Abstract

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, is an adept pathogen partly due to its intrinsic antibiotic resistance systems which make an infection difficult to treat with antibiotics. Parts of these intrinsic antibiotic resistance systems are regulated by the product of the gene *whiB7*. A yeast two-hybrid experiment suggested that WhiB7 might interact with the anti-sigma factor RsbW, indirectly implying involvement with the alternate sigma factor SigF. Co-expression of WhiB7 and RsbW in *Escherichia coli* followed by pull-down experiments did not support this hypothesis.

In order to understand mycobacterial intrinsic antibiotic resistance, a new screening method was designed and implemented to identify pairs of compounds that inhibited mycobacteria by synergistic interactions. *Mycobacterium bovis* BCG(lux) cells were exposed to pairwise compound combinations and assessed for viability. A computer program was created to identify synergistic combination leads from the screen. The synergistic interactions might provide us with a greater understanding of the intrinsic antibiotic resistance systems in mycobacteria. A sample of the leads from the screen tested further confirmed that 38% of domperidone combinations and 25% of rifampicin combinations were synergistic.

The data from the screen has also identified new potential anti-mycobacterial compounds, specifically the avermectins. The avermectins, as a family, have never been reported to demonstrate antibiotic activity, but they have been used to treat people and other mammals for parasitic infections. The antibiotic activity of the avermectins was investigated in this work and found to be specific to mycobacteria. The potential of this compound family as a possible future treatment for tuberculosis therapy warrants further study.
Preface

The protocols in this work were approved by the UBC Biosafety Committee. The certificate number is B07-0169.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Expanded</th>
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<tr>
<td>ADC</td>
<td>albumin, dextrose, catalase</td>
</tr>
<tr>
<td>ADS</td>
<td>albumin, dextrose, saline</td>
</tr>
<tr>
<td>ATCC™</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guérin</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
</tr>
<tr>
<td>FIC(I)</td>
<td>fractional inhibitory concentration (index)</td>
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<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>NAD⁺/NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>OADC</td>
<td>oleic acid, albumin, dextrose, catalase</td>
</tr>
<tr>
<td>OADS</td>
<td>oleic acid, albumin, dextrose, saline</td>
</tr>
<tr>
<td>OD</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
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</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>X-gal</td>
<td>bromo-chloro-indolyl-galactopyranoside</td>
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</table>
Acknowledgements

I would like to express my gratitude to my Supervisor, Charles Thompson, for his enthusiasm and support of my work both in the lab and outside the lab. Thank you to the members of my Committee, Dr. Yossef Av-Gay and Dr. Rachel Fernandez, for lively and supportive meetings regarding my work.

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A final thank you to my parents, my siblings and my fiancé for their unfailing support in my pursuit of this degree. The path was easier and more joyful to travel with your good company.
Introduction

1.1 The tuberculosis threat

Tuberculosis (TB) is one of the world’s greatest health challenges. One-third of the world’s population is infected with the etiological agent of TB, *Mycobacterium tuberculosis*. One in ten of these individuals will develop active TB at some point in their lifetime and, if untreated, they will infect an additional 10 to 15 people per year. In 2009 alone, 1.7 million people died of TB [30]. The emergence of multi drug-resistant, extensively drug-resistant and totally drug-resistant *M. tuberculosis* (MDR-TB, XDR-TB and TDR-TB respectively) has increased the threat of TB as a major cause of loss of human life [19,42].

*M. tuberculosis* is an adept pathogen that is easily spread through the air. Once it has infected a host it is capable of limiting and manipulating the host immune system in order to persist and it exhibits intrinsic (natural) resistance to a wide range of chemotherapeutic agents [28,45].

1.2 The intrinsic antibiotic resistance of *Mycobacterium tuberculosis*

Unlike acquired antibiotic resistance, which can be gained through random mutation or horizontal gene transfer, intrinsic antibiotic resistance results from the amalgamation of a variety of natural characteristics. *M. tuberculosis* has a unique cell wall that is different from that of most Gram-positive and Gram-negative bacteria. In addition to peptidoglycan, mycobacterial cell wall components include arabinogalactan, mycolic acids and glycolipids. There is also a mycobacterial outer membrane, or polysaccharide capsule
outside the plasma membrane and this structure includes approximately 140 putative membrane proteins [13,29]. The mycobacterial cell wall is relatively impermeable to hydrophobic and hydrophilic compounds and blocks many common antibiotics from entering the cell [27]. Porin-like protein channels may allow the passage of some antibiotic compounds into the \textit{M. tuberculosis} cell [20], but a variety of internal metabolic systems are activated that prevent many of these antibiotics from acting on their targets. These systems appear to be multi-functional, in that they are primarily responsible for generalized physiological roles, but they are also capable of providing general resistance mechanisms [28].

The concept of a “general” resistance mechanism is counter-intuitive when held up against the traditional understanding that antibiotics cause cell death by specific, not general, modes-of-action based on their chemical structure. Yet despite diversity of antibiotics, mycobacteria have a high level of intrinsic antibiotic resistance seemingly based on these general resistance mechanisms. An alternative hypothesis regarding antibiotic-mediated cell death states that, regardless of class, bactericidal antibiotics stimulate the production of hydroxyl radicals which ultimately result in cell death [21]. Hydroxyl radical formation can be an end product of a cascade of events that take place as a general response to antibiotic challenge. In this model, the stress of bactericidal antibiotics stimulates hyperactivation of the electron transport chain, which results in superoxide formation. The superoxides damage iron-sulfur clusters which frees ferrous iron for oxidation by the Fenton reaction and this leads to hydroxyl radical formation. The hydroxyl radicals damage DNA, proteins and lipids which results in bacterial cell death [21].

The general antibiotic resistance that frustrates clearance of TB infection [28] presents an attractive target for new chemotherapies. If the underlying general resistance mechanism (against antibiotics or against the toxic metabolites they induce) can be impaired or undermined, then there may be many compounds that could then be used to treat TB that are currently considered ineffective. This manuscript describes two approaches I took to learn more about how these resistance systems operate. In section 1.3 and Part I I analyze a single component of \textit{M. tuberculosis}, the WhiB7 protein, and its role as a regulator of intrinsic antibiotic resistance at the gene regulatory level. In section 1.4 and Part II I describe a broad screening method to identify compounds that interfere with intrinsic antibiotic resistance systems and may lead us to the cellular target or pathway of intrinsic antibiotic resistance in future studies.
1.3 WhiB7 regulates intrinsic antibiotic resistance

In order for general physiological systems to act effectively in a multi-functional way to confer antibiotic resistance in mycobacteria, they should be appropriately regulated in response to the presence of antibiotics. Within the Actinomycetes phylum, expression of regulatory proteins from the WhiB family is induced in response to adverse environmental conditions. The WhiB proteins then regulate the expression of many other genes to initiate events such as cell division and sporulation (in *Streptomyces* spp.) and to control expression of a set of genes known as the resistome, which determines antibiotic resistance profiles of soil-dwelling bacteria [10, 28]. Antibiotic-induced changes in gene expression may also be mediated by alternate sigma factors of the RNA polymerase holoenzyme. These alternate sigma factors are themselves regulated by specific, antagonistic binding of anti-sigma factors [35]. In actinomycetes, the combination of WhiB proteins, alternate sigma factors, anti-sigma factors and other regulatory mechanisms may result in a general resistance response when a given antibiotic challenges the bacteria. I endeavoured to explore the relationship and interactions between the *M. tuberculosis* WhiB proteins, sigma factors and anti-sigma factors to gain a more complete understanding of how intrinsic antibiotic resistance against a wide-range of antibiotic compounds is regulated.

In the context of *M. tuberculosis* multi-functional resistance systems, three genes are of particular interest: *whiB7*, of the WhiB family, the alternate sigma factor *F*, *sigF*, and the *sigF*-specific anti-sigma factor *rsbW*.

Previous studies showed that *whiB7* is intimately linked to intrinsic antibiotic resistance [6, 26]. The expression of *whiB7* is induced by sub-inhibitory concentrations of diverse antibiotics and a *whiB7*-null mutant exhibits increased sensitivity to a number of antibiotics [26]. Microarray transcription profiles suggest *whiB7* is a primary regulatory gene. Its regulon consists of at least eight *whiB7*-dependent transcripts, four of which are directly correlated with antibiotic resistance: *tapC* (*Rv1258c*), an efflux pump for aminoglycosides and tetracycline; MT1519 (*Rv1473*), a putative macrolide transporter; *erm* (*Rv1988*), a ribosomal methyltransferase; and *eis* (*Rv2416c*), an aminoglycoside acetyltransferase that confers kanamycin resistance [44]. It has been proposed that *whiB7* also autoregulates its own expression [26]. This hypothesis is supported by evidence that WhiB proteins have DNA-binding capability [36], and by the recent identification of the putative mycobacterial *whiB7* promoter target sequence [6]. The related protein WhiB3 has been shown to interact with sigma factor A (SigA), and is proposed to regulate genes via SigA [38]. It is possible that WhiB7 works...
by interacting with a sigma factor or other direct transcriptional regulator, as proposed for WhiB3.

The *M. tuberculosis* sigF gene is homologous to *Bacillus subtilis* sigF and sigB which are the sporulation and stress response sigma factors, respectively [32]. Stress conditions also seem to induce the expression of sigF in mycobacteria [35]. When *M. tuberculosis* sigF is introduced into *M. bovis* BCG, sigF expression is induced by the presence of some antibiotics [25]. Additionally, a sigF-null mutant of *M. smegmatis* demonstrates higher transformation efficiency, which suggests it plays a role in the regulation of genes responsible for the building and maintenance of the mycobacterial envelope [32].

SigF is itself regulated by the anti-sigma factor RsbW which has been proposed to regulate SigF through partner-switching between SigF and the anti-anti sigma factors RsfA and RsfB. RsbW binds RsfA under reducing conditions, as the result of a cysteine-based motif, possibly a disulfide bridge, that appears to be necessary for RsbW-RsfA interaction [21]. The other anti-anti sigma factor, RsfB, may bind RsbW in an ATP/ADP dependent manner due to a conserved serine residue at position 65 [21]. The factors that contribute to RsbW-SigF binding are currently unknown [21, 24]. Through this alternate binding, RsbW is likely a useful mechanism for *M. tuberculosis* to regulate gene expression in response to varying internal environmental conditions.

RsbW may be directly involved with the function of WhiB7. Recently, a yeast two-hybrid experiment showed that WhiB7 and RsbW bind each other [R. Morris, personal correspondence]. It is known that RsbW specifically binds SigF [24], but the binding of RsbW to WhiB7 was an unexpected finding and it begged the question whether there is a link, physical or regulatory, between WhiB7 and SigF. Part I of this thesis was based on the hypothesis that WhiB7 activity is negatively regulated by RsbW and that RsbW alternately binds WhiB7 and SigF under different stress conditions. The general aim of this project was to investigate the expression, interactions and functions of *whiB7, sigF* and *rsbW* in relation to each other. I did not see evidence of any significant interaction between these genes, or their products, with regards to mycobacterial antibiotic susceptibility.
1.4 Screening for synergistic combinations that target intrinsic antibiotic resistance and inhibit growth

Targeting intrinsic antibiotic resistance systems of *M. tuberculosis* will likely require new drug compounds. The traditional development of a new therapeutic compound has been estimated to cost over $800 million US and often takes as long as 15 years to be approved for human use [2, 9]. It would be ideal to use a more economical and timely approach for developing new tuberculosis treatments. To this end, the Thompson Lab has developed drug-drug interaction screens [34]. Our approach combines the two powerful therapeutic principles of repurposing and synergy.

Repurposing is the finding of new applications for currently approved drugs known to be effective in other therapies. We seek to repurpose existing drugs for effective TB treatment. The synergy we are interested in occurs when combinations of drugs have a greater antimycobacterial activity together than the sum of their individual antimycobacterial activities. The screen we have developed tests a library of approved compounds for synergistic antimycobacterial activity. We apply this method to identify compounds or combinations of compounds that target the systems of intrinsic antibiotic resistance and inhibit mycobacterial growth.

One challenge with performing a screen of this nature is the compromise between throughput-volume, efficiency, accuracy and reproducibility of the findings. The methodology of the procedure largely impacts the outcome of the experiment. This was pointed out in the paper by Pethe et al., where a chemical genetic screen performed in media containing glycerol gave disappointing results for drug efficacy *in vivo*. In this case, the lead compounds that were identified and optimized *in vitro*, in media containing glycerol, were not efficacious *in vivo* because the antibacterial properties of the lead compounds were linked with glycerol metabolism and *M. tuberculosis* does not metabolize glycerol during an infection [31].

