CHEMICAL COMBINATION SCREENING

CHEMICAL-CHEMICAL COMBINATIONS AS TOOLS OF BIOLOGY AND AS ROUTES TO DRUG DISCOVERY

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Abstract

With dramatic rises in bacterial resistance both in the clinic and community, there is an urgent need for new chemical matter and strategies. Over the years, antibacterial drug discovery has been centered almost entirely on identifying single agents that target essential proteins or enzymes. Recent advances in systems biology have suggested that this paradigm may not be as therapeutically effective as combinations of drugs. Further, chemical-chemical combinations, rather than single agents, can be used to probe relationships between target proteins in a biological system, maximizing insight into its functional organization. In this regard, we first demonstrate that systematic testing of chemical-chemical combinations can yield information on the functional connections that exist among molecular targets. Indeed, chemical-chemical interaction profiles with various drug classes provided powerful means to elucidate the mode of action of two uncharted compounds active against *E. coli*. In fact, this approach proved invaluable as a method for target identification in all phenotype-based screening efforts that followed. Systematic testing of combinations of chemicals can also lead to the discovery of synergistic interactions with enhanced antibacterial activity. In this respect, two unique screening strategies were adopted to identify novel combinations effective against drug-resistant pathogens. The first uncovered ticlopidine, a novel adjuvant to β-lactam antibiotics that together, synergize against MRSA through an intricate mechanism involving the concerted synthesis of wall teichoic acid polymers and cell wall. The second screening approach identified unique synergistic combinations through a search for molecules that modulate each of the compensatory pathways that regulate bacterial

proton motive force. This unconventional screening approach led to the identification of nine novel synergistic pairs effective against MRSA. Overall, this work illustrates the powerful capacity of chemical-chemical combinations as tools to probe mechanism of novel compounds and as routes to new drug discovery. Importantly, such combinatorial approaches at probing systems and targeting pathogens are consonant with the modern view of the cell.

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To my husband, Shawn. Thank you for your encouragement during graduate school and mostly, for the laughs in times of stress. Your support has meant the world to me. I love you.

To my son, Daniel, you are a true blessing. There are no words to describe how much I love you. I hope this work will one day inspire you to lead a path of innovation and scholarship.

Finally, I dedicate this work to the memory of my late father Edward Farha, who I know would be very proud of me. This is for you.

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List of Abbreviations

ATP	Adenosine triphosphate
CLSI	Clinical and Laboratory Standards Institute
DHFR	Dihydrofolate reductase
$DiSC_3(5)$	3,3'-Dipropylthiadicarbocyanine Iodide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FIC	Fractional inhibitory concentration
FICI or ΣFIC	Fractional inhibitory concentration Index
GFP	Green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
LB	Luria-Bertani
MIC	Minimum inhibitory concentration
MDR	Multi-drug resistant
MHB	Muller Hinton Broth
MOA	Mechanism of action
MRSA	Methicillin-resistant *Staphylococcus aureus*
OD	Optical density
PAD	Previously-approved drug
PBP	Penicillin-binding protein
PG	Peptidoglycan
PMF	Proton motive force
RNA	Ribonucleic acid
TSB	Tryptic soy broth
VRE	Vancomycin-resistant *Enterococcus faecium*
WTA	Wall teichoic acid

CHAPTER ONE - Introduction

Antibiotic Discovery - Successes and Failures

The introduction of β-lactam antibiotics in the 1940's revolutionized medicine and was undoubtedly a key factor in significantly expanding human lifespan (Bax, Mullan et al. 2000). Since, several new classes of antibiotics, both natural products and synthetic molecules, have been introduced. Indeed, innovation would reach its peak in the next 20 years, and antibiotic molecules, whether novel chemical structures or improvements of existing classes, were successfully uncovered and designed at a rapid rate. Most of the start of the 20th century was thus marked by declining mortality due to infectious disease (Armstrong, Conn et al. 1999). This 'golden era' of antibiotic discovery, however, did not last and ended abruptly in the 1960's. In fact, discovery efforts during the next fifty years would only lead to two novel antibiotics: linezolid and daptomycin, introduced in 2000 and 2003, respectively (Lewis 2013). What's worse is this failure in novel discoveries coincides with the emergence of multi-drug resistant bacteria (Boucher, Talbot et al. 2009).

The large majority of antibiotics target either the cell wall or a macromolecular biosynthetic process (DNA, RNA or protein) within the bacterial cell (Bax, Mullan et al. 2000). Over time, however, bacteria have developed mechanisms to outsmart antibiotic action, either by mutagenesis or by acquiring new genes from other bacteria. Resistance mechanisms have included efflux proteins that rapidly pump antibiotics out of cells, enzymes that modify or degrade the antibiotic molecule, rendering it inactive, and alterations to the cellular target in order to prevent drug binding (Wright 2005).

Remarkably, almost as soon as the discovery of penicillin, resistant bacteria were isolated.

Today, emergence of multidrug-resistant bacterial pathogens has become the main challenge facing clinicians and researchers in infectious disease medicine. Resistance has now emerged to all classes of known antibiotics diminishing their usefulness. The crisis is epitomized by the spread of multidrug-resistant 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species), highlighted by the Infectious Diseases Society of America as causing the majority of hospital infections in the United States (Boucher, Talbot et al. 2009). Rates of infection due to the Gram-positive pathogens, methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE), are rapidly increasing. In fact, MRSA, which accounts for the majority of infections, causes higher mortality than HIV/AIDS and tuberculosis combined (Klevens, Edwards et al. 2006). The threat of MDR Gram-negative pathogens rivals that of MRSA and VRE. Indeed, in the case of some Gram-negative bacteria, such as *A. baumannii*, there are strains that are resistant to all currently available antibiotics (Higgins, Dammhayn et al. 2010).

As therapeutic options dwindle, it is increasingly becoming clear that clinicians will soon face a therapeutic dead end in treating certain types of bacterial infections. The urgent need to develop novel strategies to combat MDR bacteria cannot be overstated.

Approaches to drug discovery

There are two main screening approaches to drug discovery: target-based and

phenotypic screening. The former measures the effects of compounds on a purified protein of interest in *in vitro* assays, whereas the latter looks at the effects that compounds induce in cells (Zheng, Thorne et al. 2013). Advances in chemical synthesis platforms during the 20th century have allowed progress toward more systematic approaches to drug discovery. Indeed, synthesis of large libraries of compounds has drastically changed the throughput of compound screening.

Drug development was once mostly driven by phenotypic screening (Swinney and Anthony 2011). While such efforts had considerable power in discovering new molecules that modify a disease phenotype, subsequently determining the relevant cellular target often proved difficult. Advances in molecular biology and genomics at the start of the 1980's caused a shift to target-based screening, with technologies enabling the characterization and screening of novel targets. Here, the target is defined and the challenging task of identifying the cellular target(s) becomes obsolete. Over the next two decades, this screening approach would in fact become the main paradigm for drug discovery (Zheng, Thorne et al. 2013).

In the last decade, a shift back to phenotype-based screening has taken place with the realization that target-based screening has in fact resulted in reduced success in discovering first-in-class medicines (Swinney and Anthony 2011; Zheng, Thorne et al. 2013). While compounds identified via target-based screening may potently inhibit the target of interest *in vitro*, they may not cross the bacterial envelope in an *in vivo* setting or may be victim to drug efflux (Roemer and Boone 2013). There is also the possibility that they may end up affecting other undescribed targets *in vivo* leading to unfavorable side

effects. These factors have thus placed phenotype-based screening in a more favorable light in more recent years. Target identification of hit compounds, however, remains a daunting task.

Target Identification

The challenge of target identification has long plagued early stage discovery efforts and has been simply due to a paucity of systematic methods to characterize the cellular target of small molecules (Burdine and Kodadek 2004). Classically, protein targets have been identified biochemically using labeled or immobilized molecules. The advent of genomics brought forth novel and powerful methodologies to investigate the mode of action of compounds. These have included high-throughput genomic and proteomic approaches, such as genome-scale clone sets, (e.g. gene deletions, overexpression constructs and promoter-reporter strains) and microarrays (Barker, Farha et al. 2010). Further, advances in genomics have made whole-genome sequencing of drug-resistant mutants an apt method for target identification (Roemer and Boone 2013). Although these approaches have proven useful in a number of target identification efforts, they remain far from ideal, as most require chemical modifications of the small molecules (Lomenick, Olsen et al. 2011), large-scale expression profiles (Fischer, Brunner et al. 2004) or the generation of pathogen-specific clone sets (Barker, Farha et al. 2010). Efforts to further optimize and devise novel technologies for target identification are ongoing (Kasper, Baker et al. 2009). Undoubtedly, developing reliable techniques for addressing target identification is at the foundation of successful drug development.

Chemical Genetics

The field encompassing the aforementioned small molecule libraries, phenotypic screening and target identification is known as chemical genetics. Termed in the late 1990's, chemical genetics is derived from classical genetics but makes use of chemical perturbations instead of genetic knockouts. The field aims to systematically use small-molecule techniques to understand biological processes by studying how perturbations of protein functions affect the biological system (Stockwell 2000).

Chemical genetics presents some advantages over classical mutagenesis. The use of small molecules to perturb protein function can be achieved with ease, the effects being reversible, tunable, rapid and easily combined, unlike genetic perturbations, which are effectively permanent and technically difficult (Spring 2005).

Genetic perturbations have certainly proven valuable in investigating biology and have been made further accessible by the advent of comprehensive mutant libraries in model organisms. Chemicals can provide information that is often complementary to genetic perturbations. In fact, coupling the utility of small molecules with various genomic technologies has proven a remarkable tool in adequately probing gene function, investigating biological systems as a whole and characterizing new antibacterial targets (Giaever, Flaherty et al. 2004; Parsons, Lopez et al. 2006; Pathania, Zlitni et al. 2009). Resources for such studies are growing dramatically with genome-wide libraries being constructed for various yeast and bacterial species (Lehar, Stockwell et al. 2008). Overall, chemogenomic efforts are increasingly showing considerable promise in enabling detailed studies of biological networks and in aiding in drug discovery efforts.

Challenging Erhlich's Philosophy

Over a century ago, Paul Erhlich set forth his hypothesis of creating 'magic bullets' for use in the fight against human diseases (Strebhardt and Ullrich 2008). This one-drug-one-target approach has served as the foundation of antimicrobial drug discovery since the discovery of penicillin, inspiring the development of many powerful therapeutics. In fact, this concept of 'magic bullets' that act on individual drug targets is still the guideline being followed by much of modern drug discovery, including antibacterial drug discovery.

Recent systems and network biology studies have, however, challenged this paradigm, exposing its flaws in treating infection. Large-scale gene knock-out experiments in model organisms have revealed that biological systems are very complex and remarkably robust in the face of perturbation. Indeed, a large proportion of single-gene knockouts by themselves exert little or no effect on the fitness or phenotype of the organism. In *E. coli*, for example, only approximately 7% of the genome is essential (Baba, Ara et al. 2006). Similarly, characterization of the yeast genome has revealed that approximately only 18% of the genome is essential for viability (Winzeler, Shoemaker et al. 1999). Additionally, the dispensability of a large number of yeast genes is also dependent on other genes (Winzeler, Shoemaker et al. 1999; Giaever, Chu et al. 2002; Costanzo, Baryshnikova et al. 2010). The scope of this complexity is vast; not only are networks made up of many redundant functions and alternative compensatory pathways, but it is increasingly becoming clear that most genes or proteins within the network also have several interacting partners forming numerous functional cross-connections (Jeong,

Mason et al. 2001; Ho, Gruhler et al. 2002; Butland, Peregrin-Alvarez et al. 2005; Costanzo, Baryshnikova et al. 2010). Such observations of phenotypic robustness indicate that modulating multiple nodes simultaneously is often required for modifying phenotypes (Barabasi and Oltvai 2004; Csermely, Agoston et al. 2005). As such, exquisitely selective compounds, such as Erhlich's 'magic bullets', will likely result in lower-than desired efficacy in treating disease. Certainly, the modern understanding of the cellular architecture suggests that individual drugs may not be as effective as their combinations.

Many successful antibiotics do, in fact, work by simultaneously modulating multiple targets. Some examples include β-lactam antibiotics, whose antibacterial action depends on inhibition of at least two PBPs (Denome, Elf et al. 1999); fluoroquinolone antibiotics that target the proteins ParC and GyrA (Janoir, Zeller et al. 1996); and cycloserine, which inhibits both pairs of alanine racemases and D-Ala-D-Ala ligases (Prosser and de Carvalho 2013). Combination therapy should progress as an effective strategy to overcome the staggering complexity and resilient nature of biological systems (Lehár, Krueger et al. 2008).

Combination Therapy

As mentioned, the prevailing view in antibacterial drug discovery has largely been antibiotic monotherapy. Combination therapy has mostly been an afterthought and was traditionally only a result of combining existing antibiotics in an effort to improve efficacy. More recently, this field is gaining increased attention as a result of the emerging view of the cell's complexity. In fact, drug combinations are slowly becoming

the standard of care in antiviral therapy. For example, in anti-HIV therapy, combinations of protease inhibitors and reverse transcriptase inhibitors are routinely used (Shafer and Vuitton 1999). Further, the use of drug combinations is now highly sought after in cancer chemotherapy (Bozic, Reiter et al. 2013). A number of combination therapies have in fact shown efficacy against bacterial infections, setting a precedent for this paradigm in antibacterial therapy. These include Augmentin®, which is a combination of the β-lactamase inhibitor clavulanate and the second generation penicillin, amoxicillin and co-trimoxazole, which combines two inhibitors of the folate pathway, sulfamethoxazole and trimethoprim. Both pairs are highly used in the clinic and strengthen the thesis that combinations will also be effective as antibacterial therapeutics.

While combination antibiotic therapy may be more complex to develop, requiring more time and higher costs (Bonhoeffer, Lipsitch et al. 1997), its progress is warranted given the unremitting challenges of resistance. The benefits of combination therapy over monotherapy are numerous. Drug combinations allow a superior control of the system by attacking it on multiple fronts, leading to better clinical efficacy (Zimmermann, Lehar et al. 2007; Lehar, Krueger et al. 2009). With the added perturbation over monotherapy, combinations can overcome the intrinsic robustness of the biological system in question, by targeting compensatory pathways, for example. Combinations also allow a decrease in dose-related toxicities by sparing the doses between two drugs needed for efficacy (Keith, Borisy et al. 2005). Finally, combination therapy can potentially reduce or prevent the emergence of resistance (Craig and Salamone 1988; Keith, Borisy et al. 2005; Chait, Craney et al. 2007). The underlying thesis is that therapies that inhibit more than one

target might delay or decrease the ability of the pathogen to accumulate simultaneous mutations affecting those multiple targets (Walsh 2003). Antituberculous chemotherapy provides a classic example where drug combinations have successfully prevented the emergence of resistant organisms (Johnston and Wildrick 1974). Efficacy, toxicity and resistance are limitations often faced my monotherapies and have indeed been major sources of attrition in drug development. As a result, in recent years, research groups have increased their efforts towards the search for multicomponent therapeutics via systematic screens for drug combinations (Borisy, Elliott et al. 2003; Fitzgerald, Schoeberl et al. 2006; Onyewu and Heitman 2007; Zimmermann, Lehar et al. 2007).

Classically, interactions among combinations of drugs have been categorized in one of three types: additive, synergistic or antagonistic (Keith, Borisy et al. 2005). The expected null interaction, whereby indifference upon combination is observed, is called additivity. Synergy occurs when the combination of the two drugs has a greater effect than with either drug alone. Antagonism describes a combination that has a smaller effect than either drug alone.

The most commonly used method to assess the effect of a combination in a laboratory setting is the checkerboard method (Figure 1-1a). In this assay, a combination is tested in all possible permutations of serially diluted drug concentrations. The effect is calculated by comparing a combination's response to those of its single agents. Deviations from additivity can be assessed visually on an isobologram (Figure 1-1b) or numerically as a mathematical representation of the pattern seen in the isobologram, with a fractional inhibitory concentration index (FICI) (Figure 1-1c) (Lambert and Lambert

2003). This index is most often determined using the minimum inhibitory concentration (MIC), the lowest concentration of drug that inhibits measurable growth of the bacterial culture. The FICI is calculated as the summed effective concentrations of the combined drugs at their MIC divided by the sum of the single-drug concentrations at their MIC. Typically, an index of less than 0.5 defines a synergistic interaction, whereas an index larger than or equal to 2 defines an antagonistic combination. An additive effect occurs when the sum of these fractions equates 1.

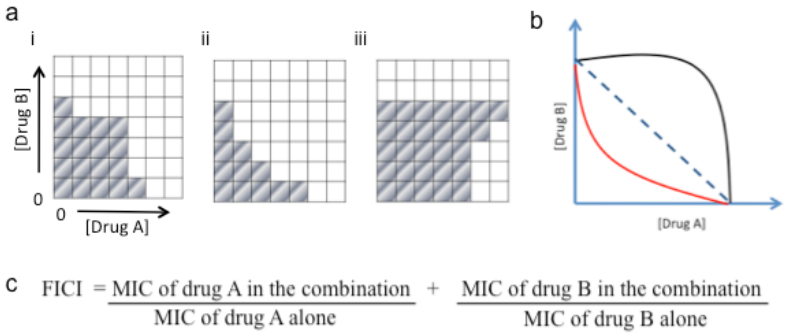

Figure 1-1. Assessing interactions between two combined drugs, Drug A and Drug B. **(a)** The checkerboard method is the most widely used technique to test combinations of drugs *in vitro*. Here, grey shading represents bacterial growth. This method allows multiple concentrations, proportional to the MICs of the drugs, to be tested, in all possible permutations. The resulting response shape reveals whether the interactions is (i) additive (ii) synergistic or (iii) antagonistic. **(b)** An isobologram provides a visual assessment of the interaction. Plotted are individual dose response curves for Drugs A and B. Points for a desired effect (eg. MIC) are connected with a straight line, representing the line of additivity (dashed line). Any combination that falls below this line (red), such that the effect is attained with smaller doses of the two drugs, is synergistic. When the combination is found above the line (black), the combination is antagonistic. **(c)** The FICI is a mathematical representation of the isobologram and is calculated as shown.

Statistical models often accompany the latter method of representing drug combinations. Two different reference models for defining additivity exist: Loewe additivity and Bliss Independence (Fitzgerald, Schoeberl et al. 2006). The former concept is based on the assumption that the components of the combination have a similar mode of action or similar target. As such, the combination effect remains constant when the first

drug is gradually replaced by increasing amounts of the second drug; hence the effect is the same at different combined concentrations. The Bliss Independence model assumes the opposite and is based on the notion of independence. The two drugs act independently from one another with the assumption that the site of action of the combined drugs is different from the sites that each of the individual compounds acts on. Of the latter two models, the most relevant for medical applications is that of Loewe additivity (Chou and Talalay 1984).

Combination chemical genetics

Two decades following the conception of the field chemical genetics, a new field has been coined as 'combination chemical genetics' (Lehar, Stockwell et al. 2008), referring to the systematic testing of multiple perturbations involving chemical agents. Given the increased appreciation of the complexity of biological systems and the wide recognition that combination therapy may be a promising therapeutic approach, combination chemical genetics is a field that allows the systematic application of combinations of perturbants as tools to study biological systems and as routes to facilitate drug discovery.

Chemical-Chemical Combinations as Tools for Biology

Since proteins rarely function in isolation within the cell, interactions from combinations of chemical perturbants can be useful tools to gain biological insight. To date, the focus of most combination perturbation studies has been on exploring drug sensitivities across large sets of genetic knockouts in various model organisms (Giaever, Flaherty et al. 2004; Lum, Armour et al. 2004; Liu, Tran et al. 2010). More recently,

studies are increasingly turning to the systematic testing of purely chemical combinations to obtain biological information.

Analogous to epistasis among genetic mutations, responses to a combination of perturbants can reveal the underlying connectivity among their cellular targets. Indeed, similar to genetic perturbations, two drugs may have no interaction, or they may exhibit synergy or antagonism, increasing or suppressing their individual effects. Thus, in principle, two compounds at sub-lethal concentrations that act synergistically likely share some underlying connectivity in their cellular targets. Similarly, there is likely a mechanistic reasoning underlying an antagonistic interaction observed between two compounds. Conversely, an additive interaction would be expected between two small molecules in a neither non-redundant nor associated pathway.

In this regard, a recent study using simulations of bacterial pathways has shown that dose matrix responses to combinations of chemical probes can be used to determine mechanistic relationships between their targets (Lehar et al. 2007). Depending on the response model obtained from a pair of perturbants, the underlying connectivity of their molecular targets and overall pathway topologies could be elucidated. This study and others (Yeh, Tschumi et al. 2006; Farha, Leung et al. 2013) have highlighted the wealth of functional information that can be obtained from chemical-chemical combinations. Further, combination studies using chemicals can provide a means to reveal the molecular target of a drug and aid in understanding its mechanism of action (Yeh, Tschumi et al. 2006; Farha and Brown 2010).

Overall, connectivities within biological pathways in the cell can be of various types. As such, the use of two small molecules can adequately probe such inter-relationships and yield rich information about the respective molecular targets. Further, the use of chemicals as a means to infer mechanisms of action of uncharted molecules presents some advantages over the more recent genomic approaches used for target identification. Indeed, the approach is independent of the organism under study and can be universally used to probe target connectivity.

Chemical-Chemical Combinations as Routes to Drug Discovery

In addition to mapping out connections within biological systems, the systematic testing of chemical-chemical combinations can uncover synergies with beneficial therapeutic responses. Recent studies have shown that cell-based screens can successfully identify unexpected synergies with enhanced antibacterial activity (Borisy, Elliott et al. 2003; Ejim, Farha et al. 2011; Tan, Hu et al. 2012; Farha, Leung et al. 2013). An added advantage of combination screening is the search space increases dramatically when drugs are used in combination. Indeed, systematic drug combination screening, among even a modest number of compounds, gives way to a vast number of possible pairwise combinations; a valuable diversity that far exceeds that from searches for single agents.

Antibiotic adjuvants

An emerging approach to identify efficacious synergistic pairs is that of antibiotic adjuvants, a strategy rationally devised to overcome the challenges of antibiotic resistance and the failure of new drug discovery. An adjuvant is a potentiator of antibiotic activity that when in combination with antibiotics, enhances their antimicrobial activity.

Antibiotic adjuvant therapies can include combinations of antibiotics, combinations of antibiotic and non-antibiotic compounds, as well as combinations of molecules that inhibit antibiotic resistance mechanisms. Adjuvants can restore antibiotic activity through several mechanisms (Kalan and Wright 2011). The adjuvant can weaken the bacteria themselves making them more vulnerable to antibiotics. This can be achieved either by inhibiting a related step within the targetted biological pathway (Figure 1-2a), or by increasing the uptake of the antibiotic (Figure 1-2b). Further, adjuvants can interfere with mechanisms of antibiotic resistance, such as blocking drug efflux (Figure 1-2c) or by inhibiting resistance enzymes that modify or degrade the antibiotic (Figure 1-2d). Finally, adjuvants can alter the physiological state of a microbe, rendering it more susceptible to antibiotic action (Figure 1-2e)

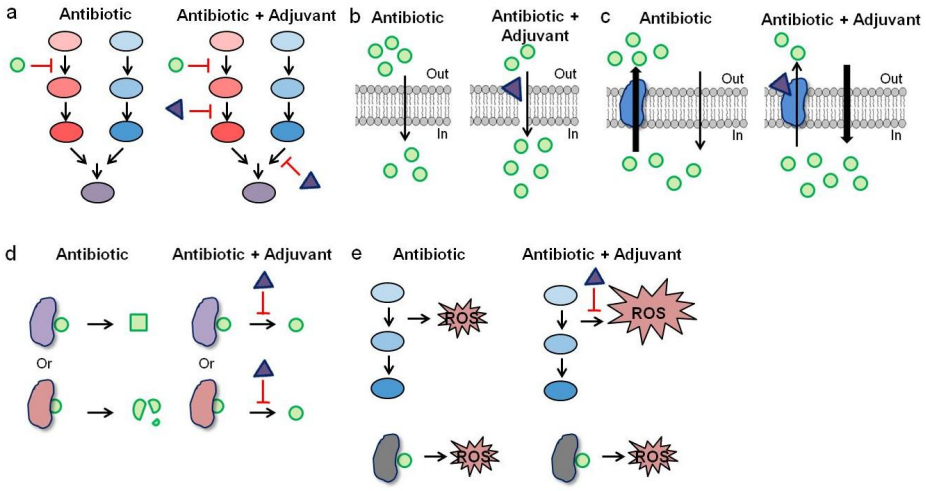

Figure 1-2. Strategies for potentiating antibiotic (green circle) activity with antibiotic adjuvants (purple triangle). **(a)** Addition of an adjuvant that targets a sequential or orthogonal step to the antibiotic within a pathway can enhance its activity. **(b)** Addition of an adjuvant can increase the uptake of an antibiotic into the cell by overcoming the physical barrier. **(c)** Adjuvants can block resistance mechanisms of drug efflux, allowing higher concentrations of antibiotic inside the cell. **(d)** Adjuvants can inhibit resistance enzymes that either modify or degrade the antibiotic. **(e)** Adjuvants can alter the physiological state of a microbe rendering it more susceptible to antibiotics e.g. by targeting an enzyme that stimulates production of reactive oxygen species, and thus enhance the activity of the antibiotic, which also leads to the formation of lethal reactive oxygen species.

Large-scale genomic studies have shown the potential of identifying novel targets for antibiotic adjuvants. A screen of a collection of 4,000 non-essential gene deletions in *E. coli* for chemical hypersensitivity revealed a large subset of genes, involved in all major cellular functions that influence drug susceptibility and therefore act as candidate adjuvant targets (Liu, Tran et al. 2010). Another recent study looked at genes that, when partially depleted, can re-sensitize MRSA to β-lactam antibiotics (Lee, Jarantow et al. 2011). Here, a number of genes, most implicated in cell wall and cell division, were identified as potential adjuvant targets to β-lactam antibiotics. Such studies reinforce the interconnectedness of cellular pathways and present endless possibilities for potential targets of antibiotic adjuvants.

Potentiating antibiotic activity with adjuvants is proving an effective method for discovering new opportunities of pairs of agents with improved antibacterial activity (Ejim, Farha et al. 2011; Tan, Therien et al. 2012; Brynildsen, Winkler et al. 2013; Farha, Leung et al. 2013). Adjuvant strategies could revolutionize the use of antibiotics, not only by reducing levels of antimicrobial resistance but also by establishing a means to preserve the efficacy of existing antibiotics.

Research objectives and organization of thesis

The objectives of the research in this thesis were to employ chemical-chemical combinations to extract mechanistic information for uncharted antibacterial molecules and to uncover novel therapeutic antibacterial combinations effective against drug-resistant pathogens. Overall, the thesis is based on the hypothesis that combinations of chemicals offer distinct advantages over the use of monotherapy, both as probes of

biology and as therapeutics. This stems from the growing realization that the most useful paradigm to target bacterial processes is no longer via a single molecule in a relevant pathway, but instead with a set of compounds that can cooperate to produce an effective response.

