Design and synthesis of 4-[4-formyl-3-(2-naphthyl)pyrazol-1-yl] benzoic acid derivatives as potent growth inhibitors of drug-resistant Staphylococcus aureus

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Abstract
We report the synthesis and antimicrobial studies of a new series of naphthyl-substituted pyrazole-derived hydrazones. Many of these novel compounds are potent growth inhibitors of several strains of drug-resistant bacteria. These potent compounds have inclined growth inhibitory properties for planktonic Staphylococcus aureus and Acinetobacter baumannii, and its drug-resistant variants with minimum inhibitory concentration (MIC) as low as 0.78 and 1.56 µg ml⁻¹, respectively. These compounds also show potent activity against S. aureus and A. baumannii biofilm formation and eradication properties. Time Kill Assay shows that these compounds are bactericidal for S. aureus and bacteriostatic for A. baumannii. The probable mode of action is the disruption of the bacterial cell membrane. Furthermore, potent compounds are nontoxic to human cell lines at several fold higher concentrations than the MICs.

Introduction
Antibiotic resistance is one of the leading health crises of our time. It can affect people at any stage of life. Antibiotic-resistant infections often lead to longer hospital stays, disability, and even death. Each year more than 2.8 million people get an antibiotic-resistant infections and more than 35,000 die from this problem [1]. Staphylococcus aureus is a Gram-positive bacterium. Methicillin-resistant S. aureus (MRSA) is resistant to β-lactam antibiotics including methicillin. S. aureus and MRSA causes nearly 325,700 infections and more than 10,600 deaths every year in the United States alone. Carbapenem-resistant Acinetobacter baumannii, a Gram-negative bacterium, causes thousands of nosocomial infections and it has been classified as an urgent threat bacterium (https://www.cdc.gov/drugresistance/pdf/threats-report/mrsa-508.pdf).

Bacterial biofilms are small bacterial communities held together by an extracellular matrix. The biofilm matrix makes bacteria tolerant to harsh conditions and more resistant to antibacterial treatments [2, 3]. An estimated 17 million new biofilm-associated infections are reported each year, resulting in up to 550,000 fatalities. Biofilm forming bacteria cause ~80% bacterial infections. In addition, the presence of bacterial biofilm in medical devices is a major concern and causes numerous fatal infections [4]. The Infectious Disease Society of America has designated the most problematic antibiotic-resistant bacterial species as ESKAPE pathogens (Enterococcus faecium, S. aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa, and Enterobacter species) owing to their prominence as a cause of both nosocomial and community-acquired infections and the lack of effective antibiotics to combat these infections. Four of these species (S. aureus, P. aeruginosa, A. baumannii, and K. pneumoniae) are also among the most common causes of biofilm-associated infections, particularly in healthcare settings [5]. Due to the importance of controlling biofilms to manage microbial infections, there
have been increased efforts towards the inhibition of biofilm formation by small molecules [4, 6].

Naphthalene derivatives are known for their wide range of biological activities including antimicrobial properties [7, 8]. Several naphthalene containing drugs have been approved by the Food and Drug Administration and being marketed as therapeutics such as, nafcillin [9], naftifine [10, 11], and tolnaftate [12] to treat microbial infections. There are many naphthalene-derived bioactive phytoconstituents present in nature such as rifampicin (antitubercular agent) [13]. Similarly, the pyrazole ring is present as the core structure of several leading drugs such as celecoxib, a potent anti-inflammatory [14], the antidepressant agent fezolamine [15], the antiobesity rimonabant [16], and difenamizole [17] (an analgesic). Pyrazole derivatives exhibit several biological activities including antibacterial properties [18, 19]. In addition, hydrazone derivatives have a wide variety of biological and pharmacological properties [20–22].

We have reported the synthesis and antimicrobial studies of phenyl-substituted pyrazole-derived hydrazone derivatives as potent growth inhibitors of MRSA and A. baumannii [23, 24]. Fluoro-substitutions in the phenyl ring have increased the activity of the resultant molecules [25, 26]. Replacement of the phenyl ring with the coumarin moiety also has shown significant potency of the molecules against MRSA, A. baumannii, and other tested strains [27]. Based on the literature precedence and our experience on pyrazole derivatives as potent antimicrobial agents, we designed and synthesized naphthalene-substituted pyrazole-derived hydrazones. Excitingly, we found several molecules in this series as potent anti-MRSA agents.

