A novel bi-functional chalcone inhibits multi-drug resistant *Staphylococcus aureus* and potentiates the activity of fluoroquinolones

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**ABSTRACT**

*Staphylococcus aureus* is the leading cause of bacteremia and the dwindling supply of effective antibacterials has exacerbated the problem of managing infections caused by this bacterium. Isoliquiritigenin (ISL) is a plant flavonoid that displays therapeutic potential against *S. aureus*. The present study identified a novel mannich base derivatives of ISL, IMRG4, active against Vancomycin intermediate *S. aureus* (VISA). IMRG4 damages the bacterial membranes causing membrane depolarization and permeabilization, as determined by loss of salt tolerance, flow cytometric analysis, propidium iodide and fluorescent microscopy. It reduces the intracellular invasion of HEK-293 cells by *S. aureus* and decreases the staphylococcal load in different organs of infected mice models. In addition to anti-staphylococcal activity, IMRG4 inhibits the multidrug efflux pump, NorA, which was determined by molecular docking and EtBr efflux assays. In combination, IMRG4 significantly reduces the MIC of norfloxacin for clinical strains of *S. aureus* including VISA. Development of resistance against IMRG4 alone and in combination with norfloxacin was low and IMRG4 prolongs the post-antibiotic effect of norfloxacin. These virtues combined with the low toxicity of IMRG4, assessed by MTT assay and haemolysis, makes it an ideal candidate to enter drug development pipeline against *S. aureus*.

**1. Introduction**

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of community-acquired (CA-MRSA) and health care associated (HA-MRSA) infection outbreaks that range from severe to being potentially fatal [1]. MRSA and the recently emerged vancomycin-intermediate and resistant *S. aureus* (VISA and VRSA, respectively) have been listed as 'high-priority' deadly bacterial pathogens by the World Health Organization (WHO) [2]. Despite introduction of numerous drugs for the treatment of staphylococcal infections, the bacterium has been successful to resist the action of almost all classes of antibiotics including the latest classes, linezolid and daptomycin [3]. The mechanisms by which *S. aureus* evades the antibacterial action of antibiotics include, (i) enzymatic drug modification/inactivation, (ii) modification of drug binding site, (iii) acquisition of novel drug resistant target and (iv) over-expression of endogenous efflux pumps [4]. Efflux pumps are integral membrane based transport proteins which are involved in the extrusion of a broad range of structurally diverse antimicrobial agents. This results in reduced intracellular concentration to an ineffective level and subsequent cell survival [5]. Efflux pump transporters belong to five different families namely, Resistance Nodulation Division (RND) family; the Major Facilitator Superfamily (MFS); the ATP Binding Cassette (ABC) superfamily; the Small Multidrug Resistance (SMR) family; and the Multidrug And Toxic compound Extrusion (MATE) family. In Gram-positive bacteria, including *S. aureus*, MFS is the predominant family that utilizes an electrochemical gradient across the bacterial membrane for transporting drugs [6]. The well-studied native MFS efflux pump in *S. aureus*, NorA, is a proton motive force (PMF) dependent pump and is overexpressed in more than 50% of the resistant clinical isolates [7]. NorA confers resistance to a wide range of unrelated substrates such as hydrophilic fluoroquinolones (ciprofloxacin, norfloxacin), biocides (acriflavine, cetrimide, benzalkonium chloride) and dyes (ethidium bromide) [6]. Inhibition of NorA, therefore, is a possible strategy to combat the infections caused by MDR *S. aureus*. Natural plant products like reserpine [8], capsaicin [9], coumarins [10], chalcones [11], Boeravinone B [12] and some synthetic novel derivatives such as 1-(1H-indol-3-yl)ethanamine [13], Dithiazole thiones [14] have been reported as potent NorA efflux pump inhibitors.

