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14. ABSTRACT

New approaches are required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections.

2-Aminoimidazole compounds that, at low micromolar levels and in conjunction with conventional antibiotics, will inhibit and disperse biofilms from both Gram-positive and Gram-negative pathogens, and re-sensitize MDR variants of these pathogens to FDA approved antibiotics are being developed and a number of analogues that suppress antibiotic resistance in MRSA, *A. baumannii* and *P. aeruginosa* have been identified. The activity of lead compounds has been characterized and investigations into the mechanisms of action carried out.

The lead compounds do not exhibit hemolytic activity or toxicity in cell line assays. Large-scale synthesis and formulation has been performed to allow *in vivo* toxicity and efficacy to be evaluated.

15. SUBJECT TERMS
Multi-drug resistance, biofilm, antibiotic resistance suppression, 2-amnoimidazole,

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INTRODUCTION:

New approaches are required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections. MDR bacterial infections can cause sepsis, cellulitis and skin abscesses, pneumonia, toxic shock syndrome, osteomyelitis, and endocarditis among other symptoms. Serious cases result in organ failure (especially kidney), loss of limbs (via amputation) and death.

Presenting a tremendous impediment for the treatment of MDR infections are bacterial defense mechanisms such as biofilm formation and antibiotic resistance. Bacteria within a biofilm are upwards of 1000-fold more resistant to antibiotics than their planktonic (free-floating) counterparts and they are inherently insensitive to the host immune response. Antibiotic treatment is further compromised by the acquisition of antibiotic resistance genes such as ß-lactamases, multidrug efflux pumps, and antibiotic modifying enzymes.

Objective: We are developing a powerful new therapeutic approach to tackle multi-drug resistant infections that the injured Warfighter is especially susceptible to. The overarching goal of this proposal is to identify and develop a 2-aminoimidazole (2-AI) derived small molecule that, at sub-micromolar levels and in conjunction with conventional antibiotics, will: 1) disperse protective biofilms from both gram-positive and gram negative pathogens, focusing on *Pseudomonas aeruginosa*, MRSA, and MDRAB; 2) re-sensitize multi-drug resistant variants of these pathogens to the effects of at least one conventional, FDA-approved antibiotic; 3) exhibit acceptable toxicological properties for use on the injured Warfighter; and 4) be an effective adjuvant for the treatment of infected wounds in multiple animal models. Based upon this goal, the Aims of this research proposal as described in the statement of work are to:

Aim 1. Develop highly active 2-AI derivatives based upon a current lead compound. (months 1 – 30).

Aim 1A. Synthesis of 2-AIT conjugates using boronic acids as the diversity pool (months 1-12).
Aim 1B. Synthesis of 2-AIT conjugates using amines as the diversity pool (months 1-12).
Aim 1C. Synthesis of 2-AIT conjugates with diversity introduced at each of the four positions of the 2-AI (months 1-12).
Aim 1D.Screening of the existing 2-AI library to identify further lead compounds, and subsequent analogue synthesis to augment activity (months 1-30).

Aim 2. Evaluate 2-AI derivatives for their ability to inhibit and disperse bacterial biofilms, as well as suppress antibiotic resistance (months 1-30).

Aim 2A. Evaluate 2-AI conjugates for their ability to inhibit and disperse biofilms (months 1-30).
Aim 2B. Synthesis of 2-AI conjugates for their ability to suppress antibiotic resistance (months 1-30).

Aim 3. Evaluate active 2-AI derivatives for in vitro toxicity. Models will include epidermal cell toxicity, model organism toxicity (Caenorhabditis elegans), and hemolysis.

Aim 3A. Evaluate 2-AI derivatives for epidermal cell toxicity (months 18-36).
Aim 3B. Evaluate 2-AI derivatives for their model organism toxicity (months 18-36).
Aim 3C. Evaluate 2-AI conjugates for hemolytic potential (months 18-36).

Aim 4 Evaluate active 2-AIT derivatives for in vivo activity in collaboration with COL. Craft (Director, Wound Infections/Diagnostics) and his team at Walter Reed Army Institute of Research (WRAIR). The models we will employ are the mouse cutaneous puncture model, the pig partial thickness model and the rat open fracture model.

Aim 4A. Evaluate 2-AI derivatives in the mouse cutaneous puncture model (months 13-18), 960 mice.
Aim 4B. Evaluate 2-AI derivatives in the pig partial thickness model (months 19-24), 12 Yorkshire pigs.
Aim 4C. Evaluate 2-AI derivatives in the rat open fracture model (months 25-30), 120 Norwegian brown rats.

The **WRAIR group** will be responsible for all *in vivo* evaluation.

RESEARCH ACCOMPLISHMENTS

We have identified lead compounds through library screening and analogue synthesis that suppress antibiotic resistance in drug resistant strains of each of the three bacteria described in the introduction: MRSA, *A. baumannii* and *P. aeruginosa*. The activity of lead compounds has been characterized across numerous strains, suitability for in vivo applications has been examined, and investigations into the mechanism of action have been performed. Toxicity screening is underway and large-scale synthesis of material for *in vivo* studies, which are scheduled to begin shortly, has been performed. These accomplishments are detailed below.

Aim 1. Develop highly active 2-AI derivatives based upon a current lead compound and Aim 2. Evaluate 2-AI derivatives for their ability to inhibit and disperse bacterial biofilms, as well as suppress antibiotic resistance.

MRSA

In the 2012 annual report, we described progress towards the synthesis and *in vitro* testing of libraries of novel analogues of the initial lead compound **2-AIT-1**[1] and a second lead compound **2-AIT-2**[2] (Figure 1) that was identified from screening of our in-house library for suppression of MRSA resistance to penicillin G and oxacillin.

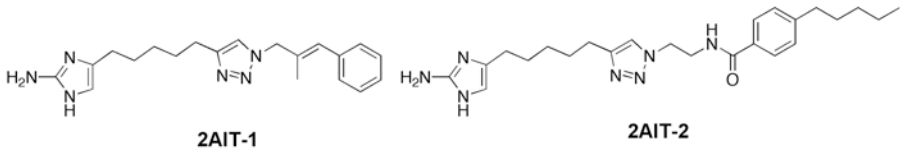

Figure 1. Lead compounds **2AIT-1** and **2AIT-2**

Synthetic methodologies to access seven scaffolds (Figure 2)[3-8] based upon **2-AIT-1** and **2-AIT-2** have been developed and 106 novel compounds were synthesized. Synthetic routes and compound characterization are documented in the references cited in Figure 2.

