bacterial pathogens is predominantly conferred by Major Facilitator Superfamily (MFS) of efflux pumps comprised of either 12 or 14 transmembrane segments. NorA, NorB, MdeA and Tet38 are examples of MFS pumps in *S. aureus* of which; NorA has been widely studied.[18-20] Sampling of MRSA from different geographical locations revealed that these strains typically upregulate NorA efflux pumps and exhibit spat002 genotype.[21] Many instances of treatment failure in MRSA infections was attributed to the poor bactericidal activity of vancomycin,[22] hence enhancing bactericidal activity of antimicrobial agents like ciprofloxacin (CPX) by combination therapy with putative EPI's, will help in better therapeutic management of MRSA infections.

NorA functions as a proton mediated antiporter and uses diverse structurally unrelated molecules including fluoroquinolones,[23] biocides and dyes as its substrates. Diverse classes of molecules including plant metabolites reserpine,[24] piperine,[25,26a] capsaicin,[26] flavones,[27] flavonolignans,[28] heterocyclic organic compounds phenothiazines and thioxanthines[29] piperazinyl linked fluoroquinolones[30] and even biogenic metal nanoparticles[31] were earlier identified as NorA/efflux inhibitors in *S. aureus*. The structural diversity of molecules that serve as NorA substrates/inhibitors reveal its broad specificity and implies that NorA should bind their substrates through hydrophobic and electrostatic interactions rather than a precise network of non-covalent interactions as suggested earlier.[32,33] Broad substrate specificity in turn affords credibility for screening novel classes of inhibitors. In addition, many of the molecules reported earlier were not taken up further due to their toxicity and/or less potency to employ them clinically as observed with reserpine.[34] Thus there is need for potent NorA inhibitors with lower inherent toxicity and better minimum effective concentration (MEC) values.

Benzochromene derivatives have been identified to have pronounced impact on multi-drug resistant tumor cells.[35] In general, multi-drug resistance mechanism in cancer cells were circumvented by shutting-off ATP dependant efflux pumps. Hence, it is of interest to investigate the efflux pump inhibitory activity of benzochromene derivatives in a highly drug resistant *S. aureus* model strains.

Benzochromene derivatives can be synthesized by low molecular weight triethylamine catalysed reaction under sonication or reflux conditions.[36] The previously reported amine catalysts have numerous disadvantages such as difficulty in isolation of catalyst from the reaction medium, toxic and volatile nature, difficulty in tuning the catalyst properties etc. Hence, we are also evaluating hyperbranched polyethylenimine (PEI) as new catalyst to synthesise benzochromene derivatives. PEI shown in Figure 1 is non-toxic, hydrophilic, less-volatile highly branched polymeric amine derivative with good catalytic potential. Moreover, the PEI catalyst displayed good stability towards air and moisture and its properties can be altered favourably by functionalizing the amine groups.

Herein, we report hyperbranched PEI catalyzed novel benzochromene derivatives by facile one-pot synthesis and its subsequent evaluation as a NorA efflux pump inhibitor in *S. aureus* relative to reserpine using *in silico* & *in vitro* methods. Mutation prevention concentration and mutation frequency were used to assess ability of putative EPI to resist generation of drug resistant mutants. Membrane permeability and membrane integrity studies were used to discern mechanism of action of non-toxic putative EPI. Finally as a proof of concept, the selected non-toxic EPI were evaluated to potentiate the effect of CPX against ATCC culture of MRSA and a clinical isolate of MRSA. We have employed both cell culture based and zebrafish based toxicity testing to evaluate the toxicity of the selected molecules.

B Results and Discussion

Chemistry

The PEI with number average molecular weight (M_n) 1200 was used for our synthesis. The number of primary, secondary and tertiary amine groups (NH_2, NH and N) present in the PEI was calculated from the inverted gate ^{13}C-NMR spectroscopy. The PEI was converted to the corresponding secondary PEI derivatives (PEI-Bz) and tertiary PEI derivatives (PEI-Me) by closely following the method previously developed in our laboratory.[37] The structure of the catalyst was given in Figure 1. Representative synthesis of benzochromene derivative was given in Figure 1. The detailed information on characterization of PEI and synthesis of PEI derivatives were given in the supplementary information.

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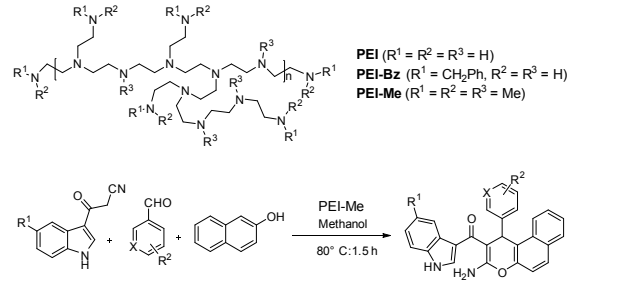

R^1 = H; X = CH; R^2 = 4-Cl (**BC1**); 4-Br (**BC2**); 3-NO_2 (**BC3**); 2,4-dichloro (**BC4**); 3-OMe (**BC5**)
R^1 = Br; X = CH; R^2 = 4-(2-naphthyl) (**BC6**); 4-Cl (**BC7**); 2,4-dichloro (**BC8**); 3-N (**BC9**)
R^1 = Br; X = N; R^2 = H (**BC9**)

Figure 1. PEI catalysed synthesis of benzochromene derivatives.

