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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 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.

Keywords

Antimicrobial; Naphthalene; Pyrazole; Hydrazone; *A. baumannii*; *S. epidermidis*; MRSA

1. 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

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including methicillin. *S. aureus* and MRSA causes nearly 325,700 infections and more than 10,600 deaths every year in the US alone. Carbapenem-resistant *Acinetobacter baumannii* (CRAB), 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. Additionally, the presence of bacterial biofilm in medical devices is a major concern and causes numerous fatal infections [4]. The Infectious Disease Society of America (IDSA) 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 anti-microbial properties [7, 8]. Several naphthalene containing drugs have been approved by the Food and Drug Administration (FDA) 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 (anti-tubercular 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 anti-depressant agent fezolamine[15], the anti-obesity rimonabant[16], and difenamizole[17] (an analgesic). Pyrazole derivatives exhibit several biological activities including anti-bacterial properties [18, 19]. Additionally, 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.

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2. Materials and Methods

2.1. 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). ^{1}H and ^{13}C NMR spectra were recorded with a Varian Mercury −300 MHz and 75 MHz respectively in DMSO-d_6 solvent with TMS as internal standard. ESI-FTMS mass spectra were recorded in Brucker 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, *S. 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.

2.2. 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 hours 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 minutes, phosphorus oxychloride ($POCl_3$, 10 mmol, 5.43 mL) was added dropwise to form the Vilsmeier reagent. The reaction mixture was heated at 90 °C for 8 hours. After the completion of the reaction, the reaction mixture was poured onto ice and stirred for 12 hours 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 hours (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.

2.3. 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. 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 $1x10^6$

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cfu/mL. 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 in the first well to 0.4 μg/mL 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 hours and results were observed and recorded. Positive wells remained blue/non-turbid (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/non-turbid) were recorded as the MIC.

For some compounds, MIC determination was followed by MBC determination. Five non turbid (blue) wells in a column above and including the MIC well were diluted (10^0, 10^1, and 10^2) and plated in blood agar plates by using the 6x6 drop plate method as described by C.-Y. 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%.

2.4. 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 methodlology 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 hours, they were diluted in sterile fresh warm broth to a final concentration of approximately $3x10^6$ cfu/mL and exposed to compounds at 4xMIC for *A. baumannii* ATCC 19606 and 4xMIC for *S. aureus* ATCC 33599 (in triplicates). Colistin and vancomycin 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 hours 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-hour intervals for viable counts.

A viable count for each sample were performed using the 6x6 drop plate method as described above (C.-Y. Chen et al. (2003)) [28]. Blood agar plates for viable count were incubated for 18-24 hours at 35 °C prior to counting. Bacterial concentrations in log cfu/mL were calculated from colonies counts and their respective dilutions and plotted against the incubation time.

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2.5. 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 1×10^6 cfu/mL. Aliquots of this suspension (200μL) was transferred to wells of a 96-well polystyrene flat bottom plate. The plates were incubated at 35 °C for 24 hours. 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 minutes and 0.1% (w/v) crystal violet (250 μL) was added to each well and left for 15 minutes 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 minutes. 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™ 5 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.

2.5.1. Biofilm inhibition assay—In this assay, the biofilm-forming strain *S. aureus* ATTC 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 1×10^6 cfu/mL. Bacterial broth suspension (195 μL) was transferred to each well in 96-well polystyrene flat bottom plate. 2xMIC, MIC, and 0.5xMIC 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 hours. After incubation, washing, drying, and staining followed by resolublization 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.

2.5.2. Biofilm destruction assay—This assay was performed to test weather 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 hours 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 2xMIC, MIC and 0.5xMIC concentrations of compounds or DMSO in triplicate and the plate was incubated at 35 °C for 24 hours. After incubation, washing, drying, staining, dissolving stained dye and measuring optical density in a plate reader were performed as described above.

