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Nitro Sulfonyl Fluorides are a new pharmacophore for the development of antibiotics

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Abstract

The development of antibiotics against Gram-negative bacteria is a central problem in drug discovery. In this report, we demonstrate that aromatic sulfonyl fluorides with a nitro group in their ortho position have remarkable antibacterial activity and are active against drug-resistant pathogens, such as methicillin-resistant Staphylococcus aureus (MRSA), multidrug resistant Acinetobacter baumannii, and Pseudomonas aeruginosa.

Infections caused by drug-resistant bacteria are a severe threat to global public health and will cause a public health crisis if new antibiotics are not developed.[1–3] For example, in 2017, the Centers for Disease Control and Prevention (CDC) estimated that more than 2 million people in the world were infected with bacteria that were resistant to antibiotics.[4] Despite significant efforts, no new classes of antibiotics have been clinically approved during the last 25 years. Developing new antibiotics has been challenging because of the low permeability of Gram-negative bacteria (GNB) to antibiotics,[5] which dramatically restricts the types of molecules that can be considered for future antibiotic development. Therefore, there is a great need for the development of novel pharmacophores that are both permeable to GNBs and have efficacious antibacterial activity.

Covalent irreversible inhibitors have tremendous potential as antibiotics because of their long residence time and ability to kill dormant bacteria.[6,7] For example, the most effective class of antibiotics, the beta lactams, are covalent inhibitors,[8] and represent 65% of the world's antibiotic usage. However, existing covalent inhibitors such as beta lactams, were identified from natural products, and developing new covalent inhibitors, de-novo,[9] has been challenging due to a lack of suitable electrophiles as starting fragments for antibiotic discovery.

Sulfonyl fluorides (SFs) have great potential as electrophiles[10] for future antibiotic development because they have low reactivity toward nucleophiles and low rates of hydrolysis, and because their electrophilicity is dramatically increased in the presence of local hydrogen bonding.[11] This allows SFs to react selectively with protein targets that provide the proper localized hydrogen-bonding environment. SFs, therefore, have the

potential to be highly selective for their target and cause low levels of off-target damage and toxicity. In addition, SFs are small in size and should be permeable to cells with the potential to be active against Gram-negative bacteria. Despite their promise as irreversible antibiotics, the ability of sulfonyl fluorides to act as scaffolds for future antibiotic development has never been investigated.

Herein, we screened the antibacterial activity of a library of SF-containing compounds against *E. coli* to determine if SFs had any intrinsic antibacterial activity. The library contained a variety of chemical structures including aliphatic, aromatic, and heterocyclic SFs, such as thiophene and imidazole SFs, as well as Michael acceptors, such as ethenesulfonyl fluoride (ESF). We also included two known serine protease inhibitors, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)[12] and phenylmethylsulfonyl fluoride (PMSF)[13]. All compounds were tested against a Kanamycin-resistant strain of *E. coli* (plasmid transfected BL21) at an initial concentration of 100 μM. From this screen, 2-nitrobenzenesulfonyl fluoride (**1**), emerged as the most potent antibacterial compound (Figure 1), and had an MIC_{99} value of 5.15 μg/mL (see Figure S1 for detailed structures of the initial library).[14] The MIC_{99} of compound **1** is fairly potent. For example, the FDA-approved nitroaryl-containing drugs metronidazole and ornidazole[15] have MICs against *E. coli* between 16 – 28 μg/mL and 8 – 128 μg/mL, respectively. We also investigated the mammalian cell toxicity of compound **1** against RAW macrophage cells to determine its selectivity for bacteria. Compound **1** had an IC_{50} value of 290 μM (Figure S2), suggesting that compound **1** has potential as a future antibiotic.[16] However, there is a possibility that compound **1** will alkylate proteins in vivo and generate nitro aromatic based antigens, which would cause an immune response (see reference for a discussion on the toxicity of nitroaromatic-containing drugs).[17]

