SHANNON BETH FALCONER - ANTIBACTERIAL ACTIVITY THROUGH METAL CHELATION

CHEMICAL-GENETICS IDENTIFIES TWO MECHANISTICALLY UNIQUE SPIRO-ANALOGS: AN INHIBITOR OF BACTERIAL IRON HOMEOSTASIS AND A ZINC CHELATOR THAT RE-SENSITIZES A METALLO-β-LACTAMASE-PRODUCING PATHOGEN TO CARBAPENEM ANTIBIOTICS

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TITLE: Chemical-genetics identifies two mechanistically unique spiro-analogs: an inhibitor of bacterial iron homeostasis and a zinc chelator that re-sensitizes a metallo-β-lactamase-producing pathogen to carbapenem antibiotics

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Abstract

Concomitant with antibiotic use is the development of bacterial strains that are resistant to such compounds. Presently, the rate at which antibiotic-resistant pathogenic bacteria are emerging is outpacing our resupply of new antibacterials; therefore, renewed efforts to identify novel therapies are urgently needed. Transition metals are required by all life forms and, for bacteria, an adequate supply of nutrient metal is necessary to establish infection in a host. Indeed, as an antibacterial defense mechanism, eukaryotes have developed various means by which to restrict the availability of metal to the invading pathogen, thereby limiting its chances for successful colonization. As such, bacterial metal acquisition and homeostasis have been suggested as potential antibiotic targets to explore for the identification of new antibacterial small molecules. In this thesis I discuss my development of a high-throughput screening assay that specifically selects for compounds that perturb bacterial iron homeostasis. The results of this work led to the identification of a series of spiro-indoline-thiadiazole compounds that are toxic to bacteria via iron chelation. In addition to molecules that perturb the availability of bacterial intracellular iron, we present a series of spiro-indoline-thiadiazole analogs that inhibit bacterial growth by limiting zinc availability. Furthermore, we show that the respective zinc-perturbing analogs re-sensitize an otherwise drug-resistant strain of NDM-1-harbouring *Klebsiella pneumoniae* to carbapenem antibiotics. We discuss the potential for this class of compounds to serve as carbapenem adjuvants for treating infections caused by metallo-β-lactamase-containing pathogens.

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List of Abbreviations

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTPA	diethylenetriaminepentaacetic acid
EDTA	ethylenediaminetetraacetic acid
ESBL	extended-spectrum beta-lactamase
FIC	fractional inhibitory concentration
GFP	green fluorescent protein
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IMP	imipenem resistant
K_d	dissociation constant
LB	Luria Bertani
M	molar
MBL	metallo beta lactamase
MIC	minimum inhibitory concentration
mM	millimolar
MOA	mechanism of action
MRSA	methicillin-resistant *Staphylococcus aureus*
NDM-1	New Delhi metallo-beta-lactamase-1
nm	namometer
NMR	nuclear magnetic resonance
OD	optical density

PHEN	1,10-phenanthroline
RNA	ribonucleic acid
SIT	spiro-indoline-thiadiazole
TPEN	N,N,N',N'-Tetrakis(2-pyridylmethyl)ethylenediamine
μM	micromolar
VIM	Verona integron-encoded metallo-beta-lactamase

CHAPTER 1 – INTRODUCTION

1.1 Antibiotics: the current state of affairs

Since the introduction of antibacterials in the 1940s, efforts to combat microbial pathogens have been mired by the emergence of drug-resistant bacteria. Indeed, for each antibiotic, identification of a bacterial strain resistant to the respective compound has occurred within a decade of (and, in certain instances, even sooner than) the drug's entry into the clinic (Lewis, 2013). Antibiotic resistance by bacteria results from the unavoidable force of evolutionary pressure; hence, the need to identify new ways to target bacterial pathogens will continue in perpetuity (Fischbach & Walsh, 2009). However, the swiftness with which such resistance develops has been suggested to be a component of antibiotic use that can be better controlled. Among the explanations for the rapid succession between deployment of an antibiotic and the emergence of a bacterial strain resistant to the given compound are 1) widespread and injudicious antibiotic use; 2) a paucity of antibiotic targets; and 3) the existence of a limited number of antibacterial chemical scaffolds. Where addressing the first argument is largely beyond the work of research scientists, identifying both new aspects of bacterial physiology that can be exploited as drug targets, as well as antibacterial compounds with wholly novel chemical scaffolds, are necessary scientific pursuits lest the incidence of morbidity and mortality due to bacterial infection return to those observed prior to the discovery of antibiotics.

The majority of clinical antibiotics consist of a limited chemical diversity that target a very narrow spectrum of essential bacterial processes (Figure 1-1). Exceptions can be found, for example, in the folate-biosynthesis targeting antibiotics, sulfamethoxazole and trimethoprim; the polymyxins, which damage the bacterial

membrane; daptomycin, which exerts its antibacterial activity via depolarization of the cell membrane; and the ATP synthase proton pump inhibitor, bedaquiline (Lewis, 2013; Walsh, 2003). However, most antibiotics in clinical use are derivatives of roughly a half-dozen antibiotics that utilize one of only the three main mechanisms of action. Specifically, inhibition of 1) cell wall biosynthesis, which is accomplished by the β-lactam and glycopeptide antibiotics; 2) protein biosynthesis, which occurs via the action of aminoglycosides and tetracyclines, which target the 30S subunit of the ribosome, or the macrolide antibiotics, which block the action of the 50S ribosomal subunit; and 3) DNA replication, which is achieved by the fluoroquinolone class of antibiotics (Walsh, 2003). Thus, the small number of antibacterial targets, coupled with the relatively few chemical scaffolds upon which most antibiotics are comprised, have contributed to the development of strains of multi-drug resistant pathogenic bacteria that are recalcitrant to all classes of antibiotics (Payne, Gwynn, Holmes, & Pompliano, 2007).

Where the time period between 1940 and 1960 was extraordinarily fruitful by way of antibiotic drug discovery, the past five decades have seen the introduction of only three new classes of antibiotics; namely, linezolid, daptomycin and bedaquiline (Lewis, 2013; Roemer & Boone, 2013). Indeed, although drug-resistant pathogens were present during the so-called "golden era" of antibacterial drug discovery, at that time the emergence of such antibiotic-resistant strains was outpaced by the rate at which new antibacterials were identified—a scenario that is in stark contrast to what has been observed over the past 50 years. Therefore, with such pronounced stalling in the antibiotic pipeline, combined with the increasing prevalence of infection caused by multi-drug resistant pathogenic bacteria,

there is an urgent need to identify new antibiotics with both novel chemical scaffolds and mechanisms of action.

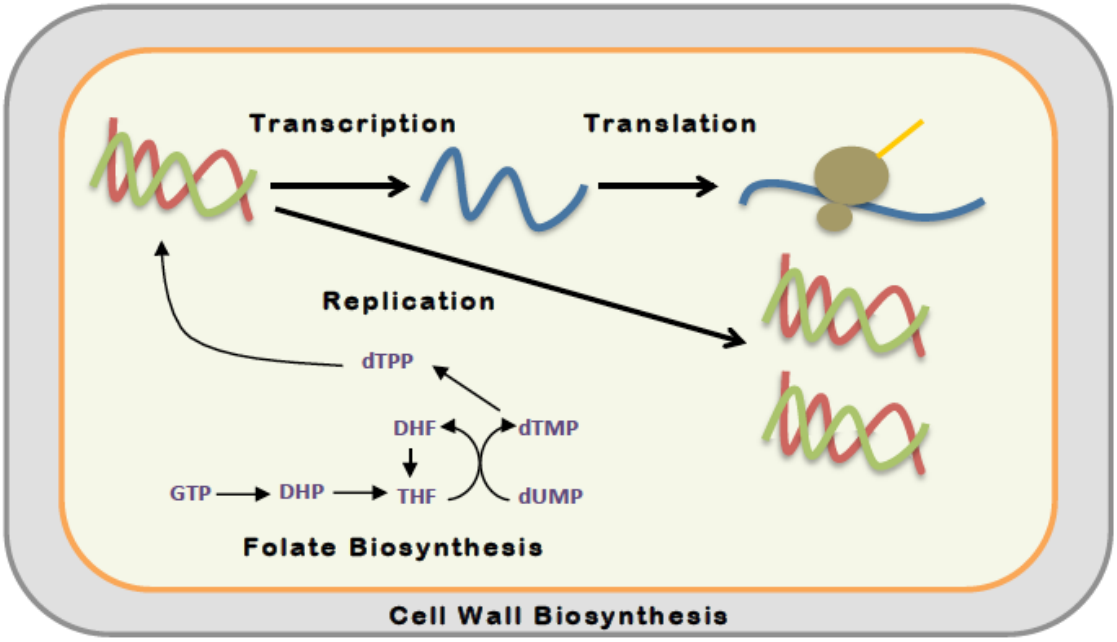


Figure 1-1. Bacterial processes serving as antibiotic targets. Current clinical antibiotics target DNA biosynthesis, RNA biosynthesis, protein translation, cell wall biosynthesis and folate biosynthesis.

1.2 Screening approaches to antibacterial drug discovery

Antibiotic drug discovery has been approached from two principal methodologies: namely, whole-cell and target-based screening. Indeed, it was via the former strategy during the 1940s-1960s that identified the bulk of antibiotic classes, where rather than high-throughput screens of small molecule libraries—which is the currently the norm for whole-cell phenotypic screens—strains of streptomycetes were investigated for their ability to inhibit the growth of a given test organism by monitoring zones of inhibition (Lewis, 2013). However, by the 1970s, the success of this process had waned, where

previously identified compounds continued to be rediscovered (Livermore, 2011). As the following decades yielded dismal returns on antibiotic discovery, the explosion of the genomic era in the 1990s was met with tremendous optimism that a new methodology—specifically, a target-centric approach—would revitalize antibiotic drug discovery. Following the sequence determination of the *Haemophilus influenzae* genome in 1995, sequencing of various pathogens was initiated, essential and conserved genes encoding targets that were absent in mammalian cells were identified, and small-molecule compound libraries were screened against such proteins in a high-throughput fashion (Payne et al., 2007). However, almost a decade later, and after enormous financial investment from various major pharmaceutical companies, not a single antibiotic from such target-based screening endeavors has entered the clinic.

1.2.1 Genomics and whole-cell phenotypic screening

Among the greatest limitations of the target-centric genomic approach is that it does not account for bioactivity of a given compound. Target-based screening selects for compounds that result in protein inhibition during *in vitro* biochemical screens; however, this strategy does not consider the critical requirements that the small molecule of interest also be able to 1) penetrate the cell, and 2) evade both efflux as well as any hydrolytic or modifying mechanisms (Roemer & Boone, 2013). As evidenced by the failure of the numerous target-based screening campaigns during the 1990s (Payne et al., 2007), identifying a small molecule that both inhibits a given target and displays bioactivity is an unlikely feat. Thus, whole-cell screening assays—which intrinsically select for

compounds that are active against bacteria—are being increasingly revisited as the more attractive option to antibiotic drug discovery (Andries et al., 2005; Mak et al., 2012; Wang et al., 2013).

Although a powerful methodology, phenotypic screens are not without their challenges. Specifically, the very reason that such assays were initially abandoned was due to the seeming inability to identify new antibacterial chemical matter (Livermore, 2011). An additional and significant disincentive of whole-cell screening is that, upon identification of a lead compound, the next step is target identification, where linking bioactivity to molecular mechanism is an immensely daunting task (Burdine & Kodadek, 2004). However, with inventive new screening strategies that avoid antibiotic rediscovery, coupled with novel sources of small molecules—such as large synthetic libraries or natural products from underexplored bacterial taxa and ecological niches—there is reason for optimism that new antibiotic compounds await discovery (Wright, 2012).

Ironically, although the advent of genomics has been relatively unsuccessful with respect to target-centric antibiotic screening, the field has brought great advancement to phenotypic screening and compound mechanism of action (MOA) studies. (Barker, Farha, & Brown, 2010; Roemer & Boone, 2013). Examples of such genomic techniques to investigate compound MOA include drug-resistant mutant analysis coupled with whole-genome sequencing (Andries et al., 2005), transcriptional profiling (Gardner, di Bernardo, Lorenz, & Collins, 2003), protein microarrays (MacBeath & Schreiber, 2000), and genome-scale clone sets of non-essential gene deletions (Baba et al., 2006),

overexpression constructs (Kitagawa et al., 2005; Pathania et al., 2009), and promoter-reporter strains (Zaslaver et al., 2006). Thus, incorporation of the coveted features of both target and whole-cell based assays has resulted in mechanism-based screens that simultaneously select for molecules that are bioactive.

1.2.1.1 Chemical genetics and antibacterial screening

In a marriage between genomics and small-molecule probes—termed chemical genetics—much progress has been achieved towards deepening our understanding of genetic networks and potential new antibacterial targets, as well as deciphering compound mechanism of action (Barker et al., 2010). The utility of small molecules—in particular, antibiotics—has largely been centered on their therapeutic application; however, these agents have also been vital in dissecting various aspects of bacterial physiology, gene-gene and chemical-gene interactions (Falconer, Czarny, & Brown, 2011). The use of small molecules as probes of bacterial mutant constructs has led to MOA identification of novel antibacterial compounds (Farha et al., 2013; Pathania et al., 2009); a greater mechanistic understanding of known but poorly described drugs (Nichols et al., 2011); as well as elucidating the means by which non-antibiotic drugs potentiate the action of known antibiotics (Ejim et al., 2011). Thus, genomic technologies coupled with the use of small molecules have been invaluable as a game-changing resource for strengthening our understanding of bacterial genetic networks, whole-cell screening and creating new possibilities for antibacterial drug discovery.

1.2.2 Novel assays that select for new antibacterial targets and new inhibitors

The modern paradigm by which most antibiotic research is conducted is via a 'magic bullet' approach; namely, emphasis is placed on identifying a single compound that targets a single cellular entity that is essential and exclusive to the physiology of the pathogen (Keith, Borisy, & Stockwell, 2005). However, the relentless emergence of pathogens resistant to such monotherapy antibiotics requires that new treatment options be considered, with a multipoint therapeutic approach being one such strategy (Kalan & Wright, 2011). Antibiotic adjuvant therapies can take the form of combinations of antibiotics, synergy between nonantibiotic and antibiotic compounds, as well as molecules that inhibit antibiotic resistance mechanisms, where recent research has focused on screening methodologies to identify such adjuvant compounds.

Traditionally, assays for antibacterial lead compounds have selected for molecules active against a given pathogen or model bacterium under standard laboratory media conditions, which is an environment rich in vitamins, essential metals, amino acids and sugars. However, such a nutrient-rich milieu is vastly different from that of the nutrient-limited environment of a host, for which the physiology of a pathogen must adapt in order to establish infection. Thus, there is a large subset of bacterial genes that are dispensable under laboratory conditions that are essential for both survival in a nutrient-deplete environment (Baba et al., 2006), as well as to cause infection in a host (Clatworthy, Pierson, & Hung, 2007). Thus, these gene products represent a source of currently untapped potential targets for new antibacterials.

1.2.2.1 Targeting nutrient metabolism and homeostasis

In an effort to circumvent one of the greatest challenges associated with current-day screening strategies—in particular, antibiotic rediscovery—researchers recently begun developing new assays that select against the identification of compounds that are lethal in rich-media conditions (where all clinical antibiotics fall into this category), but that lead to growth inhibition within a nutrient-limited environment. An example of one such screen was recently reported by Zlitni *et al*., where the authors identified an inhibitor of each *p*-aminobenzoic acid, biotin, and glycine biosynthesis (Zlitni, Ferruccio, & Brown, 2013). The novelty of the screening conditions, coupled with the unique secondary assays employed to investigate mechanism of action, enabled the authors to identify both new antibacterial chemical matter and antibiotic targets.

An additional example of an essential nutrient that bacteria must actively acquire when challenged with nutrient-replete conditions is that of iron. Under standard screening conditions culture media is rife with iron, hence the metal concentration is ample enough such that it is able to passively diffuse into the cell (Fischbach, Lin, Liu, & Walsh, 2006; Ma, Jacobsen, & Giedroc, 2009); however, when iron is limited, compounds that interfere with the ability to either actively import, or maintain homeostatic equilibrium of the element, can lead to inhibition of bacterial growth. Two recent reports illustrate screening conditions that permitted the identification of compounds that interfere with iron homeostasis (Falconer et al., 2013) and acquisition (Yep, McQuade, Kirchhoff, Larsen, & Mobley, 2014).

1.2.2.2 Screening for inhibitors of bacterial virulence

To be successful as a pathogen, bacteria must be capable of causing disease in a host. The mechanisms by which infection is accomplished is via the production of virulence factors that ultimately lead to damage of the host tissue (Clatworthy et al., 2007). Where antibiotics traditionally function by either destroying bacteria (bactericidal) or halting their growth (bacteriostatic), the concept of antivirulence compounds is distinct from this. Rather than inhibiting functions that are essential for *in vitro* logarithmic growth, such molecules instead disarm bacteria of their virulence determinants rendering them no longer pathogenic and susceptible to clearing by the host immune system (Clatworthy et al., 2007). Cholera toxin (CT) is an example of a virulence factor, which is manufactured by the pathogen, *Vibrio cholera*, and leads to acute vomiting, diarrhea and severe dehydration. To identify small-molecules that prevent production of this toxin, a recent study reported the development of a high-throughput screen that selects for compounds that are non-lethal to bacteria, but that inhibit expression of the virulence regulator, ToxT (Anthouard & DiRita, 2013). Using a whole-cell screening approach, where the strain employed harboured a plasmid containing the *toxT* promoter element upstream of the green fluorescent protein gene (*gfp*), researchers monitored expression of GFP in response to its subjection to a library of small molecules. After a series of secondary assays the authors identified three compounds that led to decreased production of ToxT, where one of the molecules was found to reduce colonization of *V. cholera* during an *in vivo* infection model (Anthouard & DiRita, 2013). Thus, screens for

antivirulence compounds as a potential strategy to treat bacterial infection is an avenue that merits further exploration.

1.2.2.3 Multi-point therapeutic strategies

Multipoint therapy, including combinations of antibiotics, is not a new concept (Borisy et al., 2003); however, it has not been until recently that researchers have begun screening specifically for compounds that potentiate the action of antibiotics that are otherwise ineffective against a given pathogen due to drug resistance mechanisms. In a screen to identify compounds that synergize with the known antibiotic, novobiocin—a drug that, alone, has negligible activity against Gram-negative organisms—researchers identified four compounds that sensitized *E. coli* to novobiocin (Taylor, Rossi, De Pascale, & Wright, 2012). The authors found that each of the compounds led to altered cell morphology, which in turn permitted the accumulation of molecules that would have otherwise been excluded from the cell. Thus, the work showed that perturbation of bacterial cell shape promoted increased sensitivity to antibiotics, suggesting that continued efforts to identify small molecules of this mechanistic class may prove useful as antibiotic adjuvants for the treatment of Gram-negative infections (Taylor et al., 2012).

In a similar study aimed at identifying small molecules capable of sensitizing Gram-negative organisms to antibiotics that are otherwise ineffective, researchers leveraged the historical success of actinomycete extracts, which are typically a rich source of bioactive small molecules (Cox & Wright, 2013). Specifically, in combination with rifampicin—an antibiotic with poor activity against Gram-negative organisms—the

authors screened a collection of fermentation extracts from a number of strains of *Actinomyces*, and identified a natural product that is synergistic with the respective antibiotic. Although the original screen was performed against a non-pathogenic laboratory strain of *E. coli*, the authors showed that the compound combination was also active against the clinical Gram-negative pathogen, *Acinetobacter baumannii*. Thus, this work suggests that screening natural products for antibiotic adjuvants may be another potential avenue to further explore for novel small-molecule therapeutics.