The specific aims of this research were two-fold. First, a methodology to elucidate the mode of action of uncharted chemicals was developed using chemical-chemical combinations, with the purpose of aiding in target identification following cell-based screening. Chapter 2 describes this approach and its subsequent use in characterizing the mode of action of two novel antibacterial compounds active against *E. coli*. Next, two screening strategies were devised to uncover new synergistic combinations. Chapter 3 describes the identification and detailed mechanism of synergy of a novel antibacterial combination effective against MRSA, made up of ticlopidine and β-lactam antibiotics. Chapter 4 describes a unique approach at identifying synergistic interactions against drug-resistant pathogens. By targeting the two opposing forces that control the proton motive force across the bacterial membrane, synergy was observed and as such, cytotoxicity averted. Finally, Chapter 5 discusses the future of chemical-chemical combinations as probes of biology and as therapeutics.

References

Armstrong, G. L., L. A. Conn, et al. (1999). "Trends in infectious disease mortality in the United States during the 20th century." JAMA : the journal of the American Medical Association 281(1): 61-66.

Baba, T., T. Ara, et al. (2006). "Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection." Molecular systems biology 2: 2006 0008.

Barabasi, A. L. and Z. N. Oltvai (2004). "Network biology: understanding the cell's functional organization." Nature reviews. Genetics 5(2): 101-113.

Barker, C. A., M. A. Farha, et al. (2010). "Chemical genomic approaches to study model microbes." Chemistry & biology 17(6): 624-632.

Bax, R., N. Mullan, et al. (2000). "The millennium bugs--the need for and development of new antibacterials." International journal of antimicrobial agents 16(1): 51-59.

Bonhoeffer, S., M. Lipsitch, et al. (1997). "Evaluating treatment protocols to prevent antibiotic resistance." Proceedings of the National Academy of Sciences of the United States of America 94(22): 12106-12111.

Borisy, A. A., P. J. Elliott, et al. (2003). "Systematic discovery of multicomponent therapeutics." Proceedings of the National Academy of Sciences of the United States of America 100(13): 7977-7982.

Borisy, A. A., P. J. Elliott, et al. (2003). "Systematic discovery of multicomponent therapeutics." Proc Natl Acad Sci U S A 100(13): 7977-7982.

Boucher, H. W., G. H. Talbot, et al. (2009). "Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 48(1): 1-12.

Bozic, I., J. G. Reiter, et al. (2013). "Evolutionary dynamics of cancer in response to targeted combination therapy." eLife 2: e00747.

Brynildsen, M. P., J. A. Winkler, et al. (2013). "Potentiating antibacterial activity by predictably enhancing endogenous microbial ROS production." Nature biotechnology 31(2): 160-165.

Burdine, L. and T. Kodadek (2004). "Target identification in chemical genetics: the (often) missing link." Chemistry & biology 11(5): 593-597.

Butland, G., J. M. Peregrin-Alvarez, et al. (2005). "Interaction network containing conserved and essential protein complexes in Escherichia coli." Nature 433(7025): 531-537.

Chait, R., A. Craney, et al. (2007). "Antibiotic interactions that select against resistance." Nature 446(7136): 668-671.

Chou, T. C. and P. Talalay (1984). "Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors." Advances in enzyme regulation 22: 27-55.

Costanzo, M., A. Baryshnikova, et al. (2010). "The genetic landscape of a cell." Science 327(5964): 425-431.

Craig, W. A. and F. R. Salamone (1988). "Do antibiotic combinations prevent the emergence of resistant organisms?" Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America 9(9): 417-419.

Csermely, P., V. Agoston, et al. (2005). "The efficiency of multi-target drugs: the network approach might help drug design." Trends in pharmacological sciences 26(4): 178-182.

Denome, S. A., P. K. Elf, et al. (1999). "Escherichia coli mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis." Journal of bacteriology 181(13): 3981-3993.

Ejim, L., M. A. Farha, et al. (2011). "Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy." Nature chemical biology 7(6): 348-350.

Farha, M. A. and E. D. Brown (2010). "Chemical probes of Escherichia coli uncovered through chemical-chemical interaction profiling with compounds of known biological activity." Chemistry & biology 17(8): 852-862.

Farha, M. A., A. Leung, et al. (2013). "Inhibition of WTA synthesis blocks the cooperative action of PBPs and sensitizes MRSA to beta-lactams." ACS chemical biology 8(1): 226-233.

Fischer, H. P., N. A. Brunner, et al. (2004). "Identification of antibiotic stress-inducible promoters: a systematic approach to novel pathway-specific reporter assays for antibacterial drug discovery." Genome Res 14(1): 90-98.

Fitzgerald, J. B., B. Schoeberl, et al. (2006). "Systems biology and combination therapy in the quest for clinical efficacy." Nature chemical biology 2(9): 458-466.

Fitzgerald, J. B., B. Schoeberl, et al. (2006). "Systems biology and combination therapy in the quest for clinical efficacy." Nat Chem Biol 2(9): 458-466.

Giaever, G., A. M. Chu, et al. (2002). "Functional profiling of the Saccharomyces cerevisiae genome." Nature 418(6896): 387-391.

Giaever, G., P. Flaherty, et al. (2004). "Chemogenomic profiling: identifying the functional interactions of small molecules in yeast." Proceedings of the National Academy of Sciences of the United States of America 101(3): 793-798.

Higgins, P. G., C. Dammhayn, et al. (2010). "Global spread of carbapenem-resistant Acinetobacter baumannii." The Journal of antimicrobial chemotherapy 65(2): 233-238.

Ho, Y., A. Gruhler, et al. (2002). "Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry." Nature 415(6868): 180-183.

Janoir, C., V. Zeller, et al. (1996). "High-level fluoroquinolone resistance in Streptococcus pneumoniae requires mutations in parC and gyrA." Antimicrobial agents and chemotherapy 40(12): 2760-2764.

Jeong, H., S. P. Mason, et al. (2001). "Lethality and centrality in protein networks." Nature 411(6833): 41-42.

Johnston, R. F. and K. H. Wildrick (1974). "The impact of chemotherapy on the care of patients with tuberculosis." The American review of respiratory disease 109(6): 636-664.

Kalan, L. and G. D. Wright (2011). "Antibiotic adjuvants: multicomponent anti-infective strategies." Expert reviews in molecular medicine 13: e5.

Kasper, A. C., J. B. Baker, et al. (2009). "The end game of chemical genetics: target identification." Future medicinal chemistry 1(4): 727-736.

Keith, C. T., A. A. Borisy, et al. (2005). "Multicomponent therapeutics for networked systems." Nature reviews. Drug discovery 4(1): 71-78.

Keith, C. T., A. A. Borisy, et al. (2005). "Multicomponent therapeutics for networked systems." Nat Rev Drug Discov 4(1): 71-78.

Klevens, R. M., J. R. Edwards, et al. (2006). "Changes in the epidemiology of methicillin-resistant Staphylococcus aureus in intensive care units in US hospitals, 1992-2003." Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 42(3): 389-391.

Lambert, R. J. and R. Lambert (2003). "A model for the efficacy of combined inhibitors." Journal of applied microbiology 95(4): 734-743.

Lee, S. H., L. W. Jarantow, et al. (2011). "Antagonism of chemical genetic interaction networks resensitize MRSA to beta-lactam antibiotics." Chemistry & biology 18(11): 1379-1389.

Lehár, J., A. Krueger, et al. (2008). "High-order combination effects and biological robustness " Mol Syst Biol 4: 215.

Lehar, J., A. S. Krueger, et al. (2009). "Synergistic drug combinations tend to improve therapeutically relevant selectivity." Nat Biotechnol 27(7): 659-666.

Lehar, J., B. R. Stockwell, et al. (2008). "Combination chemical genetics." Nature chemical biology 4(11): 674-681.

Lewis, K. (2013). "Platforms for antibiotic discovery." Nature reviews. Drug discovery 12(5): 371-387.

Liu, A., L. Tran, et al. (2010). "Antibiotic sensitivity profiles determined with an Escherichia coli gene knockout collection: generating an antibiotic bar code." Antimicrobial agents and chemotherapy 54(4): 1393-1403.

Lomenick, B., R. W. Olsen, et al. (2011). "Identification of direct protein targets of small molecules." ACS chemical biology 6(1): 34-46.

Lum, P. Y., C. D. Armour, et al. (2004). "Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes." Cell 116(1): 121-137.

Onyewu, C. and J. Heitman (2007). "Unique applications of novel antifungal drug combinations." Anti-Infect Agents Med Chem 6: 3-15.

Parsons, A. B., A. Lopez, et al. (2006). "Exploring the mode-of-action of bioactive compounds by chemical-genetic profiling in yeast." Cell 126(3): 611-625.

Pathania, R., S. Zlitni, et al. (2009). "Chemical genomics in Escherichia coli identifies an inhibitor of bacterial lipoprotein targeting." Nature chemical biology 5(11): 849-856.

Prosser, G. A. and L. P. de Carvalho (2013). "Kinetic mechanism and inhibition of Mycobacterium tuberculosis D-alanine:D-alanine ligase by the antibiotic D-cycloserine." The FEBS journal 280(4): 1150-1166.

Roemer, T. and C. Boone (2013). "Systems-level antimicrobial drug and drug synergy discovery." Nature chemical biology 9(4): 222-231.

Shafer, R. W. and D. A. Vuitton (1999). "Highly active antiretroviral therapy (HAART) for the treatment of infection with human immunodeficiency virus type 1." Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie 53(2): 73-86.

Spring, D. R. (2005). "Chemical genetics to chemical genomics: small molecules offer big insights." Chemical Society reviews 34(6): 472-482.

Stockwell, B. R. (2000). "Chemical genetics: ligand-based discovery of gene function." Nature reviews. Genetics 1(2): 116-125.

Strebhardt, K. and A. Ullrich (2008). "Paul Ehrlich's magic bullet concept: 100 years of progress." Nature reviews. Cancer 8(6): 473-480.

Swinney, D. C. and J. Anthony (2011). "How were new medicines discovered?" Nature reviews. Drug discovery 10(7): 507-519.

Tan, C. M., A. G. Therien, et al. (2012). "Restoring methicillin-resistant Staphylococcus aureus susceptibility to beta-lactam antibiotics." Science translational medicine 4(126): 126ra135.

Tan, X., L. Hu, et al. (2012). "Systematic identification of synergistic drug pairs targeting HIV." Nature biotechnology 30(11): 1125-1130.

Walsh, C. (2003). "Where will new antibiotics come from?" Nature reviews. Microbiology 1(1): 65-70.

Winzeler, E. A., D. D. Shoemaker, et al. (1999). "Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis." Science 285(5429): 901-906.

Wright, G. D. (2005). "Bacterial resistance to antibiotics: enzymatic degradation and modification." Advanced drug delivery reviews 57(10): 1451-1470.

Yeh, P., A. I. Tschumi, et al. (2006). "Functional classification of drugs by properties of their pairwise interactions." Nature genetics 38(4): 489-494.

Zheng, W., N. Thorne, et al. (2013). "Phenotypic screens as a renewed approach for drug discovery." Drug discovery today 18(21-22): 1067-1073.

Zimmermann, G. R., J. Lehar, et al. (2007). "Multi-target therapeutics: when the whole is greater than the sum of the parts. ." Drug Discov Today 12: 34-42.

CHAPTER TWO - Chemical Probes of *Escherichia coli* Uncovered through Chemical-Chemical Interaction Profiling with Compounds of Known Biological Activity

Preface

The work presented in this chapter was previously published in:

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For this work, I performed all of the experiments and wrote the manuscript, with edits provided by Eric D. Brown.

Summary

While cell based screens have considerable power in identifying new chemical probes of biological systems and leads for new drugs, a major challenge to the utility of such compounds is in connecting phenotype with a cellular target. Here, we present a systematic study to elucidate the mechanism of action of uncharacterized inhibitors of the growth of *Escherichia coli* through careful analyses of interactions with compounds of known biological activity. We studied growth inhibition with a collection of 200 novel antibacterial compounds when systematically combined with a panel of 14 known antibiotics of diverse mechanism and chemical class. Our work revealed a high frequency of synergistic chemical-chemical interactions where the interaction profiles were unique to the various compound pairs. Thus the work revealed that chemical-chemical interaction data provides a fingerprint of biological activity and testable hypotheses regarding the mechanism of action of the novel bioactive molecules. In the study reported here, we determined the mode of action of a novel inhibitor of folate biosynthesis and a novel DNA gyrase inhibitor. Moreover, we identified 8 membrane-active compounds, found to be promiscuously synergistic with known bioactives.

Introduction

Phenotype based small molecule screening has emerged as a dominant approach for the discovery of new probes of complex biology and of leads for new drugs. While cell based screens have considerable power in the discovery of new chemical matter with biological activity, the major challenge to the utility of such molecules is an understanding of mechanism of action.

Nowhere is the discovery of new bioactive chemical matter more important than in antibacterial research. With existing antibiotics directed at a small number of targets, principally cell wall, DNA and protein biosynthesis, multidrug resistance among bacterial pathogens is thought to be due in large part to the limited repertoire of antibacterial chemical matter that eradicate bacteria using a narrow range of mechanisms. Indeed, multidrug resistance in bacteria continues to be a health-care burden in both hospital and community settings where strains of some pathogens, e.g., *Pseudomonas aeruginosa, Staphylococcus aureus* and *Mycobacterium tuberculosis,* resist the action of every antibiotic in use (Boucher, et al., 2009).

In addition to the well-recognized value of small bioactive molecules as leads for new drugs, there is an emerging demand for new chemical perturbants of biological complexity (Peterson, 2008). While genetic perturbation, either by mutagenesis or targeted gene deletion, is the conventional route to probe cellular function it has drawbacks (Alaimo, et al., 2001; Stockwell, 2000). Genetic inactivation is permanent, frequently 'all or none' in scope and for genes that are essential is fraught with the

difficulty of creating conditional alleles. Further, the introduction of multiple mutations in the same cell is tedious in even the most tractable systems.

A considerable obstacle to the use of small molecules as probes of biological systems is the limited availability of highly-characterized probes. While cell based screens have considerable power in identifying new chemical perturbants, a major challenge to the utility of such probes is in understanding mechanism of action (Burdine and Kodadek, 2004). There is simply a paucity of systematic methods to reveal the cellular target or mechanism of action of phenotype-altering small molecules. Classically, protein targets have been identified biochemically using labelled or immobilized molecules. Among the most exciting advances in systematic approaches has been the development of a competitive growth assay using a pool of barcoded genome-wide heterozygous yeast strains to identify mutants that fail to grow in the presence of growth inhibitory drugs (Baetz, et al., 2004; Giaever, et al., 1999; Lum, et al., 2004; Parsons, et al., 2004). More recently, with the explosion of genomic sequence information and associated tools, efforts to identify cellular targets have turned to genome-scale clone sets for the systematic identification of protein targets of small molecules of interest (Hillenmeyer, et al., 2008; Pathania, et al., 2009). Such approaches have largely been limited to model microbes (Baetz, et al., 2004; Giaever, et al., 1999; Lum, et al., 2004; Parsons, et al., 2004), and in a recent application, to the pathogen *Staphylococcus aureus* (Donald, et al., 2009). While the aforementioned tools have proven their utility in characterizing both existing and novel bioactives, their biggest drawback lies in the requirement for genome-scale clone sets. These approaches are

specific to the organisms under study, and are virtually impossible to transfer to even closely related species, highlighting the need for systematic methods that are universally applicable to understanding the mechanism of action of small molecules, independent of the biological system of interest.

Out of recognition of the complexity and redundancy of biological networks, chemical combinations are increasingly touted as having special utility as both therapies and probes of biological systems (Lehar, et al., 2008; Zimmermann, et al., 2007). The biological impact of combinations of chemicals can be classified as synergistic, additive, or antagonistic, depending on whether the combined effect of the compounds is larger than, equal to or smaller than the effects that might be predicted from the individual drugs, respectively. The potential for efficacious drug synergy has long led to the routine testing and use of drug combinations, especially in antimicrobial therapies, but largely as an afterthought to the discovery of antibiotics (Moellering, 1983). A renaissance in interest in exploiting the power of chemical combinations in drug discovery has been accompanied by an emerging awareness of the value of simultaneous application of two molecular probes to gain biological insight (Lehar, et al., 2008; Yeh and Kishony, 2007; Yeh, et al., 2006). It is nevertheless early days in chemical combination research and there have been no systematic applications of chemical-chemical interaction profiling to understand the mechanism of action of novel bioactive molecules discovered in high-throughput screening.

In the work described herein, we have taken a systematic approach to elucidating the mechanism of action of uncharacterized inhibitors of the growth of *Escherichia coli*

through meticulous analyses of interactions with compounds of known biological activity. We have examined growth inhibition of *E. coli* using a collection of 200 novel antibacterial compounds of unknown mechanism when combined with a panel of 14 known bioactive antibiotics of diverse mechanism and chemical class. Our work revealed a surprising frequency of synergistic chemical-chemical interactions where the interaction profiles were unique to the various compound pairs. Thus these studies revealed that chemical-chemical interaction data can provide a fingerprint of biological activity and testable hypotheses regarding the mechanism of action of the novel bioactive molecules. We determined the mode of action of two novel antibacterial compounds. One molecule was found to be a novel inhibitor of folate biosynthesis and the other a novel DNA gyrase inhibitor. Further, the method allowed for the identification of membrane-active compounds. These compounds showed promiscuous synergistic behaviour in combination with various known bioactives. Of interest, we identified 8 compounds that were capable of depolarizing the membrane of *E. coli*.

Results

A screen for growth inhibitory small molecules

Our work began with a high-throughput screen to identify bioactive molecules from a library of approximately 50,000 small molecules that were growth inhibitory against *E. coli* strain MC1061 (Li, et al., 2004). *E. coli* MC1061 is an outer membrane hyperpermeable mutant, making it hypersensitive to known antibiotics (Casabadan and Cohen, 1980). A subset of actives, namely 203 compounds (Table S1), was selected based on structural diversity, solubility, and resupply (Figure 2-1). Further prioritization based on minimum inhibitory concentration (MIC) determination against *E. coli* MC1061 excluded 17 compounds with high minimum inhibitory concentrations (≥102.4 μg/mL) (Figure 2-1). These remaining 186 molecules were further subjected to combination profiling with known bioactives, 14 antibiotics of diverse mechanism and chemical class, to elucidate mechanism of action(s).

Combination profiling screen

This comparatively small number of priority actives (Figure 2-1) generates a large number of possible experiments when combined with 14 known bioctives, namely 2,604 pairwise combinations. Indeed, chemical-chemical interaction studies to detect synergy typically employ standard checkerboard methodology using a 64-point dose matrix (Krogstad and Moellering Jr., 1986). Thus a single replicate of the checkerboard methodology would require more than 166,000 wells, excluding controls, to examine our 186 priority actives in combination with 14 known bioactives of diverse chemical class and antibacterial mechanism. Instead, we developed a high-throughput method for the

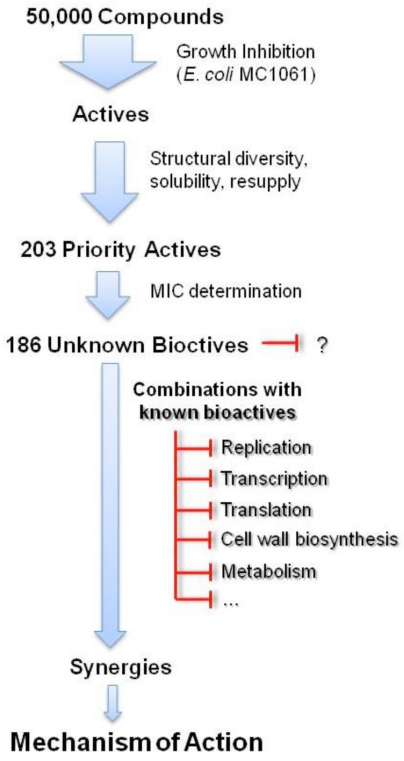


Figure 2-1. Chemical-chemical interaction profiling to characterize novel growth inhibitory compounds derived from small molecule screening. Summary of the approach to understand mechanism(s) of action of a novel active chemical matter. Synergies uncovered through combination studies, where priority actives are systematically combined with a panel of known bioactive compounds of diverse mechanism and chemical class, provide clues about the pathways and targets.

efficient identification of synergistic interactions, whereby two small molecules at sub-lethal concentrations become growth inhibitory when combined. We opted to combine compounds only at a quarter and eighth of their minimum inhibitory concentrations (MIC). This stems from the widely recognized definition of synergy, as requiring a minimum of fourfold reduction in the MIC of both compounds in combination, compared with each used alone (Krogstad and Moellering Jr., 1986). Combining bioactives at an

eighth of their MICs allows for the ready identification of highly synergistic interactions. Additionally, since there is an inherent 1-dilution variability when determining the MICs of the compounds alone (Rand, et al., 1993), combinations at an eighth of the MIC allow for a more conservative approach. This systematic two-point dose matrix allowed us to test for synergistic interactions for all of the priority actives when combined with the known bioactives in just 5,200 wells. In addition, all combinations were tested in triplicate, allowing the assignment of a standard error to all percent growth values, and the inclusion of controls accounting for 20% of all test wells ensured that all test samples could be normalized on a plate by plate basis to cells to high controls. To check that this high-throughput approach was as sensitive as the checkerboard method in detecting synergy, combination studies using both methods were conducted on a random subset of 240 pairs of small molecules and a 96% rate of agreement was calculated, revealing the reliability of the two-point dose matrix in detecting synergy (Figure S1).

Figure 2-2 highlights our two-point dose matrix approach (panel A) and shows average data from the combination profiling screen of combinations at both 1/4 and 1/8 MIC (panel B). For this work, we defined the "combination ratio" as the ratio of the average percent growth (from three replicates) of cells exposed to the various combinations divided by the average percent growth in the presence of only the known bioactives. Although the data were normalized to the percent growth in the presence of the known bioactive as a single agent, the activity of each of the 186 priority actives alone was also controlled for in the assay. In all cases, growth in the presence of the priority actives as single agents resulted in over 85% growth relative to the high control. A pair of

compounds with a combination ratio of 0.25 or lower was considered synergistic. This represents a growth inhibition of at least 75%, corresponding to the statistical threshold based on the high controls in the screen (Zlitni, et al., 2009). Figure 2-2A shows detailed sample data from the two-point dose matrix approach, where three possible chemical-chemical interaction scenarios are depicted. Figure 2-2A panel i shows an instance where the combination of the priority active and known bioactive have no interactions. In panel ii synergy is manifested at 1/4 the MIC for the two compounds but not at 1/8 the MIC and in panel iii synergy is evident at both 1/4 and 1/8 the MIC. This analysis allowed a straightforward assessment of the various chemical-chemical interactions. Synergistic pairings were evident when the effect on percent growth was significantly reduced when in combination, as compared to their effects individually. These compounds were considered hits and further evaluated in a full fingerprint of biological activity with the other known bioactives.

Figure 2-2B shows average combination ratios for each of the 186 priority actives with each of the 14 known bioactives. For the most part, combinations led to only occasional synergy, evident as a combination ratio of less than 0.25. Interestingly, triclosan was found to be synergistic with a large number of molecules, particularly at 1/4 MIC. This promiscuous behaviour is presumably due to the mechanism of triclosan, well known as disruptor of bacterial membranes (Schweizer, 2001). The next greatest preponderance of synergistic interactions of the priority actives was with fosmidomycin, sulfamethoxazole, and trimethoprim, where combination ratios were comparatively low overall relative to the other known bioactives. We attribute this trend to the shape of the

dose-response curves for these known bioactives which revealed a gradual inhibitory effect compared to steeper dose response curves for the other known bioactive compounds (Figure S2A). The shallower dose-response curve makes these compounds more prone to synergistic interactions. At a concentration of 1/4 MIC, the activity of the known bioactives would fall within the slope of the dose-response curve such that it would be more inclined to a drastic change in inhibitory activity upon combination with a second agent. And while the combination ratios of fosmidomycin, sulfamethoxazole, and trimethoprim are relatively low, the effect of the combination is not strong enough to fall into our statistically defined zone of synergy (<0.25). Indeed, we confirmed that this dose response behaviour leads to more frequent synergy when using the standard 64-point dose matrix checkerboard analyses (Figure S2B).

Evaluation of uncovered synergistic interactions

The combination profiling screen revealed that 45 of the 186 priority compounds had synergistic interactions with the 14 known bioactive compounds. At 1/4 MIC, a total of 112 compound combinations (excluding triclosan) were shown to be capable of reducing the growth of *E. coli* MC1061 by at least 75%. Triclosan showed an additional 143 synergistic combinations. These results are presented in the form of a heat map, where interactions of the priority actives with the panel of known bioactives are coloured

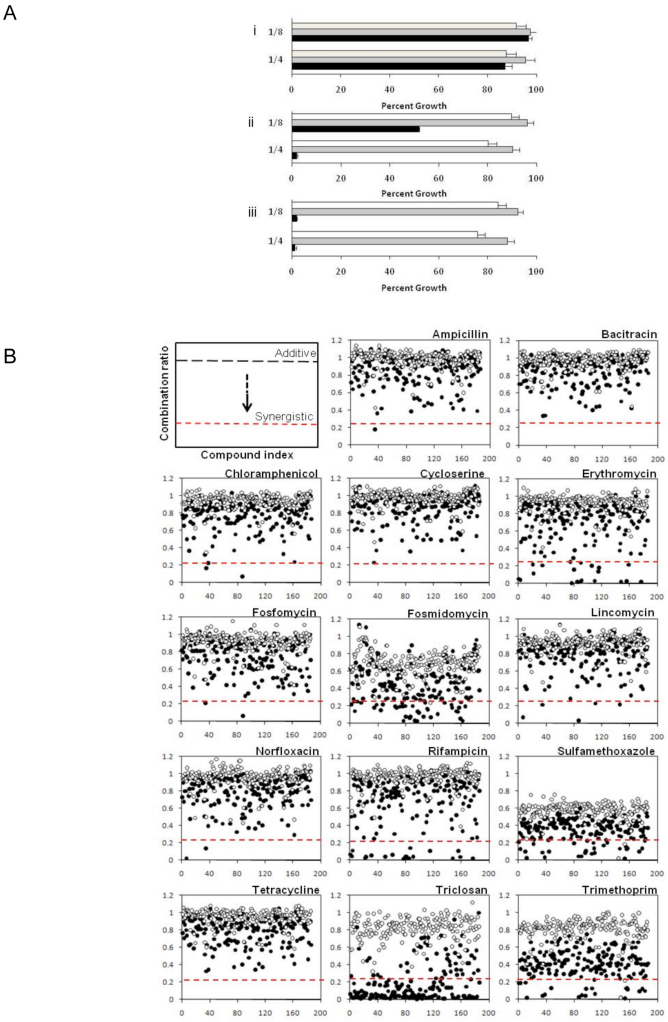


Figure 2-2. Chemical-chemical interaction profiling screen with known bioactives. Panel (A) shows sample two-point dose matrix data showing three possible outcomes of chemical-chemical interaction screens. The panels depict the effect of combining a known bioactive compound with a priority active. Growth of the combinations is represented by a black horizontal bar where the compound concentrations are at one quarter (1/4) and one eighth (1/8) of their MICs as indicated. Growth of the known bioactive compounds alone is described by a white horizontal bar and that of the novel priority active alone is a grey bar. All growth is compared to uninhibited controls and expressed as percent growth. (i) Example of an indifferent interaction, where the growth of the combination of ampicillin and MAC-0002408 was not altered significantly relative to that obtained with the compounds individually. (ii) A highly synergistic interaction is observed when norfloxacin and MAC-0003199 are combined at 1/4 of their MIC's. (iii) MAC-00038968 and sulfamethoxazole show profound synergy at both at 1/4 and 1/8 of their MICs. Panel (B) shows all the result of chemical-chemical interaction profiling of 2,604 possible pairwise combinations of 186 priority actives with 14 known antibiotics tested at 1/4 (●) and 1/8 (○) MIC values to identify synergistic interactions. Graphed are the average combination ratios (triplicate data) where percent growth recorded for the compound combinations is normalized to the percent growth found for the known bioactives as single agents for all 186 test compounds. A statistical threshold of inhibition of 75% (normalized ratio of 0.25) was established some 3 standard deviations away from the mean of the high controls (DMSO). Compounds found below this line were judged as hits.

based on the extent of synergy measured (Figure 2-3). The heat map reveals a rich interaction matrix of the 45 synergistic priority actives with known bioactives consistent with the thesis that chemical-chemical interactions can uncover valuable functional connections for uncharted small molecule inhibitors of bacterial growth.