Isoliquiritigenin (ISL), a natural chalcone, was first isolated from the root of *Glycyrrhiza glabra* [15]. It has been reported for antimicrobial,
anticancer, antioxidant, and anti-inflammatory activities [16]. Interestingly, the drug resistance reversal potential of ISL was recently reported [15] prompting us to synthesize novel derivatives of ISL using mannich base reaction and evaluate their antimicrobial and drug reversal potential against clinical strains of *S. aureus*. In the present study, we describe the role of a novel chalcone derivative as an antibacterial and an inhibitor of NorA efflux pump capable of rejuvenating the activity of fluoroquinolones.

2. Material and methods

2.1. Chemistry

Melting point was determined on a Toshniwal melting point apparatus. IR spectra were recorded on a Perkin Elmer 1719 FT-IR spectrophotometer. NMR spectra were obtained in CDCl3, acetone-d6, and pyridine-d5 on a Bruker Avance, 500 MHz instrument using TMS as internal standard. The chemical shift values are reported in ppm and coupling constants in Hz. ESI-MS spectra were recorded on a Perkin Elmer Turbo Mass/Shimadzu LC-MS. TLC analyses were carried out on precoated silica gel 60 F254 plates (Merck) using solvent system, hexane:ethyl acetate(1:1). The compounds were identified by their spectral IR, ID, (1H, 13C, DEPT) and 2D(COSY, HSQC, HMBC) ESIMS) NMR and ESIMSanalysis.

**Isoliquiritigenin** was isolated according to the reported procedure [15]. ISL derivatives IMRG1-IMRG6 were synthesized according to the procedure described below (Fig. 1).

**N-Phenyl-N, 1a-dihydro-2H-O, N-Isoliquiritigeninoxazine (IMRG1):** It was obtained by refluxing ISL (0.256 g, 1 mmol), formaldehyde (0.2 ml, 2 mmol) and aniline (0.1 ml, 1 mmol) in methanol at 60°C as a yellow solid (0.217 g, 85%), mp 70–72°C. IR max (neat): 3448 (OH), 1654 (CO), 1602, 1562, 1510, 1366, 1223, 1032, 984 (aromatics), 1284, 1103 (ether) cm−1; 1H, COSY-NMR (300MHz, Acetone-d6): δ 4.63 (2H, s, H2-1a), 5.52 (2H, s, H2-2a), 6.38 (1H, d, J = 8.38 Hz, H-5″), 6.94 (3H, m, H-3′, H-5′, H-6a), 7.18 (2H, dd, J = 8.80, 2.0 Hz, H-4a, H-8a), 7.27 (2H, ddd, J = 8.76, 7.28, 2.16 Hz, H-5a, H-7a), 7.24 (2H, d, J = 8.88 Hz, H-2′, H-6′), 7.75 (1H, d, J = 14.96 Hz, H-2), 7.87 (1H, d, J = 15.32 Hz, H-3), 8.02 (1H, d, J = 9.04 Hz, H-6), 1H, COSY-NMR (100 MHz, Acetone-d6): δ 4.06 (C-1a), 80.68 (C-2a), 109.04 (C-5″), 109.66 (C-3″, q), 114.21 (C-1″, q), 116.81 (C-4a, C-8a), 117.97 (C-2′, C-6′), 122.18 (C-6a), 127.44 (C-1′, q), 130.40 (C-5a, C-7a), 130.15 (C-6′), 131.90 (C-2′, C-6′), 145.59 (C-3), 149.07 (C-3a, q), 161.18 (C-4′, q), 161.81 (C-2″, q), 163.29 (C-4″, q), 193.28 (C-1, q); ESI-MS (positive) (m/z): 374 [M+H]+, molecular formula C23H19O4N.

