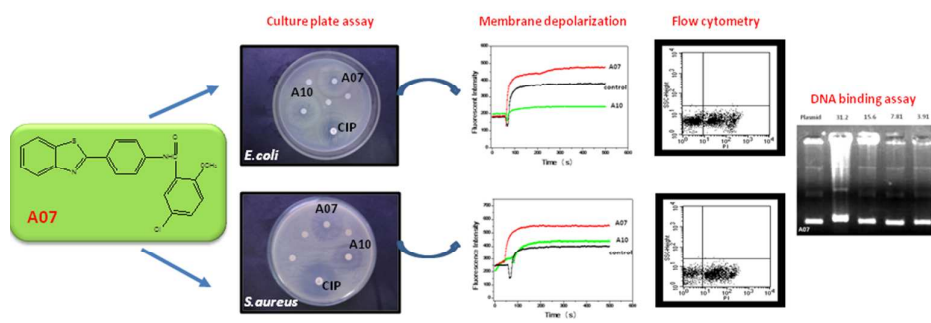




Design, Synthesis and mode of action of some benzothiazole derivatives bearing amide moiety as antibacterial agents

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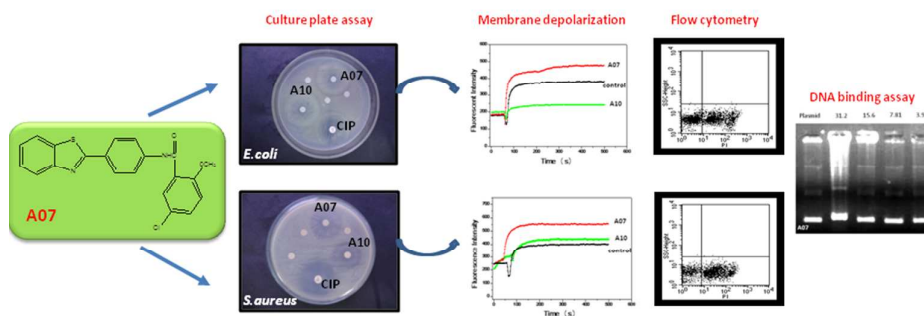
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Schematic outline of most potent compound, benzothiazole bearing amide moiety **A07** showing antibacterial activity and its mode of action.

Abstract

In this study ten benzothiazole derivatives bearing amide moiety were designed, synthesized and evaluated for their antibacterial activity and possible mode of action. Structures of the synthesized compounds were elucidated by spectral data. Four different gram-negative and two different gram-positive bacterial strains were used in antibacterial activity tests. Among all the synthesised compounds, compound **A07** displayed most potent inhibitory activity with minimum inhibitory concentration (MIC) values of 15.6, 7.81, 15.6, 3.91 µg/ml against *S.aureus*, *E.coli*, *S.typhi* and *K.pneumoniae* respectively. Structure–activity relationship (SAR) studies revealed that electronic and lipophilic factors of phenyl ring had a significant effect on the antimicrobial activity of the designed compounds. The benzothiazole bearing amides (**A01-A10**) series exhibited different modes of action based on aryl group substitution as revealed by studies on intact bacterial cells and plasmid DNA. The present study provides us two active compounds (**A07** and **A10**) with membrane perturbing mode of action, intracellular mode of action due to binding with DNA along with potent activity against clinically relevant pathogen *E.coli* and *S.aureus*.

1. Introduction

The growing prevalence of multidrug resistance to therapeutic antibiotics poses a challenge to the identification of novel targets and drugs for the treatment of infectious disease therefore, constitutes a serious public health threat. These organisms possess the ability to withstand attack by antimicrobial drugs currently available, and the uncontrolled rise in resistant pathogens threatens lives. The major concern is the emergence of methicillin resistant strains of *Staphylococcus aureus* (MRSA), fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), fluoroquinolone-resistant *Enterococcus faecalis* (QREF) and vancomycin-resistant *Enterococci* (VRE) results in increased rate of mortality.¹⁻³ Among discovery strategies, the empirical screening of chemical entities that are structurally distinct from clinically established agents represents an effective approach to developing novel antibiotics. Therefore, infections caused by the multidrug resistance bacteria create a pressing need to design and synthesis of more potent and novel antimicrobial agents. Thus, the development of novel targets and drugs for the treatment of infectious diseases are at the forefront.

A review of the recent literature revealed that many effective antimicrobial agents show a heterocyclic moiety within their structure⁴, in particular, substituted benzimidazole, benzoxazole and benzothiazole derivatives received special attention as they belong to a class of compounds with proven utility in medicinal chemistry. They possess different biological properties such as chemotherapeutical, antibacterial, antifungal, and antiviral activities, with low toxicity for the antimicrobial therapeutic use in man.⁵⁻⁷ Structure-activity relationship (SAR) studies carried out on these types of heterocycles have shown that positions 2 and 6 are crucial for antibacterial activity against Gram-positive and Gram-negative bacteria strains.⁸ Our attention was focused on the benzothiazole nucleus. Benzothiazole nucleus is present in a wide variety of therapeutically interesting drugs including antitumor,⁹⁻¹² antimicrobial,¹³⁻¹⁵ schistosomicidal,¹⁶ anti inflammatory,^{17,18} anticonvulsants,¹⁹⁻²¹ antidiabetic,^{22,23} antipsychotic,^{24,25} neuroprotective,²⁶ and diuretic²⁷ activities. Amides, RCONHR' moiety are known to play a pivotal role in molecular recognition, being important component in supramolecular chemical anion sensor²⁸⁻³⁰ technology. Furthermore, in nature, the selective binding with anion substrates such as DNA is achieved via the positional alignment of the amide hydrogen bonds. Many investigations indicated that the presence of hydrogen bonding domain e.g., amide (-CONH-) seems to be valuable in the structures of antimicrobials.³¹ Recently, Patel et al.³² identified an agent (compound 3, Fig. 1), comprises of Benzothiazole-acetamide system which plays an essential role to furnish promising antimicrobial activities.

These findings pave a way for research to be carried out in this area and prompted us to continue our investigation towards synthesis of amide bearing benzothiazole ring system as antimicrobial agents.

The main objective of our program was to investigate how the potency and selectivity against different Gram-positive (*Staphylococcus aureus*, *Enterococcus faecalis*) and Gram-negative (*Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*) bacteria belonging to American Type Culture Collection (ATCC) can be modulated by replacement of hydrogen at position-2 of the heterocyclic nucleus with groups that can generate electronic and electrostatic effects 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 amide moiety.