Lastly, our group recently reported an alternative screening approach to search for novel antibiotic adjuvants. Namely, we employed a collection of previously-approved drugs and assayed these compounds in combination with the known antibiotic, minocycline, for activity against the opportunistic pathogen, *Pseudomonas aeruginosa* (Ejim et al., 2011). As resistance mechanisms render minocycline ineffective against *P. aeruginosa*, we vied to identify compounds that could potentiate the action of the antibiotic. The inherent bioactivity of previously-approved drugs, in combination with their known toxicology profiles, suggested that screening such a collection for an alternative therapeutic use could prove to be highly fruitful. Indeed, we identified several drugs that synergized with minocycline, including the known anti-diarrheal medication, loperamide (Imodium®). Further exploration of this particular compound revealed that loperamide not only potentiates the action of minocycline, but that it possesses cryptic antibacterial activity (Ejim et al., 2011). Thus, repurposing known drugs for novel uses holds promise for future studies aimed at identifying new antibacterial therapeutic strategies.

1.3 The relationship between loperamide and our motivation to conduct a screen to identify inhibitors of bacterial iron homeostasis

Our objective to identify small-molecule antibacterial leads that function by inhibiting bacterial iron acquisition and/or homeostasis was inspired by the results of a chemical-genetic screen, where we probed the mechanism of action of loperamide using the Keio genomic library. Specifically, we vied to investigate the intracellular effects of loperamide on *E. coli*; however, due to the inability of the compound to penetrate the bacterial cell, we employed EDTA to permeabilize the outer membrane and, in turn, facilitate entry of loperamide into the cell. As our experimental design required the concurrent presence of EDTA in our screen of loperamide against the Keio collection, it was necessary to identify and eliminate those deletion strains that showed growth sensitization resulting from EDTA alone (Figure 1-2). To our intrigue, we observed that close to half of the deletion strains showing sensitization to EDTA were genes encoding proteins involved in bacterial iron acquisition (Table 1-1) (Falconer et al., 2013). Since EDTA is a strong iron chelator (with a log stability constant of 25.1 and 14.3 for Fe^{3+} and Fe^{2+}, respectively (Liu & Hider, 2002a), we reasoned that EDTA created an iron-limited environment such that bacteria were required to synthesize and secrete siderophores in order to survive; thus, for strains deficient in such processes, EDTA led to growth inhibition. Hence, we saw this as an opportunity to use EDTA to screen for small molecules that perturbed bacterial iron acquisition and/or homeostasis: in other words, we sought to identify chemical-enhancer equivalents of the Keio-EDTA genetic-enhancer screen.

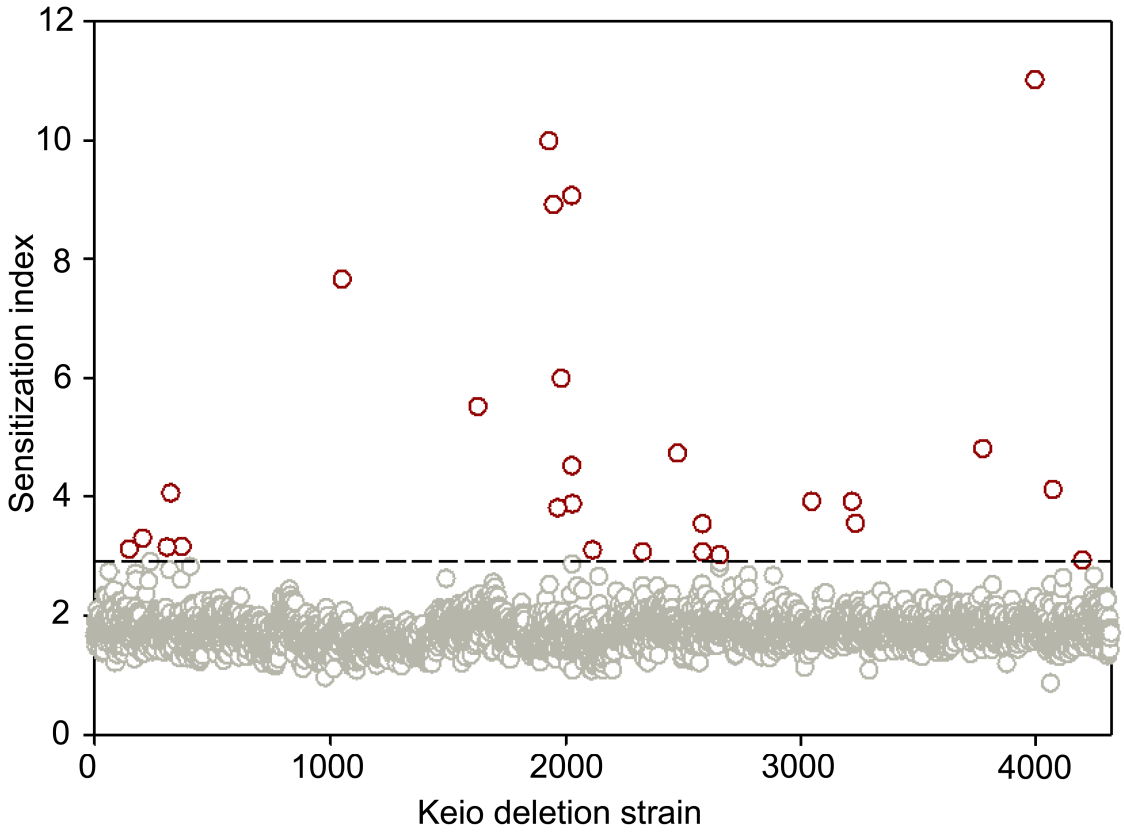


Figure 1-2. Non-essential gene-deletion strains show sensitization to EDTA. *E. coli* gene deletion strains (numbering 4320) were grown aerobically in LB broth in the presence of a subinhibitory concentration of EDTA (0.8 mM) and growth sensitivity of each strain to EDTA was measured and expressed as the sensitization index. Sensitization index refers to the normalized value that results from dividing bacterial growth at $OD_{600\ nm}$ in the absence of EDTA by growth of the same strain in the presence of EDTA. Average growth of *E. coli* grown in the presence of the chelator was approximately half that of bacteria grown in the absence of EDTA. The majority of *E. coli* gene deletion strains revealed similar growth (grey circles), where fewer than 1% of deletion strains showed a statistically significant heightened sensitization to the presence of EDTA (red circles). Hashed black line represents 3σ from the mean.

Table 1. List of all *E. coli* deletion strains sensitive to EDTA, where those strains carrying a deletion in genes involved in iron homeostasis are indicated with an asterisk[a].

Deletion strain	Sensitization index	Gene description
*entA**	11.02	2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase
*fes**	9.99	Enterochelin esterase
*entF**	9.07	Enterochelin synthetase, component F
*aroA**	8.92	3-Enolpyruvylshikimate-5-phosphate synthetase
yfgA	7.66	Transmembrane component of cytoskeleton
*aroC**	5.99	Chorismate synthase
yraP	5.52	Lipoprotein
yibP	4.81	Murein hydrolase
*aroB**	4.73	3-Dehydroquinate synthase
*entC**	4.52	Isochorismate synthetase
*fepG**	4.12	Ferric enterobactin ABC transporter – membrane subunit
ompC	4.06	Outer membrane protein
tatA	3.92	Membrane translocation of periplasmic proteins
tolB	3.92	Tolerance to colicins E2, E, A and K
*entB**	3.88	2,3-Dihydro-2,3-dihydroxybenzoate synthetase
*aroD**	3.81	3-Dedydroquinate dehydratase
*tonB**	3.55	Uptake of chelated Fe and cyanocobalamin; energy transducer
*fepD**	3.54	Ferric enterobactin ABC transporter – membrane subunit
ompR	3.30	Activator protein for osmoregulation of OmpC and OmpF
clpP	3.16	ClpP ATP-dependent protease proteolytic subunit
pal	3.15	Peptidoglycan lipoprotein
gpml	3.12	2,3-bisphosphoglycerate-independent phosphoglycerate mutase
slyB	3.10	Putative lipoprotein
*fepB**	3.07	Ferric enterobactin ABC transporter – periplasmic binding protein
wzxE	3.07	Lipid III flippase
znuC	3.02	High-affinity ABC transport system for zinc
znuA	2.93	High-affinity ABC transport system for zinc

[a] Gene descriptions were identified using Ecocyc and Colibri.

1.4 Bacteria and nutrient iron

Iron is vital for the growth of nearly all known organisms, with the exceptions being *Borrelia burgdorferi* (Posey & Gherardini, 2000), *Lactobacillus plantarum* (Weinberg, 1997) and *Streptomyces suis* (Niven, Ekins, & al-Samaurai, 1999), which bypass this need by having eliminated the majority of genes that encode proteins utilizing iron and, for those few remaining metalloproteins, replacing the required cofactor with manganese instead of iron. Despite that in all other known organisms iron remains essential for a range of biological processes, including respiration, DNA biosynthesis and gene regulation (Andrews, Robinson, & Rodríguez-Quiñones, 2003), much of the iron found in the environment or host organism is difficult to obtain, existing as insoluble ferric oxide/hydroxide complexes or bound to and stored within proteins, respectively (Fischbach et al., 2006). As such, iron concentrations required by microbes to conduct various metabolic processes far exceeds what is typically physiologically available. Specifically, bacteria require an intracellular iron concentration of 10^{-6} M (Raymond, Dertz, & Kim, 2003), though the concentration of ferric iron in both water and in most tissue is approximately 10^{-18} M (Fischbach et al., 2006), which is even further restricted in the human host during infection (Bullen, Rogers, Spalding, & Ward, 2005). Given the essentiality of the nutrient for pathogen survival, processes responsible for acquiring and maintaining iron homeostasis have been suggested as potential antibacterial targets. Indeed, such mechanisms are inherently targeted by the host immune system where, during courses of bacterial infection, the eukaryotic host produces the siderophore-

binding proteins, siderocalin and lipocalin that aid in preventing bacteria from acquiring the iron that is needed for their survival (Cassat & Skaar, 2013).

1.4.1 Bacterial iron acquisition

Siderophores are high-affinity ferric-iron chelators that are synthesized and secreted by microbes into the extracellular milieu under iron-limiting conditions in order to acquire the poorly available, yet essential nutrient, iron (Figure 1-3). Once bound, the siderophore-iron complex is recognized by cognate bacterial receptor proteins and is actively imported into the cell, where ferric iron is then released and made available for use by the bacterium (Barry & Challis, 2009). Enterobactin is perhaps the most widely studied siderophore and is synthesized by the Gram-negative organisms *E. coli*, *Salmonella enterica*, *Klebsiella pneumoniae* and *Shigella dysenteriae* (Fischbach et al., 2006). Following synthesis in the cytoplasm via a series of *ent* gene products, enterobactin is then exported first by the EntS inner membrane protein and then the outer membrane protein, TolC. Due to the siderophore's unparalleled affinity for ferric iron (K_d of 10^{-49} M), enterobactin is capable of successfully competing with, and extracting ferric iron from, virtually any iron chelator. The subsequently formed ferric iron-enterobactin complex is then recognized by the outer membrane porin, FepA, where the energy required for this active transport is provided via a TonB-dependent mechanism (Fischbach et al., 2006). Upon accompaniment through the periplasm by FepB, the complex is transported through the inner membrane by the FepDG protein channel, where ferric iron is released from the siderophore via degradation of enterobactin by the

enzymatic activity of Fes. Expression of all proteins required for enterobactin-mediated iron acquisition is under transcriptional control of the iron-dependent repressor protein, Fur (ferric uptake regulation) (Hantke, 2001).

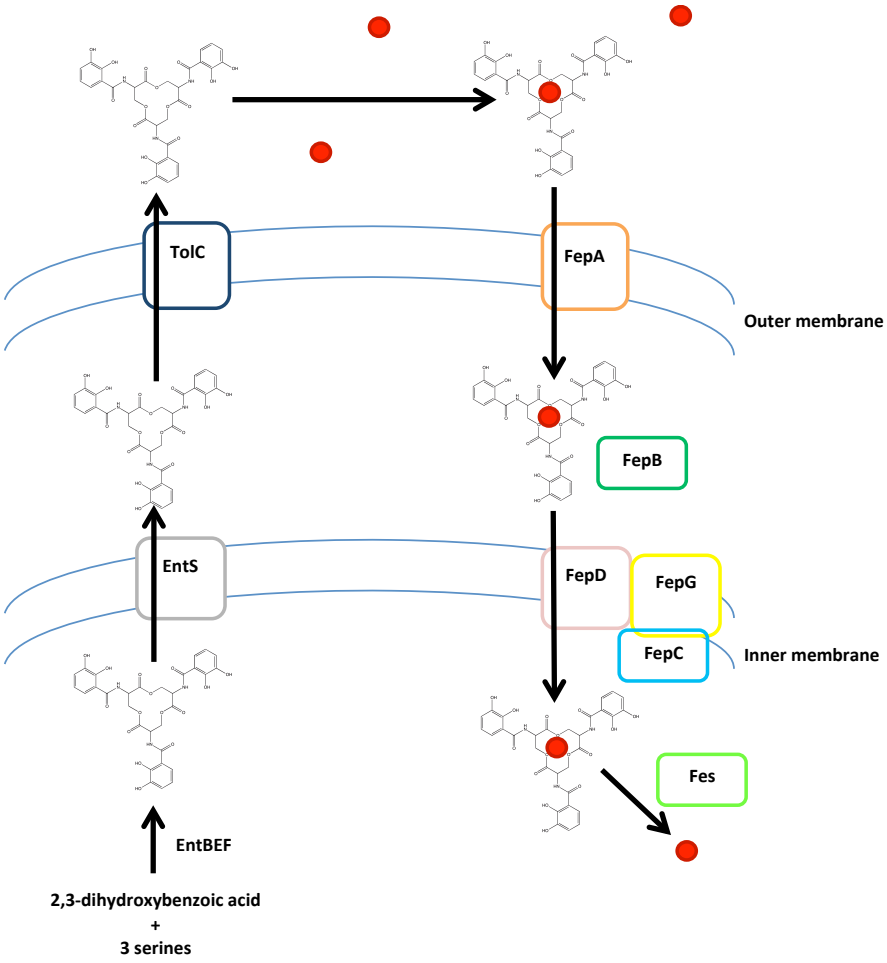


Figure 1-3. Enterobactin-mediated iron acquisition in *E. coli*. Enterobactin is synthesized in the cytosol, from where it is exported through a series of membrane proteins to the environment. Once outside the cell, enterobactin complexes with iron (red circles), which is then actively imported back into the cell. The iron molecule is then released from enterobactin and made available for use by the cell.

1.4.2 Bacterial iron homeostasis

Despite being necessary for growth, iron has long been known to contribute to the formation of reactive oxygen species and toxicity to the cell (Fenton, 1894; Haber & Weiss, 1934). Therefore, in addition to actively acquiring and maintaining an adequate

concentration of iron, bacteria must also engage in mitigating the deleterious effects of the element, which is accomplished via careful regulation of both the intracellular concentration and subcellular location of iron (Cassat & Skaar, 2013). Specifically, when the cytosolic iron concentration exceeds its quota, genes encoding proteins responsible for iron import are transcriptionally repressed, and any free intracellular iron is sequestered by iron-binding proteins, and/or is effluxed from the bacterial cell (Ma et al., 2009). Such careful tuning of the intracellular iron level is largely controlled by the transcriptional regulator, Fur, which is ubiquitous in Gram-negative bacteria and regulates the transcription of over 90 genes (Andrews et al., 2003). As a regulator of iron homeostasis in *E. coli*, Fur senses the intracellular concentration of iron by binding to the ferrous species under conditions where iron is plentiful, which in turn promotes binding of the holoprotein to the cognate DNA operator sequence, thereby halting transcription of downstream genes involved in iron uptake (such as the *ent* genes responsible for enterobactin biosynthesis) (Ma et al., 2009). Upon depletion of intracellular iron stores, the cofactor is released from Fur and, as the apoprotein has little affinity for the operator sequence, Fur is released and transcription of genes required for iron acquisition is resumed (Ma et al., 2009). Given that strict homeostasis of the metal is essential for bacterial viability, any small molecules that interfere with this process—such as intracellular chelators that inhibit the availability of free iron—serve as potential leads for new antibacterial development.

1.4.3 Small molecule inhibitors of bacterial iron acquisition and homeostasis

There exist two main mechanistic classes of compounds that function via the general disruption of bacterial iron acquisition and/or homeostasis: namely, those that inhibit a protein that is required for bacterial iron uptake under iron-limited conditions; and molecules that perturb the homeostatic concentration of the metal via iron chelation (Foley & Simeonov, 2012). In regards to the former, several groups have reported the identification of small molecules that target enzymes involved in siderophore biosynthesis in various pathogenic bacteria, including *Mycobacterium tuberculosis* and *Yersinia pestis* (Ferreras, Ryu, Di Lello, Tan, & Quadri, 2005), and *Staphylococcus aureus* and *Bacillus anthracis* (Tripathi et al., 2014). Although protein targets—in particular, those that are present in prokaryotes but absent in eukaryotes, such as those involved in siderophore biosynthesis—are the more desirable of the two modes of inhibition, identification of a compound that shows efficacy during an *in vivo* animal infection model has yet to be reported. The most parsimonious explanation for this observation is that bacteria possess redundant pathways for iron assimilation; hence, inhibition of one route of iron acquisition may result in up-regulation of a compensatory pathway, in turn voiding the effect of the given inhibitor (Foley & Simeonov, 2012). From this perspective, disruption of iron homeostasis via iron chelation—where such small molecules directly compete with bacteria rather than inhibit only one of many possible routes to acquire iron—carries appeal over a protein-targeted approach. The caveat to employing chelators as an antibacterial therapy is their increased potential to

cause host toxicity, since the essentiality of iron is not exclusive to prokaryotes, but is also required by eukaryotic host cells.

1.4.3.1 The current use and future potential of chelators in medicine

Chelators are generally thought of as small-molecule ligands that bind and remove metal toxins from the body (Franz, 2013). Specifically, those limited chelators approved for current clinical use are employed to sequester and excrete toxic concentrations of either iron, copper or other heavy metals. However, there is increasing research to suggest that chelators may have therapeutic utility beyond metal removal. In particular, cancer (Torti & Torti, 2013), neurodegenerative diseases (Perez & Franz, 2010), as well as metalloprotein-associated disease states (Jacobsen, Fullagar, Miller, & Cohen, 2011) are all under active investigation for treatment options where chelators might be of benefit. Although such compounds have traditionally been employed with the sole objective being to eliminate metals from the body, depending on the chemical features of a given chelator—such as size, lipophilicity and solubility—their biological outcomes can widely differ. Indeed, chelators can facilitate the redistribution of metals by chaperoning them into cells (Treiber et al., 2004), passivate metalloenzymes through chelation of a given metal to disrupt enzyme catalytic function (Jacobsen et al., 2011), as well as generate cytotoxic effects, such as through the formation of a chelate complex that leads to the production of reactive oxygen species and apoptosis of cancer cells (Lovejoy et al., 2011). Thus, given the urgency with which new strategies to combat bacterial infection are needed, combined with preliminary empirical data suggesting the potential

therapeutic use of chelators that goes beyond the removal of heavy metals, such classes of compounds are well worth exploring in antibacterial development.

1.5 Gram-negative pathogenic bacteria and antibiotic resistance

In a recent report by the United States Centers for Disease Control and Prevention, Gram-negative pathogens were indicated as posing the greatest challenge in our efforts to treat bacterial infections (Centers for Disease Control and Prevention (CDC), 2013). Although extensive antibiotic resistance also applies to certain Gram-positive pathogens, treatment options are not as limited as they are for Gram-negative bacteria. In particular, two new antibiotic classes to reach the clinic in the past half-century—namely, daptomycin and linezolid—target serious Gram-positive organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA); however, these therapeutics have no effect against Gram-negative bacteria (Bush, 2010). Indeed, in a recent study of 13,796 patients in 1265 intensive care units from 75 countries, Gram-positive bacteria were present in 47% of patients with bacterial infections, where Gram-negative bacteria were found to be more prevalent and in 62% of patients (Vincent et al., 2009).

