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Evaluation of Ethyl *N*-(2-Phenethyl) Carbamate Analogues as Biofilm Inhibitors of Methicillin Resistant *Staphylococcus aureus*

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Abstract

A small molecule library consisting of 45 compounds was synthesized based on the bacterial metabolite ethyl *N*-(2-phenethyl) carbamate. Screening of the compounds revealed a potent analogue capable of inhibiting several strains of Methicillin Resistant *S. aureus* biofilms with low to moderate micromolar IC₅₀ values.

Introduction

Over the last several years antibiotic resistance has increasingly become a grave threat to human health.¹ Antimicrobial resistant bacteria accounts for more than two million infections and approximately 23,000 deaths in the U.S. each year.² One of the most serious antibiotic resistant bacteria is Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA is the causative agent of more than 400,000 hospitalizations and even more alarming is that it is responsible for nearly 50% of all deaths in the U.S. that stem from antibiotic resistant infections.³

MRSA is a genetically evolved form of *S. aureus* and is resistant to the β -lactam class of antibiotics (penicillin, amoxicillin, oxacillin, methicillin and others).⁴ Outside of genetic resistance markers, many strains of MRSA have the ability to colonize surfaces and adapt a biofilm mode of growth.⁵ Biofilms are defined as a surface attached community of bacteria encased in an extracellular matrix of biomolecules and bacteria within a biofilm are upwards of 1000-fold more resistant to antibiotics than their free-floating brethren.⁶ This is especially problematic since as much as 33% of the human population is persistently colonized with *S. aureus* biofilms, with 2% colonized by MRSA, providing essentially a continuous reservoir for which this opportunistic bacteria can spread and cause infection.^{7, 8}

Relatively, few classes of MRSA biofilm inhibitors have been discovered. Inspiration for biofilm inhibitors is often drawn from marine organisms, which have evolved the ability to produce and secrete molecules that are capable of modulating biofilm attachment and growth.⁹ Discovery of new classes of MRSA biofilm inhibitors is of great interest as only a

relatively small number of molecular scaffolds are known to inhibit MRSA biofilms including: 2-aminoimidazole derivatives,^{10–14} flustramine analogues,^{15, 16} menthyl carbamates,^{17, 18} bromopyrrole analogues,¹⁹ and cembranoids (Fig 1).²⁰

Previously, it was shown that the bacterial metabolite ethyl *N*-(2-phenethyl) carbamate (**2b**) displays moderate antibiofilm activity against several strains of bacteria.²¹ An initial structure activity relationship (SAR) study performed by Yamada et al. also revealed that this scaffold could be tuned for enhanced activity. Subsequently, our group expanded these studies and identified a number of structurally unrelated carbamates that were potent inhibitors of biofilm formation.¹⁷ Recently, we also established that **2b** was able to sensitize cancer cells to the effects of marine toxins.²² This result has prompted us to further investigate the SAR of **2b** in the context of MRSA biofilm inhibition. Herein, we report a second generation SAR study of **2b** in the context of MRSA biofilm inhibition.

Results and discussion

Biofilm inhibition testing of the natural product **2b**

Initial testing of the metabolite **2b** for bacterial biofilm inhibition of medically relevant MRSA strains was performed at a 200 μ M concentration. The lead compound (**2b**) displayed weak biofilm inhibiting properties, inhibiting only 30.7%, 27.6%, 37.9%, 7.7%, and 22.2% for MRSA strains 44, 1685, 43300, 700789, and 1753 as determined by a crystal violet reporter assay (Table 1).²³ Interestingly, compound **2b** induced biofilm formation in strains 1556, 1770, 811 and 33591.

SAR studies through analogue synthesis of **2b**

An SAR study was performed to identify more potent compounds capable of inhibiting multiple strains of MRSA biofilm development with the caveat of focusing on structural space close to the natural product itself. The SAR study was performed by a strategy of modifying the aryl head, tail region and carbamate linkage (Fig 2). An analogue library was constructed by carrying out matrix syntheses consisting of the displayed amines and various chloroformates (Fig 3). The aryl head was modified by shortening/extending the tail length to methyl, ethyl, propyl, butyl, hexyl, octyl and decyl chloroformates, as well as incorporating aryl components such as anisole, phenyl and tolyl functionalities. Each compound of the library was screened at a concentration of 200 μ M against 9 different strains of MRSA. From the screen, analogue **3j** consisting of the propyl length aryl amine head and tolyl tail was the most active analogue across the spectrum of MRSA strains. Compound **3j** was more potent at 200 μ M than the natural product, inhibiting biofilm formation by 80.8%, 77.3%, 91.9%, 91.6%, 93.9%, 61.1%, 50.8%, 65.2% and 90.2% for MRSA strains 44, 1556, 1685, 43300, 700789, 1770, 811, 33591 and 1753 (Table 1).