Among the three molecules, PEI-Bz was identified as suitable catalyst for the synthesis of pyrazole (PZ), pyranopyrazole (PP), and cyclic ketone (CK) derivatives and PEI-Me for the synthesis of benzochromene (BC) derivatives. The catalytic efficiency of PEI-Me in BC1 synthesis was found to be superior to the conventional organic and inorganic bases (Ex: Piperidine, DBU, pyridine, K_2CO_3 etc.). The detailed analyses of the results were given in of the supporting information (Table S1). PEI derivatives readily sequester acidic protons from active methylene group substrates and thereby facilitate the facile formation of PZ and CK in good yields. Similarly, three-component reaction of active methylene group substrates, aldehydes with malononitrile or 2-naphthol yielded PP and BC respectively. After completion of reaction, the catalyst was easily isolated from the reaction medium by filtration and was reused up to five times without significant loss in catalytic activity. The detailed information on screening of the reaction conditions, title compounds synthesis, recyclability studies on representative compounds were given in the supplementary information. The synthesized target molecules are given in figure 2.

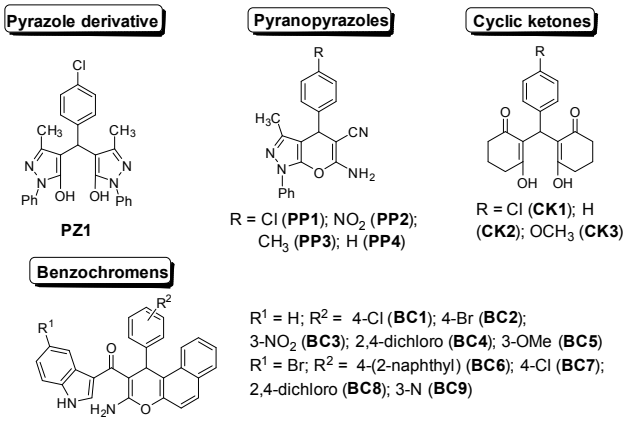

Figure 2. Synthesized target molecules by polyamine catalysis

Evaluation of EPI effect

All the 17 derivatives synthesized belonging to 4 different series viz., pyrazole (PZ), pyranopyrazole (PP), benzochromene (BC), and cyclic ketone (CK) were screened for inhibitory activity against efflux pump NorA by virtual screening. Earlier report showed that 49 % (151/309) of clinical *S. aureus* strains including MRSA typically overexpress NorA efflux pump[38] hence, targeting NorA efflux pump could effectively target MRSA strain of *S. aureus*. Since crystal structure of NorA, an efflux pump belonging to MFS family has not yet been determined, *in silico* screening was performed using model generated by homology modelling. The structure was modelled using its closest resembling homologue MFS (PDB ID: 3WDO). Based on *in silico* screening results, top 6 compounds (binding energy higher -9.5 kcal/mol) and 3 compounds with low binding energy (for validating the docking results) were chosen for further *in vitro* studies (Table S2). Among the selected compounds, two compounds belonged to cyclic ketone (CK) series, one compound pertained to pyrazole (PZ) series and rest of the five belonged to benzochromene (BC) series. Compounds CK1, BC1 and BC3 binds to one site while compounds BC2 & BC4 bind to a different site about 15 Å away from this. One compound BC9 binds to a third site that is in between to these two sites with a slight overlap to those sites (Figure S1). As proposed earlier,[27] hydrophobic interactions contribute mostly to ligand binding with aromatic π-π interactions as preferred interactions. Compound BC9 forms a hydrogen bond with Ser215 side chain (Figure 3).

Anti-Staphylococcal Effect

While screening the chosen compounds for their EPI effect, it becomes essential that they need to have lower intrinsic anti-bacterial effect since it could interfere with the EPI measurements. Hence, the selected 9 compounds were evaluated for its anti-staphylococcal activity. All compounds displayed an MIC of 64 µg/ml or > 64 µg/ml (Table S3) implying that the all the selected compounds displayed minimal anti-staphylococcal activity.

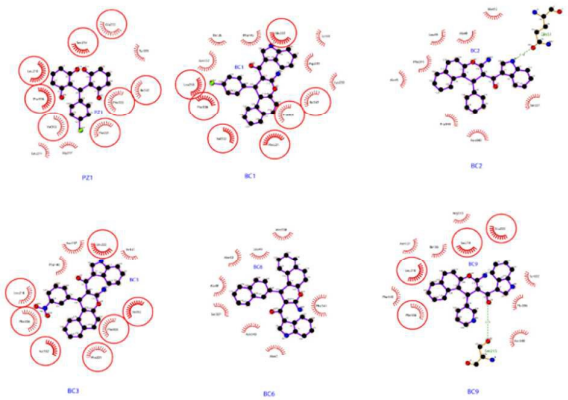

Figure 3. Benzochromene derivative BC9 interacts favorably with NorA homolog.

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Ciprofloxacin MIC reversal

All nine chosen compounds were assessed for its ability to potentiate the antibacterial effect of ciprofloxacin (CPX), fluoroquinolone antibiotic and a known substrate for NorA efflux pump.[23] *S. aureus* strain SA 1199B (NorA ++, A116E Grl A) that overexpressed NorA efflux pump was chosen for this study, apart from overexpression of NorA efflux pump, this strain also carries a mutation in its gyrase gene making it more resistant to CPX. We surmised that if the tested compound(s) could potentiate effect of CPX in pump overexpressed mutant (SA-1199B), it can effectively mitigate other *S. aureus* strains. The selected compounds were evaluated at 16 µg/ml, which is 1/4 of MIC of putative EPIs. Among the nine compounds evaluated, five benzochromene compounds (BC1, BC2, BC3, BC6, BC9) displayed a very high modulation factor (MF) of 32, BC5 displayed a MF of 8. Cyclic ketone (CK1) showed a very low MF of 2 whereas CK2 and pyrazole derivative (PZ1) did not exhibit any change in CPX MIC and hence the MF was 1 (Table 1).