Over the past few decades, a number of several synthetic and semi synthetic antimicrobial agents have been discovered and used in clinical practice such as sulphonamides, nitrofurans, penicillins, cephalosporins, oxazolidinones, fluoroquinolones, fluconazole, ketoconazole and miconazoles. In spite of availability of potent antimicrobial agents, problems against microbial infections caused by *S.aureus*, *B.subtilis*, *E.coli*, *E.aerogenes*, *A.niger* and *C.albicans* remain unresolved due to their potency, toxicity and resistance development.³³ Although these antibiotics were discovered more than 50 years ago, their mode of action is still not precisely known. We studied the ability of antimicrobial inhibitors to bind with Lipopolysaccharides (LPS), to depolarize both the outer and cytoplasmic membranes, to interact with lipid monolayers, and to kill different bacterial strains. To explore the hypothesis that permeabilization of the cytoplasmic membrane is responsible for killing, we monitored cell viability via FACS (fluorescence assisted cell cytometer) analysis and cytoplasmic membrane permeabilization at the same time. Our data suggests that cytoplasmic membrane depolarization did not correlate well with bacterial cell lethality. We further evaluated the interaction of lead molecules with plasmid DNA to probe into the molecular mode of action.

In continuation to our previous work on the synthesis, and biological properties of various Schiff base-benzothiazole hybrids³⁴ and 4-Thiazolidinones-Benzothiazole conjugates,³⁵ herein we report the design and synthesis of a small library of benzothiazole bearing amide moiety (**A01-A10**, some of them are commercially available)³⁶ to extend the research to achieve potential antibacterial agents by structural optimization, in order to obtain comprehensive SAR indications.

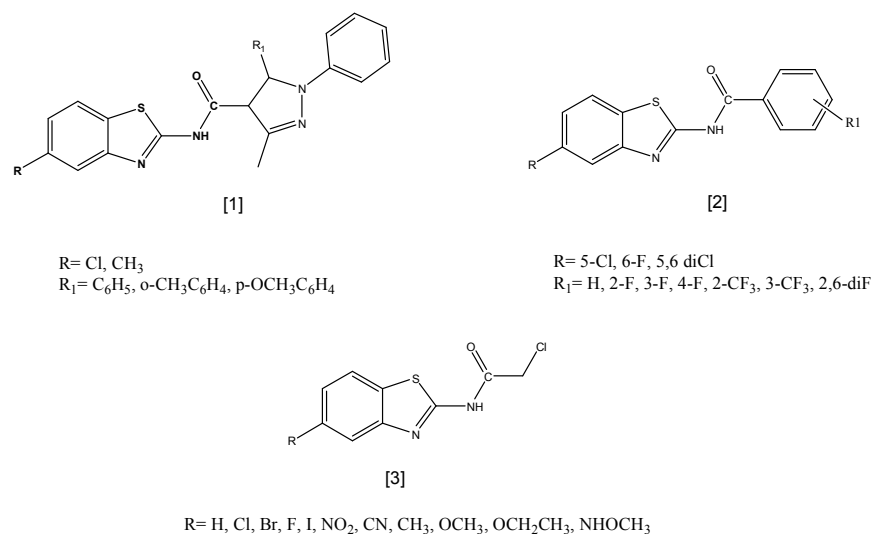


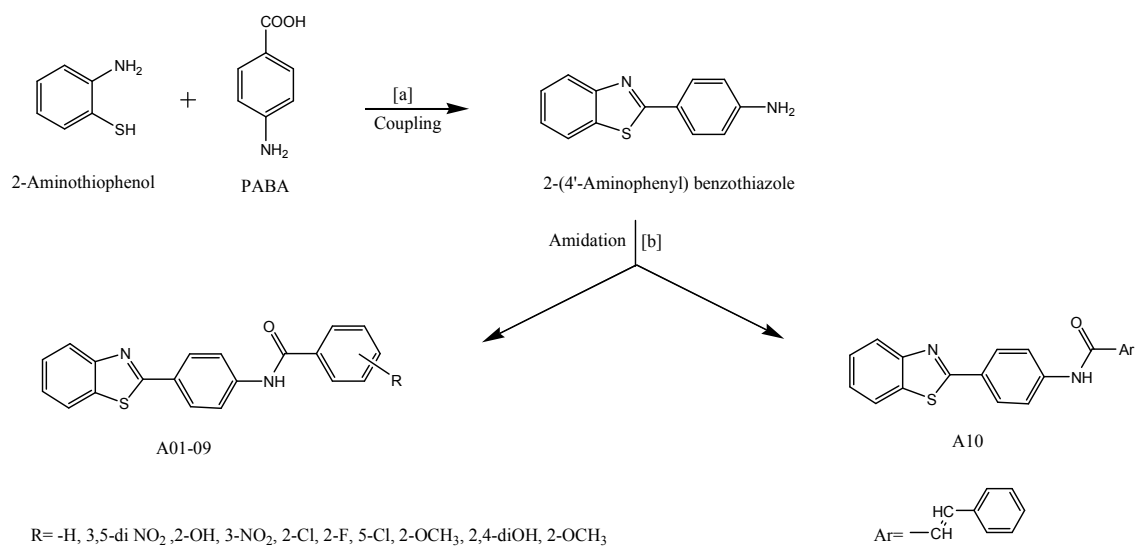
Fig. 1. Recently disclosed Benzothiazole-carboxamido as antibacterial inhibitors.

2. Results and discussion

2.1. Chemistry

The synthesis of compounds (**A01–A10**) followed the general pathway outlined in Scheme 1 and the structures of these compounds are shown in Table 1. The final products benzothiazole bearing amides (**A01–A10**) were prepared in two steps. Firstly, we synthesized key intermediate from 2-amino thiophenol and para amino benzoic acid (PABA) as the starting materials, in a satisfactory yield, according to published procedure³⁷ with minor modifications. Finally, to a solution of key intermediate i.e., 2-(4'-aminophenyl) benzothiazole in acetonitrile, different benzoic acid derivatives were added, together with double equivalent of N,N'-Dicyclohexylcarbodiimide (DCC). DCC is mostly used as a coupling-dehydrating agent in peptide synthesis but we have used it directly in heterocyclic coupling for the amide preparation, inspite of activating aromatic acids to acyl chlorides or esters. Moreover, due to the direct reaction the yield of products is quite appreciable (90-96%). DCC was used to enhance the electrophilicity of carboxylate group by activating it and hence facilitating the nucleophilic attack by the terminal amino group more efficiently and therefore affords a better leaving group (Fig. 2).

The reaction mixtures were refluxed with stirring for 4-10 h to obtain the desired compounds (**A01–A10**). All the IR, Mass, ¹H-NMR, ¹³C-NMR spectral data of compounds (**A01–A10**) were in accordance with the proposed molecular structures. The purity of the synthesized compounds was monitored by TLC and ascertained by elemental analysis.