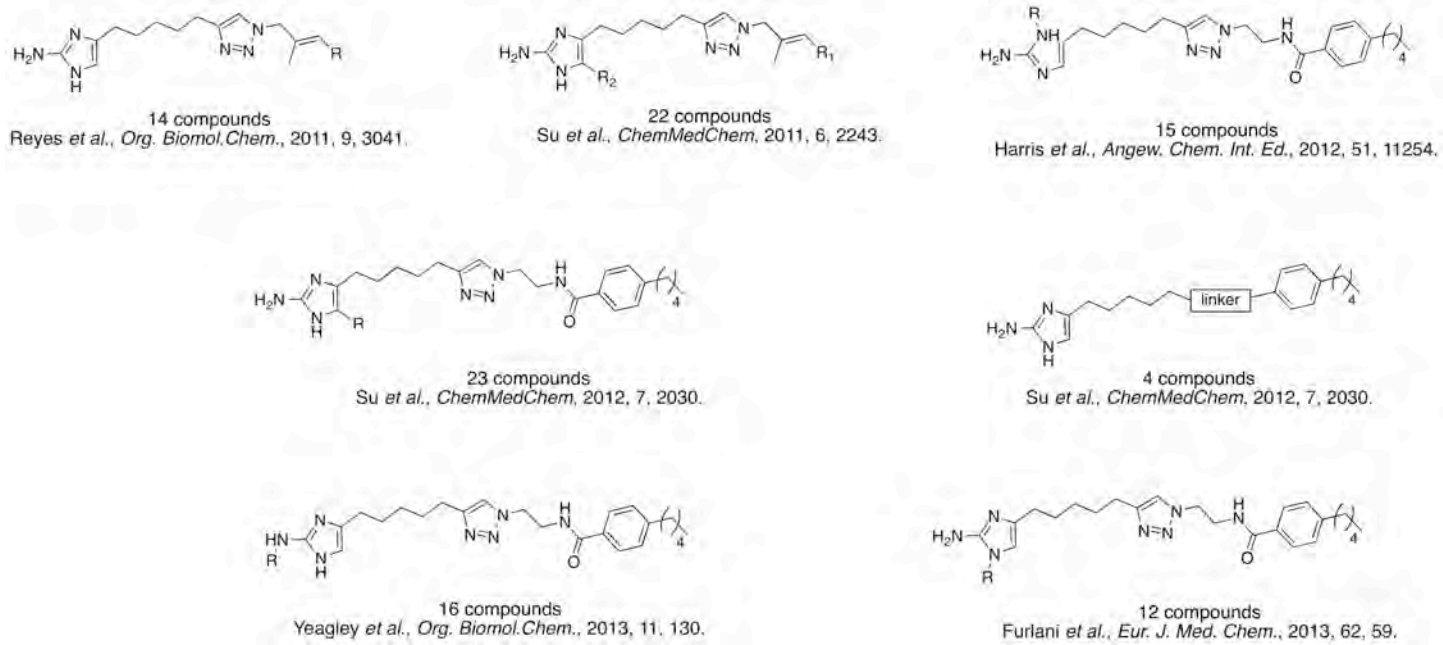

Figure 2. Analogue synthesis of **2AIT-1** and **2AIT-2**

Compounds were examined for the ability to inhibit biofilm formation and lower antibiotic minimum inhibitory concentrations (MICs) in MRSA to obtain a detailed structure activity relationship (SAR) for this series of compounds. The most active compound identified from this medicinal chemistry program was **2-AIT-**

3 (Figure 3), which lowered oxacillin MICs by up to 512-fold against a number of MRSA strains at a concentration of 5 μM (3.1 μg/mL) (Table 1).[5]

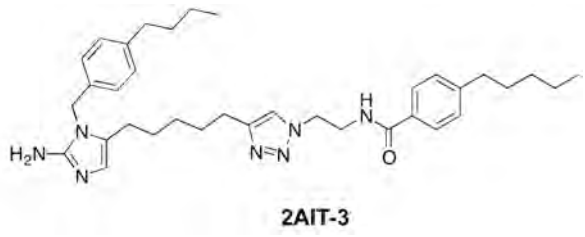

Figure 3. New lead MRSA compound **2AIT-3**

Table 1. Oxacillin MICs against several MRSA strains in the presence and absence of 2-AIT-3 (5μM)

Strain	Oxacillin MIC (μg/mL)	Oxacillin MIC (μg/mL) in the presence of 2-AIT-3 (5 μM)	Fold reduction in oxacillin MIC
43300	32	1	32
33591	256	≤0.5	512
700789	64	8	8
BAA-1753	256	64	4
BAA-811	64	1	64
BAA-1770	32	≤0.5	64
BAA-1685	256	64	4
BAA-44	512	microbicidal	-
BAA-1556	32	0.25	128
NARSA JE2	32	0.5	64

The activity of **2-AIT-3** was further characterized by the construction of time kill curves (Figure 4)[5] to allow quantitative measurement of the magnitude of bacterial growth reduction as a function of time. Time-kill curves were constructed for strain JE2 cultured in the presence of combinations of oxacillin and compound **2-AIT-3**. Compound **2-AIT-3**, when dosed alone at 5 μM (3.1 μg/mL), is bactericidal at early time points (up to 8 h); however bacterial growth is similar to that of the control by the 24 h time point. When bacteria are cultured in the presence of combinations of oxacillin and compound **2-AIT-3**, a large reduction in the number of colony forming units (CFU) compared to treatment with oxacillin alone is observed. A considerable synergistic effect can be observed at the 24 h time point. Compound **2-AIT-3** alone, at 5 μM, effected a 1.08 log reduction in CFU after 24 h, and oxacillin effected less than 0.4 log reduction at concentrations of 16 μg/mL

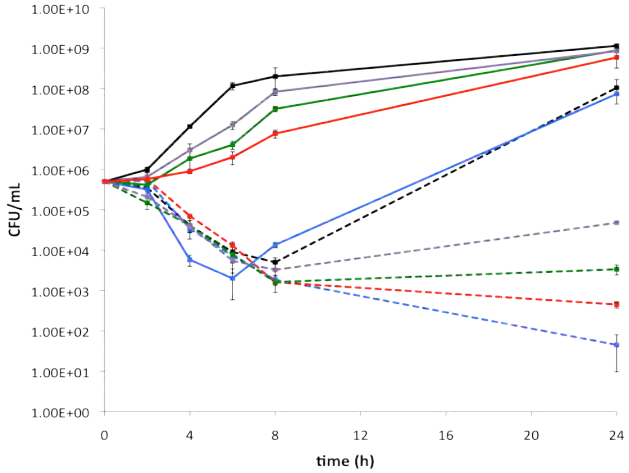

Figure 4. Time-kill curves for USA300 MRSA strain JE2. Solid lines: no 2-AIT, broken lines: 5 μM **2-AIT-3**. Black: no oxacillin, blue: 64 μg/mL oxacillin, red: 16 μg/mL oxacillin, green: 4 μg/mL oxacillin, purple: 1 μg/mL oxacillin.

and below. Combining **2-AIT-3** (5 μM) and oxacillin resulted in log CFU reductions of 6.41, 5.54 and 4.38 for oxacillin concentrations of 16, 4, and 1 μg/mL respectively.