Materials and methods

General consideration

All of the reactions were carried out under an air atmosphere in round-bottom flasks. Commercially available solvents, reagents, and the substrates were bought from Fisher Scientific (Hanover Park, IL, USA.) and Oakwood chemical (Estill, SC, USA). 1H and 13C NMR spectra were recorded with a Varian Mercury −300 and 75 MHz, respectively, in DMSO-d6 solvent with TMS as internal standard. ESI-FTMS mass spectra were recorded in Bruker Apex II-FTMS system. Growth media and bacterial broth were purchased from Fisher Scientific or ATCC. Following bacterial strains are used to evaluate the potency of the novel compounds. S. aureus ATCC 25923, S. aureus BAA-2312, S. aureus ATCC 33591, S. aureus ATCC 700699, S. aureus ATCC 33592, Staphylococcus epidermidis 700296, Bacillus subtilis ATCC 6623; A. baumannii ATCC 19606 (type strain), A. baumannii ATCC BAA-1605, A. baumannii ATCC 747, Escherichia coli ATCC 25922, Enterobacter aerogenes ATCC 13048, P. aeruginosa 27833, and K. pneumoniae ATCC 700603.

General procedures

Synthesis of the pyrazole-derived aldehyde (4)

The aldehyde derivative (4) was synthesized according to our reported procedure [23]. The reaction of 4-hydrazinobenzoic acid (1, 10 mmol, 1.521 g) with 2-acetonaphthalene (2, 10.5 mmol, 1.787 g) was performed in ethanol (Scheme 1). Refluxing the reaction mixture for 8 h led to obtaining the hydrazone derivative (3). The solvent was evaporated under reduced pressure at 60 °C to get the solid product, which was used for further reaction without isolation or purification. The hydrazone derivative (3) was dissolved in N,N-dimethyl formamide (DMF, 30 ml) and the flask was sealed with a rubber septum. The solution was stirred at 0 °C in an ice bath. After 10 min, phosphorus oxychloride (POCl3, 10 mmol, 5.43 ml) was added dropwise to form the Vilsmeier reagent. The reaction mixture was heated at 90 °C for 8 h. After the completion of the reaction, the reaction mixture was poured onto ice and stirred for 12 h to obtain a solid product in very good yield, which was filtered and washed with water followed by drying the final product under vacuum.

Synthesis of hydrazone derivatives (5–34)

Novel naphthalene-derived hydrazones were synthesized by reacting the aldehyde derivatives (4, 1 mmol, 342 mg) with commercially available substituted hydrazines (1.1 mmol) in ethanol and refluxing for 8 h (Scheme 1). Sodium acetate (1.1 mmol, 0.088 g) and acetic acid were added in case of the hydrochloride salt of hydrazine derivatives. The resulting product was filtered and washed with ethanol (~15 ml) followed by washing with water (~20 ml) to get the pure product.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The prepared compounds were weighed and dissolved in dimethyl sulfoxide (DMSO) to make concentration of 2 mg ml−1. Overnight bacterial cultures on blood agar plates were used to prepare bacterial suspensions at the 0.5 McFarland standard in PBS. Suspensions were diluted in Mueller Hilton broth to make a final concentration of approximately 1 × 105 cfu ml−1. Resazurin dye was added for the detection of bacterial viability (1:1000 dilution of a 0.1% w/v stock solution in sterile PBS).
Microdilution plates (sterile 96-well polystyrene flat bottom plates) were prepared by diluting compounds 1:2 in columns from top to bottom using DMSO as diluent. Bacteria suspension (195 µl) was combined with 5 µl of dissolved compound in each well. This 1:40 dilution of compounds produced starting concentrations of 50 µg ml\(^{-1}\) in the first well to 0.4 µg ml\(^{-1}\) in the eighth well and kept the DMSO concentration at 2.5%, below a cytotoxic level. Microplates were incubated at 35 °C for 16–20 h and results were observed and recorded. Positive wells remained blue/nonturbid (growth inhibition by compounds) and negative wells were pink/turbid due to bacterial growth (no inhibition). The lowest concentration of a compound that completely inhibited the growth (blue color/nonturbid) were recorded as the MIC.