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

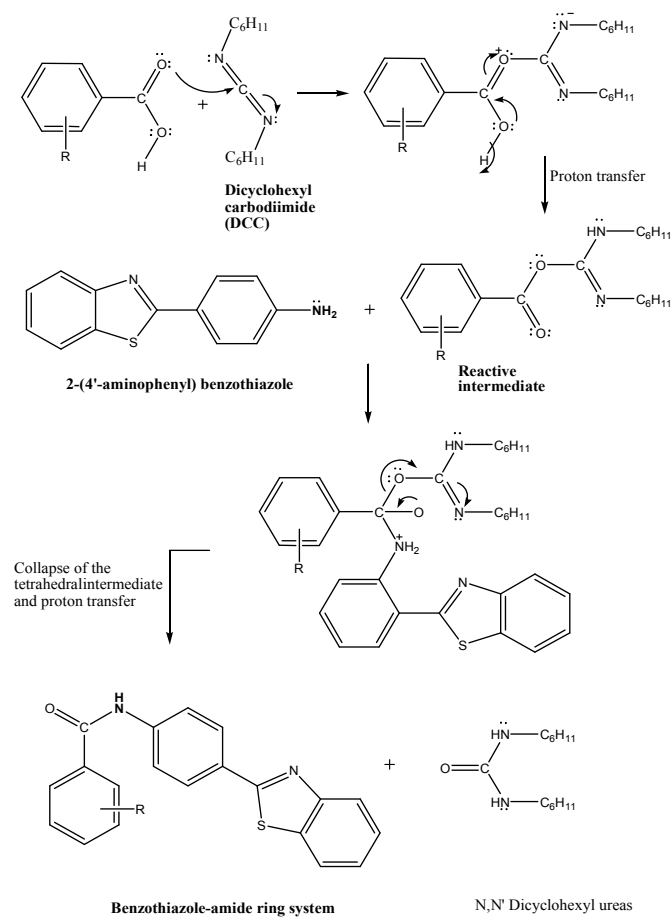
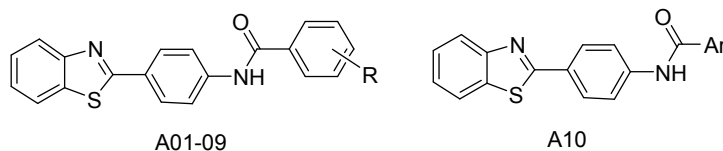


Fig. 2. Proposed reaction mechanism of formation of benzothiazole-amide ring system

Table 1: Structure of compounds **A01-A10**

Compound code	R, Ar
A01	H
A02	3,5-di NO ₂
A03	2-OH
A04	3-NO ₂
A05	2-Cl
A06	2-F
A07	5-Cl, 2-OCH ₃
A08	2,4-diOH
A09	2-OCH ₃
A10	

2.2. Antibacterial activity. The antibacterial activity of the compounds were evaluated against two Gram-positive bacterial strains: *Staphylococcus aureus* (*S.aureus*) ATCC 25323 and *Enterococcus faecalis* (*E. faecalis*) clinical isolate; and four Gram-negative bacterial strains: *Escherichia coli* (*E.coli*) ATCC 35218, *Salmonella typhi* (*S.typhi*) MTCC 3216, *Pseudomonas aeruginosa* (*P.aeruginosa*) ATCC 27893, and *Klebsiella pneumonia* (*K.pneumoniae*) ATCC 31488, using the method recommended by National Committee for Clinical Laboratory Standards (NCCLS).³⁸ The MIC (minimum inhibitory concentration) values of these compounds against the above bacteria are presented in Table 2. Penicillin and standard antibacterial agent ciprofloxacin were also screened under identical conditions for comparison.

The results revealed that most of the compounds exhibited good to moderate antibacterial activity with MIC values ranging between 3.91 and 125 µg/ml in DMSO. Out of the 10 benzothiazole derivatives, compound N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-methoxy-5-chloro-benzamides (**A07**) which is carrying chloro and methoxy groups on aryl ring displayed broad-spectrum antimicrobial activity against all the tested bacterial strains with MIC values of 3.91–62.5 µg/ml. It also displayed the most potent activity with MIC values of 15.6, 7.81, 15.6, 3.91 µg/ml against *S.aureus*, *E.coli*, *S.typhi* and *K.pneumoniae* respectively which was comparable with penicillin and ciprofloxacin with corresponding MIC values of 3.12, 1.56, 1.56, 1.56 µg/ml and 6.25, 6.25, 6.25,

6.25 µg/ml, respectively. Compound N-(4-(benzo[d]thiazol-2-yl)phenyl)-styrene-amides (**A10**) appears to exhibit maximum antibacterial activity against *E.coli* and *P.aeruginosa* (zone of inhibition up to 16–19 mm at concentration of 15.6 µg/ml) while compounds **A01**, **A02**, **A03**, **A04**, **A05**, **A06**, **A08** and **A09** showed moderate activity against few bacterial strains. Among the potent compounds (**A07** and **A10**) against *S.aureus*, it can be clearly seen that the compound **A07** with optimum lipophilicity displayed higher activity. In addition, the above mentioned two derivatives were also found to contribute highest inhibition of *E.coli* at good MIC's.

Subsequently structure–activity relationship (SAR) studies were performed to determine how the substituents on the benzene ring affected the antimicrobial activity. Compounds **A02**, **A04**, **A05**, **A06** with electron withdrawing group (NO₂) and halogen substitutions (Cl, F) were found to be more active than the compounds with electron releasing (OH, OCH₃) groups **A03**, **A08**, **A09** against both the Gram positive and negative strains. In addition, compounds **A05** with chlorine and **A06** with fluorine on phenyl ring appeared with almost same potential inhibitory efficacy against *E. faecalis* and *E.coli* at 31.2 µg/ml of MIC.

Hence, it can be inferred that chloro, fluoro and methoxy substituents bearing derivatives are 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 that electron-donating groups increase the electron density which makes the compounds effective against microorganisms and enhances the antibacterial activity.³⁹ However, high electron density causes more difficult diffusion through the bacteria cell and substantial activity loss may occur.⁴⁰ Thus, for a compound an optimum electron density is inevitable to gain significant antibacterial activity. Hence, the combined electron donating ability of chloro and methoxy groups contributes to the activity of compound **A07** and aryl group bearing compound **A10** reaches an optimum electron density which is important for its significant antibacterial activity.

Table 2: Antibacterial activities of the synthesized benzothiazole bearing amide compounds (**A01-A10**).