We performed chemical modifications on the hit compound **1** to determine the structural requirements needed for antibacterial activity, and to determine if we could improve its antimicrobial activity (Figure 2). We first investigated if the nitro group in compound **1** had to be *ortho* to the sulfonyl fluoride, and if the electrophile had to be a sulfonyl fluoride, as opposed to other electrophiles such as an epoxide. Sulfonyl fluoride compounds that contained a nitro group in the *meta*- and *para*-position were therefore tested for their antibacterial activity. In addition, compounds containing sulfonyl chlorides and epoxides as the electrophile with a nitro group in the *ortho* position were also examined. Figure 2 demonstrates that sulfonyl fluorides with a nitro group either in the *meta* (**4**) or *para* (**5**) position had no antibacterial activity against *E. coli*. In addition, Changing the electrophile to either a sulfonyl chloride or an epoxide also destroyed antibacterial activity. From the SAR study, we therefore conclude that the nitro group has to be *ortho*-positioned to the sulfonyl fluoride for the NSFs to be active, and that the electrophile has to be a sulfonyl fluoride.

We measured the water hydrolysis rate of **1** to determine its stability in aqueous environments. Compound **1** is remarkably stable in neutral pH aqueous environments, and showed no signs of hydrolysis after three weeks at pH 7.4. In contrast, compound **10**, which represents the chloride variant, hydrolyzed orders of magnitude faster, and had a $t_{1/2}$=33 h at pH 7.3.

We performed additional studies to determine the tolerance of compound **1** towards electron withdrawing and donating groups. We synthesized 9 new *ortho*-nitrobenzenesulfonyl fluoride derivatives[17] and screened them against *E. coli* (Figure 3). Among these molecules, 5 compounds which contained *para*-substituents to either the sulfonyl fluoride or the nitro group were identified as inhibiting *E. coli* growth within the range of 5 – 28 μg/ mL concentration. However, no compounds with *ortho*-substituents to either the sulfonyl fluoride or nitro group showed antibacterial activity. The compound bearing a 4-methyl group (compound **18**) was the most potent, exhibiting MIC values of 2.74 μg/mL and 5.45 μg/mL with Kanamycin and Ampicillin-resistant *E. coli* strains, respectively. Additionally, there was no potency difference between a chloride or a bromide group (compounds **19**-**22**), and *para* substituents to the sulfonyl fluoride (compounds **18**, **19**, and **22**) had better activity than *para* substituents to a nitro group (compounds **20** and **21**). Sulfonyl fluoride para-substitutions with strong electron withdrawing groups might serve to enhance the electrophilicity of the sulfonyl fluoride. Interestingly, a nitro para-substituent (compound **28**, see Figure S3) showed no cytotoxic activity in the MTT assay, although this electrophilic compound and similar electron withdrawing substitutions could harbor undesired off-target reactivity in vivo due to increased broad protein reactivity.

The mechanism by which compound **1** kills bacteria is unknown. We performed random barcode transposon-site sequencing (RB-TnSeq)[19] on bacteria that had been treated with compound **1**, using the *E. coli* strain BW25113, to identify bacterial genes that either protected or sensitized *E.coli* towards compound **1**. The results of our screen with the *E. coli* BW25113 library are shown in Table 1, with the top 5 genes for both positive and negative fitness listed. Table 1 demonstrates that the inactivation of nitroreductase A confers protection against compound **1**, which is similar to other nitro-aromatic drugs such as metronidazole and nitrofurantoin, and suggests that the nitro group in compound **1** is being reduced by nitroreductase A to a toxic intermediate.[20]

Compound **1** has two potential mechanisms by which it can kill bacteria (Figure 4). It either reacts with an essential cellular protein via the sulfonyl fluoride moiety to generate intermediate **40**, and then becomes reduced via nitroreductases, generating reactive intermediates that then destroy the modified protein or neighboring biomolecules (Figure 4, Route A). Or, compound **1** is reduced by nitroreductases first, to generate a hydroxylamine intermediate (**15**), which attacks the neighboring sulfur atom to generate the oxathiazole intermediate **16** (Figure 4, Route B). The oxathiazole intermediate **16** subsequently reacts with essential bacterial proteins causing cell death. In support of the 2nd hypothesis is the observation that the nitro group and the sulfonyl fluoride must be *ortho* to each other for antibacterial activity. In addition, compound **6**, which contains an amine *ortho* to the sulfonyl fluoride was inactive. Compound **6** represents the final reduction product of compound **1**, and demonstrates that the active intermediate of **1** is not the fully reduced compound. Attempts were made to synthesize the hydroxyl amine intermediate **16**, via reduction of **1** with hydrogen gas, but were unsuccessful, and the reduction reactions always generated the amine (compound **6**).[21]