**N-(p-Methoxyphenyl)-N, 1a-dihydro-2H-O, N-Isoliquiritigeninoxazine (IMRG2):** It was obtained by the reaction of ISL (0.256 g, 1 mmol), formaldehyde (0.2 ml, 2 mmol) and p-anisidine (0.107 g, 1 mmol) in methanol at 60°C as a yellow solid (0.217 g, 85%), mp 70–72°C. IR max (neat): 3367 (OH), 1654 (CO), 1590, 1545, 1460, 1043 (aromatics), 1113 (ether) cm−1; 1H, COSY-NMR (300 MHz, Acetone-d6): δ 2.27 (3H, s, CH3), 4.52 (2H, s, H2-1a), 5.45 (1H, s, H2-2a), 6.31 (1H, d, J = 8.7 Hz, H-5″), 6.65 (2H, d, J = 8.4, H-3′, H-5′), 7.02 (5H, m, H-2a, H-4a, H-5a, H-7a, H-8a), 7.54 (1H, d, J = 16.20 Hz, H-3), 7.80 (2H, d, J = 8.4 Hz, H-2′, H-6′), 8.01 (1H, d, J = 8.4 Hz, H-5″), 14.03 (1H, s, 4′-OH); 13C, DEPT-NMR (75 MHz, Acetone-d6): δ 20.03 (CH3), 45.78 (C-1a), 54.79 (OCH3), 80.94 (C-2a), 108.56 (C-5″), 109.12 (C-3′, q), 114.08 (C-1″, q), 115.31 (C-3′, C-5′), 116.29 (C-2′, C-6′), 118.76, 119.05 (C-4a, C-8a), 126.01 (C-1′, q), 129.96 (C-6a, q), 130.03 (C-5a, C-7a, C-2″, C-6″), 131.39 (C-6′), 144.31 (C-3), 152.12 (C-3a, q), 160.05 (C-4′), 162.34 (C-2′), 166.02 (C-4″), 192.10 (C-1, q); ESI-MS (positive) (m/z): 388 [M+H]+, molecular formula C24H21O4N.

![Fig. 1. Synthesis scheme and molecular structures of novel synthetic chalcone derivatives of ISL.](image-url)
2.3. In vitro antimicrobial susceptibility assay

Minimum inhibitory concentration (MIC) of ISL and its novel derivatives was determined by the broth microdilution method as per CLSI guidelines [18].

2.4. Time-kill studies

Time-kill kinetic study was performed to assess the bactericidal potential and synergistic interaction between most active derivative (IMRG4) and norfloxacin against S. aureus (SA-1199B, VISTA-STI745) as described earlier [19–21]. The criterion for a bactericidal effect was ≥3-log10 decrease in CFU count at a specified time while, a decline of less than 3-log10 CFU/ml was interpreted as bacteriostatic activity.

2.5. Salt tolerance assay

The salt tolerance of VISTA-STI745 was assessed in presence and absence of IMRG4 by observing the bacterial growth in agar medium supplemented with NaCl [22]. Detailed methodology can be found in Supplementary material SM1.

2.6. Cytoplasmic membrane depolarization assay

Fluorescent membrane-potential sensitive cyanine dye 3,3-dipropylthiacyanine [DiSC3(5)] was used to study membrane depolarization by IMRG4 [23]. Detailed methodology is mentioned in Supplementary material SM2.

2.7. Membrane permeabilization assay

The ability of IMRG4 to permeabilize the bacterial membrane was examined by PI uptake assay as per method described earlier and detailed in Supplementary material SM3 [24]. Microscopic determination of membrane integrity was made by using the LIVE/DEAD BacLight assay kit as per the manufacturer instructions.

2.8. Fluorescence microscopy

The mid log culture of VISTA-STI745 cells (OD600 = 0.6) were treated with 4× MIC of IMRG4, centrifuged and stained with 0.5-1 μg/ml N-(3-tribromomethylpropyl)-4-(p-diethylaminomethyl-hexa-triaryl) pyridinium dibromide (FM4–64, Molecular Probes). Samples were spotted on 2% agarose pads for imaging. Images were captured using a Zeiss Scope A1 fluorescence microscope, using the FM4–64 fluorescent protein filter (Nikon, Tokyo, Japan) as per method described earlier [25].