In this work I used the model organisms *M. smegmatis* and *M. bovis* BCG. I tested a variety of primary compounds in pairwise combination with a library of 594 compounds (the Sweet Library) in three different screening methods. The analysis of synergy was based on the FICI analysis, which is a popular method that has been shown to work well to identify synergy [14] but it does rely on assumptions that may limit its scope [23], and appear to have impacted the results of this screen, as will be addressed in the Discussion (Part II).
Through these screens and follow up experiments I have identified five combinations of compounds that act in synergy *in vitro* against *M. bovis* BCG, which gives a validation rate of 11% for the best optimized screen. The combinations include: ethambutol + compound Sweet-1F4; rifampicin + compound Sweet-5H10; rifampicin + compound Sweet-5H11; rifampicin + compound Sweet-2E9; and rifampicin + compound Sweet-8A2.

This work also shows that ivermectin, an anthelmentic, demonstrates antimycobacterial activity. This is surprising because no avermectin has previously been reported to demonstrate significant antibacterial activity [5]. Ivermectin is particularly promising as a potential TB treatment because it is mass-produced in oral tablet form and has practically no contra-indications in humans [18].

Overall, this work has provided a strong, multi-faceted basis for future investigations into specific targets, mechanisms-of-action and perhaps inspiration for *in vivo* testing for new TB drug treatment options.
Part I

Regulation and interactions of WhiB7
2

Materials and methods

2.1 Bacterial strains and culture conditions

The mycobacterial species and strains used in this work are described in Table 2.1.

All *Mycobacteria spp.* were grown in Difco Middlebrook 7H9 Broth (BD; per 180 mL: 0.94 g), with 2% glycerol (Sigma) and supplemented with 20 mL Albumin Dextrose Catalase enrichment (per 1000 mL: 8.5 g NaCl [Sigma], 50 g Bovine Serum Albumin [Sigma], 20 g D-Glucose [Bio Basic Inc.], 0.03 g catalase [Sigma]) or on Difco Middlebrook 7H10 agar (BD; 3.8 g per 180 mL) supplemented with 20 mL Difco BBL Middlebrook OADC Enrichment (BD).
Table 2.1: Mycobacterial strains used in these studies

<table>
<thead>
<tr>
<th>Species or strain</th>
<th>Purpose</th>
<th>Source</th>
</tr>
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<tr>
<td><em>Mycobacterium tuberculosis</em> H37Rv</td>
<td>genomic DNA as template</td>
<td>ATCC 25618&lt;sup&gt;TM&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em> mc&lt;sup&gt;2&lt;/sup&gt;6</td>
<td>wildtype</td>
<td>ATCC 607&lt;sup&gt;TM&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. smegmatis mc&lt;sup&gt;2&lt;/sup&gt;6 Δ<em>sigF</em></td>
<td><em>sigF</em> mutant</td>
<td>Dr. Jean-Marc Reyrat</td>
</tr>
<tr>
<td>M. smegmatis mc&lt;sup&gt;2&lt;/sup&gt;6 Δ<em>whiB7</em></td>
<td>null-whiB7 mutant</td>
<td>Thompson Lab (Carol Ng)</td>
</tr>
<tr>
<td>M. smegmatis mc&lt;sup&gt;2&lt;/sup&gt;6+pMV361</td>
<td>vector control</td>
<td>this work</td>
</tr>
<tr>
<td>M. smegmatis mc&lt;sup&gt;2&lt;/sup&gt;6 <em>rsbW</em>-OV</td>
<td>approximate 4x overexpression of <em>rsbW</em></td>
<td>this work</td>
</tr>
<tr>
<td>M. smegmatis mc&lt;sup&gt;2&lt;/sup&gt;6 Δ<em>whiB7</em>+<em>rsbW</em>-OV</td>
<td>null-whiB7 mutant and approximate 4x overexpression of <em>rsbW</em></td>
<td>this work</td>
</tr>
</tbody>
</table>
The *Escherichia coli* strains used for cloning and protein expression in these studies are described in Table 2.2.

All *E. coli* strains were routinely grown in LB broth (Sigma; per 1000 mL: 20 g LB Broth) and on LB 1.5% agar (Sigma; per 1000 mL: 20 g LB Broth and 15 g Agar). For protein expression, strains were cultured in 2x YT medium (per 1000 mL: 16 g Bacto-Tryptone [BD], 10 g Bacto-Yeast extract [BD], 5 g NaCl [Sigma], pH 7.0).

### 2.2 Primers and plasmids

#### 2.2.1 Primers

Primers were designed using the *M. tuberculosis* H37Rv *rsbW* and *whiB7* gene sequences obtained from the TubercuList Server (http://genolist.pasteur-fr/TubercuList/). All primers were synthesized at the Nucleic Acid Protein Service unit-Integrated DNA Technologies (University of British Columbia). Table 2.3 describes primers used in cloning for protein expression in *E. coli* and for gene over-expression in *M. smegmatis*. 
Table 2.2: *E. coli* strains used in these studies

<table>
<thead>
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<th>Strain</th>
<th>Purpose</th>
<th>Source</th>
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<tr>
<td><em>E. coli</em> JM107</td>
<td>cloning with pGEX4T-1 plasmid</td>
<td>gift from Dr. John Smit</td>
</tr>
<tr>
<td><em>E. coli</em> TOP10</td>
<td>cloning with the pET29a plasmid</td>
<td>Invitrogen</td>
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<tr>
<td><em>E. coli</em> BL21 (DE3)</td>
<td>protein expression</td>
<td>Invitrogen</td>
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<tr>
<td><em>E. coli</em> LIM-1</td>
<td>carrying pLNG1</td>
<td>this work</td>
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<td><em>E. coli</em> LIM-2</td>
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<td>this work</td>
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<td><em>E. coli</em> LIM-8</td>
<td>carrying pLNG6</td>
<td>this work</td>
</tr>
<tr>
<td><em>E. coli</em> LIM-10</td>
<td>carrying pLNG4</td>
<td>this work</td>
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</tbody>
</table>
Table 2.3: Primers used in these studies

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers:</td>
<td>Protein expression in <em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEL-1</td>
<td>forward</td>
<td>ATAA<strong>GGATCC</strong>CATGGCCGACTCG-GATTTACC</td>
<td>upstream of <em>M. tuberculosis</em> H37Rv rsbW, inserted BamHI site</td>
</tr>
<tr>
<td>LEL-2</td>
<td>reverse</td>
<td>ATAA<strong>CTCGAG</strong>TCACCTGCTG-GATGCCGC</td>
<td>downstream of <em>M. tuberculosis</em> H37Rv rsbW, inserted XhoI site</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers:</td>
<td>Over-expression in <em>M. smegmatis</em></td>
<td></td>
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</tr>
<tr>
<td>LEL-5</td>
<td>forward</td>
<td>ATCAGAATTCGTCAGATGGC</td>
<td>upstream of <em>M. smegmatis mc</em>155 rsbW, inserted EcoRI site</td>
</tr>
<tr>
<td>LEL-6</td>
<td>reverse</td>
<td>ATCCAGCTTTATCCGACGTCAC</td>
<td>downstream of <em>M. smegmatis mc</em>155 rsbW, inserted HinDIII site</td>
</tr>
<tr>
<td>361fwd</td>
<td>forward</td>
<td>GGAATCACTTCGCAATGG</td>
<td>sequencing primer, upstream of pMV361 multiple cloning site</td>
</tr>
<tr>
<td>361rev</td>
<td>reverse</td>
<td>CCCGTGGAATATGGGCTCATAAC</td>
<td>sequencing primer, downstream of pMV361 multiple cloning site</td>
</tr>
</tbody>
</table>
2.2.2 Plasmids

All plasmids were constructed using standard restriction enzyme digestion and ligation techniques (described in section 2.3). The plasmids used in these studies are listed in Table 2.4.

2.3 Cloning methods

2.3.1 Cloning GST-rsbW

The *M. tuberculosis* rsbW gene was amplified by PCR from *M. tuberculosis* H37Rv genomic DNA (prepared by Carol Ng, Thompson Lab). The PCR mixture included 5 µL DyNAzyme® II Hot Start Reaction Buffer, 1 µL 10mM dNTPs, 1 µL of forward primer LEL-1, 1 µL of reverse primer LEL-2 (see Table 2.3), 0.5 µL *M. tuberculosis* genomic DNA (40 ng/µL), 0.6 µL DyNAzyme® II Hot Start DNA Polymerase (New England BioLabs) and 40.9 µL H2O. The PCR conditions were: 94°C for 10 minutes followed by thirty repeats of 94°C for 20 seconds, 65°C for 20 seconds and 72°C for 30 seconds, after which there was a final incubation of 72°C for 7 minutes 30 seconds.

The PCR products were purified using the illustra GFX® PCR DNA and Gel Band Purification Kit, according to the manufacturer’s instructions (GE Healthcare) and ligated into pGEM®-T Easy. The ligation mixture included 5 µL 2X Rapid Ligation Buffer, 1 µL pGEM®-T Easy Vector, 1 µL PCR Product (24.2ng/µL), 1 µL T4 DNA Ligase and 2 µL H2O. The mixture was incubated overnight at 4°C. The ligation products were run on a 0.7% agarose gel electrophoresis (stained with SYBR Safe), the 3473 bp band was excised and purified (using the illustra GFX® PCR DNA and Gel Band Purification Kit). One µL of the plasmid prep was transformed into 50 µL electrocompetent *E. coli* JM107 cells by electroporation (using the GenePulse XCell® (Bio-Rad) at 1800 V, 25 µF, 200 Ω, 1 mm cuvette), plated on LB agar + 100 µg/mL ampicillin + X-gal and incubated at 37°C overnight.

White colonies were inoculated into 5 mL LB + 100 µg/mL ampicillin and incubated at 37°C for 6 hours. A plasmid miniprep was performed using the PureYield® Plasmid Miniprep System, according to the manufacturer’s instructions. The plasmids were sequenced by GENEWIZ, Inc. and one clone with the correct insertion was named pLNG1 (Table 2.4), the *E. coli* strain carrying pLNG1 was named LIM-1 (Table 2.2).

The plasmids pLNG1 and pGEX4T-1 were digested with restriction en-
Table 2.4: Plasmids used in these studies

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM®-T Easy</td>
<td>cloning PCR products</td>
<td>Promega</td>
</tr>
<tr>
<td>pLNG1</td>
<td>amplify PCR product of <em>M. tuberculosis</em> <em>rsbW</em></td>
<td>this work</td>
</tr>
</tbody>
</table>

**Protein expression in *E. coli***

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEX4T-1</td>
<td>parental plasmid for GST-fusion</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pLNG2</td>
<td>GST-fused RsbW protein expression</td>
<td>this work</td>
</tr>
<tr>
<td>pCR1</td>
<td>template of 6x his-tagged, GB1-fused [17] WhiB7</td>
<td>Carol Ng (Thompson Lab)</td>
</tr>
<tr>
<td>pET29a</td>
<td>parental plasmid with kanamycin resistance</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pLNG4</td>
<td>His-G-WhiB7 protein expression, kanamycin resistance marker</td>
<td>Jan Burian (Thompson Lab)</td>
</tr>
</tbody>
</table>

**Over-expression in *M. smegmatis***

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV361</td>
<td>parental plasmid for mycobacterial cloning</td>
<td>[39]</td>
</tr>
<tr>
<td>pLNG6</td>
<td><em>M. smegmatis</em> RsbW gene overexpression</td>
<td>this work</td>
</tr>
</tbody>
</table>
endonucleases (New England BioLabs) in the following mixtures: 25 µL 50 ng/µL pLNG1 or pGEX4T-1 DNA, 0.8 µL BamHI 20,000 Units/mL and 0.8 µL 20,000 Units/mL XhoI, 5 µL NE Buffer 2 and 21.4 µL H2O. The digestion mixture was incubated for 1 hour at 37°C. The pLNG1 digest was run through a 1% agarose gel electrophoresis (stained with SYBR Safe) and the 438 bp band of rsbW was excised. Both this rsbW DNA fragment and the linearized pGEX4T-1 were purified, then put in a ligation mixture (3 µL rsbW fragment, 1 µL linearized pGEX4T-1, 4 µL 5X buffer, 1 µL T4 DNA Ligase [USB Corp.]) and incubated overnight at room temperature.

