

## ORIGINAL ARTICLE

# Coumarin–benzimidazole hybrids as a potent antimicrobial agent: synthesis and biological evaluation

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Molecular hybridization approach is an emerging tool in drug discovery for designing new pharmacophores with biological activity. A novel, new series of coumarin–benzimidazole hybrids were designed, synthesized and evaluated for their broad spectrum antimicrobial activity. Among all the synthesized molecules, compound (*E*)-3-(2-(1*H*-benzo[*d*]imidazol-1-yl)-1-((4-chlorobenzyl)oxy)imino)ethyl)-2*H*-chromen-2-one showed the most promising broad spectrum antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis* and *Proteus vulgaris*. In addition, it has showed no cytotoxicity and hemolysis at 10 times the MIC concentration. SAR studies indicate that position of the chlorine atom in the hybrid critically determines the antibacterial activity.

*The Journal of Antibiotics* advance online publication, 21 June 2017; doi:10.1038/ja.2017.70

## INTRODUCTION

Despite the significant progress in medicine, treating the infectious diseases remains a serious concern to the clinics due to the emergence of multidrug resistance in most of the pathogens.<sup>1,2</sup> According to World Health Organization (WHO), annually 57 million deaths occur globally due to infectious diseases alone and this number is likely to rise in the coming years due to wide spreading of resistance genes across the bacterial species. Globally, multidrug-resistant pathogens especially the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) pose a great challenge in clinics as they are capable of 'escaping' from the lethal action of existing antimicrobial agents and are able to cause nosocomial infections. In addition to natural acquisition or lateral transfer of antibiotic resistance gene in pathogens, the decrease in new antimicrobials discovery has failed to keep the check on the rise in antibiotic resistance worldwide. It is now accepted in general that lack of new antibiotics will have a serious corollary on the universal public health, security or survival of human race in coming years.

There is an immense and urgent need for a new class of chemical entities with a different mode of action to replace or augment the existing therapeutics. In order to cope up with this problem, various governments as well as agencies such as Infectious Diseases Society of America (IDSA), the European Centre for Disease Prevention and Control (ECDC) and the WHO have started incentives for the research on novel antimicrobial agents

against life-threatening infections caused by the multidrug-resistant pathogens.

It is widely reported that the natural compounds can play a major role as lead structures for the development of synthetic molecules.<sup>3,4</sup> Coumarins (2*H*-chromen-2-one) are naturally occurring compounds with a wide range of biological activities.<sup>5–11</sup> Coumarin-based Novobiocin (**1**, Figure 1) and Clorobiocin (**2**, Figure 1) are identified as potent inhibitors of bacterial DNA gyrase.<sup>12–14</sup> Recently, Li *et al.*<sup>15</sup> have reported coumarin-based inhibitors (**3**, Figure 1) with potent antibacterial activity against multiple ciprofloxacin and methicillin-resistant *S. aureus* strains. Sahoo and Paidshetty<sup>16</sup> reported that the cobalt complexes of 3-aryl-azo-4-hydroxy coumarin analogs have a significant antimicrobial, wound healing and antioxidant properties. Paul *et al.*<sup>17</sup> reported anticancer activity of coumarin–benzimidazole hybrids. Al-Majedy *et al.*<sup>18</sup> has reviewed the previous studies on coumarins with respect to the antimicrobial activity and reported that coumarins analogs exhibited potential antimicrobial activity that is stronger than that of their parent molecules. This work and others have motivated us to design and synthesize new coumarin-based hybrid analogs with enhanced antibacterial activity. Molecular hybridization approach allows integrating two or more pharmacophoric units, possessing different or similar bioactivity, into one molecular scaffold with novel or improved biological activities with respect to the corresponding pharmacophoric units.<sup>19–21</sup> It is often reported that the hybrids offer favorable pharmacokinetics, avoid undesired drawbacks such as side effects and low oral bioavailability, in addition to

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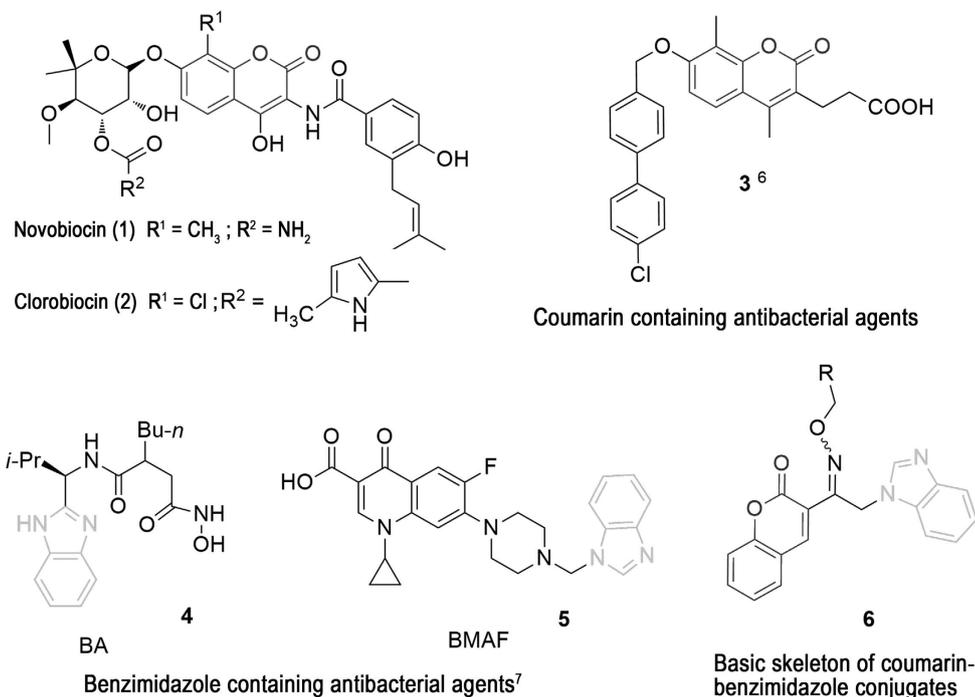
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Received 19 April 2017; revised 11 May 2017; accepted 21 May 2017