Table 1. Putative EPI's identified by virtual screening potentiated the effect of CPX against NorA overexpressed mutant (SA1199B).

Compound designation	Ciprofloxacin MIC (µg/ml)	Ciprofloxacin MIC in combination with BC 9 (µg/ml)	Fold reduction in Ciprofloxacin MIC (Modulation Factor)
CK1	8	4	2
CK2	8	8	1
BC1	8	0.25	32
BC2	8	0.25	32
BC3	8	0.25	32
BC5	8	1	8
BC6	8	0.25	32
BC9	8	0.25	32
PZ1	8	8	1

Since five out of six BC compounds were highly effective in potentiating antibacterial effect of CPX by 32 fold; it is likely that benzochromene backbone itself contributes to highly specific NorA inhibitory effect. This corroborates with docking results as these compounds have the highest binding energy and, presence of benzochromene group could help in forming the π-π interactions (Figure 3).

Previous report showed that 2- phenylquinone derivatives caused a 16 fold CPX MIC reversal in SA-1199B.[28] Similarly, an earlier study had showed that celecoxib derivative Pyrazolo[4,3c][1,2]benzothiazines 5,5-dioxide also caused a 16 fold MIC reversal for ciprofloxacin in SA-1199B [39] On the other hand with another *S. aureus* strain SA-K2378 that only overexpresses NorA efflux pump, without possessing a mutation in gyrase gene like SA-1199B, a drastic 32 fold reversal was observed.[33]

Toxicity Evaluation

We evaluated toxicity of the highly effective compounds (BC1, BC2, BC3, BC6 and BC9) at a concentration range of 2-64 µg/ml by MTT assay in human pancreatic cell lines. Our results (Figure S2) showed that BC3 was least toxic followed by BC2 and BC6 for the entire concentration range tested. Both BC1 and BC9 were toxic beyond 8 µg/ml (Figure S2). Since testing toxicity in a whole animal model would be a holistic approach relative to cell culture based testing,[40] toxicity of all five chosen compounds were evaluated in zebrafish model, which we had recently adopted for evaluating toxicity and efficiency of putative EPI's. Fishes in groups of 5 were exposed to six heterocyclic ring derivatives at a concentration of 16 µg/ml [8 times its minimum effective concentration (MEC)] the compounds were dissolved in methanol and later dispersed in water and toxicity due to compound was assessed by mortality/ survival of the fishes within the group, 48 h post exposure. Based on mortality based scoring BC9 was observed to be non-toxic whereas rest of the compounds either displayed reduced solubility and or exhibited varying level of toxicity (Table S4). Interestingly BC2 and BC3 that was non-toxic in cell culture testing were observed to be toxic in zebrafish model. Whereas BC1, BC6 and BC9 that displayed varying levels of toxicity were observed to be non-toxic in zebrafish model (Table S4). This could be partly attributed to the metabolism of the compounds by liver enzymes and partly by excretion, which highlights the previous observation that many compounds identified as non-toxic in cell culture fail in clinical trials due to their toxicity in animal models.[40] As BC9 was found to exhibit toxicity beyond 8µg/ml in cell culture but was observed to be non-toxic in zebrafish model, we performed detailed toxicity analysis of liver carboxylesterase and brain acetylcholinesterase activities for BC9 in zebrafish as reported in methods section. As shown in (Figure S3), BC9 did not have any adverse effect on liver α- and β-carboxylesterase activities (Figure S3.1) and brain acetylcholinesterase activities (Figure S3.2) relative to their respective untreated control, implying lack of any significant toxicity to zebrafish exposed to BC9. Despite the fact that BC9 was toxic beyond 8 µg/ml in cell culture, BC9 was chosen further because it was non-toxic in both mortality based scoring and by liver and brain enzyme assays in zebrafish model, which can be further accounted by the fact that cell culture is a static system where the compound does not get eliminated whereas whole animal models are dynamic where the half-life of the compound is determined by its metabolism/excretion from the system. In addition minimum effective concentration (MEC) determined by checkerboard method[42] showed that BC9 had an MEC of 2µg/ml (Corresponding to ~4.03 µM), which is 4 times lower than the concentration at which BC9 might pose toxicity to cultured cells but not in a zebrafish model.

EtBr MIC reversal

As efflux is the only known mechanism of resistance to EtBr[41] reversal of EtBr MIC indicates role of tested compound as an efflux pump inhibitor. Efflux inhibitory potential of the non-toxic benzochromene derivative BC9 was evaluated by EtBr MIC reversal studies in following *S. aureus* strains viz., NorA overexpressed strain (SA-1199B), NorA knock out strain (K-1758) and wild type strain of the same genotype (SA-1199). EtBr displayed an MIC of 64 µg/ml with NorA overexpressed

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strain, 32 µg/ml with wild type and 4 µg/ml with NorA knock out strain. Based on the previous report that EtBr MIC of > 12.5 µg/ml implies increased probability for the overexpression of efflux pump,[21] it is likely that even wild type strain might be upregulating efflux pumps. As expected, BC9 caused increased intracellular accumulation of EtBr and caused a maximal MF of >32 in NorA overexpressed strain followed by MF of 32 in wild type strain and relatively lower MF of >4 in NorA knock out strain (Table 2). By virtue of being more effective with NorA overexpressed strain and least effective with NorA knock out strain, it is evident that BC9 is highly specific inhibitor of NorA efflux pump.

