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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Design, synthesis and mode of action of novel 2-(4-aminophenyl)benzothiazole derivatives bearing semicarbazone and thiosemicarbazone moiety as potent antimicrobial agents

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Abstract A novel series of substituted benzothiazoles, bearing semicarbazone and thiosemicarbazone moieties, was designed, synthesized and evaluated for their antimicrobial activity and possible mode of action. Structures of the synthesized compounds were elucidated by ^{1}H NMR, ^{13}C NMR, IR and Mass spectral data. The results revealed that compounds **SC06, SC09, TS05** and **TS07** have potent antibacterial activity against both Gram-positive and Gram-negative strains. Compound **TS05** displayed most potent activity with MIC values of 3.91, 7.81 and 1.56 μg/ml against *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. The results from cytoplasmic membrane permeabilization assay, FACS study as well as DNA-binding assays, evaluated against clinically relevant pathogens *S. aureus* and *E. coli,* suggest membrane perturbing as well as intracellular mode of action of this class of compounds. In addition, hemolytic activity of the compounds was measured which indicated their low cytotoxicity.

Keywords Antimicrobials · Benzothiazole · DNA binding · Membrane permeabilization · Semicarbazone/thiosemicarbazone

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Introduction

Treating infectious diseases, caused by bacteria or fungi, remains an important and challenging public health problem (Jones *et al.*, 2008). Such infections most commonly affect patients with decreased immunity, neoplastic disorders and undergoing organ transplantation (Nathan, 2004). This was mainly due to increase in the number of immunocompromised hosts because of advances in medical technology and the human immunodeficiency virus pandemic (Wills *et al.*, 2000). Moreover, common pathogens and new pathogenic species that possess intrinsic primary resistance are rapidly developing secondary resistance to the current antimicrobial agents (White *et al.*, 1998). The availability of complete microbial genome sequence has led to devise concerted strategy to look at novel antibacterials. Nevertheless, in spite of the identification of many new potential drug targets, novel antimicrobial agents have not yet emerged from such efforts to the satisfaction of researchers (Schimd, 2004).

Nowadays, multidrug resistance (MDR) to therapeutic antibiotics constitutes a serious public health threat. The prevalence of extremely resistant bacteria such as methicillin-resistant *Stayphlococcus aureus* (MRSA), fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), fluoroquinolone-resistant *Enterococcus faecalis* (QREF) and vancomycin-resistant *Enterococci* (VRE) has greatly increased in some hospitals, resulting in higher rate of mortality (Dessen *et al.*, 2001; Tenovera, 2001; Leclercq, 2009). Development in biological evaluation of heterocyclic molecules has undergone manifold changes, and the advancement in molecular biology has eased the design of new molecules based on their mechanism of action. Therefore, for a more efficient control, the discovery of

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novel drugs with novel mode of action will be imperative to meet the threats by the emergence of resistance.

The exploration of new heterocycles that can accommodate potency to multiple biological targets remains an intriguing scientific endeavor. The benzothiazole ring system in numerous biologically active molecules has been recognized, which shows important pharmacological activities. Various benzothiazole bearing compounds possess activities such as antitumor (Shi *et al.*, 1996), antimicrobial (Catalano *et al.*, 2013), schistosomicidal (Mahran *et al.*, 2007), anti-inflammatory (Gupta and Rawat, 2010), anticonvulsants (Siddiqui *et al.*, 2007), antidiabetic (Navarrete-Vazquez *et al.*, 2009), antipsychotic (Arora *et al.*, 2010), neuroprotective (Anzini *et al.*, 2010) and diuretic (Yar and Ansari, 2009). 2-(4-Aminophenyl) benzothiazole moiety is present in various bioactive molecules such as antitumor **I**, **II** (Singh and Singh, 2014), orexin receptor antagonist **III** (Bergman *et al.*, 2006) and the Gram-positive selective antibacterial **IV** (Ali *et al.*, 2001) (Fig. 1). Structure–activity relationship (SAR) carried out on these heterocycles has shown that positions 2 and 6 are crucial for antibacterial activity against Gram-positive and Gram-negative bacterial strains (Yildiz-Oren *et al.*, 2004). The chemistry of thiosemicarbazone and semicarbazone has received considerable attention because of their variable bonding modes, promising biological implications, structural diversity and ion-sensing ability (Casas *et al.*, 2000; Mishra *et al.*, 2006; Kizilcikli *et al.*, 2004). Semicarbazones and thiosemicarbazones have been known to show significant pharmacological profiles against important pathogens. Owing to this, a large number of organic and metal–organic compounds derived from these have been the subject of most structural and medicinal studies. Semicarbazones are among the most relevant nitrogen–oxygen donor ligands, which seem to be valuable in the structure of antimicrobials (Gingrass *et al.*, 1961). In addition to these, there has been a growing attention toward thiosemicarbazones and semicarbazones related to their wide range of biological properties, specifically as antifungal (Pandeya *et al.*, 2012), antibacterial (Laxmi and Rajitha, 2012; Kumar *et al.*, 2001), anticonvulsants (Siddiqui *et al.*, 2007), anti-HIV (Mishra *et al.*, 2002), antitubercular (Cocco *et al.*, 2002) and anticancer (Shukla *et al.*, 2013) agents. Keeping this in view, we thought to develop newer analogs of the benzothiazole nucleus, i.e., semicarbazone and thiosemicarbazone derivatives expecting better biological activity owing to rationale in design of the target molecules.

The main objective of our study was to investigate how the potency and selectivity against different Gram-positive (*S. aureus*, *E. faecalis*) and Gram-negative (*E. coli, S. typhi, K. pneumoniae, P. aeruginosa*) bacteria can be modulated by replacement of carbonyl oxygen of the benzothiazole amide ring system with groups that can generate electronic, electrostatic as well as different steric properties. In addition, we also investigated the role of lipophilicity on the antibacterial activity through the synthesis of a series of benzothiazole bearing semicarbazone and thiosemicarbazone moieties, **SC01-10** and **TS01-10**, respectively (Table 1).

Herein, we explored the possibilities of above-synthesized compounds to bind with lipopolysaccharides (LPS), to hyperpolarize both outer and cytoplasmic membranes, to interact with lipid monolayers and to kill different bacterial strains. To circumvent this mechanism, we have employed the fluorescence polarization assay using the cationic membrane potential-sensitive dye $DiSC_3(5)$ to monitor the changes associated with membrane permeability of intact bacteria *E. coli* and *S. aureus*. Furthermore, to understand the hypothesis that induced uptake of propidium iodide (PI) is responsible for membrane rupture and hence killing, fluorescence-assisted cell cytometer (FACS) analysis was

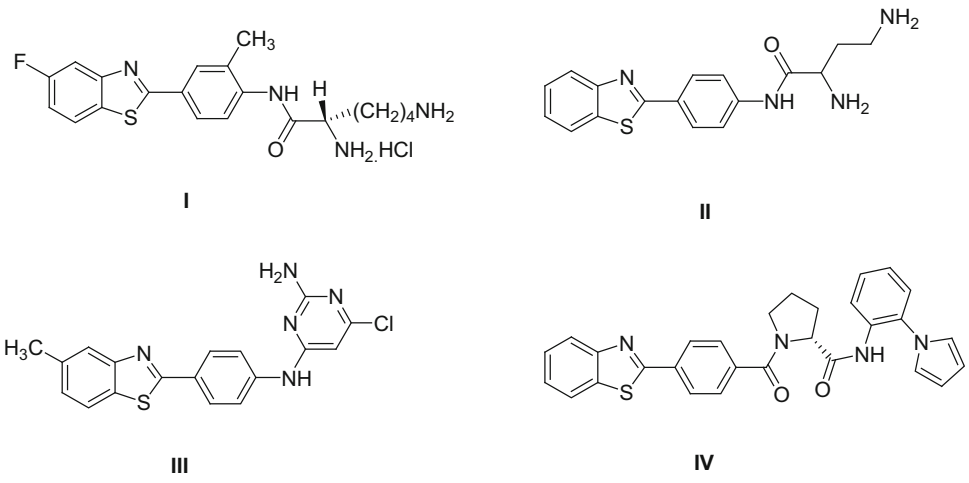
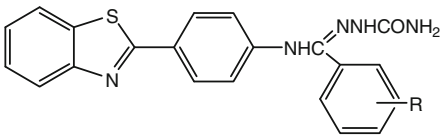
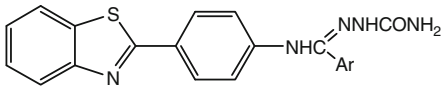


Fig. 1 Chemical structures of pharmacologically relevant benzothiazoles

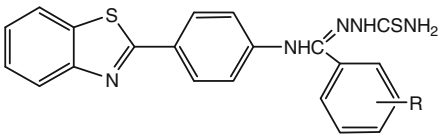
Table 1 Structure of compounds **SC01-10** and **TS01-10**



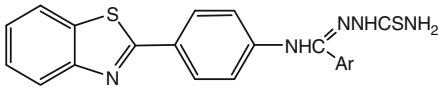
SC01-09



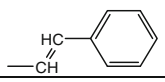
SC10



TS01-09



TS10

Compound code	R, Ar
SC01, TS01	H
SC02, TS02	3,5-di NO_2
SC03, TS03	2-OH
SC04, TS04	3-NO_2
SC05, TS05	2-Cl
SC06, TS06	2-F
SC07, TS07	5-Cl, 2-OCH_3
SC08, TS08	2,4-diOH
SC09, TS09	2-OCH_3
SC10, TS10	

performed. Outcome of both the data strongly suggested that cytoplasmic membrane hyperpolarization did correlate well with bacterial cell lethality. We further evaluated interaction of the lead molecules with bacterial plasmid DNA (pUC19) to probe the molecular mode of action. In addition, hemolytic activity of the compounds measured was used as an indicator of their cytotoxicities.

Our research group is working on this benzothiazole scaffold (Singh *et al.*, 2014a, b, 2015a, b). Recently, we reported the synthesis and design of analogs of benzothiazole amide system, their antimicrobial activity and the possible mode of action (Singh *et al.*, 2014b). In continuation to our efforts, it was thought worthwhile to design and synthesize thiosemicarbazone and semicarbazone

derivatives hoping to go a step forward in the field of antimicrobial agents. We report here design, synthesis, evaluation of antimicrobial activity and the possible mode of action of novel benzothiazole–semicarbazone and thiosemicarbazone as antimicrobial agents.