1.5.1 β-Lactamase resistance mechanisms

In addition to their outer membrane, which precludes the entry of many small molecules, the heightened antibiotic resistance exhibited by Gram-negative pathogens can also be attributed to the impressive ease by which such organisms acquire resistance genes (Livermore, 2012). Indeed, among the classes of antibiotics against which

resistance is most prominent are the β-lactams, where inactivation of the antibiotic via β-lactamase-mediated hydrolysis is the common form of β-lactam resistance in Gram-negative pathogens (Bush, 2010). β-Lactams are a class of structurally diverse antibiotics that, despite their decades of use, continue to play a pivotal role in treating bacterial infections. However, concomitant with such heavy clinical application has been the development of resistance mechanisms on behalf of pathogens. There are four major structural types of β-lactamases: class A, C and D enzymes, which utilize serine to accomplish antibiotic cleavage; and class B metalloenzymes, where zinc is required for activity (Bush & Jacoby, 2010). Classes A and D represent the largest number of β-lactamases and include the penicillinases (which encompass a relatively limited spectrum of activity) and the extended-spectrum β-lactamases (ESBLs) that, in addition to the ability to hydrolyze penicillins and cephalosporins, also inactivate extended-spectrum cephalosporins, cefotaxime and ceftazidime (Bush & Jacoby, 2010). Class C enzymes consist of cephalosporinases, which are often chromosomally encoded in many Gram-negative pathogens and, in contrast to the ESBLs, are not susceptible to the β-lactamase inhibitor, clavulanic acid (Bush, 2010). Lastly, the Class B metallo-β-lactamases (MBLs)—which are also referred to as the carbapenemases—are considered to be the most worrisome β-lactamase as they 1) hydrolyze nearly all β-lactam antibiotics (with the exception of aztreonam), 2) are not sensitive to the presence of clavulanic acid, and 3) are often found on plasmids that co-express other β-lactamases, including the ESBLs, which are capable of aztreonam inactivation.

1.5.1.1 Metallo-β-lactamases

The carbapenem antibiotics (meropenem, imipenem, doripenem and ertapenem) were the last β-lactams to be developed to retain activity against nearly all Gram-negative bacteria, and have been invaluable in remedying infections that could otherwise be untreated (Nordmann, Dortet, & Poirel, 2012; Nordmann, Poirel, Walsh, & Livermore, 2011). However, emergence of the three major classes of MBL enzymes—namely, the imipenem resistant (IMP), Verona integrin-encoded (VIM) and New Delhi metallo-β-lactamase (NDM-1)—and the subsequent spread of such resistance factors have resulted in pathogenic bacteria that are recalcitrant to such last-resort antibiotics (Worthington, Bunders, Reed, & Melander, 2012).

1.5.1.2 New Delhi-1-type metallo-β-lactamase

The New Delhi metallo-β-lactamase type-1 (NDM-1) gene, bla(NDM-1), was initially identified in 2008 from a strain of *Klebsiella pneumoniae* isolated from an individual who had acquired a urinary tract infection while in India (Yong et al., 2009). NDM-1 is the most recent MBL to be discovered and, although expression of the enzyme has not been shown to result in heightened virulence in comparison to other MBLs, the tremendous concern regarding the NDM-1-harbouring plasmids is both the rapidity and ease by which the gene is transferred, as well as their propensity to co-harbour an array of other antibiotic resistance determinants (Nordmann et al., 2011). Thus, for individuals infected with NDM-1-containting pathogens, treatment options are extremely limited, and include the use of antibiotics with significant toxicity, such as colistin. NDM-1 enzymes

have been found predominantly in isolates of Enterobacteriaceae—specifically, *K. pneumoniae* and *E. coli*—where such pathogenic strains have been isolated from disease-causing states as varied as urinary tract infections, pulmonary infections, soft-tissue infections and septicemia (Kumarasamy et al., 2010; Nordmann et al., 2011).

1.5.1.3 *Klebsiella pneumoniae* and antibiotic resistance

The threat of infection by *K. pneumoniae* has become of increasing clinical significance, as the pathogen is now the most commonly observed nosocomial infection caused by Gram-negative bacteria (Worthington et al., 2012). Infection resulting from *K. pneumoniae* was once successfully treated using carbapenem antibiotics; however, for more recent isolates harbouring NDM-1, treatment via such last-resort antibiotics is ineffective, where resistant strains of *K. pneumoniae* have been observed for virtually every antibiotic. Indeed, in 2013 in the United States alone, carbapenem-resistant strains of *Klebsiella* were estimated to cause 7,900 infections, where 520 of these resulted in death (Centers for Disease Control and Prevention (CDC), 2013). Thus, identification of new therapeutic avenues to treat such pathogens is desperately needed.

1.6 Research objectives and organization of thesis

My PhD work focuses on the hypotheses that: 1) bacterial iron homeostasis is an essential process that should be explored as an antibiotic target; and 2) zinc chelation is a potential therapeutic strategy that re-sensitizes NDM-1-producing pathogenic bacteria to carbapenem antibiotics. To test these hypotheses I employed chemical-genomic strategies

to each: aid in the design and validation of a novel high-throughput screening assay to select for small-molecule perturbants of bacterial iron homeostasis; and to verify that zinc chelation is the mechanism by which an unknown compound synergizes with carbapenem-class antibiotics against an NDM-1-harbouring strain of *K. pneumonieae*. Chapter 2 describes the use of a non-essential gene-deletion library to validate the sensitivity of iron-limited conditions to detect small-molecule inhibitors of iron homeostasis in a screen of ~30,000 small molecules against *E. coli*. This screen led to the identification of two spiro-indoline-thiadiazole analogs that inhibit bacterial growth via intracellular iron chelation. We showed that these are bistable compounds and that in only one of their two isomeric states do the compounds exhibit antibacterial activity. In particular, we show that the presence of divalent transition metals induce isomerization from the closed spiro to an open merocyanine form, where it is the latter isomeric form that exhibits antibacterial activity. Although such metal-mediated isomerization and subsequent chelation is not specific to ferrous iron under *in vitro* conditions, we show that antibacterial activity of the analogs is specific to their chelation of bacterial intracellular iron. Chapter 3 explores additional spiro-indoline-thiadiazole analogs in an attempt to identify compounds that, rather than iron, are specific to zinc chelation *in vivo*. As zinc is required for metallo-β-lactamase activity, we sought to determine whether we might identify any analogs that re-sensitive an otherwise antibiotic-resistant strain of NDM-1-producing *K. pneumoniae* to carbapenem antibiotics. In this work I discuss our identification of a series of analogs whose antibacterial activity is suppressed by zinc, but not iron. We then show that such analogs potentiate the action of carbapenems against a

clinical isolate of *K. pneumoniae* via isomerization to a merocyanine form and subsequent zinc chelation. Chapter 4 discusses potential structural explanations for the observed differences between the various analogs and their respective *in vivo* metal specificities, as well as future experiments that are planned to aid in determining the possible suitability of a metal chelator for therapeutic use.

CHAPTER 2 – SMALL-MOLECULE PERTURBATION OF IRON HOMEOSTASIS IS GROWTH INHIBITORY TO *E. COLI*

2.1 Preface

This chapter was adapted from the following publication:

Shannon B. Falconer, Wenliang Wang, Sebastian S. Gehrke, Jessica D. Cuneo, James F. Britten, Gerard D. Wright, and Eric D. Brown. Metal-induced isomerization yields an intracellular chelator that disrupts bacterial iron homeostasis. Chemistry and Biology. 2014. 21(1):136-145.

All experiments in this chapter were performed by myself with the exception of the compound crystallization and crystal structure determination, which were performed by Wenliang Wang and James F. Britten, respectively. Sebastian S. Gehrke assisted me with determining the stability constants between the ligand-metal complexes, and Jessica D. Cuneo provided technical assistance with the chemical-chemical interaction experiments. The manuscript was written by myself and edits were provided by Gerard D. Wright and Eric D. Brown.

2.2 Summary

The dwindling supply of antibiotics that remain effective against drug-resistant bacterial pathogens has precipitated efforts to identify new compounds that inhibit bacterial growth using novel mechanisms of action. Herein we report both 1) a high-throughput screening methodology designed to discover chemical perturbants of the essential, yet under-exploited, process of bacterial iron homeostasis and 2) our findings from a small-molecule screen of more than 30,000 diverse small molecules that led to the identification and characterization of two novel spiro-indoline-thiadiazoles that disrupt iron homeostasis in bacteria. We show that these compounds are unique intracellular chelators with the capacity to exist in two isomeric states. Notably, these spiroheterocyles undergo a transition to an open merocyanine chelating form with antibacterial activity that is specifically induced in the presence of its transition-metal target.

2.3 Introduction

The role of iron in sustaining life bears a complexity that is unique among micronutrients. Although essential for the survival of virtually all organisms, under aerobic conditions iron has highly limited bioavailability, where it is insoluble at neutral pH and is tightly bound by proteins in vertebrate hosts (Fischbach et al., 2006). As such, biology has elaborated important systems to acquire iron and, in the case of bacterial infection, there is an intense competition between the host and pathogen for this nutrient. Indeed, documentation exemplifying the struggle between host and pathogen dates back to the 1940s, where an iron-sequestering agent in human blood plasma (now known to be

transferrin) was found to inhibit bacterial growth (Schade & Caroline, 1946). Humans maintain levels of free iron that are on the stringent order of 10^{-18} M in most tissues—a concentration that is even further reduced during infection (Bullen et al., 2005). Such a strategy on behalf of the host to deny essential nutrients to the invading pathogen is referred to as nutritional immunity (Hood & Skaar, 2012), where numerous studies support that interfering with this host defense mechanism by increasing the amounts of available iron during infection results in increased bacterial virulence for a number of different pathogens both in the laboratory (Bullen, Leigh, & Rogers, 1968; Forsberg & Bullen, 1972; Wright, Simpson, & Oliver, 1981) and during human cohort studies (Murray, Murray, Murray, & Murray, 1978; Sazawal et al., 2006).

Since the requirement for iron far exceeds concentrations that are typically biologically available, bacteria produce high-affinity chelating agents known as siderophores, where pathogens carrying mutations in genes responsible for siderophore biosynthesis have been found to display attenuated virulence in animal-infection models (Crouch, Castor, Karlinsey, Kalhorn, & Fang, 2008; Dale, Doherty-Kirby, Lajoie, & Heinrichs, 2004; Meyer, Neely, Stintzi, Georges, & Holder, 1996). Thus, strategies aimed at interfering with bacterial iron homeostasis through either the application of synthetic chelators or by interfering with siderophore-mediated iron acquisition are widely regarded as having high potential as therapeutic interventions for bacterial infection (Foley & Simeonov, 2012). Nevertheless, there are currently no antibacterial therapies targeting iron homeostasis in bacteria.

The widespread emergence of drug-resistant bacterial infections is commonly attributed to a lack of innovation in modern antibacterial drug discovery and a consequent decline in the development pipeline for new antibiotics. In the work reported here we describe a new approach to screen for chemical perturbants of bacterial iron assimilation and report on the discovery of two novel and structurally related antibacterial chemicals that interfere with iron homeostasis in *Escherichia coli*. Remarkably, these compounds are bistable, spiro-indoline-thiadiazoles that are selectively triggered by transition metals to undergo a switch from a closed spiro to open merocyanine isomer capable of intracellular metal chelation.

2.4 Results

2.4.1 Systems approach validates iron-sensitive conditions

Prior to initiating our high-throughput chemical screen we first established conditions for which small-molecule inhibitors of iron homeostasis could be detected. The strategy was to make *E. coli* susceptible to such perturbants with a sub-lethal concentration of the extracellular metal chelator EDTA. Using an ordered *E. coli* gene-deletion collection of some 4,320 strains (Baba et al., 2006), we established that an otherwise sub-inhibitory concentration of EDTA (0.8 mM) could profoundly perturb the growth of strains with deletions in genes involved in iron homeostasis. Indeed, 27 strains showed significantly reduced growth at this concentration of EDTA, where 13 of those strains contained deletions in genes involved in iron assimilation (Table 1-1) (Keseler et al., 2013; Medigue, Viari, Henaut, & Danchin, 1993).

2.4.2 High-throughput chemical screen identifies novel spiro-indoline-thiadiazole inhibitors of *E. coli* iron homeostasis

Having demonstrated through our validation screen of EDTA against the non-essential gene-deletion collection the ability to detect iron-sensitive targets under such iron-deplete conditions, we conducted a high-throughput screen of *E. coli* BW25113 against 30,880 compounds in the presence of EDTA and identified 280 compounds that were growth inhibitory (Figure 2-1, Figure 2-2). The primary screening data showed good reproducibility along with high quality Z'-factor statistical scores of 0.81 and 0.82, for replicates 1 and 2, respectively (Zhang, Chung, & Oldenburg, 1999).

As we were only interested in selecting compounds that inhibited bacterial growth in an iron-limited environment (specifically, in the presence of EDTA), we further narrowed this list of molecules by eliminating compounds that were also growth inhibitory in the absence of EDTA, yielding 136 compounds. EDTA has been shown to adversely affect the integrity of the outer membrane of Gram-negative organisms leading to sensitization towards toxic compounds otherwise unable to penetrate the cell (Ejim et al., 2011). To select against compounds that inhibited the growth of *E. coli* due to the membrane-compromising, versus the iron-limiting, effects of EDTA, we used a hyperpermeable *E. coli* strain (MC1061) (Casadaban & Cohen, 1980; Li et al., 2004) against which to test the active molecules. Specifically, to identify those compounds that resulted in growth inhibition due to the membrane-permeabilizing conditions created by EDTA, we tested the 136 compounds against the genetically hyperpermeable strain, MC1061, with the rationale that any compounds producing a growth-inhibitory effect when grown under metal-replete conditions would not likely be involved in disrupting

iron homeostasis. From this assay we determined that the majority of the 136 molecules showed activity against MC1061 under metal-replete conditions; hence, we eliminated those compounds from further study and focused on the remaining 13 molecules, which were not active against MC1061 in LB media, yet showed activity against BW25113 in the presence of EDTA in our primary screen. Next, to further avoid any confounding effects resulting from the use of EDTA, we conducted assays in minimal media treated with the transition-metal-chelating resin Chelex 100, where we verified that 6 of the remaining 13 compounds were growth inhibitory to the wild-type strain under the respective metal-deplete conditions. Lastly, we investigated whether activity from the 6 compounds could be suppressed by added iron or apo-enterobactin and found that growth inhibition was reversed in the presence of each iron source for 3 compounds (Figure 2-3).

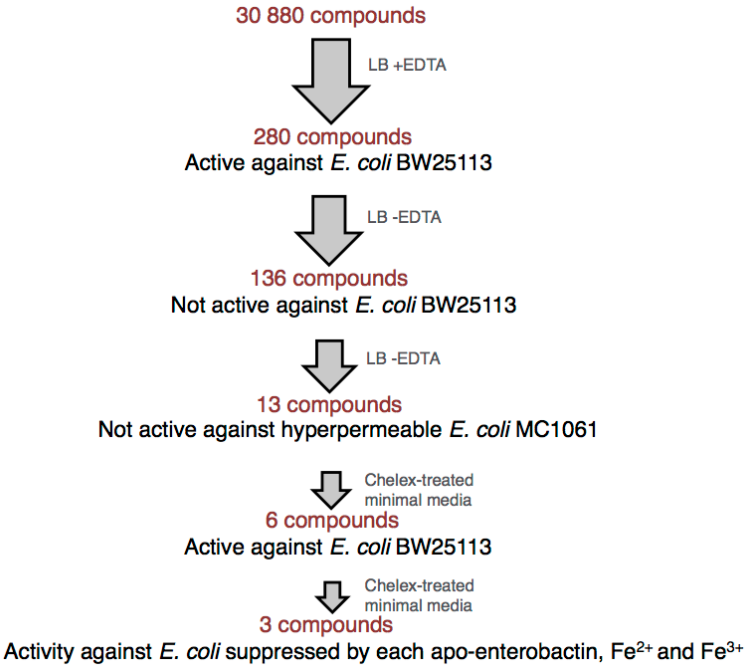


Figure 2-1. Strategy to identify inhibitors of bacterial iron homeostasis.

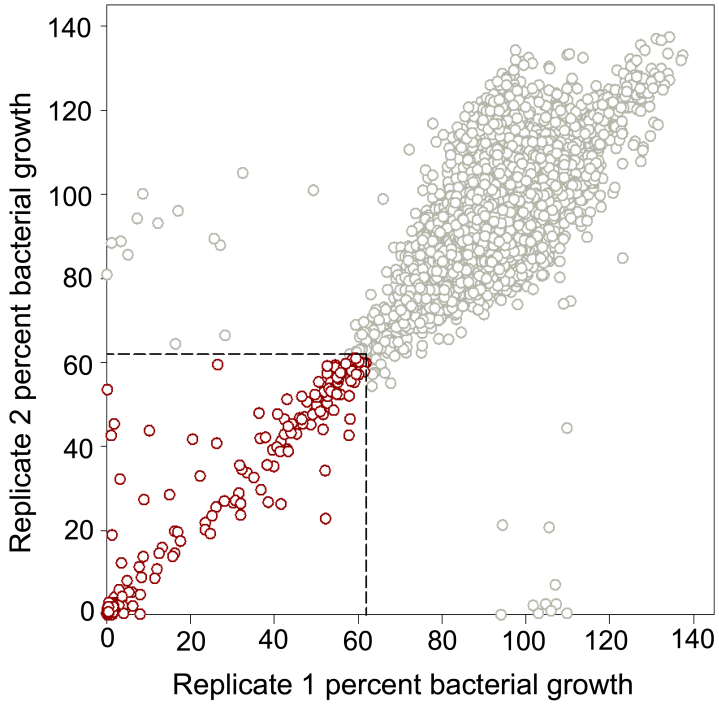


Figure 2-2. Replicate plot of 30,880 compounds screened against *E. coli* in the presence of a subinhibitory concentration of EDTA (0.8 mM). Bacteria were grown aerobically for 18 hours in LB media. Dashed lines represent 3σ below the average growth of bacteria against all compounds, where molecules resulting in growth inhibition are represented by red circles.

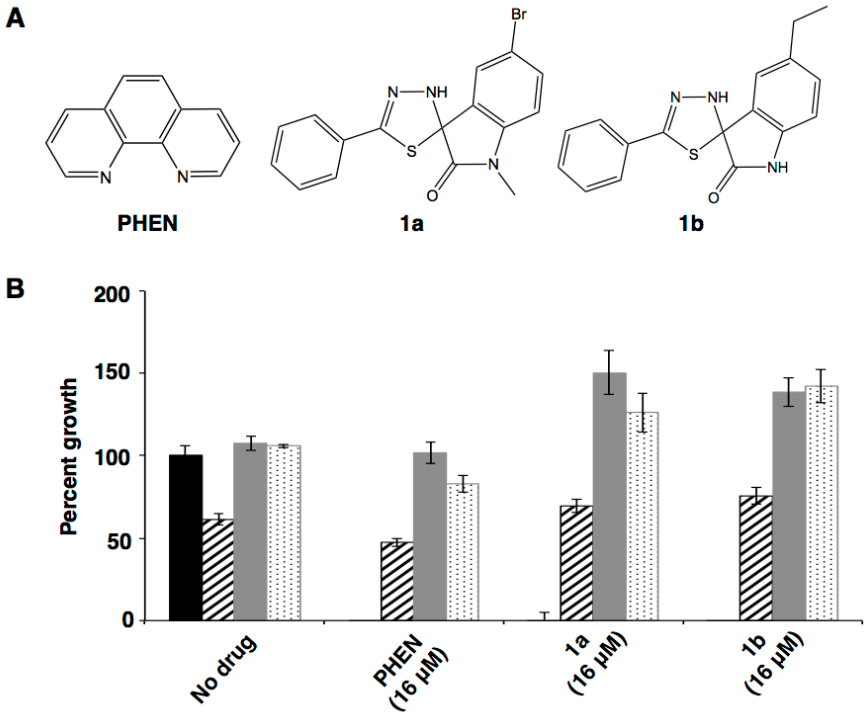


Figure 2-3. Activity of compounds is suppressed by each apo-enterobactin, ferrous chloride, and ferric chloride. (A) Chemical structures of active molecules (1,10-phenanthroline abbreviated to PHEN). (B) Growth of *E. coli* (black bar) in Chelex 100-treated minimal media was inhibited in the presence of active compounds. Lethal activity of the respective drugs was suppressed by the addition of 16 μM of each apo-enterobactin (diagonal lined bar), ferrous chloride (gray bar), and ferric chloride (dotted bar). Bar graphs represent the mean and ±SD for three samples.