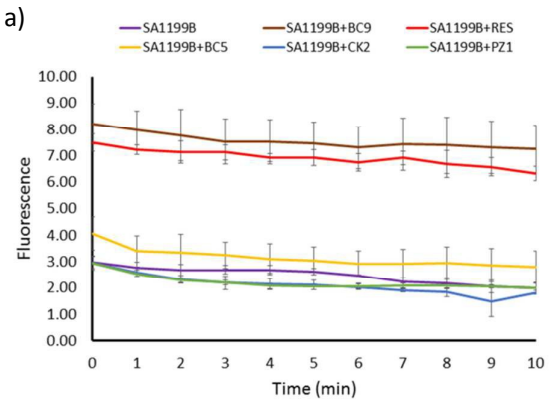
Table 2. Benzochromene derivative BC9 causes maximal reversal of EtBr MIC in NorA overexpressed strain

Strains	EtBr MIC (µg/mL)	EtBr + BC9 MIC (µg/mL)	Modulation Factor
SA1199 (Wild type)	32	1	32
K1758 (NorA knock out)	4	< 1	> 4
SA1199 B (NorA Overexpressed)	64	< 2	> 32

EtBr MIC reversal for Nor A overexpressed strain by CK2, PZ1 and BC5 showed that CK2 and PZ1 caused a 2 fold reversal whereas BC5 caused a 16 fold reversal in EtBr MIC which is in line with *in silico* studies wherein compounds with lower binding energies were on an average less effective in their efflux inhibitory effect relative to those with higher binding energy.

Real Time Efflux

In order to confirm and quantitate the EPI effect of BC9, Real time efflux (RTE) studies were performed with both wild type *S. aureus* (SA-1199) and NorA overexpressed strain of *S. aureus* (SA-1199B). Reserpine a known a NorA inhibitor[24] was used as a positive control. Inhibition of EtBr efflux by the screened compound results in higher residual fluorescence relative to untreated cells. RTE studies show that BC9 causes enhanced



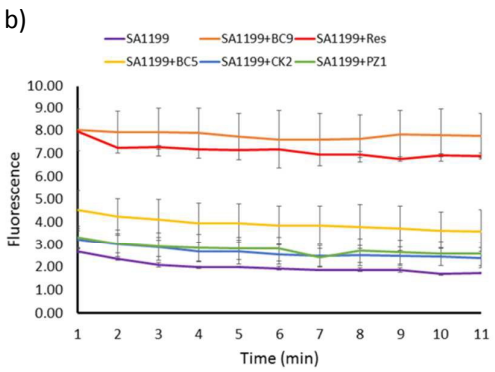
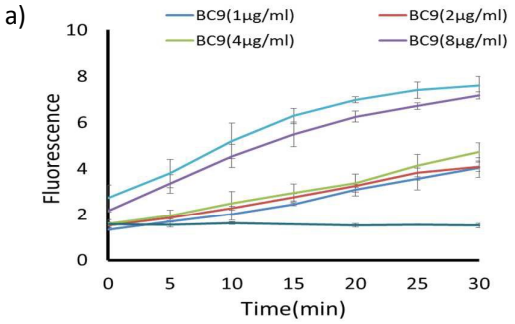


Figure 4: Benzochromene derivative BC9 inhibits EtBr efflux in wild type and NorA overexpressed strain of S. aureus. Residual fluorescence of EtBr in a) NorA overexpressed strain of *S.aureus* (SA1199B) and b) wild type *S. aureus* (SA1199) over the time course of 0-10 min after 1h treatment with BC9,BC5,CK2,PZ1 and reserpine. Data presented is the average from three independent experiments. Error bars represent standard error of the mean. (BC – Benzochromene, CK- cyclic ketone, PZ- Pyrazole and Res – reserpine.)

residual fluorescence both in wild type and NorA overexpressed strain, which was higher than that affected by reserpine (Figure 4). On the other hand, among derivatives with lower binding energy BC5 caused discernible efflux inhibition in both wild type and NorA overexpressed strain but the efflux inhibitory potential of BC5 was much lower than that caused by either reserpine or BC9. Whereas PZ1 and CK2 failed to inhibit efflux in NorA overexpressed strain but in wild type strain a low but perceptible efflux inhibition was noted, implying that both PZ1 and CK2 might inhibit efflux pump other than NorA in *S.aureus.* Thus our RTE studies reveal that BC9 is a better and non- toxic inhibitor of NorA relative to reserpine.

Dose response curve for EtBr accumulation showed that BC9 relative to reserpine caused 2-3 fold increase in EtBr accumulation in the entire concentration range tested (2-16µg/ml), which is 1/32 to 1/4 of its MIC (Figure 5a and 5b). Lower minimum effective concentration (MEC) of 2 µg/ml (4.03µM) displayed by BC9, its enhanced efflux inhibitory effect relative to reserpine and its less toxic nature even at higher concentrations tested (16 µg/ml) in zebrafish model imply therapeutic potential of BC9.