With the active compound in hand further modifications were performed to better understand the compounds structural needs for activity (Fig 4). The first set of changes made to **3j** were swapping the *para*-methyl substituent for *para*-ethyl (**5**) and *para*-propyl (**6**) substituents. The ethyl and propyl substituted compounds **5** and **6** gave similar results to the lead compound **3j** for strains 1685, 43300, 1556, 44 and 33591 (Table 2). The analogues

displayed repressed biofilm activity for 1753, 700789 and 1770, however both derivatives showed superior activity for MRSA strain 811 inhibiting biofilm formation 75.1% and 73.4% for **5** and **6** respectively. At this point we opted to swap the electron donating aliphatic substituents for an electron withdrawing trifluoromethyl group (**7**). Changing the alkyl chains to a trifluoromethyl group resulted in a drastic loss in biofilm inhibition for all bacterial strains compared to parent compound **3j**. The carbamate linkage was modified to a urea derivative (**8**) resulting in a significant decline of activity against all MRSA strains. The directionality of the carbamate linkage was then reversed and compared to lead compound **3j**. The reverse carbamate **9** displayed comparable biofilm inhibiting properties as the lead **3j** for MRSA strains 1685, 43300, 1770 and 44. For strains 1753, 700789, 1556 and 33591 biofilm inhibition capabilities were substantially abrogated. Analogue **9** did, however, inhibit biofilm formation of strain 811 by 61.0%, which is more potent than lead compound **3j**. None of the previous modifications were capable of inhibiting biofilm formation with as much potency as compound **3j** across the spectrum of MRSA strains.

Dose response study of analogue **3j**

Dose response studies were performed with **3j** against each MRSA strain to generate IC₅₀ values and quantify activity (Table 1 and See SI for IC₅₀ graphs). The IC₅₀ values for **3j** were determined to be 72.0 ± 5.0 µM, 18.2 ± 4.0 µM, 73.6 ± 2.9 µM, 15.7 ± 4.0 µM, 39.1 ± 5.8 µM, 151.9 ± 4.4 µM, 215.0 ± 7.6 µM, 111.9 ± 7.3 µM and 60.5 ± 3.2 µM for MRSA strains 44, 1556, 1685, 43300, 700789, 1770, 811, 33591 and 1753.

Time kill curve of analogue **3j**

In order to determine whether analogue **3j** was acting through a toxic or non-toxic mechanism, bacterial growth was measured as a function of time. Methicillin resistant *S. aureus* strain 43300 was grown in the presence/absence of **3j** (15.7 µM). Bacterial growth was checked at 2, 4, 6, 8, and 24 h time points (SI Fig 1). Based on the analysis of the time kill curve, bacterial growth devoid of analogue **3j** and bacterial growth in the presence of **3j** appear to be nearly identical throughout the 24 h time period. This result suggests that compound **3j** is most likely acting through a non-toxic mechanism.

Confirmation of **3j** biofilm inhibiting properties

Carbamates are readily known to be susceptible to hydrolysis in solution. To confirm that **3j** is responsible for the biofilm inhibition and not any potential degradation by-products, the corresponding amine (**3**) and hydrolyzed chloroformate (**j**) were tested for biofilm inhibition against MRSA strain 43300 (SI Table 1). **3** and hydrolyzed **j** were tested at 200 µM and **3j**'s IC₅₀ value (15.7 µM). The amine (**3**) and hydrolyzed **j** were tested separately and together. Neither compound separately or in combination at either concentration inhibited biofilm formation of any significant amount. This suggests that indeed **3j** is inhibiting biofilm formation and not any hydrolyzed degradation by-products.

Experimental section

Materials and methods

All reagents and solvents for synthesis were obtained from Sigma-Aldrich, St. Louis, MO, USA. Triethylamine (TEA) was dried by refluxing over CaH_2 , followed by distillation and storage over 4 Å molecular sieves. Deuterated solvents were acquired from Cambridge Isotope Laboratories (CIL). Purification was performed using 60-mesh standard silica gel from Sorbtech. ^1H NMR (300 MHz) and ^{13}C NMR (100 MHz) spectra were recorded at 25 °C on Varian Mercury spectrometers. Chemical shifts (δ) are given in ppm relative to the respective NMR solvent. Mass spectra were recorded on Thermo Fisher Scientific Exactive Plus MS (ESI).

Synthesis

General procedure for compounds 1a–4j—A flame dried round bottom flask under N_2 atmosphere was filled with anhydrous CH_2Cl_2 (5 mL). To this flask was added amine (1.0 mmol) and TEA (3.0 mmol). The reaction mixture was cooled to 0 °C and the chloroformate (0.9 mmol) was slowly added. The reaction was stirred until completion as determined by TLC. The solvent was removed *in vacuo* and the resulting residue was purified by flash chromatography to furnish pure product.