For some compounds, MIC determination was followed by MBC determination. Five nonturbid (blue) wells in a column above and including the MIC well were diluted (100, 101, and 102) and plated in blood agar plates by using the 6 × 6 drop plate method as described by Chen et al. [28]. Plates were incubated and the number of colonies in each 5 µl spot were counted to determine the percentage of surviving cells compared to the initial cfu/ml. The MBC was defined as the lowest concentration that reduced the bacterial concentration by at least 99.9%.

**Time Kill assay**

Time kill assay was performed using two strains of bacteria (A. baumannii ATCC 19606 and S. aureus ATCC 33599)
using our most potent compounds. First, growth curves for each bacterium were plotted by using methodology described by Foerster et al. [29] using optical density for estimating the log phase for each bacterium. Once bacteria were in log phase after shaking in Muller Hilton broth II for around 2 h, they were diluted in sterile fresh warm broth to a final concentration of ~3 × 10⁶ cfu ml⁻¹ and exposed to compounds at 4 × MIC for A. baumannii ATCC 19606 and 4 × MIC for S. aureus ATCC 3599 (in triplicates). Colistin and vancomycin (VC) served as positive controls for Acinetobacter and Staphylococcus species, respectively. Bacteria in broth (195 μl) plus compound (5 μl) were inoculated in 8 different columns for each compound in 96-well polystyrene flat bottom plates and marked 0, 2, 4, 6, 8, 10, 12, and 24 h, respectively. These columns were used to take samples for the respective time points for viable counts. Each plate was incubated at 35 °C and samples were taken at 2-h intervals for viable counts.

A viable count for each sample was performed using the 6 × 6 drop plate method as described above (Chen et al. [28]). Blood agar plates for viable count were incubated for 18–24 h at 35 °C prior to counting. Bacterial concentrations in log cfu ml⁻¹ were calculated from colonies counts and their respective dilutions and plotted against the incubation time.

**Biofilm**

*S. aureus* strains in our collection were screened to determine which produced the greatest measurable amount of biofilm under our laboratory conditions using methodology proposed by Sabrina et al. with some modifications [30]. The strains were grown on blood agar plate and incubated at 35 °C overnight. From isolated colonies, a bacterial suspension in PBS was prepared using the 0.5 McFarland standard. The bacterial suspension was diluted 1:100 into yeast extract casamino acid broth to give an approximate final concentration of 1 × 10⁶ cfu/ml. Aliquots of this suspension (200 μl) was transferred to wells of a 96-well polystyrene flat bottom plate. The plates were incubated at 35 °C for 24 h. Biofilm formation was quantified using the method proposed by Halicki et al. with some modifications [31]. After incubation, the contents of wells were removed and wells were washed with 1xPBS solution for three times to remove any planktonic cells. The plate was dried in an oven at 60 °C for about 15 min and 0.1% (w/v) crystal violet (250 μl) was added to each well and left for 15 min for staining biofilms. Excess crystal violet was removed by draining and washing three times with deionized water, and the plate was again dried in oven for 10 min. After drying, 33% acetic acid (250 μl) was added to each well to dissolve the stained biofilm. The optical density of the solubilized crystal violet in each well was measured at 620 nm using a Bio Tek™ Cytation™ five-plate reader. Bacteria were grouped according to the magnitude of the absorbance, and strains being classified as strong, moderate or weak biofilm former. Only strong biofilm formers were used for further studies.

**Biofilm inhibition assay**

In this assay, the biofilm forming strain *S. aureus* ATCC 25923 was grown with different concentrations of naphthalene derivatives prior to the formation of biofilm to determine if the compounds were capable to inhibit biofilm formation. Overnight blood agar culture of bacteria was suspended in PBS solution to maintain 0.5 McFarland standard and was diluted 1:100 into yeast extract casamino acid broth to give an approximate final concentration of 1 × 10⁶ cfu ml⁻¹. Bacterial broth suspension (195 μl) was transferred to each well in 96-well polystyrene flat bottom plate. 2 × MIC, MIC, and 0.5 × MIC of the compounds (5 μl) were added to wells in triplicate along with broth only and bacteria along with DMSO controls and plates were incubated at 35 °C for 24 h. After incubation, washing, drying, and staining followed by resolubilization of crystal violet. Quantification was performed by reading plates in the plate reader at 620 nm wavelength. The compounds with best MIC values for planktonic strains of *S. aureus* were chosen for this assay.