Materials and methods

Chemistry

All the chemicals and solvents were of analytical grade and procured from Merck, HiMedia (Mumbai) and Aldrich. Plasmid DNA (pUC19) was purchased from Invitrogen. $DiSC_3(5)$ and propidium iodide (PI) were from Aldrich. The synthesized compounds were characterized by IR, ^{1}H NMR, ^{13}C NMR, Mass spectral and elemental analysis. Melting points were determined in open glass capillaries on Electrothermal Stuart-SMP10 melting point apparatus and were uncorrected. IR absorption spectra were recorded on Shimadzu FTIR-8400 s, FTIR spectrometer. ^{1}H NMR spectra were recorded on the Bruker DRX-300; FT-NMR and ^{13}C NMR spectra were recorded on the JEOL AL300, FT-NMR spectrometer. Both NMR instruments operate at 300 MHz/25 °C using DMSO-d6 as solvent and TMS as internal standard. Chemical shifts were reported in parts per million (ppm, δ:0 units). ESI–MS spectra were recorded on Micromass Quattro II spectrometer. Elemental analysis was performed with CE-440 elemental analyzer (Exeter Analytical Inc., USA). All the IR, Mass, ^{1}H-NMR, ^{13}C-NMR spectral data of compounds (**SC01-10** and **TS01-10**) were in accordance with the proposed molecular structures. The purity of the synthesized compounds was monitored by TLC and ascertained by elemental analysis. Analytical and physicochemical properties of the compounds are given in Table 2.

General procedure for the synthesis of compounds (SC01-10) and (TS01-10)

Semicarbazide/thiosemicarbazide hydrochloride (1.0 mM) dissolved in 5 ml hot distilled water and catalyst NaOAc were added to a suspension of *N*-(4-(benzo[d]thiazol-2-yl)phenyl)-substituted benzamides **A01-10** (1.0 mM) reported earlier (Singh *et al.*, 2014a, b) in 50.0 ml ethanol. The reaction mixture was refluxed on a water bath for 4–10 h (Scheme 1). The progress of the reaction was monitored by TLC at appropriate time intervals. After completion of the reaction, the reaction mixture was cooled to room temperature. The precipitate formed was filtered, washed several times with warm ethanol and dried. Compounds were purified by either re-crystallization or column chromatography as required.

Semicarbazide/thiosemicarbazide hydrochloride reacts with benzothiazole amide derivatives through nucleophilic addition reaction. Amino group of semicarbazide/thiosemicarbazide hydrochloride acts as a nucleophile, and addition of nucleophilic nitrogen occurs on the active carbonyl group of benzothiazole amide derivatives, resulting in formation of target compounds as shown in Fig. 2. Characterization data of the compounds are given below.

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)benzamides ***(SC01)***

IR (KBr, υmax cm^{-1}): 3248.06 (–NH str.), 3367.04, 3343.63 (–NH_2 str.), 1673.50 (C=O str. of semicarbazone), 1572.57 (C=N str.), 3078.12 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.21–7.88 (m, 13H, Ar–H), 13.56 (s, 1H, NH), 9.35 (s, 1H, –CNH), 8.95, 8.93 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 165.28 (C=O of semicarbazone), 151.32 (–C=N), 156.23 (benzothiazole–C–2), 118.16–141.67 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 388 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-3,5-dinitro-benzamides ***(SC02)***

IR (KBr, υmax cm^{-1}): 3143.81 (–NH str.), 3214.09, 3358.10 (–NH_2 str.), 1631.42 (C=O str. of semicarbazone), 1565.38 (C=N str.), 1320.31, 1555.28 (NO_2 str.), 3079.23 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.12–7.78 (m, 11H, Ar–H), 13.11 (s, 1H, NH), 9.41 (s, 1H, –CNH), 8.42, 8.36 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 162.97 (C=O of semicarbazone), 152.64 (–C=N), 157.06 (benzothiazole–C–2), 121.36–138.42 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 478 (M + 1, 100).

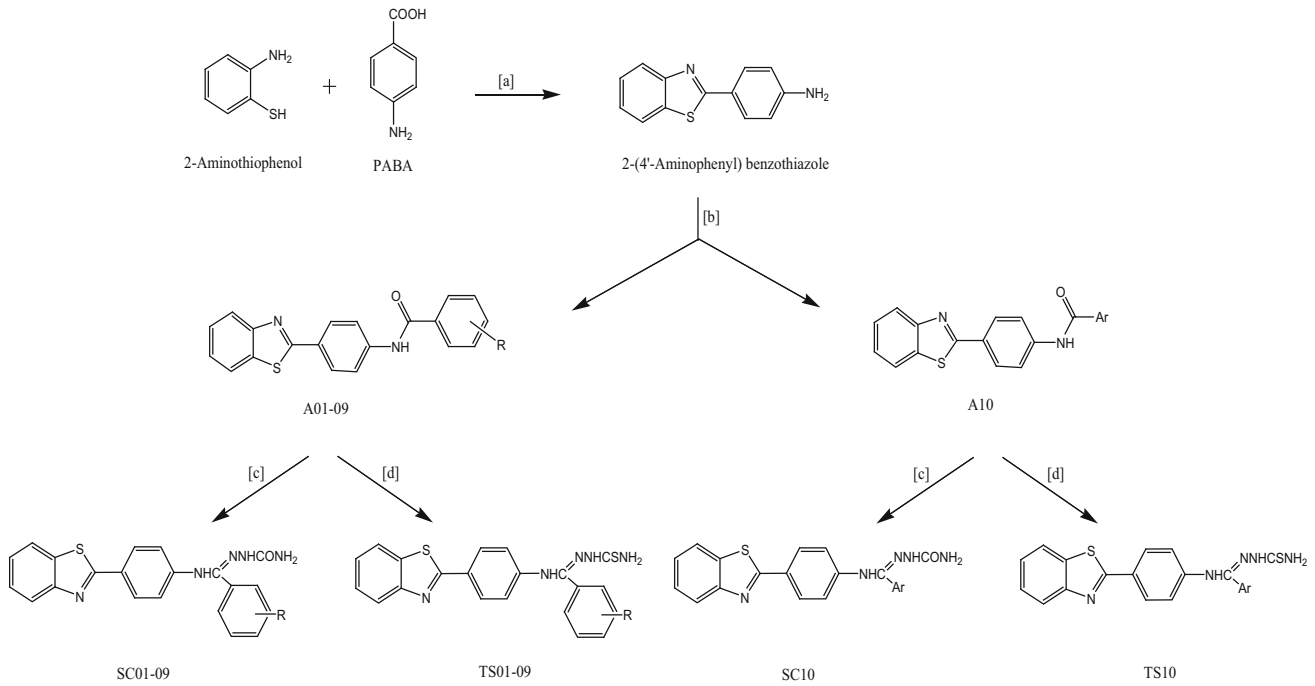
N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-hydroxy-benzamides ***(SC03)***

IR (KBr, υmax cm^{-1}): 3231.06 (–NH str.), 3359.21, 3375.03 (–NH_2 str.), 1655.85 (C=O str. of semicarbazone), 1571.18 (C=N str.), 3491.83 (–OH str.), 3047.33 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.18–7.80 (m, 12H, Ar–H), 13.42 (s, 1H, NH), 9.33 (s, 1H, –CNH), 8.85, 8.78 (2 s, 2H, NH_2), 10.46 (br, s, 1H, –OH); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 166.08 (C=O of semicarbazone), 152.54 (–C=N), 155.13 (benzothiazole–C–2), 113.10–142.52 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 404 (M + 1, 100).

Table 2 Antimicrobial activity and MIC values (μg/ml) against the tested bacteria and hemolytic activity of the synthesized compounds

Compound code	Bacteria						% Hemolysis
	Gram-positive bacteria		Gram-negative bacteria				
	S. aureus (ATCC 25323)	*E. faecalis* (clinical isolate)	*E. coli* (ATCC 35218)	*S. typhi* (MTCC 3216)	*K. pneumonia* (ATCC 31488)	*P. aeruginosa* (ATCC 27893)	
SC01	–	13.33 ± 0.57 (62.5)	12.45 ± 0.47 (62.5)	–	–	11.28 ± 0.66 (62.5)	11.96 ± 0.30
SC02	19.43 ± 0.46 (15.6)	12.63 ± 1.16 (62.5)	15.16 ± 0.68 (31.2)	–	14.20 ± 0.41 (31.2)	–	8.71 ± 0.45
SC03	13.30 ± 0.48 (62.5)	–	12.73 ± 0.66 (62.5)	–	–	14.38 ± 0.40 (31.2)	13.09 ± 0.18
SC04	11.20 ± 0.61 (62.5)	–	13.36 ± 0.97 (31.2)	–	18.34 ± 0.27 (15.6)	–	12.14 ± 0.67
SC05	14.23 ± 0.44 (31.2)	11.33 ± 0.60 (62.5)	14.53 ± 0.37 (31.2)	15.23 ± 0.69 (31.2)	–	11.80 ± 0.35 (62.5)	12.03 ± 0.33
SC06	15.20 ± 0.43 (31.2)	–	18.30 ± 0.43 (15.6)	11.82 ± 0.43 (62.5)	18.40 ± 0.16 (15.6)	–	7.20 ± 0.28
SC07	11.57 ± 0.37 (62.5)	–	15.10 ± 0.58 (31.2)	–	17.80 ± 0.45 (15.6)	13.15 ± 0.37 (31.2)	12.32 ± 0.50
SC08	–	10.23 ± 0.45 (125)	11.63 ± 0.72 (62.5)	–	11.67 ± 0.40 (62.5)	–	15.02 ± 0.27
SC09	25.23 ± 0.77 (7.81)	10.86 ± 0.29 (62.5)	14.23 ± 0.39 (31.2)	–	14.70 ± 0.17 (31.2)	18.10 ± 0.43 (15.6)	4.36 ± 0.82
SC10	12.10 ± 0.66 (62.5)	10.15 ± 0.36 (125)	11.67 ± 0.18 (62.5)	–	10.70 ± 0.45 (125)	–	32.01 ± 0.33
TS01	–	13.40 ± 0.50 (62.5)	12.35 ± 0.44 (62.5)	–	13.67 ± 0.17 (31.2)	11.44 ± 0.38 (62.5)	10.62 ± 0.28
TS02	15.20 ± 0.75 (31.2)	14.73 ± 0.48 (31.2)	15.48 ± 0.28 (31.2)	–	14.56 ± 0.46 (31.2)	–	8.11 ± 0.39
TS03	14.6 ± 0.46 (31.2)	–	–	–	–	–	14.09 ± 0.54
TS04	11.60 ± 0.37 (62.5)	–	13.76 ± 0.82 (31.2)	–	–	–	6.94 ± 0.14
TS05	29.26 ± 0.63 (3.91)	18.19 ± 0.40 (15.6)	24.21 ± 0.55 (7.81)	15.29 ± 0.19 (31.2)	14.29 ± 0.50 (31.2)	35.30 ± 0.33 (1.56)	3.21 ± 0.67
TS06	12.03 ± 0.61 (62.5)	–	11.72 ± 0.45 (62.5)	–	14.23 ± 0.25 (31.2)	–	7.90 ± 0.39
TS07	25.06 ± 0.34 (7.81)	18.33 ± 0.19 (15.6)	18.34 ± 0.47 (15.6)	15.16 ± 0.50 (31.2)	17.17 ± 0.40 (15.6)	13.33 ± 0.61 (31.2)	5.38 ± 0.45
TS08	–	10.26 ± 0.34 (125)	11.39 ± 0.18 (62.5)	–	–	11.50 ± 0.16 (62.5)	13.59 ± 0.20
TS09	15.15 ± 0.86 (31.2)	12.30 ± 0.31 (62.5)	11.62 ± 0.33 (62.5)	–	15.27 ± 0.40 (31.2)	–	12.83 ± 0.50
TS10	12.51 ± 0.26 (62.5)	11.93 ± 0.28 (62.5)	14.82 ± 0.72 (31.2)	–	14.30 ± 0.26 (31.2)	18.39 ± 0.94 (15.6)	9.42 ± 0.58
Penicillin	34.38 ± 0.63 (3.12)	34.21 ± 0.39 (3.12)	38.68 ± 0.25 (1.56)	37.40 ± 0.30 (1.56)	38.27 ± 0.90 (1.56)	38.20 ± 0.45 (1.56)	–
Ciprofloxacin	31.60 ± 0.40 (6.25)	29.27 ± 0.77 (6.25)	34.61 ± 0.27 (6.25)	34.15 ± 0.72 (6.25)	29.30 ± 0.30 (6.25)	34.52 ± 0.45 (3.12)	–