Among the active compounds identified was the known intracellular iron chelator, 1,10-phenanthroline, providing an internal positive control for the screen's sensitivity to detect perturbants of iron homeostasis; in addition to 2 spiro-indoline-thiadiazole analogs, 5-bromo-1-methyl-5'-phenyl-3'*H*-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one (compound **1a**) and 5-ethyl-5'-phenyl-3'*H*-spiro[indoline-3,2'-[1,3,4]thiadiazol]-2-one (compound **1b**). To confirm that our assay conditions specifically identified compounds

involved in perturbing the homeostasis of iron versus that of another biological metal, we employed the known intracellular zinc chelator, N,N,N'N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN)—which binds both iron and zinc with log stability constants of 14.61 and 15.58, respectively (Anderegg, Hubmann, Podder, & Wenk, 1977)—and found that growth inhibition of *E. coli* by TPEN could not be suppressed by the addition of apo-enterobactin (data not shown).

2.4.3 Compound *1a* is a specific chelator of divalent transition metals and is selective for Fe^{2+} over Fe^{3+}

To determine whether the spiro-indoline-thiadiazole analogs acted in a similar fashion to 1,10-phenanthroline through metal chelation, we conducted growth-suppression assays using various metals. Although 1,10-phenanthroline primarily exerts its intracellular effects on the chelation of Fe^{2+}, the chelator is also capable of binding a range of other transition metals. Thus, we reasoned that if antibacterial activity of **1a** and **1b** could be suppressed by metals other than iron, in particular by transition metals that are known to form greater relative stabilities when coordinated with a ligand, such as described by the Irving-Williams series (Irving & Williams, 1953), then chelation rather than inhibition of a protein target would be a compelling hypothesis for compound mode of action. We found that, like that for 1,10-phenanthroline, addition of a transition metal other than iron could achieve full suppression of activity of each **1a** and **1b**; however, supplementation with alkaline earth metals, Ca^{2+} and Mg^{2+}, resulted in suppression that was 10 percent or less (Figure 2-4). Together, these investigations suggested that the

mode of action of these analogs was due to metal chelation where the specificity was for transition metals.

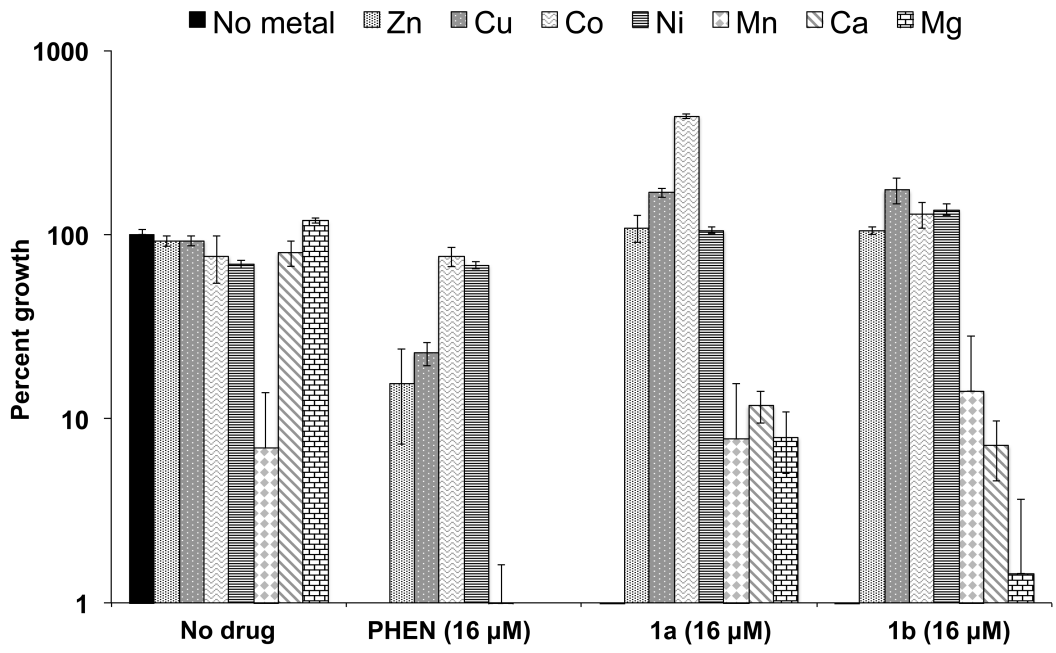


Figure 2-4. Antibacterial activity of spiro-indoline-thiadiazole analogues is specifically suppressed by transition metals. Growth inhibition of *E. coli* in Chelex-treated M9 minimal media by each 1a and 1b was suppressed by transition metals (16 µM) other than iron; however, alkaline earth metals $MgCl_2$ and $CaCl_2$ (16 µM) were unable to suppress compound activity of 1a and 1b by more than approximately 10%. Activity of the intracellular iron chelator, 1,10-phenanthroline (abbreviated to PHEN) is similarly ablated by transition metals except $MnCl_2$, and is not suppressed by $MgCl_2$ and $CaCl_2$. Bar graphs represent the mean and ± SD for triplicate samples.

An observation during the suppression experiments was that the addition of certain transition metals to each **1a** and **1b** resulted in an immediate bathochromic shift. Although the active compounds have not been previously reported to display inducible chromophoric behaviour, similar classes of stimuli-triggered chromophoric compounds are the spiropyrans and the spirooxazines.

Based on availability for resupply, we chose **1a** as the representative analog to explore whether metal complexation resulted in compound isomerization and colour change. To accomplish this we employed visible region spectrophotometry and showed that where **1a** alone absorbs in the 420 nm region, increasing concentrations of Fe^{2+} resulted in a dramatic redshift in absorption with a λ_{max} of 501 nm (Figure 2-5). The corresponding titration indicated a stoichiometry of two molecules of **1a** with one Fe^{2+} (Figure 2-5, inset). Using the Benesi-Hildebrand method (Benesi, 1949) we determined the stability constant between **1a** and Fe^{2+} to be $10^{14.3}$ M^{-2}. Interestingly, probing the spectrophotometric behaviour of **1a** exposed to other metals revealed a redshift to the 450-550 nm range upon the addition of each Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+} and Mn^{2+} (data not shown), where we determined the stability constants between **1a** and each metal to be $10^{13.8}$ M^{-2}, $10^{13.3}$ M^{-2}, $10^{13.7}$ M^{-2}, $10^{13.9}$ M^{-2} and $10^{14.5}$ M^{-2}, respectively. However, we were unable to detect a change in absorbance of **1a** when in the presence of Fe^{3+}, Ca^{2+} or Mg^{2+}.

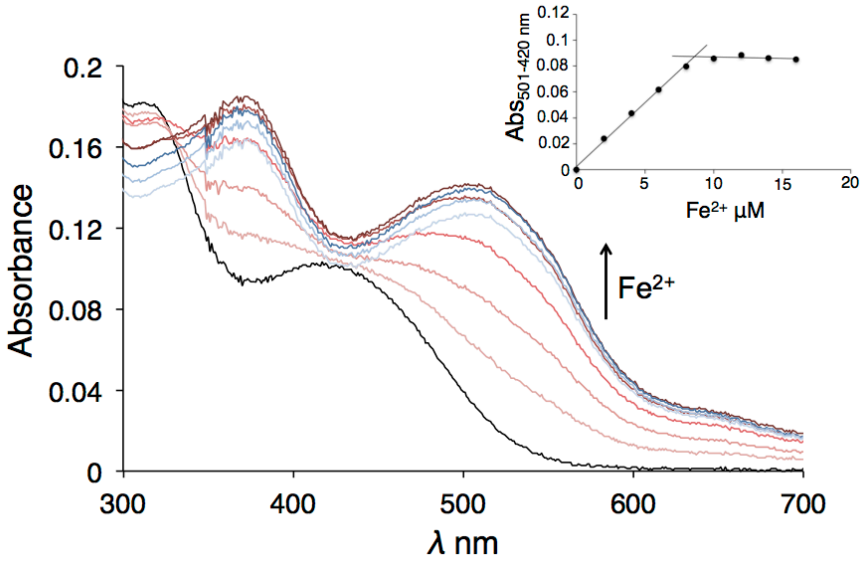


Figure 2-5. Compound 1a binds ferrous iron. Compound 1a undergoes a bathochromic shift in the presence of Fe^{2+}. Visible absorbance spectroscopy of 20 µM compound 1a (black line) with increasing concentrations of Fe^{2+} from 2 µM (light red) to 10 µM (dark red). Concentrations of Fe^{2+} from 12 µM (dark blue) to 16 µM (light blue) resulted in precipitation of the ligand-Fe^{2+} complex. Inset: titration of compound 1a at lmax 501 nm with increasing Fe^{2+} concentrations. To account for the precipitate formed after the addition of 10 µM Fe^{2+}, the titration curve was generated by plotting Fe^{2+} concentration against the differential absorbance (A501 nm – A420 nm).

2.4.4 X-ray crystallography reveals binding nature of ligand-metal complex

To characterize the means by which **1a** chelates transition metals we conducted x-ray single crystal analysis. Maroon crystals of **1a**-Fe^{2+} and **1a**-Zn^{2+} complexes were obtained by slow evaporation in a methanol-chloroform (1:1, v/v) solution. Single crystals were isolated and used for slow diffraction data collections. Structure analyses gave similar molecular structures for **1a** when complexed to either Fe^{2+} (Figure 2-6) or Zn^{2+} (data now shown). In contrast to the closed structure, we observed that when bound to Fe^{2+} or Zn^{2+}, **1a** displayed an open merocyanine isomer generated via heterocyclic

cleavage through the tetrahedral spirocarbon. ^{1}H and ^{13}C NMR spectroscopy of **1a** in the absence of metal confirmed an intact C_{spiro}—S bond (data not shown), indicating that **1a** exists in the closed isomeric state under conditions where transition metals are absent. Due to the paramagnetic properties of iron we used $ZnCl_2$ to show that addition of the transition metal to **1a** resulted in a reduced intensity of the spiro signal and the appearance of an imine signal, consistent with the formation of the **1a** merocyanine isomer. Thus, isomerization of **1a** from the closed spiro to open merocyanine form is induced by the transition metal that it binds to (Figure 2-7).

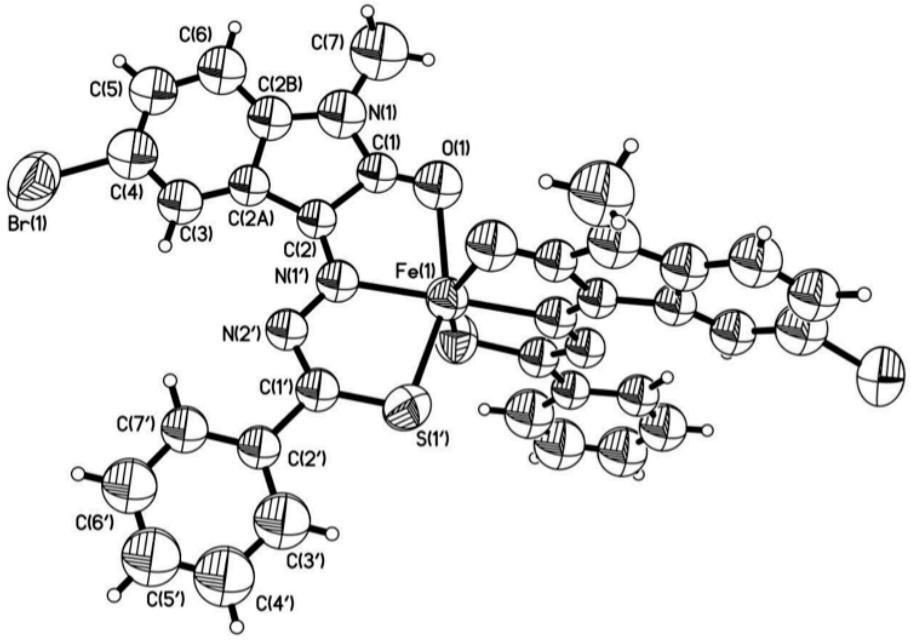


Figure 2-6. Single crystal structure of compound 1a-Fe^{2+}. Complex Ellipsoids are at 50% probability and spheres representing hydrogen atoms are of arbitrary size. The structure of the **1a**-Fe^{2+} complex is deposited in Cambridge Crystallographic Data Centre, registry number 931217.

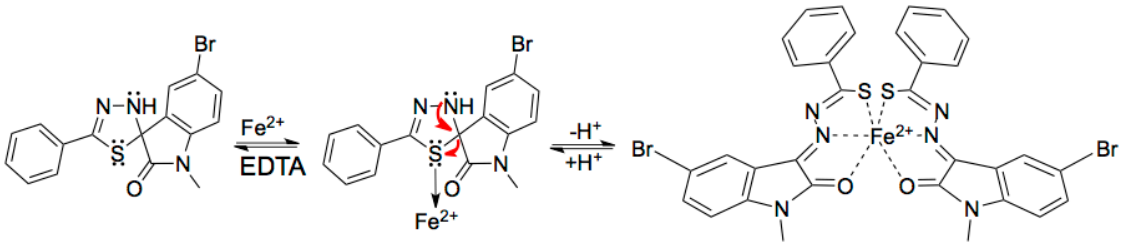


Figure 2-7. Proposed mechanism of Fe^{2+}- induced isomerization of compound 1a to the open merocyanine form.

To investigate the potential reversibility of **1a** between its open and closed isomers we demonstrated via NMR spectroscopy analysis that the addition of EDTA (which has a reported log stability constant for Zn^{2+} of 16.5 (Liu & Hider, 2002a) to the **1a**-Zn^{2+} complex resulted in removal of the metal from the merocyanine ligand and isomerization of **1a** back to its closed spiro form (data not shown).

2.4.5 The merocyanine isomer of *1a* is responsible for antibacterial activity

The finding that **1a** could exist in one of two distinct chemical states prompted us to question whether its antibacterial activity was the result of the closed or open spiro isomer. To address this we tested two chemical analogs of **1a**: compound **1c**, a spiro-indole-thiadiazole analog that lacks the bromine and methyl substituents of **1a**; and compound **1d**, a spiro-indole-diazole, which differs from **1c** by replacement of the metal-coordinating sulphur with a methylene substituent (Figure 2-8a). We hypothesized that if chelation was responsible for the mechanism of action then substituting sulphur with carbon should abolish compound opening and, in turn, antibacterial activity. Growth inhibition assays of *E. coli* grown in Chelex 100-treated minimal media revealed an MIC

of 32 μM and >128 μM (limited by compound solubility) for **1c** and **1d**, respectively, versus the MICs of 16 μM for each **1a** and **1b**. Further, upon addition of Fe^{2+} to each **1c** and **1d** in a 1:2 ratio, absorbance spectroscopy detected a redshift in absorbance with a λ_{max} at 490 nm for the former derivative. In contrast, the latter had a featureless visible spectrum that was unaffected by addition of Fe^{2+} (Figure 2-8b). Since **1d** lacked a visible spectrum, we sought to confirm our conclusion regarding the inability of **1d** to bind Fe^{2+} by performing a competition assay between **1d** and the known iron-binding agent, ferrozine, where the latter produces a visible spectrum with a λ_{max} of 562 nm upon complexation with Fe^{2+} (Stookey, 1970). We showed that pre-incubation of Fe^{2+} with **1d** followed by treatment with ferrozine resulted in formation of the Fe^{2+}-ferrozine complex, indicating that **1d** was unable to compete with ferrozine for Fe^{2+}, further demonstrating that **1d** is incapable of chelating Fe^{2+} (Figure 2-8c). Together, these results support a model where the sulphur residue of **1c** is required for compound activity such that, in the presence of transition metals, the spiroheterocycle undergoes isomerization to the open metal-bound form, where chelation is the mechanism of antibacterial action of **1a**.

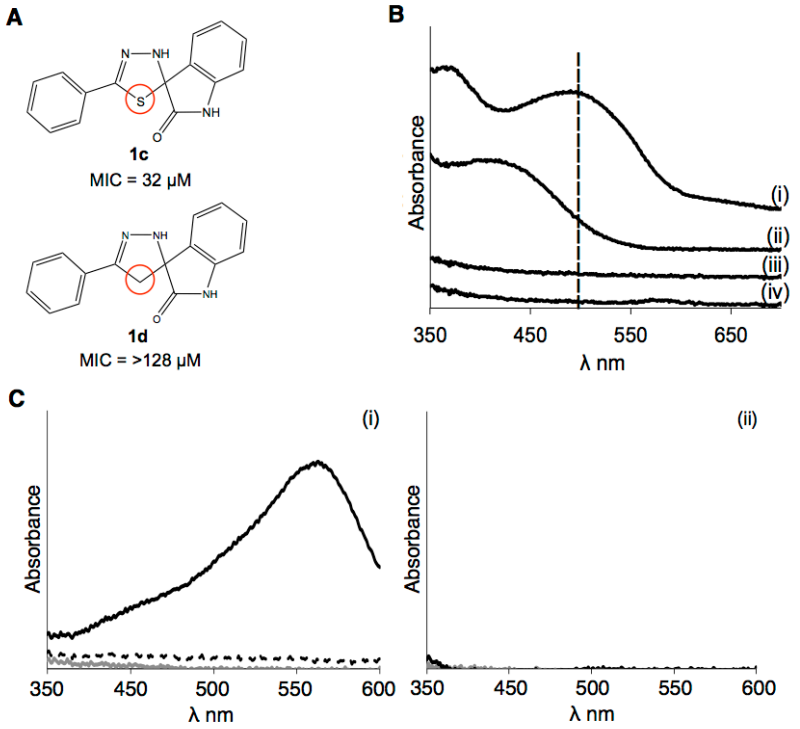


Figure 2-8. The merocyanine form of 1a is responsible for antibacterial activity. (A) Chemical structure of compounds **1c** and **1d**. (B) Vertical stacking of the visible spectra of 20 mM compound **1c** with 10 mM Fe^{2+} (i); 20 mM compound **1c** alone (ii); 20 mM compound **1d** with 10 mM Fe^{2+} (iii); 20 mM compound **1d** alone (iv). Hashed line indicates lmax of compound 1c with Fe^{2+} at 490 nm. Samples were assayed in Chelex 100-treated HEPES (10 mM, pH 7.4). (C) Compound **1d** (40 mM, hashed black line) was incubated for 10 min at room temperature with ferrous iron solution (30 mM, solid gray line) (10 mM FeSO4 stock in 0.5N HCl) in buffer (8 mM hydroxyl amine, 75 mM ammonium acetate, pH 9.5). Following incubation of **1d** and FeSO4, ferrozine (40 mM) was then added to the reaction mixture, where spectra monitoring formation of the ironferrozine complex (solid black line) were collected between 600 and 400 nm (i). In a control experiment to show that preincubation of iron with a bona fide iron chelator prevents subsequent formation of the iron-ferrozine complex (solid black line), EDTA (40 mM, hashed black line) was preincubated with ferrous iron solution (30 mM, solid gray line) for 10 min at room temperature. In contrast to (i) the addition of ferrozine (40 mM) to EDTA resulted in a featureless visible spectrum (ii).