**Biofilm destruction assay**

This assay was performed to test whether our compounds could destroy preformed biofilm in vitro. For this assay, 195 μl bacterial broth culture was inoculated in each well of 96-well plate and incubated overnight at 35 °C for 24 h to allow the formation of enough biofilm along with triplicate wells with the growth media only. After incubation, well contents were carefully removed, and wells were washed with sterile 1xPBS solution to remove any unadhered cells. Next, 195 μl sterile Yeast extract casamino acid broth was added to each well with 5 μl of 2 × MIC, MIC, and 0.5 × MIC concentrations of compounds or DMSO in triplicate and the plate was incubated at 35 °C for 24 h. After incubation, washing, drying, staining, dissolving stained dye and measuring optical density in a plate reader were performed as described above.

**Processing of data**

As these biofilm assays were performed in triplicates mean and standard deviation of plate reading data were processed. Results were expressed as percentage by using the formula:

\[
\text{Percentage biofilm inhibition/destruction} = \left(1 - \frac{\text{OD}_{\text{compound}} - \text{OD}_{\text{DMSO}}}{\text{OD}_{\text{broth}} - \text{OD}_{\text{DMSO}}} \right) \times 100
\]
hydrazinobenzoic acid (by 1H and 13C NMR spectroscopy and high resolution mass
substituted hydrazines in ethanol in very good overall yields
reaction of the aldehyde derivative (naphthalene-derived hydrazones were synthesized by the
molecule, ODbroth = OD of well with bacteria broth + DMSO.
The data were processed and represented in graphical form in Microsoft Excel for Office 365 MSO.

Results and discussions

Chemistry

In our efforts to get potent pyrazole-derived hydrazone as antimicrobial agents, we designed and synthesized naphthyl-substituted pyrazole-derived hydrazones. To synthesize these molecules, the starting material—the aldehyde derivative (4)—was synthesized in multigram scale by reacting hydrazinobenzoic acid (1) with 2-acetylnaphthalene (2) in ethanol to form hydrazone (3) followed by treatment with POCl3/DMF in a one-pot reaction (Scheme 1). Novel naphthalene-derived hydrazones were synthesized by the reaction of the aldehyde derivative (4) with commercially substituted hydrazines in ethanol in very good overall yields (66–91%). These new compounds have been characterized by 1H and 13C NMR spectroscopy and high resolution mass spectrometry.

Reaction of hydrazine with the aldehyde derivative (4) afforded the product (5) in 83% yield. N-Phenyl substituent (6) was formed in very good yield (89%). N,N-Disubstituted hydrazone derivatives reacted smoothly to give corresponding products (7, 8, 9, and 10) in efficiently. Electron-donating group on the aryl ring of hydrazone provided the desired product (11) in 91% yield as well as ethyl- and methoxy-derivatives (12 and 13) were formed in very good yield. Similarly, electron-withdrawing groups on the phenyl ring of hydrazone such as, fluoro-, chloro-, and bromo-substituted were reacted with aldehyde derivative (4) to obtain the pure products (14, 15, 16, 17, and 18). Dihalo-substituted naphthyl-derived hydrazone products (19, 20, 21, and 22) were formed in 86%, 83%, 81%, and 84% yields, respectively. Reaction of tetrafluoro and pentafluoro-substituted hydrazone gave the desired compounds (23 and 24) in very good yield. Strong electron-withdrawing substituents on the phenyl ring also reacted with aldehyde derivative (4) and delivered products (25, 26, and 27) in 80%, 79%, and 89% yields, respectively. Carboxylic acid-substituted naphthyl-derived hydrazone product (28) was formed in very good yield. The reaction N,N-dimethyl hydrazone substituent with corresponding aldehyde derivative (4) gave the desired product (29) in 89% yield. Aliphatic N-heterocyclic hydrazine derivatives also reacted to give products (30 and 31) in good yields. Triazole and imidazoline naphthyl-derived hydrazone products (32 and 33) were formed in very good yield. Methyl hyrazinocarboxylate substituent gave the pure product (34) in 81% yield. All the synthesized compounds are novel and stable at ambient condition.