The value of each compound calculated in triplicate, consisted of zone of inhibition (in mm) ± SEM with MIC values in brackets. Level of significance $P < 0.05$



Scheme 1 Reagents and conditions: *a* polyphosphoric acid (PPA), 220 °C, 1–2 h reflux with stirring *b* ArCOOH, Acetonitrile, DCC, reflux 4–10 h *c* Semicarbazide hydrochloride, CH_3OH/C_2H_5OH, NaOAc, reflux 6–8 h *d* Thiosemicarbazide hydrochloride, CH_3OH/C_2H_5OH, NaOAc, reflux 4–8 h

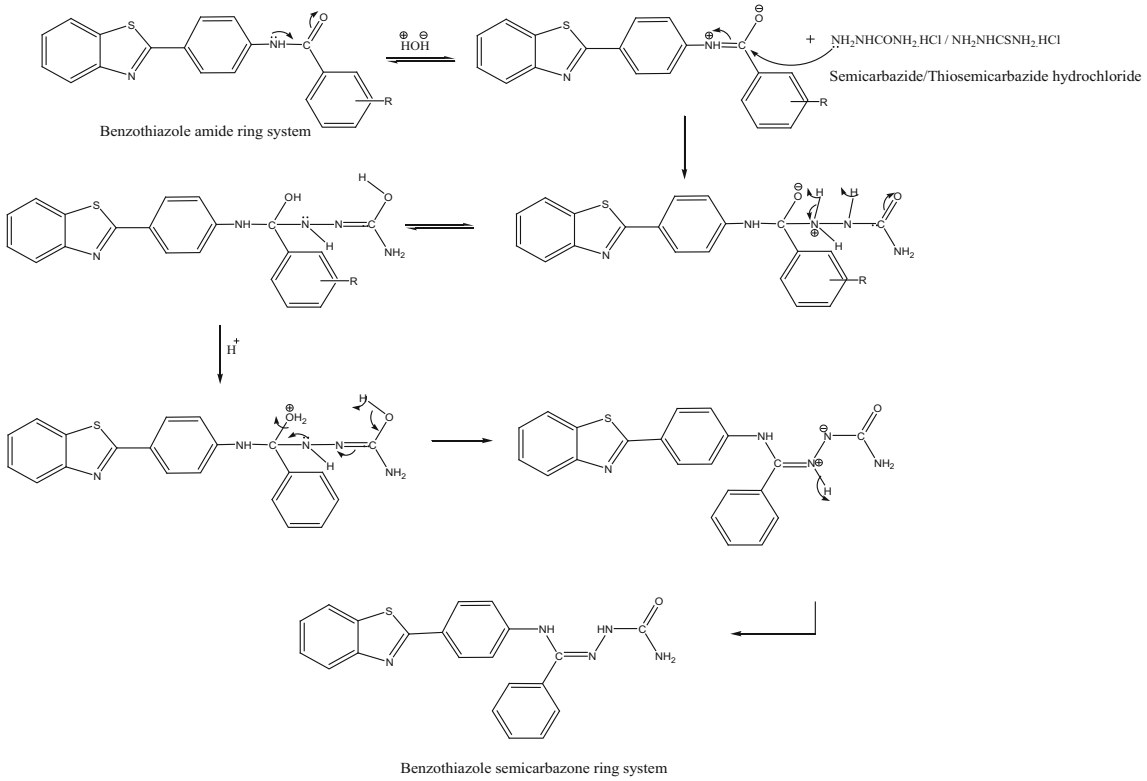


Fig. 2 Proposed reaction mechanism of formation of benzothiazole–semicarbazone/thiosemicarbazone ring system

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-3-nitro-benzamides ***(SC04)***

IR (KBr, υmax cm^{-1}): 3180.72 (–NH str.), 3267.52, 3327.32 (–NH_2 str.), 1624.12 (C=O str. of semicarbazone), 1577.82 (C=N str.), 1313.57, 1533.46 (NO_2 str.), 3037.99 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.00–7.91 (m, 12H, Ar–H), 13.17 (s, 1H, NH), 9.36 (s, 1H, –CNH), 8.43, 8.31 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 163.27 (C=O of semicarbazone), 153.00 (–C=N), 156.56 (benzothiazole–C–2), 124.56–133.94 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 433 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-chloro-benzamides ***(SC05)***

IR (KBr, υmax cm^{-1}): 3226.81 (–NH str.), 3315.94, 3365.03 (–NH_2 str.), 1678.30 (C=O str. of semicarbazone), 1566.32 (C=N str.), 1052.67 (–C–Cl str.), 3082.92 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.01–7.75 (m, 12H, Ar–H), 13.46 (s, 1H, NH), 9.43 (s, 1H, –CNH), 8.76, 8.85 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 163.88 (C=O of semicarbazone), 152.12 (–C=N), 156.06 (benzothiazole–C–2), 121.15–144.21 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″; MS (*m/z*, %): 422 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-fluoro-benzamides ***(SC06)***

IR (KBr, υmax cm^{-1}): 3298.38 (–NH str.), 3421.83, 3506.70 (–NH_2 str.), 1647.26 (C=O str. of semicarbazone), 1543.10 (C=N str.), 3107.43 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.07–7.88 (m, 12H, Ar–H), 13.32 (s, 1H, NH), 9.56 (s, 1H, –CNH), 8.53, 8.51 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 167.31 (C=O of semicarbazone), 153.17 (–C=N), 159.73 (benzothiazole–C–2), 115.79–139.17 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 406 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-methoxy-5-chloro-benzamides ***(SC07)***

IR (KBr, υmax cm^{-1}): 3253.12 (–NH str.), 3365.31, 3387.73 (–NH_2 str.), 1673.30 (C=O str. of semicarbazone), 1532.92 (C=N str.), 1062.17 (–C–Cl str.), 2917.66 (–C–H str. of OCH_3), 3069.92 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.27–7.98 (m, 11H, Ar–H), 13.31 (s, 1H, NH), 9.54 (s, 1H, –CNH), 8.37, 8.74 (2 s, 2H, NH_2), 3.81 (s, 3H, OCH_3); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 55.60 (OCH_3), 162.38 (C=O of semicarbazone), 154.26 (–C=N), 156.49 (benzothiazole–C–2), 122.62–140.61 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 452 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2,4-dihydroxy-benzamides ***(SC08)***

IR (KBr, υmax cm^{-1}): 3242.16 (–NH str.), 3374.13, 3379.03 (–NH_2 str.), 1645.08 (C=O str. of semicarbazone), 1562.78 (C=N str.), 3361.83 (–OH str.), 3054.63 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.16–7.70 (m, 11H, Ar–H), 13.23 (s, 1H, NH), 9.26 (s, 1H, –CNH), 8.71, 8.79 (2 s, 2H, NH_2), 10.21, 10.34 (s, 2H, –OH); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 162.15 (C=O of semicarbazone), 153.64 (–C=N), 156.38 (benzothiazole–C–2), 111.54–138.02 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 420 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-methoxy-benzamides ***(SC09)***

IR (KBr, υmax cm^{-1}): 3206.41 (–NH str.), 3295.14, 3342.83 (–NH_2 str.), 1666.13 (C=O str. of semicarbazone), 1573.72 (C=N str.), 2948.30 (–C–H str. of OCH_3), 3074.22 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.13–7.69 (m, 12H, Ar–H), 13.28 (s, 1H, NH), 9.48 (s, 1H, –CNH), 8.64, 8.82 (2 s, 2H, NH_2), 3.80 (s, 3H, OCH_3); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 55.62 (OCH_3), 162.14 (C=O of semicarbazone), 153.23 (–C=N), 155.10 (benzothiazole–C–2), 118.35–148.10 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 418 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-styrene-amides ***(SC10)***

IR (KBr, υmax cm^{-1}): 3237.47 (–NH str.), 3335.14, 3371.39 (–NH_2 str.), 1652.92 (C=O str. of semicarbazone), 1563.12 (C=N str.), 3083.02 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.19–7.75 (m, 13H, Ar–H), 9.51 (s, 1H, –CNH), 6.70, 6.73 (dd, –CH=CH); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 165.28 (C=O of amide), 153.13 (–C=N), 156.00 (benzothiazole–C–2), 117.32–138.75 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 414 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)benzamides ***(TS01)***

IR (KBr, υmax cm^{-1}): 3176.56 (–NH str.), 3326.12, 3210.73 (–NH_2 str.), 1271.60 (C=S str.), 1542.17 (C=N str.), 3048.15 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.32–7.98 (m, 13H, Ar–H), 13.61 (s, 1H, NH), 9.28 (s, 1H, –CNH), 9.15, 8.92 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 181.08 (C=S), 149.02 (–C=N), 157.13 (benzothiazole–C–2), 121.16–141.63

(Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 404 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-3,5-dinitro-benzamides ***(TS02)***

IR (KBr, υmax cm^{-1}): 3122.86 (–NH str.), 3321.53, 3184.58 (–NH_2 str.), 1242.20 (C=S str.), 1575.89 (C=N str.), 3036.06 (Ar–C–H str.), 1311.64, 1348.29, 1533.46 (NO_2 str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.26–8.23 (m, 11H, Ar–H), 13.68 (s, 1H, NH), 9.72 (s, 1H, –CNH), 9.21, 8.91 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 182.49 (C=S), 149.53 (–C=N), 158.11 (benzothiazole–C–2), 124.07–139.42 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 494 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-hydroxy-benzamides ***(TS03)***

IR (KBr, υmax cm^{-1}): 3183.15 (–NH str.), 3363.13, 3217.80 (–NH_2 str.), 1239.10 (C=S str.), 1562.19 (C=N str.), 3079.16 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.12–8.02 (m, 12H, Ar–H), 13.93 (s, 1H, NH), 9.67 (s, 1H, –CNH), 9.10, 8.89 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 183.45 (C=S), 145.72 (–C=N), 156.14 (benzothiazole–C–2), 121.37–138.83 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 420 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-3-nitro-benzamides ***(TS04)***