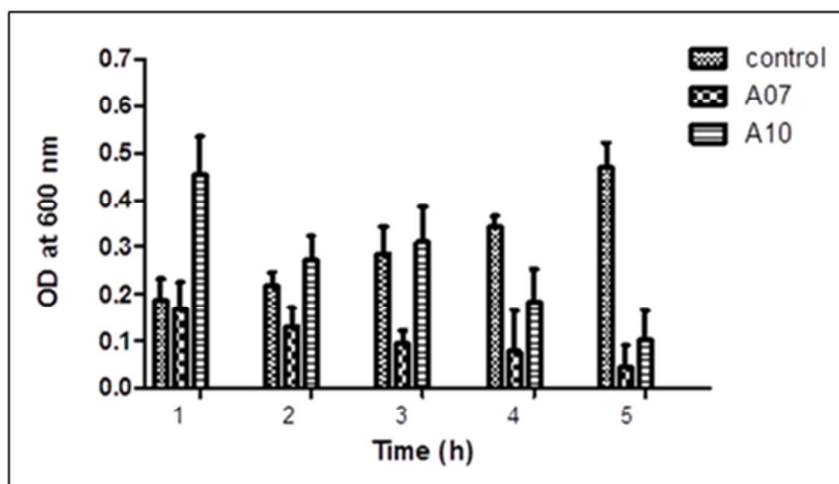
Compound code	Bacteria					
	Gram positive bacteria		Gram negative bacteria			
	<i>S. aureus</i> (ATCC 25323)	<i>E. faecalis</i> (Clinical isolate)	<i>E. coli</i> (ATCC 35218)	<i>S. typhi</i> (MTCC 3216)	<i>K. pneumonia</i> (ATCC 31488)	<i>P. aeruginosa</i> (ATCC 27893)
A01	12-14(62.5)	11-13(62.5)	-	-	10-12(62.5)	-
A02	11-14(62.5)	12-15(31.2)	11-13(62.5)	-	10-13(62.5)	-
A03	<10(125)	-	<10(125)	-	<10(125)	-

A04	11-13(62.5)	-	12-13(62.5)	12-15(31.2)	-	-
A05	-	12-15(31.2)	14-16(31.2)	-	-	-
A06	-	13-15(31.2)	13-15(31.2)	-	-	-
A07	17-19(15.6)	12-15(31.2)	24-25(7.81)	16-18(15.6)	28-31(3.91)	10-12(62.5)
A08	10-13(62.5)	-	11-13(62.5)	<10(125)	<10(125)	-
A09	<10(125)	11-13(62.5)	10-13(62.5)	-	-	-
A10	14-17(31.2)	-	16-19(15.6)	13-15(31.2)	13-15(31.2)	16-19(15.6)
Penicillin	33-35(≥ 3.12)	33-35(≥ 3.12)	36-40(≥ 1.56)	36-40(≥ 1.56)	36-40(≥ 1.56)	36-40(≥ 1.56)
Ciprofloxacin	30-32(≥ 6.25)	28-30(≥ 6.25)	33-35(≥ 6.25)	34-35(≥ 6.25)	29-30(≥ 6.25)	33-35(≥ 3.12)

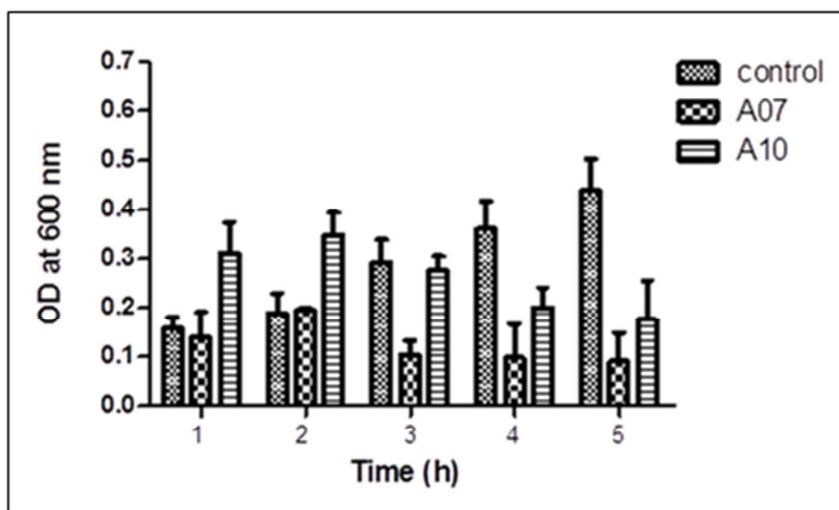
The value of each compound consisted of 'zone of inhibition range (MIC)' of 03 replicates.
Level of significance $p < 0.05$

2.3. Bactericidal kinetics

The bactericidal activity of designed compounds was determined at regular time intervals so as to evaluate the potential killing effects of bacterial cells within minutes at concentrations higher than MIC. In order to monitor the rapidity of mode of action of designed compounds, we incubated compounds **A07** and **A10** with log-phase Gram positive *S. aureus* and Gram negative *E. coli* at 37 °C and monitored the course of change in optical density (OD₆₀₀) at different time intervals. The results showed a significant reduction in numbers of gram positive and negative bacteria (90 to 99%) within the first hour after addition of the compound **A07** at 4 × MIC (Fig. 3). Compound **A10** was found to be less effective as compared to compound **A07** in *E. coli* and *S. aureus* even up to 5 h. At 4 × MIC both 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. At 4 × MIC compound **A07** exhibited the most potent inhibition of growth compared to compound **A10** in both the strains. Thus the designed compounds are capable of inhibiting bacterial growth within hours of initial interactions.



(A)



(B)

Fig. 3. Time dependent killing of (A) *S. aureus* (B) *E. coli* upon treatment with compounds A07 and A10 at $4 \times \text{MIC}$.

2.4. Cytoplasmic Membrane depolarization assay

To study the mode of action of designed analogs the assay for depolarization of the cytoplasmic membrane was done with both Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. A family of fluorescent cyanine dyes has been developed that can be used to monitor membrane depolarization.⁴¹ These dyes lose fluorescence intensity in polarized membranes and become highly fluorescent once polarization is lost. Therefore, the effects of the designed analogs on the membrane of respective strains by using DiSC3(5) dye was determined. In this experiment, if the analogs alter the membrane potential as a result of pore formation/membrane destabilization, an increase in fluorescence intensity is observed. Fig. 4 shows membrane depolarization, by an increase in fluorescence units, as a function of time. An increase of approximately 150 fluorescence unit was observed

using triton 2% as a positive control. Compounds **A07** and **A10** shows similar profile with respect to positive control. At the highest concentration of designed analogs **A07** and **A10** tested (i.e., 4 x MIC), the level of depolarization appeared to reach a maximum at times from about 2 min to 5 min. All of the designed analogs studied here had the ability to depolarize the cytoplasmic membrane of *S.aureus* and *E.coli*; however, compounds with different structures had different concentration-activity profiles (data not shown here). Comparisons were made between the maximum levels of depolarization indicated by the fluorophore to levels of depolarization reached at various time points and concentrations of designed analogs. Compounds **A07** and **A10** completely depolarized the membrane at lower concentrations than those of the other compounds studied.