Antimicrobial studies

All the synthesized derivatives were tested for their growth inhibition ability against Gram-positive and Gram-negative bacterial strains. Several of the designed molecules were found to be the potent growth inhibitors of several tested strains specifically S. aureus and A. baumannii (Table 1). The unsubstituted hydrazone (5) and phenyl-substituted (6) derivatives showed moderate activity against S. aureus strains with the MIC value of 25 µg/ml. The N-phenyl-N-methyl derivative (7) showed excellent potency against the methicillin-resistant strains of Staphylococcus (S. aureus ATCC 700699 (Sa99), and S. aureus ATCC 33592 (Sa92)) with MIC values as low as 1.56 µg ml−1, but no activity against other Gram-positive strains. N,N-Diphenyl substituted hydrazone (8) found to be an excellent antimicrobial agent for the tested Gram-positive strains. This novel molecule showed the growth inhibition of antibiotic susceptible strain (S. aureus ATCC 25923 (Sa23)) and two MRSA strains with an MIC value as low as 1.56 µg ml−1. It also inhibited the growth of S. epidermidis with an MIC value of 1.56 µg ml−1. N,N-Diphenyl substituted hydrazone (8) is also a moderate growth inhibitor of B. subtilis. N-Benzyl-N-phenyl derivative (9) showed similar activity against the tested strains but weaker activity against B. subtilis. Substituted N-phenyl derivatives with electron-donating groups such as methyl (11), ethyl (12), and methoxy (13) reduced the potency drastically. Fluoro substitution (14 and 15) showed very good activity against some the Staphylococcus strains with MIC values as low as 1.56 µg ml−1. Chloro (16) and bromo (17) substituted compounds also showed similar activity against the tested strains. The 4-bromo derivative (18) showed better activity against Sa99 than other tested Gram-positive strains with an MIC value as low as 0.78 µg ml−1. Difluoro (19) and dichloro (20) derivatives showed enhanced activity against all the tested strains. Mixed halide derivatives (21 and 22) are also potent inhibitors of tested Gram-positive strains. Polyfluorinated derivatives such as tetrafluoro (23) and pentafluoro (24) showed very potent activity against some of the strains with an MIC value as low as 0.78 µg ml−1, but no activity against other strains. Trifluoromethyl substituted derivative (25) showed broad and potent activity against the tested strains with MICs value as low as 0.78 µg ml−1 for three S. aureus strains. Very strong electron-withdrawing groups such as cyano (26), nitro (27), and carboxylic acid (28) eliminated the
activity of the resultant compounds. \(N,N\)-Dimethyl derivative (29) did not show any activity against the tested bacterial strains. Aliphatic and aromatic heterocycles and other derivatives (30, 31, 32, 33, and 34) failed to show any remarkable antimicrobial activity against the tested strains.

Based on the MIC values, we can derive the following structure activity correlations. \(N,N\)-Diphenyl, \(N,N\)-dibenzyl, and \(N\)-benzyl-\(N\)-phenyl (8, 9, and 10) without substitution in the phenyl ring showed potent activity against the tested Gram-positive bacterial strains with MIC value as low as 1.56 \(\mu g ml^{-1}\). Among all the other substitutions in the \(N\)-phenyl ring, halogens showed the most prominent activity. Dihalo (19–22) and trifluoromethyl (25) substitution gave the best results for their antimicrobial properties.

All the synthesized compounds were tested against the following Gram-negative bacterial strains: three \(A.\) baumannii strains: \(A.\) baumannii ATCC 19606 (type strain, Ab06), \(A.\) baumannii ATCC BAA-1605 (Ab05), and \(A.\) baumannii ATCC 747 (Ab47); \(E.\) coli ATCC 25922, \(E.\) aerogenes ATCC 13048, \(P.\) aeruginosa 27833, and \(K.\) pneumoniae ATCC 700603. None of the compounds showed any significant activity against the tested \(E.\) coli, \(E.\) aerogenes, \(P.\) aeruginosa, and \(K.\) pneumoniae strains. Six compounds (14–18 and 22) showed good activity against