IR (KBr, υmax cm^{-1}): 3212.95 (–NH str.), 3381.15 (–NH_2 str.), 1246.90 (C=S str.), 1573.29 (C=N str.), 3099.26 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.14–7.87 (m, 12H, Ar–H), 13.72 (s, 1H, NH), 9.84 (s, 1H, –CNH), 9.21, 8.76 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 181.44 (C=S), 147.84 (–C=N), 156.39 (benzothiazole–C–2), 122.46–139.03 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 449 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-chloro-benzamides ***(TS05)***

IR (KBr, υmax cm^{-1}): 3122.86 (–NH str.), 3329.25 (–NH_2 str.), 1244.13 (C=S str.), 1575.89 (C=N str.), 3034.13 (Ar–C–H str.) 1087.89 (C–Cl); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.31–7.88 (m, 12H, Ar–H), 13.87 (s, 1H, NH), 9.66 (s, 1H, –CNH), 8.93, 8.81 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 181.30 (C=S), 157.42 (–C=N), 164.24 (benzothiazole–C–2), 124.56–139.90 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 438 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-fluoro-benzamides ***(TS06)***

IR (KBr, υmax cm^{-1}): 3168.13 (–NH str.), 3336.25, 3214.65 (–NH_2 str.), 1258.12 (C=S str.), 1566.39 (C=N str.), 3078.54 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.19–7.79 (m, 12H, Ar–H), 13.97 (s, 1H, NH), 9.42 (s, 1H, –CNH), 9.83, 8.78 (2 s, 2H, NH_2); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 182.10 (C=S), 154.76 (–C=N), 158.14 (benzothiazole–C–2), 123.66–141.78 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 422 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-methoxy-5-chloro-benzamides ***(TS07)***

IR (KBr, υmax cm^{-1}): 3223.12 (–NH str.), 3358.41, 3327.73 (–NH_2 str.), 1273.30 (C=S str.), 1562.02 (C=N str.), 1065.15 (–C–Cl str.), 2932.56 (–C–H str. of OCH_3), 3071.62 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.15–7.90 (m, 11H, Ar–H), 13.63 (s, 1H, NH), 9.47 (s, 1H, –CNH), 9.32, 8.90 (2 s, 2H, NH_2), 3.82 (s, 3H, OCH_3); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 56.43 (OCH_3), 182.08 (C=S), 153.06 (–C=N), 156.37 (benzothiazole–C–2), 118.62–140.01 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 469 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2,4-dihydroxy-benzamides ***(TS08)***

IR (KBr, υmax cm^{-1}): 3147.46 (–NH str.), 3338.52, 3312.63 (–NH_2 str.), 1245.75 (C=S str.), 1568.64 (C=N str.), 3376.53 (–OH str.), 3085.63 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.26–7.87 (m, 11H, Ar–H), 13.46 (s, 1H, NH), 9.62 (s, 1H, –CNH), 8.91, 8.73 (2 s, 2H, NH_2), 10.33, 10.13 (s, 2H, –OH); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 182.55 (C=S), 154.09 (–C=N), 156.68 (benzothiazole–C–2), 113.80–141.62 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 436 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-methoxy-benzamides ***(TS09)***

IR (KBr, υmax cm^{-1}): 3134.96 (–NH str.), 3395.14, 3238.03 (–NH_2 str.), 1256.13 (C=S str.), 1558.42 (C=N str.), 2936.13 (–C–H str. of OCH_3), 3075.32 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.24–7.88 (m, 12H, Ar–H), 13.58 (s, 1H, NH), 9.42 (s, 1H, –CNH), 8.97, 8.84 (2 s, 2H, NH_2), 3.83 (s, 3H, OCH_3); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 55.48 (OCH_3), 182.54 (C=S), 151.23 (–C=N), 155.78 (benzothiazole–C–2), 121.65–144.32

(Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 434 (M + 1, 100).

N-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-styrene-amides ***(TS10)***

IR (KBr, υmax cm^{-1}): 3175.87 (–NH str.), 3373.74, 3277.39 (–NH_2 str.), 1252.64 (C=S str.), 1558.49 (C=N str.), 3068.32 (Ar–C–H str.); ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm): 7.14–7.65 (m, 13H, Ar–H), 9.70 (s, 1H, –CNH), 6.71, 6.69 (dd, –CH=CH); ^{13}C NMR (*DMSO–d6*, 300 MHz, δ ppm): 184.27 (C=S), 154.16 (–C=N), 156.95 (benzothiazole–C–2), 119.13–135.67 (Aromatic–C, C4–C9 C1′–C6′ C1″–C6″); MS (*m/z*, %): 430 (M + 1, 100).

Antimicrobial assay

Disc diffusion method

Antimicrobial activities of newly synthesized compounds were evaluated on different Gram-positive and Gram-negative human pathogens viz. *Staphylococcus aureus* (ATCC 25323), *Escherichia coli* (ATCC 35218), *Pseudomonas aeruginosa* (ATCC 27893), *Klebsiella pneumonia* (ATCC 31488), *Enterococcus faecalis* (clinical isolate) and *Salmonella typhi* (MTCC 3216) according to the guidelines of National Committee for Clinical Laboratory Standards (Wayne, 1997) using the agar disc diffusion method (Bharti *et al.*, 2010a, b). Briefly, a 24-/48-h-old culture of selected bacteria was mixed with sterile physiological saline (0.85 %), and the turbidity was adjusted to the standard inoculums of MacFarland scale 0.5 [$\sim 10^6$ colony-forming units (CFUs) per milliliter]. Petri plates containing 20 ml of Mueller–Hinton Agar (MHA, HiMedia) were used for all the bacteria tested. The inoculums were spread on the surface of the solidified media, and Whatman no. 1 filter paper discs (6 mm in diameter) impregnated with the test compound (20 µl/disc) were placed on the plates. Ciprofloxacin (5 µg/disc, HiMedia) was used as positive control for bacteria. A paper disc impregnated with dimethyl sulfoxide (DMSO) was used as negative control. Plates inoculated with the bacteria were incubated for 24 h at 37 °C. The inhibition zone diameters were measured in millimeters. All the tests were performed in triplicate.

Determination of MIC

Minimum inhibitory concentration (MIC) of any compound is defined as the lowest concentration, which completely inhibits visible growth (turbidity on liquid media). MIC values were determined by broth micro-dilution method, according to NCCLS guidelines document M27-A (Wayne, 1997). Equal volume of test compounds with different dilutions and nutrient broth was mixed in wells of microtiter plate, which were serially twofold diluted to determine the MIC. Specifically 0.1 ml with approximately 5×10^5 CFU/ml of actively dividing bacterial cells was inoculated in each well. The standard antibiotic, ciprofloxacin (10 µg/ml) for bacteria was used as positive controls, and 100 µl of DMSO was used as a negative control. All the inoculated plates were incubated at 37 °C, and the results were evaluated by visible turbidity in each well after 24 h. All determinations were done in triplicate, and the average was taken as final MIC value.

Bactericidal kinetics

Overnight cultures of *S. aureus* ATCC 25323 and *E. coli* ATCC 35218 were diluted 10^{-2} times in fresh MHB and allowed to grow to exponential phase (optical density at 600 nm of 0.6). The time-course killing activity of synthesized compounds was determined using the 96-well plates of plate reader as described previously (Joshi *et al.*, 2012). The lead compounds were added at 4 times their MICs, and this suspension was incubated at 37 °C at 200 rpm. After addition of the compounds, the absorbance of the plates was recorded at 600 nm at regular time intervals, i.e., 0, 1, 2, 3, 4 and 5 h. The experiment was repeated on three different days, and values are plotted as mean ± SD.

Hemolytic activity

Hemolytic activity assay was carried out according to the procedure described previously (Singh *et al.*, 2013). Briefly, freshly collected 100 µL hRBC was washed thrice in sterile phosphate-buffered saline (PBS) solution. The hRBC suspension was re-suspended in 4 % v/v PBS. The final concentration was adjusted to 5×10^8 cells/ml at pH 7.4. Compound solution containing 100 µM of test compound (100 µl) was mixed with 100 µl of hRBC suspension, and final volume was made to 1 ml with buffer and was placed in a 96-well plate. The cell suspensions were incubated for 1 h at 37 °C with constant shaking. The samples were then centrifuged for 5 min at 1300 rpm, and the release of hemoglobin was monitored by measuring the absorbance (A_{sample}) of the supernatant at 540 nm. For negative and positive controls, hRBC in PBS (A_{blank}) and in 0.2 % (final concentration v/v) Triton X-100 (A_{triton}) were used, respectively. The percentage of hemolysis was calculated according to the following equation.

$$\text{Percentage of hemolysis} = \left[\left(A_{sample} - A_{blank}\right) / \left(A_{triton} - A_{blank}\right)\right] \times 100$$

Cytoplasmic membrane permeabilization assay

Compound-induced polarization of *S. aureus* ATCC 25323 and *E. coli* ATCC 35218 membrane measures the efficacy to dissipate the potential across these cell membranes and was determined using the membrane potential-sensitive cyanine dye $DiSC_3(5)$ described previously (Friedrich *et al.*, 2000). Briefly, exponential-phase bacteria were harvested by centrifugation, washed and re-suspended in 5 mM HEPES–20 mM glucose buffer (pH 7.2) to an optical density of 0.06. This cell suspension was incubated with 100 mM KCl (to equilibrate cytoplasmic and external K^+ concentration) and $DiSC_3(5)$ at a concentration of 1 μM for 1 h at room temperature. When the fluorescence level (excitation at 622 nm and emission at 670 nm wavelength) of the bacterial suspension became stable (approximately 90 % reduction in fluorescence due to $DiSC_3(5)$ uptake and quenching in the cell in response to an intact membrane potential), a 400 μl aliquot of cell suspension and the 4 × MIC concentration of lead compounds were added in the cuvette in order to record the membrane hyperpolarization of bacterial cell membrane. All samples were then measured on PerkinElmer Life Sciences LS 50-B spectrofluorimeter (PerkinElmer Corp., Norwalk, Conn.) in a 5-mm path length quartz cell at 25 °C. Samples were stirred during the experiment at a constant temperature of 37 °C. An increase in fluorescence due to partitioning of samples into the membrane was recorded as a function of time until no further increase in intensity was observed.

Flow cytometry analysis

The membrane damage of *S. aureus* ATCC 25323 and *E. coli* ATCC 35218 was examined by flow cytometer after employing suitable fluorescent probes (Novo *et al.*, 2000). Briefly, the cells at exponential phase were stained with propidium iodide after the treatment with the lead compounds at 37 °C for 1 h with constant shaking. The cells were centrifuged, washed two times with PBS and incubated further with propidium iodide (PI) at 4 °C for 30 min, followed by removal of the unbound dye through washing with an excess of PBS and re-suspended in buffer. These cells were then analyzed by flow cytometry in the form of dots (excitation and emission wavelength set at 488 and 617 nm, respectively, for propidium iodide) and plots with respect to the control without PI treated, with PI treated cells without preincubation with test compounds and with test compound treated cells.