**Figure 1** Some potent coumarin, benzimidazole antibacterial compounds and general structure of our synthesized coumarin–benzimidazole conjugates. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

interesting multiple biological activities, high selectivity. These advantages have made hybrid molecules a rationally attractive source for current drug discovery. Benzimidazole is an important heterocyclic pharmacophore structure with a very strong application in the synthesis of drug-like molecules.<sup>22,23</sup> Literature review indicates that many derivatives of benzimidazole have been successfully developed as clinical drugs and have been widely used in the treatment of various diseases. Recent work indicated that benzimidazoles (4 and 5, Figure 1) possess much potential to inhibit the growth of bacteria.<sup>22</sup>

Even though, hybrid approach has given excellent results in the drug discovery program for antimalarial and anticancer agents,<sup>24,25</sup> not much work is done in the area of antimicrobial agents especially coumarin benzimidazoles. These scanty reports regarding the coumarin–benzimidazole hybrids and success stories of hybrid approach lead us to synthesize the novel coumarin–benzimidazole hybrids with special reference to antimicrobial activity.<sup>17,26,27</sup> We here report the synthesis of the coumarin–benzimidazole hybrids as well as their antimicrobial activity, especially against the ESKAPE pathogens.

## RESULTS AND DISCUSSION

A simple and efficient method for the preparation of coumarin–benzimidazole conjugates was developed. The synthesized hybrids were evaluated for the antibacterial activity against ESKAPE pathogens as well as cytotoxicity against eukaryotic cells by using the FDA-approved protocols. We observed that the compound 12 ((*E*)-3-(2-(1*H*-benzo[*d*]imidazol-1-yl)-1-(((4-chlorobenzyl)oxy)imino)ethyl)-2*H*-chromen-2-one) has shown a broad spectrum antimicrobial activity. SAR studies showed that in comparison to the others, halogen containing coumarin–benzimidazole conjugates had a broad spectrum activity over the once, which do not have.

## Chemistry

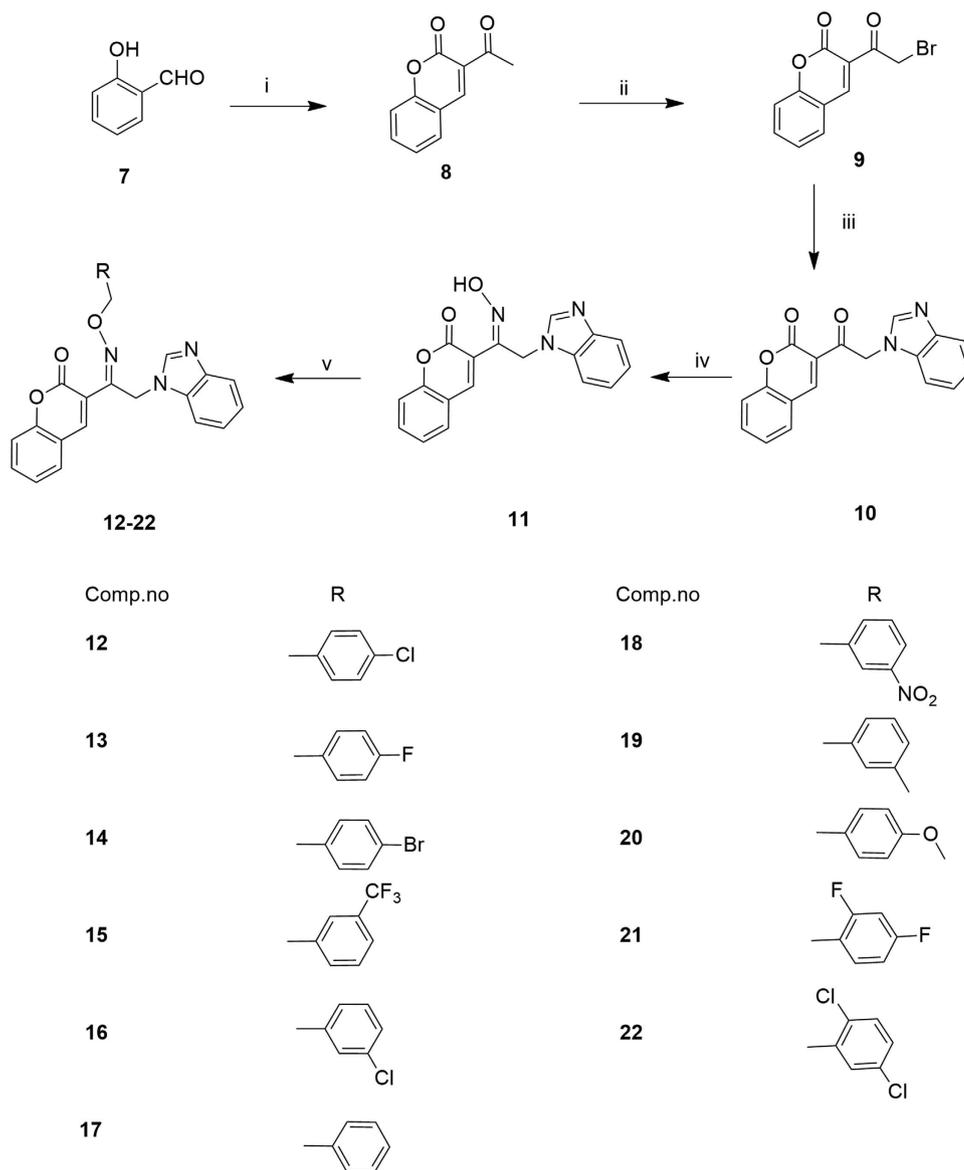
The route followed for the synthesis of coumarin–benzimidazole conjugates is illustrated in Figure 2. The Knoevenagel condensation

between salicylaldehyde and ethyl acetoacetate in the presence of catalytic amount of piperidine gave intermediate 8, which on further bromination in dry chloroform resulted in the formation of compound 9.<sup>28</sup> Further, the nucleophilic substitution on compound 9 with benzimidazole in acetonitrile at room temperature gave compound 10. Subsequently, condensation of compound 10 with hydroxyl amine hydrochloride in ethanol under reflux conditions gave compound 11.<sup>29</sup> Finally, the targeted conjugates (12–22) were achieved by etherification of compound 11 with different substituted benzyl halides in DMSO and potassium *tert*-butoxide as a base. All the new synthesized coumarin–benzimidazole conjugates were characterized using NMR, IR and MS (Supplementary Figure 1). The characterization details were provided in the Supplementary section.