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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:

$$\text{Percentage biofilm inhibition/destruction} = [1 - \frac{OD\, compound - OD\, broth}{OD\, dmso - OD\, broth}] \times 100$$

Where, $OD_{compound}$ = Optical density of well with compound, OD_{broth} = OD of well with broth only

OD_{dmso} = OD of well with bacteria broth + DMSO

The data was processed and represented in graphical form in Microsoft® Excel® for Office 365 MSO.

3. Results and Discussions

3.1. 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 multi-gram scale by reacting hydrazinobenzoic acid (**1**) with 2-acetylnaphthalene (**2**) in ethanol to form hydrazone (**3**) followed by treatment with $POCl_3$/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 ^{1}H and ^{13}C NMR spectroscopy and High Resolution Mass Spectrometry (HRMS).

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 hydrazine 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 hydrazine 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 napthyl-derived hydrazone products (**32** and **33**) were formed in very good yield. Methyl hyrazinocarboxylate substituent gave the pure

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product (**34**) in 81% yield. All the synthesized compounds are novel and stable at ambient condition.

3.2.1. 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 (Sa 99), and *S. aureus* ATCC 33592 (Sa 92)) with MIC values as low as 1.56 μg/mL, 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 (Sa 23)) and two MRSA strains with an MIC value as low as 1.56 μg/mL. It also inhibited the growth of *S. epidermidis* with an MIC value of 1.56 μg/mL. *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*. *N,N*-Dibenzyl derivative (**10**) showed similar activity against the tested *Staphylococcus* strains but no 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. 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. 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, 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 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 μg/mL. 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.

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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 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. Chloro-substitution (**16**) showed activity with an MIC value of 3.125 μg/mL 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. Chlorofluoro substituted compound (**22**) was a moderate growth inhibitor of *A. baumannii* strains (Table 1). Thus, we found a good structure activity relationship (SAR) 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.

3.2.2. 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 2xMIC, MIC and 0.5xMIC concentrations (Figure 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 2xMIC concentrations but their potency decreased at lower concentrations (Figure 1a). The positive control, vancomycin, showed potent inhibition at 2xMIC and MIC concentration but showed weak inhibition at 0.5xMIC 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 (Figure 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 2xMIC but its ability decreased at lower concentrations. The positive control, vancomycin, almost failed to show any activity against the preformed biofilm.[32]

3.2.3. 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

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greater of planktonic bacteria in the test. Compounds **20**, **21**, and **25** showed MIC values as low as 0.78 μg/mL and MBC for these compounds were seen to be 6.25, 12.5 and 25 μg/ml 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 and test drug vancomycin (VC) with MIC value 3.125 showed MBC value 6.25 μg/mL.

Compounds **15**, **16**, **18**, **22** and the test drug, colistin, were tested against *A. baumannii* ATCC 19606 and respective MIC and MBC values were determined and were observed as listed (Table 2). 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 and 12.5 μg/mL.

3.2.4. Time Kill Assay

Time Kill Assay: Time kill assays were performed with some of our potent compounds at 4xMIC 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 hours, there was no growth in the presence of compounds (**16** and **18**) with a continued bacteriostatic effect through 24 hours. Growth never exceeded a 2-fold increase in log_{10} cfu/mL of the bacterial population. Colistin showed an immediate bactericidal effect, completely killing the starting population within 4 hours of incubation.

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

3.2.5. 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 (Figure 3a). Similarly, compounds **19**, **20**, **21**, and **22** have shown more potent membrane disruption activity than vancomycin, the positive control, for *S. aureus* ATCC 33599 (MRSA) (Figure 3b).