2.4.6 Antibacterial activity of *1a* occurs intracellularly

To ascertain whether chelation and, in turn, growth inhibition by compound **1a** occurs extra- or intracellularly we used combinations of known chelators and investigated signature interactions between these molecules. Chemical-chemical interaction profiles using known bioactive compounds have been identified as a powerful means to probe mechanism of novel compounds (Farha & Brown, 2010; Yeh, Tschumi, & Kishony, 2006). Therefore, using the known extracellular chelators, EDTA and pentetic acid (DTPA); the intracellular iron chelator, 1,10-phenanthroline; and the intracellular zinc chelator, TPEN, we generated interaction profiles that we then compared to those between **1a** and the known chelators. We observed synergy between EDTA and each 1,10-phenanthroline, TPEN and **1a**, and detected slight antagonism between the two extracellular chelators, EDTA and DTPA (Figure 2-9a) (Pillai, Moellering, & Eliopoulos, 2005). Interaction profiles between each of the two known intracellular chelators and **1a** showed slight synergy, where **1a** in combination with DTPA produced a synergistic interaction. Combining 1,10-phenanthroline and TPEN resulted in synergy. We hypothesized that if **1a** were acting as an extracellular chelator then we would have expected to see a similar antagonistic interaction signature with each EDTA and DTPA as was produced upon combination of the two respective extracellular chelators; however, we instead observed synergy between **1a** and each EDTA and DTPA—where synergy was also observed between EDTA and each 1,10-phenanthroline and TPEN—suggesting **1a** to behave more similarly to the intra- versus extracellular chelators. Indeed, these conclusions are consistent with the screening conditions under which these chelators were

discovered. With a concentration of 0.8 mM EDTA in the media, growth inhibition upon addition of 10 μM compound **1a** would be difficult to explain were the molecule simply another extracellular chelator.

To further confirm our hypothesis that **1a** functions intracellularly, we performed a time-course assay where the supernatant of **1a**-treated *E. coli* was monitored via visible spectroscopy (Figure 2-9b). We show that upon immediate treatment of cells with **1a** the amount of compound (based on the absorbance spectrum of **1a** between 600 and 350 nm) in the supernatant was identical to the absorbance spectrum of **1a** from that of the cell-free sample. At 1 hour post-treatment, the amount of **1a** from the cell-containing supernatant was approximately half that of the spectrum observed from the cell-free sample; and at 2 hours post-treatment the absorbance of **1a**-treated cells was identical to that of the spectrum shown for the untreated-cell sample, indicating that **1a** was no longer detectable in the supernatant.

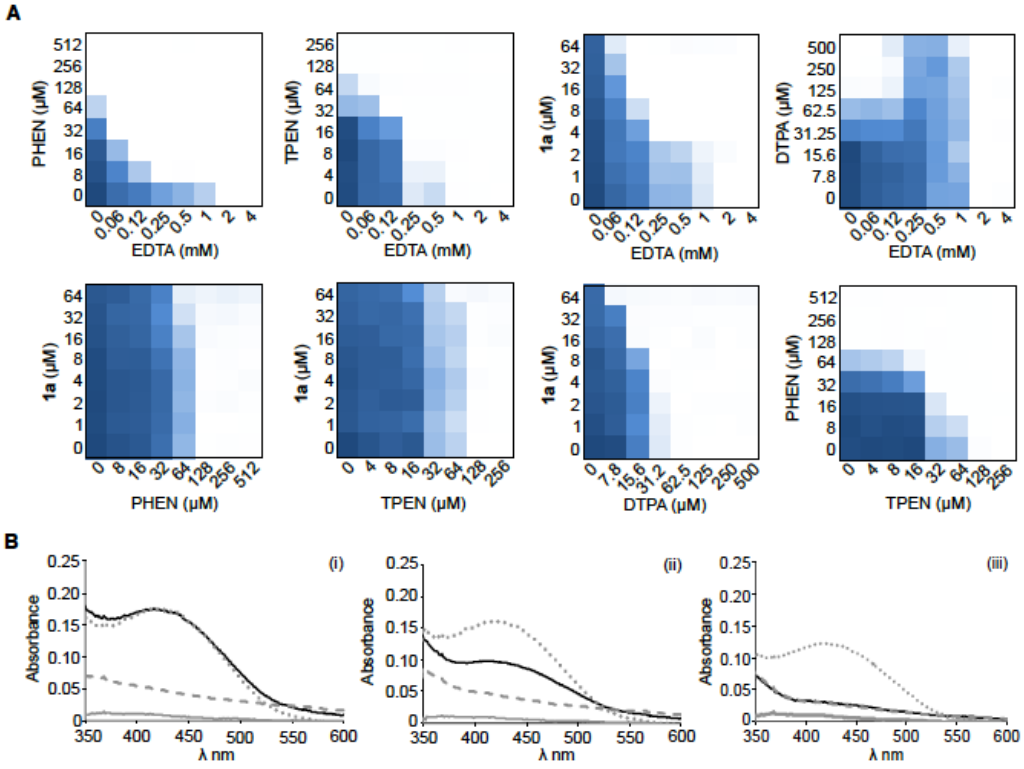


Figure 2-9. Compound 1a functions as an intracellular versus an extracellular chelator. (A) Checkerboard growth assays of the effect of chemical combinations on the growth of *E. coli.* Dark blue squares to white squares represent full growth and complete growth inhibition, respectively. The plots are described succinctly by FIC indexes (Pillai et al., 2005) where values of <0.5, 1, and >2.0 indicate synergistic, additive, or antagonistic compound interactions, respectively. EDTA combined with each 1,10-phenanthroline, TPEN, and 1a resulted in synergy, with FIC indexes of 0.188, 0.313, and ≤0.156, respectively. EDTA combined with DTPA produced a slightly antagonistic effect, with an FIC index of 1.125. **1a** combined with each 1,10-phenanthroline and TPEN produced slightly synergistic interactions, with FIC indexes of 0.75 in both cases. **1a** and DTPA resulted in asynergistic interaction with an FIC index of 0.5, and the combination of 1,10-phenanthroline and TPEN yielded synergy, resulting in an FIC index of 0.5. (B) Samples (1.5 ml) of saturated *E. coli* cultures in Chelex 100-treated M9 minimal media were prepared separately for 0, 1, and 2 hr time points according to the following: bacteria treated with 40 mM **1a** (black solid line); bacteria treated with DMSO solvent (hashed gray line); media alone with 40 mM **1a** (gray dotted line); and media alone with DMSO solvent (gray solid line). Samples (1 hr and 2 hr) were shaken at 250 rpm at 37°C, where supernatant from 0 hr samples were measured immediately. Supernatants were obtained by centrifugation of samples for 6 min at 15,000 rpm, after which the absorbance of each sample was measured between 600 and 350 nm using quartz cuvettes and a Varian Cary Bio 300 UV-Vis spectrophotometer.

2.5 Discussion

Among the greatest hurdles for lead identification in antibiotic drug discovery is the identification of compound mechanism of action. Yet, at a time when the scarcity of effective antibiotics is hindering the treatment of multi-drug resistant bacterial infections, it is incumbent upon researchers to identify novel antibacterials and to accelerate the means by which mechanism is deciphered. Herein, we describe a mechanism-guided screen for inhibitors of bacterial iron homeostasis and the discovery of novel spiro-indoline-thiadiazole intracellular chelators with antibacterial activity.

Iron is an essential micronutrient whose availability is well documented to be inextricably linked to successful pathogen-mediated host infection; however, antibacterial agents that target bacterial iron homeostasis are absent from the clinic. Studies using the fungal pathogen, *Aspergillus fumigatus* (Pinto & Moore, 2009), and the yeast, *Saccharomyces cerevisiae* (Simm, Luan, Weiss, & O'Halloran, 2011) have previously demonstrated the capability of high-throughput screens to detect small molecules that inhibit iron and zinc homeostasis in each of these respective organisms; however, such investigations have not been reported for bacteria. In this study we have both validated a screening methodology that specifically selects for compounds that inhibit bacterial iron homeostasis, and report on a series of small molecules that are toxic to *E. coli* via a novel metal-mediated isomerization and iron chelation.

Iron can exist in a range of oxidation states, though it is primarily found in either the Fe^{3+} ferric form under aerobic conditions, or in the reduced Fe^{2+} ferrous state in environments where oxygen is absent (Andrews et al., 2003). Siderophores, such as

enterobactin found in *E. coli*, are highly specific for Fe^{3+}. Under iron-limiting conditions *E. coli* synthesizes and excretes enterobactin into the extracellular milieu where the siderophore scavenges iron and forms a tight association with Fe^{3+}. The resulting ferri-siderophore complex is subsequently imported into the bacterial cell where Fe^{3+} is reduced to Fe^{2+} and in turn released from enterobactin (Andrews et al., 2003). In our validation screen employing the non-essential gene-deletion library in *E. coli*, we observed that strains most sensitive to iron depletion were those mutants incapable of siderophore biosynthesis or uptake—stated otherwise, these are strains unable to accomplish Fe^{3+} acquisition. Although the chemical library screened in this study did not yield compounds targeting enterobactin biosynthesis or import, those deletion strains sensitive to the presence of EDTA comprise a potential target list for future high-throughput screens conducted under parameters identical to those reported here. In particular, barring chelation as the mechanism of action, likely potential targets of any compounds identified from such screening conditions are those enzymes and proteins absent from the deletion strains reported here to be sensitive to an iron-limited environment.

Although Fe^{3+} is the predominant iron species found in aerobic environments, within the bacterial cell iron has been shown to exist primarily in the reduced Fe^{2+} state (Keyer & Imlay, 1996). Thus, when bacteria are grown in an oxygen-rich environment, although it is the ferric form that enters the cell, iron is rapidly converted to and maintained in the ferrous state. We therefore hypothesized that, in addition to having the potential to detect compounds that inhibit Fe^{3+} acquisition, our screening conditions

would be amenable to the identification of compounds that perturb the intracellular Fe^{2+} iron pool. Thus, in our final assay following the high-throughput screen, in order to confirm that the molecules of interest were specifically involved in disrupting iron homeostasis—either by inhibiting Fe^{3+} acquisition or through perturbation of intracellular Fe^{2+} homeostasis—we asked whether compound activity could be suppressed by supplementing with each apo-enterobactin, ferrous iron and ferric iron. Our rationale for using apo-enterobactin as a secondary assay was to select for compounds that specifically perturb iron homeostasis. Namely, we sought to preclude 1) compounds capable of metal chelation that disrupted the homeostasis of an essential transition metal other than iron, such as Mn^{2+} or Zn^{2+}, or 2) compounds that might be inactivated by iron in our counter screen through the formation of a chelate complex. Chelex-treated minimal media is iron deplete; however, as iron is absolutely required for bacterial growth, a limited amount of iron sufficient to permit growth of *E. coli* nonetheless remained in the media. Thus, we sought to answer whether the addition of apo-enterobactin, which would scavenge trace iron in the Chelex-treated media and be imported into the cell as the holo-siderophore, would be sufficient to suppress an inhibitor-induced lethal phenotype. The concentration of intracellular iron is stringently regulated; therefore, only under circumstances where intracellular iron was being depleted would holo-enterobactin be imported into the cell. Therefore, if growth inhibition by **1a** resulted from homeostatic disruption of a metal other than iron then we would not expect to see suppression by enterobactin as the holo-siderophore would not be imported into the cell if the availability of iron was not disturbed. Indeed, we showed that growth inhibition by TPEN—whose principal

mechanism of inhibition is through perturbation of zinc homeostasis, but which also binds other transition metals, including iron—was not suppressed with supplementation of apo-enterobactin. Thus, the use of apo-enterobactin as a counter screen permitted assurance that compound activity of our molecules of interest was indeed the result of perturbing either iron acquisition or homeostasis.

Our identification of the known intracellular Fe^{2+} chelator, 1,10-phenanthroline, served as initial confirmation that the employed screening conditions were indeed capable of identifying chemical matter that disrupts Fe^{2+} homeostasis. Although we found the activity of **1a** and **1b** was suppressed by a series of biological transition metals—which therefore suggested these spiro-indoline-thiadiazole analogs to function as chelators—it was still unclear whether the mechanism of action was through chelation of Fe^{2+} or Fe^{3+}. However, by conducting visible region spectrophotometry and x-ray crystallography analyses we were able to show unequivocally that **1a** binds Fe^{2+} in addition to other divalent transition metals. We were unable to identify complex formation between **1a** and the non-transition divalent metals, Ca^{2+} or Mg^{2+}; and, more interestingly, we did not detect binding between **1a** and Fe^{3+}. Where even the known chelator 1,10-phenanthroline is considered to be highly selective for ferrous iron with log stability constants for Fe^{2+} and Fe^{3+} of 21.0 and 14.1, respectively (Liu & Hider, 2002b), we report here an unusual example of a synthetic chelator that, while capable of binding Fe^{2+}, appears to be unable to chelate the oxidative form of iron.

The stability constants of **1a** with transition metals implied the following binding order: $Cu^{2+} < Ni^{2+} < Zn^{2+} < Co^{2+} < Fe^{2+} < Mn^{2+}$. Although these findings suggest Mn^{2+} to

be the preferred substrate for **1a** in a mixed-metal *in vitro* environment, a key consideration when contemplating target metal under biological conditions is differential metal bioavailability. Specifically, *E. coli* has been found to accumulate 10-100 times more iron than manganese (Outten & O'Halloran, 2001), suggesting that the greater cellular concentration of the former metal may predispose it over manganese to chelation by **1a**. Furthermore, the EDTA-LB conditions under which **1a** was initially identified were identical to those employed to screen the non-essential gene-deletion collection, where although we detected many strains carrying mutations in genes involved in iron homeostasis, we did not identify any such mutants with deletions in genes responsible for maintaining manganese homeostasis. Hence, although we found **1a** to have a slightly higher stability constant for Mn^{2+} over Fe^{2+}, in light of the argument regarding cellular metal bioavailability as well as our findings from the non-essential gene-deletion screen—which served as a proxy for the target pathways that we could expect to inhibit during a small-molecule screen—we argue that **1a** is indeed perturbing the homeostasis of Fe^{2+} over other biological metals.

Pertaining to the metal-binding chemistry and antibacterial activity of these spirocompound analogs, of particular interest to us was the observation that **1a** is able to exist in two isomeric states. There is an extensive literature regarding the spiropyrans and spirooxazines which, similar to **1a**, are bistable compounds susceptible to isomerization to the merocyanine form when faced with a specific environmental cue; however, in the absence of a trigger will spontaneously revert back to the more stable, closed spiro state (Tian et al., 2010). Indeed, when zinc-exposed **1a** was treated with EDTA we observed

reversion of **1a** from the merocyanine to the closed state, suggesting the spiro form of **1a** to also be the more stable isomer. In experiments with the chemical analogs, **1c** and **1d,** we were able to show that antibacterial activity of **1a** and **1b** is contingent on the presence of a sulphur moiety within the diazole, thus inferring that it is the open merocyanine, rather than the stable spiro isomer that is responsible for antibacterial activity. To our knowledge, this is the first report of a bistable compound that is specifically induced by its transition-metal target to assume an active, antibacterial form.

Our demonstration that the antibacterial activity of **1a** results from the ability of the compound to chelate Fe^{2+} is, alone, strongly suggestive of **1a** functioning as an intra- versus extracellular chelator. In order for **1a** to be active in the extracellular environment we would expect the compound to be capable of binding the form of iron that predominates in the aerobic surroundings outside of the cell—which is Fe^{3+}. However, as we've shown **1a** to be incapable of complexing with Fe^{3+}, the most parsimonious conclusion is that **1a** exercises its antibacterial activity by chelating the Fe^{2+} intracellular iron pool. As evidence to more conclusively prove this hypothesis, we showed that chemical-chemical interaction profiles generating signature relationships between **1a** and known intra- and extracellular chelators were similar to those interaction profiles observed for other known intracellular chelators, yet were quite disparate from those profiles revealed by known extracellular chelators. Although we found 1,10-phenanthroline and TPEN to be synergistic upon combination with one another, where **1a** with each of the intracellular chelators was only very mildly synergistic, we surmise that such observations result from **1a** being saturated by the extracellular metal in the LB

media, in turn rendering **1a** unable to adequately chelate iron within the cell. Conversely, EDTA and DTPA sequestered much of the extracellular metal, thereby permitting **1a** to remain functional intracellularly. Further, to more convincingly show that **1a** is an intracellular chelator, we demonstrated that the presence of **1a** in the supernatant of **1a**-treated cells had completely disappeared over the course of 2 hours, suggesting that **1a** migrated from the outside into the intracellular space.

2.6 Experimental procedures

Bacterial strains, growth media and reagents. *E. coli* K-12 strain BW25113 was employed for all experiments unless stated otherwise. The non-essential gene-deletion collection was a kind gift from Hirotada Mori, where the genetic background of all deletion strains in this library is *E. coli* BW25113. All growth experiments were conducted in liquid media under aerobic conditions. Luria Bertani (LB) medium contained 10 g of Bacto Tryptone (BD Biosciences), 10 g of sodium chloride and 5 g of yeast extract (BD Biosciences) per liter of distilled water. All experiments, including those involving EDTA, were conducted in LB media, unless stated otherwise. Minimal media was comprised of 1X M9 minimal salts prepared from 5X M9 minimal salts stock (Sigma-Aldrich), 0.2% v/v Casamino acids (BD Biosciences), 10 μM of each aromatic amino acid, L-tyrosine, L-tryptophan and L-phenylalanine, 1 μM thiamine, and 0.4 % v/v glucose. Transition metal-depleted media was generated by passing M9 minimal media over a column containing Chelex 100 resin (Bio-Rad). The high-throughput small-molecule screen was conducted using the Canadian Compound Collection (CCC), which

was assembled and is maintained by McMaster University's High-throughput Screening Facility. The CCC is comprised of 30,880 small molecules, which are sourced from various vendors, including ChemBridge, Maybridge, MicroSource Discovery Systems, Inc., Prestwick Chemicals and Biomol-Enzo Life Sciences. Unless stated otherwise, all chemicals and reagents, including the metal chelators 1,10-phenanthroline, EDTA, and TPEN, and metal salts ferrous chloride, ferric chloride, zinc chloride, manganese chloride, cobalt chloride, nickel chloride, cupric chloride were purchased from Sigma-Aldrich. Resupply of compound **1a** and compound **1b** were from ChemBridge. Compounds **1c** and **1d** were purchased from ChemBridge and Vitas-M Laboratory, respectively.

LB-EDTA high-throughput small-molecule screen. *E. coli* BW25113 was grown overnight at 37°C with shaking at 250 rpm in 5 mL LB media to a saturated culture. Bacteria were diluted 1:100 into fresh LB media, grown to an $OD_{600\ nm}$ of 0.3 and diluted again 1:1000 in LB media containing 0.8 mM EDTA. A Beckman Biomek FX (Beckman Coulter Inc.) was used for all subsequent liquid handling experiments. EDTA-diluted cells were transferred at a volume of 198 µl to 96-well plates (Costar) followed by the addition of 2 µl of each compound dissolved in DMSO (Caledon Laboratory Chemicals) to achieve a final screening concentration of 10 µM. Alternating positive and negative controls replaced CCC compounds in both the first and last column of each plate, where controls consisted of ampicillin (64 µg ml^{-1}) and DMSO, respectively. Plates were read at $OD_{600\ nm}$ before and after incubation for 16 hours at 37°C, where the initial read was later subtracted from the final read in order to account for compounds that

contributed to absorbance at 600 nm. Each compound was screened in duplicate. Bacterial growth was measured via the following equation:

$$\%\ growth = \left(\frac{OD_{600} - \mu OD_{600low}}{\mu OD_{600high} - \mu OD_{600low}}\right) x\ 100 \quad (1)$$

where μOD_{600low} and $\mu OD_{600high}$ represent the average sterility and bacterial growth controls, respectively.

Growth curves and minimum inhibitory concentration determinations. All bacterial strains were grown overnight at 37°C with shaking at 250 rpm in 5 mL of media, which consisted of either LB or M9 minimal media (where the latter was the overnight media used for compounds tested in either M9 or Chelex 100-treated M9 media). Saturated cultures were subsequently diluted 1:100 into either LB or M9 minimal media accordingly (where the latter was the subculture media used for compounds tested in either M9 or Chelex 100-treated M9 media) and grown to an $OD_{600\ nm}$ of 0.3 or 0.2 for growth in LB and M9 minimal media, respectively. Bacteria were again diluted 1:100,000 into media containing the final concentration of compound to be tested, where LB or M9 media was used for experiments conducted under transition metal-replete conditions, and Chelex 100-treated M9 media was employed for metal-deplete experiments. Bacteria with compound were incubated under stationary conditions at 37°C where growth at $OD_{600\ nm}$ was measured after 18 hours unless stated otherwise.