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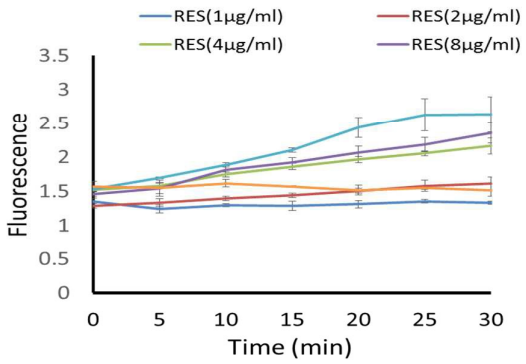

Figure 5: Benzochromene derivative BC9 causes dose dependent accumulation of EtBr in Nor A overexpressed mutant strain of *S.aureus* (SA1199B). a) Dose-dependent uptake of EtBr in NorA overexpressed strain of *S.aureus* (SA-1199B) in the presence of a) BC9 and b) Reserpine (RES). The test compounds have been tested at the following concentrations (1, 2, 4, 8 and 16 µg/ml). The experiments were carried out over 30min of time and the residual fluorescence quantified by spectrofluorimetry with an Ex 530nm and Em 580nm. The error bars represents standard error of the mean from three independent experiments.

Time-Kill studies

Time kill curve studies with NorA overexpressed strain showed that CPX at 4 µg/ml concentration (Half the MIC) did not cause significant bactericidal effect although it prevented further growth for 24 h. However, when CPX (4µg/ml) was used in conjunction with BC9 (8 µg/ml), more than 3 log fold reduction in Colony forming units (CFU) was observed proving that BC9 has synergistic interaction with CPX and potentiates bactericidal effect of CPX. The positive control reserpine (25µg/ml) also potentiated the bactericidal effect of CPX (4µg/ml), however, the potentiation of CPX caused by BC9 was consistently higher than reserpine for the entire time range

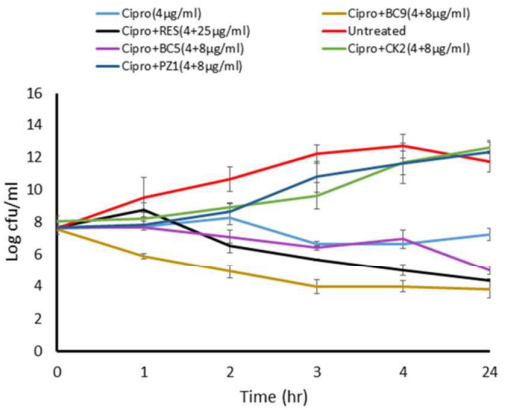

Figure 6: Benzochromene BC9 potentiates the bactericidal effect of ciprofloxacin in NorA overexpressed mutant *S. aureus* (SA-1199B). NorA overexpressed *S.aureus* strain was incubated along with Ciprofloxacin (4µg/ml) and Cipro in combination with various derivatives. Cells were plated at various time intervals on TSA plates and colony counts were estimated after 24h of incubation. (BC- Benzochromene; PZ- Pyrazole; CK- Cyclic ketone)

evaluated even though BC9 was used at 3 fold lower concentration relative to reserpine (Figure 6). As expected, among derivatives with lower binding energy, BC5 was less effective than reserpine but was better than treatment with CPX alone, whereas derivatives CK2 and PZ1 were unable to potentiate the effect of CPX for the entire duration evaluated and in fact by 24h, they displayed CFU equivalent to that of untreated control. Thus based on time-kill studies, increased efficacy displayed by BC9 at a 3 fold lower concentration and its low toxicity in zebrafish model imply that BC9 is a more potent and safe EPI than reserpine.

Synergy testing

Synergistic interactions of BC9 with CPX was determined by checkerboard assay for all three *S. aureus* strains (NorA overexpressed, knock out and wild type), and the results show that BC9 did not exhibit any synergy with both NorA knock out (FIC index =8.00) and wild type strain (FIC Index = 25.03), whereas it displayed synergy with CPX against NorA overexpressed strain (FIC Index = 0.14). BC9 exhibiting synergy only with NorA overexpressed strain but not with NorA knock out and wild type strain reveals that BC9 is highly specific EPI of NorA efflux pump. During synergy testing by checkerboard method we observed that BC9 at its MEC of 2µg/ml (4.03µM) was observed to reduce MIC of CPX in NorA overexpressed strain from 8µg/ml to 1µg/ml which underscores the NorA efflux inhibitory potential of BC9

EPI effect of BC9 against MDR strain

Since BC9 functioned as a highly specific inhibitor of NorA and did not display synergy with NorA knock out strain, we were interested in evaluating if it could indeed curtail MRSA strains. Towards this end, ability of BC9 to cause CPX MIC reversal in an ATCC culture of MRSA and a clinical isolate of MRSA was evaluated. Our results showed that as expected, BC9 at 2X MEC (4 µg/ml) re-sensitized MRSA to CPX and reduced MIC of CPX in ATCC culture by 128 fold (from 32 to 0.25µg/ml) and in clinical isolate by 4 fold (from 128 to 32 µg/ml) proving its utility in a clinical setting to tackle MDR MRSA strains. Although based on our data we can surmise that BC9 would be effective as an EPI with NorA overexpressing strains of MRSA, its efficacy against MRSA *in vivo* and also against strains that overexpresses efflux pumps other than NorA remains to be explored.