When the known bioactives were combined with themselves, very few combinations were synergistic. This was not unexpected, however, as these compounds were chosen to probe diverse aspects of bacterial physiology. However, a small number of known bioactives, fosmidomycin, sulfamethoxazole, trimethoprim and triclosan, were found to yield synergies. Among these was trimethoprim and sulfamethoxazole, a signature interaction that has been long exploited in antibacterial therapy (Rubin and Swartz, 1980). Additionally, fosmidomycin was found to be synergistic with both trimethoprim and sulfamethoxale (Neu and Kamimura, 1982).

Having selected for priority actives that were synergistic with known bioactives, the heat map details a high density of interactions for these 45 molecules. Many of these are unique interactions that reveal a fingerprint of selective biological activity. On the other hand, many of the compounds demonstrated promiscuous synergistic interactions with several known antibiotics. For example, MAC-0010522 was strongly synergistic with all chosen known antibiotics. Such behaviour suggested that this compound might act on multiple pathways or affect cellular permeability, perhaps enhancing the uptake of the known bioactives. Thus, our screening approach had an ability to identify non-selective molecules that would otherwise prove quite challenging in follow-up experiments to identify cellular target(s). The promiscuous nature of these molecules can

limit their utility as chemical probes or as leads for drugs. Such molecules were further assessed for their ability to permeabilize bacterial membranes as described below.

Clustering of chemical-chemical interaction profiles

To assess whether there was a correlation of chemical-chemical interaction profiles and chemical structures, we performed hierarchical clustering of the priority synergistic actives based on their profiles (Figure 2-3) and on chemical similarity (Figure S3). More than half the time, clustering by structural similarity tracked with clustering by chemical-chemical interaction profile (Figure S3). Examples of related compounds are shown in Table 2-I. MAC-0007715 and MAC-0007720 share chemical functionality, only differing in one of the thiourea side chains possessing either a morpholine or 2-methylpiperdine ring, and show a unique interaction fingerprint that includes erythromycin, rifampicin, sulfamethoxazole and triclosan. MAC-0019671 and MAC-0020001 show a similar profile but with an additional signature interaction with fosmidomycin, and only differ in their substituents in the *para* position of the *N*-phenylurea functionality. MAC-002303 and MAC-0024645 share a 1-(4-chlorophenyl)urea moiety and interacted uniquely with fosmidomycin, sulfamethoxazole and triclosan. These observations reinforce a well-established concept that chemical structure dictates biological activity and reveal the predictive power of our approach in assigning the potential chemical-chemical interaction profile of a novel molecule. There are however examples of molecules showing similar chemical-chemical interactions when in combination, without being structurally similar.

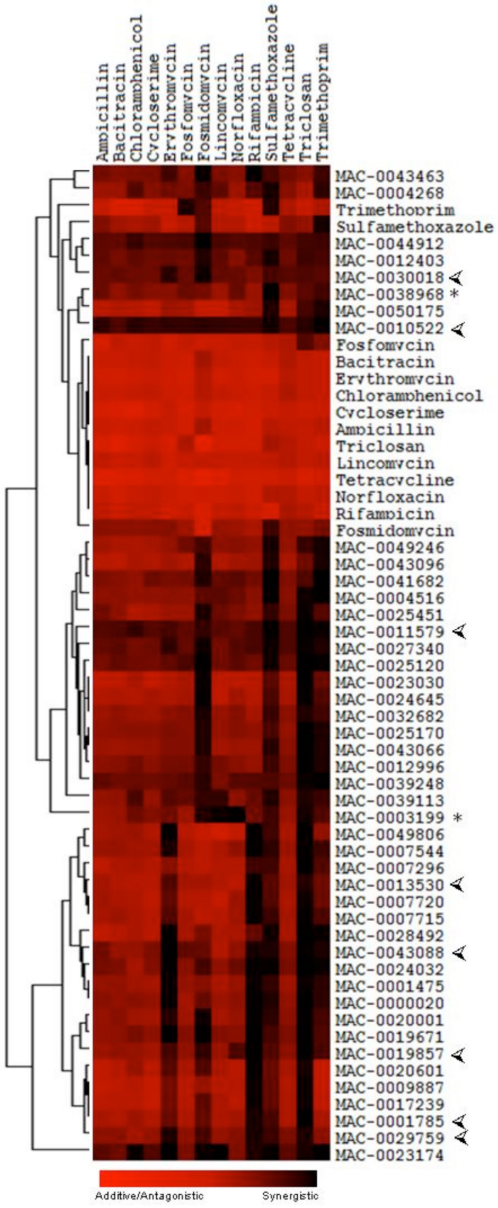
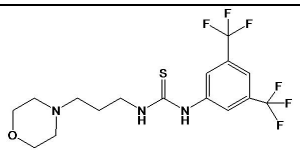
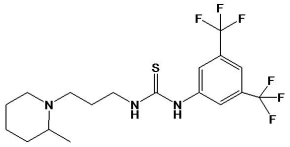
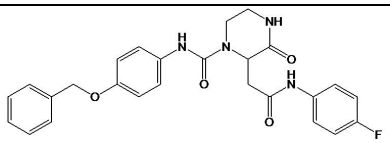
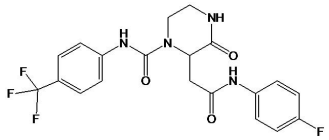
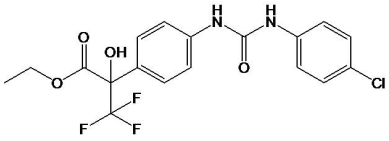
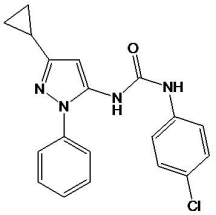


Figure 2-3. Hierarchical clustering of chemical-chemical interaction profiles. Priority actives found to be synergistic with at least one known bioactive are clustered according to their response when combined with the panel of known antibiotics. Hierarchical cluster analysis was performed based on relative percent residual growth using Cluster software and displayed using Treeview software (Eisen, et al., 1998). Highly synergistic interactions are represented in black. The asterisks (*) denote the two molecules of high interest in this work: MAC-0038968, a dihydrofolate reductase inhibitor, and MAC-0003199, a DNA gyrase inhibitor. The arrows (⋖) to the right of the fingerprints represent the molecules found to be membrane active as judged by the $DiSC_3$ fluorescence assay described herein.

Table 2-1. Examples of molecules with similar structures and biological fingerprints.

Compound	Structure	Biological Fingerprints
MAC-0007715		
MAC-0007720		
MAC-0019671		
MAC-0020001		
MAC-0023030		
MAC-0024645		

Biological fingerprints derived from the combination profiling screen with a panel of known antibiotics for the various molecules are shown, where highly synergistic combinations are represented in black. The biological fingerprints were taken out of the heat map in Figure 2-3 to illustrate the correlation between activity and chemical structures.

In this work, we were especially interested in following up on compound combinations that showed unique synergies. Two high interest compounds, MAC-0038968 and MAC-0003199, were selected for follow-up experiments to characterize cellular targets and mechanisms of action. We also investigated the activity of a subset of priority actives that were found to be promiscuously synergistic.

MAC-0038968 is synergistic with sulfamethoxazole and active against dihydrofolate reductase (DHFR)

One compound of interest uncovered in our screen was MAC-0038968 (Figure 2-4A). This small molecule was found to be uniquely synergistic with sulfamethoxazole, an antibiotic that inhibits tetrahydrofolate biosynthesis in bacteria by acting as an analog to one of the pathway intermediates (Walsh, 2003). A detailed checkerboard analysis of the compound combination confirmed strong synergy between MAC-0038968 with sulfamethoxazole with a fractional inhibitory index (∑FIC) of 0.187 (Figure 2-4B). Interestingly, we previously reported on this compound in a biochemical screen to identify inhibitors of dihydrofolate reductase (DHFR), the enzyme responsible for the reduction of dihydrofolate to tetrahydrofolate but had not investigated its cellular activity (Zolli-Juran, et al., 2003). The observed synergy served to validate the proposed method, as MAC-0038968, which was found to be highly synergistic with sulfamethoxazole, inhibits a later step in a common cellular pathway. To investigate the cellular target of MAC-0038968, we sought to suppress the action of this compound by expression of the putative target DHFR at high copy. Indeed, we have previously found high copy suppression to be a valuable chemical-genetic tool to probe mechanism of novel

antibacterial compounds (Li, et al., 2004; Pathania, et al., 2009). Figure 2-4C reveals that increased expression of DHFR led to a steady increase in the MIC for MAC-0038968 and trimethoprim without any impact on the MIC of the control compound tetracycline (Figure 2-4C). These results are consistent with the conclusion that the growth inhibition by MAC-0038968 was due to inhibition of DHFR. Trimethoprim, one of the known bioactives in this study is of course a celebrated DHFR inhibitor (Baccanari and Kuyper, 1993). The lack of synergy with trimethoprim (Figure 2-3A) suggests that MAC-0038968 and trimethoprim bind to similar sites on DHFR; otherwise a synergistic interaction would be expected if the two bound to separate sites on the target (Krogstad and Moellering Jr., 1986). In fact, we have previously shown that this DHFR inhibitor, like trimethoprim, is competitive with the substrate dihyrofolate and inhibits the enzyme with a reasonable potency (K_i = 65 nM) (Zolli-Juran, et al., 2003). Importantly MAC-0038968 represents a new chemical class of DHFR inhibitors with cellular activity.

MAC-0003199 is a DNA gyrase inhibitor, uncovered through synergy with norfloxacin

From the combinatorial screen the quinoline carboxylic acid, MAC-0003199, was found to have a unique interaction fingerprint that included lincomycin, triclosan and norfloxacin. The latter interaction was particularly noteworthy as this compound had a selective and profound interaction with this DNA gyrase inhibitor. MAC-0003199 represents a novel structure but is reminiscent of the quinolone family of synthetic antibiotics that are well known for their inhibitory action on bacterial DNA gyrase and topoisomerase IV and associated lethal impact on DNA replication and transcription.

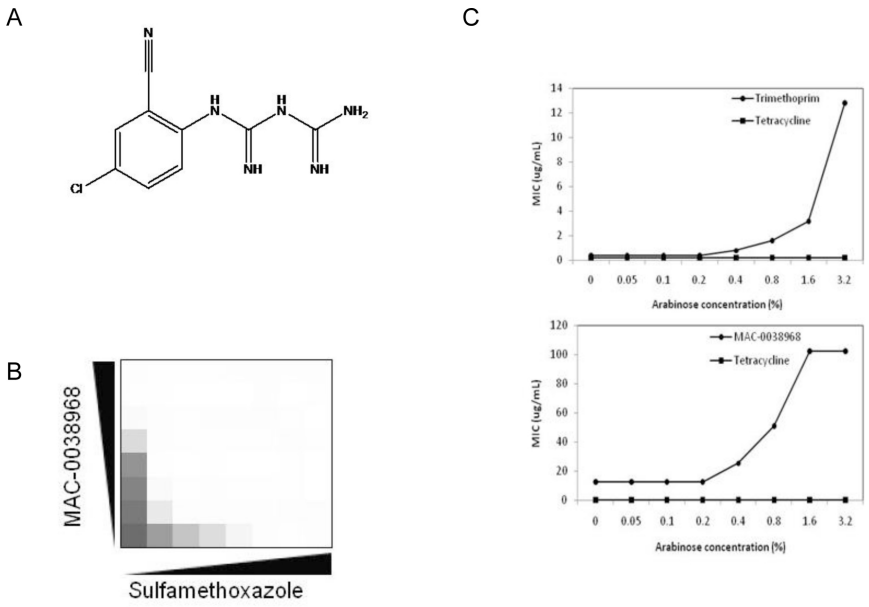


Figure 2-4. MAC-0038968 is uniquely synergistic with sulfamethoxazole and active against dihydrofolate reductase (DHFR). (A) Chemical structure of MAC-0038968. (B) Checkerboard analysis of MAC-0038968 in combination with sulfamethoxazole, showing a strong synergistic interaction with a ΣFIC=0.187. (C) Suppression of growth inhibition by MAC-0038968 on increasing expression of DHFR encoded in the *folA* gene. Here expression is manipulated by the arabinose-inducible promoter of copy of pBAD18-*folA* in *E. coli* strain MC1061. Arabinose dependence of the MIC is shown for the positive control trimethoprim (circles, top panel) and the test compound MAC-0038968 (circles, bottom panel). Also shown is the negative control tetracycline (squares).

A detailed checkerboard analysis of the interaction between norfloxacin and MAC-0003199 confirmed the synergistic interaction with a ∑FIC of 0.312 (Figure 2-5B). In an effort to assess the capacity of MAC-0003199 to induce DNA damage we characterized the *in vivo* phenotypic response of *E. coli* in response to the compound, employing a DNA damage-inducible reporter construct that is based on LexA repression for regulation of *gfp* transcription (Dwyer, et al., 2007). Thus an output in fluorescence is indicative of RecA-stimulated autocleavage of LexA following recognition of DNA-damage (Dwyer, et al., 2007). The control compound norfloxacin led to the induction of high levels of fluorescence (Figure 2-5C). Norfloxacin is known to stabilize a tripartite interaction with DNA gyrase and cleaved DNA thus stimulating the formation of DNA breaks (Walsh, 2003). We also observed a large shift in fluorescence upon treatment of the cells with MAC-0003199 (Figure 2-5C), as compared to the negative control, tetracycline (Figure 2-5C). We next sought to determine whether MAC-0003199's mode of action in the cell was similar to that of norfloxacin, acting by inhibiting DNA gyrase's ability to supercoil DNA (Drlica and Zhao, 1997). MAC-0003199 inhibited DNA gyrase at a relatively high concentration, compared to the potent inhibitor norfloxacin (Figure 2-5D) but nevertheless was consistent with the relative cellular potencies of these compounds (6.4 μg/mL for MAC-0003199 compared to 0.025 μg/mL for norfloxacin).

Interestingly, the synergistic nature of the interaction between MAC-0003199 and norfloxacin suggests that the two molecules do not work with exactly the same mechanism. For example, it seems unlikely that the two compounds bind to exactly the same site on DNA gyrase as this would lead to an antagonistic interaction (Krogstad and

Moellering Jr., 1986). Further, MAC-0003199 was found to be synergistic with triclosan and lincomycin, while norfloxacin showed no such interaction. Triclosan is known to disrupt the inner membrane of bacteria (Schweizer, 2001) and this interaction may relate to inherent permeability problems for MAC-0003199. Lincomycin is an inhibitor of protein synthesis that binds the 50S ribosome and inhibits peptidyl transferase activity, thus preventing translation termination (Lin, et al., 1997; Walsh, 2003). The mechanistic connection between lincomycin and MAC-0003199 remains unclear but suggests nevertheless that MAC-0003199 may not function simply as an inhibitor of DNA gyrase. Its striking similarity in structure to the quinolone class of molecules, however strongly suggests that MAC-0003199 is likely an inhibitor of DNA gyrase, such that its interaction with norfloxacin would expectedly be antagonistic in nature. However, quinolones have a unique mechanism of action in that they inhibit both bacterial DNA gyrase and topoisomerase IV, with varying target preference, depending on the organism in question. In *E. coli*, the inhibition of topoisomerase IV becomes more apparent when DNA gyrase is mutated to be resistant to the quinolone (Khodursky, et al., 1995). Moreover, quinolones can in fact prefer topoisomerase IV over DNA gyrase, and vice-versa, or target both enzymes at nearly the same level (Takei, et al., 2001). This dual-targeting property of quinolones may help explain the synergy between MAC-0003199 and norfloxacin, in that the observed antibacterial activities are involved not only in DNA gyrase inhibition but also in topoisomerase IV inhibition.

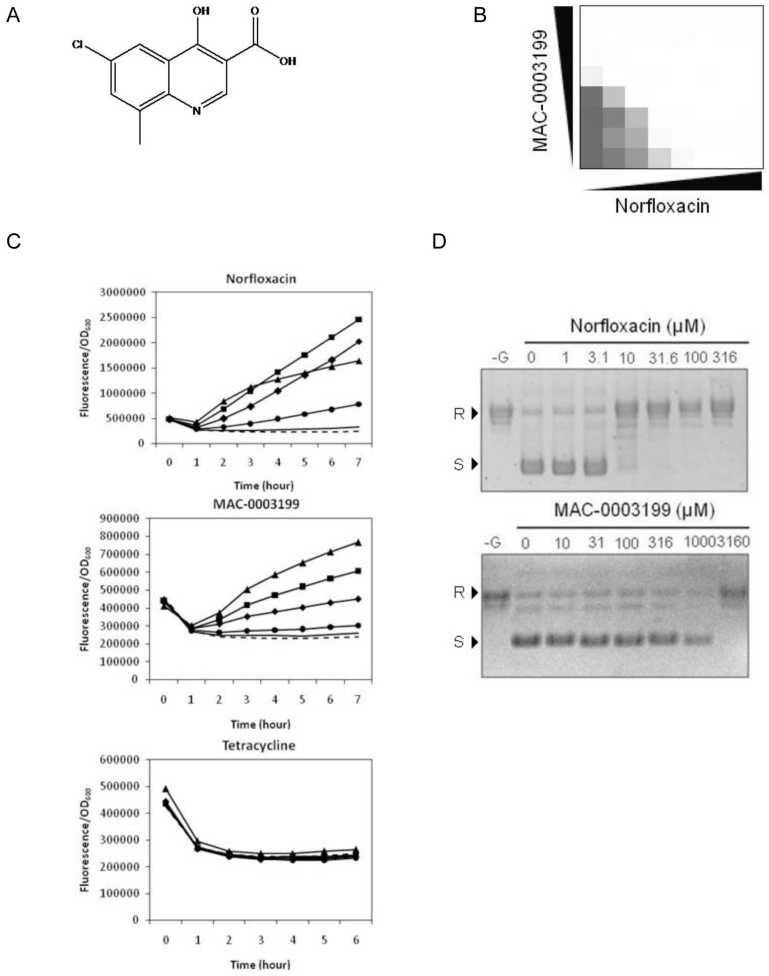


Figure 2-5. MAC-0003199, synergistic with norfloxacin, inhibits DNA gyrase. (A) Chemical structure of MAC-0003199. (B) Checkerboard analysis of MAC-0003199 in combination with norfloxacin, showing synergy with a ΣFIC=0.312 (C). *E. coli* MC1061 harbouring pL(lexO)-GFP, a promoter-GFP reporter gene construct used to report on DNA lesion formation, was grown to an OD_{600} of 0.3-0.4 and treated with various concentration of norfloxacin (positive control), MAC-0003199, and tetracycline (negative control). Optical density at 600 nm and fluorescence at 535 nm were monitored for 7 hours. Induction of DNA damage was evident for norfloxacin and MAC-0003199. (D) Inhibition of supercoiling of DNA by MAC-0003199 and norfloxacin. *E. coli* gyrase was incubated with relaxed pBR322 in the presence of various concentrations of norfloxaxin (top panel) and MAC-0003199 (bottom panel).The lane labelled –G is relaxed pBR322 in the absence of *E. coli* gyrase . R and S represent relaxed and supercoiled pBR322, respectively.

Promiscuously synergistic molecules

As discussed above, a particular advantage of systematic screening for chemical-chemical interactions was the identification of promiscuously synergistic molecules. In an effort to understand the mode of action of such molecules, we investigated the possibility that these were membrane active and resulted in a breach in bacterial membrane potential. This was assessed with a membrane potential-sensitive fluorescent probe, 3,3'-dipropylthiacarbocyanine ($DiSC_3$) (Sims, et al., 1974). Once inside the cell, this dye becomes concentrated and self-quenches its own fluorescence. Membrane active compounds that depolarize the $\Delta\psi$ component of the proton motive force will lead to the release of the dye and a consequent increase in fluorescence. Normally, this dye is incapable of penetrating Gram-negative bacteria; however, we were able to establish this assay using our screening strain (*E. coli* strain MC1061) because of its documented hyperpermeability. In fact, we tested all compounds found to be synergistic in our screen in our combination profiling screen for their capacity to alter membrane potential at MIC concentrations. All 14 of the known bioactives tested negatively in this assay, as expected, whereas polymyxin B, a lipopeptide known to be membrane active (Daugelavicius, et al., 2000), led to a significant increase in $DiSC_3$ fluorescence in our assay (Figure S4A). Of the 45 synergistic priority actives, 8 molecules caused a significant increase in $DiSC_3$ fluorescence, suggesting that these molecules have the capacity to depolarize the cytoplasmic membrane. These are highlighted with an arrow in Figure 2-3 to the right of their respective chemical-chemical interaction profiles and the data are presented in Figure S4B.

It is tempting to conclude that these eight molecules represent non-selective compounds that are unsuitable probes of biology or as leads for new drugs. Membrane active molecules, such as the peptide-drugs, daptomycin and polymyxin B, have proven invaluable in treating drug resistant infections (Evans, et al., 1999; Fuchs, et al., 2002; Giamarellou and Poulakou, 2009; King and Phillips, 2001). Regardless of the disposition toward membrane-active and promiscuous chemical matter, the combination profiling approach used here was quite successful in identifying strong candidates for membrane depolarizing activity among priority actives.

Discussion

The systematic determination of the mechanism of actives derived from phenotype-based small molecule screening remains a very significant challenge to the discovery of useful probes of biology or leads for new drugs. Chemical-genetic approaches have dominated for this purpose in recent years and, while the success of these has been tangible, there remain some significant drawbacks. Forward chemical-genetic methods rely on enhancement or suppression of growth phenotypes associated with novel actives by genetic perturbation. The latter has the potential to inform on mechanism and provide testable hypotheses particularly when there is depth of knowledge on the interacting genes. Classical chemical-genetic approaches are powerful but require the tedious isolation and characterization of suppressing or enhancing mutants (Eggert, et al., 2001; Gitai, et al., 2005; Heitman, et al., 1991). Modern chemical-genomic approaches have typically relied on genome-scale deletion or overexpression clone-sets in model microbes such as yeast or *E. coli* to screen for suppressing or

enhancing genotypes (Baetz, et al., 2004; Giaever, et al., 1999; Li, et al., 2004; Lum, et al., 2004; Pathania, et al., 2009). Such clonesets are currently unavailable in all but the most tractable model microbes.

Herein, we describe an alternative and complementary approach where a small library of known bioactives is systematically combined with novel actives to detect chemical-chemical interactions. Almost 200 growth inhibitory and novel active compounds were systematically combined with a library of some 14 well-known antibiotics of diverse chemical class and mechanism. Combination profiles so discovered were examined in the context of a deep knowledge of the mechanism of interacting chemicals. These profiles generated hypotheses that were testable with biochemical and physiological experiments and revealed new probes of folate metabolism, DNA gyrase and the cell membrane. As such, chemical-chemical interaction profiles can be generated for any phenotype-inducing compound in any biological system without a need for genetic manipulations provided there are existing well characterized chemical perturbants available.

The work reported here suggests that phenotypic responses to combinations of known and novel bioactives can reveal information about the pathways and targets affected by the latter. The approach was data-rich but nevertheless allowed for efficient testing of combinations of chemicals, making it readily adaptable as a secondary screening approach in high-throughput screening efforts. In a validating discovery, the bi-guanidine-containing compound MAC-0038968 was found to be synergistic with sulfamethoxazole. Sulfamethoxazole limits the supply of PABA precursor available to

subsequent steps in the tetrahydrofolate biosynthesis pathway and is well known for its synergy with trimethoprim, an inhibitor of DHFR (Rubin and Swartz, 1980). Further, we demonstrated that MAC-0038968, previously shown to be a potent inhibitor of DHFR *in vitro* (Zolli-Juran, et al., 2003), could be suppressed by high copy DHFR, demonstrating that his compound had cellular actvitiy and was on target. Interestingly, MAC-0038968 showed no synergy with trimethoprim, presumably because these compounds bind to the same site on DHFR. MAC-0003199, a quinoline carboxylic acid, was shown to be a DNA-damaging agent that targets DNA gyrase. This molecule, similar in structure to quinolones, was suitably found to be highly synergistic with norfloxacin, a potent inhibitor of DNA gyrase. The two novel inhibitors of bacterial physiology uncovered through the use of chemical-chemical interactions highlight the utility of the proposed approach in facilitating the search for cellular targets of novel biological probes.

In cases where a novel compound shows promiscuous interactions with multiple known bioactives, this non-selective behaviour can be used as a filter to eliminate nuisance compounds. Other methods to identify such molecules include computational means based on chemical functionality (Roche, et al., 2002; Walters, et al., 1999). Another is a biochemical approach that uses a detergent-based assay to counter-screen for non-selective aggregating compounds (Feng, et al., 2005; McGovern, et al., 2002; Seidler, et al., 2003). There are however few systematic counter screens for non-selective compounds that are cell-based. Interestingly, further study of such compounds in the work reported here led to the discovery of a subset of compounds capable of dissipating the transmembrane potential of *E. coli*. Given recent clinical success of the antibiotic

daptomycin, also know to depolarize membranes (Silverman, et al., 2003), promiscuous behaviour might well be viewed as both a strength and a weakness. Nevertheless, combination profiling proved to be a powerful tool in identifying these molecules.

Interactions between bioactive chemicals can be synergistic or antagonistic (Krogstad and Moellering Jr., 1986). In this study, we limited our focus to synergistic interactions. In the same manner that synergies were specifically used to elucidate mechanisms of action, a screen looking for antagonistic interactions with known bioactives could also yield rich information about associated cellular pathways. Briefly, antagonistic interactions are most often observed when two compounds compete between binding sites or when one alters the binding site of the other (Krogstad and Moellering Jr., 1986). Antagonism might also be the result of complex genetic interactions propagated through the cellular genetic network (D'Elia, et al., 2009; Motter, et al., 2008). Thus, looking for antagonistic interactions among novel and know bioactives, though rare, could surely also provide useful mechanistic insights and testable hypotheses regarding the mechanism of action of novel actives.

Herein we have demonstrated real promise for chemical-chemical interactions in understanding mechanism of the interacting components. So characterized, these interactions also have the potential to perturb the complex and redundant nature of biological pathways. It is increasingly becoming evident that biological systems are composed of dense networks of interacting components that are characterized by redundancy (Ho, et al., 2002; Tong, et al., 2004). Thus, the simultaneous use of multiple perturbants will ultimately be required to adequately describe this complex cellular matrix

(Lehár, et al., 2008). As the compendium of interacting combinations grows, these will become an increasingly powerful tool for assessing mechanism of action.