EDTA screen against *E. coli* non-essential gene deletion library. Strains from the non-essential gene deletion library arrayed in 96-well plates were inoculated from frozen stocks into fresh LB media containing 50 μg ml^{-1} kanamycin using the Duetz

cryoreplicator (Duetz et al., 2000). Inoculated plates were shaken for 18 hours at 37 °C and 250 rpm to achieve saturated cultures. Strains were then subcultured at a 1:100 dilution by transferring 2 µl of culture into 198 µl LB containing 50 µg ml^{-1} kanamycin and returned to shake at 37 °C and 250 rpm until bacteria reached an $OD_{600\ nm}$ of 0.3. Strains were diluted 1:200 where samples were either treated with 0.8 mM EDTA or left to grow in LB media alone. Bacteria were incubated at 37 °C for 16 hours and growth was measured and recorded at $OD_{600\ nm}$. Each condition was performed in duplicate.

Metal- and apo-enterobactin-suppression experiments. *E. coli* BW25113 was treated as described above for MIC experiments; however, rather than testing the final 1:100,000 dilution of bacteria against a range of compound concentrations, here we added the bacteria to the previously-established MIC of our compound of interest combined with an equimolar concentration of apo-enterobactin or the test metal of interest. Samples were then incubated under stationary conditions at 37°C and bacterial growth was measured at $OD_{600\ nm}$ after 18 hours. Results are represented as the mean and ± SD for three samples.

Crystallization conditions and crystal structure determination. Maroon crystals of the **1a**-Fe^{2+} and **1a**-Zn^{2+} complexes were each generated by slow evaporation at ambient temperature of a 1:1 (v/v) methanol:chloroform solution containing 1:1.2 (molar ratio) of **1a** powder and either ferrous chloride or zinc chloride. Crystals of each **1a**-Fe^{2+} and **1a**-Zn^{2+} were observed after 3 weeks and single-crystal X-ray crystallographic analyses were conducted. Diffraction data were obtained using ω-scans via a Bruker SMART6000 CCD area detector mounted on a fixed-χ 3-circle D8

goniometer using a Rigaku Cu rotating anode with cross-coupled mirrors and CuKα radiation (λ=1.54178 Å). Data collection, processing and refinement were accomplished using APEX2 (Bruker-AXS), SHELXS (Sheldrick, solution) and SHELXL (Sheldrick, refinement), respectively. Details of data collection and refinement for **1a**-Fe^{2+} and **1a**-Zn^{2+} are presented in Table S1 and are deposited with the Cambridge Crystallographic Data Centre, registry number 931217 and 931218, respectively.

Spectroscopic measurements. Studies were conducted using quartz cuvettes and a Varian Cary Bio 300 UV-Vis spectrophotometer. Samples were prepared in Chelex 100-treated HEPES (10 mM) at pH 7.4 and assayed according to conditions as stated in the results. Each experiment was conducted in triplicate and averaged for accuracy. To maintain iron in a reduced oxidative state experiments involving Fe^{2+} were performed under an N_2 atmosphere.

Ferrozine competition assay. All solutions were prepared as described previously (Viollier, Inglett, Hunter, Roychoudhury, & Van Cappellen, 2000). Briefly, ferrozine (10^{-2} M) was prepared in ammonium acetate solution (10^{-2} M). The reducing agent, hydroxylamine hydrochloride (1.4 M) was prepared in analytical grade hydrochloric acid (2 M). Ammonium acetate buffer (10 M) was prepared at pH 9.5. Experiments were performed as described by Figure 6c.

Chemical-chemical interaction experiments. For each chemical combination an 8x8 matrix was prepared at the appropriate drug concentrations. An *E. coli* BW25113 subculture grown in LB media to an $OD_{600\ nm}$ of 0.3 and was added to the compound assay plate at a final dilution of 1:200,000. Growth was measured after 18 hours at 37 °C.

Each compound combination was assayed in duplicate where averaged values were used to calculate the FIC index.

Time-course assay monitoring intracellular uptake of *1a* by *E. coli.* A single colony of *E. coli* BW25113 was grown in 5 mL of M9 minimal media for 18 hours. The saturated culture was then pelleted via centrifugation for 6 minutes at 15000 rpm, washed 3 times with Chelex 100-treated M9 minimal media, and resuspended in 10 mL of Chelex-treated M9 minimal media. 1.5-mL aliquots of culture were added to separate acid-washed flasks and samples were prepared as described for Figure 7b.

CHAPTER 3 – ZINC SEQUESTRATION BY A SMALL-MOLECULE CHELATOR POTENTIATES CARBAPENEM ACTIVITY AGAINST A METALLO-β-LACTAMASE-PRODUCING STRAIN OF *K. pneumoniae*

3.1 Preface

This chapter was adapted from the following manuscript in preparation:

Shannon B. Falconer, Sebastian S. Gehrke, Wenliang Wang, James F. Britten, Gerard D. Wright, and Eric D. Brown. Identification of a small molecule zinc chelator that re-sensitizes NDM-1-producing *Klebsiella pneumoniae* to carbapenem antibiotics. *In preparation.*

All experiments in this chapter were performed by myself with the exception of compound synthesis, which was conducted by Sebastian S. Gehrke, and compound crystallization and crystal structure determination, which were performed by Wenliang Wang and James F. Britten, respectively. I wrote the manuscript with input from Eric Brown.

3.2 Summary

β-Lactams are a diverse class of antibiotics that are very effective in the treatment of bacterial infections; however, their extensive use has been a driver for pervasive and and concerning β-lactam resistance. Among the most important resistance determinants are the metallo-β-lactamase enzymes, which are capable of inactivating nearly all classes of β-lactams, including the last-resort carbapenem antibiotics. A distinguishing feature of the metallo-β-lactamase enzymes is a requirement for zinc for catalytic activity. We recently reported on the discovery of two spiro-indoline-thiadiazole chelator analogs that were toxic to bacteria via perturbation of intracellular iron homeostasis; yet, *in vitro*, were also capable of complexing with a series of bidentate transition metals, including zinc. In the work described herein, we sought to explore the potential of altering the specificity of the spiro-indoline-thiaidazole series for zinc chelation. Our rationale was that, by disturbing bacterial zinc homeostasis, we might be capable of re-sensitizing a zinc-dependent metallo-β-lactamase-carrying strain of bacteria to the action of β-lactam antibiotics. Indeed, we report here the identification of zinc-selective spiro-indoline-thiaidazole analogs that potentiate β-lactam antibiotics *in vitro* by limiting the action of metallo-β-lactamase enzymes.

3.3 Introduction

The ability to effectively treat bacterial infections is indispensable to modern medicine, where antibiotics serve not only to remedy common infections, but are pivotal to ensuring the success of a range of crucial medical procedures, such as organ

transplantation and cancer chemotherapy (Laxminarayan et al., 2013). Widespread antibiotic drug resistance, however, has mired the efficacy of these agents and created a looming health crisis that affects individuals worldwide. Although multidrug-resistant strains of bacteria exist for both Gram-positive and Gram-negative pathogens, concern regarding the former has been temporarily assuaged by the relatively recent introduction of linezolid (Leach, Brickner, Noe, & Miller, 2011) and daptomycin (Steenbergen, Alder, Thorne, & Tally, 2005)—two antibiotic classes that target Gram-positive bacteria. In contrast, attempts to identify new compounds capable of treating infections resulting from Gram-negative organisms have been bleak. Coupled with the paucity of antibiotics available to treat such infections, strains of Gram-negative pathogens that are resistant to virtually every clinical antibiotic have become increasingly prevalent in hospital-acquired infections (Laxminarayan et al., 2013), making the identification of new strategies to treat such infections a priority in antibiotic research.

Among the most alarming Gram-negative pathogens are those that harbor β-lactamase-resistance mechanisms. β-lactams are a large class of antibiotics that, despite decades of use, continue to be a mainstay for treating infections caused by Gram-negative bacteria. However, when employed against bacteria that have acquired the ability to produce β-lactamase enzymes, such forms of treatment are severely compromised. The most worrisome are the metallo-β-lactamases—a mechanistically distinct class of β-lactamases enzymes that employ an active site zinc atom to help catalyze the hydrolysis of the β-lactam ring (Nordmann et al., 2011). Although metallo-β-lactamase-producing organisms have not been shown to be any more virulent than non-metallo-β-lactamase-

producing pathogens, the concern regarding the former is their recalcitrance to the carbapenem-class β-lactam antibiotics (Kumarasamy et al., 2010). Carbapenems are the most recent β-lactam to be developed for activity against a broad range of Gram-negative pathogens, and have been invaluable as a last resort antibiotic to remedy infections that could otherwise be untreated (Nordmann et al., 2011; 2012). In addition to their insusceptibility to the action of carbapenems, strains of metallo-β-lactamase-producing pathogens tend to harbor multiple drug resistance determinants, where insensitivity to aminoglycosides and quinolones is particularly common (Cornaglia, Giamarellou, & Rossolini, 2011). Thus, for individuals infected with a metallo-β-lactamase-producing pathogen, treatment options are extremely limited and the use of antibiotics with significant toxicity, such as colistin, is not uncommon (Worthington et al., 2012).

The most recent β-lactamase to emerge is the New Delhi metallo-β-lactamase type-1 (NDM-1); an enzyme that has been predominantly found in Enterobacteriaceae, isolates of *Klebsiella pneumoniae* and *Escherichia coli* from disease-causing states as varied as urinary tract infections, pulmonary infections, soft-tissue infections and septicemia (Kumarasamy et al., 2010; Nordmann et al., 2011). Plasmids carrying the NDM-1-encoding gene, $bla_{\text{NDM-1}}$, have been found to contain up to 14 additional antibiotic resistance determinants, and are easily transferred to confer such resistance mechanisms to other bacteria (Walsh, Weeks, Livermore, & Toleman, 2011). Pathogenic strains that harbor $bla_{\text{NDM-1}}$-containing plasmids have been detected on virtually every continent and, coupled with multiple other antibiotic resistance determinants common to such pathogens, a global health crisis is imminent if new therapies are not soon met.

Although antibiotic monotherapies are the traditional course of treatment for bacterial infection, an additional strategy is the use of compound combinations (Laxminarayan et al., 2013). Such combinations might include two antibiotics that when co-administered result in a synergistic interaction; or, a non-antibiotic combined with an antibiotic, where the former acts as an adjuvant to potentiate the action of the latter. Indeed, there is a precedent for the success of adjuvant therapy, such as seen in the clinical use of Zosyn® and Augmentin®—antibacterial compound combinations that are comprised of a β-lactam antibiotic (piperacillin and amoxicillin, respectively) and a β-lactamase inhibitor (tazobactam and clavulanic acid, respectively). However, where the majority of β-lactamase-class enzymes are susceptible to the action of tazobactam and clavulanic acid, the unique catalytic mechanism of metallo-β-lactamases render these enzymes insensitive to such compounds (Shlaes, 2013). Attempts to identify clinically relevant inhibitors of the metallo-β-lactamases have thus far been unsuccessful. Interestingly, a class of compounds that has shown promise are thiol-containing compounds, where interaction of the active site zinc with the respective sulfur residue was shown to be an important determinant for metallo-β-lactamase inhibition (Watkins, Papp-Wallace, Drawz, & Bonomo, 2013).

Examples of chelators in clinical use are limited, and are generally reserved for disease states that require metal removal from the body, such as iron and copper overload, and heavy metal toxicity (Franz, 2013). However, there is increasing research towards the potential application of chelators for medicinal purposes that don't conform to their conventional employment as metal detoxifiers. In particular, the development of

chelators for the treatment of cancer (Whitnall, Howard, Ponka, & Richardson, 2006), disease states associated with metalloenzymes (Jacobsen et al., 2011), as well as various neurodegenerative diseases (Perez & Franz, 2010), is under active investigation. Thus, we speculated that zinc chelation may have therapeutic potential as an antibiotic adjuvant. Specifically, we reasoned that limiting zinc availability in the bacterial cell should compromise activity of zinc-dependent metallo-β-lactamases rendering the bacterium susceptible to β-lactam antibiotics.

We recently reported on the discovery of two related spiro-indoline-thiadiazole divalent transition metal chelators that were toxic to bacteria through a mechanism of intracellular iron chelation (Falconer et al., 2013). Indeed, while the antibacterial activity of these chelators could be suppressed with excess iron, we found that addition of the high affinity iron siderophore enterbactin was sufficient to reverse the activity of these compounds in growth media depleted for transition metals. Further, despite their iron selectivity *in vivo*, these compounds also showed affinity *in vitro* to a series of bidentate transition metals, including zinc. In the work reported here, we sought to further investigate the potential for developing zinc specificity in the bacterial cell through systematic structural modification of these spiro-indoline-thiadiazole leads. That goal was realized with a series of spiro-indoline-thiadiazole metal chelators that selectively perturb zinc homeostasis in a lab strain of *E. coli*. Further, we show that when tested in concert with carbapenem-class antibiotics against an NDM-1-producing clinical isolate of *K. pneumoniae*, the compound combination results in a synergistic interaction that re-sensitizes the pathogen to the action of carbapenems.

3.4 Results

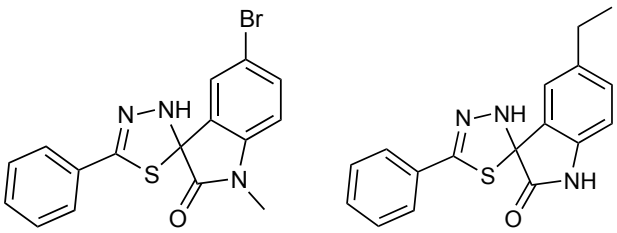
3.4.1 Identification of a small molecule series that perturbs zinc homeostasis in *E. coli* K-12

Motivated by our recent discovery of two spiro-indoline-thiadiazole analogs (**SIT-1** and **SIT-2**) that bound various transition metals, yet selectively perturbed iron homeostasis in *E. coli* (Figure 3-1), we assembled a series of antibacterial spiro-indoline-thiadiazole analogs to investigate their potential for zinc selectivity (Table 3-1). We sought to identify small-molecule analogs that, rather than iron, preferentially bind and disrupt the cellular equilibrium of zinc. Our rationale to identify zinc-specific perturbants by exploring spiro-indoline-thiadiazole analogs was predicated on the hypothesis that chemical modifications to a known transition metal chelator may result in a molecule that, within the complex environment of a bacterial cell, favours zinc rather than iron chelation. Iron and zinc are the most abundant transition metals in bacteria, where *E. coli* has been shown to accumulate concentrations of these metals in the range of 10^{-4} M (Finney & O'Halloran, 2003; Outten & O'Halloran, 2001). Our previous study revealed that antibacterial activity of the chelators **SIT-1** and **SIT-2** could be suppressed by adding exogenous zinc or iron to metal-deplete media. Nevertheless, we found that the high affinity iron siderophore enterbactin was sufficient to reverse the activity, leading to the conclusion that the compounds were selective perturbants of iron homeostasis in cells. In these followup studies, we reasoned that if exogenous zinc, but not iron, could suppress the lethal phenotype of a given analog, then bacterial growth inhibition could be attributed to the selective perturbation of zinc homeostasis. We tested 11 spiro-indoline-thiadizole analogs that were active against *E. coli* K-12 under transition metal-deplete

conditions and showed that 5 of those analogs bore activity that could be suppressed by either ferrous chloride or zinc chloride, where toxicity of the remaining 6 analogs showed selective suppression by zinc chloride (Table 3-1). As previously shown, the presence of the sulfur moiety within the diazole backbone was required for both metal chelation and antibacterial activity (Falconer et al., 2013); hence, all analogs retained this structural core.

Having observed that the iron perturbants **SIT-1** and **-2**, could be suppressed with the addition of either iron or zinc, we investigated the activity of the phenyl-spiro-indoline-thiadiazole skeleton alone (**SIT-3**) and found likewise. As compound **SIT-1** possesses substituents at both the R1 and R3 positions, we sought to investigate the metal suppression profiles for analogs with a single modification off either the R1 or R3 carbon. Suppression of antibacterial activity by both iron and zinc was retained in the absence of a methyl group at R1 and the presence of a bromide atom at R3 (**SIT-4**). Compound **SIT-Z1**, which retained the R1 methyl group yet lacked the R3 bromide, showed a zinc-specific suppression profile. The respective zinc-specific suppression of the antibacterial activity of **SIT-Z1** prompted us to explore additional substitutions at the R1 position. In particular, analogs **SIT-Z2**, **-Z3**, **-Z4**, **-Z5** and **-Z6**, possessed various substitutions at the R1 position, and all showed zinc-specific suppression of antibacterial activity. Given that the additions at the R1 position of the **Z**-series of analogs are carbon-based moieties, we sought to determine whether increasing the overall compound lipophilicity, rather than substitutions specifically at the R1 position, contributed to the observed compound suppression profiles. Subsitution of the R2 benzyl ring with a

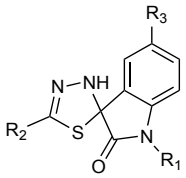
naphthyl moiety increased the logP from 3.52 (**SIT-3)** to 4.51 (**SIT-5)** but imparted no metal specifity to the suppression of antibacterial activity. Thus, substitution at the R1 position alone appeared to confer zinc-specific suppression.

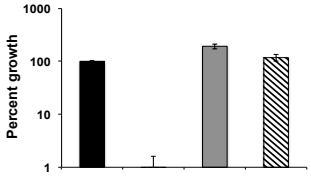
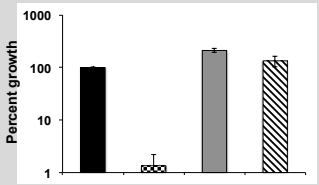
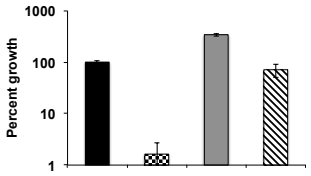
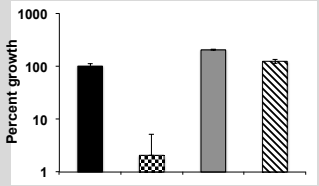
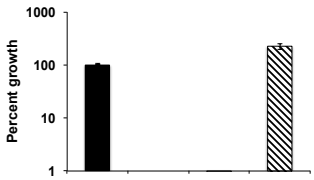
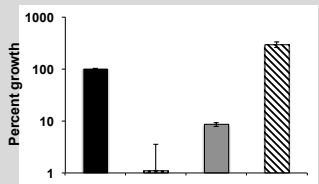


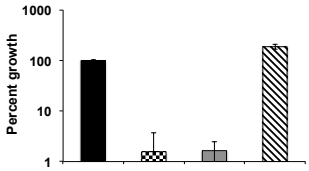
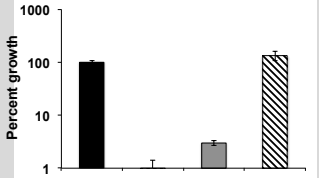
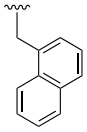
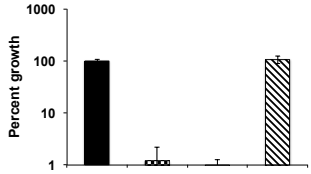
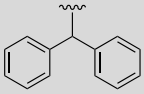
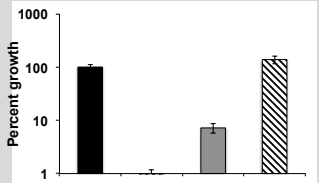
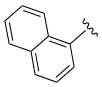
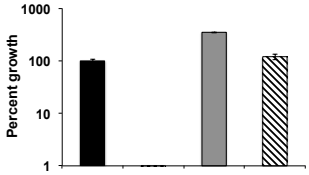
SIT-1 **SIT-2**

Figure 3-1. Chemical structures of two spiro-indoline-thiadiazole (SIT) analogs that specifically perturb bacterial iron homeostasis.