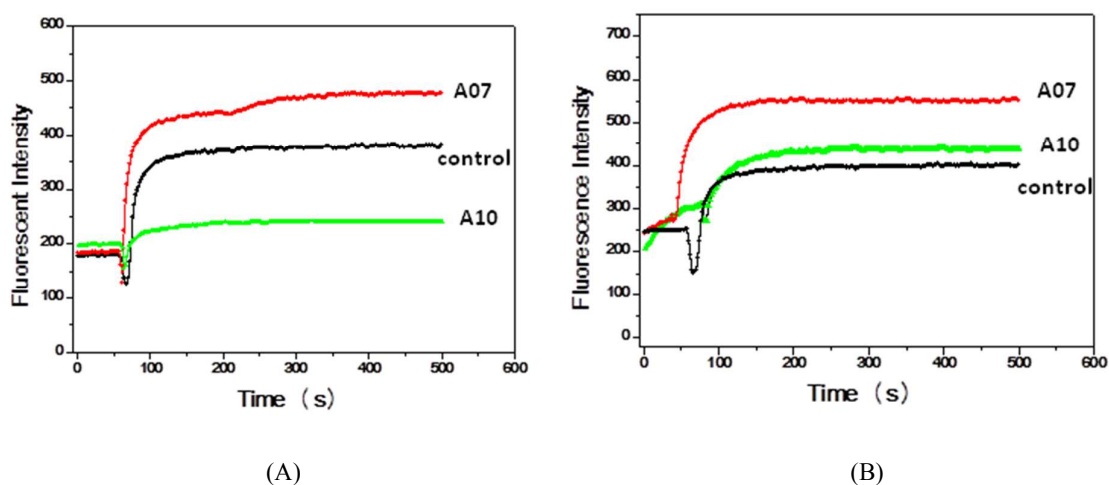


Fig. 4. Membrane depolarization ability of designed compounds on (A) *E.coli* and (B) *S.aureus*. Cultures were grown to log phase ($OD_{600} = 0.05$) and treated with 4xMIC concentration of compounds **A07** and **A10**. Compounds are presented as (red curve) **A07**, (green curve) **A10** and (black curve) Triton 2% as positive control.

2.5. Flow Cytometry analysis

The change in membrane potential may leads to rupture of bacterial cells. So to further explore the permeabilization of the cytoplasmic membrane responsible for cell killing, we monitored cell viability via FACS (fluorescence assisted cell cytometer) analysis by quantifying the amount of DNA released using propidium iodide (PI) as DNA probe. Similar to the fluorometric analysis, flow cytometric assays were performed concurrently with cell viability measurements. The large number of dots in lower right quadrant fluorescing with propidium iodide after exposure to compounds might indicate defective outer-membrane repair and consequent leakiness of more DNA and RNA.⁴²

Consistent with the permeabilization of bacteria, designed analogs **A07** and **A10** induced damages to the membrane organization of *S.aureus* but induced significantly lesser damage to the membrane organization of *E. coli* as indicated by PI staining of the cells following the treatments of these compounds (Fig. 5). However, the number of PI-stained cells in both strains decreased significantly in **A10** analogs. Overall this suggests that the mode of action of designed analogs **A07** and **A10** and related compounds might result from disruption of the membrane potential that is utilized for cellular energy production. However, this is unlikely to be the sole mechanism, since compound **A10** was slightly less active than **A07**. The results further indicated that the ability of **A10** analog to damage the organization of both bacterial cell membranes decreased appreciably due its aryl-substitution. Thus the results showed that the **A10** analog exhibited contrasting lytic activity towards the bacterial cells.

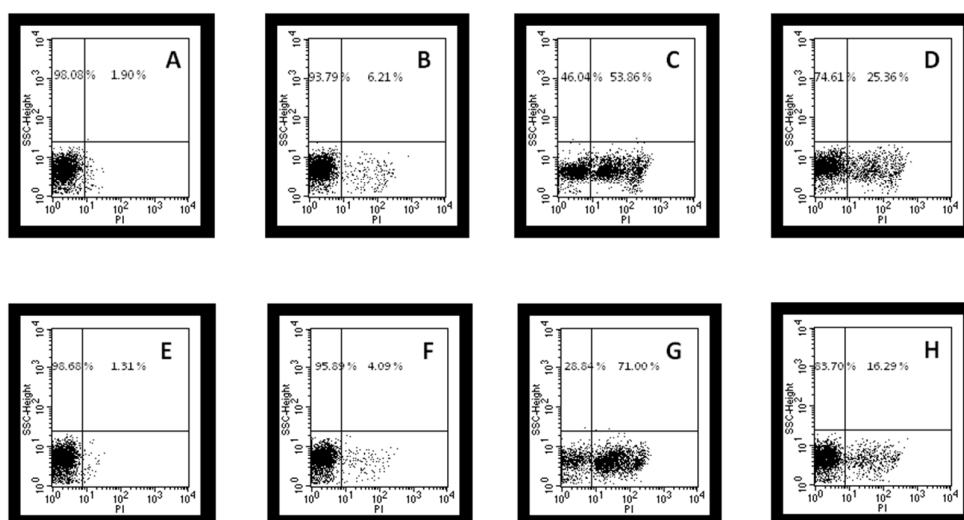


Fig. 5. Determination of compound-induced membrane damage of *E. coli* and *S.aureus* cells by flow cytometric studies. (A–H) PI staining of *E. coli* (A) control without PI treatment (B) control with PI treatment (C) **A07** (D) **A10** and on *S.aureus* (E) control without PI treatment (F) control with PI treatment (G) **A07** (H) **A10** respectively.

2.6. DNA binding

Antimicrobial potency of various classes of DNA binding agents is well reported in the literature.^{43,44} We determined the DNA binding ability of designed analogs **A07** and **A10**. Compound **A07** showed excellent binding with DNA (200 ng) causing retardation at 31.2 $\mu\text{g/ml}$ whereas for **A10** no retardation was observed at all the concentrations (Fig. 6). However it was intriguing that DNA binding was influenced by overall structure of the compounds as chloro and methoxy containing compound **A07** showed the most potent DNA retardation ability as compared to styrene analog **A10**.

Regardless to the actual mechanism of staining, the MG/DAPI technique is of great value to both research cytogeneticists and clinical investigators in the identification of major and minor groove regions of DNA. To probe the interacting site of **A07** with pUC19, the plasmid DNA was treated with DAPI or MG (methyl green)⁴⁵ prior to the addition of **A07**. DAPI (minor groove binder) was added to the reaction mixture, no significant inhibition was observed in the cleavage pattern. Whereas in presence of methyl green (major groove binder), the cleavage pattern was also not affected (Fig. 7). The electrophoretic pattern demonstrates that compound **A07** shows non-specific affinity towards both the major and the minor grooves, and this is only possible if the compound extend through the DNA double helix, as seen from an intercalation binding mode. Thus, in the present study upon comparing DNA binding and antimicrobial potency (compound **A10** showed no DNA binding though was equipotent as **A07**) it was evident that there were no direct correlations between DNA retardation and antimicrobial potency/ mode of action.