As a preliminary investigation into the mechanism by which **2-AIT-3** is able to lower the oxacillin MIC against MRSA, we obtained a number of mutant strains belonging to the Nebraska Transposon Mutant Library from the Network on Antimicrobial Resistance in *S. aureus* (NARSA). These mutants are all derived from JE2, allowing us to probe non-essential pathways that may be involved in suppression of oxacillin resistance. MICs of oxacillin and **2-AIT-3** were established for each mutant strain, three of the strains tested exhibited considerably lower oxacillin MIC values than the parent strain: NE481, an unidentified DNA-binding response regulator mutant, NE554 (*vraR* mutant), and NE823 (*vraS* mutant) exhibited oxacillin MICs that were reduced 16-fold, eight-fold, and eight-fold respectively, which is in agreement with previous studies that show expression of VraSR contributes to oxacillin resistance.[9] The ability of **2-AIT-3** to lower the oxacillin MIC against the mutant strains was then examined in an identical manner to that used for the parent strain (at 40% of the MIC). Of the mutants that exhibited altered oxacillin MIC values compared to the parent, **2-AIT-3** failed to lower the MIC of both the VraSR two-component system (TCS) mutant strains NE554 and NE823, suggesting the mode of action of oxacillin resistance suppression activity of compound **2-AIT-3** involves VraSR. The VraSR TCS in MRSA is capable of sensing perturbation of cell wall synthesis and coordinating a response involving expression of a number of genes involved in antibiotic resistance. Expression of VraSR is induced upon exposure to cell wall-acting antibiotics including ß-lactams, glycopeptides, daptomycin, and bacitracin, and it has been shown that VraSR mutants are treatable with an oxacillin regimen *in vivo*.[10]

As the molecules are amphipathic, we also investigated the effect of the compounds on cell membrane integrity. The ability of **2-AIT-3** to permeabilize the bacterial cell membrane was quantified using the BacLight assay. After exposure of strain JE2 to compound **2-AIT-3** for one hour, the ratio of intact/damaged cells was measured and compared to control (DMSO only treated) bacteria. At 4x the MIC, 96% of cells were damaged, while at 1x, 0.4x, and 0.25x the MIC, only 33%, 21%, and 9% of cells were damaged respectively. An inactive compound from the same library (that did not lower the oxacillin MIC) was found to be comparable, with 83%, 24%, 23%, and 16% of cells damaged at 4x, 1x, 0.4x, and 0.25x the MIC respectively, suggesting that cell membrane permeabilization is not the mechanism by which **2-AIT-3** suppresses resistance to oxacillin.

We also identified compounds that are able to inhibit and disperse MRSA biofilms. **2-AIT-2** inhibits biofilm formation by (ATCC BAA-44) MRSA with an IC_{50} (concentration at which the compound inhibits 50% of the biofilm compared to an untreated control) of 4.3 μM, and disperses pre-formed biofilms of this strain with an EC_{50} (concentration at which the compound disperses 50% of the biofilm compared to an untreated control) of 80.6 μM.[5] The activity of the newer generation analogues was described in the 2013 annual report, with the most active compound exhibiting an IC_{50} value of 1.42 μM.[4]

A. baumannii

Screening of our in house library of 2-AI compounds has identified **2-AI-4**[11] (Figure 5) as having the ability to potentiate the response of MDR isolates of *A. baumannii* to the polymyxin antibiotic colistin. Due to increasing isolation of carbapenem resistant strains of Gram-negative bacteria, colistin is now essentially the last line of defense against MDR Gram-negative infections, especially in wounded soldiers returning from combat operations in Iraq/Afghanistan. This increase in use of colistin therapy has however led to the isolation of highly colistin-resistant strains of Gram-negative bacteria including *A. baumannii*.

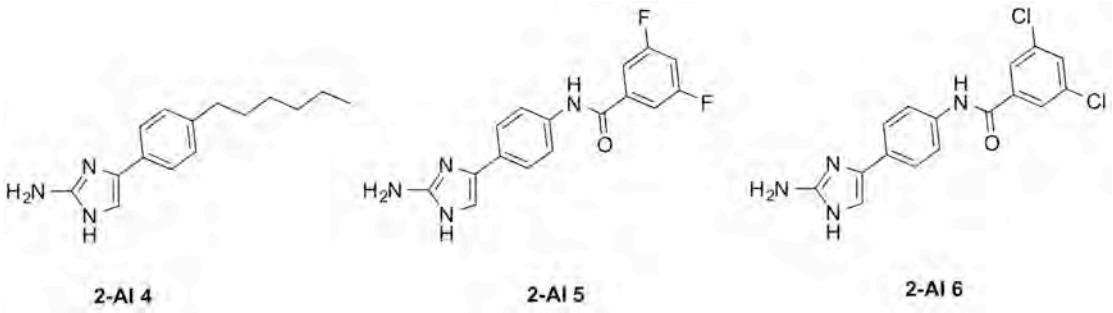

Figure 5. Compounds **2-AI 4, 2-AI 5 and 2-AI-6** that suppress antibiotic resistance in MDR *A. baumannii*.

2-AI-4 was found to suppress colistin resistance in a number of clinical *A. baumannii* isolates obtained from WRAIR (Table 3), at 20 μM (6 μg/mL) MICs were reduced by 16- to 128-fold, while at 30 μM (9 μg/mL), **2-AI-4** lowered MICs by 128- to 1024-fold, taking the MIC of most (3/5) strains to below the colistin breakpoint of 2 μg/mL.

Several additional *A. baumannii* clinical isolates were obtained from the Ernst laboratory at The University of Maryland, Baltimore, and **2-AI 4** was screened for colistin resistance suppression (Table 4). Again, **2-AI 4** lowered the MICs of all strains, lowering the MIC below the breakpoint for colistin resistance in all but one strain. The MIC_{90} values (MIC for 90% of the strains tested) for all 29 strains tested in the absence and presence of **2-AIT-4** are 512, >128, 32 and 2 respectively.

The activity of **2-AI-4** against strain 4106 was further characterized by constructing time kill curves (Figure 6.) The addition of **2-AI-4** to the colistin treated bacteria reduced the number of CFUs considerably, particularly at early time points (2-8 h). For example, after 4 h, 10 μM **2-AI-4** alone effected less than 0.1 log reduction in CFUs compared to untreated bacteria, as did colistin alone at 8 μg/mL, while the combination resulted in a 3.8 log reduction at this time point. Similarly, the addition of 20 μM **2-AIT-4** (which effected a 1.2 log reduction in CFUs after 4 h when administered alone) to 8 μg/mL colistin resulted in a 4.9 log reduction in CFUs. The combination of 30 μM **2-AIT-4** (which effected a 3.4 log reduction in CFUs after 4 h when administered alone) and 8 μg/mL colistin resulted in a value below the limit of detection after 4 h, while the combination of 30 μM **2-AIT-4** and concentrations of colistin below the clinical breakpoint level resulted in log reductions in CFU counts of 5.0 and 4.1 for 2 μg/mL and 0.5 μg/mL colistin respectively. After 8 h **2-AIT-4** did not appreciably reduce CFU counts when administered alone, even at 30 μM (log reduction of 0.47), while the combination of 30 μM **2-AIT-4** and 2μg/mL colistin still effected a log reduction in CFUs of 2.9.