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3.3. 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 potent antimicrobial agents did not show any significantly *in vitro* toxicity (Figure 4). The *N,N*-dibenzyl (**10**) derivative, one of the most potent compounds, did not show any significant toxicity (IC_{50} ~50 μg/mL) for this human cell line. Fluoro and chloro substituted compounds (**19**, **20**, & **21**) also showed high IC_{50} values compared to their MIC values with a selectivity factor (IC_{50}/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. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Aknowledement:

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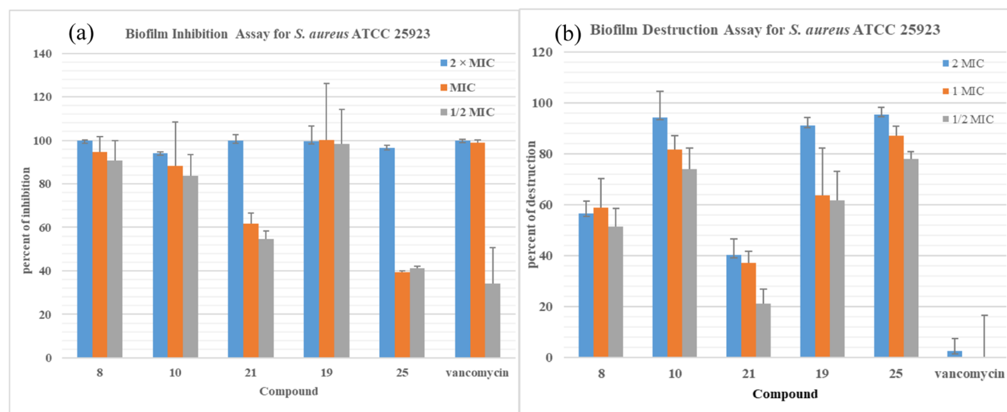

Figure 1.

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.

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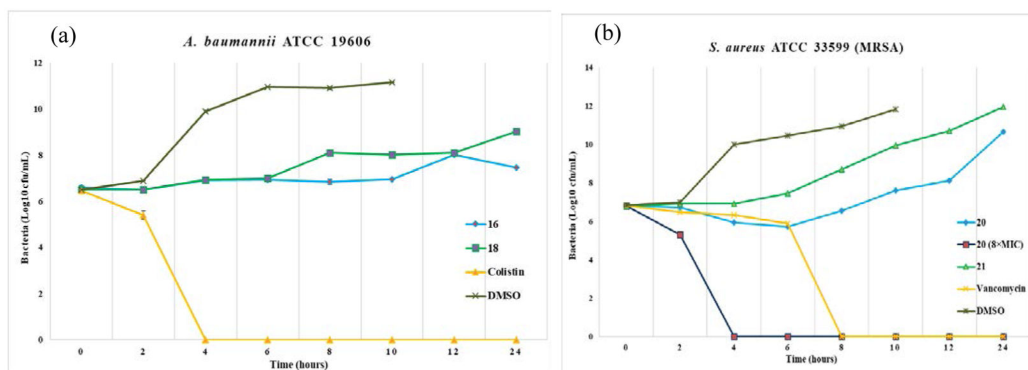

Figure 2.
Time Kill Assay. Compounds were tested at 4xMIC (except indicated) against **(a)** *A. baumannii* ATCC 19606 and **(b)** *S. aureus* ATCC 33599 (MRSA) over an incubation period of 24 hours at 35 °C.

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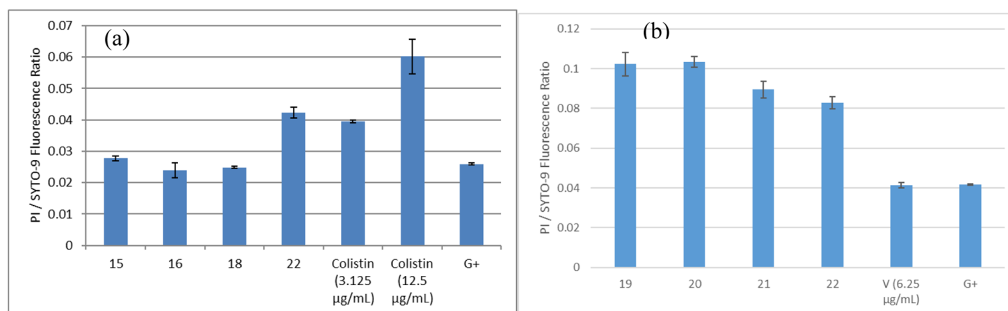

Figure 3.