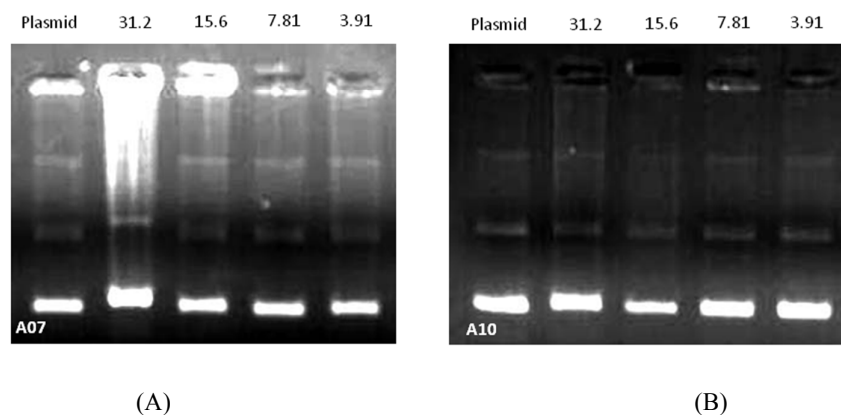


Fig. 6. Agarose gel electrophoresis patterns of pUC19 (200 ng) cleaved by (A) **A07** (31.2–3.91 µg/ml), after 1 h incubation time (concentration dependent) Lane 1: control; Lane 2: 31.2 µg/ml **A07** + DNA; Lane 3: 15.6 µg/ml **A07** + DNA; Lane 4: 7.81 µg/ml **A07** + DNA; Lane 5: 3.91 µg/ml **A07** + DNA, and (B) **A10** (31.2–3.91 µg/ml), after 1 h incubation time (concentration dependent) Lane 1: control; Lane 2: 31.2 µg/ml **A10** + DNA; Lane 3: 15.6 µg/ml **A10** + DNA; Lane 4: 7.81 µg/ml **A10** + DNA; Lane 5: 3.91 µg/ml **A10** + DNA, in buffer (5 mM Tris–HCl/50 mM NaCl, pH 7.2 at 25 °C).

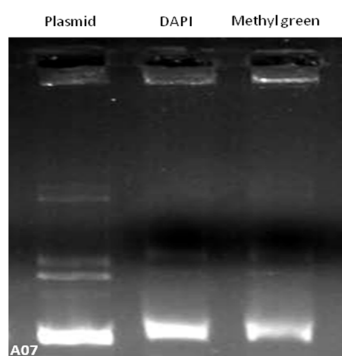


Fig. 7. Agarose gel electrophoresis pattern for the cleavage of supercoiled DNA (200 ng) by **A07** in presence of minor groove (DAPI) and major groove binding agent (methyl green).

3. Conclusion

This work focused on the development of new potentially active antibacterial agents based on benzothiazole-amides compact system. In general, the results of the *in vitro* pharmacological activity are encouraging, as out of 10 benzothiazole bearing amide derivatives, **A07** compound with chloro and methoxy groups on aryl ring showed the most potent antibacterial activity with MIC values of 3.91–62.5 µg/ml against the tested bacterial strains, while compound **A10** with styrene moiety was found almost comparable in few bacterial strains. Based on the results described above, such compounds with benzamide ring can be highlighted as new active leads that may have a multi-targeted mode of action, resulting from its ability to target the bacterial membrane. Such agents are increasingly becoming attractive therapeutic options owing to their potent actions, likely multi-target effects and limited potential for resistance development. Compounds **A07** and **A10** showed excellent activity and membrane perturbing mode of action at concentration higher than MIC. These compounds were also able to alter the electrophoretic mobility of DNA, which was not directly related to activity, may as well be responsible for further enhanced potency of these compounds due to intracellular mode of action. The characterization of benzothiazole bearing amides as a membrane-targeting molecule therefore presents a promising lead for further optimization in an attempt to identify advanced experimental candidates with antimicrobial therapeutic potential.

4. Experimental section

4.1. Materials and measurements

All chemicals (reagent grade) used were commercially available. The reactions were monitored with the help of thin-layer chromatography using pre-coated aluminium sheets with GF₂₅₄ silica gel, 0.2 mm layer thickness (E. Merck) and visualized under ultraviolet (UV) light (254 nm). Melting points were determined in open-glass capillaries on Stuart-SMP10 melting point apparatus and were reported uncorrected. IR absorption spectra were recorded on Shimadzu FTIR-8400s. ¹H NMR spectra were recorded on the Bruker DRX-300 FTNMR and ¹³C NMR spectra were recorded on the JEOL AL300 FTNMR spectrometer operating at 300 MHz, with TMS and solvent signals allotted 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. IR, ¹H NMR, ¹³C NMR and Mass spectra were consistent with the assigned structures. Elemental analyses (C, H, N) were performed on Exeter Analytical Inc., USA, CE-440 elemental analyzer and were within 0.03% of the theoretical values.

4.2. General procedure for the synthesis of compounds [N-(4-(benzo[d]thiazol-2-yl)phenyl)-substituted-benzamides (A01-10)]

A reaction mixture in equimolar quantities of 2-(4'-aminophenyl) benzothiazole (0.01mol) and the appropriate substituted aromatic acids (0.01mol) in acetonitrile (ACN, 20ml) was added together with DCC (2 equiv.). The mixture was refluxed under stirring for 4-10 hr. The completion of reaction was monitored by TLC at appropriate time intervals. Following completion, the reaction mixture was cooled to room temperature. The reaction mixture was filtered through a medium frit. The solution was then given aqueous wash several times, solid thus separated was filtered and dried and the rest was flashed away. The residue was crystallized from ethanol to obtain the target compounds.

4.3. Characterization data of the compounds are given below

4.3.1. N-(4-(benzo[d]thiazol-2-yl)phenyl)benzamides (A01). White solid, mp: 126–128 °C, yield: 91%. IR (KBr, ν_{\max} cm^{-1}): 3309.02 (–NH str.), 1668.12 (C=O str. of amide), 3048.14 (Ar–C–H str.); ^1H NMR (CDCl_3 , 300 MHz) δ (ppm): 7.02–7.98 (m, 13H, Ar–H), 9.48 (s, 1H, –CONH); ^{13}C NMR (DMSO- d_6) δ (ppm): 163.34 (C=O of amide), 155.09 (benzothiazole–C–2), 118.98–138.17 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 331 ($\text{C}_{20}\text{H}_{14}\text{N}_2\text{OS}$, $[\text{M} + \text{H}]^+$). Anal $\text{C}_{20}\text{H}_{14}\text{N}_2\text{OS}$. Calc. for: C, 72.70; H, 4.27; N, 8.48. Found: C, 72.68; H, 4.25; N, 8.48%.