2.9. Uptake of fluorescently labelled vancomycin

The mid log culture of VISTA-STI745 cells (OD600 = 0.6) was treated with 50 mg/L IMRG4 (4× MIC). After 4 h, treated cells as well as untreated control cells were harvested by centrifugation, washed thrice and resuspended in PBS. Cells were incubated with BODIPY-vancomycin (0.5× MIC) for 30 min at room temperature. After incubation, the cells were washed again with PBS to remove unbound BODIPY-vancomycin and resuspended in fresh PBS. The localization of BODIPY-vancomycin was analyzed by fluorescence microscopy using a green fluorescent protein filter (Nikon, Tokyo, Japan) as per method described by earlier [26].
2.10. Invasion assay

To ability of *S. aureus* cells (SA-1199B, VISA-ST1745 and SA-K1758) to invade HEK 293 (Human embryonic kidney cell lines) cells was assessed in presence and absence of IMRG4 as described earlier [9]. The details of the procedure are mentioned in Supplementary material SM4.

2.11. In vivo efficacy of IMRG4 in mice

The therapeutic efficacy of IMRG4 was evaluated through oral administration using Swiss albino mice model of *S. aureus* infection as described earlier [27]. Supplementary material SM5 contains the detailed methodology.

2.12. Checkerboard titration assay

The fractional inhibitory concentration (FICs) of ISL derivatives with different antibiotics was evaluated by modified broth checkerboard method [19,20]. The modified methodology has been detailed in Supplementary material SM6.

2.13. Computational assessment of NorA-IMRG4 interaction

The NorA sequence was used to prepare a 3D model and the using information of other known inhibitors of NorA, docking studies were performed to study interaction of IMRG4 with NorA. The detailed methodology has been given in Supplementary material SM7.

2.14. Ethidium bromide accumulation and efflux assay

Inhibition of NorA efflux pump by IMRG4 was studied by spectrofluorometric and flow cytometric determination of ethidium bromide accumulation and efflux assay previously described [28]. The methodology has been detailed in Supplementary material SM8.

2.15. In vitro ciprofloxacin/H⁺ antiport assay

Everted membrane vesicles were prepared from NorA overexpressing cells (SA-1199B) as described previously with some modifications [29]. Ciprofloxacin/H⁺ antiport activity was measured by the quinacrine fluorescence quenching method. The methodology has been detailed in Supplementary material SM9 and SM10.

2.16. Resistance development studies

The propensity of bacteria (VISA-ST1745) to develop resistance against IMRG4 alone and in combination with norfloxacin was evaluated as described earlier [30]. The methodology has been detailed in Supplementary material SM11.

2.17. Post antibiotic effect (PAE)

PAE was determined as described previously [29] and is detailed in Supplementary material SM 12.

2.18. Toxicity studies

The toxicity of IMRG4 was determined by haemolysis and MTT assay using HEK293 cell lines [29,31]. Detailed methodology has been given in Supplementary material SM13 and SM14 respectively.

3. Results

3.1. IMRG4, an ISL derivative, has potent anti-staphylococcal activity

Six novel ISL derivatives (IMRG1-6) were synthesized by Mannich base reaction method (Fig. 1). The antibacterial activity of ISL and its novel derivatives was assessed against different clinical strains of *S. aureus* (Table 1).

Among the six derivatives, IMRG4 [N-(5a-chloro, 8a-triflouroanyl)-benzyl-N, 1a-dihydro-2H-O, N-iso liquiritigeninoxazine] exhibited significant antibacterial activity against all the tested strain of *S. aureus* with MIC values ranging from 25 to 50 mg/L. Since VISA-ST1754 displayed a wider antibiotic resistance profile among all the clinical isolates, it was used in further experiments (Supplementary Table S1). Time-kill assay, using the strain VISA-ST1745, showed that at MIC concentration, IMRG4 causes a 2-log reduction in the bacterial viability (in terms of bacterial CFU/ml) for upto 24 h. At higher concentrations of 2× and 4× MIC, as high as 4-log reduction in CFU/ml was observed after 24 h of incubation (Fig. 2a).