Table 3-1. Structure-activity relationship between spiro-indoline-thiadiazole analogs and their antibacterial activity metal-suppression profiles[*].



Compound	LopP	MIC (μM)	R_1	R_2	R_3	$FeCl_2$ and $ZnCl_2$ suppression profiles in *E. coli*
SIT-1	4.15	16				
SIT-2	4.47	16				
SIT-3	3.52	32				
SIT-4	4.29	16				
SIT-Z1	3.38	32				
SIT-Z2	3.74	32				

Compound	LopP	MIC (μM)	R_1	R_2	R_3	$FeCl_2$ and $ZnCl_2$ suppression profiles in *E. coli*
SIT-Z3	4.70	16				
SIT-Z4	5.04	8				
SIT-Z5	6.09	4				
SIT-Z6	6.89	4				
SIT-5	4.51	16				

***** Black bars indicate growth of *E. coli* alone, in the absence of either metal or drug; checkerboard bars show growth of *E. coli* in the presence of an inhibitory concentration of drug; grey bars indicate growth of *E. coli* in the presence of both an inhibitory concentration of drug and an equimolar concentration of ferrous chloride; and hashed bars reveal growth of *E. coli* in the presence of both an inhibitory concentration of drug and an equimolar concentration of zinc chloride.

3.4.2 *E. coli* toxicity by Z5 occurs specifically via chelation and perturbation of zinc homeostasis

Our observation that the antibacterial activity of the **Z**-series of analogs could not be suppressed by iron prompted us to ask whether this resulted from an inability of such compounds to chelate iron; or, if chelation does occur, whether the **Z**-series exhibit a ligand-metal stoichiometry that is unique between iron and zinc. We showed via crystal structure determination that, similarly to the known iron perturbant and metal chelator, **SIT-1** (Falconer et al., 2013), the merocyanine isomer of **SIT-Z5** chelates ferrous iron in a bidentate fashion and via the same coordinating N, S and O atoms as occurs in the **SIT-1**-Fe^{2+} complex (data not shown). Establishing the capability of **SIT-Z5** to chelate iron, we then determined the crystal structure of **SIT-Z5**-Zn^{2+}, and revealed the same bidentate mechanism of binding and N and S coordinating ligands as was observed for the **SIT-Z5**-Fe^{2+} complex (Figure 3-2), suggesting a more subtle explanation regarding the zinc-specificity conferred by the **Z**-series of analogs than could be gleaned from the structural interactions between ligand and metal. As **SIT-Z5** was the analog that we were able to determine a crystal structure for, and given that it displayed the lowest minimum inhibitory concentration (MIC) against *E. coli*, we chose this as the representative ligand to probe the antibacterial mechanism of the **Z**-series of compounds.

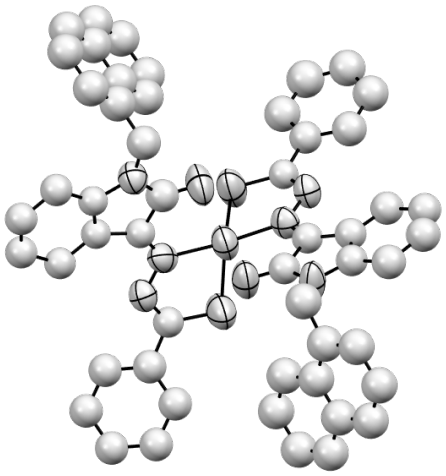


Figure 3-2. Single crystal structure of SIT-Z5-Zn^{2+} coordination complex. Ellipsoids are at 50% probability. Hydrogen atoms are not shown.

Although we determined the capability of **SIT-Z5** to form a coordination complex with zinc, whether the chelator had another biological effect on the bacterial cell remained unclear. Thus, to aid us in answering this question we employed a subinhibitory concentration of compound and screened it against a genomic library comprised of approximately 2,000 bacterial strains, each of which harbored a plasmid containing a single *E. coli* K-12 promoter fused to the green fluorescent protein gene (*gfp*) (Zaslaver et al., 2006) (Figure 3-3). In performing this experiment we hypothesized that we would be able to detect any bacterial processes affected by the presence of **SIT-Z5** via induction of promoter activity and, in turn, fluorescence production. Interestingly, we found that of the ~2,000 strains only two promoter-*gfp* containing strains resulted in fluorescence activity: namely, we observed a five- and eight-fold induction of promoter activity for the *znuA*

and *ykgM* genes, respectively. ZnuA is a component of the *E. coli* high-affinity zinc uptake system that has been shown to be activated under zinc-deplete conditions (Patzer & Hantke, 1998). YkgM is a paralog of the ribosomal protein, RpmE, that has lost the zinc-binding motif displayed by the latter protein (Makarova, Ponomarev, & Koonin, 2001), and which has been shown to undergo increased expression under conditions of zinc limitation (Graham et al., 2009; Hensley et al., 2012). It has been proposed that YkgM replaces the function of RpmE under zinc-deplete conditions. Indeed, the selective activation of these zinc-dependent promoters suggested that the mechanism of toxicity of **SIT-Z5** against *E. coli* was due to a specific ability to chelate and perturb zinc homeostasis.

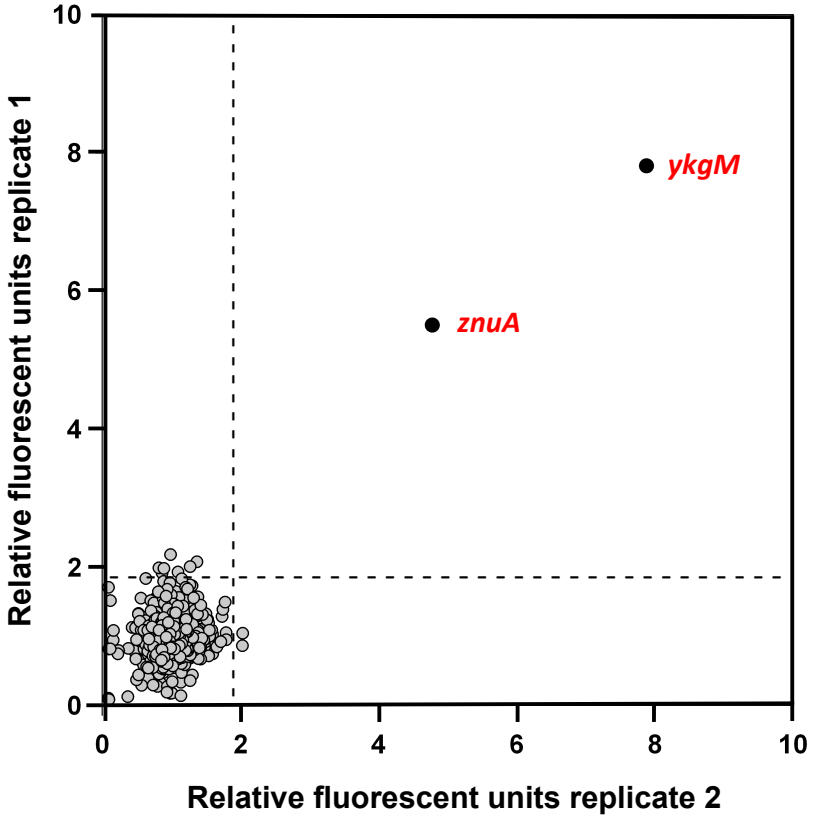


Figure 3-3. Replicate plot of *gfp*-promoter library screened against a subinhibitory concentration of compound SIT-Z5. Dashed lines represent 3σ above the average relative fluorescent units of all strains.

3.4.3 Compound activity susceptible to zinc-specific suppression correlates with analog-induced fluorescence of *ykgM* and *znuA* promoter-*gfp* strains

To confirm that *ykgM* and *znuA* promoter induction was the specific result of our proposed role for **SIT-Z5** in perturbing bacterial zinc homeostasis, we next assayed all analogs against the *ykgM* and *znuA gfp*-promoter strains. In particular, we aimed to verify that the respective promoters were not simply sensitive to any compound with a spiro-indoline-thiadiazole backbone, such as compounds **SIT-1** and **SIT-2**, which are known to be primarily involved in disrupting bacterial iron homeostasis. We observed that

induction of both of the *ykgM* and *znuA gfp*-promoter strains occurred in the presence of each **Z**-series analog; in contrast however, none of the non-**Z**-series of compounds led to increased expression of either of the respective *gfp*-promoter strains (Figure 3-4). Thus, only those analogs showing zinc-specific suppression of toxicity were found to activate promoters involved in responding to zinc limitation, thus further reinforcing the conclusion that **Z**-series of compounds were involved in perturbing bacterial zinc homeostasis.

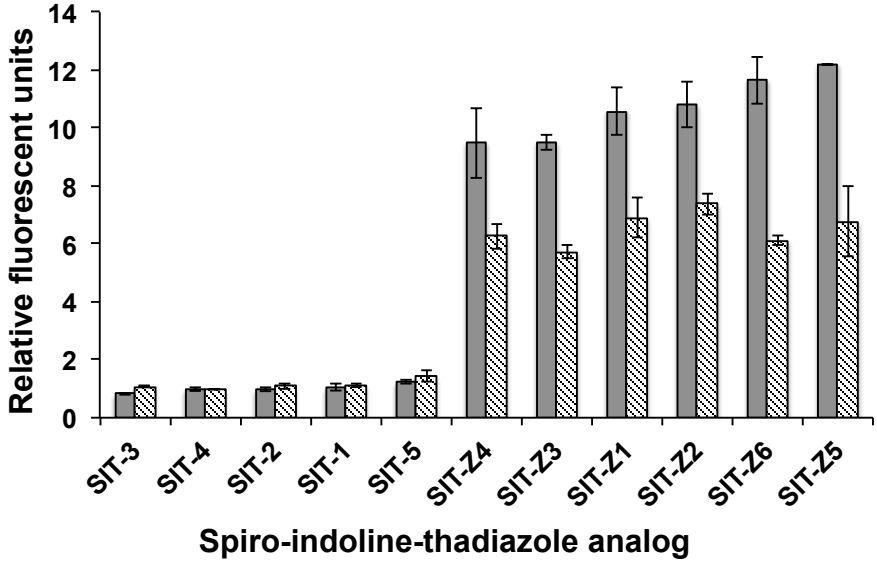


Figure 3-4. Perturbation of bacterial zinc homeostasis is specific to the Z-series of analogs. Individual strains of *gfp*-promoter strains, *ykgM* (grey bars) and *znuA* (hashed bars), grown in Chelex 100-treated minimal media were subjected to a subinhibitory concentration of the various analogs, where activation of the promoter strains was monitored via fluorescence. Bar graphs represent the mean and ±SD for three samples.

3.4.4 Compound SIT-Z5 potentiates carbapenem activity against an NDM-1-positive strain of *Klebsiella pneumonia*

Next, we endeavored to determine whether **SIT-Z5** might be capable of re-sensitizing a metallo-β-lactamase-producing strain of bacteria to carbapenem-class antibiotics. Thus, we performed checkerboard assays of **SIT-Z5** in combination with the carbapenem antibiotic, meropenem, against an NDM-1-harbouring clinical isolate of *K. pneumoniae*. Our results indicated that, when used in combination with **SIT-Z5**, meropenem activity against *K. pneumoniae* was potentiated, where the MIC of the antibiotic against *K. pneumoniae* decreased from >2 μM to 0.25 μM (Figure 3-5). To confirm that the observed potentiation of meropenem by **SIT-Z5** was specific to the zinc-chelating properties of **SIT-Z5**, we assayed whether such synergism between the compound pair could also be observed in the non-NDM-1-harbouring bacterial strain, *E. coli* BW25113. Interestingly, we showed that, although profoundly synergistic against *K. pneumoniae*, the **SIT-Z5**-meropenem combination showed an additive antibacterial phenotype against *E. coli* (Figure 3-5).

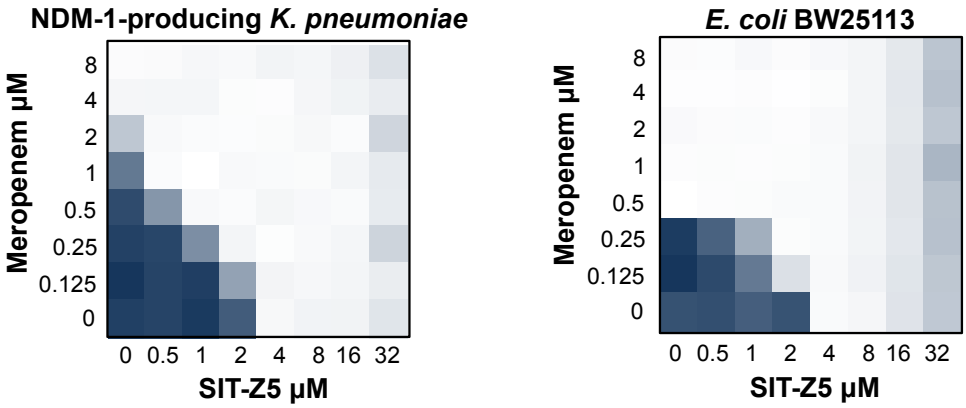


Figure 3-5. Potentiation of meropenem activity by SIT-Z5 is specific to bacteria harbouring metallo- β -lactamase resistance. The effects of chemical-chemical interactions between meropenem and compound **SIT-Z5** against a carbapenem-resistant strain of *K. pneumoniae*, and a non-pathogenic lab strain of *E. coli*, are represented by full bacterial growth (blue squares) to complete growth inhibition (white squares). Interactions are quantified by fractional inhibitory concentration (FIC) indexes (Pillai et al., 2005), where values <0.5, 1, and >2 indicate compound synergy, additivity and antagonism, respectively. The meropenem-**SIT-Z5** profile agasint an NDM-1-harbouring strain revealed a profoundly synergistic chemical-chemical interaction with an FIC index of 0.375. The compound pair against non-pathogenic *E. coli* yielded an additive response represented by an FIC index of 1.

Next, we addressed whether synergy between **SIT-Z5** and meropenem against *K. pneumoniae* was specific to carbapenem-class compounds, or whether **SIT-Z5** was also capable of potentiating the action of additional antibiotics. We found that, although not as pronounced as when added in combination with meropenem, compound **SIT-Z5** also potentiated the action of the carbapenem antibiotics, imipenem, doripenem and biapenem, as evidenced by the signature staircase pattern of synergy (Figure 3-6). However, such potentiation was not detected between **SIT-Z5** and non-carbapenem β-lactams or non-cell wall active antibiotics.

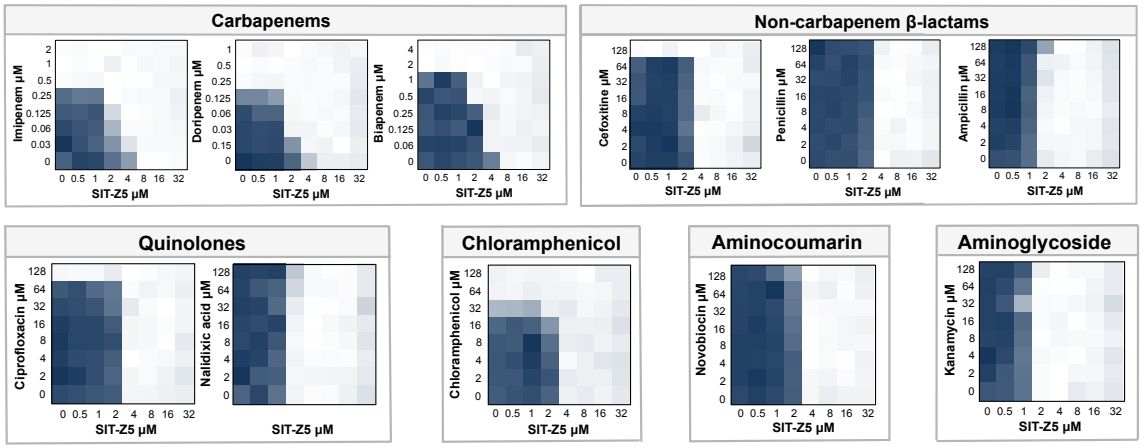


Figure 3-6. Chemical-chemical interaction studies between various antibiotics and compound SIT-Z5 against a carbapenem-resistant strain of *K. pneumoniae*. Compound interaction growth assays against NDM-1-harbouring *K. pneumoniae*. Dark blue squares to white squares represent full growth and complete growth inhibition, respectively. Compound **SIT-Z5** in combination with imipenem, doripenem and biapenem resulted in synergistic FIC indexes of 0.62, 0.37 and 0.5, respectively. Synergy was not observed between **SIT-Z5** and any other antibiotic combination, where the resulting FIC indexes were ≥1.

3.4.5 Analogs specific to zinc perturbation potentiate meropenem activity against an NDM-1-positive strain of *K. pneumoniae*

In addition to compound **SIT-Z5** we wanted to determine whether those analogs with antibacterial activity that was specific to zinc perturbation—namely, the **Z**-series of analogs—were capable of synergizing with meropenem against *K. pneumoniae*. With the exception of compound **SIT-Z1,** we observed synergy between meropenem and all zinc-specific perturbants when tested against *K. pneumoniae***,** though we did not detect potentiation of meropenem activity when combined with any of the non-**Z**-series of analogs (Figure 3-7). With respect to the lack of synergy between **SIT-Z1** and meropenem, a possible explanation for this may be the relatively low logP value

displayed by the respective analog as compared to all other compounds in the **Z**-series. Specifically, it may be that **SIT-Z1** is capable of infiltrating the outer membrane of *E. coli* K-12, however, is unable to do so against *K. pneumoniae*. This hypothesis would be consistent with our observations that all other analogs in the **Z**-series display greater hydrophobicity than **SIT-Z1**.

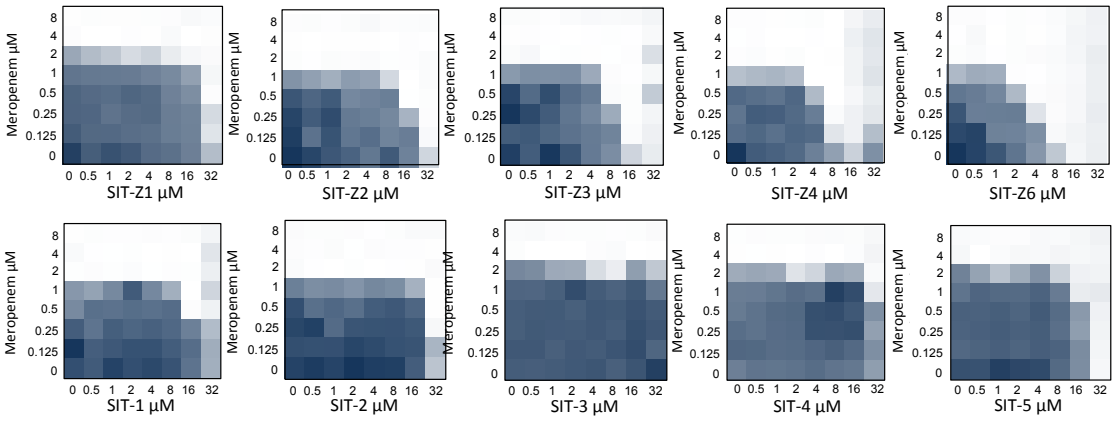


Figure 3-7. Chemical-chemical interaction studies between meropenem and each of the non-Z-series and Z-series of analogs against carbapenem-resistant *K. pneumoniae*. Dark blue squares to white squares represent full growth and complete growth inhibition, respectively. FIC indexes for meropenem in combination with compounds **SIT-Z1**, **-Z2**, **-Z3**, **-Z4** and **-Z6** were 0.75, 0.5, 0.5, 0.63 and 0.5, respectively. Meropenem in combination with **SIT-1**, **SIT-2**, **SIT-3**, **SIT-4** and **SIT-5** resulted in FIC indexes of 0.75, 0.75, 1.5, 1 and 0.75, respectively.