4.3.2. N-(4-(benzo[d]thiazol-2-yl)phenyl)-3,5-dinitro-benzamides (A02). White solid, mp: 122–124 °C, yield: 90%. IR (KBr, ν_{\max} cm^{-1}): 3327.32 (–NH str.), 1627.92 (C=O str. of amide), 3066.92, 3109.35 (Ar–C–H str.) 1342.50 (–NO₂ str.); ^1H NMR (CDCl_3 , 300 MHz) δ (ppm): 7.31–7.87 (m, 11H, Ar–H), 9.13 (s, 1H, –CONH); ^{13}C NMR (DMSO- d_6) δ (ppm): 162.48 (C=O of amide), 156.42 (benzothiazole–C–2), 121.67–140.17 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 421 ($\text{C}_{20}\text{H}_{12}\text{N}_4\text{O}_5\text{S}$, $[\text{M} + \text{H}]^+$). Anal $\text{C}_{20}\text{H}_{12}\text{N}_4\text{O}_5\text{S}$. Calc. for: C, 57.14; H, 2.88; N, 13.33. Found: C, 57.12; H, 2.85; N, 13.31%.

4.3.3. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-hydroxy-benzamides (A03). White solid, mp: 132–134 °C, yield: 94%. IR (KBr, ν_{\max} cm^{-1}): 3312.68 (–NH str.), 1681.42 (C=O str. of amide), 3096.10 (Ar–C–H str.); ^1H NMR (CDCl_3 , 300 MHz) δ (ppm): 7.18–7.79 (m, 12H, Ar–H), 9.32 (s, 1H, –CONH), 10.48 (br, s, 1H, –OH); ^{13}C NMR (DMSO- d_6) δ (ppm): 163.90 (C=O of amide), 154.17 (benzothiazole–C–2), 110.28–135.46 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 347 ($\text{C}_{20}\text{H}_{14}\text{N}_2\text{O}_2\text{S}$, $[\text{M} + \text{H}]^+$). Anal $\text{C}_{20}\text{H}_{14}\text{N}_2\text{O}_2\text{S}$. Calc. for: C, 69.35; H, 4.07; N, 8.09. Found: 69.33; H, 4.08; N, 8.10%.

4.3.4. N-(4-(benzo[d]thiazol-2-yl)phenyl)-3-nitro-benzamides (A04). White solid, mp: 136–139 °C, yield: 91%. IR (KBr, ν_{\max} cm^{-1}): 3325.82 (–NH str.), 1648.12 (C=O str. of amide), 3037.77 (Ar–C–H str.) 1356.50 (–

NO₂ str.); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.24–7.69 (m, 12H, Ar–H), 9.58 (s, 1H, –CONH); ¹³C NMR (DMSO-d₆) δ (ppm): 164.04 (C=O of amide), 157.23 (benzothiazole–C–2), 116.20–148.07 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 376 (C₂₀H₁₃N₃O₃S, [M + H]⁺). Anal C₂₀H₁₃N₃O₃S. Calc. for: C, 63.99; H, 3.49; N, 11.19. Found: 63.97; H, 3.47; N, 11.17%.

4.3.5. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-chloro-benzamides (A05). White solid, mp: 136–139 °C, yield: 92%. IR (KBr, ν_{max} cm⁻¹): 3333.10 (–NH str.), 1647.26 (C=O str. of amide), 3066.92 (Ar–C–H str.), 1070.63 (C–Cl str.); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.26–7.61 (m, 12H, Ar–H), 9.49 (s, 1H, –CONH); ¹³C NMR (DMSO-d₆) δ (ppm): 162.71 (C=O of amide), 156.57 (benzothiazole–C–2), 119.64–145.85 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 365 (C₂₀H₁₃ClN₂O₂S, [M + H]⁺). Anal C₂₀H₁₃ClN₂O₂S. Calc. for: C, 65.84; H, 3.59; N, 7.68. Found: C, 65.82; H, 3.60; N, 7.67%.

4.3.6. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-fluoro-benzamides (A06). White solid, mp: 140–143 °C, yield: 90%. IR (KBr, ν_{max} cm⁻¹): 3309.96 (–NH str.), 1653.05 (C=O str. of amide), 3061.13 (Ar–C–H str.); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.08–7.42 (m, 12H, Ar–H), 9.18 (s, 1H, –CONH); ¹³C NMR (DMSO-d₆) δ (ppm): 162.88 (C=O of amide), 156.78 (benzothiazole–C–2), 115.83–143.86 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 349 (C₂₀H₁₃FN₂O₂S, [M + H]⁺). Anal C₂₀H₁₃FN₂O₂S. Calc. for: C, 68.95; H, 3.76; N, 8.04. Found: C, 68.93; H, 3.75; N, 8.05%.

4.3.7. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-methoxy-5-chloro-benzamides (A07). White solid, mp: 150–143 °C, yield: 96%. IR (KBr, ν_{max} cm⁻¹): 3319.60 (–NH str.), 1637.62 (C=O str. of amide), 3061.13 (Ar–C–H str.), 2852.81 (C–H str., OCH₃ group); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.11–7.78 (m, 11H, Ar–H), 9.39 (s, 1H, –CONH); ¹³C NMR (DMSO-d₆) δ (ppm): 162.49 (C=O of amide), 58.11 (OCH₃), 153.01 (benzothiazole–C–2), 122.36–136.75 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 379 (C₂₁H₁₅ClN₂O₂S, [M + H]⁺). Anal C₂₁H₁₅ClN₂O₂S. Calc. for: C, 66.57; H, 3.99; N, 7.39. Found: C, 66.54; H, 3.96; N, 7.37%.

4.3.8. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2,4-dihydroxy-benzamides (A08). White solid, mp: 150–143 °C, yield: 96%. IR (KBr, ν_{max} cm⁻¹): 3353.18 (–NH str.), 1664.73 (C=O str. of amide), 3085.10 (Ar–C–H str.); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.22–7.86 (m, 11H, Ar–H), 9.56 (s, 1H, –CONH), 10.48, 9.86 (s, 2H, –OH); ¹³C NMR (DMSO-d₆) δ (ppm): 162.79 (C=O of amide), 155.44 (benzothiazole–C–2), 121.78–145.26 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 363 (C₂₀H₁₄N₂O₃S, [M + H]⁺). Anal C₂₀H₁₄N₂O₃S. Calc. for: C, 66.28; H, 3.89; N, 7.73. Found: C, 66.24; H, 3.86; N, 7.71%.