3.2. IMRG4 perturbs cytoplasmic membrane and causes membrane permeabilization

Preliminary assays were carried out to determine a probable mode of action of IMRG4. A significant reduction in the viability of *S. aureus* was found when the cells were grown in medium supplemented with 7.5% NaCl in the presence of IMRG4 (Fig. 2b). There was a mean 4-log reduction in bacterial salt tolerance (in terms of bacterial growth after 24 h in CFU/ml) when IMRG4 was added to the medium at MIC and 0.5× MIC concentrations. Since salt tolerance is a function of bacterial cell membrane, this observation hinted at damage to the membrane. Treatment of bacterial cells with IMRG4 resulted in an increased fluorescence of a cationic dye, DiSC3(5), similar to that in case of a known energy uncoupler, CCCP (Fig. 2c, d). In addition to membrane damage, this observation indicated dissipation of the bacterial membrane potential (ΔΨ) by IMRG4. Further fluorescent microscopic assays using propidium iodide (PI) showed an increased fluorescence and concomitant uptake by the bacterial cells. Untreated cells of *S. aureus* carried a little PI fluorescent signal, suggesting that they had intact and viable cell membranes (Fig. 2e). By contrast, a significant increase in PI fluorescent signal was observed in *S. aureus* cells treated with IMRG4, implicating that the membrane was permeabilized (Fig. 3a). The membrane disruption potential of IMRG4 was later validate by fluorescent microscopic assays using the membrane stain FM4-64. Deformed membranes with small bulge have been observed in IMRG4 treated cells as compared to untreated control cells (Fig. 3b). In addition, an increased in bodipy-vancomycin staining was observed at the septum in IMRG4 treated cells as compared to untreated control cells (Fig. 3c).

3.3. IMRG4 reduces the bacterial adhesion to eukaryotic cells and decreases bacterial load in infected organs in mice

The effect of IMRG4 on *S. aureus* was determined in terms of cellular
invasion and bacterial load in S. aureus infected mice model. The invasiveness of S. aureus cells was examined in the presence of IMRG4 at its subsub-MIC (0.25 × MIC) concentration using HEK-293 cell lines. In the presence of IMRG4 there was more than 1-log reduction in invasiveness of S. aureus (Fig. 4a).

3.4. IMRG4 potentiates the activity of antibiotics when used in combination

The combinations of IMRG4 with different antibiotics and dyes like norfloxacin, ciprofloxacin, tetracycline and EtBr showed profound synergy (Table 2, Supplementary Tables S4–S6).

There was a 4- to 20-fold reduction in MIC of norfloxacin when used in combination with IMRG4 against four clinical isolates, including norA overexpressor (SA-1199B) and knockout strain (SA-K1758) with fractional inhibitory concentration index (FICI) ranging from 0.30 to 0.49 (Supplementary Table S4). The IMRG4/ciprofloxacin combination showed synergistic interaction with 4- to 8-fold reduction against all tested clinical isolates and FICI ranging from 0.18 to 0.50 (Supplementary Table S6). Since IMRG4...
Fig. 3. Permeabilization of VISA-ST1745 membranes by IMRG4. (a) Fluorescence microscopy analysis for viability of bacterial cells using LIVE/DEAD BacLight bacterial viability assay kit. Bacterial cells were exposed to IMRG4 (4 × MIC) for 2 h at 37 °C. Subsequently, the cells were washed with PBS and analysed by LIVE/DEAD BacLight bacterial viability assay kit. Microscopic images of representative IMRG4 treated and untreated bacterial cells were captured using Zeiss Scope A1 microscope. Green fluorescence corresponds to the cells with intact membranes while red fluorescence corresponds to cells with permeabilized/damaged membranes cells. The data was normalized against the drug-free control. (b) Fluorescence microscopy analysis for membrane disruption using the membrane stain FM4-64. Bacterial cells were exposed to IMRG4 (4 × MIC) for 8 h at 37 °C. Subsequently, the cells were washed with PBS and strain with FM4-64 (1 µg/mL). Deformed membranes with small bulge have been observed in IMRG4 treated cells as compared to untreated control cells. Scale bar corresponds to 2 μm. (c) Fluorescence microscopic analysis for localization of BODIPY-vancomycin in VISA-ST1745. Bacterial cells were treated with BODIPY-Van at 0.5 × MIC concentration for 30 min, after incubation, the cells were washed with PBS and again resuspended in the same buffer. An increase in fluorescence was observed indicating the binding of BODIPY-Van only at bacterial cell surface. Bacterial cells were treated with IMRG4 (4 × MIC) for 4 h and were further treated with BODIPY-Van (0.5 × MIC) for another 30 min. After incubation, the cells were washed with PBS and again resuspended in the same buffer. An increase in bodipy-vancomycin staining was observed at the septum in IMRG4 treated cells as compared to untreated control cells (indicated by red arrows) which indicates that vancomycin might be diffuse towards septum (even at 0.5 × MIC) in IMRG4 treated S. aureus cells. Scale bar corresponds to 2 μm.
exhibited up to 20-fold reduction in MIC of norfloxacin, the combination was used in further experiments. Bacterial kill kinetics revealed that IMRG4, in combination with norfloxacin at 0.25× MIC concentration, diminished the viability of SA-1199B (Fig. 5a) and VISA-ST1745 (Fig. 5b) by up to 3-log while at 0.5× MIC concentration, viability was reduced up to 7-log after 24h of incubation. This is equivalent to the efficacy of norfloxacin alone at 4× MIC concentration (Fig. 5a and b).