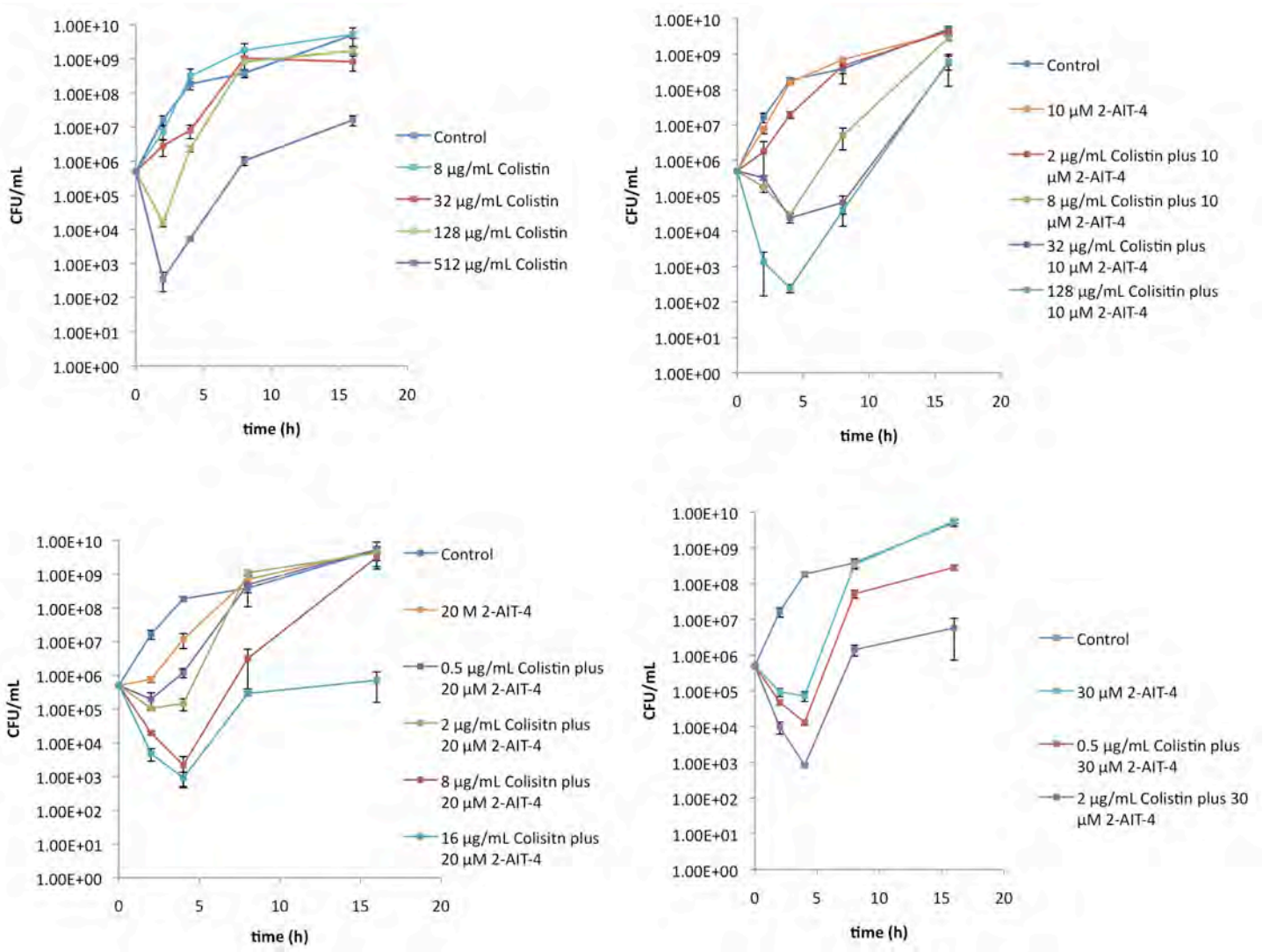

Figure 6. Time kill curves for *A. baumannii* strain 4106 with varying concentrations of colistin in the presence and absence of **2-AI 4**

We also investigated the ability of **2-AI-4** to inhibit the ability of *A. baumannii* to evolve resistance to colistin. *A. baumannii* (ATCC 19606) was serially passaged in the presence of increasing colistin concentrations for seven days, during this time the colistin MIC rose from 1 μg/mL to 1028 μg/mL (Table 2). Measuring the colistin MIC of this evolved strain in the presence of **2-AI-4**, at a concentration of 2.5 μM, resulted in a reduced MIC of 128 μg/mL, while increasing the concentration of **2-AI-4** to 5 μM resulted in a colistin MIC of 16 μg/mL. The evolved strain became more susceptible to the compound alone, with MIC of **2-AI-4** dropping from 25 μM to 6.25 μM upon evolution of colistin resistance. Repeating the serial passage in the presence of **2-AI-4** considerably suppressed the ability of the bacteria to evolve resistance to colistin (Table 2). In the presence of **2-AI-4** at a concentration of 5 μM, the colistin MIC rose from 1 μg/mL to just 16 μg/mL over the seven days, while at 10 μM the colistin MIC did not increase at all, remaining at 1 μg/mL even after seven days of serial passage.

Table 2. Evolution of resistance to colistin on the presence and absence of 2-AIT-4

	Colistin MIC (μg/mL)		
Day	Exposed to colistin only	Exposed to colistin plus 5 μM **2-AI-4**	Exposed to colistin plus 10 μM **2-AI-4**
1	1	1	1
2	4	1	1
3	32	2	1
4	256	2	1
6	>1028	8	1
7	>1028	16	1