## Antimicrobial studies

In this present study, 11 novel coumarin–benzimidazole conjugates were screened for their *in vitro* antibacterial activity against reported Gram-positive and Gram-negative pathogenic bacterial strains by microdilution method as per CLSI guidelines (formerly known as NCLS).<sup>30–32</sup> MIC values were determined by using ampicillin, kanamycin, tetracycline, ciprofloxacin as a reference. The *in vitro* antibacterial results confirmed that some of the coumarin–benzimidazole conjugates exhibited significant antibacterial activity against two Gram-positive strains such as *B. subtilis* (MTCC 1789), *S. aureus* (ATCC 6538P) and three Gram-negative *P. aeruginosa* (ATCC 25668), *Proteus vulgaris* (ATCC 29905) and *Escherichia coli* (MTCC 739) bacterial strains Table 1.

In the antimicrobial assay, out of 11 compounds, compounds 12 and 14 effectively inhibited the growth of the *B. subtilis* (MTCC 1789) with MIC = 0.95 and 6.25  $\mu\text{g ml}^{-1}$ , respectively, whereas reference drug ampicillin had MIC = 25  $\mu\text{g ml}^{-1}$ . In the case of *P. vulgaris* (ATCC 29905), compounds 14, 17, 21 and 22 displayed strong inhibition at MIC 1.56, 3.12, 1.56 and 1.56  $\mu\text{g ml}^{-1}$  respectively, while



**Figure 2** Synthesis of coumarin–benzimidazole conjugates.

ampicillin inhibited at  $\text{MIC} > 50 \mu\text{g ml}^{-1}$ . Compound **12** showed better antibacterial activity against *P. aeruginosa* (ATCC 25668) with  $\text{MIC} 3.12 \mu\text{g ml}^{-1}$  compared to reference drug ampicillin ( $\text{MIC} = 25 \mu\text{g ml}^{-1}$ ), tetracycline and kanamycin ( $\text{MIC} > 50 \mu\text{g ml}^{-1}$ ). Furthermore, compound **12** showed remarkable activity at  $\text{MIC} 1.56$  and  $3.12 \mu\text{g ml}^{-1}$  against *S. aureus* (ATCC 6538P) and *E. coli* (MTCC 739) bacterial strains, respectively. The structure–antimicrobial activity relationship of the synthesized compounds revealed that compounds **14**, **17**, **21** and **22** inhibited the growth of *P. vulgaris* ATCC 29905. *P. vulgaris* belongs to the Enterobacteriaceae family of Gram-negative bacilli and is often implicated in serious sepsis and urinary tract infections in humans, along with ESKAPE species. Plech *et al.*<sup>33</sup> have reported 1,2,4-triazole-ciprofloxacin hybrids as potential antibacterial agents against drug-susceptible and drug-resistant bacteria. CBR-2092, a rifamycin-quinoline hybrid exhibited potent antibacterial activity.<sup>34</sup> Coumarin thiosemicarbazone hybrid derivatives were found to have antimicrobial activity against *E. coli*.<sup>35</sup> Furthermore, a series of coumarin-thiazole hybrids were reported

as significant antibacterial agents against Gram-positive and Gram-negative bacteria.<sup>36</sup>

Molecular structural analysis of compounds showed that molecule with a halogen atom has antibacterial activity. We observed that the position and number of halogen atoms in the molecule are also having a significant effect on the activity and host spectrum. Coumarin–benzimidazole conjugate with chlorine atom at *para* position (compound **12**) was found to have highest broad spectrum antimicrobial activity. Interestingly, compound with chlorine atom at *meta* or *ortho* position (**16** and **22**) did not show any antibacterial activity. It is widely known that the molecules biophysical properties can also influence the action spectrum of various therapeutic molecules. EL Hage *et al.*<sup>37</sup> has shown that the number of chlorine atoms and their position on the aromatic ring had a notable effect on the antifungal activity in the bifonazole derivatives. Our results are in consistency with the results obtained with halogenated 1, 2-benzothiazine derivatives compounds, where it was demonstrated that compounds with chlorine or bromine atom in the *para* position

have higher antimicrobial activity over the others.<sup>38</sup> Recently it has been demonstrated that the presence of halogen atoms in molecules increases the target binding ability and also stabilizes the ligand–target complex.<sup>31</sup> Our observation including others clearly indicates that the position of the chlorine atom is very much important for antibacterial activity.

### Hemolysis study

Hemolysis assay is a simple, robust and high throughput assay to detect the toxicity of the compounds. To investigate the toxicity profile, we selected compound **12** as it has shown a broad spectrum antibacterial activity. As these molecules were primarily intended to develop as human therapeutic agents, we tested the toxicity against

human erythrocytes. The hemolysis assay results clearly indicate that the selected compounds are not toxic against human erythrocytes even at 10 times the MIC concentration (Figure 3a). Given this, we believe that this molecule is safe and have the potential to become a lead molecule.

### Viability assay

Benzimidazoles are structural homolog to purines and their mode of action is by inhibiting the nucleic acid synthesis. As our compound **12** is obtained by the fusion of coumarin and benzimidazole, we tested these molecules in both lactate dehydrogenase (LDH) and MTT assay. We used actively dividing L929 cells (mouse fibroblast cell line) as target cells, LDH assay as cytotoxicity indicator, MTT as an indicator for the cell viability and proliferation. Initially, we exposed the L929 cell lines to the compound **12** at 1× and 10× times the MIC concentration for 24 h and then measured the LDH release from dying cells (Figure 3b) and MTT conversion to formazan by metabolically viable cells (Figure 3c). Our results clearly show that the compound **12** is not toxic to L929 cells and does not induce any morphological changes in the structure of the cell (Figure 4). Some of the compounds (**14**, **16**, **17**, **18**, **21** and **22**) synthesized showed detectable antibacterial activity against Gram-positive bacteria *B. subtilis* and *S. aureus*. However, their activity was found to be weaker as compared to the reference standard. On the basis of these results, *in vitro* cytotoxicity test was not conducted for these compounds. Mantu *et al.*<sup>39</sup> reported that benzimidazole and pyridine hybrids have the anticancer activity. Novel series of 1, 2, 3-triazolo-phenanthrene hybrids synthesized by employing Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) showed *in vitro* cytotoxic against various human cancer cell lines.<sup>40</sup>