3.5. IMRG4 inhibits NorA efflux pump

Synergy of IMRG4 with antibiotics that are substrates of an MDR efflux pump, NorA, hinted at interaction between IMRG4 and NorA. The NorA structure was modelled on 1PW4 and 3WD0 PDB structures (Supplementary Fig. S2A). The quality of model was ensured by using Ramachandran plot and the model had 94% residues in the favoured region and no residue in disallowed region (Supplementary Fig. S2B).
Docking studies performed using ciprofloxacin, norfloxacin, reserpine, IN-142, IN-203 and capsaicin on this model suggested that these substrates and inhibitors bind around the central hydrophilic cleft of trans-membrane domain. Our docking analysis suggests that IMRG4 also binds in the same hydrophobic cleft, interacting with Gln51, Val44, Ser337 (hydrogen bonding) Leu17, Ala48, Ala105, Met109 (hydrophobic interactions) with similar energetics like the known inhibitors (Supplementary Fig. S3 and Table S7).

To assess the inhibition of NorA, fluorometric assays using ethidium bromide (EtBr) were carried out. In presence of IMRG4 (0.25× and 0.5× MIC), the accumulation of EtBr increased inside the bacterial cells over expressing NorA (Fig. 6a). A significant shift in the intensity of red fluorescence in the presence of IMRG4 as compared to untreated control was observed (Fig. 6b). The ability of IMRG4 (0.25× and 0.5× MIC) to inhibit the efflux of EtBr from bacterial cells was also evaluated. The NorA expressing cells were loaded with EtBr and the fluorescence was measured in presence and absence of IMRG4, once glucose was added to energize the pump. There was a rapid decrease in the fluorescence of untreated cells due to NorA-mediated EtBr efflux (Fig. 6c). In the presence of IMRG4, however, the loss of fluorescence was significantly reduced, reflecting inhibition of the efflux by IMRG4. No significant shift in the intensity of red fluorescence was observed inside the cells in the presence of IMRG4 (Fig. 6d). A similar effect was also recorded with a known efflux pump inhibitor, reserpine.

3.6. IMRG4 inhibits the drug/H+ antiport movement across the bacterial membrane

NorA pump of S. aureus is driven by the H+ gradient across bacterial membrane. To assess the effect of IMRG4 on drug/ H+ antiport movement across the membrane, a quinacrine-based fluorescence quenching assay was carried out using NorA enriched everted membrane vesicles of SA-1199B cells. The pumps were energized by the addition of lactate, resulting in an influx of H+ ions and decrease in the fluorescence of quinacrine (Fig. 6e). Upon addition of ciprofloxacin, a substrate of NorA, the drug/ H+ antiport caused ciprofloxacin to move inside the vesicles in exchange for H+ ions, resulting in an increase in quinacrine fluorescence. This antiport was inhibited by addition of IMRG4, seen as a sharp increase in fluorescence of quinacrine. Thus, IMRG4 disrupts the proton gradient across the membrane causing an efflux of H+ ions from the everted membrane vesicles.