Figures 2–3 and Figure S3 demonstrate that our attempts at improving the MIC of our lead compound **1,** using a benzene core, were unsuccessful. We therefore investigated if replacing the benzene core with a hetero-aromatic core would lead to enhanced activity. The rationale for using a hetero-aromatic core was based on the success of hetero-aromatic nitro-containing drugs, such as nitrofurantoin, which appear to have a similar mechanism of activity as compound **1**. We therefore synthesized[18] the heteroatom-containing aromatic sulfonyl fluorides, 5-chloro-4-nitrothiophene-2-sulfonyl fluoride **32**, 5-methyl-2-nitrothiophene-3-sulfonyl fluoride **33**, 5-methyl-4-nitrothiophene-2-sulfonyl fluoride **34**, and 2-nitrothiophene-3-sulfonyl fluoride **2**, and tested their antibacterial activity (Structures shown in Figure 5). Of these new compounds, we discovered compound **33** had better activity against Kanamycin-resistant *E. coli* (2.0 μg/mL) compared to compound **1**, and we identified 2-nitrothiophene-3-sulfonyl fluoride (compound **2**) as a new lead hit with superior performance compared to all previous NSF derivatives. Compound **2** had an MIC value of 0.66 μg/mL and 10.6 μg/mL against Kanamycin and Ampicillin-resistant *E. coli* strains, respectively (Figure 5). The synthesis of compound **2** was straightforward and readily accessible from the commercially available sulfonyl chloride derivative, via treatment with KFHF in a 1:1 mixture of acetone and water (Scheme S1).

We determined the MIC of compound **2** against a panel of drug-resistant bacteria, such as drug-resistant *A. baumannii*, *P. aeruginosa*, MRSA, and compared its MIC against nitrofurantoin. These pathogens were selected for investigation because of the significant health risk they pose. The MICs of compound **2** and nitrofurantoin against our panel of bacteria are summarized in Table 2. Compound **2** had an MIC of 21 and 42 μg/mL against two different *A. baumannii* clinical isolates, which were resistant to almost all antibiotics currently used, including colistin (a last-resort antibiotic). Nitrofurantoin had no activity against these pathogens. Compound **2** also had a better antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) than nitrofurantoin. Moreover, compound **2** had better efficacy against both *P. aeruginosa* strains tested than nitrofurantoin, and had an MIC value of 42 μg/mL, against one of these strains, whereas nitrofurantoin had no activity up to 95 μg/mL. Compound **2** was also active against a standard *E. coli* strain (ATCC 25922) as well as a drug resistant *E. coli* clinical isolate obtained from San Francisco General Hospital (SFGH; Extended-Spectrum Beta Lactamase-producer).

In conclusion, we present a new electrophile fragment for antibacterial drug discovery based on the nitrosulfonyl fluoride (NSF) fragment. Compound **2**, which contains the NSF fragment, was active against MRSA and a variety of drug resistant pathogens, and is also exceptionally atom economical with molecular weights between 200 – 250 Daltons. Compound **2** and the NSF fragment therefore have tremendous potential for further optimization and future applications in drug discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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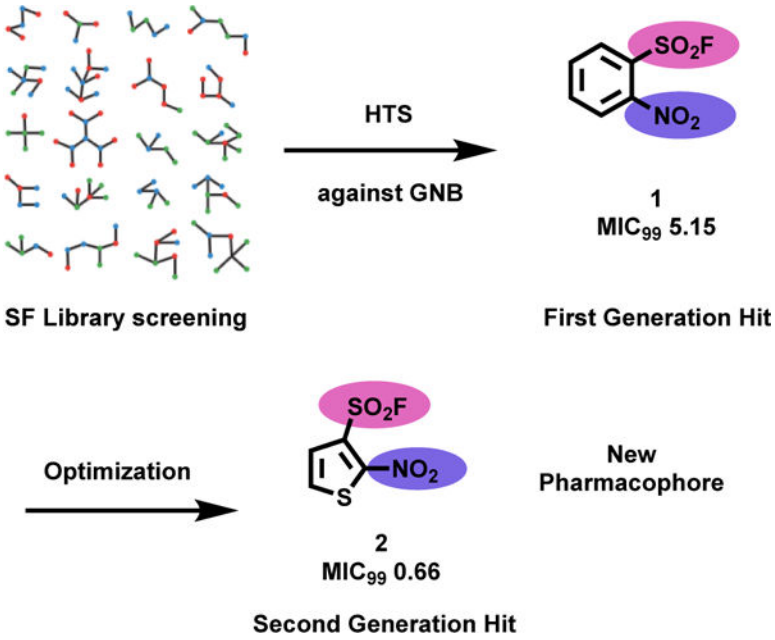

Figure 1. Ortho-nitro sulfonyl fluorides have antibacterial activity.