Significance

Phenotype based screening provides a powerful tool for the discovery and characterization of new probes of biology and leads for new drugs, however significant challenges remain for connecting compounds to their targets. Here, we report the application of systematic chemical-chemical combination profiling to understand the mechanism of action of novel antibacterial compounds. The interaction of newly discovered compounds with a panel of known bioactives of diverse mechanism and chemical class was used to gain insight into mode of action. Combination profiling for synergistic chemical-chemical interactions resulted in a data-rich map of interactions and led to testable hypotheses regarding the mechanism of action of the novel bioactive molecules. One such molecule was found to be a novel inhibitor of folate biosynthesis, (MAC-0038968) and another was shown to be a new DNA gyrase inhibitor (MAC-0003199). The approach also proved to be useful in identifying non-selective molecules that showed promiscuous interaction behaviour where a subset of these compounds was shown to depolarize the bacterial membrane.

Experimental Procedures

Bacterial strains, reagents and general methods. *E. coli* MC1061 was used for all experiments and follow-up work. Growth media was liquid or agar Luria-Bertani Broth (LB). The small molecule library was purchased from Maybridge (Cornwall, England) and was dissolved in DMSO at a concentration of 6.4 mg/ml. All chemicals were purchased from Sigma Aldrich (Oakville, ON).

Determination of minimum inhibitory concentrations of chemical compounds using E. coli strain MC1061. The minimum inhibitory concentrations of priority actives and known antibiotics were determined to characterize their growth inhibition. Typically, *E. coli* MC1061 was grown overnight in 5 ml of LB media. The cells were then diluted 1:100 in fresh media and allowed to grow until the OD_{600} reached 0.4. The cells were then diluted 1:100,000 and exposed to two-fold serial dilutions of the compounds at final concentrations ranging from 0-204.8 µg/mL from stock solutions of 6.4 mg/mL. These were tested in 96-well microwell plates with a total volume of 200 µl and incubated at 37°C with 80% humidity for 18 hours before determining optical density at 600 nm. The concentration where the optical density was less than 0.05 was deemed the MIC of the test compound.

Combination profiling screen. To screen the various combinations, 80-fold stock solutions of chemicals were placed into polypropylene 96-well master plates in two consecutive columns. Aliquots of 2.5 µl from both master plates were dispensed into the assay plate using a Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, CA) in the McMaster High Throughput Screening Laboratory. Subsequently, 195 µl of a mid-

log subculture of *E. coli* MC1061 cells diluted 1:100,000 were added. These plates were incubated for 18 hours at 37˚C before being read for optical density at 600 nm. All combinations were done in triplicate, allowing the assignment of a standard error to all percent growth values.

Checkerboard analysis of synergy. A mid-log subculture of *E. coli* MC1061 cells was diluted 1:100,000 before adding to an 8 x 8 matrix of priority actives and known antibiotics. The plates were incubated for 18 hours at 37 ˚C before reading for optical density at 600 nm. All combinations were done in duplicate. Heat maps of the averages were created in Excel and used to illustrate growth compared to the high controls (DMSO only). To evaluate the effect of the combinations, the fractional inhibitory concentrations (∑FIC) indices were calculated. This metric is defined as the sum of the MIC of each drug when used in combination divided by the MIC when used alone. Chemical-chemical interactions with ∑FIC of less than 0.5 were deemed synergistic.

High copy suppression of growth inhibition. *E. coli* MC1061 harbouring pBAD18-*folA* was grown overnight in LB supplemented with 100 μg/mL ampicillin, subcultured the following day (1:100 dilution in the same media) and grown to mid log (OD_{600} = 0.4) with aeration at 250 rpm at 37ºC. The cells were then diluted 1:100,000 and exposed to two-fold serial dilutions of the compounds ranging from 0-102.4 μg/mL in the presence of various concentration of arabinose (0-3.2%). These were tested in 96-well microwell plates (total volume of 200 μl) and incubated at 37°C with 80% humidity for 18 hours before determining optical density at 600 nm. The concentration where the optical density was less than 0.05 was deemed the MIC.

Promoter-reporter construct experiments. The DNA-damage reporter construct was a kind gift from Dr. James Collins (Boston University). In all experiments, *E. coli* MC1061 harbouring pL(lexO)-GFP were grown overnight and then diluted 1:100 in 50 mL LB supplemented with 100 mg/ml ampicillin. Cells were grown at 37˚C, 250 rpm, until an OD_{600} of 0.3-0.4. Cells were aliquoted into black clear bottom 96-well plates (Corning Life Sciences, New York, USA) and drug, previously serially diluted in stock plates, was added at various concentrations. Optical density at 600 nm and fluorescence at 535 nm were monitored for 7 hours using the EnVision from Perkin Elmer (Massachusetts, USA).

DNA supercoiling assay. *E. coli* gyrase was incubated with 0.5 µg of relaxed pBR322 DNA in a 30 µl reaction mixture at 37˚C for 1 h under the following conditions: 35 mM Tris-HCL (pH 7.5), 24 mM KCl, 4mM $MgCl_2$, 2 mM DTT, 1.75 mM ATP, 5 mM spermidine, 0.1 mg/mL BSA, and 6.5% glycerol. Reactions were analyzed by electrophoresis through a 0.8% agarose gel (run at 130V for 1.5 h).

Dissipation of transmembrane potential. The transmembrane potential was determined with the fluorescent probe , 3'-dipropylthiacarbocyanine ($DiSC_3(5)$). *E. coli* MC1061 cells were washed twice with Tris buffer (10 mM, pH 7.5), and then resuspended to an optical density at 600 nm of 0.35. $DiSC_3(5)$ was added at a final concentration of 0.4 µM, and the cells were incubated, with constant stirring to let the dye stabilize. Compounds were then injected. Fluorescent traces were measured in a fluorimeter (Photon Technology International) at the excitation and emission wavelengths of 622 and 660 nm, respectively.

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References

Alaimo, P.J., Shogren-Knaak, M.A., and Shokat, K.M. (2001). Chemical genetic approaches for the elucidation of signaling pathways. Curr Opin Chem Biol. 5, 360-367.
Baccanari, D.P., and Kuyper, L.F. (1993). Basis of selectivity of antibacterial diaminopyrimidines. J Chemother 5, 393-399.
Baetz, K., McHardy, L., Gable, K., Tarling, T., Reberioux, D., Bryan, J., Andersen, R.J., Dunn, T., Hieter, P., and Roberge, M. (2004). Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. Proc Natl Acad Sci U S A 101, 4525-4530.
Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., Scheld, M., Spellberg, B., and Bartlett, J. (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis 48, 1-12.
Burdine, L., and Kodadek, T. (2004). Target identification in chemical genetics: the (often) missing link. Chem Biol 11, 593-597.
Casabadan, M.J., and Cohen, S.N. (1980). Analysis of gene control signals by DNA fusion and cloning in Escherichia coli. . J Mol Biol. 138, 179-207.
D'Elia, M.A., Pereira, M.P., and Brown, E.D. (2009). Are essential genes really essential? Trends Microbiol 17, 433-438.
Daugelavicius, R., Bakiene, E., and Bamford, D.H. (2000). Stages of polymyxin B interaction with the Escherichia coli cell envelope. Antimicrob Agents Chemother 44, 2969-2978.
Donald, R.G., Skwish, S., Forsyth, R.A., Anderson, J.W., Zhong, T., Burns, C., Lee, S., Meng, X., LoCastro, L., Jarantow, L.W., et al. (2009). A Staphylococcus aureus fitness test platform for mechanism-based profiling of antibacterial compounds. Chem Biol 16, 826-836.
Drlica, K., and Zhao, X. (1997). DNA gyrase, topoisomerase IV, and the 4-quinolones. Microbiol Mol Biol Rev 61, 377-392.

Dwyer, D.J., Kohanski, M.A., Hayete, B., and Collins, J.J. (2007). Gyrase inhibitors induce an oxidative damage cellular death pathway in Escherichia coli. . Mol Syst Biol. 3, 1-15.
Eggert, U.S., Ruiz, N., Falcone, B.V., Branstrom, A.A., Goldman, R.C., Silhavy, T.J., and Kahne, D. (2001). Genetic basis for activity differences between vancomycin and glycolipid derivatives of vancomycin. Science 294, 361-364.
Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A 95, 14863-14868.
Evans, M.E., Feola, D.J., and Rapp, R.P. (1999). Polymyxin B sulfate and colistin: old antibiotics for emerging multiresistant gram-negative bacteria. Ann Pharmacother 33, 960-967.
Feng, B.Y., Shelat, A., Doman, T.N., Guy, R.K., and Shoichet, B.K. (2005). High-throughput assays for promiscuous inhibitors. Nat Chem Biol 1, 146-148.
Fuchs, P.C., Barry, A.L., and Brown, S.D. (2002). In vitro bactericidal activity of daptomycin against staphylococci. J Antimicrob Chemother 49, 467-470.
Giaever, G., Shoemaker, D.D., Jones, T.W., Liang, H., Winzeler, E.A., Astromoff, A., and Davis, R.W. (1999). Genomic profiling of drug sensitivities via induced haploinsufficiency. Nat Genet 21, 278-283.
Giamarellou, H., and Poulakou, G. (2009). Multidrug-resistant Gram-negative infections: what are the treatment options? Drugs 69, 1879-1901.
Gitai, Z., Dye, N.A., Reisenauer, A., Wachi, M., and Shapiro, L. (2005). MreB actin-mediated segregation of a specific region of a bacterial chromosome. Cell 120, 329-341.
Heitman, J., Movva, N.R., and Hall, M.N. (1991). Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. Science 253, 905-909.
Hillenmeyer, M.E., Fung, E., Wildenhain, J., Pierce, S.E., Hoon, S., Lee, W., Proctor, M., St Onge, R.P., Tyers, M., Koller, D., et al. (2008). The chemical genomic portrait of yeast: uncovering a phenotype for all genes. Science 320, 362-365.
Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., et al. (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415, 180-183.
Khodursky, A.B., Zechiedrich, E.L., and Cozzarelli, N.R. (1995). Topoisomerase IV is a target of quinolones in Escherichia coli. Proc Natl Acad Sci U S A 92, 11801-11805.
King, A., and Phillips, I. (2001). The in vitro activity of daptomycin against 514 Gram-positive aerobic clinical isolates. J Antimicrob Chemother 48, 219-223.
Krogstad, D.J., and Moellering Jr., R.C. (1986). Antibiotics in Laboratory Medicine. (Baltimore: Williams and Wilkins).
Lehar, J., Krueger, A., Zimmermann, G., and Borisy, A. (2008). High-order combination effects and biological robustness. Mol Syst Biol 4, 215.
Lehar, J., Stockwell, B.R., Giaever, G., and Nislow, C. (2008). Combination chemical genetics. Nat Chem Biol 4, 674-681.
Lehár, J., Stockwell, B.R., Giaver, G., and Nislow, C. (2008). Combination chemical genetics. Nat. Chem. Biol. 4, 674-681.

Li, X., Zolli-Juran, M., Cechetto, J.D., Daigle, D.M., Wright, G.D., and Brown, E.D. (2004). Multicopy suppressors for novel antibacterial compounds reveal targets and drug efflux susceptibility. Chem Biol. 11, 1423-1430.
Lin, A.H., Murray, R.W., Vidmar, T.J., and Marotti, K.R. (1997). The oxazolidinone eperezolid binds to the 50S ribosomal subunit and competes with binding of chloramphenicol and lincomycin. . Antimicrob Agents Chemother 41, 2127-2131.
Lum, A.M., Huang, J., Hutchinson, C.R., and Kao, C.M. (2004). Reverse engineering of industrial pharmaceutical-producing actinomycete strains using DNA microarrays. Metab Eng 6, 186-196.
Lum, P.Y., Armour, C.D., Stepaniants, S.B., Cavet, G., Wolf, M.K., Butler, J.S., Hinshaw, J.C., Garnier, P., Prestwich, G.D., Leonardson, A., et al. (2004). Discovering modes of action for therapeutic compounds using a genome-wide screen of yeast heterozygotes. Cell 116, 121-137.
McGovern, S.L., Caselli, E., Grigorieff, N., and Shoichet, B.K. (2002). A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening. J Med Chem 45, 1712-1722.
Moellering, R.C., Jr. (1983). Rationale for use of antimicrobial combinations. Am J Med 75, 4-8.
Motter, A.E., Gulbahce, N., Almaas, E., and Barabasi, A.L. (2008). Predicting synthetic rescues in metabolic networks. Mol Syst Biol 4, 168.
Neu, H.C., and Kamimura, T. (1982). Synergy of fosmidomycin (FR-31564) and other antimicrobial agents. Antimicrob Agents Chemother 22, 560-563.
Parsons, A.B., Brost, R.L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G.W., Kane, P.M., Hughes, T.R., and Boone, C. (2004). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. Nat Biotechnol 22, 62-69.
Pathania, R., Zlitni, S., Barker, C., Das, R., Gerritsma, D.A., Lebert, J., Awuah, E., Melacini, G., Capretta, F.A., and Brown, E.D. (2009). Chemical genomics in Escherichia coli identifies an inhibitor of bacterial lipoprotein targeting. Nat Chem Biol 5, 849-856.
Peterson, R.T. (2008). Chemical biology and the limits of reductionism. Nat Chem Biol 4, 635-638.
Rand, K.H., Houck, H.J., Brown, P., and Bennett, D. (1993). Reproducibility of the microdilution checkerboard method for antibiotic synergy. Antimicrob Agents Chemother 37, 613-615.
Roche, O., Schneider, P., Zuegge, J., Guba, W., Kansy, M., Alanine, A., Bleicher, K., Danel, F., Gutknecht, E.M., Rogers-Evans, M., et al. (2002). Development of a virtual screening method for identification of "frequent hitters" in compound libraries. J Med Chem 45, 137-142.
Rubin, R.H., and Swartz, M.N. (1980). Trimethoprim-sulfamethoxazole. N Engl J Med 303, 426-432.
Schweizer, H.P. (2001). Triclosan: a widely used biocide and its link to antibiotics. . FEMS Microbiol Lett. 202, 1-7.

Seidler, J., McGovern, S.L., Doman, T.N., and Shoichet, B.K. (2003). Identification and prediction of promiscuous aggregating inhibitors among known drugs. J Med Chem 46, 4477-4486.
Silverman, J.A., Perlmutter, N.G., and Shapiro, H.M. (2003). Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus. Antimicrob Agents Chemother 47, 2538-2544.
Sims, P.J., Waggoner, A.S., Wang, C.H., and Hoffman, J.F. (1974). Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry 13, 3315-3330.
Stockwell, B.R. (2000). Chemical genetics: ligand-based discovery of gene function. Nat Rev Genet 1, 116-125.
Takei, M., Fukuda, H., Kishii, R., and Hosaka, M. (2001). Target preference of 15 quinolones against Staphylococcus aureus, based on antibacterial activities and target inhibition. Antimicrob Agents Chemother 45, 3544-3547.
Tong, A.H., Lesage, G., Bader, G.D., Ding, H., Xu, H., Xin, X., Young, J., Berriz, G.F., Brost, R.L., Chang, M., et al. (2004). Global mapping of the yeast genetic interaction network. Science 303, 808-813.
Walsh, C. (2003). Antibiotics: Actions, Origins, Resistance. ((Washington: ASM Press).
Walters, W.P., Ajay, and Murcko, M.A. (1999). Recognizing molecules with drug-like properties. Curr Opin Chem Biol 3, 384-387.
Yeh, P., and Kishony, R. (2007). Networks from drug-drug surfaces. Mol Syst Biol 3, 85.
Yeh, P., Tschmu, A., and Kishony, R. (2006). Functional classification of drugs by properties of their pairwise interactions. . Nat. Gen. 38, 489-494.
Zimmermann, G.R., Lehar, J., and Curtis, K.T. (2007). Multi-target therapeutics: when the whole is greater than the sum of the parts. . Drug Discov Today 12, 34-42.
Zlitni, S., Blanchard, J.E., and Brown, E.D. (2009). High-throughput screening of model bacteria. Methods Mol Biol 486, 13-27.
Zolli-Juran, M., Cechetto, J.D., Hartlen, R., D.M., D., and Brown, E.D. (2003). High throughput screening identifies novel inhibitors of Escherichia coli dihydrofolate reductase that are competitive with dihydrofolate. . Bioorg. Med. Chem. Lett. 13, 2493-2496.

CHAPTER THREE - Inhibition of WTA Synthesis Blocks the Cooperative Action of PBPs and Sensitizes MRSA to ß-lactams

Preface

The work presented in this chapter was previously published in:

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For this work, I performed all experiments with technical assistance from Leung A, with the exception of: creation of *tarO* deletion strains and PG analysis (D'Elia, MA and Pereira, PM), creation of the *tarH* conditional deletion strain (Allison, SE) and TarO *in vitro* assay (Sewell, EW). Ejim L provided technical assistance with the *G. mellonella* model. I wrote the manuscript with edits provided by Brown, ED.

Abstract

Rising drug resistance is limiting treatment options for infections by methicillin-resistant *Staphylococcus aureus* (MRSA). Herein we provide new evidence that wall teichoic acid (WTA) biogenesis is a remarkable antibacterial target with the capacity to destabilize the cooperative action of penicillin-binding proteins (PBPs) that underlie ß-lactam resistance in MRSA. Deletion of gene *tarO,* encoding the first step of WTA synthesis, resulted in the restoration of sensitivity of MRSA to a unique profile of β-lactam antibiotics with a known selectivity for penicillin binding protein 2 (PBP2). Of these, cefuroxime was used as a probe to screen for previously-approved drugs with a cryptic capacity to potentiate its activity against MRSA. Ticlopidine, the antiplatelet drug Ticlid®, strongly potentiated cefuroxime and this synergy was abolished in strains lacking *tarO*. The combination was also effective in a *Galleria mellonella* model of infection. Using both genetic and biochemical strategies, we determined the molecular target of ticlopidine as the *N*-acetylglucosamine-1-phosphate transferase encoded in gene *tarO* and provide evidence that WTA biogenesis represents an Achilles heel supporting the cooperative function of PBP2 and PBP4 in creating highly cross-linked muropeptides in the peptidoglycan of *S. aureus*. This approach represents a new paradigm to tackle MRSA infection.

Introduction

Since their first appearance in the early 1960s, methicillin-resistant *Staphylococcus aureus* (MRSA) strains have spread worldwide and have become one of the most menacing of human pathogens (*1-2*). For much of this period, outbreaks of MRSA were confined to hospitals, however, over the last decade, the prevalence of MRSA in the community has increased alarmingly. USA300 and USA400 isolates now represent the most predominant cause of community-acquired infections in the United States, Canada and Europe (*3-4*). While penicillin and other β-lactams such as methicillin were once very effective antibiotics in treating staphylococcal infections, the widespread resistance of MRSA to this class of antibiotics has made treatment increasingly difficult. Besides common resistance to methicillin and β-lactams in general, *S. aureus* has also become resistant to so-called 'drugs of last resort' including vancomycin, daptomycin and linezolid (*5-7*).

β-lactams target the synthesis of peptidoglycan (PG), a stress-bearing cell wall polymer of a disaccharide of *N*-acetylmuramic acid and *N*-acetylglucosamine. The former is bonded to a short amino acid stem (L-alanine-D-*iso*-glutamine-L-lysine-D-alanine-D-alanine) that is bridged to neighboring stems via a pentaglycyl segment in *S. aureus* (Supplementary Figure 1). Transglycosylases and transpeptidases mediate the final assembly of peptidoglycan, forming glycosyl bonds between the disaccharides and cross-links between the neighboring stem peptides using pentaglycine bridges, respectively. These enzymes are collectively known as penicillin-binding proteins

(PBPs), with their transpeptidase region making up the cellular target of β-lactam antibiotics. β-lactam resistance in MRSA involves the acquisition of PBP2A, encoded in *mecA*, to the complement of the four native staphylococcal PBPs. The function of the transpeptidase domain of PBP2A is similar to that of the bifunctional transglycosylase/transpeptidase PBP2 but has a remarkably low affinity for β-lactam antibiotics (*8*). PBP2A is thought to take over the biosynthetic role of β-lactam-inhibited PBP2 in cross-linking peptidoglycan when assisted by the transglycosylase domain of native PBP2 (*9*).

The bacterial cell wall remains a prime target for anti-MRSA drug discovery and the β-lactam class of antibiotics continues to be explored, albeit with limited success, for example, with the development of analogues capable of inhibiting PBP2A (*10*-*11*). Others strategies to treat MRSA have centered on the development of new drug classes with orthogonal mechanisms to the β-lactams such as linezolid, daptomycin and new glycopeptides (*12*). Nevertheless resistance to these novel classes has already been reported (*5*-*7*). With limited treatment options for MRSA, there is a pressing need for new agents that will avoid existing resistance mechanisms.

To this end, there is a growing interest in identifying auxiliary genes that are required for β-lactam resistance as potential novel targets. Previous work has suggested that PBP2A is not the sole determinant for β-lactam resistance (*13*-*16*). Specifically, although not essential for viability, PBP4 has been shown to play a key role in β-lactam resistance in strains of community-acquired MRSA (CA-MRSA), which was linked to its unique function in producing highly-cross linked peptidoglycan species during cell wall

synthesis (*17*). Thus cooperative functions among PBPs, in particular PBP2 and PBP2A (*9*) as well as PBP2 and PBP4 (*17-18*), during cell wall synthesis are also critical to the expression of β-lactam resistance in MRSA. Moreover, growing evidence suggests that a complex network of gene products involved in PG metabolism (*14*), synthesis (*19-20*) and regulation (*21-22*) play key roles in the expression of β-lactam resistance in strains of MRSA. Most recently, antisense technology was used to systematically probe 245 essential genes in MRSA for their role in β-lactam resistance (*23*). Several non-obvious genes were noted including *ftsZ,* an important cell division protein. A follow up study revealed that the known FtsZ inhibitor, PC190723, was in fact synergistic with imipenem and effective against MRSA (*24*).

There is likewise emerging evidence that wall teichoic acids (WTAs), anionic polyol phosphate polymers covalently attached to PG, have a role in the expression of β-lactam resistance in MRSA. In 1994, a transposon mutant (*llm*) that suppressed methicillin resistance in *S. aureus* was isolated and mapped to the 3'-terminal region of *tarO*, which encodes the first step of WTA synthesis (*25*). In *S. aureus*, the assembly of WTA polymers begins with two non-essential steps for cell viability, encoded in *tarO* and *tarA*, and continues with late-acting gene products that are indispensible upon initiation of polymer synthesis (*26*). Specifically, the TarO protein initiates the assembly of WTAs with the transfer of *N*-acetylglucosamine-1-phosphate to a membrane-anchored undecaprenyl-phosphate carrier lipid while TarA successively adds an *N*-acetylmannosamine moiety. This product is ultimately elaborated into a long polymer containing ribitol phosphate repeats that is attached to PG (*26*) (Supplementary Figure 1).

The function of WTAs is incompletely understood but shown to be important in processes such as cell division (*27-29*), virulence (*30*) and, more recently, in the expression of β-lactam resistance. In the aforementioned antisense study, gene *tarL,* encoding a late acting WTA biosynthetic enzyme was identified to lead to β-lactam sensitivity upon depletion (*23*). In addition, recent evidence has shown that TarS, a glycosyltransferase that attaches β-O-N-acetyl-D-glucosamine residues to WTA polymers, is also required in maintaining β-lactam resistance in MRSA (*31*). Finally, tunicamycin, a common probe of WTA synthesis, has been shown to sensitize MRSA to β-lactam antibiotics (*28*). Here, sensitization was attributed to defects in the assembly of PG machinery, due to possible mislocalization of either PBP2 or PBP2A (*28*). While tunicamycin is an imperfect inhibitor of wall teichoic acid synthesis – it is a non-selective glycosyltransferase inhibitor that also inhibits PG synthesis and has significant eukaryotic toxicity (*32*) – these observations are intriguing and consistent with a role for WTA in β-lactam resistance.

Herein we provide new evidence that wall teichoic acid (WTA) biogenesis is indeed a remarkable antibacterial target in MRSA. We found that deletion of gene *tarO* restored the sensitivity of MRSA to cefuroxime and other β-lactam antibiotics with signature selectivity for PBP2. In addition, we have discovered a novel inhibitor of wall teichoic acid synthesis in *S. aureus* that strongly potentiates β-lactam antibiotics against MRSA *in vitro* and *in vivo*. Where previous studies have suggested that WTA has an important role in localizing PBP4, we provide evidence here that WTA biogenesis plays a key role among the cooperative function of PBPs 2 and 4. The present work offers new

insights into the complex biology underlying cell wall synthesis in *S. aureus* and provides a promising example of how antibiotic drug resistance may be targeted with existing drugs.

Results and Discussion

Deletion of tarO sensitizes MRSA to β-lactams

Given their intimate link to β-lactam resistance, we sought to gain a better understanding of the precise mechanism by which WTA polymers mediate β-lactam resistance. We generated a deletion of the *tarO* gene in MRSA strains, both community- (CA-) and hospital-acquired (HA-), to investigate their sensitivity to β-lactams. Phosphate analysis of isolated cell wall of the epidemic strains CA-MRSA USA300 *ΔtarO* and HA-MRSA EMRSA 15 *ΔtarO*, which measures the levels of phosphate-rich WTA polymers, confirmed that the strains were devoid of WTA (Supplementary Table 1). Comparison of the parental strains to their respective *ΔtarO* deletion strains following treatment with an extensive panel of antibiotics revealed a high sensitivity to β-lactams, while the activity of other classes of antibiotics remained unaffected (Figure 3-1). Interestingly, only certain β-lactams were highly sensitized in the deletion background, while others retained their resistant phenotype. For example, we observed a greater than 64-fold change in the CA-MRSA *ΔtarO* strain with cefuroxime and oxacillin and as high as a 512-fold change in the HA-MRSA *ΔtarO* strain with ceftizoxime. Very minor changes in MIC values were obtained with β-lactams such as cefsulodin and meropenem.

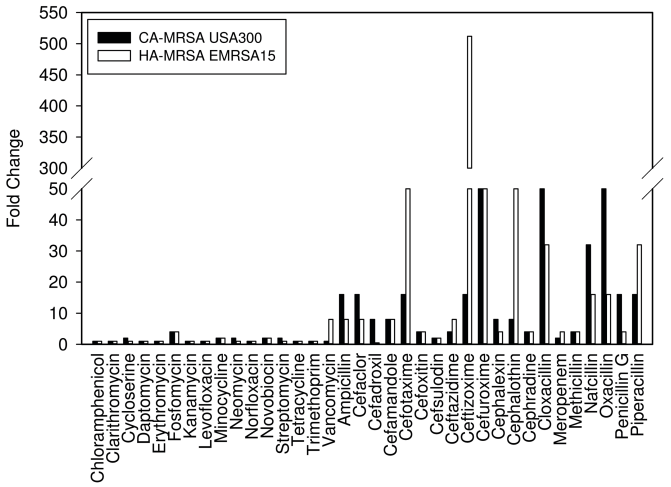

Figure 3-1. CA- and HA-MRSA ΔtarO deletion strains impaired for WTA synthesis are sensitized to β-lactam antibiotics. Sensitivity profiles of diverse antibiotics in CA-MRSA USA300 (black bars) and HA-MRSA EMRSA15 (white bars) relative to their ΔtarO deletion strains. Fold change refers to the MIC of the antibiotic in the parent strain divided by MIC in the deletion strain. The highest sensitivity was exclusively observed with certain β-lactam antibiotics.