General procedure for compounds 5–9—A flame dried round bottom flask under N_2 atmosphere was filled with anhydrous CH_2Cl_2 (5 mL). To this flask was added sequentially triphosgene (0.4 mmol), TEA (3.0 mmol), and amine (1.0 mmol). The reaction was stirred for 15 min and then the solvent was removed *in vacuo*. The resulting solid was dissolved in THF (5 mL) followed by TEA (3.0 mmol). The flask was cooled to 0 °C and the corresponding alcohol or amine (1.0 mmol) was slowly added. The reaction was allowed to stir for 5 min before being refluxed overnight. After refluxing, the solvent was removed *in vacuo* and the resulting residue was purified by flash chromatography affording pure product.

Biological

Bacteria and media—*Staphylococcus aureus* strains were obtained from the American Type Culture Collection (ATCC), Manassas, VA, as ATCC # (BAA-44, BAA-1556, BAA-1685, 43300, 700789, BAA-1770, BAA-811, 33591 and BAA-1753). *S. aureus* was grown in tryptic soy broth with 0.5% glucose supplement (TSB-G) with shaking or on tryptic soy agar at 37 °C.

Biofilm inhibition assay—One day cultures (24 h) were subcultured to an OD_{600} of 0.01 into TSB-G. Aliquots (1 mL) were taken from the subcultured media and added to small culture tubes for each concentration of each compound to be tested. Compound from a 100 mM stock solution was added to the small culture tubes to give the desired concentration of compound (50–200 μM). Aliquots (100 μL) of each sample were added into

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each well of a row of a microtiter plate with two control lanes containing no compound and one row containing only water. The plate was covered in a damp plastic container and incubated under stationary conditions at 37 °C for 24 h. The media and planktonic bacteria were discarded and the plates were washed with water and 110 µL of a 0.1% crystal violet solution was added to each well and left to stand for 30 min. The crystal violet was discarded and the plate was washed with water and 200 µL of EtOH was added to each well and was allowed to stand for 10 min, then 125 µL from each well was transferred to a polystyrene microtiter plate and the OD₅₄₀ of each well was measured and the water lanes were subtracted out. The percentage of biofilm inhibition was calculated.

Conclusions

In summary, an analogue library of the bacterial metabolite **2b** was constructed in a matrix fashion. Testing of the compounds revealed a single analogue (**3j**) that was potent against several strains of medically relevant MRSA. Compound **3j** inhibited bacterial biofilm formation by 50.8 – 93.9% at 200 µM against MRSA strains respectively and low to moderate micromolar IC₅₀ values ranging from 15.7 ± 4.0 µM – 215.0 ± 7.6 µM. The ease of synthesis and simplistic nature of **3j** make it an attractive option for bacterial biofilm control of MRSA. We are currently testing these analogues for their ability to potentiate cancer chemotherapeutics to determine if there is a substantial correlation between these two apparently disparate activities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Flustramine

Bromopyrrole

2-Aminoimidazole

Menthyl carbamate

Fig 1.
Various MRSA biofilm inhibitor classes.

2b, ethyl *N*-(2-phenethyl) carbamate

3j

Fig 2.

Derivatization strategy of the natural product (**2b**) and active compound (**3j**) from SAR study.

Fig 3.
Matrix syntheses of analogue library.

Fig 4.
Further SAR studies of compound **3j**.

Table 1Biofilm inhibition and IC₅₀ data for **2b** and **3j**.

MRSA Strain	% Inhibition (200 μ M 2b)	% Inhibition (200 μ M 3j)	IC ₅₀ (μ M 3j)
44	30.7	80.8	72.0 $\pm$ 5.0
1556	−40.0	77.3	18.2 $\pm$ 4.0
1685	27.6	91.9	73.6 $\pm$ 2.9
43300	37.9	91.6	12.8 $\pm$ 4.3
700789	7.7	93.9	39.1 $\pm$ 5.8
1770	−6.6	61.1	151.9 $\pm$ 4.4
811	−66.2	50.8	215.0 $\pm$ 7.6
33591	−10.1	65.2	111.9 $\pm$ 7.3
1753	22.2	90.2	60.5 $\pm$ 3.2

Table 2

(%) Biofilm inhibition data for compounds **5–9** at 200 μ M.

MRSA Strain	5	6	7	8	9
44	76.4	59.5	39.9	48.2	70.4
1556	60.9	60.3	4.2	-63.2	43.7
1685	81.9	79.7	59.5	48.2	87.8
43300	76.1	69.8	69.5	30.4	84.4
700789	-13.3	4.5	5.3	-49.5	40.7
1770	-14.2	-16.0	-41.0	-43.9	45.8
811	75.1	73.4	56.5	10.4	61.0
33591	54.2	52.8	-139.3	3.9	39.6
1753	22.1	28.9	38.4	29.7	43.8