A library screen of sulfonyl fluorides (SFs) against *E.coli* identified 2-nitrobenzenesulfonyl fluoride (**1**) as a lead fragment with antibacterial activity. Further chemical optimization led to the development of compound **2**, which had an MIC of 0.66 μg/mL against *E.coli*, and was active against multidrug resistant *Acinetobacter baumannii* (MRSA)*, and Pseudomonas aeruginosa*. Ortho-nitro sulfonyl fluorides are a new class of pharmacophores with great potential for drug discovery, given their low molecular weights and high efficacy.

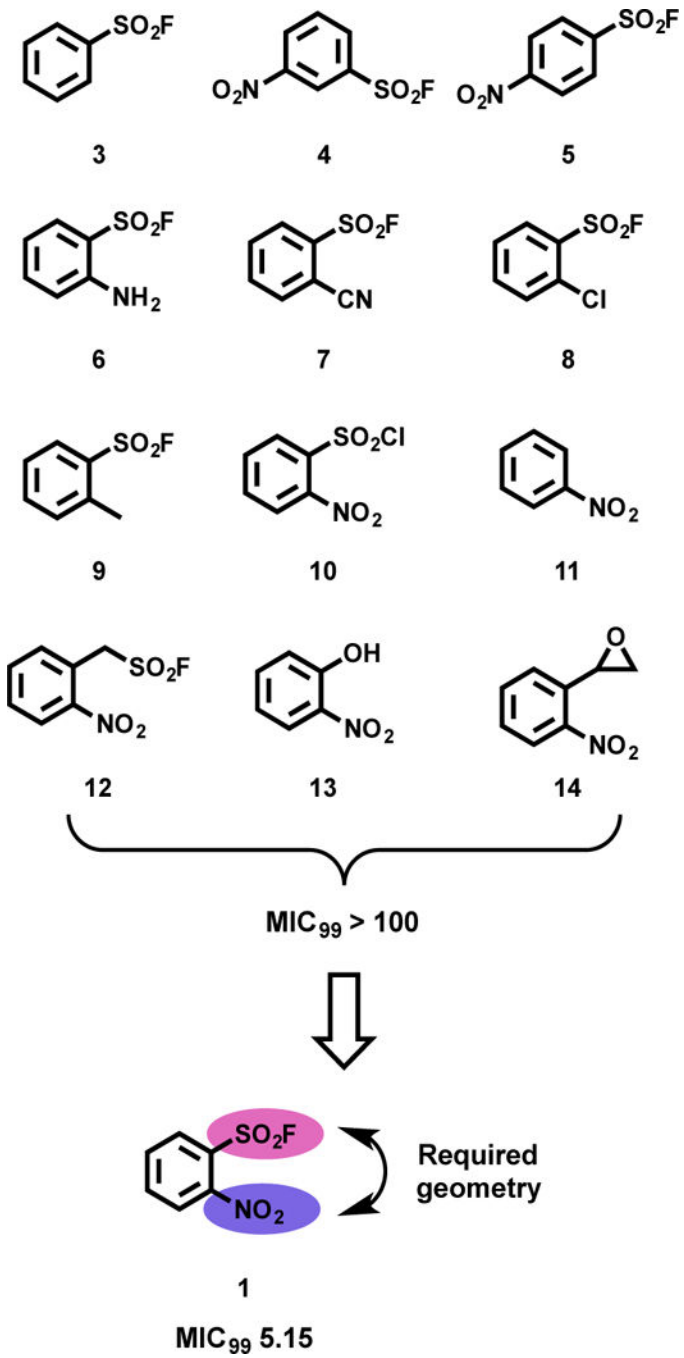

Figure 2. The nitro group and the sulfonyl fluoride group in compound 1 need to be in the ortho configuration to have antibacterial activity, and the electrophile needs to be a sulfonyl fluoride.