While a potential connection between WTA expression and PG assembly has been inferred (*28, 33-34*), only recently has a possible mechanism been uncovered. In the absence of WTA synthesis, PBP4 of *S. aureus* RN4220, was shown to be mislocalized away from the division septum, and thus unable to perform its role of cross-linking PG (*35*). Concordantly, in CA-MRSA strains, PBP4 was shown to be responsible for the production of highly cross-linked peptidoglycan and essential for β-lactam resistance (*17, 36*). These two observations suggested a possible mechanism for the β-lactam sensitivity seen in Δ*tarO* strains, namely the impairment of PBP4 function in peptidoglycan cross-linking. Thus, we examined the level of PG cross-linking in CA-, HA-MRSA and respective Δ*tarO* deletion strains. To ensure the observations were due specifically to the deletion of *tarO* and therefore the loss of WTA, a plasmid expressing *tagO*, the *B. subtilis*

orthologue, was used to complement the HA-MRSA EMRSA15 Δ*tarO* deletion strain (Supplementary Figure 2). Indeed, the Δ*tarO* strain was found to have decreased levels (approximately 30%) of highly-cross linked muropeptide species as compared to the parental strain and a higher amount of monomeric, dimeric and trimeric muropeptides (Supplementary Figure 2), establishing a link between WTA synthesis and β-lactam sensitivity. We posit that a strain devoid of WTA leads to the mislocalization of PBP4, compromising its role as a transpeptidase in PG cross-linking and, specifically in the case of CA-MRSA USA300, results in sensitivity to certain β-lactams.

Combination screening identifies ticlopidine

Given the therapeutic potential of blocking WTA biogenesis in restoring the efficacy of β-lactams against MRSA, we set out to identify a novel inhibitor of WTA synthesis. As a source of chemical matter we employed a library of approximately 2,080 previously-approved drugs (PADs). There has been considerable interest in recent years in the concept of screening for new uses for previously-approved drug molecules. The interest stems from a growing understanding that small molecules with proven therapeutic activity for a particular use often have uncharacterized potential for alternate therapeutic uses (*37*-*38*). Implicit is the advantageous potential of any such molecule to rapidly advance into clinical development by leveraging a proven track record in humans and a deep history of study. Importantly, potentiators of β-lactam activity would be highly desirable components of therapeutic combinations against MRSA.

Thus, we mounted a screen of 2,080 previously-approved drugs (Supplementary Table 2) for compounds capable of potentiating the activity of cefuroxime, a broad spectrum β-lactam that was highly sensitized in the *ΔtarO* strain, against the clinically relevant CA-MRSA strain USA300 (Supplementary Figure 3a). Our screening efforts yielded several active compounds (Supplementary Figure 3b). To identify potential inhibitors of WTA, active combinations were tested against the CA-MRSA USA300 *ΔtarO* strain. Here, we were looking for suppression of synergy since this strain lacks WTA and is not susceptible to WTA inhibitors. One compound, ticlopidine (Figure 3-2a), an antiplatelet drug (Ticlid®) that inhibits the binding of adenosine 5'-phosphate to its platelet receptor in humans, was effective in restoring the efficacy of cefuroxime against MRSA (Figure 3-2b) and this synergistic interaction was reversed in the Δ*tarO* strain (Figure 3-2c). Ticlopidine, while not active on its own as an antibiotic, was potently synergistic with cefuroxime. Indeed, the Fractional Inhibitory Concentration (FIC) Index, a common measure of synergy (*39*), for this pair against CA-MRSA USA300 was ≤0.063 (Figure 3-2b). Further, ticlopidine dramatically potentiated the activity of cefuroxime against 9 of 10 MRSA strains (*40*), including CA-MRSA strain USA300 (64-fold) and HA-MRSA strain USA200 (32-fold) (Table 3-1). Notably, we did not observe synergy in the common lab strain of *S. aureus*, RN4220 (Table 3-1), consistent with a lack of sensitivity to β-lactams in a Δ*tarO* deletion of this strain (Supplementary Table 3). Strong synergies with cefuroxime were also observed with commercially available on-patent analogs of ticlopidine, namely clopidogrel (Plavix®) and prasugrel (Effient®), against CA-MRSA USA300 (FIC Index ≤0.125 and ≤0.5,

respectively) (Supplementary Table 4). To assess *in vivo* efficacy, we administered ticlopidine and cefuroxime in a *Galleria mellonella* model of MRSA infection. The larval stage of the Greater Wax Moth is a widely used model to assess *S. aureus* virulence and allows for a testing throughput that is otherwise impossible with small mammals but nevertheless representative of *in vivo* activity (*41-42*). In this model, a significantly (P<0.001) higher fraction of larvae survived MRSA infection following combined treatment, compared to that with cefuroxime and ticlopidine alone (Figure 3-2d). After 14 days, the survival rate increased from 3.3% in untreated larvae to 53.3% when treated with the combination; treatment with cefuroxime and ticlopidine alone led to 16.7% and 10% survival, respectively (Figure 3-2d). Thus, cefuroxime and ticlopidine at sub-efficacious doses acted synergistically to provide *G. mellonella* protection from bacterial infection.

Table 3-1. *In vitro* interactions between ticlopidine and cefuroxime in *S. aureus* species

Strain [a]	MIC cefuroxime (μg/mL)	FIC[b] cefuroxime	MIC ticlopidine (μg/mL)	FIC[b] ticlopidine	FIC Index[c]
Newman [d]	2	0.125	>256	0.125	≤0.250
HA-MRSA USA600 [e]	≥1024	0.008	>256	0.032	≤0.040
HA-MRSA USA100/800/NY [e]	1024	0.125	>256	0.125	≤0.250
HA-MRSA [e]	>2048	0.250	>256	0.032	≤0.282
HA-MRSA USA200/EMRSA16 [e]	1024	0.032	>256	0.063	≤0.095
HA-MRSA USA500 [e]	32	1	>256	1	≤2
HA-MRSA [e]	>2048	0.250	>256	0.016	≤0.266
CA-MRSA USA400/MW2 [e]	256	0.063	>256	0.063	≤0.125
HA-MRSA EMRSA15 [e]	512	0.063	>256	0.063	≤0.125
HA-MRSA [e]	2048	0.125	>256	0.063	≤0.188
CA-MRSA USA300 [e]	512	0.032	>256	0.032	≤0.063
RN4220 [f]	0.5	1	>128	1	≤2
SA178R1 [g]	0.5	1	>128	1	≤2

[a] HA, hospital-associated isolate, CA, community-associated isolate
[b] FIC = [X]/MICX, where [X] is the lowest inhibitory concentration of drug in the presence of the co-drug.
[c] FIC index = FICcefuroxime + FICticlopidine
[d] *Ref* (*50*)
[e] *Ref* (*40*)
[f] *Ref* (*51*)
[g] *Ref* (*26*)

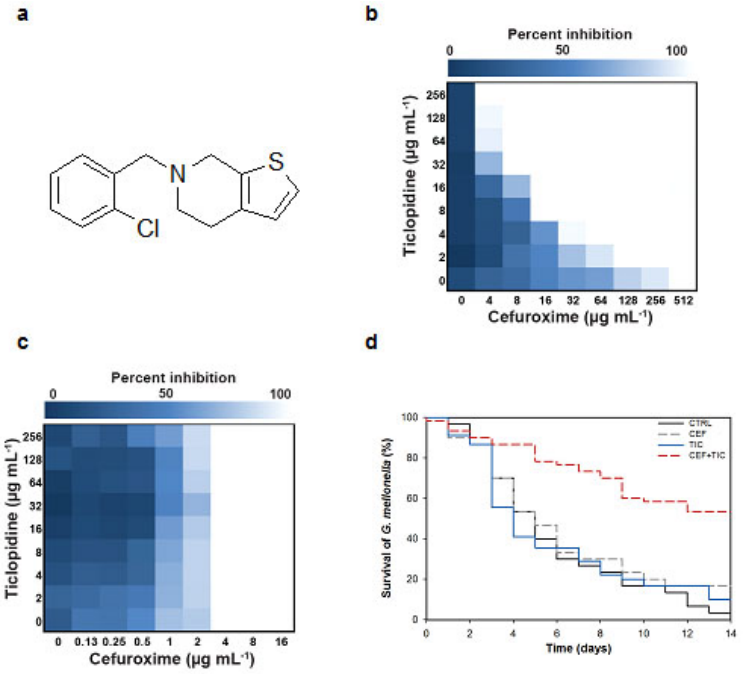

Figure 3-2. Ticlopidine potentiates the activity of the β-lactam antibiotic cefuroxime against CA-MRSA USA300, but not CA-MRSA USA300 ΔtarO. (a) Chemical structure of ticlopidine. (b) Microdilution checkerboard analysis showing the combined effect of cefuroxime and ticlopidine against CA-MRSA USA300 where the extent of inhibition is shown as a heat plot. Synergistic effects are evident as both molecules alone have MICs that exceed 256 µg mL-1 and result in an FIC Index of ≤0.063. (c) Suppression of the synergy in CAMRSA USA300 ΔtarO, leading to an additive interaction with FIC Index of ≤2. (d) *Galleria mellonella* virulence assay. Survival curve of *G. Mellonella* infected with CA-MRSA USA300 receiving no drug treatment control (CTRL) or a treatment with 0.3 mg/kg cefuroxime (CEF) or 0.3 mg/kg ticlopidine (TIC), or a combination of both at 0.3 mg/kg each (CEF+TIC). After 14 days, treatment with the combination lead to significantly increased survival, compared to no drug control or antibiotic treatment alone ($P<0.001$).

Characterization of the mode of action of ticlopidine

To further probe the mechanism of action of ticlopidine, we investigated its interactions with an extensive panel of diverse antibiotics using CA-MRSA USA300. Ticlopidine restored the efficacy of several β-lactam antibiotics, while the activity of other classes of

antibiotics remained unaffected (Supplementary Table 5). Indeed, the sensitization profile was strikingly similar to that seen in the Δ*tarO* strains (Figure 3-1), although the sensitivity was generally higher in the deletion strain, likely because it was completely devoid of WTA (as shown by phosphate analysis in Supplementary Table 1). Most remarkable was the lack of antibacterial activity of ticlopidine on its own and its capacity to render MRSA highly susceptible to a number of β-lactams. We reasoned that if ticlopidine inhibited WTA synthesis, its lack of antibacterial activity would be consistent with inhibition of the early steps in the pathway catalyzed by TarO or TarA. WTA biosynthetic genes exhibit complex dispensability patterns (*26, 43*). WTA genes encoding the initiating enzymes, TarO and TarA, are dispensable for growth *in vitro* while the downstream late-acting genes have an essential phenotype. Idiosyncratically, the late-acting genes become dispensable in strains with a deletion in either *tarO* or *tarA,* presumably because accumulation of WTA intermediates is toxic to the cell (*26, 29, 33, 43*).

To test the hypothesis that ticlopidine inhibited either of these initiating enzymes we initially employed genetic and physiological approaches. First, we took advantage of the conditional dispensability patterns of late steps in WTA assembly. As an early step inhibitor, ticlopidine should have an antagonistic interaction with the late step (TarG) inhibitor, targosil. (*44*) TarG, an essential gene product, is the transmembrane component of the ABC transporter that exports WTAs to the cell surface. Indeed, ticlopidine rendered targosil completely inactive against both CA-MRSA USA300 and the *S. aureus* lab strain RN4220 (Figure 3-3a and Supplementary Figure 4). In additional genetic

experiments, we tested the capacity of ticlopidine to suppress the lethal phenotype associated with late gene *tarH*. Using a strain where *tarH* expression was under the control of a xylose-induced promoter, we found that ticlopidine could partially rescue of growth in the absence of xylose (40% relative to growth in 2% xylose) at the highest concentration tested (Figure 3-3b). We next investigated ticlopidine`s ability to directly decrease WTA synthesis by measuring phosphate content in the cell wall of *S. aureus* with increasing concentrations of ticlopidine. In both CA-MRSA USA300 (Figure 3-3c) and SA1781 (a RN4220 derivative) (Supplementary Figure 5), WTA incorporation began to decrease at ticlopidine concentrations of 64 μg/mL, similar to the concentration at which it begins to potentiate the activity of cefuroxime. Greater inhibition was noted at 200 μg/mL of ticlopidine but residual WTA remained (~50%), while Δ*tarO* strains were completely devoid of phosphate. We note here that the partial suppression seen was consistent with the partial inhibition of WTA synthesis evident in our cell wall phosphate content assays. Furthermore, bacteriophage Ø11, which uses WTA as receptor-binding sites, infected *S. aureus* RN450 but not ticlopidine- or tunicamycin-treated RN450 (Supplementary Figure 6). WTA is known to contribute to the resistance of PG against lysozyme (*45*). We found that wild-type PG was completely resistant to degradation by lysozyme while PG from ticlopidine-treated cells and *ΔtarO* cells were indeed sensitive to degradation (Supplementary Figure 7). In sum, our genetic and physiological experiments were consistent with ticlopidine targeting either TarO or TarA.

To test directly the hypothesis that ticlopidine inhibited either TarO or TarA, we used *in vitro* biochemical assays. While ticlopidine showed no impact on the transferase

activity of TarA (Supplementary Figure 9) we found substantial inhibition of TarO activity (Figure 3-3d). Further, the relatively weak inhibition constant (IC_{50} of 238 μM or 71 μg/mL) was consistent with the partial phenotypes noted in our phenotypic and genetic assays.

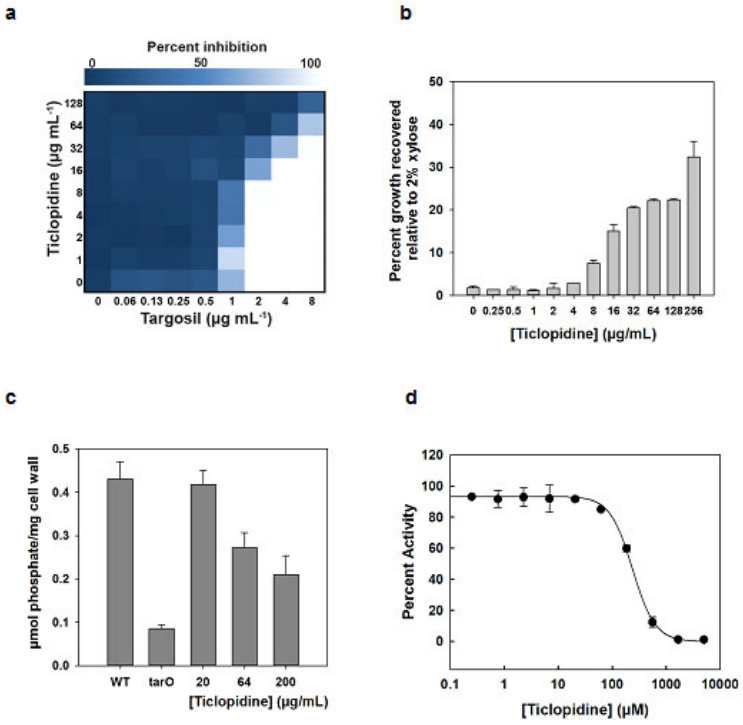

Figure 3-3. Ticlopidine inhibits the initiation of wall teichoic acid biosynthesis in *S. aureus*. (a) At concentrations of 8 μg mL^{-1}, ticlopidine begins to antagonize the activity of targosil, a late-stage inhibitor, against CA-MRSA USA300. (b) Ticlopidine can suppress the lethality associated with late WTA stage deletion. Shown is the percent growth of a tarH conditional deletion strain normalized to the growth upon induction with 2% xylose in the presence of increasing concentrations of ticlopidine. Ticlopidine can recover approximately 40% of the growth at the highest concentration of 256 μg mL^{-1}. (c) Ticlopidine shows a dose-dependent decrease in the phosphate content of cell wall isolated from CA-MRSA USA300, with approximately 50% less phosphate when ticlopidine is present at 200 μg mL^{-1}. CA-MRSA USA300 ΔtarO is completely devoid of WTA compared to the parental strain. (d) Membrane-based in vitro assay following the generation of a radiolabeled reaction product of TarO, undecapreny-P-P-[^{14}C]GlcNAc, as a result of the incorporation of [^{14}C]GlcNAc onto undecaprenyl-P-P. Assay was performed on membranes derived from E. coli cells (ΔwecA) expressing recombinant TarO from *S. aureus*. The reaction product was monitored by thin layer chromatography (TLC) and shown to be dependent on the presence of recombinant TarO (Supplementary Figure 8). Ticlopidine inhibited the activity of TarO, yielding an IC50 value of 238 ± 14 μM.

A basis for the synergy among ticlopidine and ß-lactams

To further investigate the basis of synergy of ticlopidine and cefuroxime we sought to understand the characteristic β-lactam sensitivity profiles that were shared on treatment of MRSA in combination with ticlopidine or alone using the Δ*tarO* MRSA strains. A survey of the PBP selectivity of β-lactams tested in our studies revealed that sensitization was seen particularly with those having high binding affinities for PBP2 of *S. aureus* (Figure 3-1 and Supplementary Table 5). Given the impact of the loss of WTA on PBP4 localization (*35*), PBP2 selectivity can be rationalized based on previous reports of cooperativity between PBP2 and PBP4 in creating highly cross-linked PG in MRSA strains contributing to β-lactam resistance (*18*) (Figure 3-4, panel i). A strain lacking WTA, such as with treatment with ticlopidine, would be compromised in its PG cross-linking due to mislocalized PBP4 and be highly sensitive to β-lactams such as cefuroxime that target its partner protein PBP2 (Figure 3-4, panel iii). Resistance would persist if the β-lactam in combination with ticlopidine targets PBP1 or PBP3, as sufficient cross-linking would maintain the resistant phenotype (Figure 3-4, panel ii). Further, this rationale is consistent with the idea that PBP2A is not the sole determinant for β-lactam resistance in MRSA (*9, 15, 17*). It suggests that the synergy between ticlopidine and cefuroxime might also be observed in a wild-type, methicillin-sensitive strain such as *S. aureus* Newman, which lacks PBP2A. Indeed, synergy is evident in this strain (Table 3-1). Remarkably, this work also suggests that β-lactam resistance might be reversed by targeting PBP2 and PBP4 with combinations of existing β-lactams. We tested this using

the PBP4-selective β-lactam cefoxitin (*46*). When cefoxitin was combined with the PBP2-selective β-lactams cefuroxime, ceftizoxime, oxacillin and penicillin, the MICs of these antibiotics decreased 32 to 128-fold. Conversely, combining cefoxitin with β-lactams having a low affinity for PBP2 led to only a 2-8 fold change in the MIC (Supplementary Table 6).

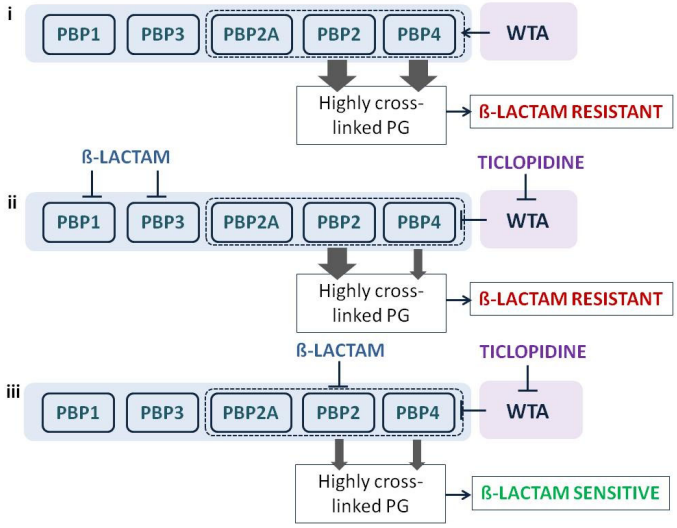

Figure 3-4. A synthetic lethal interaction when targeting TarO and native PBP2. Indirect Inhibition of PBP4, via inhibition of TarO, and inhibition of PBP2 function account for the synergistic interaction among ticlopidine and cefuroxime. i. When not challenged with β-lactams, WTA synthesis will guide the proper localization of PBP4, and together with PBP2, will provide highly cross-linked muropeptide species (thick arrow) that contribute to high-level β-lactam resistance. ii. Treatment with a β-lactam with low affinity for PBP2 and ticlopidine leads to an additive interaction as sufficient highly cross-linked peptidoglycan are present to maintain β-lactam resistance (one thick arrow), even when PBP4 function is affected by the lack of WTA (one thin arrow). iii. Due to their cooperative function, PBP4 (when challenged with ticlopidine) and PBP2 (when challenged with β-lactam with high affinity for PBP2), will be impaired in their capacities to produce a highly cross-linked cell wall (two thin arrows), contributing to enhanced β-lactam susceptibility and thus a synergistic interaction.

Conclusions

The WTA biosynthetic pathway has long been speculated as a viable target for antibacterial intervention. While dispensable for viability, WTA is known to be a critical determinant of cell shape in *Bacillus subtilis (27, 29)*, virulence in *S. aureus (30)* and recently, for proper cell division (*28*). Furthermore, the essential phenotypes of late WTA biosynthetic genes, although paradoxical, suggest that these enzymes may well be reasonable targets for new antibiotics. Emerging evidence of a role for wall teichoic acids in β-lactam resistance in MRSA has fueled further interest in targeting this pathway, including a renewed interest in tunicamycin, a natural product nucleoside antibiotic that has been shown to inhibit TarO (*28*). Unfortunately, tunicamycin is a promiscuous inhibitor of bacterial and eukaryotic phosphosugar transferases, hindering its use as a selective probe and therapeutic. In the work reported here, we have carried out a meticulous chemical genetic study of the importance of wall teichoic acids to community- and hospital-acquired MRSA strains to reveal signature interactions with β-lactam antibiotics having a known selectivity for PBP2. These findings led to the discovery that ticlopidine, a well-known antiplatelet drug, had the cryptic capacity to block WTA synthesis through the inhibition of TarO. Importantly, synergistic interactions of ticlopidine and PBP2-binding β-lactams provide for efficacious combinations to limit the growth of MRSA strains *in vitro* and *in vivo*. Thus ticlopidine represents a promising new lead with a strong record of examination in humans but also provides an exciting

new probe of WTA synthesis. In our hands, ticlopidine was an extremely useful probe along with existing β-lactam antibiotics to further elaborate the role of WTA in supporting the cooperative role of PBP2 and 4 in the expression of β-lactam resistance in MRSA. As such, this work offers new insights into the complex biology underlying cell wall synthesis in *S. aureus* and provides a promising example of how antibiotic drug resistance might be targeted with existing drugs.

Methods

Peptidoglycan purification and analysis. Peptidoglycan from CA-MRSA USA300, HA-MRSA EMRSA15, their Δ*tarO* deletion and HA-MRSA EMRSA15Δ*tarO* pLI50-*tagO* was prepared from exponentially growing cells as previously described (*47*). The purified peptidoglycan was digested with mutanolysin (Sigma), an N-acetylmuramidase that cuts glycan strands between the N-acetylmuramic and N-acetylglucosamine residues. The resulting muropeptides were reduced with sodium borohydride (Sigma) and analyzed by reverse-phase HPLC using a Hypersil ODS column (Thermo Electron Corporation). The eluted muropeptides were detected and quantified by determination of their ultraviolet absorption at 206 nm, using the Shimadzu LC solution software. The area of eluted UV-absorbing peaks, corresponding to the different muropeptides, was quantified and shown as a percentage of the total area of the chromatogram.

Combination screening. CA-MRSA USA300 was screened against the PAD library in the presence of cefuroxime. The screening protocol was based on CLSI guidelines. Screening was carried out in 96-well plates, in duplicate, using Mueller Hinton Broth (MHB) with 2% DMSO and a library compound concentration of 10 μM. The

concentration of cefuroxime was 16 μg ml^{-1}, a quarter of its MIC value obtained under the same conditions. Background controls (8 wells per plate) contained only media and DMSO and growth controls, also 8 wells per plate, contained media, DMSO and inoculum. Plates were incubated at 37°C for 20 hours and optical density read at 600nm using an EnVision plate reader (Perkin Elmer). The percentage growth for each test well was calculated as (OD – mean background) / (mean growth – mean background) * 100 and normalized to the percent growth attributed by the PAD alone to obtain a growth ratio such that a ratio of 1.0 is indicative of no difference.

Checkerboard analyses and FIC index determination. FICs were determined by setting up standard checkerboard broth microdilution assays with serially diluted 8 (or 9) concentrations of each drug, using the same conditions as screening. At least 3 replicates were done for each combination and the means used for calculation. The MIC for each drug was the lowest [drug] showing <10% growth. The FIC for each drug was calculated as the [drug in the presence of co-drug] for a well showing <10% growth, divided by the MIC for that drug. The FIC index is the sum of the two FICs. Interactions with FIC Index of less than 0.5 were deemed synergistic.

Phosphate analysis. Strains were inoculated from an overnight culture and grown in 100 ml MHB to OD600 0.8-0.9 at 37°C, 250 rpm with various compound treatment. Isolation of cell wall and phosphate analysis were performed as previously described (*48*).

In vitro **TarO inhibition assay**. For this assay, UDP-[^{14}C]GlcNAc (0.1 mCi mL^{-1}) was purchased from American Radiolabelled Chemicals, Ultima Gold liquid scintillation cocktail from Perkin Elmer and Silica gel 60 TLC plates from EMD Chemicals. TarO

activity was assayed in 100 μL reactions containing Reaction Buffer (50 mM Tris pH = 8, 10 mM $MgCl_2$, 1 mM EDTA), 300 μM UDP-GlcNAc, 0.1 μCi UDP-[^{14}C]GlcNAc, 0.1 % (w/v) TritonX-100, 0.8 % (v/v) DMSO and varied amounts of membranes (75 μg - 1 mg of protein). Reactions were quenched with the addition of 1250 μL $CHCl_3$:MeOH (3:2). Lipid-linked products were extracted according to the following method: Quenched reactions were incubated for 2.5 hrs followed by vortexing for 3 min. Insoluble material was removed by centrifugation (13 000 x g, 2 minutes), and 150 μL of 40 mM $MgCl_2$ was added to the supernatant. Samples were vortexted for 5 min, and the upper aqueous layer was removed. The lower, organic layer was washed twice with 400 μL of pure solvent upper phase ($CHCl_3$:MeOH:H_2O:1 M $MgCl_2$ in H_2O [18:294:282:1]). The final product was analyzed by either TLC or scintillation counting. TLC analysis was preformed as in reference (*49*).

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References

1. Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B., and Bartlett, J. (2009) Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America, *Clin Infect Dis 48*, 1-12.
2. Klevens, R. M., Morrison, M. A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Zell, E. R., Fosheim, G. E., McDougal, L. K., Carey, R. B., and Fridkin, S. K. (2007)

Invasive methicillin-resistant Staphylococcus aureus infections in the United States, *JAMA 298*, 1763-1771.