The ligation products were transformed directly into *E. coli* JM107 by electroporation (as described previously) then plated on LB agar + 100 µg/mL ampicillin and incubated at 37°C overnight. The remaining culturing, miniprep and sequencing was performed as described. The plasmid that was confirmed for the correct sequence was named pLNG2 (Table 2.4), the *E. coli* strain carrying pLNG2 was named LIM-6 (Table 2.2).

### 2.3.2 Cloning His-G-WhiB7

The plasmid pCR1 contains a 6x His-tagged, GB1-fusion whiB7 sequence (His-G-WhiB7), however the selection of this plasmid is ampicillin resistance, the same as pLNG2. For selection of co-transformants using two selection criteria, the His-G-WhiB7 sequence was incorporated into the pET29a plasmid backbone which offers kanamycin resistance. Jan Burian (Thompson Lab) generated this plasmid, named pLNG4. The *E. coli* TOP10 strain carrying pLNG4 (Table 2.4) was named LIM-10 (Table 2.2).

### 2.3.3 Cloning *M. smegmatis* rsbW for overexpression

The *rsbW* gene from *M. smegmatis* genomic DNA was PCR amplified using the primers LEL-5 and LEL-6 (Table 2.3). The cloning steps followed the same procedure as described in subsection 2.3.1, except the restriction endonucleases used were EcoRI and HindIII (20,000 Units/mL each, New England BioLabs), the plasmid backbone used was the integrative pMV361 [39], conferring kanamycin resistance (Table 2.3) and the host strain was *E. coli* TOP10 (carrier was renamed LIM-8 [Table 2.2]).

The plasmid was later isolated from LIM-8 by plasmid miniprep and transformed by electroporation into *M. smegmatis mc²6*, then renamed *M. smegmatis mc²6 rsbW-OV* (Table 2.1). Similarly, an empty pMV361 was cloned for the vector control strain.
2.4 Protein binding experiments in *E. coli*

2.4.1 Protein co-expression and pull-down in *E. coli*

The plasmids pLNG2 and pLNG4 were electroporated into *E. coli* BL21 (DE3) cells (as described previously), plated on LB agar + ampicillin (100 µg/mL) + kanamycin (30 µg/mL) and incubated overnight at 37°C. Colonies were then inoculated into 5 mL LB + ampicillin (100 µg/mL) + kanamycin (30 µg/mL) and incubated overnight at 37°C with shaking. Protein expression was induced by adding IPTG to a final concentration of 0.1 mM and the culture was incubated overnight at 23°C with shaking.

**Purification by 6xHis-tag**

For purification by the 6xHis-tag, a protein miniprep was performed using a modified protocol from the QIAexpressionist™ ([33]). Briefly, cells were harvested by centrifuging 3 mL of culture at 14,000 × g and resuspended in 300 µL lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0), with lysozyme added to 1 mg/mL and DTT added to 10 mM, the mixture was incubated on ice for 30 minutes. After incubation cells were lysed by vortexing, the lysate was centrifuged for 10 minutes at 14,000 × g and the supernatant isolated. To this supernatent, 20 µL of 50 % slurry of Ni-NTA resin (Qiagen) was added and the mixture incubated for 30 minutes at 4°C with gentle rotation. After 30 minutes, the resin was washed twice with wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl and 20 mM imidazole, pH 8.0), with brief centrifugations between washes. Finally, the protein was eluted from the resin by 3 elutions with 20 µL elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl and 250 mM imidazole, pH 8.0).

**Purification by GST-tag**

For purification by the GST-tag, a protein miniprep was performed as follows: Cells were harvested by centrifuging 3 mL of culture at 14,000 × g and resuspended in 100 µL of BugBuster™ 10x Protein Extraction Reagent (Novagen) and mixed gently at room temperature for 15 minutes. Next, 200 µL cold PBS was added, then the lysate was centrifuged for 10 minutes at 14,000 × g and the supernatant was transferred to a fresh microfuge tube where 20 µL of a 50% slurry of Glutathione Sepharose 4B (GE) was added. This was mixed gently at room temperature for 5 minutes. The beads were washed 3 times by adding 100 µL PBS, centrifuging briefly and discarding the supernatent. The protein was eluted by adding 10 µL elution buffer
(Sigma; 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0) and incubating at room temperature for 5 minutes, then the mixture was centrifuged briefly and the supernatent collected for SDS-PAGE and western blotting.

2.4.2 SDS-PAGE and western blotting

Whole cell lysates, supernatent and aliquots of the purified protein preps were mixed with equal volumes SDS Loading Buffer (10 mL: 2.5 mL 0.5 M Tris-HCl pH 6.8, 2 mL glycerol, 4 mL 10% w/v SDS, 0.5 mL 0.1% w/v bromophenol blue, 1 mL H₂O and add 2-Mercaptoethanol to 2.5%), incubated in boiling water for 5 minutes and 12 µL loaded on a 10% tricine gel (prepared as described in [37]). The SDS-PAGE was run using the PowerPac HC (Bio-Rad) for .03 A at 45 minutes, then 185 V for 1 hour immersed in anode and cathode buffers [37]. The products were transferred to nitrocellulose using the Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) at 15 V for 30 minutes, soaked in electrode buffer (300 mM Tris, 100 mM acetic acid, pH 8.6).

The nitrocellulose was incubated with Blocking Buffer for Near InfraRed Fluorescent Western Blotting (Rockland) for 1 hour at room temperature with rocking and incubated with Blocking Buffer + 0.5 µg/mL Anti-GST (Rabbit) Antibody IRDye 700DX® Conjugated (Rockland) + Anti 6X HIS EPITOPE TAG (MOUSE) Monoclonal Antibody IRDye 700DX® Conjugated (Rockland) for 1 hour. The membrane was washed six times with PBS-T (Sigma; per 1000 mL: 8 g NaCl, 0.2 g KCl, 2.68 g Na₂HPO₄-7H₂O, 0.24 g KH₂PO₄ and 1% v/v Tween 20 adjusted to pH 7.4) before visualization using the Odyssey® Imager (LI-COR Biosciences, settings used were Resolution: 169 µm, Quality: medium, Focus Offset: zero, Intensity: 5.0), made available for use by Dr. Ninan Abraham.

2.5 Antibiotic sensitivity assays

2.5.1 Resazurin and MTT assays

Microtiter 96-well clear Flat Bottom Assay Plates (Costar) were prepared with eight serial two-fold dilutions of antibiotics in 7H9. *M. smegmatis* strains (Table 2.1) were grown to exponential phase (OD₆₀₀=0.4-0.8) then inoculated to a final OD₆₀₀=0.0025 to the prepared microtiter wells. The antibiotics tested are listed in Table 2.5.

The assay plates were wrapped in aluminum foil to prevent vapour loss and incubated for 3 days at 37°C then 100 µL of 10 mg/mL resazurin
Table 2.5: Antibiotics tested in these studies

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Highest concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clindamycin</td>
<td>512</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>128</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>1024</td>
</tr>
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<td>Spectinomycin</td>
<td>512</td>
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<td>Tetracyclin</td>
<td>2</td>
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<td>Imipenem</td>
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<td>Azithromycin</td>
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<td>Roxythromycin</td>
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<td>Capreomycin</td>
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<td>Clofazimine</td>
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<td>Isoniazid</td>
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<td>Rifampicin</td>
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<td>Streptomycin</td>
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<td>Ethambutol</td>
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<tr>
<td>Triclosan</td>
<td>32</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>32</td>
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</tbody>
</table>

(Sigma) or 50 µL of 5 mg/mL MTT (Sigma) was added to each well. The plates were incubated overnight at 37°C. Plates with resazurin were read by photometric measurement of wavelength 570 nm, Bandwidth 5 nm and Measurement time 100 ms Varioskan Flash (Thermo Scientific), made available by Dr. Steven Hallam. The plates with MTT had an additional 50 µL of 10% SDS (w/v) added, another overnight incubation at 37°C followed by a photometric measurement of wavelength 560 nm, bandwidth 5 nm and measurement time 100 ms Varioskan Flash.

2.5.2 Disc diffusion assays

*M. smegmatis* liquid culture was diluted to an OD$_{600}$=0.0125 and spread evenly on 7H10 + ADC agar plates. A paper disc (BBL™, 6 mm diameter) was placed on the surface of the agar and 2.5 µL antibiotic solution was applied to each disc. Plates were incubated at 37°C for 4 days, and the halo of inhibition was measured (in mm).
3

Results

3.1 Protein binding experiments in \textit{E. coli}

The protein co-expression and purification experiments were carried out as described in section 2.4. Both GST-RsbW and His-G-WhiB7 were successfully expressed in \textit{E. coli} BL21(DE3) cells along with empty vector controls (see Figure 3.1). The GST-RsbW was predicted to be approximately 44 kDa, the His-G-WhiB7 band was predicted to be approximately 16 kDa (although it typically runs >20 kDa, [unpublished data]) and the GST-tag is reported to be 26 kDa [4].

The His-purification procedure was capable of pulling down the His-G-WhiB7 but the GST-RsbW did not co-elute with it (Figure 3.2). The GST-purification was successful for purifying GST-RsbW, and one out of four trials did pull-down the His-G-WhiB7 as well (Figure 3.3). This result could not be reproduced and may be due to a technical error.
Figure 3.1: WhiB7 and RsbW were co-expressed in *E. coli*. A western blot of various *E. coli* BL21(DE3) whole cell lysates probed with anti-His and anti-GST antibodies show successful co-expression. L is the protein standard ladder and the numbers indicate the kDa weight of the standard bands and + is the positive His-control (His-tagged *Caulobacter spp.* S-layer [provided by Jan Mertens, Smit Lab]). Lanes A-C are whole cell lysates from *E. coli* cultures where A expresses His-G-WhiB7 (WhiB7) and GST-RsbW (RsbW), B expresses GST-RsbW and 6xHis peptide and C expresses GST and His-G-WhiB7. The strain expressing both fusion proteins GST-RsbW and His-G-WhiB7 is the co-expression strain to test binding between RsbW and WhiB7 (A) and the other strains are the purification controls (B and C). The red arrow indicates the GST-RsbW band, the green arrow indicates the His-G-WhiB7 band and the blue arrow indicates the GST band.
Figure 3.2: RsbW does not co-purify along with WhiB7. A western blot of *E. coli* BL21(DE3) cells expressing GST-RsbW and the His-G-WhiB7 fusion proteins: Lane 1 was loaded with whole cell lysate and lane 2 was loaded with the His-purified fraction. The membrane was probed with anti-His and anti-GST antibodies. The r indicates the expected GST-RsbW band and the w indicates the expected His-G-WhiB7 band. The GST-RsbW fusion protein is present in the whole cell lysate but not in the His-purified fraction as predicted.

Figure 3.3: Does WhiB7 co-purify with RsbW? A western blot of *E. coli* BL21(DE3) cells expressing GST-RsbW and the His-G-WhiB7 fusion proteins: Lane 1 was loaded with whole cell lysate and lane 2 was loaded with the GST-purified fraction. The membrane was probed with anti-His and anti-GST antibodies. The r indicates the expected GST-RsbW band and the w indicates the expected His-G-WhiB7 band.
3.2 Antibiotic sensitivity profiles of *M. smegmatis* *sigF* and *rsbW* strains

3.2.1 Generating *M. smegmatis* vector control and *rsbW*-OV

The cloning method for generating the vector control and *rsbW*-OV strains was described in subsection 2.3.3. The parent vector, pMV361, is an integrative vector that inserts one copy of the gene into the genome. The inserted gene is overexpressed via the heat shock promoter, hsp60. The success of the transformation was measured by performing a PCR on a genomic prep from each clone to amplify the insertion site of pMV361. Figure 3.4 shows the agarose gel with the expected bands present in Lane 1 at 329 bp and Lane 2 at 761 bp, confirming the transformations were successful.

![Agarose gel showing PCR products](image)

Figure 3.4: Transformation and integration of *rsbW* into *M. smegmatis* was successful. Two clones, one transformed with *rsbW* (under control of the hsp60 promoter) and one with an empty-vector control were grown. A genomic prep from each strain was subjected to PCR and the products were run on a 1% agarose gel, shown here. L is the ladder, 1 is the PCR from the clone transformed with pMV361 (empty-vector control) and 2 is PCR of the clone expressing *rsbW*. 
3.2.2 Comparing antibiotic susceptibilities in liquid and agar cultures

The antibiotic sensitivities of the *M. smegmatis* mc²6 wildtype strain and *M. smegmatis* mc²6 ∆sigF were determined using the resazurin or MTT assay in liquid microtiter culture. MICs were identical between *M. smegmatis* mc²6 wildtype strain and *M. smegmatis* mc²6 ∆sigF for different antibiotics (see Table 3.1).