DNA-binding assay

Gel retardation experiments were performed on synthesized compounds by agarose gel electrophoresis as described previously (Bharti *et al.*, 2000). Briefly, 200 ng of plasmid DNA (pUC19) was mixed with increasing amounts of lead compounds in 20 μL of binding buffer (5 % glycerol, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 μg/ml bovine serum albumin). The reaction mixtures were incubated for 1 h at 37 °C. Consequently, loading buffer containing 25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol was added and then subjected to 1 % agarose gel electrophoresis at 60 V for 1 h in 10 mM Tris–HCl buffer. The migration of DNA was detected by the fluorescence of ethidium bromide. The illuminated gel was photographed by Alpha Innotech Corporation Instrument. Besides, the reaction was also monitored upon addition of various groove binders—methyl green (MG) and DAPI.

Statistical analysis

Each experiment was repeated three times, and the results were expressed as mean ± standard deviations. The data were analyzed statistically using Prism 5.1 and Origin 6.0 software. Statistical analysis was performed using analysis of variance with the Dunnett's test. A P value of <0.05 was considered statistically significant, whereas a P value of >0.05 was insignificant.

Results and discussion

Antimicrobial activity

The structures of the compounds are given in Table 1. The antibacterial activity of the synthesized compounds was determined using the method recommended by National Committee for Clinical Laboratory Standards (NCCLS) (Wayne, 1997) for a panel of two Gram-positive and four Gram-negative bacteria. The antimicrobial results are presented in Table 3. Standard antibacterial agent penicillin and ciprofloxacin were also screened under identical conditions for comparison. The results revealed that most of the compounds exhibited good-to-moderate MIC values ranging between 1.56 and 125 μg/ml in DMSO. Out of the 20 benzothiazole derivatives, compound *N*-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-floro-benzamides (**SC06**) exhibited maximum antibacterial activity against *E. coli* and *K. pneumoniae* (zone of inhibition up to 18–19 mm at concentration of 15.6 μg/ml). Similarly, compound *N*-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-semicarbazone)-2-methoxy-benzamides (**SC09**) showed maximum activity against *S. aureus* (zone of inhibition up to 24–26 mm at concentration of 7.81 μg/ml) and *P. aeruginosa* (zone of inhibition up to 17–19 mm at concentration of 15.6 μg/ml). Compound *N*-(4-(benzo[d]thiazol-2-yl)phenyl)-(1′-thiosemicarbazone)-2-

Table 3 Analytical and physicochemical data of the synthesized compounds

Sr. No.	Compound code	Molecular formula	M.W.[a]	M.p.(°C)[b]	Yield (%)	% Analysis of C, H,N found (calc.)[c]		
						C	H	N
1	**SC01**	$C_{21}H_{17}N_5OS$	387.46	198–200	76	65.10 (65.10)	4.40 (4.42)	18.06 (18.08)
2	**SC02**	$C_{21}H_{15}N_7O_5S$	477.45	222–224	56	52.81 (52.83)	3.15 (3.17)	20.54 (20.54)
3	**SC03**	$C_{21}H_{17}N_5O_2S$	403.46	218–220	78	62.51 (62.52)	4.23 (4.25)	17.35 (17.36)
4	**SC04**	$C_{21}H_{16}N_6O_3S$	432.46	192–194	46	58.30 (58.32)	3.72 (3.73)	19.43 (19.43)
5	**SC05**	$C_{21}H_{16}ClN_5OS$	421.92	248–250	81	59.76 (59.78)	3.80 (3.82)	16.60 (16.60)
6	**SC06**	$C_{21}H_{16}FN_5OS$	405.45	240–242	67	62.20 (62.21)	3.96 (3.98)	17.26 (17.27)
7	**SC07**	$C_{22}H_{18}ClN_5O_2S$	451.93	259–260	84	58.45 (58.47)	4.00 (4.01)	15.50 (15.50)
8	**SC08**	$C_{21}H_{17}N_5O_3S$	419.46	210–212	72	60.12 (60.13)	4.07 (4.09)	16.70 (16.70)
9	**SC09**	$C_{22}H_{19}N_5O_2S$	417.48	227–229	66	63.27 (63.29)	4.57 (4.59)	16.76 (16.78)
10	**SC10**	$C_{23}H_{19}N_5OS$	413.49	201–203	52	66.80 (66.81)	4.61 (4.63)	16.92 (16.94)
11	**TS01**	$C_{21}H_{17}N_5S_2$	403.52	189–190	70	62.49 (62.51)	4.24 (4.25)	17.34 (17.36)
12	**TS02**	$C_{21}H_{15}N_7O_4S_2$	493.52	187–189	59	51.10 (51.11)	3.04 (3.06)	19.88 (19.87)
13	**TS03**	$C_{21}H_{17}N_5OS_2$	419.52	211–213	84	60.12 (60.12)	4.06 (4.08)	16.68 (16.69)
14	**TS04**	$C_{21}H_{16}N_6O_2S_2$	448.52	224–225	68	56.22 (56.23)	3.60 (3.60)	18.72 (18.74)
15	**TS05**	$C_{21}H_{16}ClN_5S_2$	437.97	261–263	85	57.57 (57.59)	3.67 (3.68)	15.97 (15.99)
16	**TS06**	$C_{21}H_{16}FN_5S_2$	421.51	236–238	79	59.84 (59.84)	3.81 (3.83)	16.60 (16.61)
17	**TS07**	$C_{22}H_{18}ClN_5OS_2$	467.99	239–240	91	56.43 (56.46)	3.86 (3.88)	14.95 (14.96)
18	**TS08**	$C_{21}H_{17}N_5O_2S_2$	435.52	227–229	86	57.91 (57.91)	3.90 (3.93)	16.06 (16.08)
19	**TS09**	$C_{22}H_{19}N_5OS_2$	433.55	206–208	72	60.93 (60.95)	4.40 (4.42)	16.14 (16.15)
20	**TS10**	$C_{23}H_{19}N_5S_2$	429.56	215–218	82	64.30 (64.31)	4.44 (4.46)	16.29 (16.30)

[a] Molecular weight of the compound

[b] Melting point of the compound at their decomposition

[c] Elemental analyses for C, H and N were within ±0.03 % of the theoretical value

chloro-benzamides (**TS05**) and *N*-(4-(benzo[d]thiazol-2-yl) phenyl)-(1′-thiosemicarbazone)-5-chloro-2-methoxy-benzamides (**TS07**) displayed broad-spectrum antimicrobial activity against all tested bacterial strains with MIC values in the range of 1.56–31.2 μg/ml. Compound **TS05** displayed the most potent activity with MIC values of 3.91, 15.6, 7.81 and 1.56 μg/ml against *S. aureus*, *E. faecalis, E. coli* and *P. aeruginosa*, respectively, and compound **TS07** displayed MIC values of 7.81, 15.6, 15.6 and 15.6 μg/ml against *S. aureus*, *E. faecalis*, *E. coli* and *K. pneumoniae*, respectively, which was comparable with penicillin and ciprofloxacin with corresponding MIC values of 3.12, 3.12, 1.56, 1.56 μg/ml and 6.25, 6.25, 6.25, 6.25 μg/ml, respectively, used as standards. Some of the compounds **SC02**, **SC04**, **SC07** and **SC10** exhibited good activity against few bacterial strains having MIC value of 15.6 μg/ml, while rest of the compounds showed moderate activity. In addition, it was observed that the potent compounds (**SC06, SC09, TS05** and **TS07**) displayed greater activity against *S. aureus* and *E. coli.*

Hemolytic activity

To assess toxicity of synthesized compounds **SC01-10** and **TS01-10**, hemolytic activity was carried out according to the procedure described previously (Singh *et al.*, 2013) on human hRBC at a fixed concentration of 100 μM. The results showed that all the tested compounds caused 3–15 % hemolysis, which signifies non-toxicity of the series. Compounds **SC02**, **SC06, SC09, TS02, TS04, TS05, TS06, TS07** and **TS10** showed less than 10 % hemolysis, while rest of the compounds except compound **SC10** showed maximum of 15 % hemolysis. The highest hemolysis (32 %) of compound **SC10** owes its greater toxicity. Compound **TS05** showed only 3.21 % hemolysis at very high concentration, i.e., 100 μM. The results of the in vitro hemolytic study of the compounds are shown in Table 2 along with its antibacterial activity.

Bactericidal kinetics

The bactericidal activity of lead compounds **SC06, SC09, TS05** and **TS07** was directed in confirming its potency toward the specific *S. aureus* and *E. coli* strains to be used in the evaluation. It was determined at regular time intervals so as to see the potential killing effects of bacterial cells within minutes at concentrations fourfold MICs. Time–kill studies demonstrated that compounds **SC06** and **TS05** were rapid with 90–99 % lethality within 2–5 h

(Fig. 3). Compound **SC09** was found to be less effective as compared to compounds **SC06, TS05** and **TS07** in both strains even up to 5 h. At 4 × MIC, compounds inhibited bacterial growth from 2 h onwards keeping growth arrested till 5 h. However, complete eradication of bacterial growth by any of the tested compounds was not observed up to 5 h. Compound **TS05** exhibited the most potent inhibition of growth in both the strains at 4 × MIC. Based on these data, studies were designed to learn the bactericidal mechanism of test compounds against *S. aureus* and *E. coli*.