3. Baba, T., Takeuchi, F., Kuroda, M., Yuzawa, H., Aoki, K., Oguchi, A., Nagai, Y., Iwama, N., Asano, K., Naimi, T., Kuroda, H., Cui, L., Yamamoto, K., and Hiramatsu, K. (2002) Genome and virulence determinants of high virulence community-acquired MRSA, *Lancet 359*, 1819-1827.
4. Diep, B. A., Gill, S. R., Chang, R. F., Phan, T. H., Chen, J. H., Davidson, M. G., Lin, F., Lin, J., Carleton, H. A., Mongodin, E. F., Sensabaugh, G. F., and Perdreau-Remington, F. (2006) Complete genome sequence of USA300, an epidemic clone of community-acquired meticillin-resistant Staphylococcus aureus, *Lancet 367*, 731-739.
5. Howden, B. P., Johnson, P. D., Charles, P. G., and Grayson, M. L. (2004) Failure of vancomycin for treatment of methicillin-resistant Staphylococcus aureus infections, *Clin Infect Dis 39*, 1544; author reply 1544-1545.
6. Mangili, A., Bica, I., Snydman, D. R., and Hamer, D. H. (2005) Daptomycin-resistant, methicillin-resistant Staphylococcus aureus bacteremia, *Clin Infect Dis 40*, 1058-1060.
7. Tsiodras, S., Gold, H. S., Sakoulas, G., Eliopoulos, G. M., Wennersten, C., Venkataraman, L., Moellering, R. C., and Ferraro, M. J. (2001) Linezolid resistance in a clinical isolate of Staphylococcus aureus, *Lancet 358*, 207-208.
8. Lim, D., and Strynadka, N. C. (2002) Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant Staphylococcus aureus, *Nat Struct Biol 9*, 870-876.
9. Pinho, M. G., de Lencastre, H., and Tomasz, A. (2001) An acquired and a native penicillin-binding protein cooperate in building the cell wall of drug-resistant staphylococci, *Proc Natl Acad Sci U S A 98*, 10886-10891.
10. Llarrull, L. I., Fisher, J. F., and Mobashery, S. (2009) Molecular basis and phenotype of methicillin resistance in Staphylococcus aureus and insights into new beta-lactams that meet the challenge, *Antimicrob Agents Chemother 53*, 4051-4063.
11. Turk, S., Verlaine, O., Gerards, T., Zivec, M., Humljan, J., Sosic, I., Amoroso, A., Zervosen, A., Luxen, A., Joris, B., and Gobec, S. (2011) New noncovalent inhibitors of penicillin-binding proteins from penicillin-resistant bacteria, *PLoS One 6*, e19418.
12. Aksoy, D. Y., and Unal, S. (2008) New antimicrobial agents for the treatment of Gram-positive bacterial infections, *Clin Microbiol Infect 14*, 411-420.
13. Berger-Bachi, B. (1999) Genetic basis of methicillin resistance in Staphylococcus aureus, *Cell Mol Life Sci 56*, 764-770.
14. de Lencastre, H., and Tomasz, A. (1994) Reassessment of the number of auxiliary genes essential for expression of high-level methicillin resistance in Staphylococcus aureus, *Antimicrob Agents Chemother 38*, 2590-2598.
15. De Lencastre, H., Wu, S. W., Pinho, M. G., Ludovice, A. M., Filipe, S., Gardete, S., Sobral, R., Gill, S., Chung, M., and Tomasz, A. (1999) Antibiotic resistance as a stress response: complete sequencing of a large number of chromosomal loci in

Staphylococcus aureus strain COL that impact on the expression of resistance to methicillin, *Microb Drug Resist 5*, 163-175.

16. Labischinski, H., Ehlert, K., and Berger-Bachi, B. (1998) The targeting of factors necessary for expression of methicillin resistance in staphylococci, *J Antimicrob Chemother 41*, 581-584.
17. Memmi, G., Filipe, S. R., Pinho, M. G., Fu, Z., and Cheung, A. (2008) Staphylococcus aureus PBP4 is essential for beta-lactam resistance in community-acquired methicillin-resistant strains, *Antimicrob Agents Chemother 52*, 3955-3966.
18. Leski, T. A., and Tomasz, A. (2005) Role of penicillin-binding protein 2 (PBP2) in the antibiotic susceptibility and cell wall cross-linking of Staphylococcus aureus: evidence for the cooperative functioning of PBP2, PBP4, and PBP2A, *J Bacteriol 187*, 1815-1824.
19. Gardete, S., Ludovice, A. M., Sobral, R. G., Filipe, S. R., de Lencastre, H., and Tomasz, A. (2004) Role of murE in the Expression of beta-lactam antibiotic resistance in Staphylococcus aureus, *J Bacteriol 186*, 1705-1713.
20. Sobral, R. G., Ludovice, A. M., Gardete, S., Tabei, K., De Lencastre, H., and Tomasz, A. (2003) Normally functioning murF is essential for the optimal expression of methicillin resistance in Staphylococcus aureus, *Microb Drug Resist 9*, 231-241.
21. Berger-Bachi, B., and Rohrer, S. (2002) Factors influencing methicillin resistance in staphylococci, *Arch Microbiol 178*, 165-171.
22. Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., and Hiramatsu, K. (2003) Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in Staphylococcus aureus, *Mol Microbiol 49*, 807-821.
23. Lee, S. H., Jarantow, L. W., Wang, H., Sillaots, S., Cheng, H., Meredith, T. C., Thompson, J., and Roemer, T. (2011) Antagonism of chemical genetic interaction networks resensitize MRSA to beta-lactam antibiotics, *Chem Biol 18*, 1379-1389.
24. Tan, C. M., Therien, A. G., Lu, J., Lee, S. H., Caron, A., Gill, C. J., Lebeau-Jacob, C., Benton-Perdomo, L., Monteiro, J. M., Pereira, P. M., Elsen, N. L., Wu, J., Deschamps, K., Petcu, M., Wong, S., Daigneault, E., Kramer, S., Liang, L., Maxwell, E., Claveau, D., Vaillancourt, J., Skorey, K., Tam, J., Wang, H., Meredith, T. C., Sillaots, S., Wang-Jarantow, L., Ramtohul, Y., Langlois, E., Landry, F., Reid, J. C., Parthasarathy, G., Sharma, S., Baryshnikova, A., Lumb, K. J., Pinho, M. G., Soisson, S. M., and Roemer, T. (2012) Restoring Methicillin-Resistant Staphylococcus aureus Susceptibility to beta-Lactam Antibiotics, *Sci Transl Med 4*, 126ra135.
25. Maki, H., Yamaguchi, T., and Murakami, K. (1994) Cloning and characterization of a gene affecting the methicillin resistance level and the autolysis rate in Staphylococcus aureus, *J Bacteriol 176*, 4993-5000.
26. D'Elia, M. A., Pereira, M. P., Chung, Y. S., Zhao, W., Chau, A., Kenney, T. J., Sulavik, M. C., Black, T. A., and Brown, E. D. (2006) Lesions in teichoic acid biosynthesis in Staphylococcus aureus lead to a lethal gain of function in the otherwise dispensable pathway, *J Bacteriol 188*, 4183-4189.

27. Bhavsar, A. P., Beveridge, T. J., and Brown, E. D. (2001) Precise deletion of tagD and controlled depletion of its product, glycerol 3-phosphate cytidylyltransferase, leads to irregular morphology and lysis of Bacillus subtilis grown at physiological temperature, *J Bacteriol 183*, 6688-6693.
28. Campbell, J., Singh, A. K., Santa Maria, J. P., Jr., Kim, Y., Brown, S., Swoboda, J. G., Mylonakis, E., Wilkinson, B. J., and Walker, S. (2011) Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in Staphylococcus aureus, *ACS Chem Biol 6*, 106-116.
29. D'Elia, M. A., Millar, K. E., Beveridge, T. J., and Brown, E. D. (2006) Wall teichoic acid polymers are dispensable for cell viability in Bacillus subtilis, *J Bacteriol 188*, 8313-8316.
30. Weidenmaier, C., and Peschel, A. (2008) Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions, *Nat Rev Microbiol 6*, 276-287.
31. Brown, S., Xia, G., Luhachack, L. G., Campbell, J., Meredith, T. C., Chen, C., Winstel, V., Gekeler, C., Irazoqui, J. E., Peschel, A., and Walker, S. (2012) Methicillin resistance in Staphylococcus aureus requires glycosylated wall teichoic acids, *Proc Natl Acad Sci U S A*.
32. Price, N. P., and Tsvetanova, B. (2007) Biosynthesis of the tunicamycins: a review, *J Antibiot (Tokyo) 60*, 485-491.
33. D'Elia, M. A., Millar, K. E., Bhavsar, A. P., Tomljenovic, A. M., Hutter, B., Schaab, C., Moreno-Hagelsieb, G., and Brown, E. D. (2009) Probing teichoic acid genetics with bioactive molecules reveals new interactions among diverse processes in bacterial cell wall biogenesis, *Chem Biol 16*, 548-556.
34. Schlag, M., Biswas, R., Krismer, B., Kohler, T., Zoll, S., Yu, W., Schwarz, H., Peschel, A., and Gotz, F. (2010) Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl, *Mol Microbiol 75*, 864-873.
35. Atilano, M. L., Pereira, P. M., Yates, J., Reed, P., Veiga, H., Pinho, M. G., and Filipe, S. R. (2010) Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in Staphylococcus aureus, *Proc Natl Acad Sci U S A 107*, 18991-18996.
36. Henze, U. U., and Berger-Bachi, B. (1995) Staphylococcus aureus penicillin-binding protein 4 and intrinsic beta-lactam resistance, *Antimicrob Agents Chemother 39*, 2415-2422.
37. Chong, C. R., and Sullivan, D. J., Jr. (2007) New uses for old drugs, *Nature 448*, 645-646.
38. Ejim, L., Farha, M. A., Falconer, S. B., Wildenhain, J., Coombes, B. K., Tyers, M., Brown, E. D., and Wright, G. D. (2010) Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy, *Nat Chem Biol 7*, 348-350.
39. Odds, F. C. (2003) Synergy, antagonism, and what the chequerboard puts between them, *J Antimicrob Chemother 52*, 1.

40. Christianson, S., Golding, G. R., Campbell, J., and Mulvey, M. R. (2007) Comparative genomics of Canadian epidemic lineages of methicillin-resistant Staphylococcus aureus, *J Clin Microbiol 45*, 1904-1911.
41. Gao, W., Chua, K., Davies, J. K., Newton, H. J., Seemann, T., Harrison, P. F., Holmes, N. E., Rhee, H. W., Hong, J. I., Hartland, E. L., Stinear, T. P., and Howden, B. P. (2010) Two novel point mutations in clinical Staphylococcus aureus reduce linezolid susceptibility and switch on the stringent response to promote persistent infection, *PLoS Pathog 6*, e1000944.
42. Purves, J., Cockayne, A., Moody, P. C., and Morrissey, J. A. (2010) Comparison of the regulation, metabolic functions, and roles in virulence of the glyceraldehyde-3-phosphate dehydrogenase homologues gapA and gapB in Staphylococcus aureus, *Infect Immun 78*, 5223-5232.
43. D'Elia, M. A., Henderson, J. A., Beveridge, T. J., Heinrichs, D. E., and Brown, E. D. (2009) The N-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in Bacillus subtilis and Staphylococcus aureus, *J Bacteriol 191*, 4030-4034.
44. Swoboda, J. G., Meredith, T. C., Campbell, J., Brown, S., Suzuki, T., Bollenbach, T., Malhowski, A. J., Kishony, R., Gilmore, M. S., and Walker, S. (2009) Discovery of a small molecule that blocks wall teichoic acid biosynthesis in Staphylococcus aureus, *ACS Chem Biol 4*, 875-883.
45. Bera, A., Biswas, R., Herbert, S., Kulauzovic, E., Weidenmaier, C., Peschel, A., and Gotz, F. (2007) Influence of wall teichoic acid on lysozyme resistance in Staphylococcus aureus, *J Bacteriol 189*, 280-283.
46. Nair, S. R., and Cherubin, C. E. (1978) Use of cefoxitin, new cephalosporin-like antibiotic, in the treatment of aerobic and anaerobic infections, *Antimicrob Agents Chemother 14*, 866-875.
47. Filipe, S. R., Tomasz, A., and Ligoxygakis, P. (2005) Requirements of peptidoglycan structure that allow detection by the Drosophila Toll pathway, *EMBO Rep 6*, 327-333.
48. Bhavsar, A. P., Erdman, L. K., Schertzer, J. W., and Brown, E. D. (2004) Teichoic acid is an essential polymer in Bacillus subtilis that is functionally distinct from teichuronic acid, *J Bacteriol 186*, 7865-7873.
49. Schaffer, C., Wugeditsch, T., Messner, P., and Whitfield, C. (2002) Functional expression of enterobacterial O-polysaccharide biosynthesis enzymes in Bacillus subtilis, *Appl Environ Microbiol 68*, 4722-4730.
50. Duthie, E. S., and Lorenz, L. L. (1952) Staphylococcal coagulase; mode of action and antigenicity, *J Gen Microbiol 6*, 95-107.
51. Kreiswirth, B. N., Lofdahl, S., Betley, M. J., O'Reilly, M., Schlievert, P. M., Bergdoll, M. S., and Novick, R. P. (1983) The toxic shock syndrome exotoxin structural gene is not detectably transmitted by a prophage, *Nature 305*, 709-712.

CHAPTER FOUR – Collapsing the Proton Motive Force to Identify Synergistic Combinations Against *Staphylococcus aureus*

Preface

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For this work, I performed all of the experiments (with the exception of the cytotoxicity assays, performed by Verschoor, CP) and wrote the manuscript, with edits provided by Brown, ED.

Summary

Pathways of bacterial energy metabolism, such as the proton motive force, have largely remained unexplored as drug targets, owing to toxicity concerns. Here, we elaborate a methodical and systematic approach for targeting PMF using chemical combinations. We began with a high-throughput screen to identify molecules that selectively dissipate either component of the PMF, ΔΨ or ΔpH, in *S. aureus*. We uncovered six perturbants of PMF, three (I1, I2 and I3) that countered ΔΨ and three (D1, D2 and D3) that selectively dissipated ΔpH. Combinations of dissipators of ΔΨ with dissipators of ΔpH were highly synergistic against MRSA. Cytotoxicity analyses on mammalian cells revealed that the dose-sparing effect of the observed synergies could significantly reduce toxicity. The discovery and rational combination of modulators of ΔΨ and ΔpH may represent a promising strategy for combating microbial pathogens.

Introduction

Emergence and spread of multidrug-resistant bacteria represents an increasingly serious problem and an unmet medical need due to the lack of therapeutic options. The situation is especially dire for *Staphylococcus aureus* for which methicillin-resistant (MRSA) and vancomycin intermediate resistant (VISA) strains have emerged (Chopra, 2003). The escalating concern of antibiotic resistance coupled with the dwindling antibiotic pipeline has prompted drug developers to turn to unconventional strategies for the discovery of novel drugs to combat bacterial infections, both in exploring novel compound classes and validating new target pathways.

The cell membrane of *S. aureus*, in particular, is a relatively unexplored target for the development of novel antimicrobials. This essential macromolecular structure plays a critical role in cellular activities such as active transport of substances into the cell, cellular respiration, the establishment of the proton motive force and cell-cell communication (Hurdle, et al., 2011). Yet, over the years, discovery efforts have steered clear of membrane-active agents that either disrupt the membrane's physical integrity or target its energetics, mostly given challenges with associated toxicity in mammals. In fact, a consensus has emerged in the field that active compounds discovered in whole cells screens that act primarily on the cell membrane should be excluded from further study (O'Neill and Chopra, 2004; Silver, 2011). Indeed, a number of assays have been explored to detect these undesirable compounds early enough to evade follow-up studies (Gentry, et al., 2010; Prakash Singh, 2006).

Paradoxically, this unconventional antibacterial target has gained attention, in part because of studies showing its importance in non-growing bacteria (Coates and Hu, 2008; Hu, et al., 2010; Hurdle, et al., 2011) and out of recognition of the recent success of the antibiotic medicines daptomycin (Hawkey, 2008), telavancin (Zhanel, et al., 2010), and HT61 (Hu, et al., 2010), the latter currently in clinical trials for topical use, that work by permeabilizing and depolarizing the cytoplasmic membrane of *S. aureus*. Also noteworthy is the compound TMC107, which disrupts membrane energetics by targeting the ATP synthase and is currently in phase IIb trials for the treatment of drug-resistant *Mycobacterium tuberculosis* (Balemans, et al., 2012). Nevertheless, the energetic pathways of the bacterial membrane remain largely unexplored and relatively unpopular drug targets.

In bacteria, the extrusion of protons by the electron transport chain results in an electrochemical gradient of protons, known as the proton motive force (PMF), generated across the cell membrane. The PMF is subsequently necessary for ATP synthesis by the F_1F_0-ATPase and for transport of various solutes (Mitchell, 1966). The PMF is made up of the sum of two parameters: the electric potential ($\Delta\Psi$) and the transmembrane proton gradient (ΔpH). Critical to bacterial survival, the PMF has nevertheless been largely disregarded as a target for antimicrobials, owing to toxicity concerns. Still, ionophores such as the natural product monensin, that dissipate the PMF by disrupting the flow of ions across the membrane, have found extensive use as antibacterial agents in the cattle and poultry industries (Russell and Houlihan, 2003). Interestingly, bacteria exercise exquisite control over $\Delta\Psi$ and ΔpH in order to maintain a constant value of PMF.

Dissipation in either component is compensated for by a counteracting increase in the other (Bakker and Mangerich, 1981). We sought in this work to exploit this compensation mechanism as an Achilles heel in bacteria that would be exquisitely susceptible to a combination of perturbants, one that specifically disrupted the electric potential ($\Delta\Psi$) and another, the transmembrane proton gradient (ΔpH).

We set out to identify specific disrupters of the electrical and chemical components of PMF through a fluorescence-based high-throughput screen. In particular, the screen identified three compounds that specifically dissipated $\Delta\Psi$ and three that selectively disrupted ΔpH. We demonstrated profound synergy between these two classes of compounds when used in combination against methicillin resistant *S. aureus* (MRSA). Thus the concerted dissipation of $\Delta\Psi$ and ΔpH had a synergistic effect in collapsing the PMF and, importantly, this synergy spares the doses that are necessary for each of the individual agents. We show that this dose-sparing phenomenon likewise reduces the toxicity of the respective agents. Indeed, drug combination therapy is gaining favour (Keith, et al., 2005) on account of increased efficacy, lower toxicity and reduced propensity for resistance (Cottarel and Wierzbowski, 2007). Herein, we report on the first systematic effort in discovering synergistic antibacterial chemical combinations that exploit the interdependence of the electric potential and chemical gradient of the membrane in maintaining the proton motive force. These findings suggest new opportunities to target energetic systems within bacterial pathogens as a means to develop novel therapeutic strategies.

Results

Development of a high-throughput screen for alterations in PMF.

A widely used probe to study the effect of small molecules on membrane potential is the cationic, membrane potential-sensitive dye, 3,3'-dipropylthiacarbocyanine iodide ($DiSC_3(5)$) (Wu, et al., 1999). In this fluorescence-based assay, bacteria are grown and loaded with dye, which accumulates in the cytoplasmic membrane in response to $\Delta\Psi$ and self-quenches its own fluorescence. When $\Delta\Psi$ is disrupted or the membrane permeabilized upon treatment with a small molecule, the dye is released into the medium resulting in an increase in fluorescence. Interestingly, this dye can also inform on dissipations in the other component of PMF, the transmembrane ΔpH, through observed decreases in fluorescence. Upon dissipation of their pH gradient, bacterial cells will compensate by increasing $\Delta\Psi$ to maintain a constant PMF. As such, this increased membrane potential further concentrates $DiSC_3(5)$ dye in the membrane, such that high local concentrations lead to decreased fluorescence intensity due to further quenching. As validation for the assay, we measured the effect of valinomycin (in the presence of K^+) and nigericin. Valinomcyin, a K^+ ionophore which specifically dissipates the $\Delta\Psi$ component of the PMF, led to a significant increase in fluorescence (Figure 4-1A), while nigericin, an electroneutral antiporter for H^+ and K^+, caused a decrease in fluorescence as it selectively dissipates ΔpH (Figure 4-1A). Solvent (DMSO)-treated $DiSC_3(5)$ loaded cells did not lead to changes in fluorescence consistent with an intact membrane potential (Figure 4-1A). Having established $DiSC_3(5)$ as a suitable tool to identify molecules that

selectively dissipate both components of the PMF, we adapted the methodology to a microwell plate format to allow screening of large numbers of compounds. In this assay, after loading *S. aureus* cells with $DiSC_3(5)$ and treating with compound, dissipation of either $\Delta\Psi$ or ΔpH was immediate, but stable for at least 30 minutes, which allowed a sufficient window of time for recording fluorescence intensity. Overall, the optimized $DiSC_3(5)$ microwell assay had robust signal and noise characteristics, with average Z' values (Zhang, et al., 1999) of 0.88 and 0.80, using known probes valinomycin and nigericin, for increases and decreases in fluorescence intensity, respectively.

We carried out a high-throughput screen (HTS) for $DiSC_3(5)$ fluorescence using a diverse collection of synthetic chemicals and known bioactive compounds (30,000 compounds) assayed at 10 μM against *S. aureus* (strain Newman). Results of the screen are shown in Figure 4-1B, plotted as a normalized fluorescence ratio (Experimental Procedures) such that a ratio of 1.0 represents no change in fluorescence. We set stringent, arbitrary cut-offs for both increases and decreases in fluorescence as ratios of 1.4 and 0.25, respectively. This translated to 79 compounds that increased fluorescence and 272 compounds that decreased fluorescence (Figure 4-1C). A counterscreen of the active compounds was conducted to account for fluorescence artifacts, which could arise from intrinsically fluorescent compounds or compounds that quench $DiSC_3(5)$ fluorescence, independent of the proton motive force (Figure S1). The latter effect, assessed by reading the fluorescence intensity in the absence of cells but in the presence of dye, was a very important counterscreen and reduced the number of active compounds

that decreased fluorescence to 29. The 79 active compounds that increased $DiSC_3(5)$ fluorescence displayed no fluorescence artifacts (Figure 4-1C).

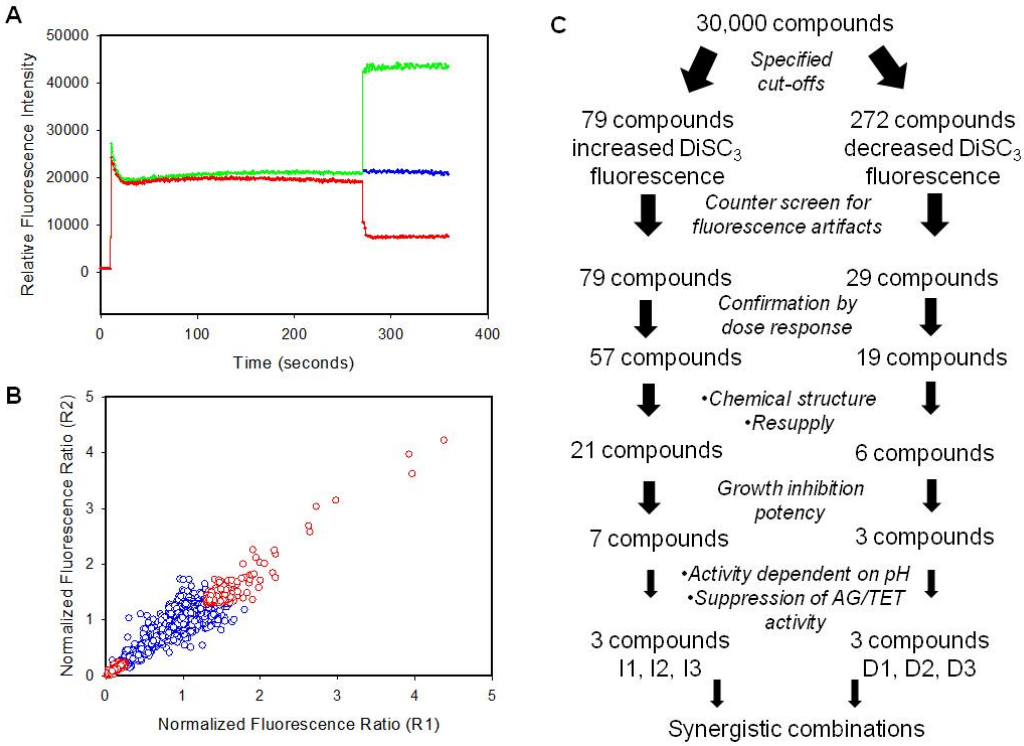

Figure 4-1. A high-throughput screen to identify dissipaters of ΔΨ and ΔpH. (A) Fluorescence of $DiSC_3(5)$ over time following addition of DMSO (blue), valinomycin (5 μM), which disrupts ΔΨ (green) and nigericin (5 μM), which disrupts ΔpH (red). $DiSC_3(5)$ dye was first added at 10 sec followed by self-quenching and stabilization. Solvent or compounds were added at 270 sec. (B) Replicate plot of the $DiSC_3(5)$ primary screen. *S. aureus* was grown and loaded with $DiSC_3(5)$. Cells were added to one of each of the 30,000 compounds at 10 μM. Shown are the normalized ratios of fluorescence values of two replicate samples. The cutoff score for molecules that increased $DiSC_3(5)$ fluorescence was chosen as ≥1.4 and for molecules that decreased fluorescence ≤0.25. Active compounds are shown in red. (C) Schematic diagram of the work-flow from the high-throughput screen of 30,000 compounds to yield the active compounds that increased fluorescence, I1, I2 and I3 as well as thosed that decreased fluorescence, D1, D2 and D3. Compounds were eliminated at each stage according to the criteria indicated. Specifically, after confirming a dose-dependent fluorescence response, the various compounds were tested for the effect of pH on their activity and their ability to suppress the activity of the aminoglycoside (AG), kanamycin, and tetracycline (TET). In aggregate, 6 compounds were confirmed as modulators of components of PMF. These were investigated for synergistic activity against MRSA.

Follow-up to the HTS

In order to confirm active molecules from the HTS, we generated dose response analyses using a range of compound concentrations to assess the dose dependence of the fluorescence readout. Of the 79 compounds that increased fluorescence in the primary screen, 57 dissipated the membrane potential in a concentration-dependent manner (Figure 4-1C). We eliminated 22 compounds whose effects on $DiSC_3(5)$ fluorescence did not occur in a clear dose-dependent fashion or showed high variability in fluorescence readings among three replicates (examples in Figure S2A). Among the 29 compounds that decreased fluorescence, 19 compounds confirmed as causing a dose-dependent reduction in the pH gradient. Similarly, here, we eliminated 10 compounds that did not display clear dose-dependence or exhibited high variability among the three replicates (examples in Figure S2B). Subsequently, we narrowed our list of molecules based on conventional filters, such as visual inspection of chemical structures, ability to resupply and growth inhibitory potency (detailed process in Figure S3) against *S. aureus*. While not all dissipaters of components of the PMF have antibacterial activity, we focused on molecules that were inhibitory against *S. aureus*. Importantly, we also observed that antibacterial activity of the molecules against *S. aureus* correlated well with their ability to dissipate either $\Delta\Psi$ or ΔpH. Overall, we narrowed our working set of molecules to 10 compounds, 7 of which caused an increase in fluorescence ('I' molecules) and potential dissipaters of $\Delta\Psi$ and 3 causing a decrease in dye fluorescence ('D' molecules), potentially dissipating ΔpH (Figure 4-1C).