MPC and Mutation frequency

Mutation Prevention Concentration (MPC) is the lowest concentration of the compound that fails to give rise to mutants.[43] MPC was tested for CPX alone and CPX in combination with BC9 against wild type *S. aureus* and NorA

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overexpressed mutant (SA-1199B) and we noted that MPC for CPX was at 4 x MIC of CPX, whereas when used in combination with BC9, MPC of CPX was reduced to 2x MIC for both wild type and mutant strains. Similarly, BC9 in combination with 2x MIC of CPX exhibited a significantly lower mutation frequency ($1x\ 10^{-9}$) relative to treatment with CPX alone (8×10^{-6} for the wild type and 2.2×10^{-6} for NorA resistant mutant). By virtue of exhibiting a lower MPC and mutation frequency BC9 as an EPI can restrict the selection of CPX resistant mutants.

Membrane Permeability & Membrane Integrity

Membrane permeability assay was performed to assess whether BC9 affects the membrane permeability/ membrane integrity with propidium iodide (PI) in wild type *S.aureus* (SA-1199) strain as reported earlier.[44] Our results showed that relative to untreated cells, BC9 treatment caused 36% increase in membrane permeability and enhancement in permeability was independent of dose dependent effect of BC9 (Table 3). Thus it is likely that BC9 accesses its target efflux pump NorA by altering bacterial cell membrane permeability.

Table 3: Treatment with BC9 perturbs membrane permeability in *S.aureus*

Treatments	Average Fluorescence Intensity (AU)	Permeability (%)
Untreated (Cells +PI)	2.66 ± 0.13	-
CTAB Treated	5.59 ± 0.23	-
Normalized Fluorescence (CTAB treatment)	2.93	100
BC9 (2µg/ml)	3.77 ± 0.39	37.8
BC9 (4 µg/ml)	3.69 ± 0.21	35.1
BC9 (8 µg/ml)	3.72 ± 0.38	36.2

Membrane integrity studies revealed that BC9 did not cause significant change in extracellular concentration of DNA and protein relative to untreated bacterial cells, whereas treatment with non-ionic detergent (Triton X 100) caused a 6 fold increase in concentration of extracellular protein and nucleic acid relative to treatment with BC9, revealing that membrane integrity is not compromised by BC9.

Experimental

General

All the chemicals were purchased from Sigma Aldrich, Loba chemicals, Merck, Avra synthesis, and SD Fine chemicals and all used without any other further purification. Melting points were taken in the microscopic melting point meter and were uncorrected. Subsequently NMR analyses (H^1 & C^{13}) were determined by a Bruker Av-300MHz spectrometer. All the reactions were conducted to the 10 ML round bottom flask with a magnetic stirrer. Both PEI-Bz and PEI-Me are prepared by previously reported procedure.[37] Hyper branched polyamine with number average molecular weight (M_n) 1200 was chosen for studies. The number of primary (1^o), secondary (2^o), tertiary (3^o) amine group present in the hyperbranched polyamine is calculate based on inverted-gate ^{13}C-NMR spectroscopy.[45]

General procedure for the synthesis of cyclic ketons (CK 1-3) or pyrazole (PZ1): A mixture of substituted aldehyde (1 mmol), 1,3 cyclohexadienone (or) 3-Methyl-1-phenyl-2-pyrazoline-5-one (348.4 mg; 2 mmol) and PEI-Bz polyamine catalyst (100 mg) were taken in the 5 ml of ethanol and it was stirred at RT. The progress of the reaction was monitored through thin layer chromatography (TLC) using ethyl acetate and petroleum ether (3: 7) as eluent mixture. After completion, the reaction mixture was poured in to crushed ice and filtered through the Whatman filter paper. The solid mass was collected and further stirred in hexane (1 x 10 mL) for 10 minutes and filtered through Whatman filter paper. The product thus obtained was essentially pure.

Procedure for recyclability of PEI-Bz on CK1 synthesis

Synthesis of CK1 was carried out as mentioned in the above section. After completion of reaction, the reaction mixture was poured in to crushed ice and filtered through the Whatman filter paper. The combined filtrate was concentrated under reduced pressure and extracted with chloroform (2×15 mL). The combined organic extracts were dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The recycled catalyst was without further purification.

General procedure for the synthesis of pyranopyrazoles (PP 1-4). A mixture of aromatic substituted aldehyde (1 mmol), 3-Methyl-1-phenyl-2-pyrazoline-5-one (174.20 mg, 1mmol), malononitrile(66mg, 1 mmol) and secondary polyamine (50mg) were taken in the 3 ml of ethanol and was stirred at 80°C for 45 min to 1 h. The progress of the reaction was monitored through thin layer chromatography (TLC) using chloroform and methanol (9:1) as eluent mixture. After completion, the reaction mixture was cooled to room temperature and the reaction was quenched with distilled water (5 mL) followed by extracted with ethyl acetate (2×5 mL), washed with anhydrous brine solution and dried over sodium sulphate. The organic layer was filtered and concentrated under reduced pressure. The crude reaction mass further purified through column chromatography (Chloroform: Methanol (9:1)).further concentrated under reduced pressure. The crude reaction mass further purified through column chromatography (Chloroform: Methanol (9:1)).

General procedure for the synthesis of benzochromenes (BC1-9): A mixture of substituted aldehyde (1 mmol), Substituted 3-Cyanoacetyl indole (1 mmol) and 2-naphthol (1 mmol) containing PEI-Me catalyst (50 mg) were taken in the 5 ml of methanol and it was stirred at 80°C in appropriate time. The progress of the reaction was monitored through thin layer chromatography (TLC) using methanol and Chloroform (1:9) as eluent mixture. After completion, the reaction mixture was cooled to room temperature, the solid obtained was filtered through the Whatman filter paper and

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washed with cold ethanol. The product thus obtained was essentially pure.