3.7. IMRG4 enhances the post antibiotic effect (PAE) and delays the appearance of resistance against norfloxacin

Since IMRG4 showed synergistic effect with fluoroquinolones and inhibited fluoroquinolones efflux through NorA, we assessed its utility as a therapeutic agent. Our results suggest that the clinical strains of S. aureus (VISA-ST1745) were not able to develop resistance against IMRG4 alone as well as in combination with norfloxacin even after 20 subsequent passages (Fig. 7a). A 2- to 8-fold increase in the MIC of IMRG4 alone was observed after 12 to 20 subsequent passages. However, in case of norfloxacin alone, upto 512-fold increase in MIC was observed during the same period (Fig. 7a). The post antibiotic effect (PAE) of IMRG4 and norfloxacin alone as well as in combination was determined at 0.5× MIC against the clinical strain VISA-ST1745 (Supplementary Table S8). IMRG4 and norfloxacin alone exhibited PAE of 24 min and 17 min respectively, which further increased up to 64 min in combination.

Cytotoxicity of IMRG4 was determined against human kidney embryonic cell lines (HEK-293). The percentage inhibition of cellular viability in presence of varying concentrations of IMRG4 was determined and the IC50 was calculated to be 281.63 mg/L (Fig. 7b). The IC50 is about 5 times higher than the MIC of IMRG4 against VISA-ST1745. Release of haemoglobin from human erythrocytes (RBCs) exposed to different concentrations of IMRG4 was also assessed to determine its toxicity. IMRG4 displayed no significant haemolytic activity up to 10× MIC concentration (Fig. 7c, Supplementary Fig. S4). In contrast, Triton X-100 completely lysed RBCs (100% haemolysis) significantly at a low concentration of 1% (v/v).

4. Discussion

Emergence and spread of multi-drug resistant clinical isolates of S. aureus represents a severe problem due to lack of effective therapeutic options [32]. Therefore, the development of novel antibacterial pharmacophores, which also delay the emergence of resistance, is required to combat such MDR infections. Plant phytochemicals have, in recent past, displayed their potential as antibacterial as well as drug resistance reversal agents. These phytochemicals frequently act through different mechanisms than conventional antibiotics [33,34]. Chalcones, including isoliquiritigenin (ISL), are one of those many phytochemicals with such reported activities [15,35]. Taking advantage of its properties, we synthesized novel derivatives of ISL using

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**Table 2** In-vitro interaction studies of novel ISL derivative (IMRG 4) with various antibiotics against clinical isolates of S. aureus. (Bold numbers indicate the synergistic interaction of IMRG4 with different antibiotics in terms of Fraction Inhibitory Concentration Index (FICI))

<table>
<thead>
<tr>
<th>S. aureus strain</th>
<th>Norfloxacin</th>
<th>Ciprofloxacin</th>
<th>Tetracycline</th>
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<td>FICI</td>
<td>Fold</td>
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<td>VISA-ST1745</td>
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**Fig. 5.** Time kill kinetic study of norfloxacin in presence and absence of IMRG4. A sub-MIC concentration of norfloxacin with IMRG4 (0.25× MIC (Nor + IMRG4)) shows reduction in bacterial cells [SA-1199B (a), VISA-ST1745 (b)] by up to 3-folds (open squares) which was almost equivalent to norfloxacin (2× MIC, solid squares) treated cells, in comparison to untreated control cells (solid circles). At another sub-MIC concentration, norfloxacin in combination with IMRG4 (0.5× MIC (Nor + IMRG4)) shows bactericidal activity with reduction in growth of bacterial cells [SA-1199B (a), VISA-ST1745 (b)] by up to 7-folds (open triangles), similar to the activity of norfloxacin at 4× MIC (solid triangles). Each point represents the mean of three different observations with error bars representing SD.
mannich base reaction (Fig. 1). Out of the six derivatives, IMRG4 [N-(5a-chloro, 8a-trifluoromethyl)-benzyl-N, 1a-dihydro-2H-O, N-isoliquiritigeninoxazine] displayed a potent antibacterial activity against clinical isolates of S. aureus which included mecA positive MRSA, norA overexpressing strain (SA-1199B) and a norA deletion mutant (SA-K1758) of S. aureus. We determined that one of the strains, MRSA-ST1745, exhibits a vancomycin intermediate resistant phenotype, leading to its new designation as VISA-ST1745. This strain was used in further experimentation due to its broader antibiotic resistance profiling among all the tested clinical isolates of S. aureus (Supplementary Table S2).