Functional group modifications of compound **1** revealed that the *ortho*-nitrosulfonyl fluoride geometry is required for antibacterial activity (μg/mL). Compounds **4**–**5** and **12** are inactive and lack the ortho configuration. In addition, Compounds **10** and **14**, have electrophiles composed of epoxides and sulfonyl chlorides and are inactive.

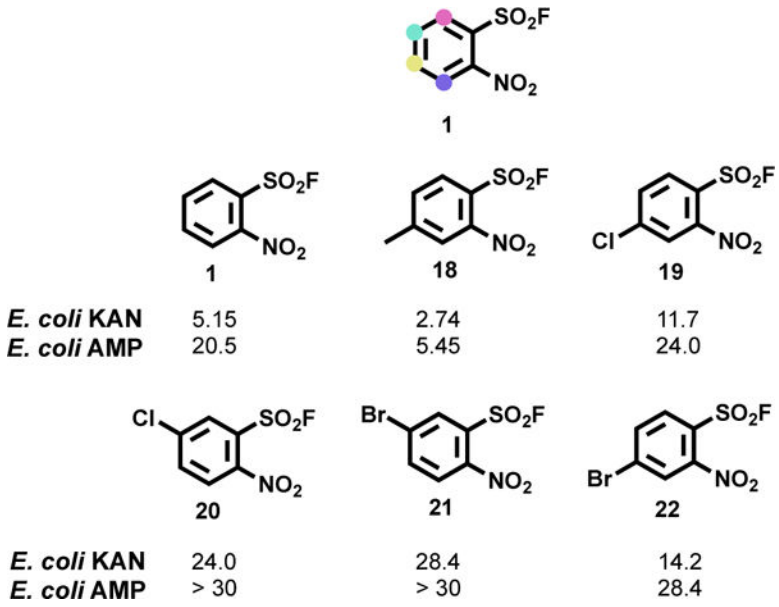

Figure 3. 2-Nitrobenzenesulfonyl fluoride derivatives and their MICs (μg/mL).

Functional group modifications of lead hit **1** were explored to further understand the electronic and steric requirements which are necessary for antibacterial activity. Compound **1** tolerates halogen modifications, however most modifications resulted in lower activity.

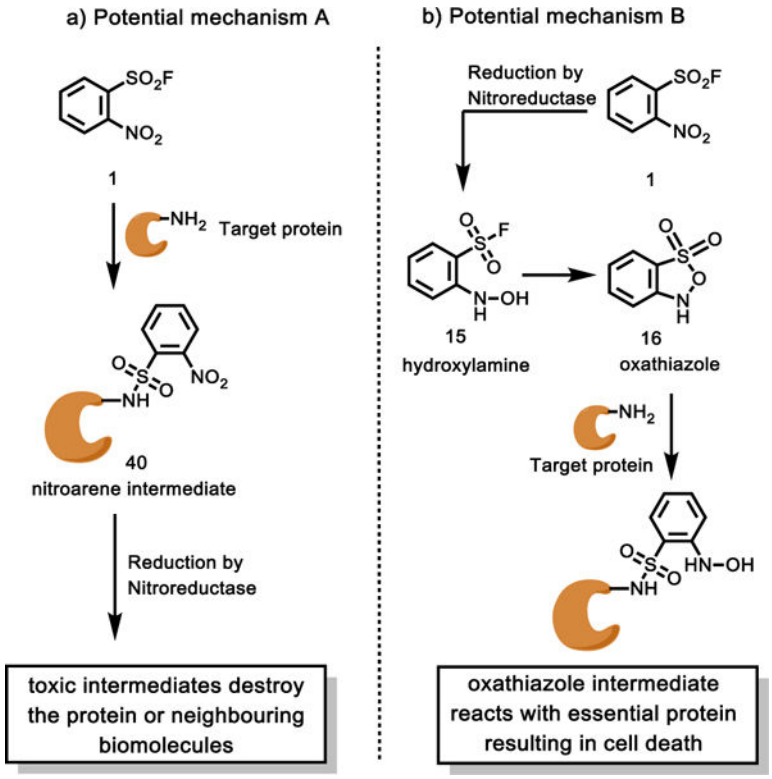

Figure 4. Potential mechanism by which nitroreductase A activates compound 1.