The antibiotic sensitivities of *M. smegmatis* vector control and rsbW-OV strains were determined using the resazurin or MTT assay in liquid microtiter culture and the disc diffusion assay on agar lawns. In almost all cases, there was no significant difference in susceptibility of the two strains to the antibiotics tested (see Figure 3.5, Figure 3.7 and Table 3.2).

Similarly, the antibiotic sensitivities of *M. smegmatis* ∆whiB7 and *M. smegmatis* ∆whiB7+rsbW-OV were determined using the resazurin or MTT assay in liquid microtiter culture and the disc diffusion assay on agar lawns. There was no significant difference between the control strain and the overexpression strain in the wildtype or whiB7-null background, with the exception of an eight-fold greater sensitivity of the *M. smegmatis* ∆whiB7+rsbW-OV strain to azithromycin compared to *M. smegmatis* ∆whiB7 (see Figure 3.6, Figure 3.7 and Table 3.2).
Table 3.1: *M. smegmatis* sensitivity to antibiotics was not impacted by *sigF*.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. smegmatis</em> mc²6 wildtype strain</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>64</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>16</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>128</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>32</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2</td>
</tr>
<tr>
<td>Imipenem</td>
<td>64</td>
</tr>
<tr>
<td>Triclosan</td>
<td>8</td>
</tr>
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<td>Azithromycin</td>
<td>64</td>
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<tr>
<td>Clofazimine</td>
<td>1</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>8</td>
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<tr>
<td>Rifampicin</td>
<td>128</td>
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<td>Streptomycin</td>
<td>0.5</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 3.5: Antibiotic susceptibility of control *M. smegmatis* mc²6+pMV361 (empty-vector) versus *M. smegmatis* mc²6 *rsbW-OV* (*rsbW-OV*) in liquid microtiter cultures. Values were determined using the resazurin susceptibility or MTT susceptibility assay (as indicated).
Figure 3.6: Antibiotic susceptibility of *M. smegmatis* mc²6 Δ*whiB7* (whiB7-null) versus *M. smegmatis* mc²6 Δ*whiB7+rsbW-OV* (whiB7-null + rsbW-OV) in liquid microtiter *M. smegmatis* Δ*whiB7* (control) strain and the *M. smegmatis* mc²6 Δ*whiB7+rsbW-OV* strain.
Figure 3.7: Antibiotic susceptibility of *M. smegmatis* mc²6 rsbW-OV (A.), empty-vector control *M. smegmatis* mc²6+pMV361 (B.), *M. smegmatis* ∆whiB7 (C.) and *M. smegmatis* mc²6 ∆whiB7+rsbW-OV (D.) Antibiotics tested were tetracycline (TET), erythromycin (ERY) and spectinomycin (SPEC) with final µg applied indicated centrally. Halos of inhibition for each assay are indicated (in mm).
Table 3.2: Comparison of halos of inhibition of various *M. smegmatis* strains in response to antibiotic treatment.

<table>
<thead>
<tr>
<th>Antibiotic (total applied)</th>
<th>Diameters of halos of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>M. smegmatis mc^2^6 strains</em></td>
</tr>
<tr>
<td></td>
<td>empty-vector control</td>
</tr>
<tr>
<td>Tetracycline (1.6 µg)</td>
<td>30</td>
</tr>
<tr>
<td>Erythromycin (5 µg)</td>
<td>9.5</td>
</tr>
<tr>
<td>Spectinomycin (80 µg)</td>
<td>11</td>
</tr>
</tbody>
</table>
Discussion

The premise for exploring the relationship between whiB7, sigF and rsbW and their gene products was four-fold: whiB7 is strongly implicated in regulating intrinsic antibiotic resistance [6,26]; an M. tuberculosis sigF-null mutant was reported to be hypersensitive to the rifamycin drugs, rifapentine and rifampicin, exclusive of changes in cell wall permeability [8]; RsbW is known to interact directly with and negatively regulate SigF [3]; and WhiB7 and RsbW demonstrated direct interaction in a yeast two-hybrid assay (R. Morris, personal communication).

Based on this cumulative evidence, I hypothesized that RsbW alternately and negatively regulated WhiB7 or SigF, depending on the cellular stress conditions. This hypothesis is depicted in Figure 4.1.
Figure 4.1: Hypothesized relationship between WhiB7, SigF and RsbW. Arrows indicate known (black) and likely (grey) protein-protein binding interactions. It is unknown what kind of relationship, if any, exists between WhiB7 and SigF (white arrow).
To test this hypothesis, I looked for evidence direct protein-protein binding in another system. I chose to clone and express *M. tuberculosis* genes in *E. coli* in order to take advantage of the expression plasmids available for *E. coli* systems. I expressed the *rsbW* with an N-terminal fusion of the GST tag and the *whiB7* with an N-terminal fusion of 6xHis-GB1 on two different plasmids carrying different resistance genes. The dual resistance of successful co-transformations allowed me to efficiently select for co-transformants.

I was successfully able to co-express the GST-RsbW and His-G-WhiB7 fusion proteins (see Figure 3.1). Because both proteins were tagged, I was able to perform a pull-down experiment by purifying either the GST tag or the 6xHis tag (Figure 3.2, Figure 3.3). These purification experiments gave conflicting results. The GST purification, expected to purify GST-RsbW and hypothesized to pull-down the His-G-WhiB7 did appear, in one instance, to do so (Figure 3.3). This result is questionable because it was seen once among eight trials (two purifications of four cultures each). It is more likely that the positive result was contamination of the whole cell lysate (containing both proteins) than a true pull-down.

Consistent with the majority of the GST-RsbW pull-down results, the His-purification, expected to purify the His-G-WhiB7 and hypothesized to pull-down the GST-RsbW, never showed the GST-RsbW present in the purified fraction.

In parallel to these pull-down experiments, I compared the antibiotic sensitivity spectrums of the various *M. smegmatis* strains: wildtype (mc²6), Δ*sigF*, Δ*whiB7*, *M. smegmatis* mc²6+pMV361 (vector control), *rsbW*-OV and Δ*whiB7*+*rsbW*-OV. Each strain was treated with 16 different antibiotics representing a variety of antibiotic classes and targeting diverse cellular processes. The Δ*whiB7* strains showed a much higher sensitivity to antibiotics, as expected (see Figure 3.7).

The MIC values measured in the resazurin or MTT studies are not predicted to be absolute. Generally, the actual MIC is expected to fall within a 2-fold range of any given experimental result. By this standard, the Δ*sigF* did not demonstrate any aberrant sensitivity (see Table 3.1), even to rifampicin, which was surprising because the Δ*sigF* strain had been reported to be hypersensitive to rifamycin drugs ([8]).

According to the hypothesis that *rsbW* negatively regulates *whiB7*, I expected to see a sensitivity profile that mimicked the Δ*whiB7* strain in the *rsbW*-OV strain. Instead, over-expression of RsbW in both the wildtype- or Δ*whiB7*-background failed to show any difference in sensitivity as compared to control strains (Figure 3.5, Figure 3.6).

The negative results of the protein pull-down experiments suggest that
RsbW and WhiB7 do not directly interact with each other on the protein level. The negative results of the antibiotic sensitivity assay further suggests that the genes or gene products do not interact with each other in a way that affects antibiotic sensitivity in \textit{M. smegmatis}.

Initially, the failure of the \textit{M. smegmatis} $\Delta$sigF strain to show hypersensitivity to rifamycin was disturbing because it conflicted with previously published results. However, in August 2010, Hartkoorn et al. reported that overexpression or deletion of \textit{sigF} did not alter susceptibility to rifampin, contrary to a previous report, consistent with my findings \cite{15}.

The Hartkoorn et al. manuscript called into question the premise that \textit{sigF} played a role in antibiotic resistance. Additionally, the susceptibility studies in this work suggest that RsbW does not play a large role in antibiotic resistance. The overexpression of \textit{rsbW} did not affect the susceptibility of \textit{M. smegmatis}, although the overexpression was only about four-fold above basal levels (C. Schumacher, qRT-PCR data not shown).

The hypothesis is further voided with the findings that GST-RsbW was unable to consistently pull-down His-G-WhiB7 and that His-G-WhiB7 was unable to pull-down GST-RsbW. These data suggest that these two proteins do not directly bind one another.

While each individual experiment could be optimized further, the deluge of negative results strongly suggests that the hypothesis that RsbW alternately and negatively regulates WhiB7 or SigF is incorrect.
Part II

Drug-drug interaction screening
Materials and methods

5.1 Bacterial strains and culture conditions

The Mycobacterial species and strains used in this work are described in Table 5.1. All Mycobacterium spp. were grown in Difco Middlebrook 7H9 Broth (BD; per 180 mL: 0.94 g), with 2% glycerol (Sigma) supplemented with 20 mL Albumin Dextrose Catalase (per 1000 mL: 8.5 g NaCl [Sigma], 50 g Bovine Serum Albumin [Sigma], 20 g D-Glucose [Bio Basic Inc.], 0.03 g catalase [Sigma]) or on Difco Middlebrook 7H10 agar (BD; 3.8 g per 180 mL: ) supplemented with 20 mL Difco BBL Middlebrook OADC Enrichment (BD).

The drug-drug interaction screens were performed on NE agar, 7H9-agar and in liquid 7H9, as described below.

For all screens, single cultures of M. smegmatis, M. bovis BCG and M. bovis BCG (lux) were grown to stationary phase, dispensed into 1 mL aliquots, frozen at -20°C overnight and stored at -80°C until use.

Other bacterial species tested for susceptibility are described in Table 5.2. All species were grown in LB Broth media (Sigma; per 1000 mL: 20 g LB Broth) and on LB 1.5% agar (Sigma; per 1000 mL: 20 g LB Broth and 15 g Agar).

5.2 Antibiotic combinations used in drug-drug interaction screening

This methodology was designed to test for drug-drug interactions between pairs of compounds. Each pair consisted of a primary compound (1°) and
Table 5.1: Mycobacterial strains used in these studies

<table>
<thead>
<tr>
<th>Species or strain</th>
<th>Purpose</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>susceptibility studies</td>
<td>Dr. Richard Stokes’ Lab</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> CDC1551</td>
<td>susceptibility studies</td>
<td>Dr. Richard Stokes’ Lab</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Erdman</td>
<td>susceptibility studies</td>
<td>Dr. Richard Stokes’ Lab</td>
</tr>
<tr>
<td><em>M. smegmatis</em> mc²155</td>
<td>NE agar screen,</td>
<td>ATCC 700084</td>
</tr>
<tr>
<td></td>
<td>susceptibility studies</td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em> BCG</td>
<td>7H9-agar screen</td>
<td>Pasteur 1173P2</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG/[lux] [34]</td>
<td>liquid 7H9 screen</td>
<td>Dr. Yossef Av-Gay’s Lab</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>susceptibility studies</td>
<td>ATCC 25291</td>
</tr>
</tbody>
</table>

Table 5.2: Other bacterial species tested for susceptibility

<table>
<thead>
<tr>
<th>Species or strain</th>
<th>Gram-positive or -negative</th>
<th>Pathogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Gram-negative</td>
<td>Pathogenic</td>
</tr>
<tr>
<td><em>Streptomyces lividans</em> 1326</td>
<td>Gram-positive</td>
<td>Non-pathogenic</td>
</tr>
<tr>
<td><em>Rhodococcus jostii</em> RHA1</td>
<td>Gram-positive</td>
<td>Non-pathogenic</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> ATCC 1960</td>
<td>Gram-negative</td>
<td>Opportunistic</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PA01(H103)</td>
<td>Gram-negative</td>
<td>Opportunistic</td>
</tr>
<tr>
<td><em>Kocuria rhizophila</em></td>
<td>Gram-positive</td>
<td>Non-pathogenic</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>Gram-positive</td>
<td>Pathogenic</td>
</tr>
</tbody>
</table>
a secondary partner compound (2°). The 1° compounds were selected because they are currently used in tuberculosis therapy or because they have particularly interesting properties that make them candidates for possible future tuberculosis therapy (see Table 5.3). The MIC of each 1° compound was determined experimentally for each method.