| Compd | Sa23 | Sa91 | Sa92 | Sa99 | Sa12 | Se | Bs | Ab05 | Ab47 | Ab06 |
|-------|------|------|------|------|------|----|----|------|------|------|
| 5     | 25   | 25   | 25   | 25   | 25   | 25 | NA | NA   | NA   | NA   |
| 6     | 25   | >25  | 12.5 | 12.5 | 25   | 25 | 25 | NA   | NA   | NA   |
| 7     | NA   | NA   | 1.56 | 1.56 | NA   | NA | NA | NA   | NA   | NA   |
| 8     | 1.56 | 3.125| 1.56 | 1.56 | 3.125| 1.56| 12.5| NA   | NA   | NA   |
| 9     | 3.125| 1.56 | 1.56 | 1.56 | 3.125| 3.125| 25 | NA   | NA   | NA   |
| 10    | 3.125| 1.56 | 1.56 | 1.56 | 3.125| 3.125| NA | NA   | NA   | NA   |
| 11    | 25   | 25   | 12.5 | 6.25 | 25   | 25 | 25 | NA   | NA   | NA   |
| 12    | 25   | 25   | 12.5 | 25   | >25  | 25 | NA | NA   | NA   | NA   |
| 13    | >25  | 25   | 12.5 | >25  | >25  | >25| NA | NA   | NA   | NA   |
| 14    | 12.5 | 12.5 | 1.56 | 3.125| 1.56 | 12.5| 6.25| 25   | 25   | 25   |
| 15    | 6.25 | 12.5 | 3.125| 1.56 | 12.5 | 6.25| 12.5| 25   | 12.5 | 6.25 |
| 16    | 6.25 | 12.5 | 3.125| 1.56 | 6.25 | 6.25| 12.5| 6.25 | 12.5 | 3.125|
| 17    | 6.25 | 6.25 | 1.56 | 1.56 | 6.25 | 6.25| 3.125| NA   | NA   | 12.5 |
| 18    | 6.25 | 12.5 | 3.125| 0.78 | 12.5 | 12.5| 6.25| 12.5 | 6.25 | 1.56 |
| 19    | 1.56 | 3.125| 1.56 | 1.56 | 3.125| 3.125| 25 | NA   | NA   | NA   |
| 20    | 3.125| 6.25 | 0.78 | 0.78 | 3.125| 3.125| 3.125| NA   | NA   | NA   |
| 21    | 1.56 | 12.5 | 0.78 | 0.78 | 3.125| 1.56 | 3.125| NA   | NA   | NA   |
| 22    | 3.125| 6.25 | 1.56 | 0.78 | 3.125| 3.125| 6.25 | 25   | 12.5 | 6.25 |
| 23    | 3.125| NA   | 1.56 | 1.56 | NA   | NA  | 3.125| NA   | NA   | NA   |
| 24    | 3.125| NA   | 0.78 | 0.78 | NA   | NA  | 1.56| NA   | NA   | NA   |
| 25    | 0.78 | 25   | 0.78 | 0.78 | 12.5 | 25  | 1.56| NA   | NA   | NA   |
| 26    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 27    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 28    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 29    | >25  | NA   | >25  | 25   | NA   | >25 | >25| NA   | NA   | NA   |
| 30    | >25  | >25  | 25   | 25   | NA   | >25 | 25 | NA   | NA   | NA   |
| 31    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 32    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 33    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| 34    | NA   | NA   | NA   | NA   | NA   | NA  | NA | NA   | NA   | NA   |
| VC    | 1.56 | 3.125| 0.39 | 3.125| 0.78 | 3.125| 0.195| NA   | NA   | NA   |
| C     | 3.125| 1.56 | 3.125| 1.56 | 3.125| 3.125| 3.125| NA   | NA   | NA   |