Route A: Compound **1** can react with target proteins to generate **40**, and then undergoes reduction to generate reactive intermediates that destroy the protein or neighbouring biomolecules. Route B: Compound **1** is reduced by nitroreductase A to form a hydroxyl amine **15**, which can attack the neighbouring sulfur and generate the five-membered oxathiazole intermediate **16**. Compound **16** can then react with essential bacterial proteins to kill bacteria.

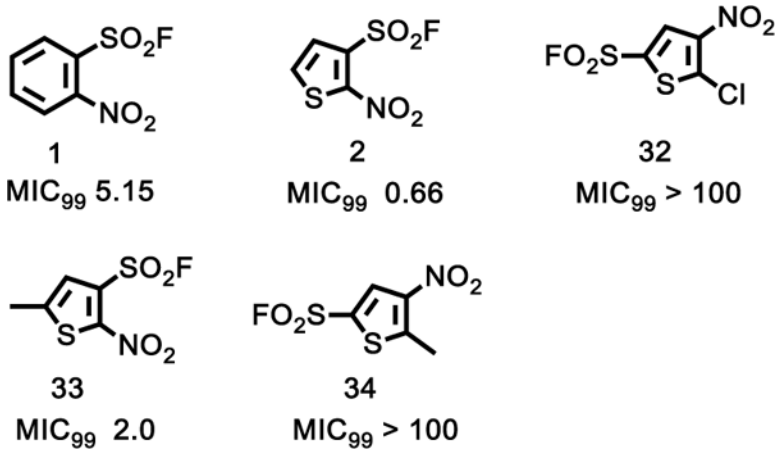

Figure 5. 2-nitrothiophene-3-sulfonyl fluoride (2) is more effective than 2-nitrobenzenesulfonyl fluoride (1) at killing bacteria.

Compound **2** has an MIC (μg/mL) against *E. coli* that is 10 times lower than compound **1**, and is a new sulfonyl fluoride-based pharmacophore for future antibiotic development. Additional modifications to compound **2** lowered its antibacterial activity (compounds **32–34**).

Table 1.

Compound 1 is activated by nitroreductase A in bacteria.

Compound **1** was incubated with the *E. coli* BW25113 library and the deletion of genes that either conferred resistance or sensitization to compound **1** were identified**.** Positive values indicate that gene deletion confers resistance, and negative values indicate that gene deletion causes sensitization to compound **1**. Nitroreductase deletion strongly protects *E.coli* against compound **1**.

Gene	Name	Description	Value
b0851	**nfsA**	**Nitroreductase A, NADPH-dependent, FMN-dependent**	**6.26**
b1531	marA	Multiple antibiotic resistance	4.37
b0464	acrR	DNA-binding transcriptional repressor	3.53
b0912	ihgB	Integration host factor subunit beta	3.05
b1217	chaB	Cation transport regulator	3.03
Gene	**Name**	**Description**	**Value**
b3961	**oxyR**	**DNA-binding transcriptional dual regulator**	**− 4.66**
b0463	acrA	multidrug efflux system	− 4.26
b0462	acrB	multidrug efflux system protein	− 4.20
b3742	mioC	flavodoxin	− 4.09
b3169	nusA	transcription elongation factor NusA	− 3.99

Table 2.

Compound 2 is active against drug resistant bacteria.

The MIC (μg/mL) values of compound **2** against a panel of clinically isolated bacterial strains was determined and compared against nitrofurantoin and colistin. Compound **2** had better antibacterial efficacy against *A. Baumannii 605A* than either colistin or nitrofurantoin.

Treatment\Strain	2	Nitrofurantoin Colistin	
E. coli KAN-RES	**0.66**	0.37	0.5
E. coli AMP-RES	**10.6**	6.0	RES
E. coli SFGH 207	**42**	6.0	0.5
E. coli ATCC 25922	**1.32**	3.0	2.0
MRSA	**5.28**	12	ND
A. baumannii 605 A	**21**	> 95	4.0
A. baumannii 597 A	**42**	> 95	256
P. aeruginosa SFGH 266	**42**	> 95	ND
P. aeruginosa SFGH 427	**84**	> 95	ND