Outer membrane permeabilization assay

To understand the basis of antimicrobial activity of designed analogs, compound-induced permeability of *S. aureus* and *E. coli* membrane was examined by determining the efficacy to dissipate the diffusion potential across the LPS membrane. The ability of designed analogs to depolarize the cytoplasmic membrane was determined by using cationic membrane potential-sensitive cyanine dye [3, 3′-dipropyl- thiadicarbocyanine iodide $DiSC_3(5)$]. The distribution of $DiSC_3(5)$ between cell membrane and periphery medium is dependent on the cytoplasmic membrane potential gradient (Wu *et al.*, 1999). This cationic dye readily partitions into the bacterial cell membrane and aggregates within the membrane, causing self-quenching. If the antimicrobial compounds perturb the cell membrane, it can lead to the loss of the membrane potential gradient, causing the dye to release into the medium. As a result, membrane depolarization occurs and the fluorescence intensity of the dye increases (Sims *et al.*, 1974). Upon hyperpolarization of the plasma membrane, the dye enters the cell, binds to intracellular proteins and undergoes quenching of its fluorescent emission with a decrease in its fluorescent signal (Plasek *et al.*, 1994). Triton (2 %) and valinomycin (0.1 μM) were found to depolarize and hyperpolarize the bacterial membrane, respectively. All compounds showed similar profile with respect to positive control proton ionophore valinomycin, which was found to hyperpolarize the membrane by specifically binding to potassium ions (K^+ ions) and thereby acts as K^+ ions carrier molecule. However, none of the above-mentioned compounds shows depolarization of the bacterial membrane as observed in case of negative control Triton (Singh

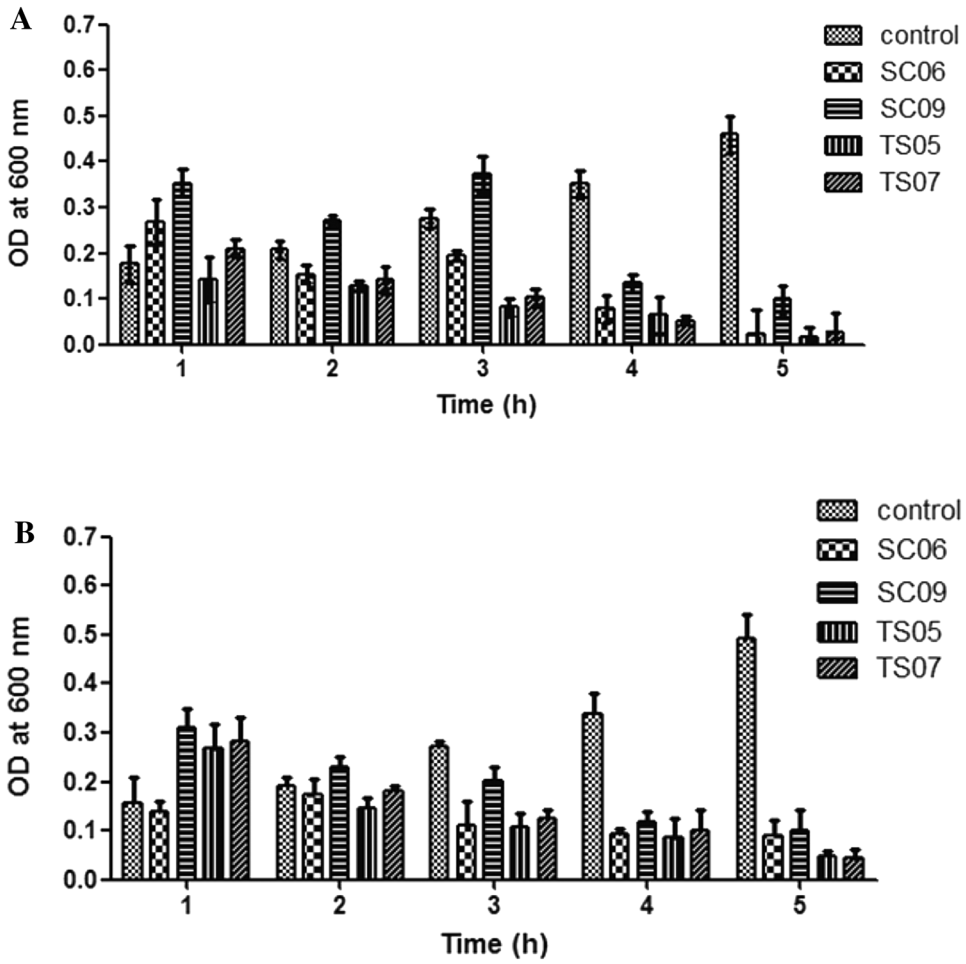


Fig. 3 Time-dependent killing of **a** *S. aureus* **b** *E. coli* upon treatment with compounds **SC06, SC09, TS05** and **TS07** at 4 × MIC concentrations

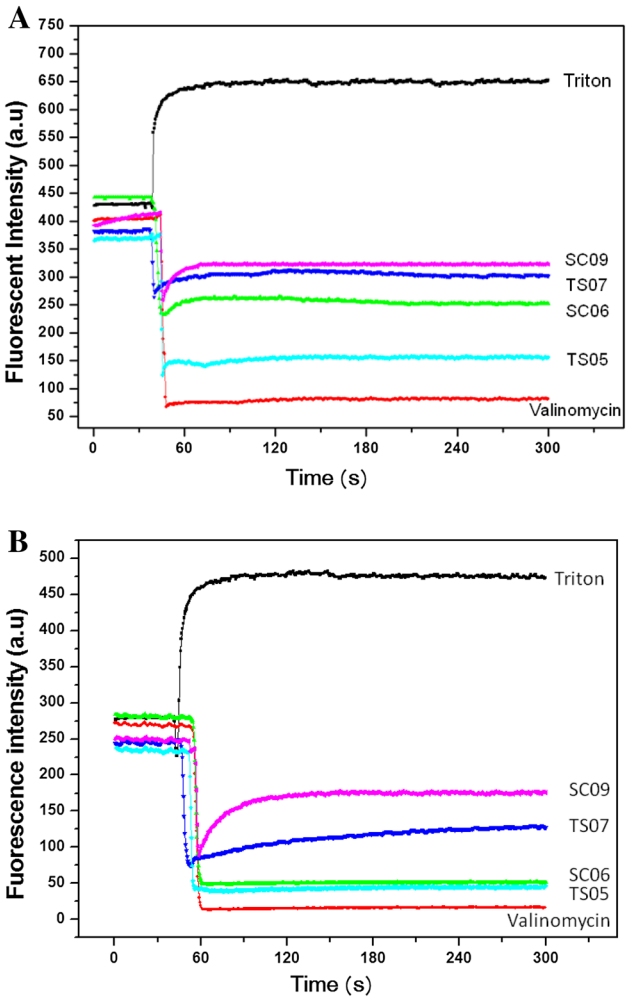


Fig. 4 Effects of designed analogs on **a** *S. aureus* and **b** *E. coli*, membrane potential at 4 × MIC μg/ml. Compounds are presented as (*cyan curve*) **TS05**, (*green curve*) **SC06**, (*blue curve*) **TS07**, (*pink curve*) **SC09**. Triton 2 % (*black curve*) and valinomycin 0.1 μM (*red curve*) were used as negative and positive controls, respectively (Color figure online)

et al., 2014b). At the highest concentration of designed analogs **SC06, SC09, TS05** and **TS07** tested (i.e., 4 × MIC), the level of hyper polarization appeared to reach a maximum at times from about 1–5 min (Fig. 4). Consistent with their cytotoxic and antimicrobial properties, **SC09** showed the minimum, while **TS05** showed maximum relative percentage fluorescence with respect to valinomycin as described in Fig. 5. Unusual pattern of emitted fluorescence was observed in the both strains. All the designed analogs studied had the ability to hyperpolarize the cytoplasmic membrane of *S. aureus* and *E. coli*; however, compounds with different structures had different concentration-activity profiles (data not shown here). Compounds **SC06** and **TS05** completely hyperpolarized the membrane at lower concentrations than those of the other compounds studied.

Permeabilization of membrane of viable bacteria

To determine the effect of test compounds to the integrity of the bacterial membrane, PI a DNA-intercalating fluorescent dye was used to incubate with *S. aureus* and *E. coli* in the presence or absence of test compounds. PI could pass through the damaged membrane and intercalate into DNA. Cell viability was examined by FACS (fluorescence-assisted cell cytometer) by quantifying the amount of DNA released in terms of PI. The large number of dots in lower right quadrant fluorescing with propidium iodide after exposure to compounds might indicate the defective outer membrane repair and consequent leakiness of more DNA and RNA (Riccardi, 2006). As shown in Fig. 6, test compounds **SC06, SC09, TS05** and **TS07** induced maximum damages to the membrane organization of *E. coli* as compared to significantly lesser damage to the membrane of *S. aureus*. Further, the number of PI-stained cells

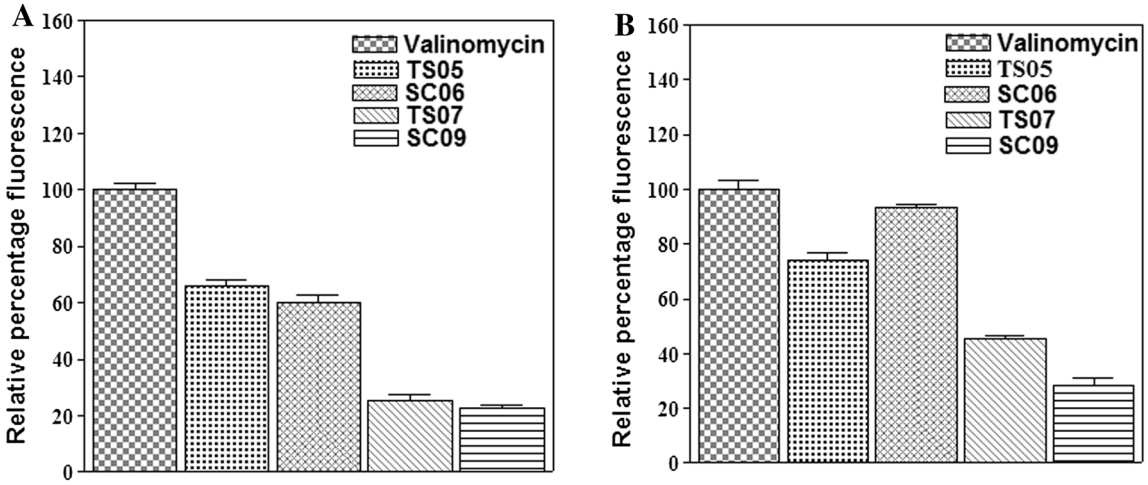


Fig. 5 *Bar diagram* showing relative percentage fluorescence of **a** *S. aureus* and **b** *E. coli*

decreased significantly in **SC09** and **TS07** in *S. aureus* (19.09 and 15.97 %, respectively) as compared to *E. coli* (60.61 and 58.29 %, respectively), implicating lesser membrane damage of *S. aureus.* However, maximum damage of bacterial membrane of both strains was observed in compounds **SC06** and **TS05** (Fig. 6).

DNA-binding activity

Together with the results of membrane permeabilization assay, FACS analysis, we can conclude that series of benzothiazole bearing semicarbazone and thiosemicarbazone moiety could disrupt the integrity of membrane, leading to the leakage of cell contents of bacteria. Simultaneously, during the interaction process with membrane, test compounds unavoidably could enter into cytoplasm, but the effect of test compounds in the cytoplasm is not certainly known. Thus, we determined the DNA-binding ability of test compounds **SC06, SC09, TS05** and **TS07** to determine the mode of action. The DNA-binding affinities of the test compounds were examined by analyzing the electrophoretic mobility of plasmid DNA bands at various compound concentrations. Compared with the test compounds **SC06, SC09, TS05** and **TS07**, compound **SC06** could not inhibit the migration of plasmid DNA at concentration even up to 31.2 μg/ml, while compounds **SC09, TS05** and **TS07** showed DNA retardation at 15.6, 7.81 and 7.81 μg/ml, respectively (Fig. 7). However, at higher concentration compounds **SC09** and **TS05** cleaved the DNA. Therefore, it can be concluded that test compounds bind and cleaved the bacterial DNA, and thus, both membrane and DNA may be its target.