4.3.9. N-(4-(benzo[d]thiazol-2-yl)phenyl)-2-methoxy-benzamides (A09). White solid, mp: 168–170 °C, yield: 95%. IR (KBr, ν_{max} cm⁻¹): 3322.68 (–NH str.), 1647.23 (C=O str. of amide), 3054.13 (Ar–C–H str.),

2850.11 (C–H str., OCH₃ group); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.28–7.91 (m, 12H, Ar–H), 9.26 (s, 1H, –CONH); ¹³C NMR (DMSO-d₆) δ (ppm): 163.25 (C=O of amide), 56.15 (OCH₃), 156.23 (benzothiazole–C–2), 122.46–146.35 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 361 (C₂₁H₁₆N₂O₂S, [M + H]⁺). Anal C₂₁H₁₆N₂O₂S. Calc. for: C, 69.98; H, 4.47; N, 7.77. Found: C, 69.96; H, 4.46; N, 7.75%.

4.3.10. N-(4-(benzo[d]thiazol-2-yl)phenyl)-styrene-amides (A10). White solid, mp: 147–149 °C, yield: 92%. IR (KBr, ν_{max} cm⁻¹): 3317.40 (–NH str.), 1653.82 (C=O str. of amide), 3087.42 (Ar–C–H str.); ¹H NMR (CDCl₃, 300 MHz) δ (ppm): 7.26–7.71 (m, 14H, Ar–H), 9.58 (s, 1H, –CONH), 6.72, 6.77 (dd, –CH=CH); ¹³C NMR (DMSO-d₆) δ (ppm): 164.18 (C=O of amide), 156.30 (benzothiazole–C–2), 116.36–138.15 (Aromatic–C, C4–C9 C1'–C6' C1''–C6''); MS (m/z, %): 357 (C₂₂H₁₆N₂OS, [M + H]⁺). Anal C₂₂H₁₆N₂OS. Calc. for: C, 74.13; H, 4.52; N, 7.86. Found: C, 74.12; H, 4.50; N, 7.85%.

4.4. *In vitro* antibacterial activity

Antimicrobial activity was determined by agar disc diffusion method and was evaluated according to the guidelines of National Committee for Clinical Laboratory Standards (NCCLS, 1997).³⁸ *In vitro* activities of the compounds were tested in nutrient broth (NB) for bacteria by serial dilution method. Seeded broth (broth containing microbial spores) was prepared in NB from 24 h-old bacterial cultures on nutrient agar (Hi-media) at 37 ± 1 °C. The bacterial suspension was adjusted with sterile saline to a concentration of 10⁵ CFU ml⁻¹.⁴⁶ 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 compounds (20 ml/disc) placed on the plates. Ciprofloxacin (5 mg/disc, Hi-Media) was used as positive control and dimethyl sulfoxide (DMSO) was used as negative control. All the inoculated plates were incubated at 37°C and results were evaluated after 24 h for bacteria. The zones of inhibition were measured in millimetres. The tested compounds and reference drugs were prepared by two-fold serial dilutions to obtain the required concentrations of 125, 62.5, 31.2, 15.6, 7.81, 3.91 µg/ml. All the dilutions were made with distilled water. All determinations were done in triplicates and the average was taken as final reading. At the end of the incubation period, the MIC values were determined. The lowest concentration of the compound that prevented visible growth (turbidity on liquid media) was considered as MIC.

4.5. Bactericidal kinetics

Bactericidal kinetics were performed as described previously.⁴⁷ Overnight cultures of *S. aureus* ATCC 25323 and *E. coli* ATCC 35218 were grown in fresh MHB up to log phase. For determining the time course of killing activity 100 µL of fresh MHB was added to all wells of the 96 well-plate. Then 90 µL of approximately 10⁵

CFU ml⁻¹ were added to the wells of the 96-well plates of plate reader (4 wells for a single concentration). Then 10 µL of appropriate concentrations of test compounds **A07** and **A10** corresponding to 4 × MIC were added to the wells. The plates were incubated at 37 °C at 200 rpm. Absorbance of the plates was read at 600 nm at various time points at 0, 1, 2, 3, 4 and 5 h. The experiment was repeated on three different days and values are plotted as mean ± SD.

4.6. Cytoplasmic-membrane depolarization assay

The depolarization of the cytoplasmic membrane of *S. aureus* ATCC 25323 and *E.coli* ATCC 35218 by the designed compounds **A07** and **A10** was determined using the membrane potential-sensitive cyanine dye DiSC3(5). Bacterial cultures were grown overnight in LB media at 37°C. Cells were harvested by centrifugation and washed in a buffer containing 5 mM HEPES–20 mM glucose buffer (pH 7.2) to an optical density of 0.06. After three washings, pellets were resuspended in the same buffer. This cell suspension was incubated with 100 mM KCl (to equilibrate cytoplasmic and external K⁺ concentration) and DiSC3(5) at a concentration of 1µM. The dye was allowed to incorporate for 1hr at room temperature, When the fluorescence level (excitation and emission wavelength set at 622 and 670 nm, respectively) of the bacterial suspension became stable, (approximately 90% reduction in fluorescence due to DiSC3(5) uptake and quenching in the cell in response to an intact membrane potential), different amounts of each of the compounds were added to these suspensions in order to record the membrane depolarization of bacterial cell membrane. A 400µl aliquot of cell suspension was placed in a cuvette, and the desired concentration of compounds was added. Fluorescence was monitored with a Perkin Elmer Life Sciences LS 50-B spectrofluorimeter (Perkin-Elmer 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.

4.7. Flow Cytometry analysis

The membrane damage of *S. aureus* ATCC 25323 and *E.coli* ATCC 35218 was determined by staining the cells with propidium iodide after the treatment with the designed compounds **A07** and **A10** at 37 °C for 1 hr. These cells were then analyzed by flow cytometry in the form of dots plot with respect to the control cells, not treated with any compounds and cells treated with propidium iodide without preincubation with tested compounds. In order to check the membrane integrity of bacteria, the cells at mid-log phase were incubated with designed compounds **A07** and **A10** for 1 hr at 37 °C with constant shaking. The cells were collected by centrifugation, washed two times with PBS and incubated further with propidium iodide 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. Compound-

induced damage of bacterial cells was then analyzed by flow cytometry (excitation and emission wavelength set at 488 and 617nm, respectively for propidium iodide).

4.8. DNA binding assay

Gel retardation experiments were performed by agarose gel electrophoresis as described previously.^{43,44} Briefly, 200 ng of pUC19 plasmid DNA was mixed with increasing amounts of test compounds **A07** and **A10** in 20 μ L of binding buffer (5% glycerol, 10 mM Tris- HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 20 mM KCl, and 50 μ g/ml bovine serum albumin). The samples were incubated for 1h at 37 °C. A loading buffer containing 25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol was added and electrophoresis was carried out at 60 V for 1h in Tris-HCl buffer using 1 % agarose gel containing 1.0 μ g/ml ethidium bromide (EB).⁴⁸ The illuminated gel was photographed by Alpha Innotech Corporation Instrument. The reaction was also monitored upon addition of various groove binders-methyl green, and DAPI.

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