Uncovering agents that selectively dissipate components of PMF

The nature of the screen not only allows for the discovery of molecules that selectively dissipate components of the PMF, but also agents that permeabilize the membrane allowing $DiSC_3(5)$ dye to be released. Further, since PMF is generated by the electron transport chain, inhibitors of this respiration process may also be uncovered in the screen. It was therefore important to further investigate the mode of action of the active compounds to ensure selective disruption of components of the PMF. To this end, we first assessed the impact of pH on the activity of these compounds. Because the two components of the PMF, the membrane potential and the transmembrane pH gradient (the difference between the intracellular and extracellular pH) are interdependent, a shift in the extracellular pH to alkaline values leads to a decrease in the pH gradient across the membrane, such that the compensatory component, $\Delta\Psi$, accounts for a greater share of the PMF (Booth, 1985). In contrast, a shift to acidic values leads to an increase in ΔpH across the membrane resulting in a compensatory fall in $\Delta\Psi$, such that ΔpH becomes the dominant component (Booth, 1985). A shift in pH to alkaline values led to potentiation of the antibacterial activity of I1, I2 and I3 but did not enhance the activity of the 4 remaining 'I' molecules, suggesting the former molecules selectively modulate $\Delta\Psi$. The chemical structures of I1, I2 and I3 are shown in Figure 4-2A along with their $DiSC_3(5)$ dose-response curves in Figure 4-2B. Specifically, a range of pH 5.5 to pH 9.5 led to a 32-fold change in the minimum inhibitory concentration (MIC) of molecule I1 (Figure 4-2Ci), a 64-fold shift for I2 (Figure 4-2Cii) and a 16-fold change for I3 (Figure 4-2Ciii). Conversely, the antibacterial activity of D1, D2 and D3, for which chemical structures

and dose-response curves are shown in Figure 4-3, increased as pH values became more acidic, consistent with selective disruption of ΔpH. D1 exhibited a 16-fold change in MIC (Figure 4-3Ci), while D2 and D3 showed a 32-fold (Figure 4-3Cii) and 8-fold change in MIC (Figure 4-3Ciii) from a pH range of 9.5 to 5.5, respectively. To further corroborate that the effects of I1-I3 and D1-D3 are specific to components of PMF, we tested their interactions with the aminoglycoside antibiotic kanamycin, whose uptake is driven by ΔΨ (Taber, et al., 1987), and with tetracycline, whose uptake is specifically driven by ΔpH (Yamaguchi, et al., 1991). Indeed, molecules I1-I3 antagonized the activity of kanamycin consistent with the hypothesis that these compounds perturb ΔΨ. Further D1-D3 suppressed the activity of tetracycline against *S. aureus* supporting the conclusion that these molecules act on ΔpH (Table S1). As confirmation for the interplay between ΔΨ and ΔpH, those molecules that potentiated aminoglycoside activity clearly also suppressed tetracycline action and vice-versa (Table S2).

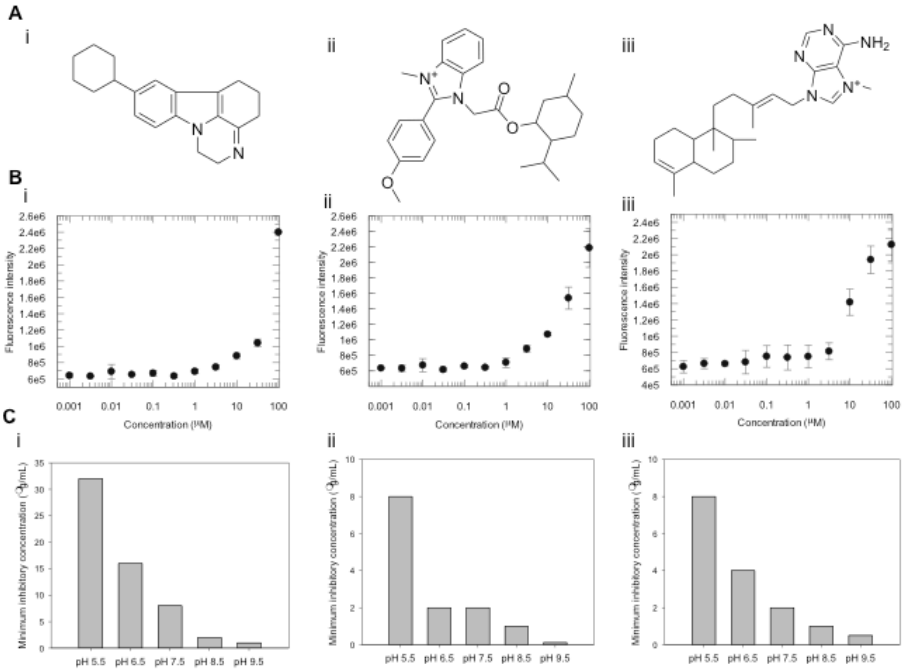

Figure 4-2. Hit compounds I1, I2 and I3 dissipate ΔΨ of *S. aureus*. (A) Chemical structures of (i) I1 (logP = 4.02), (ii) I2 (logP = 2.65) and (iii) I3 (logP = 1.56) . (B) Concentration-dependent effect of (i) I1, (ii) I2 and (iii) I3 on $DiSC_3(5)$ fluorescence. All three compounds caused a dose-dependent increase in fluorescence, indicative of dissipation of ΔΨ. (C) Effect of altering the external pH on the minimum inhibitory concentration of (i) I1, (ii) I2 and (iii) I3. The growth inhibitory activity of all three compounds is enhanced in when the external pH is shifted to an alkaline environment, where ΔΨ becomes the main component of PMF.

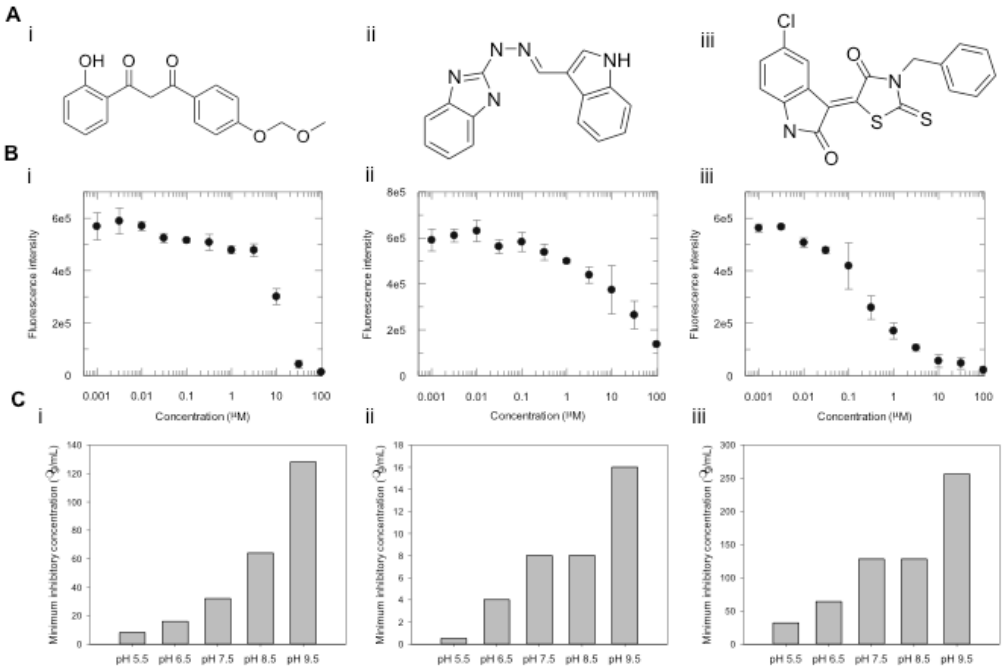

Figure 4-3. Hit molecules D1, D2 and D3 dissipate ΔpH of *S. aureus*. (A) Chemical structures of (i) D1 ((logP = 2.33), (ii) D2 (logP = 2.72) and (iii) D3 (logP = 3.02). (B) Concentration-dependent effect of (i) D1, (ii) D2 and (iii) D3 on $DiSC_3(5)$ fluorescence. All three compounds caused a dose-dependent decrease in fluorescence, indicative of dissipation of ΔpH. (C) Effect of altering the external pH on the minimum inhibitory concentration of (i) D1, (ii) D2 and (iii) D3. The growth inhibitory activity of all three compounds is enhanced in when the external pH is shifted to an acidic environment, where ΔpH becomes the main component of PMF.

Thus molecules I1-I3 and D1-D3 represented strong candidate perturbants of ΔΨ and ΔpH, where the ultimate goal was testing their efficacy in combination against *S. aureus*. Regarding the remaining molecules, I4-I7, that showed no pH dependency and did not show signature antagonism with kanamycin, we posited that these likely disrupted membrane structure or inhibited other aspects of bacterial respiration. Indeed, we tested these hypotheses as described below.

Characterizing I1, I2 and I3, potential dissipaters of ΔΨ

To further characterize the action of I1, I2 and I3, the potential modulators of ΔΨ, we first assessed their impact on the energetic condition of *S. aureus* cells by measuring their effect on the reduction of iodonitrotetrazolium chloride (INT). INT, a tetrazolium salt, can be reduced to a red insoluble formazan (INF) by components of the prokaryotic respiratory chain (Altman, 1976). Since PMF and the ETC are closely interlinked, whereby generation of an electrochemical proton gradient occurs as a result of the extrusion of protons by the electron transport chain, we reasoned that dissipation of this gradient across the cytoplasmic membrane should ultimately affect electron transport across the respiratory chain. Overall, when tested at concentrations equivalent to their MICs, all three molecules led to significant decreases in the reduction of INT to its formazan product as compared to the untreated control, suggesting that the electron transport across the cytoplamsic membrane was impaired (Figure 4-4A).

We next investigated the extent to which I1, I2 and I3, by dissipating the membrane potential, interfere with respiratory ATP production. The proton motive force is essential for the production of ATP via the F_0F_1-ATPase, such that dissipation of this electrochemical gradient should halt ATP production. Intracellular ATP levels were measured by a luciferin-luciferase bioluminescence assay at the MIC values of the various compounds. As depicted in Figure 4-4B, upon addition of I1, intracellular ATP levels drastically decreased by approximately 84% compared to DMSO-treated control. Similarly, I2 decreased ATP levels by approximately 41%, while I3 by 81% compared to the control. This is consistent with a mode of action of dissipating ΔΨ; upon treatment

with the various molecules, the intracellular ATP pool is depleted in an attempt to maintain the PMF. These results also suggest that interference with ATP synthesis or depletion of the cellular ATP pool may be the cause of bacterial killing of these molecules on their own.

We further assessed whether treatment with I1, I2 and I3 results in the efflux of cell constituents, by measuring for any loss of materials absorbing at 260 nm. Leakage of cellular constituents, such as potassium ions, amino acids and ATP, is a common mechanism shared by many small molecules that dissipate PMF. While some cause pores (Garcera, et al., 1993; McAuliffe, et al., 1998), others integrate in the membrane (Silverman, et al., 2003; Ultee, et al., 1999), similar to an ionophore-like inhibitor, and cause dissipations of the ion gradients. In this assay, molecules I2 and I3 did not cause a leak of cellular constituents, as compared to the DMSO control (Figure 4-4C). Nisin, a pore-forming lantibiotic (Garcera, et al., 1993), served as a positive control and led to a significant increase in absorbance, consistent with its mode of action. I1 caused an intermediate absorbance at a concentration equivalent to its MIC (Figure 4-4C). This increased absorbance is likely attributable to I1's high hydrophobicity, which may favor its partitioning into the cytoplasmic membrane, consequently dissipating the transmembrane ion gradient. Nucleic acids, which were leaked through a damaged cytoplasmic membrane, were more abundant with increased concentrations of I1 (data not shown), presumably as more I1 dissolves into the membrane. It should be noted that, at the pertinent tested concentration, there was no decrease in the optical density of the I1-treated cell suspensions, indicating that cell lysis did not occur. The same can be said of

treatment with I2 and I3. Thus, we suggest that the collapse of ΔΨ at the concentrations tested was caused by compound-mediated effects on the cell membrane rather than having occurred as a result of general cell lysis. While I1, I2 and I3 all act on the membrane, only I1was disruptive to its structural integrity, causing leakage of large nucleotides, while I2 and I3 likely only cause small ions to be transported or released across its surface. Overall, molecules I1, I2 and I3 all decreased membrane potential, which in turn inhibited ATP production and depleted energy in the cell, leading to reduced viability.

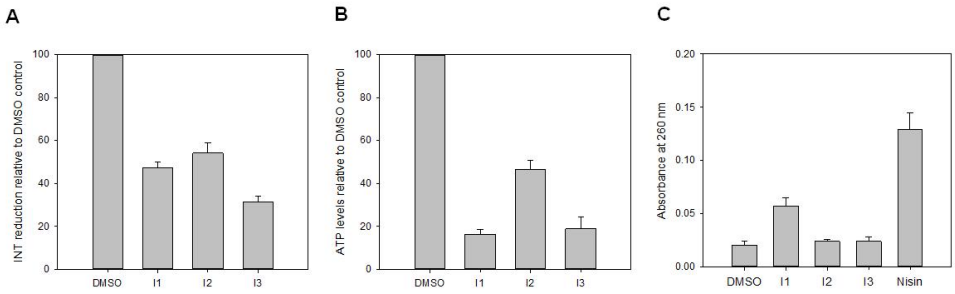

Figure 4-4. Effect of potential dissipaters of ΔΨ at MIC concentrations (I1: 8 μg/mL, I2: 2 μg/mL, I3: 2 μg/mL) on cellular respiration, ATP levels and membrane integrity. (A) Effects of I1, I2 and I3 on the reduction of INT to its formazan product, which can be detected at an absorbance of 490 nm. (B) Effects of compounds on intracellular ATP levels, measured by a luciferin-luciferase bioluminescence assay. Values are relative to DMSO control. (C) Measure of leakage of cellular constituents at 260 nm with nisin as a positive control.

Characterizing D1, D2 and D3, potential dissipaters of ΔpH

Similar to molecules I1-I3, we first assessed the effect of molecules D1, D2 and D3 on the reduction of INT (Figure 4-5A). All three molecules had adverse effects on cellular respiration, decreasing the production of formazan product, consistent with an action of dissipating PMF.

Additionally, D1, D2 and D3 also depleted cellular energy stores by causing a decrease in intracellular levels of ATP, however, to a lesser extent than 'I' molecules. Since membrane potential, but not the pH gradient, is essential for the synthesis of ATP by the F1F0-ATPase (Kaim and Dimroth, 1998), it is not surprising that D1, D2, and D3 only decreased ATP levels by 37%, 16% and 46%, respectively (Figure 4-5B). Finally, none of the dissipaters of ΔpH caused a significant leakage of cell constituents when tested at MIC values (Figure 4-5C), suggesting that they do not cause damage to the physical integrity of the membrane.

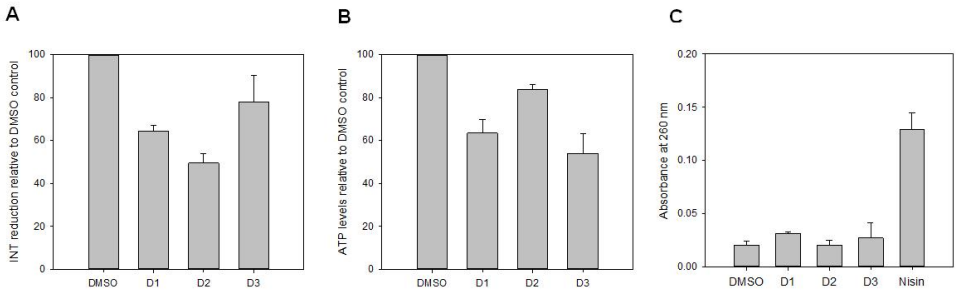

Figure 4-5. Effect of potential dissipaters of ΔpH at MIC values (D1: 32 μg/mL, D2: 8 μg/mL, D3: 128 μg/mL) on cellular respiration, ATP levels and membrane integrity. (A) Effects of D1, D2 and D3 on the reduction of INT to its formazan product, which can be detected at an absorbance of 490 nm. (B) Effects of compounds on intracellular ATP levels, measured by a luciferin-luciferase bioluminescence assay. Values are relative to DMSO control. (C) Measure of leakage of cellular constituents at 260nm. Here, nisin is a positive control.

Characterizing I4, I5, I6 and I7: other molecules that caused an increase in $DiSC_3(5)$ fluorescence

A number of molecules were uncovered in the HTS causing an increase in $DiSC_3(5)$ fluorescence but whose activity was not suppressed by an acidic external pH. We suspected that such molecules might inhibit cellular respiration, ultimately affecting

membrane potential, or simply disrupt the membrane, allowing $DiSC_3(5)$ dye to be released.

Molecule I4 (Figure S4A) caused a dose-dependent increase in $DiSC_3(5)$ fluorescence (Figure S4B) but its activity was not potentiated upon a shift of the external pH to alkaline values. I4 did, however, suppress the activity of kanamycin against *S. aureus* by 8-fold (data not show), suggesting that I4 may inhibit cellular respiration. In fact, aminoglycoside bacterial uptake not only requires intact $\Delta\Psi$, but also electron flow through the membrane-associated respiratory chain (Taber, et al., 1987). Expectedly, I4 led to decreased INT reduction as well as decreased intracellular ATP levels, as intact cellular respiration is required for both (Figures S4C and S4D). We also assessed its potential membrane-damaging properties by measuring for any loss of 260-nm-absorbing material. I4 did not cause any significant release of cell constituents, suggesting it does not inhibit growth by permeablizing the membrane of *S. aureus* (data not shown). Finally, cells treated with I4 had a significant reduction in their amount of staphyloxanthin pigment compared to untreated cells (Figure S4E). Staphyloxanthin synthesis has been shown to require intact cellular respiration (von Eiff, et al., 2006). I4 may be driving *S. aureus* into fermentative growth, which would explain the decrease in ATP yield. Additionally, since pyruvate is shunted to support fermentation, less acetyl-CoA would enter the mevalonate pathway necessary for staphyloxanthin synthesis (Pelz, et al., 2005). Interestingly, I4 synergized with I1-I3 and D1-D3 (Table S3), molecules that inhibit the generation of PMF, lending further support to the notion that I4 targets cellular respiration as a mechanism of action. In combination with these molecules, we

would expect a respiration inhibitor to further deplete energy reserves leading to reduced viability.

Other active compounds, I5, I6 and I7 (Figure S5A) induced similar effects on *S. aureus*. All three caused significant increases in $DiSC_3$ fluorescence (Figure S5B). Unlike our specific dissipaters of $\Delta\Psi$, their antibacterial activities were not potentiated upon a pH shift to alkaline values and they enhanced the activity of the kanamycin, instead of suppressing it. All three molecules did however cause a gross leakage of UV-absorbing material (Figure S5C), similar to nisin, suggesting that they may lead to structural disruption of the membrane. This permeabilization is likely the cause of the increase in $DiSC_3(5)$ dye, which is released in the medium and may also explain the observed enhanced activity of kanamycin, whose uptake would be accordingly facilitated. Interestingly, molecule I7 was structurally a bile salt, compounds known to disrupt cellular membranes (Ross, et al., 2004). By disrupting membrane integrity, I5, I6 and I7 expectedly caused decreases in INT reduction and ATP levels (Table S4), as the respiratory chain and metabolic pathways within the cytoplasmic membrane would be destroyed. Further, the membrane damage caused by I5, I6 and I7 could be large enough to allow the leakage of large compounds, such as ATP. Finally, all three compounds were also synergistic with I1-I3 and D1-D3, the selective dissipaters of $\Delta\Psi$ and ΔpH, again likely due to their facilitated uptake into the cell and likely unrelated to an energetic mechanism.

Combinations of molecules I1-I3 and D1-D3 lead to synergistic interactions

Our overriding objective for the discovery of molecules that selectively dissipate $\Delta\Psi$ and ΔpH was their combination. We hypothesized that combining antibacterial compounds belonging to these two mechanistic classes would circumvent the compensatory responses in $\Delta\Psi$ and ΔpH that maintain PMF. Accordingly, we predicted synergistic, dose-sparing interactions from such combinations. We performed all possible combinations among molecules I1-I3 and molecules D1-D3 using antibacterial checkerboard methodology (White, et al., 1996) against the highly problematic community-acquired methicillin-resistant strain of *S. aureus*, USA 300 (CA-MRSA USA300) (Figure 4-6). Here, we define synergy as having an FIC Index $\leq$0.5, additivity as 1-2 and antagonism as $\geq$2. In all cases, synergy was observed with FIC indices equal to or below 0.5. The most potent synergies were observed for combinations of I2 with D2 and I3 with D3, each resulting in an FIC index of 0.25 (Figure 4-6). As controls, we also performed all combinations among the 'I' molecules and on the other hand, among the 'D' molecules; all combinations led to FIC indices ranging between 0.75-2 (data not shown), indicative of additive interactions.

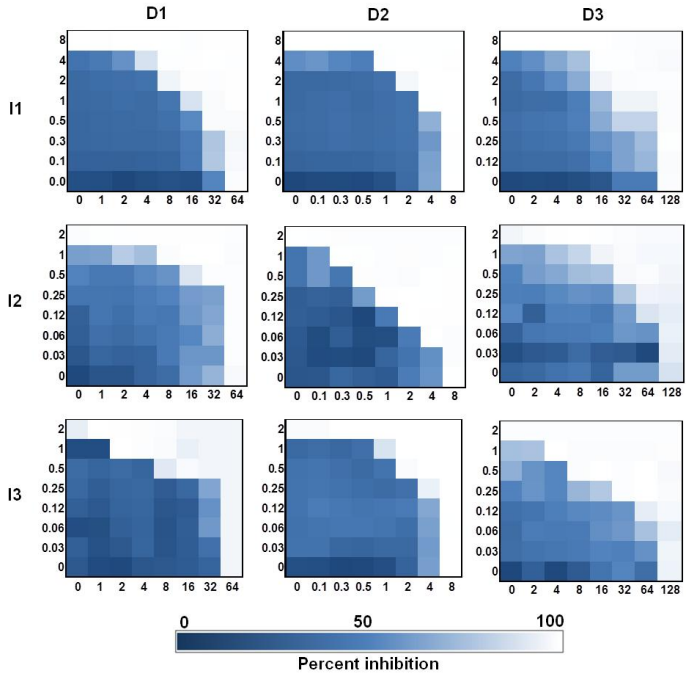

Figure 4-6. Combinations of dissipaters of ΔΨ and ΔpH exhibit synergistic interactions. Microdilution checkerboard analyses showing all possible combined effects of molecules I1-I3 with molecules D1-D3 against CA-MRSA USA300 where the extent of inhibition is shown as a heat plot. In all cases, synergistic effects are evident and result in the following FIC indices: I1-D1 0.37; I1-D2 0.5; I1-D3 0.37; I2-D1 0.5; I2-D2 0.25; I2-D3 0.5; I3-D1 0.37; I3-D2 0.5 and I3-D3 0.25. FIC Index was calculated by the sum of the FIC of each compound, calculated as such; FIC = [X]/MIC_X, where [X] is the lowest inhibitory concentration of drug in the presence of the co-drug.

Cytotoxicity studies

The cytotoxic effects of compounds I1-I3 and D1-D3 against HeLa cells were evaluated by quantifying the release lactate dehydrogenase (LDH) upon cellular necrosis (Nachlas, et al., 1960) (Figure 4-7A). In this assay, only two compounds, I1 and D1, significantly reduced cell viability by 41% and 47% at their antibacterial MIC values of 8 μg/mL and 32 μg/mL, respectively. At MIC concentrations, compounds I2 (MIC 2-4 μg/mL) and I3 (MIC 2-4 μg/mL) reduced cell viability by 9% and 6%, while D2 (MIC 8

μg/mL) and D3 (MIC 128 μg/mL) by 2% and 5%, respectively. Beyond the MIC values of the compounds, however, all but D3 showed significant cytotoxic effects on HeLa cells.

We next assessed whether toxicity at concentrations that lead to synergistic interactions could be minimized compared to toxicity of the compounds as antibacterial agents alone (Figure 4-7B). Overall, it was evident that the observed synergistic combinations would allow for reduced doses to be used, indeed minimizing cytotoxicity. We tested 3 of the 9 combinations, I1+D1, I2+D2, and I3+D3, at the concentration of each molecule that led to synergy for cytotoxicity effects (Figure 4-7B). Overall, toxicity of each compound could be reduced in combination, thus allowing for dose-sparing effects. For example, molecule I1 (which causes 41% toxicity alone) when combined with D1 (47% toxicity alone) at their effective synergistic concentrations led to 19% toxicity (Figure 4-7B). In combination, the activity of I1 is potentiated 4-fold by D1, reducing its cytotoxicity at that concentration to <25%, while D1 is potentiated 8 fold by I1, reducing its toxicity by over 20%. In other cases, such as with I2 and D2, displaying 9% and 2% cytotoxicity as single agents, respectively, resulted in very minimal cytotoxicity in combination (5%) (Figure 4-7B). Finally, as a single agent, I3 exhibited 10% toxicity at its MIC concentration and D3, 5% cytotoxicity. In combination, however, the total cytotoxicity at their effective synergistic concentrations was 7%, thus remaining almost negligible (Figure 4-7B). Overall, while all six compounds exhibited some moderate cytotoxicity, their combinations to achieve enhanced antibacterial efficacy allowed the ability to reduce cytotoxicity effects.

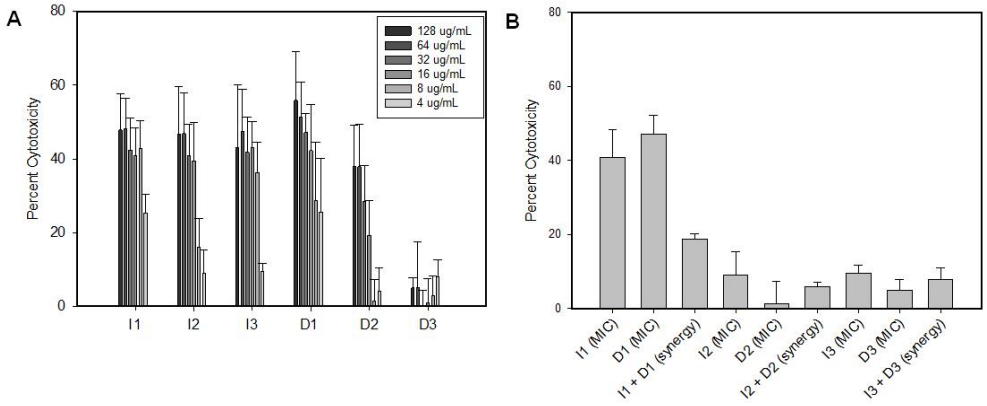

Figure 4-7. Cytotoxic effects of compounds **I1, I2 and I3 as well as D1, D2 and D3 and their combinations** on a HeLa cell line. (A) Cytotoxic effects of compounds I1-I3 and D1-D3 following exposure to different concentrations of compounds. Cell viability was assessed using LDH release method. All values are relative to the maximum LDH release control. Effect by the solvent DMSO was subtracted. (B) Cytotoxic effects of individual compounds at MIC (antibacterial) concentrations and at concentrations that are effective (antibacterial) when used in the synergistic combinations indicated.

Discussion

Bacteria establish and maintain an electrochemical gradient of protons across their cytoplasmic membrane, known as the proton motive force (Mitchell, 1966). This PMF is essential for a variety of critical bacterial processes, such as ATP synthesis, flagellar motility and nutrient import. Collapse of the PMF inhibits these important functions and thus results in the loss of bacterial viability. A unique feature of the PMF is its composition of two components that continually work together to maintain homeostasis (Bakker and Mangerich, 1981). Dissipation of one component of the gradient is buffered by a counteracting increase in the other in order to maintain a constant value for the PMF. Nevertheless, most modern antibacterial discovery efforts purposely deprioritize molecules that disrupt membrane energetics. In the work reported here, we explore the

potential of combining small molecules that perturb the ΔΨ and ΔpH components of the PMF, with the underlying hypothesis that such agents should show advantageous synergy in combination.