Antimicrobial studies: Antibacterial effect of the selected benzo chromen derivatives identified by virtual screening were discerned by evaluating its MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) by two fold broth microdilution method as reported earlier.[46] Briefly the compounds were dissolved in DMSO, serially diluted from 0.25µg/ml to 64µg/ml and inoculated with *S.aureus* strains in Mueller-Hinton broth and incubated at 37°C for 18-24h. The lowest concentration of compound which prevented growth (visible turbidity) was assumed to be MIC. Samples (10 µl) from the tubes that lacked visible turbidity were plated on MH agar plates and the concentration that gave rise to 3 or fewer colonies were deemed as MBC.

Synergy testing Checkerboard Analysis: The interactions between non-toxic putative EPI BC9 (benzochromen derivative) and fluoroquinolone antibiotic Ciprofloxacin (CPX) was discerned using checkerboard analysis on a 96-well plate by the standard method reported earlier.[47] One of the components BC9 was diluted along the y-axis and the other CPX was diluted along x-axis. The FIC was calculated according to the equation: FIC index = FIC_A + FIC_B = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/ MIC of drug B alone). An FIC index ≤ 0.5, indicated synergy, an FIC index between 0.5-2.0 indicated additive effect and an FIC ≥ 2.0 indicated antagonism.

MIC reversal: The ability of BC9 to potentiate the effect of CPX was evaluated with the following strains SA1199 (wild-type *S.aureus*), SA1199B (*S.aureus* overexpressing NorA), K1758 (NorA knock out strain of *S.aureus*). BC9 was used at 0.5X MIC along with ciprofloxacin in varying concentrations in cation adjusted Mueller-Hinton broth and incubated at 37°C for 18-24 hrs. The fold-change in MIC of ciprofloxacin due to BC9 in wild type and Nor A mutant

Real-time efflux assay was used to monitor time dependent efflux of EtBr from *S.aureus*. Overnight cultured cells of SA1199, SA1199B and K1758 were re-inoculated into nutrient broth and allowed to reach log phase (0.2-0.3 OD). Cells were harvested by centrifugation and suspended in sterile PBS (phosphate buffer saline pH 7.0) Aliquots were withdrawn and subjected to treatment with 1µg/ml of EtBr and 0.4% glucose along with BC9 at 0.5X MIC for 1 h. Post treatment, cells were harvested, washed twice and re-suspended in PBS. EtBr efflux was assessed by quantitating fluorescence for 10min using JASCO- FP-8200 spectrofluorometer (Ex 530nm and Em 585nm). Reserpine was employed as a positive control.

EtBr efflux assays: SA1199 cells were grown in nutrient broth till they reached an OD of 0.2-0.3. Cells were harvested at 6000rpm for 15min and suspended in PBS. These cells were distributed into replica tubes. BC9 was added in increasing concentrations (10µg/ml- 25µg/ml) along with EtBr at 1µg/ml and 0.4% glucose. Tubes were incubated for 1 h for maximum uptake of EtBr. Post incubation, cells were again harvested and washed twice with PBS to remove any traces of EtBr and resuspended in EtBr free PBS. The rate of EtBr efflux was immediately quantified for over 30min at 5min intervals using Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan) with Ex 530nm and Em 580nm. Reserpine was used as positive control.

Membrane Permeability studies:

PI-CTAB assay: Propidium iodide accumulates only in membrane compromised cells; hence accumulation of PI indicates enhanced membrane permeability. **L**og-phase cells of *S.aureus* suspended in PBS were treated with BC9 and propidium iodide (PI) for 1-2h. Fluoresence due to uptake and binding of PI to DNA can be quantified (Ex 500nm Em 600nm) using a Jasco FP-8200 spectrofluorimeter (Jasco, Tokyo, Japan). Cetyltrimethylammonium bromide (CTAB) was used as a positive control and the change in permeability is expressed as ratio of PI accumulation in CTAB - treated to CTAB-untreated cells.

Membrane Integrity

In order to discern membrane damage, cell membrane integrity was studied in the presence or absence of BC9 as reported earlier.[48]

Briefly, cells after treatment were collected at various time intervals (0-4h), pelleted at 13,250 rcf for 5 min. The release of DNA and proteins due to cell damage caused by BC9 was quantified at 260nm and 280nm respectively. 0.5 % triton X 100 treated cells was used as control.

Homology modeling and Protein Preparation

The Protein sequence NorA of *Staphylococcus aureus* (Accession No: P0A0J7) was retrieved from the UniProtKB database (http://www.uniprot.org/). The 3D protein structure was predicted using MUSTER (Multi-source Protein structure Threader).[49] The predicted structure had best similarity with Crystal Structure of Major facilitator superfamily MFS (PDB ID: 3WDO) having a resolution of 3.15 Å. The Predicted 3D Protein structure was validated using Ramachandran plot and Prosa. The template and target protein structures were studied for their similarity by super imposing using SuperPose Version 1.0. The validated protein structure was subjected to non-Polar hydrogens and Gasteiger charges using Autodock 4.0 (ScrippsResearch Institute, USA).

Ligand Preparation

The energy minimization for the 31 ligands drawn using chemdraw ultra 8.0 was done by Avagadro software and subsequently the ligands were subjected to addition of Gastegier charges through Autodock 4.0.