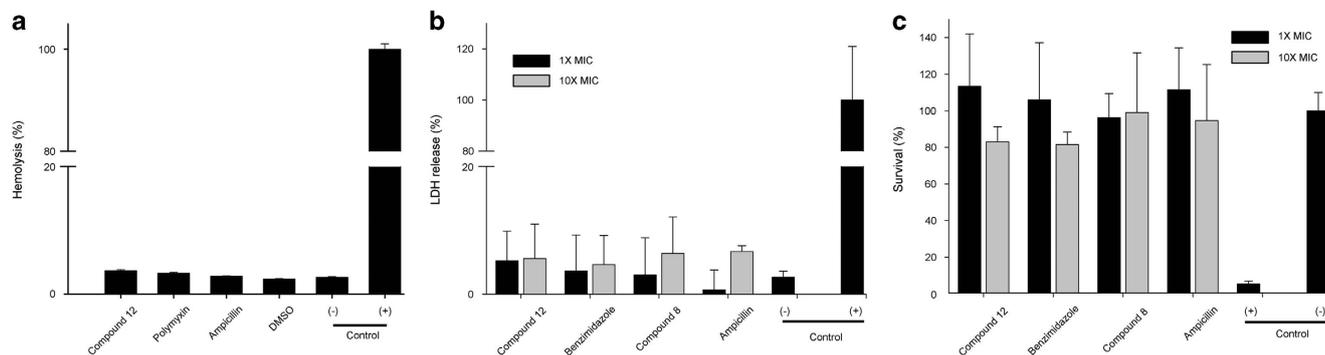
### CONCLUSION

In conclusion, we have designed and synthesized a novel series of coumarin–benzimidazole hybrids and evaluated them for their antibacterial activities. The hybrid compound **12** (*E*)-3-(2-(1*H*-benzo[*d*]imidazol-1-yl)-1-(((4-chlorobenzyl)oxy)imino)ethyl)-2*H*-chromen-2-one displayed significant activity against different Gram-positive and Gram-negative bacterial strains. Although the precise target and mode of action remains unknown, given the robust increase in the list of pathogen acquiring the resistance against antibiotics, we believe that

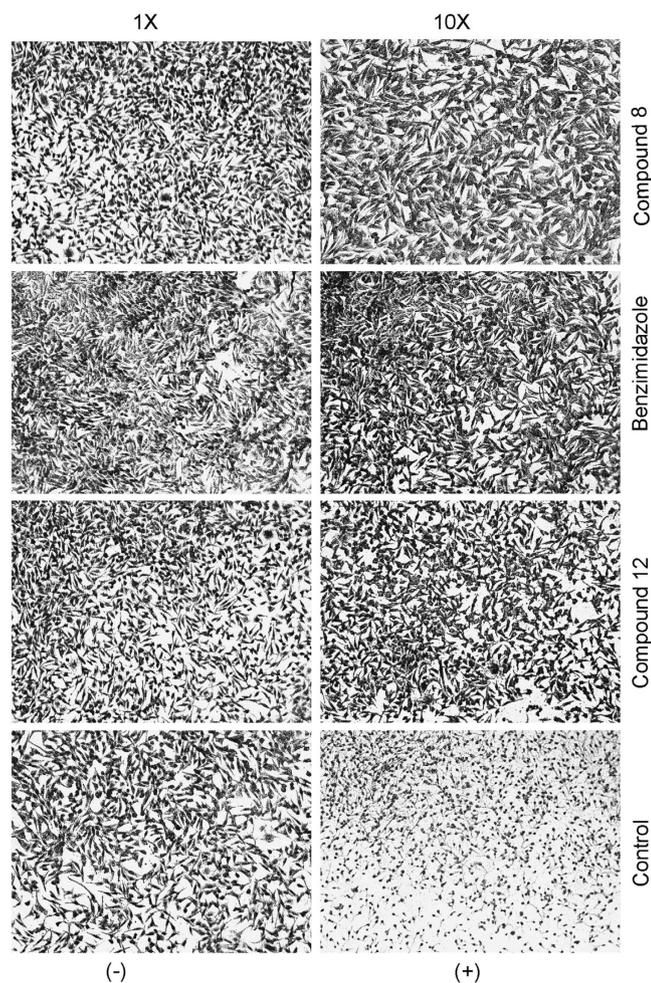
**Table 1** MIC in  $\mu\text{g ml}^{-1}$  against microbes

Compound	MIC in $\mu\text{g ml}^{-1}$ against microbes								
	Gram-positive					Gram-negative			
	I	II	III	IV	V	VI	VII	VIII	IX
<b>12</b>	<b>0.95</b>	>50	>50	<b>1.56</b>	>50	<b>3.12</b>	>50	>50	<b>3.12</b>
<b>13</b>	>50	>50	>50	>50	>50	>50	>50	>50	>50
<b>14</b>	<b>6.25</b>	>50	>50	>50	>50	>50	<b>1.56</b>	>50	>50
<b>15</b>	>50	>50	>50	>50	>50	>50	>50	>50	>50
<b>16</b>	>50	>50	>50	>50	>50	>50	<b>12.25</b>	>50	>50
<b>17</b>	>50	>50	>50	>50	>50	>50	<b>3.12</b>	>50	>50
<b>18</b>	>50	>50	>50	>50	>50	>50	<b>12.25</b>	>50	>50
<b>19</b>	>50	>50	>50	>50	>50	25	>50	>50	>50
<b>20</b>	>50	>50	>50	>50	>50	>50	>50	>50	>50
<b>21</b>	>50	>50	>50	>50	>50	>50	<b>1.56</b>	>50	>50
<b>22</b>	>50	>50	>50	>50	>50	>25	<b>1.56</b>	>50	>50
Ampicillin	25	>50	>50	0.5	>50	25	>50	>50	1.56
Kanamycin	6.25	>50	0.6	1.5	25	>50	3.15	25	3.15
Tetracycline	6.25	3.15	25	12.5	3.15	>50	>50	3.15	6.25
Ciprofloxacin	0.6	1.5	6.25	25	6.25	1.5	1.5	>50	12.5

(I) *Bacillus subtilis* MTCC 1789; (II) *Bacillus cereus* MTCC 1305; (III) *Staphylococcus aureus* ATCC 9144; (IV) *S. aureus* ATCC 6538P; (V) *Staphylococcus epidermidis* ATCC 155; (VI) *Pseudomonas aeruginosa* ATCC 25668; (VII) *Proteus vulgaris* ATCC 29905; (VIII) *Klebsiella pneumoniae* ATCC 29665; (IX) *Escherichia coli* MTCC 739.