To explore the actual mechanism of binding, the MG/DAPI dyes were used because of their preferential binding with major and minor groove regions of DNA. Therefore, to probe the interacting site of compounds **TS05, TS07** and **SC09** with plasmid DNA, the DNA was treated with DAPI or MG prior to the addition of compounds. DAPI (minor groove binder) was added to the reaction mixture containing compounds **TS05** and **TS07**; no significant inhibition was observed in the cleavage pattern. Similarly, in the presence of methyl green (major groove binder), the cleavage was also not suppressed (Fig. 8). Thus, electrophoretic pattern demonstrated that compounds **TS05** and **TS07** showed nonspecific affinity toward both the major and the minor grooves, and this was only possible if the compound extended through the DNA double helix, as seen from an intercalation binding mode. Further, in compound **SC09** significant inhibition was observed in the cleavage pattern when added with DAPI, whereas in the presence of methyl green (major groove binder), the cleavage was partially suppressed (Fig. 8). Thus, electrophoretic pattern demonstrated that compound **SC09** showed preferential affinity toward the major groove and partial affinity toward minor groove of the DNA.

Conventional antibiotics target important mechanisms or pathways of bacterial survival to inhibit their growth. However, frequent emergence of resistance to these molecules resulted in the emergence of "superbacteria," which put people in potentially dangerous situations of having no available antibiotics. Benzothiazole–semicarbazone and thiosemicarbazone as antimicrobial precursors are considered as essential lead candidates for the development of novel antimicrobial therapeutic agents. The results presented in this study showed that designed compounds have promising antimicrobial activity against both Gram-positive and Gram-negative bacteria. Structure–activity relationship (SAR) demonstrated the effect of substituents on the antimicrobial activity. Compounds **SC06, SC09, TS05** and **TS07** with electronegative groups (F, Cl) and electron-releasing (OCH_3) groups were found to be more active than the compounds with electron-withdrawing (NO_2) groups. However, compounds **SC03, TS03, SC08** and **TS08** bearing electron-releasing OH group were moderately active. Hence, it can be contingent that chloro, fluoro and methoxy substituents bearing derivatives offer the most suitable compounds for achieving the best antibacterial spectrum. The results may be explained by electron density of the compounds. It has been reported in the literature that increased electron density makes the compounds effective against microorganisms and enhances the antibacterial activity (Kumar *et al.*, 2009). Conversely, soaring electron density makes diffusion through the bacterial cell more difficult, and considerable activity loss may occur (Hania, 2009). Thus, for a compound an optimum electron density is essential for significant gain in antibacterial activity. In order to have insight into the toxicity determination of designed compounds, hemolysis on hRBCs was carried out. Almost all the tested compounds exhibited moderate-to-very low hemolysis, which indicated their low toxicity. In general, compounds with better antimicrobial activity showed negligible or very low toxicity profile (Table 2). These results further support the significance of the study. Overall, the present study provides us compounds **SC06, SC09, TS05** and **TS07** with potent activity against a broad range of bacterial strains which also accounts for very low hemolysis in the range of 3–7 %. The antibacterial activity data of compounds (**SC05**, **SC07**; MIC: 15.6–62.5 μg/ml) and (**TS05**, **TS07**; MIC: 1.56–31.2 μg/ml) showed that thiosemicarbazone derivatives exhibited more pronounced activity than semicarbazones. However, both semicarbazones and thiosemicarbazones exhibited much better activity in comparison with amides, earlier reported in our previous work (Singh *et al.*, 2014a, b).

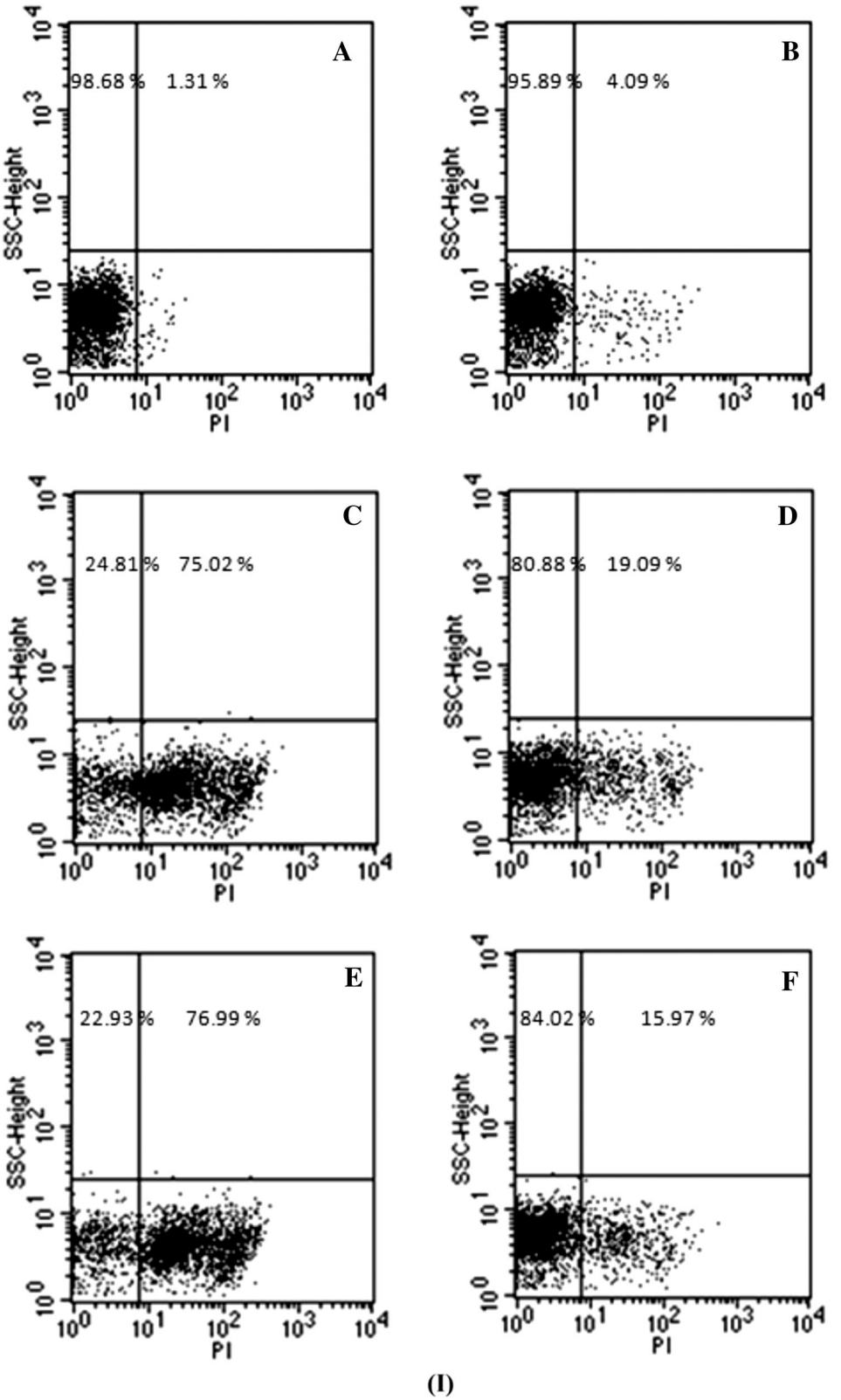
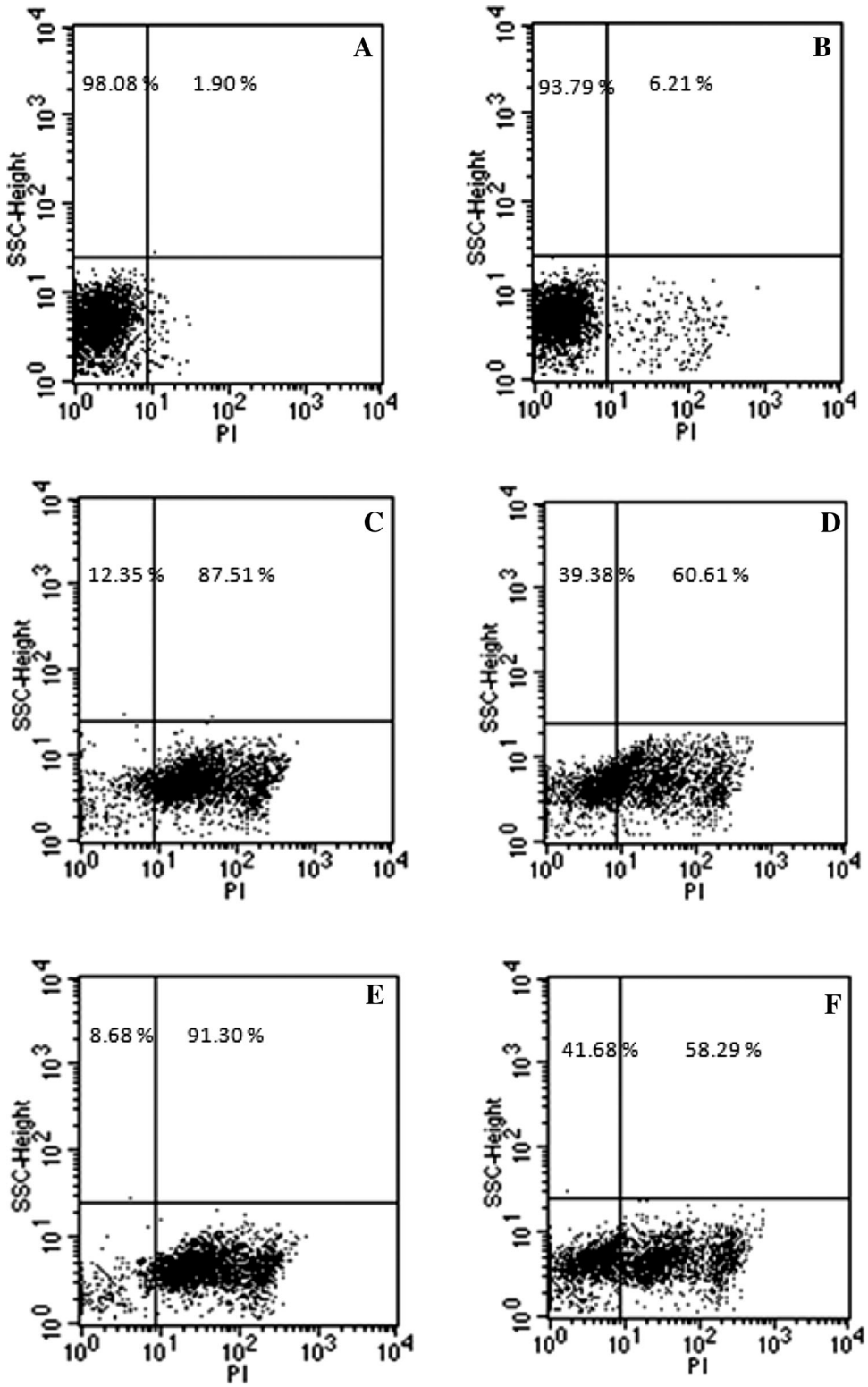


Fig. 6 Determination of compound-induced membrane damage of (**I**) *S. aureus* and (**II**) *E. coli* cells by flow cytometric studies. **a**–**f** PI staining of both strains **a** control without PI treatment **b** control with PI treatment **c SC06 d SC09 e TS05** and **f TS07**. *Lower left quadrant* of each panel depicts unstained cells, whereas the *lower right quadrant* depicts the stained cells. Concentrations of the compounds were 4 × MIC. A total of 10,000 events were recorded for each sample, and control panels show staining of the cells in the absence of any compound

Our previous observations regarding the mode of action of benzothiazole bearing amide moiety demonstrated that these compounds disrupted the integrity of cell membrane and exhibited bactericidal effects (Singh *et al.*, 2014b). Further, we wanted to know whether the designed benzothiazole–semicarbazone and thiosemicarbazone

Fig. 6 continued



(II)

compounds, that are extension of the earlier reported series (Singh *et al.*, 2014b), also targets the plasma membrane and disrupts its integrity, resulted in the killing of bacteria.