The 2° compounds came from the Sweet Library which was compiled by Dr. Gaye Sweet (Thompson Lab). This library was assembled because results from a previous screen showed that synergistic hits were enriched for antibiotics partnered with other antibiotics [34]. The Sweet Library contains 594 compounds, the majority selected because they have antimicrobial activity. Each compound was dissolved in its optimal solvent to 5 mM, aliquoted into microtiter plates and stored at -80°C.

5.3 Screening for drug-drug interactions against mycobacteria

There were three screens carried out to identify compound combinations that were effective against mycobacteria.

1. NE agar screen: combinations of 1° and 2° compounds applied to M. smegmatis grown on NE agar.

2. 7H9-agar screen: combinations of 1° and 2° compounds applied to M. bovis BCG(lux) grown on 7H9-agar.

3. Liquid 7H9 screen: combinations of 1° and 2° compounds applied to M. bovis BCG(lux) grown in liquid 7H9.

5.3.1 Screen for drug-drug interactions against M. smegmatis on NE agar

Screening was carried out by transferring microliter volumes of the Sweet Library compounds to a lawn of bacteria growing on NE agar with sub-inhibitory concentrations of the 1° compounds. For this screen, the 1° compounds were clarithromycin, domperidone, ethambutol, moxifloxacin and rifampicin.

First, the MIC, on agar, of each 1° compound was determined by the following procedure: 60 mm × 15 mm petri dish plates (Fisherbrand) were poured with two layers of agar. The bottom layer was 10 mL NE 1.5% agar (per 1000 mL: 10 g D-glucose, 2 g yeast extract [Sigma], 2 g casamino acids
Table 5.3: The primary compounds used in the drug-drug interaction screens and the rationale for including each.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Media, bacteria (MIC in µg/mL)</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarithromycin</td>
<td>NE agar, <em>M. smegmatis</em> (8) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (0.125)</td>
<td>treat TB and <em>M. avium</em> infections [1]</td>
</tr>
<tr>
<td>Clofazimine</td>
<td>7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (0.125)</td>
<td>last-resort TB drug [1]</td>
</tr>
<tr>
<td>Domperidone</td>
<td>NE agar, <em>M. smegmatis</em> (264) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (128)</td>
<td>butyrophenone, repurposing [34]</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>NE agar, <em>M. smegmatis</em> (0.5) 7H9-agar, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (4) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (2)</td>
<td>current front-line TB drug [1]</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>NE agar, <em>M. smegmatis</em> (0.5) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (2)</td>
<td>current front-line TB drug [1]</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>NE agar, <em>M. smegmatis</em> (4) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (4)</td>
<td>repurposing</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>NE agar, <em>M. smegmatis</em> (0.125)</td>
<td>last-resort TB drug [1]</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>NE agar, <em>M. smegmatis</em> (64) 7H9-agar, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (64) 7H9 liquid, <em>M. bovis</em> BCG &lt;sup&gt;(lux)&lt;/sup&gt; (0.03125)</td>
<td>current front-line TB drug [1]</td>
</tr>
</tbody>
</table>

[Sigma], 1 g Lab Lemco powder [Sigma] and 15 g agar, pH 7.0), the top layer was 5 mL NE 0.5% agar (same as NE 1.5% agar, except this includes only 5 g of agar). The 1° antibiotics were added to both layers of agar at a range of concentrations, one concentration per plate. The top layer of agar also included *M. smegmatis* diluted to OD<sub>600</sub>=0.06 from frozen stock (stationary phase). The 1° compounds tested in these studies were clarithromycin, domperidone, ethambutol, ivermectin and rifampicin.

The screen was performed with the 1° compounds at 8-fold and 4-fold below the previously determined MIC (1/8 MIC and 1/4 MIC) in combination with the Sweet Library compounds. The MIC for each compound are listed
in Table 5.3.

First, bottom agar (NE 1.5% agar) was freshly prepared and 1° antibiotic added directly to the molten agar at either $\frac{1}{8}$ MIC or $\frac{1}{4}$ MIC. Twenty-two mL of bottom agar were pipetted into a sterile, single-well omni tray (Nunc, from VWR). This layer was allowed to solidify. Next, top agar (NE 0.5% agar) was freshly prepared, 1° antibiotic added to the molten agar at either $\frac{1}{8}$ MIC or $\frac{1}{4}$ MIC and *M. smegmatis* diluted to OD$_{600}$=0.06 in the molten agar. Twenty-two mL of the top agar layer were pipetted evenly on top of the solidified bottom agar, then allowed to set. Control plates were prepared without antibiotics in the agar.

The Sweet Library compounds (thawed) were shaken on a Titer Plate Shaker (Banstead Lab-Line) for 1 minute, then centrifuged at 1000 $\times$ g for 5 minutes (Sorvall LegendRT). Using the Boekel 96 pin replicator, the Sweet Library compounds were transferred to the solidified top layer of agar. The replicator was rinsed in 70% ethanol and flame sterilized. The omnitrays were covered in foil and incubated at 37°C for 3 days. All pipetting and pinning steps were performed in a biological safety cabinet.

After incubating for 4 days, the plates were scanned with a Canon CanoScan 5600F machine, and analysis was performed using the scanned images.

### 5.3.2 Screening for drug-drug interactions against *M. bovis* BCG on 7H9-agar

The preliminary set-up and the screening was carried out similarly to the *M. smegmatis* drug-drug interaction screen, as described in subsection 5.3.1, with a few exceptions: bottom agar was 7H9-1.5% agar (per 180 mL: 0.94 g 7H9 [BD], 3 g agar [Sigma]) supplemented with OADS (20 mL OADS per 200 mL 7H9-agar. OADS per 1000 mL: 9.5 g NaCl [Sigma], 50 g Bovine Serum Albumin [Sigma], 20 g D-glucose [Bio Basic Inc.], 268 mg oleic acid [Sigma]) and top agar was 7H9-0.5% agar (same as 7H9-1.5% agar, except with 1 g agar) supplemented with 20 mL OADS. There was no glycerol in the media. *M. bovis* BCG was diluted in top agar to OD$_{600}$=0.015 from frozen stock. The 1° compounds tested in these studies were ethambutol and rifampicin. Omnitrays were incubated for 3 weeks at 37°C with 5% CO$_2$. 

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5.3.3 Screening for drug-drug interactions against *M. bovis* BCG in liquid 7H9 microtiter cultures

The MIC of the 1° compounds used in this screen (clarithromycin, clofazimine, domperidone, ethambutol, ivermectin and rifampicin) were determined in microtiter cultures by the MTT assay. Cultures were grown in 7H9 media supplemented with ADS (20 mL ADS per 200 mL 7H9. ADS per 1000 mL: 9.5 NaCl [Sigma], 50 g Bovine Serum Albumin [Sigma], 20 g D-glucose [Bio Basic Inc.] and 2% glycerol) in 96-well microtiter plates (Costar) at 100 µL total volume, with *M. bovis* BCG (lux) diluted to OD_{600} = 0.00625. The 1° compounds were prepared fresh and applied in two-fold serial dilutions. The cultures were incubated for 7 days at 37°C with 5% CO₂. After incubation, 50 µL of 5 mg/mL MTT was added to each well. The plates were incubated overnight at 37°C, then 50 µL of 10% SDS (w/v) was added and cultures were incubated overnight at 37°C. Finally, absorbance was read on the Varioskan Flash (made available by Dr. Steven Hallam) at wavelength 560 nm, bandwidth 5 nm and measurement time 100 ms (per well).

The values obtained for each well were compared to control wells. A culture was determined to be viable if the absorbance indicated growth greater than 10% (or there was less than 90% growth inhibition) of the non-treated culture. The lowest concentration that can achieve this growth inhibition is called the MIC₉₀. In this experiment, growth was evaluated using the MTT cell viability assay, where viable cultures reduce the yellow tetrazolium MTT to purple formazan. A spectrophotometer quantifies this conversion by measuring the absorbance at 560 nm. The MIC₉₀ is the lowest concentration of compound (in a series of dilutions) required to inhibit growth of 90% of the culture. To calculate the absorbance value that delineates the threshold between a growing culture (growth > 10%) from an inhibited culture (growth < 10%), the following formula is used:

\[
\text{Threshold} = [(\text{Absorbance}_{\text{viable}} - \text{Absorbance}_{\text{bkgrd}}) \times 0.1] + \text{Absorbance}_{\text{bkgrd}}
\]

The threshold is calculated by the absorbance of the control cultures (viable, no compounds applied) adjusted for background absorbance (no cells, only media).

Once the MICs for each of clarithromycin, clofazimine, domperidone, ethambutol, ivermectin and rifampicin were established, the drug-drug interaction screen was performed. For every pairwise combination of 1° and 2°, three concentrations of 1° compound (0 µg/mL or no compound, \(\frac{1}{5}\) MIC and \(\frac{1}{4}\) MIC) were combined with eight concentrations of 2° compound (four-fold dilutions starting from 100 µM and decreasing to 25 µM, 6.25 µM,...,
6.1 nM). This combination scheme is presented diagramatically in Figure 5.1 for a single combination of 1° compound + 2° compound.

The screen was carried out using 96-well microtiter plates. For every test plate a single 2° compound was applied in every column in 1/4 dilutions from A through H, starting at 100 µM. Three plates were set-up for every 2° compound in order to combine the 2° compound dilution series with the three concentrations of the 1° compound at 0 µg/mL, 1/8 MIC and 1/4 MIC. The layout of one set of 96-well plates is depicted in Figure 5.2.

This set-up was performed in batches for all combinations of six 1° compounds and 594 2° compounds. After set-up, the plates were incubated for 7 days at 37°C with 5% CO₂. After incubation, 50 µL of 5 mg/mL MTT was added to each well. The plates were incubated overnight at 37°C, then 50 µL of 10% SDS (w/v) was added and cultures were incubated overnight at 37°C. Absorbance was read on the Varioskan Flash at wavelength 560 nm, bandwidth 5 nm and measurement time 100 ms (per well). The output of the absorbance readings was one Microsoft Excel® spreadsheet per 96-well plate. To compare the MIC₉₀(2°) of the pairwise combinations for the changing 1° compound (0 µg/mL, 1/8 MIC and 1/4 MIC), a custom software program was developed (described in section 5.3.3).
Figure 5.1: A representation of how each pair of $1^\circ$ and $2^\circ$ compound were combined in the 7H9 liquid culture drug-drug interaction screen. The black drops (solid and lined) represent the $1^\circ$ compound (clarithromycin, clofazimine, domperidone, ethambutol, ivermectin or rifampicin) and the blue drops represent the $2^\circ$ compounds of the Sweet Library. The $1^\circ$ compounds were held constant at one of two different concentrations ($\frac{1}{8}$ MIC and $\frac{1}{4}$ MIC) and the $2^\circ$ compounds were used in a series of eight four-fold dilutions with concentrations ranging from 6.1 nM to 100 µM (size of the drop implies concentration). Theoretically, if the drugs interact in synergy the concentration of the $2^\circ$ compound required to inhibit bacterial growth will drop when comparing its use alone with its use in combination with the $1^\circ$ compound as illustrated by the inhibition/no inhibition labels.
Figure 5.2: Schematic representation of the drug-drug interaction screen against *M. bovis* BCG (*lux*) in liquid 7H9 microtiter culture. The wells are coloured to represent the presence of compounds and the arrows indicate the direction of the serial dilutions. The control plate has cultures of the three 1° concentrations used - 0 µg/mL or no compound (white), $\frac{1}{8}$ MIC (pale orange) and $\frac{1}{4}$ MIC (orange). The control plate also has two columns of cells treated with serial $\frac{1}{2}$ dilutions of isoniazid as a control for inhibition (yellow). Finally, the control plate has a positive control for synergy: in column 9 spectinomycin is applied in serial $\frac{1}{2}$ dilutions (green), in column 10 bromperidol is applied in serial $\frac{1}{2}$ dilutions (red) and in columns 11-12 spectinomycin is applied at 1 µg/mL and bromperidol is applied in serial $\frac{1}{2}$ dilutions (green and red).

The other three plates represent the replicate plates for a single 1° compound combined with twelve 2° compounds. Each plate contains either 0 µg/mL, $\frac{1}{8}$ MIC or $\frac{1}{4}$ MIC in all wells (white, pale orange and orange respectively). Each column in each plate contains serial $\frac{1}{2}$ dilutions, where each column (1-12) across these three plates contains the same 2° compound and dilutions (indicated by the replicate colours).
Hit analysis by custom software program

The output data from the interactions screen in liquid 7H9 microtiter culture, described in subsection 5.3.3, existed as Microsoft Excel® spreadsheets (one for every 96-well plate). To evaluate this data efficiently and consistently, I developed a custom computer program, “Screen Analysis” using the Python™ programming language, with teaching and support by Shane Mottishaw.