**Figure 3** Effect of coumarin–benzimidazole conjugates on mammalian cells: (a) Hemolytic effects of compound **12** were investigated. Erythrocytes were incubated with Compound **12** ( $100 \mu\text{g ml}^{-1}$ ), 2% Triton X-100 (Sigma-Aldrich) served as positive control. The absorbance of hemoglobin release was measured at 540 nm and is expressed as % of Triton X-100-induced hemolysis. (b) and (c) L929 cells were exposed to Compound **12**, coumarin and benzimidazole at indicated concentration for 24 h, and the cell viability was measured by LDH-based kit and MTT assay. Compound **12**, coumarin and benzimidazole did not induce any LDH release nor did they affect viability of the cells. 1% Triton X-100 was used as a positive control (yielding 100% LDH release) and buffer as negative presented as controls were present.



**Figure 4** Effect of coumarin-benzimidazole conjugates on mammalian cells morphology. Effects of compound **12**, coumarin and benzimidazole on the morphology of L929 cell line was investigated by using phase contrast microscopy. Cells were incubated for 24 h at  $\times 1$  (left column) and  $\times 10$  (right column), and stained with Giemsa stain before observing under the phase contrast microscope for the morphological changes. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

these hybrids may serve as promising scaffolds against the treatment of bacterial infections.

## EXPERIMENTAL PROCEDURES

### Analysis and instruments

All reagents were commercially procured from Sigma (Delhi, India) or Spectrochem (Hyderabad, India) India and were used without further purification. Chromatography was carried on silica gel (60–120 and 100–200 mesh). All reactions were monitored by TLC; silica gel plates with fluorescence F254 were used. Melting points were uncorrected. The  $^1\text{H}$  NMR, and  $^{13}\text{C}$  NMR spectra were determined on 200, 300, 50, 75, MHz, respectively, using  $\text{CDCl}_3$  and  $\text{DMSO}-d_6$  as solvents and tetramethylsilane (TMS) as an internal standard. All chemical shifts were given in p.p.m. IR spectra were recorded on in the range of  $500\text{--}4000\text{ cm}^{-1}$  and multiplicity (*s*=singlet, *brs*=broad singlet, *d*=doublet, *brd*=broad doublet, *dd*=double doublet, *t*=triplet, *q*=quartet, *m*=multiplet).

### Synthesis of 3-(1*H*-benzo[*d*]imidazole-1-carbonyl)-2*H*-chromen-2-one (**10**)

The intermediates **8** and **9** were synthesized according to the literature procedure with 92% and 88% yields, respectively.<sup>28</sup> Further, the brominated

compound **9** (2.0 g, 7.5 mmol) was subjected to reaction with benzimidazole (890 mg, 7.5 mmol) in the presence of 30 ml of acetonitrile at room temperature. The reaction mixture was kept stirring for 3 h. Upon complete consumption of reactants, the reaction was quenched with saturated aq.  $\text{NaHCO}_3$  solutions and extracted with 10 ml of ethyl acetate three times. The combined solvent fractions were dried over anhydrous  $\text{Na}_2\text{SO}_4$  and evaporated under reduced pressure. The resulting crude product thus obtained was purified by column chromatography (using 100–200 mesh silica gel and  $\text{MeOH}/\text{CHCl}_3$  as eluent). Subsequently pure compound **10** was obtained. Yellow solid, yield: 71%, that is, 631 mg; mp  $150\text{--}152\text{ }^\circ\text{C}$ ; IR (KBr,  $\text{cm}^{-1}$ ): 3014, 2887, 2359, 1743, 1657, 1402, 1199, 1087;  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  8.84 (s, 1H), 8.19 (s, 1H), 8.04 (d,  $J=7.8$  Hz, 1H), 7.85–7.80 (m, 1H), 7.68 (bs, 1H), 7.57 (d,  $J=8.4$  Hz, 1H), 7.50–7.45 (m, 2H), 7.23–7.21 (m, 2H), 5.89 (s, 2H); ESI-MS ( $m/z$ ): 305  $[\text{M}+\text{H}]^+$ .

### Synthesis of (*E*)-3-((1*H*-benzo[*d*]imidazol-1-yl) (hydroxyimino) methyl)-2*H*-chromen-2-one (**11**)

The reaction of compound **10** (2.5 g, 8.2 mmol) was carried out with hydroxyl amine hydrochloride (655 mg, 9.8 mmol) in the presence of 30 ml of ethanol under refluxing conditions at  $70\text{--}80\text{ }^\circ\text{C}$  for 4 h. On completion of the reaction (monitored by TLC), the excess solvent was removed under reduced pressure. The resulted crude product was purified by column chromatography (using 100–200 mesh silica gel and  $\text{MeOH}/\text{CHCl}_3$  as eluent) to obtain pure compound **11**. Yellow solid, yield: 65%, that is, 1.625 g; mp  $178\text{--}179\text{ }^\circ\text{C}$ ; IR (KBr,  $\text{cm}^{-1}$ ): 3412, 3019, 2889, 2399, 1652, 1405, 1215, 1095;  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ ):  $\delta$  12.35 (s, 1H), 8.10 (s, 1H), 8.05 (s, 1H), 7.68 (d,  $J=7.4$  Hz, 1H), 7.61–7.54 (m, 2H), 7.43 (d,  $J=7.8$  Hz, 1H), 7.36 (d,  $J=8.0$  Hz, 1H), 7.32–7.29 (m, 1H), 7.24–7.20 (m, 1H), 7.16–7.12 (m, 1H), 5.53 (s, 2H); ESI-MS ( $m/z$ ): 320  $[\text{M}+\text{H}]^+$ .