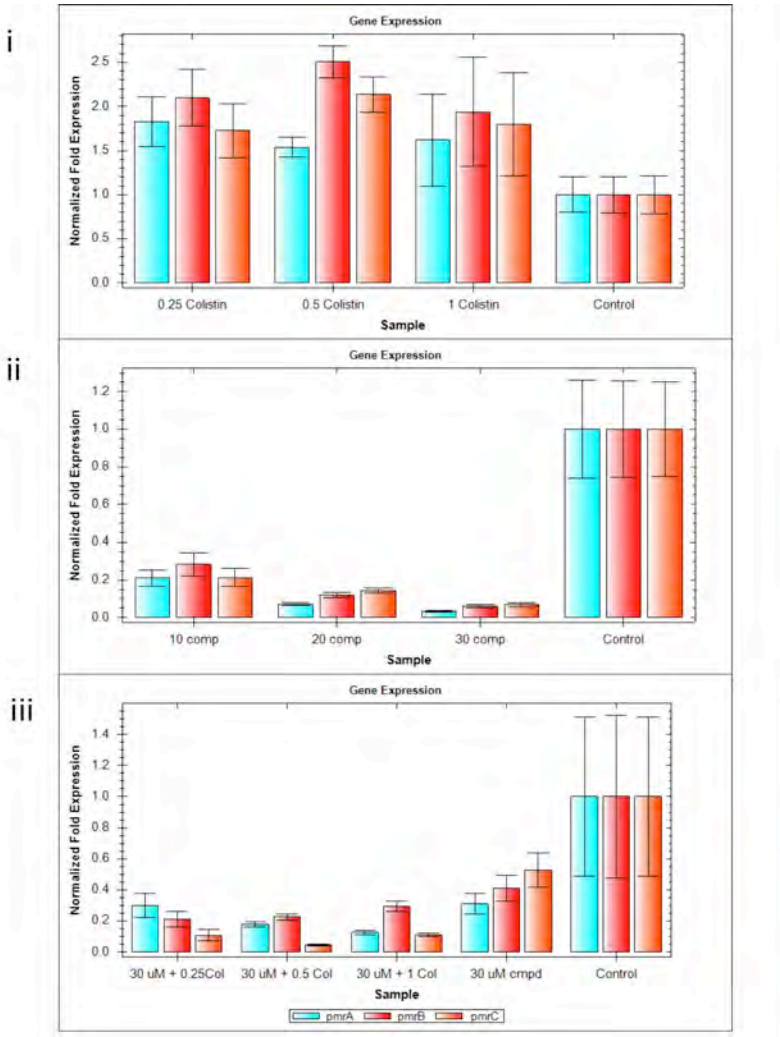

Figure 7. rtPCR analysis of the effect of colistin and **2-AIT-4** on *pmrABC* transcript levels. i) effect of colistin alone, ii) effect of **2-AIT-4** alone, iii) effect of the combination of colistin and **2-AIT-4**.

It has been established that resistance to colistin in *A. baumannii* is driven by modification of the lipid A component of the bacterial membrane, and that this modification is regulated by the PmrAB TCS.[12] To investigate the mechanism of action of **2-AIT-4**, we investigated the effect it has on the expression of the *pmrABC* genes in *A. baumannii* strain 4106 using rtPCR analysis in the absence or presence of colistin (Figure 7). This was also compared with the ability of colistin to induce the *pmrABC* system. We noted that colistin upregulated the *pmrABC* system in 4106 (Figure 7i), however this was not strictly dose responsive in concordance with previous reports. We noted potent, dose-responsive suppression of *pmrABC* expression in the presence of **1** such that at 30 μM, we observed upwards of 10-fold downregulation of the *pmrABC* system (Figure 7ii). Compound **1** also downregulated *pmrABC* expression in the presence of colistin (Figure 7iii). In collaboration with Professor Ernst, we have further validated that the modification of lipid A is absent in treated samples by analyzing the MS signature of the lipid A fraction of 4106 (Figure 8)

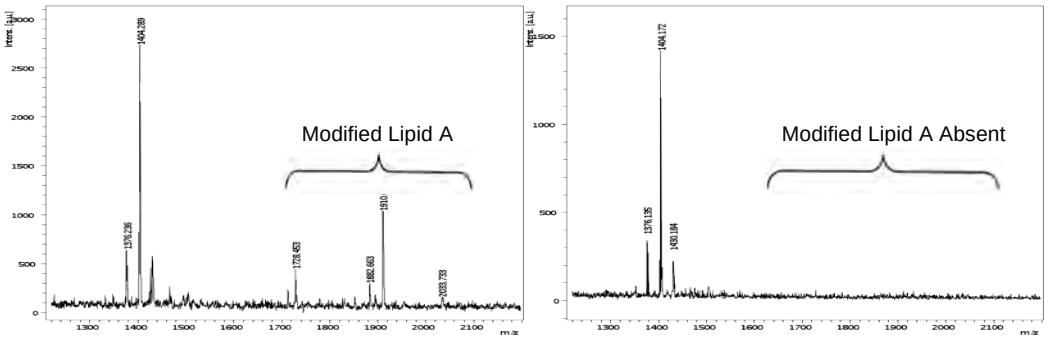

Figure 8. MALDI-ToF mass spectrum of Lipid A component of the cell membrane of *A. baumannii* isolate 4106. **Lef**t = control (non-treated) bacteria, **Right** = bacteria treated with compound **5** (30 μM). m/z 1404 = tetra-acylated Lipid A, m/z 1728 = hexa-acylated Lipid A, m/z 1910 = hepta-acylated Lipid A, m/z 2034 = phosphoethanolamine modified hepta-acylated Lipid A

Our initial screening of compounds for the ability to suppress β-lactam resistance in *A. baumannii*, led to the identification of **2-AI-5**,[13] which lowered the MIC of meropenem against *A. baumannii* ATCC BAA-1605 by 16-fold from 32 μg/mL to 2 μg/mL at a concentration of 60 μM (21 μg/mL). This compound also lowered the imipenem MIC by eight-fold from 16 mg/mL to 2 μg/mL. With the aim of identifying a more potent compound, a number of additional halide containing analogues of **2-AI 5** have been synthesized as described in the 2013 annual report. Screening of these analogues for imipenem and meropenem resistance suppression against *A. baumannii* ATCC BAA-1605 has been performed and resulted in the identification of **2-AI-6**, which lowered carbapenem MICs by 8-16 fold at a much lower concentration than that at which **2-AI 5** was active (15 μM/6 μg/mL). This compound also suppressed carbapenem resistance in a number of other *A. baumannii* clinical isolates by 8-16 fold.

Compounds with the ability to inhibit and disperse *A. baumannii* biofilms have also been identified from the libraries synthesized. Lead compound 2-AI-2 inhibits biofilm formation by *A. baumannii* strain ATCC BAA-1605 with an IC_{50} value of 15.1 μM and disperses preformed biofilms of this strain with an EC_{50} value of 17.2 μM. Of the advanced 2-AI analogues, the most potent biofilm inhibitor for this bacterial strain exhibited an IC_{50} value of 11.3 μM and an EC_{50} value of 44.6 μM.[4]

1C *P. aeruginosa*

Screening of the first generation library of **2-AI -5** and **2-AI 6** analogues for the ability to lower carbapenem and cephalosporin MICs against several MDR *P. aeruginosa* clinical isolates obtained from the Ernst lab at The University of Maryland, Baltimore has also been carried out as detailed in the 2013 annua. report. **2-AI 6** again displayed the highest activity of the compounds tested thus far, effecting reductions in the MICs of imipenem, meropenem and ceftazidime of 4-32 fold at concentrations of 30-60 μM (12-24 μg/mL).

Aim 3. Evaluate active 2-AIT derivatives for *in vitro* toxicity

Preliminary investigations into the toxicity of all lead compounds against eukaryotic cells have been carried out by measuring the hemolytic activity. Defibrinated sheep blood cells suspended in PBS was exposed to various concentrations of compound at 37 ˚C for 1 h. The percentage of lysed cells was calculated based upon a 1% solution of triton X as the 100% marker and PBS as the 0% marker. None of the lead compounds displayed notable hemolytic activity at active concentrations. **2-AIT-3** exhibited only 1% lysis at its active resistance suppression concentration (5 μM), while only 5.6% lysis was observed as high as 10x above the active concentration (50 μM). **2-AI 4** exhibited only 2.5% lysis compared to the positive control at its active resistance suppression concentration of 30 μM and exhibited less than 50% lysis at a concentration of 500 μM. **2-AI 6** was also not hemolytic, exhibiting less than 1% lysis at the highest concentration tested (400 μM).