Molecular Docking Studies:

The grid box was generated by keeping the grid points 101x101x101 in X, Y, Z directions where the target protein (NorA) was covered to let the ligand find its binding pocket in its free state. Docking studies were performed between the target protein and prepared ligands by applying Lamarckian genetic algorithm[50] in Autodock 4.0. The docking protocol was then implemented on the ligands in the dataset. The study was carried for 20 GA runs, which was found to be optimum to reproduce the pose in its crystal. The other GA parameters like the population size and the genetic operators were kept at their default values. The results were interpreted by observing the least binding energy (kcal/mol) and hydrogen bonds; stacking interactions involving between the active site residues of the target and ligand molecules were analyzed using Pymol molecular visualization tool. The RMSD was calculated to ensure that the selected ligands have conformational similarity.

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MTT Assay

Human pancreatic cells (MIA-Pa-Ca-2) were cultured in 96 well plates in the presence of varying concentrations (1-64 µg/ml) of benzochromene compounds (BC1, BC2, BC3, BC6 and BC9). After 24 h of growth MTT was added and after 4h of incubation, MTT was solubilized using DMSO and the absorbance was measured at 590 nm

Fish toxicity studies

Adult zebrafish (*Danio rerio*) irrespective of sex, measuring 4 to 5 cm in length, weighing approx. 300 mg, were purchased from a local aquarium. Animal acclimatization was performed following established protocols.[51] Since the putative EPIs exhibited low antimicrobial activity, the compounds were evaluated at 8X MEC (16 µg/ml). For toxicity evaluation, a total of 5 fish were exposed to various concentrations of the compound for 48 h and mortality of the fish was monitored. The toxicity experiment was repeated twice. The compounds were first prepared as stocks in dimethyl sulfoxide (DMSO) and later mixed directly in water for fish exposure. A separate DMSO alone control was maintained for a similar duration but there were no mortality or significant behavioural changes (data not shown). Water and compounds were renewed every day. Based on the mortality during the course of exposure and the number of fish alive at the end of 48 hours in both the exposures, selected derivatives were chosen for further analyses. The study consisted of three groups: control, DMSO alone and compounds (1 L/tank).

Tissue preparation

To check the effect of selected compounds on select biochemical parameters of zebrafish, a total of 10 fish were exposed to 8mg/L of the respective compounds for 48 h. At the end of exposure (48 h), fish were sacrificed (anesthetized by 150mM MS-222 and euthanized by decapitation), skin removed and the liver from two fish from the same group were pooled and homogenized in ice-cold buffer (Tris-HCl, 0.1M, pH 7.4). The homogenate was centrifuged (10,000 x g, 10 min, 4^{o}C) and supernatant used for all analyses in duplicates. Brain was homogenized for acetylcholinesterase (AChE) assay. From the homogenate prepared from liver or brain pooled from two fishes, duplicates were derived for each assay. Protein was estimated by the method of Lowry et al.[52] Estimation of carboxyl esterase was essentially as described by Argentine and James [53] and acetylcholinesterase (AChE) activity was measured by Edmann's degradation.[54]

Conclusions

In conclusion we have adopted a combination of *in silico* and *in vitro* studies and identified that benzochromene BC9 displayed low intrinsic anti *staphylococcal* activity and highly specific efflux inhibitory effect against NorA pump of *S. aureus*. MIC reversal studies show that even at concentrations as low as ¼ of its MIC, BC9 caused a drastic 32 fold CPX MIC reversal in NorA overexpressed strain SA-1199B. BC9 displayed a low MEC of 2µg/ml corresponding to 4.03 µM. Real time efflux and time kill studies proved that BC9 potentiated effect of CPX better than reserpine and exhibited synergy with CPX in NorA overexpressed strain. Most importantly MPC and mutation frequency showed that BC9 restricted selection of drug resistant mutants. Its hydrophobic nature might explain increased permeability and enhanced binding to NorA exhibited by BC9. As a proof of concept BC9 caused remarkable reversal in CPX MIC in two MRSA strains. Added with its ease of facile one pot synthesis and relatively low toxicity in zebrafish model, BC9 has potential to be considered as lead molecule that specifically targets NorA mediated drug efflux in *S. aureus* and is suitable for evaluating EPI effect *in vivo* in appropriate animal models.

Authors contributions

AG- synthesized compounds; LRC carried out the biological studies; HMV- carried out the docking studies; UV- designed *in silico* studies; TR designed zebra fish studies; VS- designed cell culture studies; KK- contributed for synthesis and characterization; NS and SSG designed biology and chemistry part of the work respectively and NS,SSG, UV and TR wrote the manuscript.

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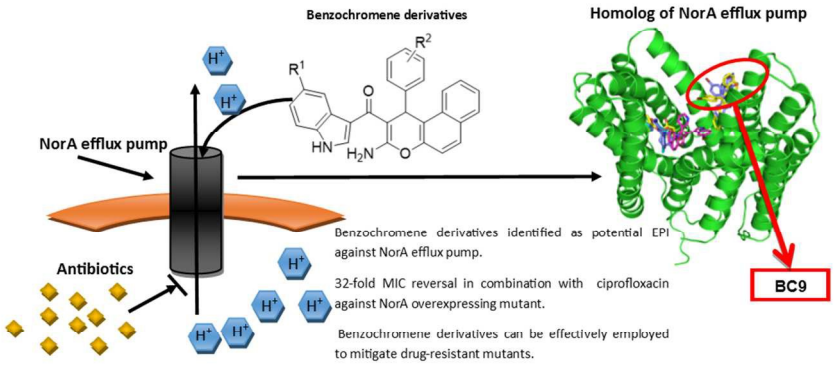

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