### Synthesis of (*E*)-3-(2-(1*H*-benzo[*d*]imidazol-1-yl)-1-(((4-chlorobenzyl)oxy)imino)ethyl)-2*H*-chromen-2-one (**12**)

Compound **11** (250 mg, 0.78 mmol) was dissolved in 4 ml of DMSO and then potassium *tert*-butoxide (130 mg, 1.1 mmol) was added to it. The resulting reaction mixture was kept stirring at room temperature. After 5 min 4-chlorobenzyl chloride (125 mg, 0.78 mmol) was added and the reaction mixture was kept stirring at room temperature for about 1h. After completion of reaction (monitored by TLC), the crude product was extracted with ethyl acetate. The combined organic layers were dried on anhydrous sodium sulfate, concentrated under reduced pressure and purified by column chromatography (100–200 mesh neutral silica gel) to afford the pure compound **12**. White solid, yield: 67%, that is, 167.5 mg; mp  $130\text{--}132\text{ }^\circ\text{C}$ ; IR (KBr,  $\text{cm}^{-1}$ ): 3021, 2401, 1718, 1616, 1494, 1427, 1215, 1025;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.97 (s, 1H), 7.71 (d,  $J=7.6$  Hz, 1H), 7.67 (s, 1H), 7.53–7.49 (m, 1H), 7.41–7.35 (m, 5H), 7.28 (d,  $J=8.3$  Hz, 1H), 7.24–7.19 (m, 4H), 5.66 (s, 2H), 5.31 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.1, 152.1, 144.0, 143.7, 143.2, 135.1, 134.6, 133.6, 133.0, 130.1, 129.0, 128.7, 124.9, 123.3, 122.5, 121.1, 120.4, 118.4, 116.7, 109.4, 76.8, 39.7; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{18}\text{ClN}_3\text{O}_3$   $[\text{M} + \text{H}]^+$  444.1115 found 444.1067. The compounds **13–22** were prepared by similar procedure as described for **12** using appropriately substituted benzyl halides.

### (*E*)-3-(2-(1*H*-benzo[*d*]imidazol-1-yl)-1-(((4-fluorobenzyl)oxy)imino)ethyl)-2*H*-chromen-2-one (**13**)

White solid, yield: 71%, that is, 177.5 mg; mp  $168\text{--}170\text{ }^\circ\text{C}$ ; IR (KBr,  $\text{cm}^{-1}$ ): 3023, 2415, 1720, 1608, 1500, 1370, 1216, 1019;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.95 (s, 1H), 7.71–7.67 (m, 2H), 7.53–7.49 (m, 1H), 7.43–7.40 (m, 2H), 7.36 (d,  $J=7.4$  Hz, 1H), 7.28–7.25 (m, 1H), 7.23–7.18 (m, 4H), 7.13–7.09 (m, 2H), 5.65 (s, 2H), 5.31 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  164.2, 161.7, 160.0, 154.1, 152.0, 144.1, 143.7, 133.7, 133.0, 132.9, 132.4, 130.8, 130.7, 128.7, 124.9, 123.2, 122.4, 121.2, 120.4, 118.5, 116.7, 115.8, 115.6, 109.4, 76.8, 39.6; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{18}\text{FN}_3\text{O}_3$   $[\text{M} + \text{H}]^+$  428.1410 found 428.1399.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((4-bromobenzyl)oxy)imino)ethyl)-2H-chromen-2-one (14)**

White solid, yield: 63%, that is, 157.6 mg; mp 124–126 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3019, 2400, 1721, 1608, 1490, 1405, 1215, 1026;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.94 (s, 1H), 7.70 (d,  $J=7.3$  Hz, 1H), 7.60 (s, 1H), 7.55 (d,  $J=8.3$  Hz, 2H), 7.53–7.49 (m, 1H), 7.36 (dd,  $J=7.7, 1.2$  Hz, 1H), 7.30 (d,  $J=8.2$  Hz, 2H), 7.27 (d,  $J=8.6$  Hz, 1H), 7.23–7.20 (m, 2H), 7.19–7.17 (m, 2H), 5.65 (s, 2H), 5.29 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.2, 152.3, 144.1, 143.7, 135.6, 133.8, 133.0, 131.9, 130.4, 128.7, 124.9, 123.2, 122.7, 122.4, 121.2, 120.5, 118.5, 116.7, 109.4, 76.7, 39.7; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{18}\text{BrN}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  488.0610 found 488.0584.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-(3-(trifluoromethyl)benzyloxyimino)ethyl)-2H-chromen-2-one (15)**

Whitish yellow solid, yield: 71%, that is, 177.2 mg; mp 146–148 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3001, 2517, 1722, 1611, 1473, 1384, 1250, 1026;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.94 (s, 1H), 7.75 (s, 1H), 7.70–7.66 (m, 2H), 7.64–7.62 (m, 2H), 7.58 (d,  $J=7.6$  Hz, 1H), 7.51–7.49 (m, 1H), 7.34 (dd,  $J=7.7$  Hz,  $J=1.1$  Hz, 1H), 7.28–7.26 (m, 1H), 7.23–7.18 (m, 2H), 7.16–7.15 (m, 2H), 5.67 (s, 2H), 5.40 (s, 2H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.2, 152.6, 144.1, 143.9, 143.5, 137.7, 133.7, 133.0, 132.0, 131.4, 130.9, 129.4, 128.7, 125.9, 125.5, 125.0, 123.3, 122.5, 121.0, 120.5, 118.4, 116.7, 109.2, 76.7, 39.7; HRMS (ESI) calcd for  $\text{C}_{26}\text{H}_{18}\text{F}_3\text{N}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  478.1379 found 478.1360.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((3-chlorobenzyl)oxy)imino)ethyl)-2H-chromen-2-one (16)**