Compound **2-AI 6** was subjected to a methylthiazolyldiphenyl-tetrazolium (MTT) assay, using the HaCaT keratinocyte cell line. We have used this assay previously to establish that related 2-AI anti-biofilm compounds do not exhibit toxicity at concentrations used to induce biofilm dispersion/inhibition effects.[14] The assay was carried out as previously reported;[14] cells were exposed to varying concentrations of **9d** and the concentrations at which 50% toxicity (TCID) was observed (full data see table S8 in the supporting information). Compound **9d** exhibited a TCID of 387 μM, 25-fold and 6.5-fold greater than the concentrations at which it suppresses ß-lactam resistance against *A. baumannii,* and *P. aeruginosa* respectively.[15]

Aim 4. Evaluate active 2-AIT derivatives for *in vivo* activity in collaboration with Walter Reed Army Institute of Research (WRAIR).

Large-scale synthesis of the three lead compounds indentified through this study **2-AI 3, 2-AI-4** and **2-AI-6)** has been carried out to obtain gram quantities of material and evaluation of in mouse models of infection by Dr Zurawski's lab at WRAIR. In preparation for in vivo studies, the activity of the compounds in the presence of blood plasma has been investigated, and gel-based formulation of the compounds (in polaxomer P407) for topical application has been investigated. Only a slight reduction in activity is observed in the presence of plasma and activity is retained when formulated in P407.

Formulation of **2-AI 3** and **2-AI 4** for *in vivo* testing in Dr Daniel Zurawski's lab at WRAIR was carried out. The compounds were formulated in Poloxamer 407 gel for topical application and provided to Dr Zurawski to be tested in combination with nafcillin and colistin respectively *in vivo*.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed synthetic routes to access various substitution patterns about the 2-aminoimidazole ring and synthesized and tested several libraries of 2-AI analogues.
- Identified several novel compounds that suppress antibiotic resistance in MRSA, MDR *A. baumannii* and MDR *P. aeruginosa*.
- Characterized the antibiotic resistance suppression activity of lead compounds.
- Synthesized gram quantities of lead compounds and formulated them for *in vivo* testing in a mouse model of infection
- Demonstrated that several lead compounds do not exhibit hemolytic activity and the lead *A. baumannii* and *P. aeruginosa* compound does not exhibit cell line toxicity.

REPORTABLE OUTCOMES:

Manuscripts (Abstracts and reprints appended at the end of the report):

- Harris, T.L., Worthington, R.J., Hittle, L.E., Zurawski, D.Z., Ernst, R.K., and Melander, C. Small Molecule Downregulation of PmrAB Reverses Lipid A Modification and Breaks Colistin Resistance. *ACS Chemical Biology*, 2013, **DOI:** 10.1021/cb400490k

- Blackledge, M.S., Worthington, R.J., and Melander, C. Biologically inspired strategies for combating bacterial biofilms. *Current Opinion in Pharmacology*, 2013, 13, 699-706.

- Worthington, R.J. and Melander, C. Overcoming Resistance to b-Lactam Antibiotics. *Journal of Organic Chemistry*, 2013, 78, 4207-13.

- Worthington, R.J., Blackledge, M.S., and Melander, C. Small Molecule Inhibition of Bacterial Two-Component Systems to Combat Antibiotic Resistance and Virulence. *Future Medicinal Chemistry*, 2013, 5,1265-1284.

- Worthington, R.J, Richards, J.J., and Melander, C. Non-Microbicidal Control of Bacterial Biofilms with Small Molecules. *Anti-Infective Agents*, 2013 Accepted

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- Yeagley, A.A., Su, Z., McCullough, K., Worthington, R., J., and Melander, C. N-Substituted 2-Aminoimidazole Inhibitors of MRSA Biofilm Formation Accessed Through Direct 1,3-Bis(tert-butoxycarbonyl)guanidine Cyclization. *Organic and Biomolecular Chemistry*, **2013**, 11 (1), 130-137.

- Harris, T.L., Worthington, R.J., and Melander, C. Potent Small Molecule Suppression of Oxacillin Resistance in MRSA. *Angewandte Chemie*, **2012**, 51 (45), 11254-11257.

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- Worthington, R.J., Richards, J.J., and Melander, C. Chemical Control of Bacterial Biofilms, *Organic and Biomolecular Chemistry*. **2012**, 10 (37), 7457 - 7474.

- Su, Z., Peng, L., and Melander, C. A Modular Approach to the Synthesis of 1,4,5-Substituted-2-Aminoimidazoles. *Tetrahedron Letters*, **2012**, 53 (10), 1204-1206.

- Su, Z., Peng, L., Worthington, R.J., and Melander, C. Evaluation of 4,5-Disubstituted-2-Aminoimidazoles for Dual Antibiofilm/Antibiotic Resensitization Activity. *ChemMedChem*, **2011**, 6, 2243-2251.

- The Angewandte Paper (Potent Small Molecule Suppression of Oxacillin Resistance in MRSA) was listed as a "HOT" paper from the journal. We were subsequently highlighted by the University and interviewed by the Triangle Business Journal for this paper.

- Bracket, C.M, Melander, R.J., An, I., Krishnamurthy, A. Thompson, R/ J., Cavanagh, J., and Melander, C, Small Molecule Suppression of ß-Lactam Resistance in Multi-drug Resistant Gram-negative Pathogens. *J. Med. Chem.*, **2014**, 57, 7450.

Presentations:

- Poster: Suppression of Antibiotic Resistance by Modulating Response Regulator Function, Roberta J. Worthington and Christian Melander, ICAAC, San Francisco, Sep 9-12, 2012.

- Poster: Suppression of Antibiotic Resistance by Modulating Response Regulator Function, Roberta J. Worthington and Christian Melander, GRC New Antibacterial Discover and Development, Lucca Italy, April 15-20, 2012.
- Poster: Small Molecule Suppression of Oxacillin Resistance in MRSA, Tyler L. Harris, Roberta J. Worthington and Christian Melander, SERMACS Regional Meeting, Raleigh, November 14-17, 2012.
- Oral Presentation: Identification of a 1,5-substituted 2-aminoimidazole/triazole conjugate that suppresses oxacillin resistance in MRSA, Tyler L. Harris, Roberta J. Worthington and Christian Melander, ACS National Meeting, New Orleans, April 7-11, 2013.

Degrees obtained that are supported by this award:

- Dr Zhaoming Su, Ph.D. 2012
- Dr Tyler Harris, Ph.D. 2013

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

- Dr Daniel Whitehead, current position: faculty, Clemson University
- Dr LingLing Peng, current position: postdoc in the Zhang lab TSRI
- Dr Andrew Yeagley, current position: Lebanon Valley College
- Dr Zhaoming Su, current position: postdoc in the Chiu Lab, Baylor College of Medicine
- Dr Tyler Harris, current position: postdoc in the Janda lab TSRI

CONCLUSION:

New approaches are desperately required to control multi-drug resistant (MDR) bacterial infections in military medical facilities, as injured Warfighters are highly susceptible to such infections.