The program took the input (the Excel spreadsheets) and user parameters (such as what MIC endpoint to use, the default was 90) to process the data and provide the analyses as a single Microsoft Excel® spreadsheet containing a list of every combination of compounds and the MIC of the $2^\circ$ compound alone and when combined with each concentration of each $1^\circ$ compound ($\frac{1}{2}$ MIC and $\frac{1}{4}$ MIC). The process of analysing the data is shown, simplified, in Figure 5.3. The code is printed in Appendix B.

Using Microsoft Excel® tools, the data was mined for $2^\circ$ compounds that partnered synergistically with multiple $1^\circ$ compounds and for combinations that were extremely potent together.
Figure 5.3: A simplified representation of the algorithm used to determine which combinations were synergistic. This algorithm works with Microsoft Excel® spreadsheets and the cells described here refer to spreadsheet cells. The input is all the raw data (in Microsoft Excel® spreadsheet form) and the output is a single Microsoft Excel® spreadsheet which lists all combinations categorized as positive/negative for a hit and the MIC values of the 2° compounds for every given combination.
5.4 Validating the results of the screens using the checkerboard assay

The checkerboard assay is a two-dimensional array of combinations of two compounds [23, p. 367]. Each compound is applied in a range of concentrations. Every concentration of one compound is combined with every concentration of the second compound. Every combination of the two compounds (within their respective ranges) exists within the checkerboard.

To evaluate the results of a checkerboard, the fractional inhibitory concentration index (FICI) is calculated. First, the fractional inhibitory concentration (FIC) if each compound was determined by:

\[
FIC_{\text{drug A}} = \frac{\text{MIC}(\text{drug A when combined with drug B})}{\text{MIC}(\text{drug A alone})}
\]

The FICI is determined by addition of the two FICs:

\[
FICI = FIC_{\text{drug A}} + FIC_{\text{drug B}}
\]

Combinations are categorized, according to their FICI, as synergistic when FICI ≤ 0.5, indifferent when 0.5 < FICI ≤ 4.0 and antagonistic when FICI ≥ 4.0 [23, p.372]. In order to identify combinations that had a mildly synergistic interaction, this work included a category called potentiation when 0.5 < FICI ≤ 1.0.

Checkerboard assay in 7H9 liquid culture

Checkerboard assays were set up as one pairwise combination per 96-well plate. The 1° compound was diluted vertically, from high to low (rows G-A) and the 2° compound was diluted horizontally, from high to low (columns 2-11). Column 12 contained control wells. The set up is depicted in Figure 5.4.

Checkerboard assays for mycobacteria were performed in 7H9 + ADS + glycerol, where checkerboard assays for other bacterial species (listed in Table 5.2) were performed in LB media. Bacteria were inoculated at a final OD\textsubscript{600}=0.00625. All M. tuberculosis and M. bovis BCG strains were incubated for 7 days at 37°C + 5% CO\textsubscript{2} and all other bacterial strains were incubated for 3 days at 37°C. After incubation, 50 µL of 5 mg/mL MTT was added to each well. The plates were incubated overnight at 37°C, then 50 µL of 10% SDS (w/v) was added and cultures were incubated overnight at 37°C.
To determine the MIC of each compound, the absorbance of each well was read using the Varioskan Flash (made available by Dr. Steven Hallam) at wavelength 560 nm, bandwidth 5 nm and measurement time 100 ms. The MIC was determined as the MIC$_{90}$, using the formula described in subsection 5.3.3. These MICs were used to calculate the FICs and FICI.

5.4.1 Agar lawn checkerboard assay

An agar lawn checkerboard for the combination of ethambutol + compound Sweet-1F4 was performed by growing _M. smegmatis_ lawns in individual 60 mm × 15 mm petri dish plates, prepared one-by-one with the various combinations of both compounds to make up the checkerboard array. Briefly, _M. smegmatis_ was diluted to OD$_{600}$=0.0125 directly into molten NE 1.5% agar. The molten agar was divided into smaller aliquots and various combinations of concentrations of ethambutol and compound Sweet-1F4 were added to each aliquot. Then 10 mL aliquots were pipetted into the petri dishes and allowed to set. Plates were incubated at 37°C for 3 days.

The MIC of ethambutol (alone), compound Sweet-1F4 (alone) and ethambutol + compound Sweet-1F4 was determined by eye, based on visual assessment of growth of an _M. smegmatis_ lawn. These MIC values were used to calculate the FICs and FICI.

5.4.2 Disc diffusion assays

_M. smegmatis_ was diluted to OD$_{600}$=0.0125 directly into molten NE 1.5% agar with and without sub-inhibitory concentrations of one drug. Ten milliliters of molten agar was pipetted into a 60 mm × 15 mm petri dish plate and allowed to set. A paper disc (BBL™, 6 mm diameter) was saturated with 28.5 μL of solution containing the second antibiotic. The disc was placed on the surface of the agar and plates were incubated at 37°C for 4 days. The halo of inhibition was measured (diameter, in mm).
Figure 5.4: Pictorial representation of the liquid culture checkerboard assay. The 1° compound is serially diluted two-fold through rows G to A (orange colour). The 2° compound is serially diluted two-fold through columns 2-11 (green colour). Column 12 and well H1 are control wells: *M. bovis* BCG(*lux*) with no treatment (red lines) and media-only (grey hatching).
Alternative liquid microtiter drug-drug interaction assay (potentiation assay)

The liquid checkerboard assay is labour intensive to set-up. To achieve efficient throughput of validating the drug-drug interactions of hits, an alternative assay was developed (potentiation assay). It uses the same culture conditions as the liquid checkerboard, but a different plate set-up. The principle of the FICI was used as the basis for this higher throughput protocol: the $1^\circ$ compounds were used at 0 µg/mL, $\frac{1}{8}$ MIC and $\frac{1}{4}$ MIC and the $2^\circ$ compound was used in combination as a dilution series. On a microtiter plate, dilution of the $2^\circ$ compound was performed vertically from A through H, while the concentration of the $1^\circ$ was varied column by column. This set-up is represented in Figure 5.5.

Using this method the $1^\circ$ compound is used in the combination at either $\frac{1}{4}$ MIC or $\frac{1}{8}$ MIC. Therefore, at the point of growth inhibition with the combination of compounds, the $1^\circ$ compound will have an individual FIC of 0.25 or 0.125. The $2^\circ$ compound can be screened for a four-fold reduction of MIC across the different concentrations of the $1^\circ$ compound (which gives an individual FIC of 0.25 or less, and a combined FICI \(\leq 0.5\), indicating synergy).
Figure 5.5: Pictorial representation of an alternative assay (to the checkerboard assay), called a potency assay, used for validating the type of interaction of two compounds. The 1° compound (red) is used in serial $\frac{1}{2}$ dilutions in column 2, and the theoretical $\frac{1}{4}$ MIC and $\frac{1}{8}$ MIC in columns 4-5 and 6-7, respectively. The 2° compound (green) is used in serial $\frac{1}{2}$ dilutions in column 1. Column 3 contains no compound, only cells (grey hatched). The 1° and 2° compounds are combined (mixed red and green), holding the 1° compound constant at either the theoretical $\frac{1}{4}$ MIC or $\frac{1}{8}$ MIC (8-9 and 10-11, respectively), while varying the 2° compound in the $\frac{1}{2}$ dilution, as in column 1. Column 12 is a control with no cells (white).
5.5 Antimycobacterial activity of ivermectin

Ivermectin and other avermectins, doramectin, moxidectin and selamectin, were tested for activity against mycobacteria.

5.5.1 Determining the MICs of four avermectins

The MIC of the avermectins (doramectin, ivermectin, moxidectin and selamectin [Sigma, Alpha Diagnostics Intl. Inc., Fluka Analytical and European Pharmacopeia,]) were determined for *M. smegmatis*, *M. avium*, *M. bovis* BCG and *M. tuberculosis*, as well as the bacterial species listed in Table 5.2, in liquid culture using the MTT assay as described in subsection 2.5.1.

The MIC of ivermectin was determined for *M. smegmatis* in NE agar by inoculating serial dilutions of the compound directly into molten NE agar. *M. smegmatis* was spread on the surface of the solid agar and incubated at 37°C for 4 days. Growth of an *M. smegmatis* lawn was determined visually.

5.5.2 Evidence for bacteriostatic versus bactericidal activity

To explore whether ivermectin acted as a bacteriostatic or bactericidal agent *M. bovis* BCG was treated with 64 µg/mL of ivermectin (8x MIC) and the OD$_{600}$ was measured over time. Briefly, 100 mL 7H9 was inoculated with *M. bovis* BCG at OD$_{600} = 0.0625$. Cultures were treated with 0 µg/mL ivermectin (control) or 64 µg/mL ivermectin and grown at 37°C with shaking. At Day 0, 4, 10 and 14, the OD$_{600}$ of the culture was measured using a BioPhotometer (Eppendorf). Additionally, following the final OD$_{600}$ measurement, an aliquot of each culture was inoculated into fresh 7H9 media and incubated at 37°C with rotation for over 10 generation periods to determine whether cells could grow when ivermectin was removed.
Results

6.1 Screen for drug-drug interactions against *M. smegmatis* and *M. bovis* BCG on agar

The interactions screening against *M. smegmatis* was carried out on NE agar as described in subsection 5.3.1. Pairs of compounds were combined by putting the 1\(^\circ\) compound in the agar with the 2\(^\circ\) compounds applied by pinning. The outcome of those experiments were plates such as those shown in Figure 6.1.

To look for interesting combinations, the halo of inhibition around each 2\(^\circ\) compound was compared under three conditions: 0 µg/mL 1\(^\circ\) antibiotic in the agar, 1/8 MIC 1\(^\circ\) antibiotic in the agar and 1/4 MIC 1\(^\circ\) antibiotic in the agar. The 1\(^\circ\) compounds were clarithromycin, domperidone, ethambutol, moxifloxacin and rifampicin. Potentiation or synergy between two compounds was identified when the halo of inhibition of a single 2\(^\circ\) compound increased from the plate with 0 µg/mL to either or both the 1/8 MIC and 1/4 MIC plates for a given 1\(^\circ\) antibiotic.

Similarly, the drug-drug interactions screen against *M. bovis* BCG on agar was carried out on 7H9-agar + OADS using the 1\(^\circ\) compounds ethambutol and rifampicin, as described in subsection 5.3.2. A hit analysis was performed by comparing halo sizes (as described above).

6.1.1 Validation of hits in a liquid culture assay

Lead compound combinations from the 7H9-agar interactions screen (for *M. bovis* BCG) were further analyzed by Keerat Sidhu in liquid microtiter culture (7H9 + ADS + glycerol) using a potentiation assay as described
Figure 6.1: An example of the *M. smegmatis* NE agar lawn drug-drug interaction screen. Halos of inhibition appear dark. On all three plates shown here $2^\circ$ compounds of the Sweet Library have been transferred onto the agar by pinning. Plate A, 0 µg/mL $1^\circ$ antibiotic in the agar; B, $\frac{1}{8}$ MIC $1^\circ$ antibiotic in the agar; and C, $\frac{1}{4}$ MIC $1^\circ$ antibiotic in the agar (C). The red arrow indicates a halo of inhibition that increases as the concentration of the $1^\circ$ increases, which is a positive hit for potentiation or synergy.
in section 5.4.2. These experiments hold the $1^\circ$ compound concentration constant at pre-established $\frac{1}{8}$ MIC and $\frac{1}{4}$ MIC and vary the $2^\circ$ compound concentration by serial dilution. This was performed in liquid microtiter culture and relied on the MTT assay to test for viability.

The results of this validation showed some combinations that acted as potentiatiors and some combinations that did not have any confirmed interaction (see Table 6.1).
Table 6.1: Validation results of selected interaction hits from the *M. bovis* BCG interactions screen in 7H9-agar. This assay was performed in liquid 7H9 microtiter culture holding the concentration of the 1° compound constant at $\frac{1}{8}$ MIC and $\frac{1}{4}$ MIC and combining the 2° compound in serial dilution fashion. Viability was determined by the MTT assay. The compound interaction of each combination was determined to be synergy (FICI $\leq 0.5$), potentiation ($0.5 < $ FICI $\leq 1.0$) or no interaction ($1.0 < $ FICI $< 4.0$).