In this study, we examined the effect of designed compounds on the *S. aureus* and *E. coli* bacterial membrane to establish the mode of action. All the designed

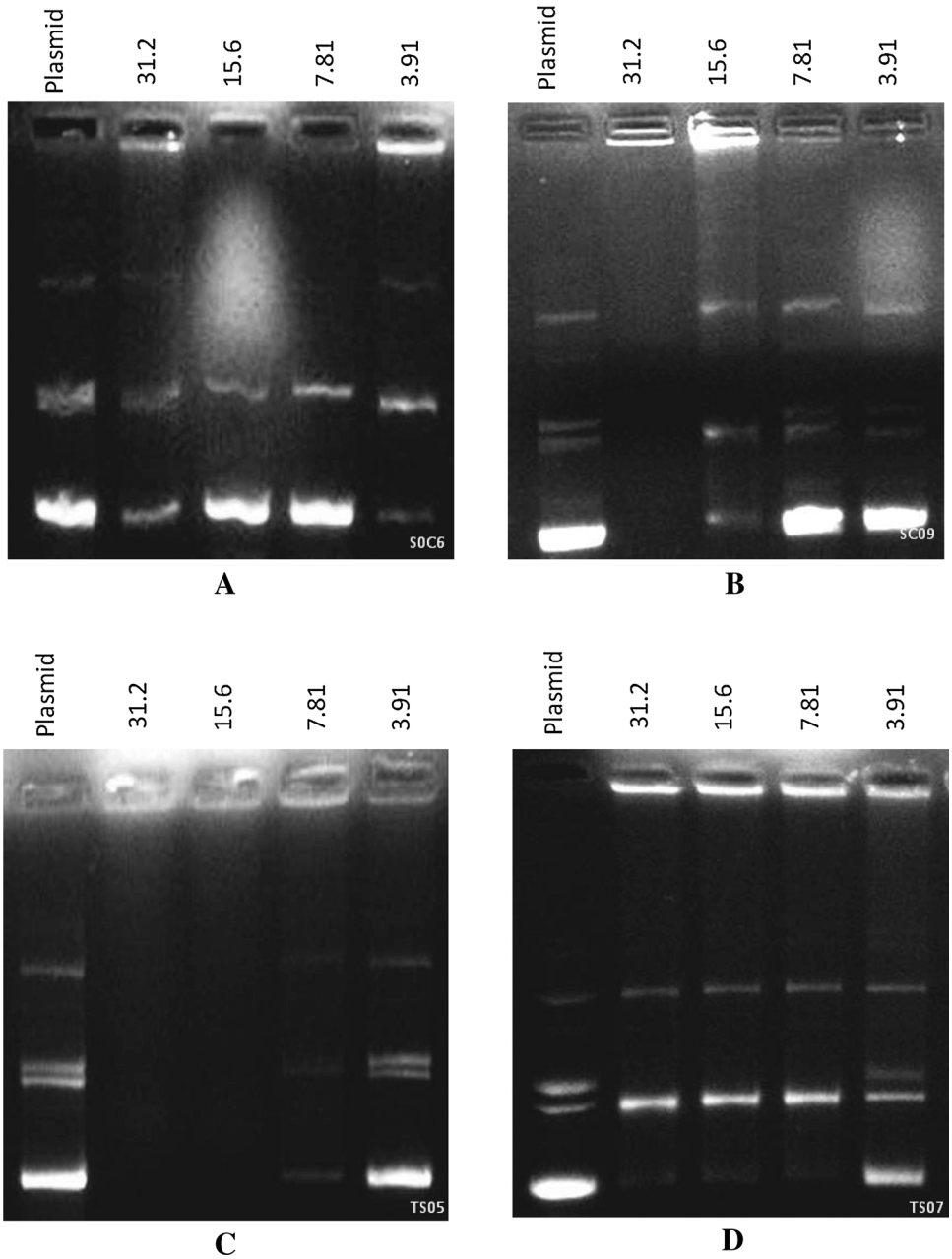


Fig. 7 Agarose gel electrophoresis patterns of pUC19 (200 ng) cleaved by **a SC06 b SC09 c TS05 d TS07** in the range of 31.2–3.91 μg/ml, after 1-h incubation time in buffer (5 mM Tris–HCl/50 mM NaCl, pH 7.2 at 25 °C)

analogs were found to rapidly hyperpolarize the *Enterococcus* and *Staphylococcal* membrane, in a manner similar to ionophore valinomycin (Fig. 4) (Orlova *et al.*, 1994). However, it seems that these compounds create further negative potential, which is observed with ionophore valinomycin. Nevertheless, it is noteworthy that the compounds **TS05** and **SC06** with higher bactericidal property create more negative potential in the bacterial membrane (Fig. 4). Valinomycin at the concentration 0.1 μM used in the study exerts its effect at the cell membrane, possibly by altering electrical properties. It selectively translocates potassium ion across the cell membrane. The increase in the K^+ permeability (Pk) may result in hyperpolarization or an impairment of the electrical behavior of the cell membrane (Spector *et al.*, 1975). Alternatively, valinomycin may act by exchanging intracellular K^+ for extracellular Na^+ or H^+. However, in the absence of clear understanding of the relative concentrations and permeabilities of sodium and potassium, as well as chloride, in the bacteria, and relative contribution to the membrane potential made by electrogenic pumps (i.e., Na+, K+ -ATPase), the present observations can be considered only indirect evidence implicating membrane potential in the events leading to bactericidal effect. Furthermore, changes

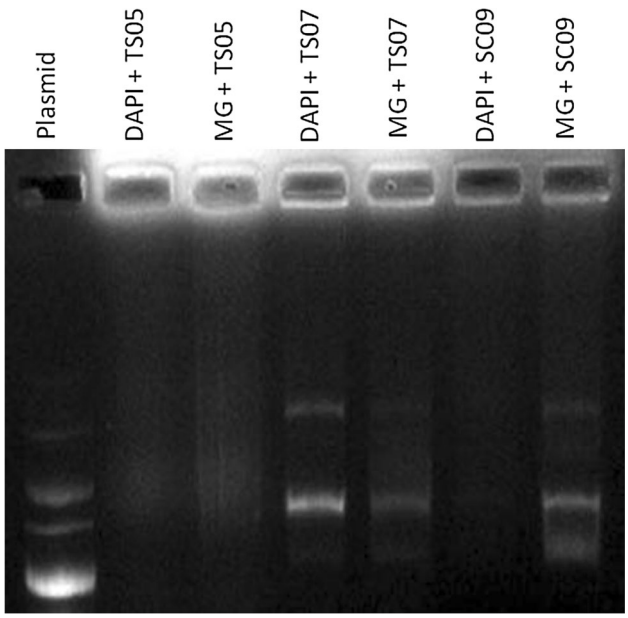


Fig. 8 Agarose gel electrophoresis pattern for the cleavage of supercoiled DNA (200 ng) by **TS05, TS07** and **SC09** in the presence of minor groove (DAPI) and major groove (methyl green) binding agent

in membrane potential status were not associated with structural damage to the membrane integrity, since hyperpolarized cells in a similar fashion to valinomycin act not by forming channels or pores through which potassium can travel, but by forming one-to-one complexes with K+ ions (Tosteson *et al.*, 1968), which can then diffuse across the hydrophobic lipid membranes directly. Overall, these observations suggest that the mode of action of benzothiazole–semicarbazone and thiosemicarbazone compounds might result from disruption of the membrane potential, which is utilized for cellular energy production and hence killing of bacterial cells.

However, this is unlikely to be the sole mechanism, since designed compounds induced the uptake of PI as shown by FACS data (Fig. 6), which is consistent with the findings for agents targeting the membrane (Singh *et al.*, 2014b). Since lead compounds showed potent activity against *E. coli* and *S. aureus*, therefore to have better insight into the mode of action of such compounds we performed gel electrophoresis experiment, to evaluate whether designed compounds could kill the bacteria through disrupting DNA genome of bacteria or not? The results showed that benzothiazole–semicarbazone and thiosemicarbazone derivatives have significant effect on the plasmid DNA. Considering this in conjunction with the above results, we concluded that the plasma membrane as well as genomic DNA of bacteria was the target of designed analogs.

Furthermore, these studies also revealed a predominant role of the electron-releasing groups in initial binding, bactericidal kinetics, membrane-disrupting ability and DNA-binding ability of designed compounds. For compound **SC06**, good level of membrane hyperpolarization and bactericidal kinetics and high level of PI uptake as well as reduced DNA-binding ability make membrane destabilization and membrane disruption, a probable mode of action. A slower bactericidal kinetics of compound **SC09** might be due to different modes of action as analyzed by lower level of membrane hyperpolarization, low level of PI uptake and preferential DNA binding toward major groove. Compound **TS05** showed high level of membrane hyperpolarization, rapid killing kinetics, high level of PI uptake and excellent DNA-binding ability. Therefore, this compound displayed membrane perturbing as well as intracellular mode of action. Similarly, compound **TS07** gave significant level of hyperpolarization, good bactericidal kinetics, low level of PI uptake and excellent DNA-binding ability, supporting its membrane and genomic DNA targeting mode of action.

Conclusion

This study was focused on the development of new therapeutically active antibacterial agents containing semicarbazone and thiosemicarbazone building blocks. We obtained four lead compounds having broad range of activity with MIC values between 1.56 and 31.2 μg/ml against Gram-positive as well as Gram-negative bacterial strains. The variation in activity of the compounds can be correlated with their lipophilic and electronic behavior. The presence of a benzothiazole ring with semicarbazone/thiosemicarbazone groups along with different R substitutions in compounds enhanced their electronic and lipophilic nature, respectively, resulting in higher capability to penetrate the microorganisms through the lipid layer of the cell membrane. Benzothiazole–semicarbazone and thiosemicarbazone derivatives showed excellent cell selectivity and killed the bacteria by disrupting the membrane. These compounds were also able to alter the electrophoretic mobility of DNA, which was not directly related to activity but may as well be responsible for enhanced potency of these compounds due to intracellular mode of action. Thus, the designed compounds serve as promising leads for further optimization and are advanced experimental candidates with antimicrobial therapeutic potential with respect to earlier reported amides (Singh *et al.*, 2014b).

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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