White solid, yield: 70%, that is, 170.0 mg; mp 148–150 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3019, 2413, 1721, 1627, 1494, 1431, 1215, 1028;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.96 (s, 1H), 7.70–7.69 (m, 1H), 7.66 (s, 1H), 7.53–7.49 (m, 1H), 7.46 (s, 1H), 7.38–7.36 (m, 2H), 7.34 (s, 1H), 7.32–7.31 (m, 1H), 7.28–7.26 (m, 1H), 7.23–7.20 (m, 4H), 5.68 (s, 2H), 5.32 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  159.9, 154.1, 152.3, 144.0, 143.8, 143.3, 138.6, 134.6, 133.7, 133.0, 130.1, 128.7, 126.7, 124.9, 123.4, 122.5, 121.0, 120.4, 118.4, 116.7, 109.4, 76.7, 39.7; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{18}\text{ClN}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  444.1115 found 444.1067.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-(benzyloxyimino)ethyl)-2H-chromen-2-one (17)**

White solid, yield: 61%, that is, 152.2 mg; mp 144–146 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3020, 2400, 1717, 1617, 1521, 1424, 1215, 1039;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.94 (s, 1H), 7.70–7.67 (m, 2H), 7.52–7.48 (m, 1H), 7.47–7.44 (m, 4H), 7.42–7.40 (m, 1H), 7.36–7.34 (m, 1H), 7.28–7.25 (m, 1H), 7.22–7.15 (m, 4H), 5.67 (s, 2H), 5.37 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.1, 151.9, 144.1, 143.7, 143.5, 136.6, 133.7, 132.9, 128.8, 124.8, 123.2, 122.3, 121.2, 120.4, 118.5, 116.7, 109.5, 76.7, 39.6; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{19}\text{N}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  410.1505 found 410.1480.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((3-nitrobenzyl)oxy)imino)ethyl)-2H-chromen-2-one (18)**

Yellow solid, yield: 72%, that is, 180 mg; mp 160–162 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3021, 2425, 1719, 1611, 1492, 1456, 1219, 1023;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.35 (s, 1H), 8.25 (d,  $J=7.3$  Hz, 1H), 8.03 (bs, 1H), 7.71 (bs, 3H), 7.63–7.59 (m, 1H), 7.55–7.51 (m, 1H), 7.39 (d,  $J=7.1$  Hz, 1H), 7.29–7.21 (m, 5H), 5.68 (s, 2H), 5.43 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  159.9, 154.1, 152.8, 148.5, 144.0, 142.8, 138.7, 134.5, 133.1, 129.9, 128.8, 125.0, 123.5, 122.7, 120.8, 120.3, 118.4, 116.7, 109.4, 76.1, 39.9; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{18}\text{N}_4\text{O}_5$  [ $\text{M} + \text{H}$ ] $^+$  455.1355 found 455.1304.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-(3-methylbenzyloxyimino)ethyl)-2H-chromen-2-one (19)**

Yellow solid, yield: 75%, that is, 187 mg; mp 157–159 °C; IR (KBr,  $\text{cm}^{-1}$ ): 2979, 2410, 1712, 1623, 1479, 1379, 1235, 1021;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.94 (s, 1H), 7.70–7.68 (m, 2H), 7.52–7.48 (m, 1H), 7.35–7.31 (m, 2H), 7.27–7.26 (m, 3H), 7.22–7.16 (m, 5H), 5.67 (s, 2H), 5.33 (s, 2H), 2.41 (s, 3H);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.1, 151.8, 144.1, 143.7, 143.4, 138.4,

136.5, 133.7, 132.8, 129.5, 129.4, 128.7, 125.8, 124.8, 123.2, 122.4, 121.2, 120.3, 118.5, 116.7, 109.5, 77.8, 39.6, 21.5; HRMS (ESI) calcd for  $\text{C}_{26}\text{H}_{21}\text{N}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  424.1661 found 424.1649.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((4-methoxybenzyl)oxy)imino)ethyl)-2H-chromen-2-one (20)**

White solid, yield: 73%, that is, 182 mg; mp 126–128 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3019, 2400, 1721, 1611, 1495, 1406, 1215, 1038;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.93 (s, 1H), 7.69–7.68 (m, 2H), 7.52–7.48 (m, 1H), 7.40 (d,  $J=8.5$  Hz, 2H), 7.35 (d,  $J=7.7$  Hz, 1H), 7.28–7.25 (m, 1H), 7.23–7.15 (m, 4H), 6.96 (d,  $J=8.6$  Hz, 2H), 5.65 (s, 2H), 5.29 (s, 2H), 3.85 (s, 3H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  160.0, 154.1, 151.6, 144.1, 143.6, 143.5, 133.7, 132.8, 130.6, 128.7, 124.8, 123.2, 122.3, 121.3, 120.3, 118.5, 116.7, 114.1, 109.5, 77.4, 55.4, 39.5; HRMS (ESI) calcd for  $\text{C}_{26}\text{H}_{21}\text{N}_3\text{O}_4$  [ $\text{M} + \text{H}$ ] $^+$  440.1610 found 440.1603.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((2,4-difluorobenzyl)oxy)imino)ethyl)-2H-chromen-2-one (21)**

White solid, yield: 68%, that is, 170 mg; mp 140–142 °C; IR (KBr,  $\text{cm}^{-1}$ ): 3020, 2411, 1719, 1615, 1499, 1457, 1277, 1019;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  7.94 (s, 1H), 7.70–7.67 (m, 2H), 7.53–7.49 (m, 1H), 7.45–7.39 (m, 1H), 7.36 (d,  $J=6.8$  Hz, 1H), 7.27 (d,  $J=8.6$  Hz, 1H), 7.24–7.18 (m, 4H), 6.95–6.89 (m, 2H), 5.67 (s, 2H), 5.38 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  165.0, 163.2, 161.7, 160.0, 154.2, 152.3, 144.0, 143.7, 143.5, 133.8, 133.0, 132.4, 128.7, 124.9, 123.2, 122.4, 121.1, 120.5, 118.5, 116.7, 109.4, 104.3, 70.8, 39.5; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{17}\text{F}_2\text{N}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  446.1316 found 446.1293.