Membrane permeability assay: PI/SYTO-9 ratio signals with the potent compounds (**15**, **16**, **18**, and **22**), colistin, and growth media (G+) against **(a)** Ab06 and compounds (**19**, **20**, **21**, **22**, and vancomycin) against **(b)** *S. aureus* ATCC 33599 (MRSA)

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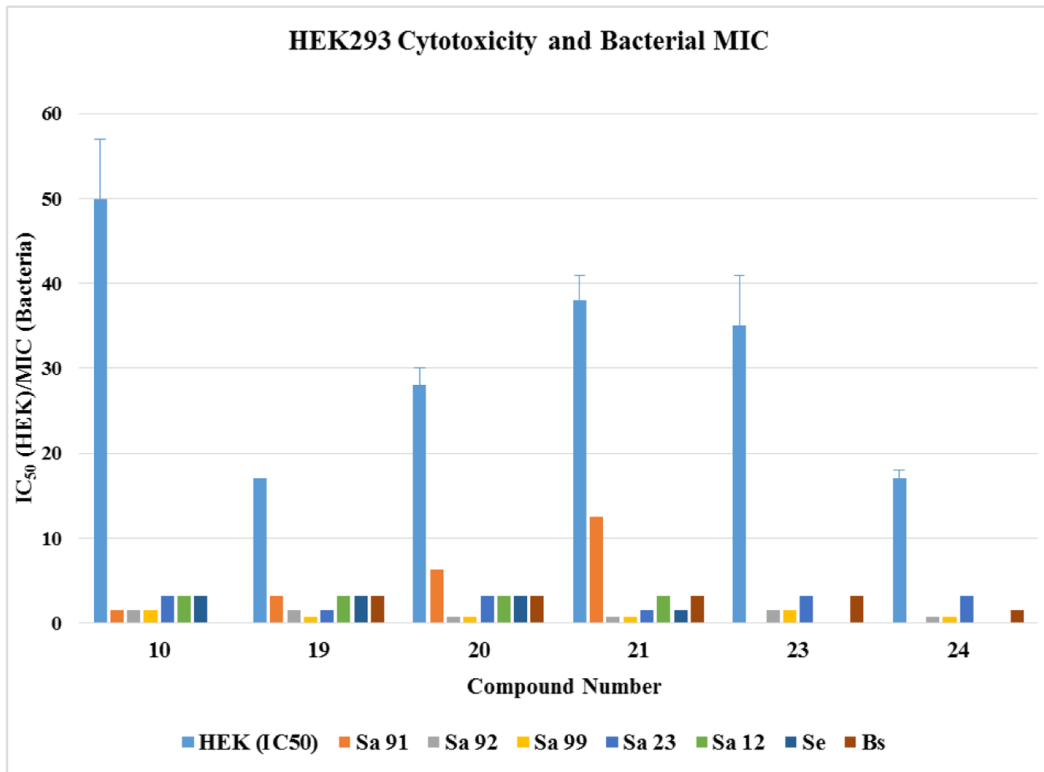