<table>
<thead>
<tr>
<th>1° compound</th>
<th>2° compound</th>
<th>Synergy, potentiation or no interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethambutol</td>
<td>Amikacin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Apramycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Chetomin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>compound Sweet-1C11</td>
<td>potentiation</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>compound Sweet-1F4</td>
<td>potentiation</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Moxidectin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>compound Sweet-2C2</td>
<td>potentiation</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Isepamicin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Ethambutol</td>
<td>Kanamycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>compound Sweet-4A6</td>
<td>potentiation</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Apramycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>BAY 11-7085</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Beauvericin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Capreomycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Carbenicillin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>compound Sweet-1C1</td>
<td>potentiation</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Chetomin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>Clopidol</td>
<td>no interaction</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Amikacin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Apramycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Capreomycin</td>
<td>no interaction</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>Clopidol</td>
<td>no interaction</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>compound Sweet-4H6</td>
<td>potentiation</td>
</tr>
</tbody>
</table>
6.1.2 M. smegmatis NE agar lawn validation of synergy between ethambutol and compound Sweet-1F4

The validation of only 6/23 combinations for potentiation and none for synergy, was unexpected (see Table 6.1). To determine whether culture conditions (agar versus liquid) may have contributed to a lack of validation, further experiments were performed on agar.

I tested the combination of ethambutol ($1^\circ$) and compound Sweet-1F4 ($2^\circ$) for synergy, although this combination showed only slight potentiation in the liquid 7H9 experiment. I used this combination of compounds in an NE agar checkerboard and in parallel with a liquid NE checkerboard. The results of the agar checkerboard validated synergy between ethambutol and compound Sweet-1F4. For ethambutol, $\text{MIC}_{\text{alone}} = 0.5 \ \mu\text{g/mL}$ but $\text{MIC}_{\text{combination}} = 0.125 \ \mu\text{g/mL}$. For compound Sweet-1F4, $\text{MIC}_{\text{alone}} = 128 \ \mu\text{g/mL}$ but $\text{MIC}_{\text{combination}} = 16 \ \mu\text{g/mL}$. This is a classical example of synergy and gives an FICI of 0.375 (see Figure 6.2). Interestingly, the liquid NE checkerboard did not validate synergy between ethambutol and compound Sweet-1F4, although it did suggest potentiation (see Appendix Table A.1).

The disc diffusion assay (on NE agar, described in subsection 5.4.2) was performed to better understand whether the method of application of the compounds affected the outcome of a synergy test. Ethambutol was applied by paper disc to an agar lawn with and without sub-inhibitory concentrations of compound Sweet-1F4. Visual comparison (see Figure 6.3) shows halos of inhibition are much greater in the presence of sub-inhibitory concentrations of compound Sweet-1F4 which indicates potentiation or synergy (this qualitative analysis was inconclusive for synergy). Interestingly, compound Sweet-1F4 alone or in combination with sub-inhibitory concentrations of ethambutol had no significant effect (see Appendix Figure C.1), which suggests that the method of application of the compounds may play a role in the strength of a compound’s effectiveness and whether synergy occurs under given conditions.
Figure 6.2: FIC ethambutol versus FIC compound Sweet-1F4 in an NE agar checkerboard. The optimal FICI (indicated by the red point) is 0.375, positive for synergy.
Figure 6.3: Disc diffusion interaction assay for ethambutol and compound Sweet-1F4. *M. smegmatis* was grown as a lawn on NE agar with and without a sub-inhibitory 2 μg/mL concentration of compound Sweet-1F4 (left and right columns, respectively). Paper discs saturated with ethambutol (total ng as indicated) were applied to the lawn.
6.2 Drug-drug interactions screening in liquid 7H9 microtiter culture

Another round of screening was performed in liquid 7H9 microtiter culture using \textit{M. bovis} BCG\textit{(lux)}, as described in subsection 5.3.3. A computer program called "Screen Analysis" was designed to process the raw data of 786 Microsoft Excel\textsuperscript{®} files. This program analysed the entire data set and produced a list of positive leads. Positive leads were those \(2^\circ\) compounds that showed an MIC\textsubscript{90} decrease of at least four-fold when combined with \(\frac{1}{5}\) MIC and \(\frac{1}{4}\) MIC of one of the \(1^\circ\) compound (clarithromycin, clofazimine, domperidone, ethambutol, ivermectin, rifampicin), as compared to the MIC of the \(2^\circ\) compound used alone.

Table 6.2 shows the list of positive leads produced by the computer program for combinations with the \(1^\circ\) compound ivermectin. For the majority of the ivermectin leads, individual checkerboard assays were performed to determine the exact interaction of the compounds. The interaction of any two compounds, such as the data from a checkerboard assay, can be represented graphically in an isobologram. An isobologram plots the FIC of each compound across a range of concentrations for the combinations of two compounds. Figure 6.4 illustrates the types of curves seen in the isobolograms. By this analysis, none of the ivermectin combinations were positive for synergy although some combinations showed potentiation (Figure 6.5).

In parallel with the ivermectin combinations, I also assayed some strongly interacting rifampicin combinations identified by the computer. Individual checkerboard assays were performed to determine the FICIs, plot the isobolograms and determine the exact interaction of the compound combinations. Four out of 16 combinations were positive for synergy and seven out of the remaining 12 combinations showed potentiation. The compounds tested and their interactions are listed in Table 6.3. The isobologram graphs for the rifampicin combinations tested by the checkerboard assay are shown in Figure 6.6.
Figure 6.4: Isobolograms are graphs that plot the FICs of two compounds used in a combination. An FIC indicates the relative change in concentration when using a compound alone versus using a compound in combination. In the isobologram, every given combination of concentrations yields an FIC for each of the partner compounds. These FICs are plotted to show an FICI curve. This is an illustration of three categories of curves seen in isobolograms, with the FIC of compound A along the y-axis and the FIC of compound B increasing along the x-axis. U-shaped lines, hugging the x-axis and y-axis, represent synergistic combinations (A); mildly U-shaped lines, where $0.5 < \text{FICI value} \leq 1.0$ represent potentiation (B); and linear lines, or slightly humped lines ($1.0 < \text{FICI} \leq 4.0$), represent no interaction (C).
Table 6.2: Positive leads with ivermectin by “Screen Analysis” were tested in a checkerboard assay for synergy. The FICI of each combination was calculated and the interaction was labelled as synergy, potentiation, or no interaction.

<table>
<thead>
<tr>
<th>2° compound</th>
<th>FICI</th>
<th>Synergy, potentiation, or no interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptopyridine</td>
<td>1.0125</td>
<td>no interaction</td>
</tr>
<tr>
<td>4-Aminosalicylic acid</td>
<td>1.5</td>
<td>no interaction</td>
</tr>
<tr>
<td>Acivicin</td>
<td>n/a</td>
<td>no data</td>
</tr>
<tr>
<td>compound Sweet-2E7</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-4B3</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Aripiprazole</td>
<td>1.5</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-1B6</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Cannabidiol</td>
<td>n/a</td>
<td>no data</td>
</tr>
<tr>
<td>compound Sweet-1C1</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Danofloxacin</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>Diacylglycerol Kinase Inhibitor II</td>
<td>1.25</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-1F3</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-2G5</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Mezerein</td>
<td>1.5</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-2H11</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>Thioridazine</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-8D3</td>
<td>0.625</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-2D12</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
</tbody>
</table>
Figure 6.5: Isobolograms, based on MIC$_{90}$, of FICs ivermectin and $2^o$ compounds in liquid 7H9 checkerboard assays. The positive control is the known synergistic combination of spectinomycin and bromperidol [34]. The FIC of ivermectin is on the vertical axes and the FIC of the $2^o$ compounds (as indicated) are on the horizontal axes, except for the control, where the FIC of spectinomycin is on the vertical axis and the FIC of bromperidol is on the horizontal axis. The FICI of each pair is indicated on each graph. The points in red indicate an FICI ($FIC_A + FIC_B$) < 0.5, which defines synergy. The control was positive but no ivermectin combinations were validated for synergy.
Table 6.3: Combinations of compounds with rifampicin identified by “Screen Analysis” were tested in a liquid 7H9 checkerboard assay. The FICI of each combination was calculated and the interaction was labelled as synergy, potentiation, or no interaction.

<table>
<thead>
<tr>
<th>2° compound</th>
<th>FICI</th>
<th>Synergy, potentiation, or no interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acivicin</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-4B3</td>
<td>0.625</td>
<td>potentiation</td>
</tr>
<tr>
<td>Balofloxacin</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>Bismuth (III) subsalicylate</td>
<td>1.0</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-5H10</td>
<td>0.15625</td>
<td>synergy</td>
</tr>
<tr>
<td>compound Sweet-5H11</td>
<td>0.14</td>
<td>synergy</td>
</tr>
<tr>
<td>compound Sweet-2E9</td>
<td>0.1875</td>
<td>synergy</td>
</tr>
<tr>
<td>Cryptotanshinone</td>
<td>1.25</td>
<td>no interaction</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>1.5</td>
<td>no interaction</td>
</tr>
<tr>
<td>compound Sweet-4G9</td>
<td>0.625</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-5A6</td>
<td>0.625</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-5B8</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-7F5</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-8A2</td>
<td>0.1875</td>
<td>synergy</td>
</tr>
<tr>
<td>compound Sweet-5G1</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
<tr>
<td>compound Sweet-2D12</td>
<td>0.75</td>
<td>potentiation</td>
</tr>
</tbody>
</table>
Figure 6.6: Isobolograms, based on MIC$_{90}$, of FICs for rifampicin and 2° compounds in liquid 7H9 checkerboard assays. The FIC of rifampicin is on the vertical axes and the FIC of the 2° compounds are on the horizontal axes (as indicated). The FICI of each pair is indicated on each graph. The points in red indicate an FICI (FIC$_A$ + FIC$_B$) < 0.5, which defines synergy. Four compounds, compound Sweet-5H10, compound Sweet-5H11, compound Sweet-2E9 and compound Sweet-8A2 showed synergy with rifampicin.
6.3 Ivermectin is bacteriostatic against some *Mycobacterium* species

Since ivermectin has never, to my knowledge, been described to be effective against any bacteria, I designed experiments to better understand its activity. Firstly, I was interested in determining whether ivermectin was effective against many bacterial species, only mycobacterial species or only *M. smegmatis*. Secondly, I wanted to know whether ivermectin was uniquely effective, or whether closely related compounds within the avermectin family might also be effective. To explore these questions, I tested the effectiveness of four avermectins: doramectin, ivermectin, moxidectin and selamectin against a variety of bacterial species (gram-positive, gram-negative, mycobacteria, pathogenic and environmental).

All mycobacterial species except *M. avium* were susceptible to ivermectin, as shown in Table 6.4. The other avermectins tested also showed varying degrees of effectiveness at inhibiting the *Mycobacterium spp.* tested. Interestingly, none of the other non-mycobacterial bacterial species tested were susceptible to any avermectin, up to 128 $\mu$g/mL (Table 6.4).

Another question about ivermectin’s activity was whether it was bacteriostatic or bactericidal against mycobacteria. A time-course measurement of OD$_{600}$ during treatment with ivermectin was performed for *M. bovis* BCG and *M. smegmatis*, and the results were that the OD$_{600}$ of the cultures remained constant over multiple generation times. Furthermore, there was obvious growth in the culture that had been treated with ivermectin, implying that ivermectin was bacteriostatic; upon reduction of the ivermectin concentration (to approximately $\frac{1}{2}$ MIC), the *M. bovis* BCG began to grow. Taken together, these data support that ivermectin is bacteriostatic against *M. bovis* BCG (shown in Figure 6.7) and *M. smegmatis* (data not shown).
Table 6.4: The MIC of the avermectins against mycobacteria and other bacteria in liquid microtiter culture. All strains or species were tested up to 128 µg/mL.