We are attempting to address this problem through the development of 2-aminoimidazole compounds that, at sub-micromolar levels and in conjunction with conventional antibiotics, will inhibit and disperse biofilms from both Gram-positive and Gram-negative pathogens, and re-sensitize MDR variants of these pathogens to FDA approved antibiotics.

We have developed synthetic routes to access various substitution patterns about a 2-aminoimidazole ring and constructed a number of focused chemical libraries. These compounds have been evaluated for antibiotic resistance suppression activity with a number of analogues exhibiting increased activity compared to the initial lead compound. Compounds that suppress resistance in MRSA, *A. baumannii* and *P. aeruginosa* have been identified. The activity of lead compounds has been characterized and investigations in to the mechanism of action have been performed.

We have also demonstrated that lead compounds do not exhibit hemolytic activity or cell line toxicity. Large scale synthesis of lead compounds has been carried out and material has been formulated and provided to the Zurawski lab at WRAIR for i*n vivo* efficacy studies.

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Appendix

Publication Abstracts

- Harris, T.L., Worthington, R.J., Hittle, L.E., Zurawski, D.Z., Ernst, R.K., and Melander, C. Small Molecule Downregulation of PmrAB Reverses Lipid A Modification and Breaks Colistin Resistance. *ACS Chemical Biology*, 2013, **DOI:** 10.1021/cb400490k

Infections caused by multi-drug resistant bacteria, particularly Gram-negative bacteria, are an ever-increasing problem. While the development of new antibiotics remains one option in the fight against bacteria that have become resistant to currently available antibiotics, an attractive alternative is the development of adjuvant therapeutics that restore the efficacy of existing antibiotics. We report a small molecule adjuvant that suppresses colistin resistance in multidrug resistant Acinetobacter baumannii and Klebsiella pneumoniae by interfering with the expression of a two-component system. The compound downregulates the pmrCAB operon and reverses phosphoethanolamine modification of lipid A responsible for colistin resistance. Furthermore, colistin-susceptible and colistin-resistant bacteria do not evolve resistance to combination treatment. This represents the first definitive example of a compound that breaks antibiotic resistance by directly modulating two-component system activity.

- Blackledge, M.S., Worthington, R.J., and Melander, C. Biologically inspired strategies for combating bacterial biofilms. *Current Opinion in Pharmacology*, 2013, 13, 699-706.

Infections caused by bacterial biofilms are a significant global health problem, causing considerable patient morbidity and mortality and contributing to the economic burden of infectious disease. This review describes diverse strategies to combat bacterial biofilms, focusing firstly on small molecule interference with bacterial communication and signaling pathways, including quorum sensing and two-component signal transduction systems. Secondly we discuss enzymatic approaches to the degradation of extracellular matrix components to effect biofilm dispersal. Both of these approaches are based upon non-microbicidal mechanisms of action, and thereby do not place a direct evolutionary pressure on the bacteria to develop resistance. Such approaches have the potential to, in combination with conventional antibiotics, play an important role in the eradication of biofilm based bacterial infections.

- Worthington, R.J. and **Melander, C.** Overcoming Resistance to ß-Lactam Antibiotics. *Journal of Organic Chemistr*y, 2013, 78, 4207-13.

β-Lactam antibiotics are one of the most important antibiotic classes but are plagued by problems of resistance, and the development of new β-lactam antibiotics through side-chain modification of existing β-lactam classes is not keeping pace with resistance development. In this JOCSynopsis, we summarize small molecule strategies to overcome resistance to β-lactam antibiotics. These approaches include the development of β-lactamase inhibitors and compounds that interfere with the ability of the bacteria to sense an antibiotic threat and activate their resistance mechanisms.

- Worthington, R.J., Blackledge, M.S., and **Melander, C.** Small Molecule Inhibition of Bacterial Two-Component Systems to Combat Antibiotic Resistance and Virulence. *Future Medicinal Chemistr*y, Accepted.

Infections caused by multi-drug resistant bacteria are a considerable and increasing global problem. The development of new antibiotics is not keeping pace with the rapid evolution of resistance to almost all clinically available drugs, and novel strategies are required to fight bacterial infections. One such strategy is the control of pathogenic behaviors, as opposed to simply killing bacteria. Bacterial two-component signal transduction systems (TCS) control many pathogenic bacterial behaviors such as virulence, biofilm formation and antibiotic resistance and are therefore an attractive target for the development of new drugs. This review presents an overview of TCS that are potential targets for such a strategy, describes small molecules inhibitors of TCS identified to date, and discusses assays for the identification of novel inhibitors. We discuss the future perspectives for the identification and use of inhibitors of TCS to potentially provide new therapeutic options for the treatment of drug-resistant bacterial infections.

- Worthington, R.J, Richards, J.J., and **Melander, C.** Non-Microbicidal Control of Bacterial Biofilms with Small Molecules. *Anti-Infective Agents*, Accepted

Bacterial biofilms are defined as a surface attached community of bacteria embedded in a matrix of extracellular polymeric substances that they have produced. When in the biofilm state, bacteria are more resistant to antibiotics and the host immune response than are their planktonic counterparts. Biofilms are increasingly recognized as being significant in human diseases such as; lung infections of cystic fibrosis (CF), colitis, urethritis, conjunctivitis, otitis, endocarditis, and periodontitis. Given the prominence of biofilms in infectious diseases, there has been an increased effort toward the development of small molecules that will modulate bacterial biofilm development and maintenance. In this review, we highlight the development of small molecules that inhibit and/or disperse bacterial biofilms through non-microbicidal mechanisms. The review provides a general overview of how bacteria develop into biofilm communities, why they are important, and the regulation of this process by quorum sensing. This is followed by a discussion of the numerous small molecules that have been identified as possessing the ability to control biofilm development.

- Worthington, R.J. and **Melander, C.** Combination Approaches to Combat Multi-Drug Resistant Bacteria. *Trends in Biotechnology*, **2013**, 31 (3), 177-184.

The increasing prevalence of infections caused by multi-drug resistant bacteria is a global health problem that is exacerbated by the dearth of novel classes of antibiotics entering the clinic over the past 40 years. Herein we describe recent developments toward combination therapies for the treatment of multi-drug resistant bacterial infections. These efforts include antibiotic-antibiotic combinations, and the development of adjuvants that either directly target resistance mechanisms such as the inhibition of b-lactamase enzymes, or indirectly target resistance by interfering with bacterial signaling pathways such as two-component systems. We also discuss screening of libraries of previously approved drugs to identify non-obvious antimicrobial adjuvants.

- Furlani, R., Yeagley, A.A., and **Melander, C.** A Flexible Approach to 1,4-Disubstituted 2-Aminoimidazoles that Inhibit and Disperse Biofilms and Potentiate the Effects of ß-Lactams against Multi-Drug Resistant Bacteria. *European Journal of Medicinal Chemistry*, **2013**, 62, 59-70.