Figure 4.
IC_{50} values of potent antimicrobial for HEK293 cell line

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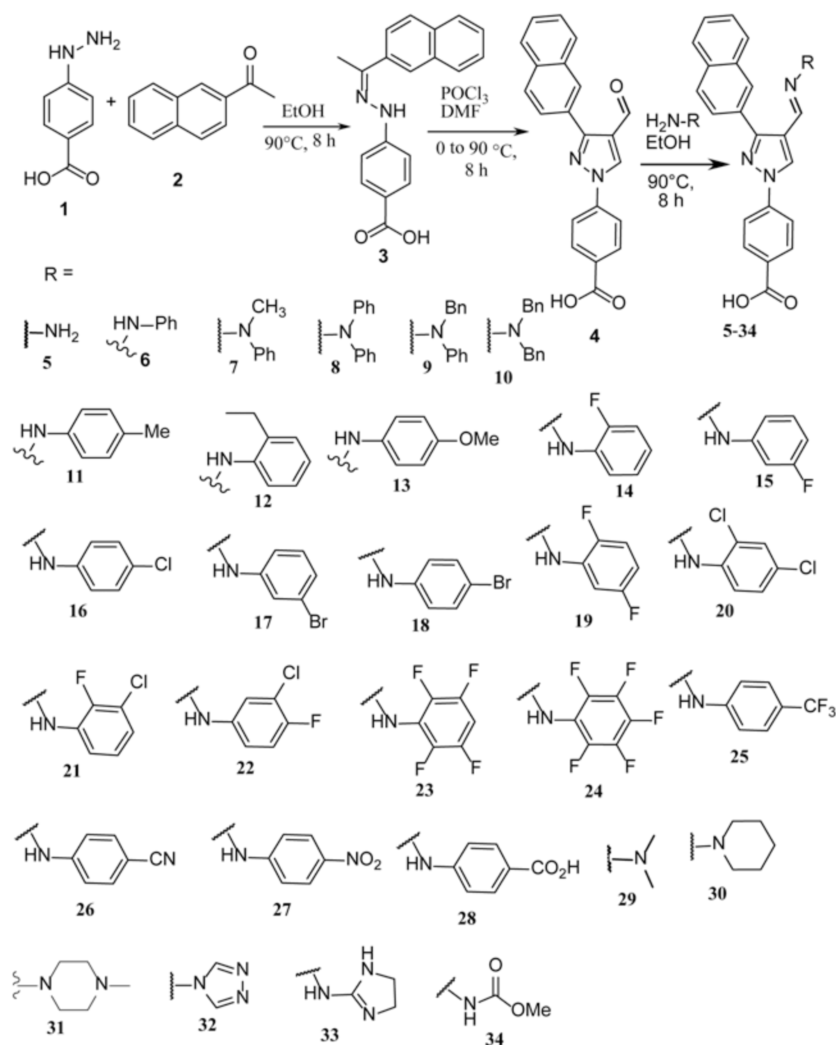

Scheme 1.
Synthesis of naphthalene-substituted pyrazole derivatives.

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Table 1:

Antimicrobial activities of novel compounds (**5-34**) against Gram-positive bacteria: antibiotic susceptible strain; *S. aureus* ATCC 25923 (Sa23), and antibiotic-resistant strains: *S. aureus* BAA-2312 (Sa12), *S. aureus* ATCC 33591 (Sa91), *S. aureus* ATCC 700699 (Sa99), *S. aureus* ATCC 33592 (Sa92), *S. epidermidis* 700296 (Se), *B. subtilis* ATCC 6623 (Bs); VC = vancomycin (positive control); *A. baumannii* ATCC 19606 (type strain, AB06), *A. baumannii* ATCC BAA-1605 (Ab05), *A. baumannii* ATCC 747 (Ab47), C = colistin (positive control), and NA = no activity up to 50 μg/mL.

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	12.5	25	>25	25	NA	NA	NA
13	>25	25	25	12.5	>25	>25	>25	NA	NA	NA
14	12.5	12.5	1.56	3.125	12.5	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	12.5	6.25	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	0.78	3.125	3.125	3.125	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

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Table 2:

MIC and MBC values (μg/mL) for *S. aureus ATCC 33599* (MRSA) and *A. baumannii* ATCC 19606

S. aureus ATCC 33599			*A. baumannii* ATCC 19606		
compd	**MIC**	**MBC**	**compd**	**MIC**	**MBC**
20	0.78	6.25	**15**	6.25	12.5
21	0.78	12.5	**16**	3.125	50
23	1.56	12.5	**18**	1.56	12.5
25	0.78	25	**22**	6.25	50
VC	3.125	6.25	C	3.125	6.25