Antibiotic susceptible strain: \(S.\) aureus ATCC 25923 (Sa23), and antibiotic-resistant strains: \(S.\) aureus BAA-2312, \(S.\) aureus ATCC 33591 (Sa91), \(S.\) aureus ATCC 700699 (Sa99), \(S.\) aureus ATCC 33592 (Sa92), \(S.\) epidermidis 700296 (Se), \(B.\) subtilis ATCC 6623 (Bs); \(A.\) baumannii ATCC 19606 (type strain, Ab06), \(A.\) baumannii ATCC BAA-1605 (Ab05), \(A.\) baumannii ATCC 747 (Ab47)\n
\(C\) colistin (positive control), NA no activity up to 50 µg ml\(^{-1}\), VC vancomycin (positive control)
the tested *A. baumannii* strains. Fluoro-substituted compounds (14 and 15) showed activity against all the tested strains with MIC value as low as 6.25 µg ml⁻¹. Chloro-substitution (16) showed activity with an MIC value of 3.125 µg ml⁻¹ against Ab06. The 3-bromophenyl derivative (17) did not inhibit the growth of Ab05 and Ab47, but moderately inhibited the growth of Ab06. 4-Bromophenyl derivative (18) is a moderate growth inhibitor of Ab05 and Ab47 but a potent inhibitor of Ab06 with an MIC value of 1.56 µg ml⁻¹. Chlorofluoro-substituted compound (22) was a moderate growth inhibitor of *A. baumannii* strains (Table 1). Thus, we found a good structure activity relationship for the compounds. Only the monohalo-substituted compounds are active against *A. baumannii* strains. Among these compounds, para-substitution with a bigger atom (e.g., bromine) gave the better result. Although, we found several potent molecules are anti-*S. aureus* agents, nonetheless we focused our further studies on compound 21 for its less lipophilicity compared to other active molecules such as compound 18. In addition, this molecule (21) has the better average potency against Gram-positive strains compared to any other molecule in the series.

**Activity against biofilm forming bacteria**

Potent compounds showing activity against planktonic bacteria were tested for their ability to inhibit the biofilm formation as well as the ability to eliminate the preformed biofilms. *N,N*-Diphenyl and dibenzyl (8 and 10) are very effective against the formation of biofilm by *S. aureus* ATCC 25923 at 2 × MIC, MIC and 0.5 × MIC concentrations (Fig. 1). The 2,5-difluoro derivative (19) is the most effective compound against the formation of biofilm at different concentration. Chlorofluoro (21) and trifluoromethyl (25) derivatives showed potent biofilm inhibition activity at 2 × MIC concentrations but their potency decreased at lower concentrations (Fig. 1a). The positive control, VC, showed potent inhibition at 2 × MIC and MIC concentration but showed weak inhibition at 0.5 × MIC value. Thus, some of the potent compounds are as good as the positive control in their ability to inhibit the growth of *S. aureus* biofilm.

These potent compounds were also tested for their ability to destroy preformed biofilms (Fig. 1b). *N,N*-Bisbenzyl (10) and 4-trifluoromethyl (25) derivatives showed excellent ability to eliminate the preformed biofilms. 2,5-Difluoro derivative (19) showed potent activity at 2 × MIC but its ability decreased at lower concentrations. The positive control, VC, almost failed to show any activity against the preformed biofilm [32].

**Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)**

Potent compounds against *S. aureus* ATCC 33599 (MRSA) were tested to find minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) (Table 2). MBC is defined as the concentration that killed 99.9% or greater of planktonic bacteria in the test. Compounds 20, 21, and 25 showed MIC values as low as 0.78 µg ml⁻¹ and MBC for these compounds were seen to be 6.25, 12.5, and 25 µg ml⁻¹ respectively. These are the concentrations at which compounds killed 99.9% of planktonic bacteria during the in vitro test. Similarly, compound 23 whose MIC value was 1.56 was bactericidal at MBC value 12.5 µg ml⁻¹ and test drug VC with MIC value 3.125 showed MBC value 6.25 µg ml⁻¹.

Compounds 15, 16, 18, 22 and the test drug, colistin, were tested against *A. baumannii* ATCC 19606 and respective MIC and MBC values are determined and are

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**Fig. 1** Representation of the biofilm inhibitory and destructive capacity of the active compounds for a *S. aureus* ATCC 25923 and b *S. aureus* ATCC 25923. Inhibition values are in percentage
observed as listed. Among the compounds tested the most effective compounds 16 and 18 with MIC values 3.125 and 1.56 µg/ml, respectively, showed MBC value 50 µg ml⁻¹ and 12.5 µg ml⁻¹.

**Time Kill Assay**

Time kill assays were performed with some of our potent compounds at 4 × MIC concentration to observe their activity against planktonic bacteria over time. Figure 2a shows the results for compounds 16 and 18 along with the positive control colistin and negative control DMSO (solvent for dissolving our compounds) against *A. baumannii* ATCC 19606. Over the first 6 h, there was no growth in the presence of compounds (16 and 18) with a continued bacteriostatic effect through 24 h. Growth never exceeded a twofold increase in log₁₀ cfu ml⁻¹ of the bacterial population. Colistin showed an immediate bactericidal effect, completely killing the starting population within 4 h of incubation.