The pyrrole-imidazole alkaloids are a 2-aminoimidazoles containing family of natural products that possess anti-biofilm activity. A library of 1,4-di-substituted 2-aminoimidazole/triazoles (2-AITs) was synthesized, and its anti-biofilm activity as well as oxacillin resensitization efficacy toward methicillin resistant *Staphylococcus aureus* (MRSA) was investigated. These 2-AITs were found to inhibit biofilm formation by MRSA with low micromolar IC_{50} values. Additionally, the most active compound acted synergistically with oxacillin against MRSA lowering the minimum inhibitory concentration (MIC) 4-fold.

- Yeagley, A.A., Su, Z., McCullough, K., Worthington, R. J., and **Melander, C.** N-Substituted 2-Aminoimidazole Inhibitors of MRSA Biofilm Formation Accessed Through Direct 1,3-Bis(tert-butoxycarbonyl)guanidine Cyclization. *Organic and Biomolecular Chemistry*, **2013**, 11 (1), 130-137.

2-amino substituted derivatives of known 2-aminoimidazole/triazole (2-AIT) biofilm modulators were prepared and examined for both biofilm activity and the ability to repress antibiotic resistance of MRSA to the β-lactam antibiotic oxacillin. Increased aliphatic functionality increased biofilm activity while shorter aliphatic chains provided greater synergy with oxacillin against MRSA (BAA-44). Increased toxicity profiles provided these 2-AIT derivatives improved synergy while making them antibiotic at concentrations that mediate biofilm formation.

- Harris, T.L., Worthington, R.J., and **Melander, C.** Potent Small Molecule Suppression of Oxacillin Resistance in MRSA. *Angewandte Chemie*, **2012**, 51 (45), 11254-11257.

No abstract

- Su, Z., Yeagley, A.A., Su, R., Peng, L., and **Melander, C.** Structural Studies on 4,5-Disubstituted-2-Aminoimidazole-Based Biofilm Modulators that Suppress Bacterial Resistance to β -Lactams. *ChemMedChem*, **2012**, 7 (11), 2030-2039.

A library of 4,5-disubstituted 2-aminoimidazole triazole amide (2-AITA) conjugates has been successfully assembled. Upon biological screening, this class of small molecules was discovered as enhanced biofilm regulators through non-microbicidal mechanisms against methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant Acinetobacter baumannii (MDRAB), with active concentrations in the low micromolar range. The library was also subjected to synergism and resensitization studies with β-lactam antibiotics against MRSA. Lead compounds were identified that suppress the antibiotic resistance of MRSA by working synergistically with oxacillin, a β-lactam antibiotic resistant to penicillinase. A further structure–activity relationship (SAR) study on the parent 2-AITA compound delivered a 2-aminoimidazole diamide (2-AIDA) conjugate with significantly increased synergistic activity with oxacillin against MRSA, decreasing the MIC value of the β -lactam antibiotic by 64-fold. Increased anti-biofilm activity did not necessarily lead to increased suppression of antibiotic resistance, which indicates that biofilm inhibition and resensitization are most likely occurring via distinct mechanisms.

- Worthington, R.J., Richards, J.J., and **Melander, C**. Chemical Control of Bacterial Biofilms, *Organic and Biomolecular Chemistry*. **2012**, 10 (37), 7457 - 7474.

Bacterial biofilms are defined as a surface attached community of bacteria embedded in a matrix of extracellular polymeric substances that they have produced. When in the biofilm state, bacteria are more resistant to antibiotics and the host immune response than are their planktonic counterparts. Biofilms are increasingly recognized as being significant in human disease, accounting for 80% of bacterial infections in the body and diseases associated with bacterial biofilms include: lung infections of cystic fibrosis patients, colitis, urethritis, conjunctivitis, otitis, endocarditis and periodontitis. Additionally, biofilm infections of indwelling medical devices are of particular concern, as once the device is colonized infection is virtually impossible to eradicate. Given the prominence of biofilms in infectious diseases, there has been an increased effort toward the development of small molecules that will modulate bacterial biofilm development and maintenance. In this review, we highlight the development of small molecules that inhibit and/or disperse bacterial biofilms through non-microbicidal mechanisms. The review discuses the numerous approaches that have been applied to the discovery of lead small molecules that mediate biofilm development. These approaches are grouped into: (1) the identification and development of small molecules that target one of the bacterial signaling pathways involved in biofilm regulation, (2) chemical library screening for compounds with anti-biofilm activity, and (3) the identification of natural products that possess anti-biofilm activity, and the chemical manipulation of these natural products to obtain analogues with increased activity.

- Su, Z., Peng, L., and **Melander, C**. A Modular Approach to the Synthesis of 1,4,5-Substituted-2-Aminoimidazoles. *Tetrahedron Letters*, **2012**, 53 (10), 1204-1206.

Diversified 1,4,5-substituted-2-aminoimidazoles were rapidly assembled via sequential N–H insertion and Grignard addition to a-diazoesters. Lead compounds were identified as antibiotics against Gram-positive bacteria with an MIC value as low as 2 μg/mL

- Su, Z., Peng, L., Worthington, R.J., and **Melander, C**. Evaluation of 4,5-Disubstituted-2-Aminoimidazoles for Dual Antibiofilm/Antibiotic Resensitization Activity. *ChemMedChem*, **2011**, 6, 2243-2251.

A library of 4,5-disubstituted-2-aminoimidazole–triazole conjugates (2-AITs) was synthesized, and the antibiofilm activity was investigated. This class of small molecules was found to inhibit biofilm formation by methicillin-resistant *Staphylococcus aureus* (MRSA) at low-micromolar concentrations; 4,5-disubstituted-2-AITs were also able to inhibit and disperse *Acinetobacter baumannii* biofilms. The activities of the lead compounds were compared against the naturally occurring biofilm dispersant cis-2-decenoic acid and were revealed to be more potent. The ability of selected compounds to resensitize MRSA to traditional antibiotics (resensitization activity) was also determined. Lead compounds were observed to resensitize MRSA to oxacillin by 2–4-fold.

- Bracket, C.M, Melander, R.J., An, I., Krishnamurthy, A. Thompson, R/ J., Cavanagh, J., and Melander, C, Small Molecule Suppression of ß-Lactam Resistance in Multi-drug Resistant Gram-negative Pathogens. *J. Med. Chem*., **2014**, 57, 7450.

Recent efforts towards combating antibiotic resistance in bacteria have focused on Gram-positive bacteria; however, multi-drug resistant Gram-negative bacteria pose a significant risk to public health. An orthogonal approach to the development of new antibiotics is to develop adjuvant compounds that enhance the susceptibility of drug-resistant strains of bacteria to currently approved antibiotics. Herein, we describe the synthesis and biological activity of a library of aryl-amide-2-aminoimidazoles based on a lead structure from an initial screen. We identified a small molecule from this library that is capable of lowering the minimum inhibitory concentration of β-lactam antibiotics by up to 64-fold.