From our experiments, it was revealed that IMRG4 acts on the bacterial membrane and its ability to depolarize the membrane was established by the membrane potential-sensitive fluorescent probe DiSC3(5) (Fig. 2). Further evidence of increase in membrane permeability and integrity were provided by microscopic visualization of PI uptake assay and membrane staining dye FM4-64 (Fig. 3a, 3b). Similar observations have been made using membrane active agents like, DNAC-1, SPI031 and DNAC-2 [25,36,37] against S. aureus suggesting that IMRG4 disrupts the bacterial membranes by combined action of membrane depolarization and membrane permeabilization. Several plant flavonoids like artonin I [38], 6,8-diprenyleriodictyol, iso-bavachalcone and 4-hydroxylonchocarpin [39] are also reported to diminish the viability of S. aureus via depolarizing the cytoplasmic membrane. Further, an increased in bodipy-vancomycin staining (even at sub-MIC) at the cell septum of virtually most of the IMRG4 treated cells (Fig. 3c), suggested that IMRG4 might affect the bacterial cell wall. Our observation led us to assume that IMRG4 may somehow also cause the thinning of the bacterial cell wall as BODIPY-vancomycin diffused easily towards septum in IMRG4 treated cells as compared to untreated control cells. However, further studies will be required to investigate the effect of IMRG4 on bacterial cell wall. Our findings are in
Similar findings have been reported earlier [9] with a synthetic branich base derivative diminishing the bacterial growth by a membrane de-polarization mechanism including a combined action of membrane depolarization and proton gradient [43]. IMRG4 affected both ΔΨ and ΔpH which might form the basis of its antibacterial as well as post-antibiotic effect of norfloxacin was delayed in presence of IMRG4 (Fig. 4a) as compared to the knockout strain (SA-K1758), an effect that can be explained by the fact that IMRG4 has a better activity against these strains due to inhibition of NorA efflux pump. Similar observations have been made by Hirakata et al. [42] with diamine compounds which reduces the invasion of *P. aeruginosa* through the invasion of MexAB-OprM pump. In addition to synergistic antibacterial activity with norfloxacin, the selection of resistant mutants as well as post-antibiotic effect of norfloxacin was delayed in presence of IMRG4. These findings, combined with the low toxicity of IMRG4, indicate that this molecule could be an ideal candidate to enter drug development pipeline.

Overall, our results collectively highlight the suitability of IMRG4 alone as well as in combination with fluoroquinolones like norfloxacin as a therapeutic agent for treatment of infections caused by MRSA/VISA. This study, for the first time, has shown that IMRG4 (novel branich base derivative) diminishes the bacterial growth by a mem-branolytic mechanism including a combined action of membrane depolarization and membrane permeabilization. In addition to this IMRG4 can also potentiate the activity of fluoroquinolones by inhibiting the NorA efflux pump.

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Technology (IIT) Roorkee, Roorkee, Uttarakhand, India for human cell lines.

Ethical clearance
The animal experiment protocols (BT/IACUC-2017-20/06) were duly approved by Institutional Animal Ethics Committee on 31-03-2017.

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Conflict of interest
None to declare.

Appendix A. Supplementary material

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