Having established $DiSC_3(5)$ fluorescence as a robust assay to identify dissipaters of ΔΨ and ΔpH, we screened a chemical library for small molecules modulators of ΔΨ and ΔpH. Compounds specific to ΔΨ and ΔpH were subsequently identified by their ability to suppress the activity of aminoglycosides or tetracyclines. Such molecules could also be modulated with changes in the external pH. This effort resulted in the identification of molecules I1, I2 and I3, which dissipate membrane potential, and D1, D2 and D3, which dissipate the pH gradient across the cell membrane of *S. aureus*. As expected, I1-I3 and D1-D3 all reduced the pool of intracellular ATP. We also uncovered a potential inhibitor of respiration, I4, as well as three membrane-damaging agents, I5, I6 and I7, which all led to increases in $DiSC_3(5)$ fluorescence.

Interestingly, Oyamada *et al.* have previously reported on molecule I1, discovered in an annucleate cell blue assay performed to identify type II topoisomerase inhibitors against *Escherichia coli* (Oyamada, et al., 2006). In their *in vitro* follow-up, however, we note that I1 exhibited a very high IC_{50} towards purified gyrase and topoisomerase IV, namely 50 μg/mL (171μM) and >100 μg/ml (>342 μM), respectively. Further, molecule I3 is a natural product, known as agelasine found in marine sponges (*Agelas* sp.). Notably, no previous characterization of its antibacterial activity has been reported, but it has been characterized as a putative inhibitor of human Na/K-ATPase (Trachtenberg, et al., 1981).

Importantly, in combination, synergistic interactions among our dissipaters of $\Delta\Psi$ and dissipaters of ΔpH were observed and proved efficacious against MRSA. One advantage afforded by combination therapy over monotherapies is the ability to circumvent toxicity with dose-sparing concentrations of two-component therapy. Indeed, in the work presented here, cytotoxicity was significantly reduced through reductions in dose that were made possible by the synergies of 'I' and 'D' molecules when used in combination.

While molecules that act on the membrane are often associated with toxicities, it is nevertheless clear that bacterial specificity is in fact attainable. The success stories of antibacterials such as daptomycin and telavancin, which depolarize the cytoplasmic membrane, reinforce the point that selective toxicity is achievable (Hurdle, et al., 2011). In our case, molecules I1-I3 and D1-D3 displayed relatively low cytotoxicity at pertinent concentrations i.e. those relating to antibacterial activity. Only some displayed high cytotoxicity against HeLa cells at very high concentrations, in many cases well above 32-fold their antibacterial inhibitory activity. Importantly, however, cytotoxicity was often negligible at MIC concentrations. In eukaryotic cells, the respiratory chain and resulting PMF is in the mitochondrial membrane instead of the cytoplasmic membrane. This distinct localization of the respiratory chain between eukaryotic and bacterial cells could partly explain the observed low toxicity (Wu, et al., 2010). Nevertheless, their combinations allowed for dose-sparing effects and further lowered cytotoxicities.

A number of molecules impart bactericidal activity by collapsing the PMF, including a variety of antimicrobial peptides (Peters, et al., 2010; Wilmes, et al., 2011),

which do so by forming pores, and uncoupling agents, which inhibit oxidative phosphorylation (Terada, 1990); fewer, however, selectively dissipate either component of the PMF (McAuliffe, et al., 1998). Discriminating between decreases in the individual components of the PMF allows for a greater control of bacterial membrane energetics and gives an opportunity for collapsing the PMF via chemical combinations. In fact, targeting redundancy and homeostasis is a critical feature of combinatorial therapy and at the root of successful combination therapies (Cottarel and Wierzbowski, 2007; Fitzgerald, et al., 2006).

We speculate that collapsing the PMF might be ideal as an antibacterial strategy against a variety of species. Compounds that collapse the PMF inhibit flagellar motility (Paul, et al., 2008), preventing invasion (Nan, et al., 2011) and swarming activities that eventually lead to biofilm formation (Ikonomidis, et al., 2008). The PMF also plays a necessary role in protein excretion and the export of many toxic secreted metabolites (Geller, 1991), which could be prevented by perturbing the PMF. Additionally, PMF-targeted molecules and their combinations could be expected to inhibit more efficiently dormant bacteria, a subpopulation of slow-growing or non-growing microorganisms that play important roles in many persistent infections (Lewis, 2007). Dormant bacteria already have a reduced cellular metabolism, with a lowered but adequate proton motive force (Hurdle, et al., 2011) and could thus be more susceptible to modulators of the PMF. Finally, many antibiotic efflux pumps are driven by the PMF (Paulsen, et al., 1996), thus dissipaters of PMF could find utility as novel adjuvants to conventional antibiotics.

In the work reported here, we have presented a proof of principle effort for a rational and methodical strategy for the discovery of chemical combinations that block PMF in a synergistic manner. This method of achieving efficacy while minimizing potential toxicities by targeting two interlinked components of a common pathway provides a foundation for subsequent development of highly potent combinations and for the application to other compensatory pathways in the cell.

Significance

Recently, we have seen a groundswell of concern over the lack of effective antibiotics to treat bacterial infection. The growing number of multi-drug resistant pathogens coupled with the retreat of the pharmaceutical sector from new antibiotic development has exacerbated this problem such that new, perhaps unconventional, strategies are needed to meet these health challenges. Here, we report on a new approach to uncover synergistic combinations effective against resistant pathogens such as methicillin-resistant *Staphylococcus aureus*. Using a fluorescence-based high-throughput screen of *S. aureus*, we have identified selective dissipaters of either $\Delta\Psi$ or ΔpH, the components that make up the proton motive force (PMF). We reasoned that combinations of selective $\Delta\Psi$ and ΔpH perturbants would be uniquely synergistic because of a requirement in bacteria to maintain a constant PMF across the cytoplasmic membrane by balancing both components. We have identified 3 novel dissipaters of $\Delta\Psi$ and 3 novel dissipaters of ΔpH and provided evidence that strategically modulating $\Delta\Psi$ and ΔpH in concert via combinations leads to profound synergy with potent activity against MRSA. Additionally, we have shown that the synergy among these membrane-active compounds

allows for considerable dose-sparing and as a result, lower toxicity. Indeed, modern antibacterial drug discovery has largely avoided membrane-active agents because of toxicity challenges. This work represents a new paradigm to tackle infectious pathogens and provides a methodical strategy to uncover novel and synergistic antibacterial drug combinations.

Experimental procedures

Materials and bacterial strains. 3,3'-Dipropylthiacarbocyanine iodide and all antibiotics used in the study were purchased from Sigma Aldrich. The strains used in the study are *S. aureus* (strain Newman strain) and CA-MRSA USA 300. Mueller Hinton Broth was used as the growth medium.

High-throughput screening for molecules that modulate PMF of *S. aureus*. Effects of compounds on the bioenergetics of the cytoplasmic membrane of *S. aureus* (strain Newman) were determined using the membrane potential-sensitive cyanine dye $DiSC_3(5)$ by a modification of the method of Epand *et al.* (Epand, et al.). Briefly, cultures of *S. aureus* were grown to exponential phase and washed 3 times and resuspended in a buffer containing 10 mM potassium phosphate, 5 mM $MgSO_4$, 250 mM sucrose (pH 7.0). After the final wash, pellets were resuspended in the same buffer to an OD_{600} of 0.085. The cells were loaded with 1 μM $DiSC_3(5)$ and allowed to stabilize before adding to assay plates containing compounds. Compounds were dispensed into 96 well assay plates (final concentration of 10 μM) using a Biomek FX liquid handler (Beckman Coulter Inc., Fullerton, CA). Cells were added using a μfill system (Biotek). Fluorescence was monitored at one time point using an EnVision plate reader (Perkin Elmer) at an excitation wavelength of 620 nm and an emission wavelength of 685 nm. The screen was completed in duplicate and each assay plate contained controls (16 wells total) including background DMSO controls, valinomycin and nigericin controls. All raw data points were normalized to the mean of the middle two quartiles computed on an individual plate basis, to account for plate-to-plate variation, resulting in a ratio, where a high ratio indicates dissipation of $\Delta\Psi$ and a low ratio, dissipation of ΔpH. Positive and negative controls on each plate were used as checkpoints that the assay was running correctly. Hit compounds were selected based on an arbitrary cut-off of 1.4 for molecules that caused an increase in fluorescence and 0.25 for molecules that caused a decrease in fluorescence.

Counter screen for fluorescence artifacts. Fluorescence artifacts were accounted for by measuring the fluorescence of the compounds, which caused an increase in fluorescence, on their own in assay buffer relative to DMSO controls, while compounds that caused a

decrease in fluorescence were screened for their ability to quench the fluorescence of $DiSC_3(5)$ in assay buffer in the absence of cells.

Dose response studies. Half-log serial dilutions of the hit compounds were prepared and aliquoted into assay plates. Fluorescence values were obtained in a similar manner as the screen. Curves were plotted on GraFit; any molecules with deviations from a clear dose-response relationship were eliminated.

MIC determinations. Protocol for MIC determinations was based on CLSI guidelines. Plates were incubated at 37°C for 18 hours and optical density read at 600 nm. The MIC for the drug was the lowest concentration showing <10% growth.

INT reduction assay. *S.aureus* cells were grown from an overnight culture to exponential phase and washed and resuspended in 0.1M potassium phosphate buffer (pH 7.5) to an optical density of 0.3 and kept on ice. In glass tubes, compound was added in buffer with 1 mM INT and 1mL of cell in a total volume of 3mL. Tubes were mixed vigorously and absorbance at 490 nm was read at 10 minute intervals. Tubes were incubated at 30°C between reads.

ATP levels. Protocol was adapted from (Patton, et al., 2006). ATP concentrations were determined using an ATP bioluminescent assay kit (Sigma) according to the manufacturer's instructions. Bioluminescence was measured using an EnVision plate reader (PerkinElmer).

Staphyloxanthin levels. *S. aureus* cell culture at an OD 0.1 was treated with compound and grown at 37°C for 24 hours. Cells were centrifuged and pellets washed twice with PBS. Pigment was extracted in methanol, placing the samples in a 40°C water bath for 20 mins. Samples were cooled and the absorbance of the extracted staphyloxanthin pigment was read at 450 nm and normalized to OD at 600 nm of the initial culture.

Checkerboard analyses and FIC index determination. FICs were determined by setting up standard checkerboard broth microdilution assays with serially diluted 8 concentrations of each drug, using the conditions based on CLSI guidelines. At least 3 replicates were done for each combination and the means used for calculation. The FIC for each drug was calculated as the [drug in the presence of co-drug] for a well showing <10% growth, divided by the MIC for that drug. The FIC index is the sum of the two FICs. Chemical-chemical interactions with an FIC index ≤ 0.5 were deemed synergistic.

Cytotoxicity assays. Cytotoxicity experiments were performed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, WI, USA), which measures the release of lactate dehydrogenase (LDH) upon cellular necrosis. Briefly, HeLa cells were seeded at 10^4 cells per well of a 96-well plate in DMEM supplemented with 10% fetal calf serum and 1% Pen-Strep, and incubated for 24-26 hours at 37°C/5% CO_2. Upon reaching 90-95% confluency, cells were washed and stimulated with each compound in duplicate or triplicate at a concentration of 1 in 100 in complete DMEM. After 18 hours, LDH release was measured according to manufacturer's instructions. Each experiment was performed in triplicate, and all values are presented as relative to the maximum LDH release control, achieved by incubating unstimulated cells with 1x lysis buffer for 45 minutes prior to the measurement of LDH release.

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References

Altman, F.P. (1976). Tetrazolium salts and formazans. Prog Histochem Cytochem 9, 1-56.

Bakker, E.P., and Mangerich, W.E. (1981). Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J Bacteriol 147, 820-826.

Balemans, W., Vranckx, L., Lounis, N., Pop, O., Guillemont, J., Vergauwen, K., Mol, S., Gilissen, R., Motte, M., Lancois, D., et al. (2012). Novel antibiotics targeting respiratory ATP synthesis in Gram-positive pathogenic bacteria. Antimicrob Agents Chemother 56, 4131-4139.

Booth, I.R. (1985). Regulation of cytoplasmic pH in bacteria. Microbiol Rev 49, 359-378.

Chopra, I. (2003). Antibiotic resistance in Staphylococcus aureus: concerns, causes and cures. Expert Rev Anti Infect Ther 1, 45-55.

Coates, A.R., and Hu, Y. (2008). Targeting non-multiplying organisms as a way to develop novel antimicrobials. Trends Pharmacol Sci 29, 143-150.

Cottarel, G., and Wierzbowski, J. (2007). Combination drugs, an emerging option for antibacterial therapy. Trends Biotechnol 25, 547-555.

Epand, R.F., Pollard, J.E., Wright, J.O., Savage, P.B., and Epand, R.M. Depolarization, bacterial membrane composition, and the antimicrobial action of ceragenins. Antimicrob Agents Chemother 54, 3708-3713.

Fitzgerald, J.B., Schoeberl, B., Nielsen, U.B., and Sorger, P.K. (2006). Systems biology and combination therapy in the quest for clinical efficacy. Nat Chem Biol 2, 458-466.

Garcera, M.J., Elferink, M.G., Driessen, A.J., and Konings, W.N. (1993). In vitro pore-forming activity of the lantibiotic nisin. Role of protonmotive force and lipid composition. Eur J Biochem 212, 417-422.

Geller, B.L. (1991). Energy requirements for protein translocation across the Escherichia coli inner membrane. Mol Microbiol 5, 2093-2098.

Gentry, D.R., Wilding, I., Johnson, J.M., Chen, D., Remlinger, K., Richards, C., Neill, S., Zalacain, M., Rittenhouse, S.F., and Gwynn, M.N. (2010). A rapid microtiter plate assay for measuring the effect of compounds on Staphylococcus aureus membrane potential. J Microbiol Methods 83, 254-256.

Hawkey, P.M. (2008). Pre-clinical experience with daptomycin. J Antimicrob Chemother 62 Suppl 3, iii7-14.

Hu, Y., Shamaei-Tousi, A., Liu, Y., and Coates, A. (2010). A new approach for the discovery of antibiotics by targeting non-multiplying bacteria: a novel topical antibiotic for staphylococcal infections. PLoS One 5, e11818.

Hurdle, J.G., O'Neill, A.J., Chopra, I., and Lee, R.E. (2011). Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9, 62-75.
Ikonomidis, A., Tsakris, A., Kanellopoulou, M., Maniatis, A.N., and Pournaras, S. (2008). Effect of the proton motive force inhibitor carbonyl cyanide-m-chlorophenylhydrazone (CCCP) on Pseudomonas aeruginosa biofilm development. Lett Appl Microbiol 47, 298-302.
Kaim, G., and Dimroth, P. (1998). ATP synthesis by the F1Fo ATP synthase of Escherichia coli is obligatorily dependent on the electric potential. FEBS Lett 434, 57-60.
Keith, C.T., Borisy, A.A., and Stockwell, B.R. (2005). Multicomponent therapeutics for networked systems. Nat Rev Drug Discov 4, 71-78.
Lewis, K. (2007). Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5, 48-56.
McAuliffe, O., Ryan, M.P., Ross, R.P., Hill, C., Breeuwer, P., and Abee, T. (1998). Lacticin 3147, a broad-spectrum bacteriocin which selectively dissipates the membrane potential. Appl Environ Microbiol 64, 439-445.
Mitchell, P. (1966). Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. Biol Rev Camb Philos Soc 41, 445-502.
Nachlas, M.M., Margulies, S.I., Goldberg, J.D., and Seligman, A.M. (1960). The determination of lactic dehydrogenase with a tetrazolium salt. Anal Biochem 1, 317-326.
Nan, B., Chen, J., Neu, J.C., Berry, R.M., Oster, G., and Zusman, D.R. (2011). Myxobacteria gliding motility requires cytoskeleton rotation powered by proton motive force. Proc Natl Acad Sci U S A 108, 2498-2503.
O'Neill, A.J., and Chopra, I. (2004). Preclinical evaluation of novel antibacterial agents by microbiological and molecular techniques. Expert Opin Investig Drugs 13, 1045-1063.
Oyamada, Y., Ito, H., Fujimoto-Nakamura, M., Tanitame, A., Iwai, N., Nagai, K., Yamagishi, J., and Wachi, M. (2006). Anucleate cell blue assay: a useful tool for identifying novel type II topoisomerase inhibitors. Antimicrob Agents Chemother 50, 348-350.
Patton, T.G., Yang, S.J., and Bayles, K.W. (2006). The role of proton motive force in expression of the Staphylococcus aureus cid and lrg operons. Mol Microbiol 59, 1395-1404.
Paul, K., Erhardt, M., Hirano, T., Blair, D.F., and Hughes, K.T. (2008). Energy source of flagellar type III secretion. Nature 451, 489-492.
Paulsen, I.T., Brown, M.H., and Skurray, R.A. (1996). Proton-dependent multidrug efflux systems. Microbiol Rev 60, 575-608.
Pelz, A., Wieland, K.P., Putzbach, K., Hentschel, P., Albert, K., and Gotz, F. (2005). Structure and biosynthesis of staphyloxanthin from Staphylococcus aureus. J Biol Chem 280, 32493-32498.
Peters, B.M., Shirtliff, M.E., and Jabra-Rizk, M.A. (2010). Antimicrobial peptides: primeval molecules or future drugs? PLoS Pathog 6, e1001067.
Prakash Singh, M. (2006). Rapid test for distinguishing membrane-active antibacterial agents. J Microbiol Methods 67, 125-130.

Ross, B.P., Braddy, A.C., McGeary, R.P., Blanchfield, J.T., Prokai, L., and Toth, I. (2004). Micellar aggregation and membrane partitioning of bile salts, fatty acids, sodium dodecyl sulfate, and sugar-conjugated fatty acids: correlation with hemolytic potency and implications for drug delivery. Mol Pharm 1, 233-245.
Russell, J.B., and Houlihan, A.J. (2003). Ionophore resistance of ruminal bacteria and its potential impact on human health. FEMS Microbiol Rev 27, 65-74.
Silver, L.L. (2011). Challenges of antibacterial discovery. Clin Microbiol Rev 24, 71-109.
Silverman, J.A., Perlmutter, N.G., and Shapiro, H.M. (2003). Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus. Antimicrob Agents Chemother 47, 2538-2544.
Taber, H.W., Mueller, J.P., Miller, P.F., and Arrow, A.S. (1987). Bacterial uptake of aminoglycoside antibiotics. Microbiol Rev 51, 439-457.
Terada, H. (1990). Uncouplers of oxidative phosphorylation. Environ Health Perspect 87, 213-218.
Trachtenberg, M.C., Packey, D.J., and Sweeney, T. (1981). In vivo functioning of the Na+, K+-activated ATPase. Curr Top Cell Regul 19, 159-217.
Ultee, A., Kets, E.P., and Smid, E.J. (1999). Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus. Appl Environ Microbiol 65, 4606-4610.
von Eiff, C., McNamara, P., Becker, K., Bates, D., Lei, X.H., Ziman, M., Bochner, B.R., Peters, G., and Proctor, R.A. (2006). Phenotype microarray profiling of Staphylococcus aureus menD and hemB mutants with the small-colony-variant phenotype. J Bacteriol 188, 687-693.
White, R.L., Burgess, D.S., Manduru, M., and Bosso, J.A. (1996). Comparison of three different in vitro methods of detecting synergy: time-kill, checkerboard, and E test. Antimicrob Agents Chemother 40, 1914-1918.
Wilmes, M., Cammue, B.P., Sahl, H.G., and Thevissen, K. (2011). Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28, 1350-1358.
Wu, G., Wu, H., Fan, X., Zhao, R., Li, X., Wang, S., Ma, Y., Shen, Z., and Xi, T. (2010). Selective toxicity of antimicrobial peptide S-thanatin on bacteria. Peptides 31, 1669-1673.
Wu, M., Maier, E., Benz, R., and Hancock, R.E. (1999). Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of Escherichia coli. Biochemistry 38, 7235-7242.
Yamaguchi, A., Ohmori, H., Kaneko-Ohdera, M., Nomura, T., and Sawai, T. (1991). Delta pH-dependent accumulation of tetracycline in Escherichia coli. Antimicrob Agents Chemother 35, 53-56.
Zhanel, G.G., Calic, D., Schweizer, F., Zelenitsky, S., Adam, H., Lagace-Wiens, P.R., Rubinstein, E., Gin, A.S., Hoban, D.J., and Karlowsky, J.A. (2010). New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. Drugs 70, 859-886.
Zhang, J.H., Chung, T.D., and Oldenburg, K.R. (1999). A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen 4, 67-73.

CHAPTER FIVE – Future Directions and Conclusions

Summary and Future Directions

Work in this thesis highlights the powerful utility of chemical-chemical combinations as probes of biology and as routes to novel drug discovery. Specifically, all three high-throughput screening campaigns discussed herein offer new methodologies that can be used in early stages of antimicrobial drug discovery. The first tackles the challenge of target identification and can be universally applicable to any pathogen under study. Indeed, the modes of action of uncharted compounds can be inferred based on interactions, whether additive, synergistic or antagonistic, with known probes of physiology. This simple approach has proven its utility in various phenotype-based screens in a variety of ways, such as identifying direct cellular target (Farha and Brown 2010), understanding unique modes of action (Ejim, Farha et al. 2011; Farha, Verschoor et al. 2013) and aiding in mapping out novel connections within biological systems (Farha, Leung et al. 2013). The potential of this strategy is only limited by the number of probes used. In this respect, the approach would benefit from the assembly of a comprehensive compendium of all probes of bacterial physiology that can be routinely screened in combination with hit compounds following phenotype-based screens. The potential to combine probes of all major cellular processes awaits the discovery of small molecule probes to all physiological pathways. Another consideration, as with all high-throughout studies, would be the need for effective methods to analyze and interpret generated profiles.

The last two screening efforts described in this study offer unique methodologies for therapeutic discovery via combination therapy to combat drug-resistant pathogens.

The first was a search for adjuvants among previously-approved drugs that can restore the activity of β-lactams, antibiotics that have lost their effectiveness with the rise of MRSA. This screening strategy has been previously established and validated, yielding another novel combination effective against *P. aeruginosa* (Ejim, Farha et al. 2011). With the evolution of antibiotic resistance, the majority of antibiotics are no longer pertinent to current practice; adjuvants can expand chemical space and effectively restore their utility. Here, the use of previously-approved drugs as adjuvants provides the added benefit of accelerating clinical development, as pharmacology and toxicology are well studied. Indeed, ticlopidine is a well-established antiplatelet drug, however, factors such as metabolic conversion limit its immediate use. To this end, SAR studies were undertaken to design analogs that can evade enzymatic conversion (Farha, Koteva et al. 2014).

The last study in this thesis represents a new paradigm in the field of antibacterial drug discovery. Modern drug discovery has largely avoided agents that act on the membrane because of toxicity challenges. In this study, identified combinations of compounds were very effective while minimizing potential toxicities. This strategy of targeting two interlinked components of a common pathway provides a foundation for subsequent development of potent combinations and can be extended towards other compensatory pathways in the cell.

Overall, development of combination therapies is not without challenges. A large limitation of combination studies is the number of possible combinations to be screened among even a modestly sized compound library. Strategies are increasingly being devised and implemented to aid in managing the overwhelming numbers (Feala, Cortes et al.

2010). Further, designs of appropriate clinical trials and efficient regulatory paths need to be established. Drug approval processes risk being further extended with combinations, whereby drugs will have to be approved individually and then tested as a combination. A sound approach for clinical approval needs to be established and will ultimately dictate the future of combination therapy.

Given the power of pairwise combinations in probing functional biological connections and their benefits as therapeutics, there is reason to believe that high-order combinations will have even greater potential in capturing biological robustness and clinical efficacy. Early research involving third order combinations have tested pairwise combinations against single mutants (Haggarty, Clemons et al. 2003) or conversely, double mutants for sensitivity to drug treatments (St Onge, Mani et al. 2007). To date, rare have been studies that investigate purely chemical combinations. The growing appreciation that biological systems are best manipulated by modulating a set of nodes that ultimately control viability predicts that higher-order combinations will exert an even more selective control of complex systems. As such, high-order combinations as probes of biology have the potential to provide a more integrated understanding of the biological system under study. From a drug discovery perspective, the benefits remain; potency, toxicity, specificity and drug resistance would all be adequately addressed. In fact, *in vitro* studies of resistant bacteria have shown that third-order combinations are more effective than drug pairs (Bhusal, Shiohira et al. 2005). Efforts to move to higher-order combinations will likely be very challenging. The complex nature of high-order

combinations will certainly bring forth technical and data management challenges, but it is, nonetheless, an area of potential activity in the foreseeable future.

Concluding remarks

While there is a long history of use of chemical-chemical combinations, only more recently have they been appreciated for their power as probes of biology and their benefits as therapeutics. Indeed, combination chemical genetics is a growing field that is benefiting from advances in systems biology and high-throughput technologies. The work reported here has identified a high-throughput approach for target identification irrespective of the organism under study. Further, two separate unique screening strategies were taken to identify novel synergistic pairs effective against highly problematic pathogens in the clinic and community. In addition to exhibiting *in vitro* and *in vivo* efficacy, the first combination has shed light on the intricate connections that underlie teichoic acid and cell wall syntheses in *S. aureus*. This adjuvant to β-lactam antibiotics shows great promise in clinical value and highlights the ability of adjuvants to repurpose existing chemical matter. The last high-throughput screen led to the identification of synergistic pairs that effectively modulate two compensatory pathways that make up the proton motive force across the bacterial cell wall. This study reinforces the power of pairwise combinations in perturbing systems and opens up the possibility to dually control other interconnected aspects of physiology via chemical-chemical combinations. Overall, the prospect of novel and better drug combinations is enticing, and screening strategies, as described herein, hold great promise in uncovering novel therapeutics.

References

Bhusal, Y., C. M. Shiohira, et al. (2005). "Determination of in vitro synergy when three antimicrobial agents are combined against Mycobacterium tuberculosis." International journal of antimicrobial agents **26**(4): 292-297.

Ejim, L., M. A. Farha, et al. (2011). "Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy." Nature chemical biology **7**(6): 348-350.

Farha, M. A. and E. D. Brown (2010). "Chemical probes of Escherichia coli uncovered through chemical-chemical interaction profiling with compounds of known biological activity." Chemistry & biology **17**(8): 852-862.

Farha, M. A., K. Koteva, et al. (2014). "Designing analogs of ticlopidine, a wall teichoic acid inhibitor, to avoid formation of its oxidative metabolites." Bioorganic & medicinal chemistry letters **24**(3): 905-910.

Farha, M. A., A. Leung, et al. (2013). "Inhibition of WTA synthesis blocks the cooperative action of PBPs and sensitizes MRSA to beta-lactams." ACS chemical biology **8**(1): 226-233.

Farha, M. A., C. P. Verschoor, et al. (2013). "Collapsing the proton motive force to identify synergistic combinations against Staphylococcus aureus." Chemistry & biology **20**(9): 1168-1178.

Feala, J. D., J. Cortes, et al. (2010). "Systems approaches and algorithms for discovery of combinatorial therapies." Wiley interdisciplinary reviews. Systems biology and medicine **2**(2): 181-193.

Haggarty, S. J., P. A. Clemons, et al. (2003). "Chemical genomic profiling of biological networks using graph theory and combinations of small molecule perturbations." Journal of the American Chemical Society **125**(35): 10543-10545.

St Onge, R. P., R. Mani, et al. (2007). "Systematic pathway analysis using high-resolution fitness profiling of combinatorial gene deletions." Nature genetics **39**(2): 199-206.