3.5 Discussion

The emergence of carbapenem-resistant Gram-negative pathogens in concert with the lean antibiotic development pipeline has resulted in a serious health crisis that will

continue to worsen unless new therapeutic measures are soon met. Of particular concern are those strains of bacteria that, in addition to being recalcitrant to the action of carbapenems, also harbour resistance determinants to an array of additional antibiotics, such as in the case of NDM-1-producing pathogens (Laxminarayan et al., 2013). Drug-resistant pathogenic Enterobacteriaceae have become a scourge to nosocomial environments, as their resilience to the current treatment strategies have afforded them the opportunity to proliferate. Furthermore, innate resistance offered by the Gram-negative outer membrane makes efforts to identify novel antibacterial compounds exceedingly difficult. In this work we explored a series of chelator analogs, whose structures are based on a spiro-indoline-thiadiazole chemical scaffold that we recently reported as being inhibitory against *E. coli* through disruption of iron homeostasis via chelation (Falconer et al., 2013). Given the susceptibility of Gram-negative bacteria to the respective chemical series, we hypothesized that the bioactive scaffold would be a good starting point to investigate additional chelator analogs that, rather than iron, chelate and perturb bacterial zinc homeostasis. Zinc is required for activity of metallo-β-lactamases, thus, we reasoned that a chelator whose antibacterial activity was due to zinc sequestration might synergize with carbapenem antibiotics and re-sensitize an otherwise antibiotic-resistant strain of Enterobacteriaceae to the action of carbapenems. Indeed, we report here the identification of a series of spiro-indoline-thiadiazole chelators that, by limiting bacterial zinc availability, potentiated carbapenem activity against a clinical isolate of NDM-1-harboring *K. pneumoniae*.

Divalent chelators are generally known to be non-specific and capable of forming complexes with various metal species (Hider, 2002). Indeed, such promiscuity with respect to metal binding is among the most prominent features of chelators limiting their therapeutic use. However, there are many features that contribute to the governing principles of metal-ligand coordination chemistry, including shape, solubility, lipophilicity and kinetics—all of which affect the biological outcome of a chelator-chelate relationship (Franz, 2013). Therefore, we reasoned that even subtle modifications to the spiro-indoline-thiadiazole ligand skeleton could influence the behaviour of a given analog when introduced to the cellular environment.

Having previously established that compound **SIT-1** was specific to perturbing iron homeostasis (Falconer et al., 2013), we began by exploring zinc- versus iron-specificity of this compound and analogs thereof. In exploring a range of chemical modifications at the R1 positions we found that, for each of the analogs tested, the presence of a moiety off the R1 carbon alone resulted in a zinc-specific activity suppression profile, therefore suggesting that a common characteristic—such as lipophilicty or ligand-metal stoichiometry—determined by the R1 moiety affected zinc-specific suppression of antibacterial activity. In the first instance, lipophilicity is a salient chemical feature that is known to correlate with compound toxicity (Leeson & Springthorpe, 2007)—a characteristic that we observed to be consistent in the case of our analogs. Since all modifications at the R1 position of the **Z**-series of compounds are carbon-based substituents, we considered whether lipophilicity also contributed to the

observed metal-suppression profiles. However, we found no correlation between lipophilicity and zinc-specific perturbation by the analogs.

To investigate the mechanism of action of the **Z**-series of ligands, we performed structural analysis, from which we obtained crystals of each the **SIT-Z5**-Zn^{2+} and **SIT-Z5**-Fe^{2+} complexes. Our results indicated that, similarly to the recently published **SIT-1**-Fe^{2+} and **SIT-1**-Zn^{2+} chelate complexes (Falconer et al., 2013), the **Z**-series of analogs were indeed capable of chelating both metals, which was accomplished via a bidentate mechanism utilizing the same S and N atoms to coordinate metal as was observed for the **SIT-1** analog.

In the absence of a difference regarding either the stoichiometry or coordinating atoms between the ligand-metal complexes, we are unable to propose a mechanistic explanation for the zinc-specific suppression of activity of the **Z**-series of analogs. Nonetheless, we have revealed a chemical trend where phenyl-spiro-indoline-thiadiazole compounds that harbour an addition exclusively at the R1 position are capable of undergoing zinc-specific suppression of antibacterial activity. Interestingly, the zinc specificity displayed by the respective **Z**-series of molecules is corroborated by our results from both *gfp*-promoter experiments, as well as chemical-chemical interaction assays against *K. pneumoniae*. Screening the *gfp*-promoter genomic collection revealed that, of the approximately 2000 strains, the only promoters to show activity in response to **SIT-Z5** were for *znuA* and *ykgM*, which are genes known to be upregulated in response to zinc limitation (Graham et al., 2009; Hensley et al., 2012; Patzer & Hantke, 1998). We demonstrated that activity of the promoter strains was unaffected by the non-**Z**-series, but

was induced by all **Z**-series of analogs. Furthermore, it was striking that, with the exception of **SIT-Z1**, our observation of zinc specificity against *E. coli* regarding the **Z**-series of analogs correlated with the potentiation of meropenem against NDM-1-producing *K. pneumoniae*. Collectively, the results of these experiments suggest a distinct mechanism of action by the respective compound series against the bacterial cell. In turn, we argue that specifically the **Z**-series of compounds disturb bacterial zinc homeostasis via chelation and limitation of the metal, and that such perturbation is their primary, if not exclusive, antibacterial mechanism of action.

In addition to establishing the **Z**-series of compounds as perturbants of bacterial zinc homeostasis, the work herein also revealed an important and potentially therapeutic application for these molecules as adjuvants for the treatment of infections caused by carbapenem-resistant *K. pneumoniae*. The use of chelators in medicine is often approached with trepidation given their complicated drug safety profiles and the many significant side effects associated with their use (Brown, Willis, Omalu, & Leiker, 2006; Drosos, Geogriou, Politi, & Voulgari, 1997; Gattermann, Finelli, Porta, & Fenaux, 2010). However, this has not prevented the long-standing use of chelators as therapeutic agents against iron overload disease (Olivieri, 1999; Olivieri & Brittenham, 1997), nor has discussion and research been thwarted regarding the development of new chelators for the treatment of various neurodegenerative diseases (Hider, Roy, Ma, Le Kong, & Preston, 2011; Li, Jankovic, & Le, 2011; Perez & Franz, 2010), and certain types of cancer (Torti & Torti, 2013). Thus, there exists a strong precedent for both current and potential future uses of chelators as pharmaceuticals, which we argue should be

considered by both researchers and medical practitioners within the field of infectious disease.

NDM-1-harbouring *K. pneumoniae* is among the most dreaded and difficult-to-treat strains of pathogenic bacteria currently plaguing the medical community. Due to the obstinacy of the outer membrane, identifying new antibacterial small molecules capable of infiltrating the respective barrier has proved exceedingly challenging. Thus, rather than continuing with the traditional antibiotic drug discovery regiment—which is to conduct chemical screens to identify new antibacterial small molecules that target a single mechanism—an alternative strategy is to develop compounds that serve as adjuvants to current antibiotics. The difficulty in achieving single-metal targeting by a given chelator, coupled with grave toxicity concerns during unsuccessful therapeutic attempts using EDTA for the treatment of various maladies, have incited staunch misgivings about the application of chelators in medicine (Franz, 2013). However, the relationship between each chelator and its chelate is highly unique such that, when applied to a complex system, such as a cell, the biological outcomes—including the degree of host toxicity—can be vastly different. In the work herein we present a series of spiro-indoline-thiadiazole compounds that, through chelation of zinc, compromise the mechanism of metallo-β-lactamase antibiotic resistance, thereby potentiating the activity of carbapenem antibiotics and reinstating efficacy of the drug class against NDM-1-harbouring *K. pneumoniae*.

3.6 Experimental procedures

Bacterial strains, growth media and reagents. *E. coli* K-12 strain BW25113 was used for all experiments with the exception of those strains contained in the *gfp*-promoter library, where the background strain for the collection is *E. coli* MG1655, and experiments involving the *K. pneumoniae* clinical isolate. The *gfp*-promoter library was a kind gift from Uri Alon and Michael Surette, and the *K. pneumoniae* strain was gifted from Michael Mulvey to Gerard D. Wright. All growth experiments were conducted in liquid media under aerobic conditions in Chelex 100 resin (Bio-Rad)-treated M9 minimal media. Minimal media was comprised of 1X M9 minimal salts prepared from 5X M9 minimal salts stock (Sigma-Aldrich), 0.2% v/v Casamino acids (BD Biosciences), 10 μM of each aromatic amino acid, L-tyrosine, L-tryptophan and L-phenylalanine, 1 μM thiamine, and 0.4 % v/v glucose. Unless stated otherwise, all reagents and chemicals, including the metal salts ferrous chloride and zinc chloride, were purchased from Sigma-Aldrich. Compounds **SIT-1**, **-2**, **-3** and **-4** were purchased from ChemBridge. Compounds **SIT-5**, **-Z1**, **-Z2**, **-Z3**, **-Z4** and **-Z5** were synthesized by Dr. Sebastian Gehrke.

Minimum inhibitory concentration determinations. All bacterial strains were grown overnight at 37°C with shaking at 250 rpm in 5 mL of M9 minimal media. Saturated cultures were subsequently diluted 1:100 into M9 minimal media and grown to an $OD_{600\ nm}$ of 0.2 for growth in Chelex 100 resin-treated M9 minimal media. Bacteria were again diluted 1:100,000 into Chelex 100 resin-treated M9 minimal media containing the final concentration of compound to be tested. Bacteria with compound were incubated

under stationary conditions at 37°C and growth at $OD_{600\ nm}$ was measured after 18 hours unless stated otherwise.

Screening analogs against *E. coli* K-12 *gfp*-promoter library. Strains from the *gfp*-promoter genomic library arrayed in 96-well plates were inoculated from frozen stocks into fresh M9 minimal media containing 25 μg ml^{-1} kanamycin using the Duetz cryoreplicator (Duetz et al., 2000). Inoculated plates were shaken for 18 hours at 37 °C and 200 rpm to achieve saturated cultures. Strains were then subcultured at a 1:100 dilution by transferring 2 μl of culture into 198 μl M9 minimal media containing 25 μg ml^{-1} kanamycin and returned to shake at 37 °C and 250 rpm until bacteria reached an $OD_{600\ nm}$ of 0.2. Strains were then diluted 1:200 in Chelex resin-treated M9 minimal media with 25 μg ml^{-1} kanamycin and samples were either treated with ¼ of the minimum inhibitory concentration of analog of interest, or were grown in Chelex resin-treated M9 minimal media alone. Bacteria were incubated at 37 °C for 16 hours and growth was measured and recorded at $OD_{600\ nm}$. Screening **SIT-Z5** against the complete genomic library was performed in duplicate; for all other analogs against *znuA* and *ykgM* promoter strains, assays were conducted in triplicate.

Metal suppression experiments. *E. coli* BW25113 was treated as described above for MIC experiments; however, rather than testing the final 1:100,000 dilution of bacteria against a range of compound concentrations, here we added bacteria to double the previously-established MIC of our compound of interest, combined with an equimolar concentration of either zinc chloride or iron chloride. Samples were then incubated under

stationary conditions at 37°C and bacterial growth was measured at $OD_{600\ nm}$ after 18 hours. Results are represented as the mean ± SD for three samples.

Crystallization conditions and crystal structure determination. Maroon crystals of the **SIT-Z5**-Zn^{2+} and **SIT-Z5**-Fe^{2+} complexes were each generated by slow evaporation at ambient temperature of a 1:1 (v/v) methanol:chloroform solution containing 1:1.2 (molar ratio) of **SIT-Z5** powder and either ferrous chloride or zinc chloride. Crystals of each **SIT-Z5**-Zn^{2+} and **SIT-Z5**-Fe^{2+} were observed after 3 weeks and single-crystal X-ray crystallographic analyses were conducted. Diffraction data for **SIT-Z5**-Zn^{2+} was obtained using ω-scans via a Bruker SMART6000 CCD area detector mounted on a fixed-χ 3-circle D8 goniometer using a Rigaku Cu rotating anode with cross-coupled mirrors and CuKα radiation (λ=1.54178 Å). **SIT-Z5**-Fe^{2+} data was collected on the Bruker Smart Apex2 CCD instrument with a Mo sealed tube source and a curved graphite monochromator. (MoKα radiation (λ=0.71073Å).) Data refinement was accomplished using SHELXL (Sheldrick, refinement).

Chemical-chemical interaction assays. For each assay an 8x8 matrix was prepared at the appropriate drug concentrations. Bacterial strains were grown for 18 hours at 37°C with shaking at 250 rpm in 5 mL of M9 minimal media. Saturated cultures were subsequently diluted 1:100 into M9 minimal media and grown to an $OD_{600\ nm}$ of 0.2. Bacteria were again diluted 1:100,000 into Chelex 100 resin-treated M9 minimal media and added to the compound assay plate. Plates were then incubated under stationary conditions at 37°C where growth at $OD_{600\ nm}$ was measured after 18 hours unless stated

otherwise. Assays for each compound combination were performed in duplicate and averaged values were used to calculate the FIC index.

CHAPTER 4 –FUTURE DIRECTIONS AND CONCLUSIONS

4.1 Screening for natural product inhibitors of bacterial iron homeostasis

There exists a rich history of success in natural product antibiotic drug discovery. Beginning with the identification of penicillin from fungi in 1929, and continuing through the next two decades with screens for bioactive small molecules produced by the actinomycete group of soil-dwelling bacteria, natural products have comprised the main source of antibiotics (Bologa, Ursu, Oprea, Melançon, & Tegos, 2013). It is estimated that there are between 200,000 and 250,000 bioactive natural products, suggesting a vast untapped chemical reservoir from which new antibacterial small molecules could be discovered (Bérdy, 2005). However, after repeated employment of the same screening techniques, rediscovery of known antibacterial small molecules, rather than the identification of new antibacterial compounds, has become the norm. As a result, the search for new antibiotics via natural products has largely fallen by the wayside.

In the face of the current antibiotic crisis, researchers have begun to reexamine the standard platform—namely, cell-based assays monitoring growth inhibition in rich media—by which antibacterial drug discovery has traditionally been conducted. Although various features of bacterial physiology essential for growth under nutrient-replete conditions are undoubtedly effective antibacterial targets (as evidenced, for example, by the clinical utility of aminoglycosides and β-lactams), such mechanisms are not the only means by which bacterial growth can be compromised. Among numerous strategies that have recently been proposed to discover both new antibacterial targets and chemical matter are screening conditions that, rather than selecting only for targets that are sensitive when grown in rich-media, reveal bacterial processes that are susceptible to

inhibition in a nutrient-limited environment, which is more closely akin to that of a host organism.

Among those essential, yet vanishingly available, nutrients that are highly coveted by nearly all microbes is iron. As discussed in Chapters 1 and 2, within a mammalian host, iron is both highly limited, and actively sequestered and denied to the invading pathogen by the immune system. Furthermore, in addition to iron being limited within a host, it is also in scarce supply in the aerobic environment. The wealth of antibacterial agents manufactured by producing organisms has been suggested to have arisen from the evolutionary advantage that such chemical warfare confers upon a given species during the arms race that exists between communities of microbes within the environment. As such, I propose that the inaccessibility of iron in the environment may render its acquisition by bacteria living within a polymicrobial community a potential target for exploitation by producing organisms. In Chapter 2 of this thesis I described screening conditions that permitted us to identify a new synthetic chemical scaffold that was toxic to bacteria via perturbation of iron homeostasis. Thus, I suggest that employing the same EDTA-LB media conditions as described in Chapter 2 to screen for bacterial growth inhibition against a collection of small-molecule natural products bears the potential to reestablish natural products as a source of new antibacterial chemical matter through the discovery of small-molecule inhibitors of iron acquisition.

4.2 The potential for metal chelators in antibacterial therapy

Presently, the medicinal utility of chelators is limited, largely due to the lack of metal specificity and general toxicity that are often associated with such compounds. However, we argue that exploring metal chelators as new potential antibiotic lead compounds is justified given that 1) there is a precedent for relatively non-toxic chelators in medicine—such as exemplified by deferoxamine for the treatment of iron overload (Olivieri & Brittenham, 1997), and penicillamine as a therapy in Wilson's disease (Ala, Walker, Ashkan, Dooley, & Schilsky, 2007)—and 2) strains of pathogenic bacteria currently exist that are resistant to virtually all clinically-used antibiotics. Therefore, as the medical community has already been forced to resort to treatment options that present significant side effects, exploring the use of chelators as antibacterial compounds may well result in the identification and development of therapeutics that prove to be more favorable than the limited options that are currently available.

4.2.1 Exploring SIT-Z5 as a potential therapeutic lead antibacterial adjuvant

Among the greatest hurdles in antibiotic discovery is identifying the mechanism of action by which a given compound executes it antibacterial activity. Although we have determined the means by which **SIT-Z5** is both itself toxic to Gram-negative bacteria, as well as able to potentiate the action of carbapenem antibiotics, we have not yet shown that such activity of **SIT-Z5** extends beyond *in vitro* assays and is also relevant in an *in vivo* infection model. Naturally, given the objective of our investigation described in Chapter 3—which was to identify a chelator that demonstrates zinc specificity with

respect to perturbation of bacterial metal homeostasis, and that in turn reinstates antibacterial activity of an antibiotic class against an otherwise drug-resistant pathogen—the application of **SIT-Z5** as a therapeutic adjuvant to meropenem would be the ideal outcome. As such, in collaboration with Brian Coombes' lab, we are exploring both the toxicity profile, as well as the capability of **SIT-Z5** to synergize with meropenem to treat *K. pneumoniae* infection in mice. An advantage of combination therapy is that lower concentrations of each drug (than might otherwise be required if administered alone) are required to achieve the desired antibacterial effect; thus, considering the potential toxicity that may arise from **SIT-Z5**, a combination strategy with respect to antibacterial treatment may be particularly beneficial in the case of chelator antibiotic adjuvants. Additionally, as compound lipophilicity is known to correlate with toxicity, should **SIT-Z5** exhibit too many side effects for it to be considered as a potential lead therapeutic, another option is to explore *in vivo* combinations between meropenem and one of the other **SIT-Z**-series of analogs that possess a logP value that is less than that of **SIT-Z5**. In particular, **SIT-Z2** both 1) exhibited similar zinc-specific suppression, and *gfp*-promoter induction profiles, against *E. coli* as that of **SIT-Z5**, as well as 2) produced a synergistic interaction with meropenem when assayed against NDM-1-harbouring *K. pneumoniae*. However, the lipophilicity value of 3.74 for **SIT-Z2** is markedly decreased from the logP value of 6.09 displayed by **SIT-Z5**, thus suggesting that a **SIT-Z**-series analog other than **SIT-Z5** may be more suitable for medicinal application.

4.3 Concluding remarks

Transition metals are essential for life, required in an array of cellular processes that include both non-catalytic and catalytic functions. However, owing to their redox properties, metal can also be extremely toxic to the cell when present in excess amounts. Thus, organisms have evolved highly sophisticated mechanisms for controlling the concentrations of a given metal within the cell, where designated cellular machinery is responsible for either acquiring or removing the particular metal of interest. During bacterial infection of a eukaryotic host, a potent defense mechanism employed by the latter is to withhold nutrient metal, therefore starving and preventing growth of the invading pathogen. Thus, considering that metal depletion is an antibacterial strategy that has already been selected by evolution, the tenant of this thesis is that we can advance the field of antibacterial small molecules that mimic such metal-withholding mechanisms, either through perturbing an enzymatic process employed by the bacteria, or via the direct sequestration of metal. Herein, we have identified, characterized and discussed two classes of small molecules that, *in vitro*, both chelate iron and zinc, yet have been found to possess distinct metal preferences and mechanisms of toxicity when acting against the bacterial cell. The work reported here has provided both 1) a tool, by way of a novel screening assay, to identify small molecules that perturb the process of bacterial iron homeostasis, as well as 2) two separate series of chemical analogs, where one is involved in perturbing bacterial intracellular iron homeostasis, and the other acts to specifically withhold zinc. From the latter class we have identified a compound that holds potential as an antibiotic adjuvant in the treatment of NDM-1-harbouring bacterial infections.

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