**(E)-3-(2-(1H-benzo[d]imidazol-1-yl)-1-((2,5-dichlorobenzyl)oxy)imino)ethyl)-2H-chromen-2-one (22)**

Yellow solid, yield: 71%, that is, 177 mg; mp 136–138 °C; IR (KBr,  $\text{cm}^{-1}$ ): 2949, 2417, 1719, 1610, 1459, 1364, 1233, 1024;  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ ):  $\delta$  8.42 (s, 1H), 7.83–7.82 (m, 2H), 7.57–7.53 (m, 1H), 7.43–7.41 (m, 2H), 7.40–7.36 (m, 2H), 7.31–7.29 (m, 4H), 7.26–7.23 (m, 1H), 5.75 (s, 2H), 5.41 (s, 2H);  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ ):  $\delta$  159.9, 154.1, 151.4, 144.3, 142.6, 138.5, 135.6, 133.3, 132.9, 132.3, 131.0, 130.6, 130.0, 128.9, 125.1, 124.7, 124.2, 120.4, 118.9, 118.4, 116.7, 110.2, 76.4, 40.4; HRMS (ESI) calcd for  $\text{C}_{25}\text{H}_{17}\text{Cl}_2\text{N}_3\text{O}_3$  [ $\text{M} + \text{H}$ ] $^+$  478.0725 found 478.0718.

**Ethical statement**

This study has the prior approval from the CSIR-CDRI institutional human ethics committee at CDRI, Lucknow (CDRI/IEC/2014/A1).

**Bacterial strains**

All the bacterial strains were obtained from the MTCC, India or ATCC, USA and were routinely grown in Nutrient agar medium (NAM; Difco/Becton & Dickinson, Franklin Lakes, NJ, USA). Just before the experiment, a loop full of bacteria was inoculated in Mueller Hinton (MH) broth and incubated at 37 °C and 5%  $\text{CO}_2$  for overnight to get the starter culture.

**MIC assay**

MIC assay for the test compounds was carried out by the microtitre broth dilution method as prescribed by CLSI (previously NCCLS).<sup>20,21</sup> For determination of MIC, test compounds were diluted from a stock solution (10 mg  $\text{ml}^{-1}$ ) such that the final concentration ranges between 50 and 0.39  $\mu\text{g ml}^{-1}$  in different wells. Bacteria grown in 5% MH broth for 12–18 h were rinsed twice with sterile phosphate-buffered saline and diluted in MH broth to a concentration of  $\sim 5 \times 10^5$  CFU per ml. About 90  $\mu\text{l}$  of bacterial suspension in MH medium was added to each well containing the test compound and incubated at 37 °C for 16 h. A positive control for growth containing no test compound was included in each assay. MIC was taken as the concentration where the growth inhibition observed was >95%. For each test compound, MIC determinations were carried independently twice using triplicate samples each time.

### Hemolysis assay

Hemolysis assay was done using human blood, collected by venipuncture technique in EDTA (2 mg ml<sup>-1</sup>) containing vacationers from the volunteers. The collected blood was centrifuged at 800 g for 10 min, followed by the removal of plasma and buffy coat from the supernatant. The erythrocytes containing pellet was washed three times in phosphate-buffered saline, pH 7.4 and resuspended in the same solution to get a 5% suspension. Later erythrocytes suspension was incubated with end-over-end rotation for 1 h at 37 °C in the presence of test compounds at (100 µg ml<sup>-1</sup>). After the incubation, samples were centrifuged at 800 g for 10 min and the supernatant was transferred to a 96-well plate. Hemoglobin release was measured by reading the absorbance at 540 nm and is expressed as a percentage of Triton X-100-induced hemolysis. For positive control, 2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) was used. The results given represent mean values from triplicate measurements.

$$\% \text{ Hemolysis} = \frac{(\text{Absorbance of sample}) - (\text{Absorbance of blank})}{\text{Highest absorbance of positive control}} \times 100$$

### MTT assay

The viability of L929 cell lines exposed to test compound was analyzed by using MTT assay. About 5 mg of MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazoliumbromide; Sigma-Aldrich, Cat No M5655) was dissolved in 1 ml of phosphate-buffered saline and filter sterilized in brown color eppendorf tubes before storing at -20 °C. Two hundred µl of L929 cell line (2.5 × 10<sup>5</sup> cells per ml) was plated in 96-well plates and grown in DMEM (Sigma, Cat. No D5525) supplemented with 2 mM Glutamine, 0.2% sodium bicarbonate and 10% fetal bovine serum for 2 h before adding the test compounds at 1 × and 10 × times of MIC concentration incubated for 18–24 h at 37 °C and 5% CO<sub>2</sub>. After the incubation, the 20 µl of MTT solution (5 mg ml<sup>-1</sup>) was added and further incubated for 3–4 h at 37 °C and 5% CO<sub>2</sub>. Medium containing the MTT was removed and 100 µl of DMSO was added to dissolve the Formazan formed from MTT by the enzymes associated with metabolic activity. The plates were gently swirled for 10 min at room temperature to dissolve the precipitate and the absorbance was monitored at 550 nm. The results presented here are mean values from the independent triplicate experiment.

### Lactate dehydrogenase assay

LDH assay was done to show that test compounds are safe and do not induce any cell death. L929 cell lines (2.5 × 10<sup>5</sup> cells per ml) were grown in 96-well plates to confluency and test compounds at 1 × and 10 × times of MIC concentration were added in triplicates to respective labeled wells of the plate, followed by incubation for 18–24 h at 37 °C and 5% CO<sub>2</sub>. After incubation, 100 µl of the medium was transferred into fresh 96-well plates and the LDH release from the cells was quantified by using Cytotoxicity Detection Kit (Roche, Indianapolis, IN, USA, Cytotoxicity Detection Kit, Cat # 11 644 793 001) as per manufacture instructions. The mean values represented here are from three independent experiments and are given as fractional LDH release compared to the positive control consisting of 1% Triton X-100 (yielding 100% LDH release).<sup>23</sup>

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### ACKNOWLEDGEMENTS

We are grateful to the Director, CDRI, Lucknow, India for constant encouragement in drug development program, Dr SP Singh for technical support, SAIF for NMR, IR and Mass spectral data. LRS, ASR, AS and GRP are thankful to CSIR, New Delhi, India for the financial support. SR is thankful to ICMR, New Delhi, India for junior research fellowship. CSIR-CDRI communication number: 302/2015/KVS.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)