Similarly, time kill assays against *S. aureus* ATCC 33599 (MRSA) for compounds 20 and 21 along with the test, drug VC, and DMSO were also performed (Fig. 2b). Compound 20 at a 4 × MIC concentration showed a mild bactericidal effect through 6 h followed by slow growth over the remaining 24 h. However, at 8 × MIC the compound was strongly bactericidal, killing all bacteria by 4 h. This is consistent with the reported MBC of this compound of 6.25 µg ml⁻¹, eight times the MIC. Compound 21 showed bacteriostatic activity until 6 h of incubation after which there was a steady increase in population throughout the incubation. Vancomycin was mildly bactericidal through 6 h after which bacteria died at a rapid rate and were eliminated by 8 h.

**Mechanism of action**

BacLight assay was used to determine the membrane permeability of *A. baumannii* ATCC 19606 following the treatment with our potent compounds according to reported procedures [26, 33]. An intact membrane of a bacterial cell is impermeable to propidium iodide (PI), whereas a damaged bacterial membrane is permeable to PI. SYTO-9, a green fluorescent protein, freely permeates through all bacterial membranes, and after binding with DNA it shows enhanced fluorescent intensity. Similarly, PI’s fluorescent intensity increases when bound to DNA and strong signal for PI is only expected when significant membrane damage occurs. One of the potent compounds (22) has shown better membrane disrupting ability for Ab06 than the positive control, colistin, at comparable MIC (Fig. 3a). Similarly, compounds 19, 20, 21, and 22 have shown more potent membrane disruption activity than VC, the positive control, for *S. aureus* ATCC 33599 (MRSA) (Fig. 3b).

**Toxicity against human embryonic kidney (HEK293) cell line**

Potent antimicrobial compounds were tested against human embryonic kidney cell line (HEK293) for their possible toxicity as we described previously [26, 27, 29]. Most of the

| Compd | MIC (µg ml⁻¹) | MBC (µg ml⁻¹) |
|-------|---------------|---------------|
| 20    | 0.78          | 6.25          |
| 21    | 0.78          | 12.5          |
| 25    | 1.56          | 12.5          |
| VC    | 3.125         | 6.25          |

| Compd | MIC (µg ml⁻¹) | MBC (µg ml⁻¹) |
|-------|---------------|---------------|
| 15    | 6.25          | 12.5          |
| 16    | 3.125         | 50            |
| 18    | 1.56          | 12.5          |
| 22    | 6.25          | 50            |
| C     | 3.125         | 6.25          |

**Table 2** MIC and MBC values (µg ml⁻¹) for *S. aureus* ATCC 33599 (MRSA) and *A. baumannii* ATCC 19606

![Fig. 2](image-url) Time Kill Assay. Compounds were tested at 4 × MIC (except indicated) against *A. baumannii* ATCC 19606 and *S. aureus* ATCC 33599 (MRSA) over an incubation period of 24 h at 35 °C.
potent antimicrobial agents did not show any significant in vitro toxicity (Fig. 4). The N,N-dibenzyl (10) derivative, one of the most potent compounds, did not show any significant toxicity (IC50 ~50 µg ml−1) for this human cell line. Fluoro and chloro substituted compounds (19, 20, and 21) also showed high IC50 values compared to their MIC values with a selectivity factor (IC50/MIC) as high as 48.7. Polyfluorinated phenyl derivatives (23 and 24) also showed selective toxicity for bacterial cells. Furthermore, all the synthesized compounds (5–34) were submitted to the National Cancer Institute (NCI) for their antineoplastic properties against NCI-60 cell lines. None of these compounds showed any significant growth inhibition activity at 10 µM concentration. High selectivity factors for these potent antimicrobial agents indicate their suitability for further antibiotic development to treat drug-resistant infections.

Conclusions

In this manuscript, we have reported the synthesis of novel naphthalene-derived pyrazole-based hydrazones. Several of these compounds are potent growth inhibitors of Gram-positive bacteria including MRSA with MIC values as low as 0.78 µg ml−1. These molecules are also potent inhibitors of bacterial biofilm formation and eliminator of preformed biofilms. Potent molecules were also tested for their potential toxicity for human cell lines and found to be very less toxic compared to their toxicity for bacteria. Potent antimicrobial activity and less human cell lines toxicity makes these molecules very good candidates for further drug development.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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