<table>
<thead>
<tr>
<th>Strain or species</th>
<th>Doramectin</th>
<th>Ivermectin</th>
<th>Moxidectin</th>
<th>Selamectin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. smegmatis</em></td>
<td>128</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>16</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> CDC1551</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> Erdman</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>S. lividans</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>R. jostii</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>A. baumannii</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
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</tr>
<tr>
<td><em>P. aeruginosa</em></td>
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<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
<tr>
<td><em>K. rhizophila</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
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</tr>
<tr>
<td><em>S. aureus</em></td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>
Figure 6.7: Ivermectin appears to be bacteriostatic. The graph in (A) shows the OD\textsubscript{600} measurements of a 100 mL culture of \textit{M. bovis} BCG treated with super-inhibitory concentrations of ivermectin (64 µg/mL) or untreated. The treated culture maintained a steady OD\textsubscript{600} throughout the experiment. Following that experiment, aliquots from both the treated and untreated culture were inoculated into fresh media (\textfrac{1}{50} dilution) and grown for one week, the results are shown in (B).
7

Discussion

7.1 The agar drug-drug interactions screen identified six potentiating combinations

The NE agar drug-drug interactions screen (described in section 6.1) yielded pairs of lead compound combinations that appeared, by qualitative analysis, to interact in potentiation or synergy against *M. smegmatis*. Twenty-three pairs of compounds were tested in liquid 7H9 microtiter culture using an assay developed in the Thompson Lab to quantitatively define their interaction (see section 5.4.2). Although none of the 23 pairs demonstrated synergistic activity under these conditions, there were six combinations that demonstrated potentiation (see Table 6.1). Initially, it was surprising that the quantitative assay validated none of the lead combinations as synergistic, but later drug-drug interaction experiments, using the same experimental method, showed that cleaning of the replicator pins between applications was inadequate, resulting in carryover of 2° compounds onto subsequent plates which confounded the results. This was likely true in the agar screens in the present study and may have led to some of the apparent false positives identified through the quantitative assay.

Another factor that may have affected the results is the differences in media used between the screen and subsequent quantitative assay. In the screen, the media was NE (for *M. smegmatis*) or 7H9-agar without glycerol, supplemented with OADS (for *M. bovis* BCG). In the quantitative assay, the media was 7H9 with glycerol, supplemented with ADS. The changing presence of glycerol and oleic acid (the “O” in OADS) may have contributed to different activity levels of the compounds. The presence of glycerol in the media changes the metabolic activity of the cell and can contribute to or
detract from the effectiveness of certain compounds [31]. Oleic acid may be used by mycobacteria as a nutrient, and may have a similar influence. In future screens, it is imperative to use the same media formulation from experiment to experiment to maximize the true hit rate.

Of the six combinations that showed potentiation using the quantitative assay, ethambutol + compound Sweet-1F4 were tested a second time in NE agar using an agar checkerboard. The agar checkerboard results gave a positive validation for synergy, with an FICI = 0.375. Repeats of both the agar checkerboard and a liquid NE microtiter checkerboard confirmed that the synergy between ethambutol and compound Sweet-1F4 is only seen on solid media. In liquid media, the interaction appears to be potentiation.

The results from the disc diffusion assays also imply that the media affects the nature of the interaction of ethambutol + compound Sweet-1F4. In these experiments, one of the partner compounds was present in the agar, and one was applied to the bacterial lawn via a paper disc saturated with the compound. Synergy was qualitatively apparent (visually) when sub-inhibitory concentrations of compound Sweet-1F4 were present in the agar and ethambutol was applied to the M. smegmatis lawn by the paper disc (Figure 6.3). Interestingly, there was no apparent synergy when ethambutol was present in the agar, and compound Sweet-1F4 applied by the paper disc (see Figure C.1). This may be a trivial artefact of chemical diffusion from the paper disc, as compound Sweet-1F4 has very limited effectiveness applied alone via the paper disc (by qualitative analysis).

The strikingly different results between liquid media and agar media, and again between applying the compounds in the agar versus via the paper disc, suggest that the interaction between two compounds, and whether the interaction is synergy or potentiation, is not a simple matter of combining drugs.

This disparity in results may be an effect of the change in media conditions. It is likely that the metabolism or growth patterns of the culture are different in agar and in liquid, which may result in the bacteria being more or less susceptible to the compound(s). It may be that the mechanism of interaction, currently unknown, between ethambutol and compound Sweet-1F4 is synergistic only under certain physiological conditions and specific compound exposures.

Alternatively, the differences between experiments may be due to length of pre-exposure to one of the compounds. In the NE agar screen, the 1° compound is added to the molten agar and the bacteria are exposed to that 1° compound for a period of time (up to 45 minutes) before the 2° compound is applied. This lag time is recognized in disc diffusion methods as the “critical
The "critical time" is a component of how bacterial growth competes with diffusion of the compound through the agar to ultimately define the edge of the zone of inhibition [23, p. 31]. By performing the compound application in this stepwise fashion, the effect (if any) of the 1° compound has time to be realized before the 2° compound is applied to the agar. This lag between application of the two compounds may allow for time-sensitive changes in the metabolism of the bacteria and these metabolic changes may be different between replicate and alternate experiments. Considering the physiological response systems of mycobacteria to antibiotics (see Part I), it is possible that preliminary exposure to the 1° compounds may have affected the way the bacteria responded to the 2° compounds.

The goal of the agar screens was to find combinations of compounds that worked in synergy in liquid culture: this successfully identified ethambutol + compound Sweet-1F4 as synergistic partners against *M. smegmatis* (under NE agar media conditions). We wanted to extract more leads from the Sweet Library, containing hundreds of antibiotics, to find more combinations. To more predictably screen for pairs of compounds that interacted in liquid 7H9 growth conditions used for validation, a new screen in liquid 7H9 microtiter culture was performed.

7.2 Four combinations from the liquid 7H9 drug-drug interactions screen were validated for synergy

The screen in liquid 7H9 microtiter culture was performed with six 1° compounds: clarithromycin, clofazimine, domperidone, ethambutol, ivermectin and rifampicin. This generated an enormous body of data to be analyzed. There were a total of 75,000 unique combinations and 786 Microsoft Excel® files generated by this screen. Subsequent data analysis (by the computer program “Screen Analysis”) yielded 266 out of 3564 pairs of compounds that appeared to be synergistic.

For follow-up, approximately ten percent of total lead combinations were selected for further experiments. Twenty lead combinations (positive for synergy) with ivermectin and 16 lead combinations with rifampicin were carried forward into checkerboard validation experiments.

The advantage of the liquid 7H9 microtiter interaction screen, as opposed to the agar interactions screen, was that fewer variables were changed between the original screen and the subsequent validation test. The media, culture conditions, volumes and bacteria tested were identical and it
was expected that this methodology would be more successful in confirming synergistic pairs of compounds that are effective in liquid 7H9 culture. Four rifampicin combinations were validated: compound Sweet-5H10, compound Sweet-5H11, compound Sweet-2E9 and compound Sweet-8A2. These four combinations represent a validation rate of 11% of all combinations tested, or 25% of rifampicin combinations tested.

7.2.1 Evaluation of the screen as a means of identifying synergistic partners

It had been our hope to have greater improvement in lead combination validation. In retrospect, we saw some potential limitations in this methodology that could be addressed in the future to achieve further improvement.

Besides outright experimental or analytical error, there were some differences between the drug-drug interactions screen and the checkerboard validation that may account for the discrepancy of the results. In the screen the *M. bovis* BCG(lux) was diluted from stationary phase frozen stock directly into media containing the 1⁰ compounds. The bacteria were exposed to these sub-inhibitory compound concentrations for at least 30 minutes before addition of the 2⁰ compound. As described previously (section 7.1), this lag between compound application may have affected the bacterial response to the 2⁰ antibiotic. In contrast, the checkerboard assay used fresh stationary-phase *M. bovis* BCG(lux) cells and exposed them to the 1⁰ and 2⁰ compounds simultaneously.

Beyond differences in the experimental set-up, the design of the screen experiment and checkerboard validation may have been in conflict. The interactions screen was performed by holding steady the 1⁰ compound at an assumed 1/8 MIC and 1/4 MIC in combination with serial four-fold serial dilutions of 2⁰ compounds. The difficulty with this set-up is twofold: first, the use of four-fold serial dilutions of 2⁰ compounds may have provided false positive hits due to general variance in MIC values between different experimental plates. This concept is illustrated in Figure 7.1. Secondly, the assumption that the concentrations used for the 1⁰ compounds were 1/8 MIC and 1/4 MIC may be incorrect due to general variance in MIC values from experiment to experiment. Future experiments should always include a serial dilution of the 1⁰ compound to confirm that the concentrations used are correct.

The checkerboard validation used two-fold serial dilutions for both the 1⁰ and 2⁰ compounds which increased the resolution of the combinatorial inhibitory activity and could differentiate between potentiation and synergy.
more clearly than four-fold serial dilutions could.

Judging by the success of the checkerboard validation experiments, where 11% of combinations tested were confirmed to be synergistic, the false positive rate appears to be close to 90%, however this assumes that the combinations selected for checkerboard validation are a fair representative of the leads from the screen and relies on the FICI analysis as the final and correct verdict of drug-drug interactions. The FICI analysis assumes that in a given combination, both compounds’ kinetics are concentration-dependent and linear within the ranges tested [23], however, the activities of many compounds are believed to inhibit bacterial growth in a concentration-dependent, exponential way [22]. The kinetics curve of the compounds could preferentially favour the FICI classification of combinations as synergistic or as no interaction, depending on the shape of the curve.

To demonstrate the variability in compound kinetic curves, Figure 7.2 illustrates linear, linear (out-of-range) and exponential concentration to activity relationship curves. In this example, an unknown compound is tested in two-fold serial dilutions, using a concentration of 3 and 6 (concentration units). The supposed effectiveness of the compound from one dilution to the next is extrapolated (indicated by the length of the double-headed arrow on each graph). In (A), if there is a linear relationship between concentration and inhibitory activity, the data points at 3 and 6 can be extrapolated to accurately predict the effect of the compound for other concentrations. In (B), the relationship between concentration and inhibitory activity is linear, but not within the concentration range tested, so the data points at 3 and 6 do not extrapolate to accurately predict the effect of the compound for other concentrations. In (C), the relationship between concentration and inhibitory activity is exponential which makes the extrapolation from the data points at 3 and 6 even less accurate in predicting the effect of the compound at other concentrations.
Figure 7.1: Concept of how \( \frac{1}{4} \) dilutions of \( 2^\circ \) compound may have led to false positive results. The liquid 7H9 microtiter screen used four-fold dilutions of compounds (A), where the checkerboard validation used two-fold dilutions (B). This example illustrates how normal, expected fluctuation of an MIC from plate to plate, in this example from 26 to 20 (arbitrary units), would have been reported as a positive hit in the screen, but a negative validation in the checkerboard assay. In (A), it appears that the MIC is reduced four-fold upon addition of sub-inhibitory concentrations of the \( 1^\circ \) compound (reported MIC of first plate compared to the reported MIC of the second plate), which appears to be a synergistic interaction, even though the MIC is only fluctuating between 26 and 20. In (B), the resolution is finer and although it appears that the MIC is reduced two-fold upon addition of sub-inhibitory concentrations of the \( 1^\circ \) compound, this is not considered synergy.
Figure 7.2: Graphical comparison of a linear versus non-linear relationship between compound concentration and its inhibitory activity, and the effect of the range of concentrations tested. A linear relationship has increasing inhibitory activity with increasing concentration (A). If the range of concentrations tested contains the concentrations where a linear relationship plateaus, the relationship between concentration and inhibitory activity may be de-emphasized (B). Non-linear relationships, such as exponential relationships, between concentration and inhibitory activity also exist (C).
The following hypothetical example illustrates how the shape of the inhibition curve is likely to affect the reliability of the screen and therefore the rate of validation: consider 2 drugs whose concentration to inhibitory activity profiles are exponential, such as in Figure 7.2 (C), and whose MIC is 6 concentration units for both compounds when used alone (ie. the culture requires an inhibition power of 6.4 inhibition units to cause no growth, which is achieved by 6 concentration units). If you combine the compounds at half the effective concentration, ie. 3 concentration units, and see an inhibition power of 6.4 inhibition units and no growth, it would appear to be no interaction, but in fact, it would be synergy. It is synergistic because the additive inhibitory activity of 3 concentrations units combined with 3 concentration units would be 0.8 + 0.8 = 1.6 inhibition units, but if the actual combinatorial effectiveness is 6.4 inhibition units then this is synergy because the combination is more effective than the sum of the individual compounds’ effectiveness. Using the FICI analysis, this type of combination would never be identified as synergistic because the assumption of linear concentration to inhibitory activity profiles would calculate the FICI as 1.0 which is interpreted as no interaction. This combination would be negative by checkerboard analysis.

Perhaps some of the combinations I tested in a checkerboard assay that were found to be negative for synergy by the FICI did have some interaction between them. To explore the possibility that the checkerboard FICI analysis was biased toward negative results by the compound’s kinetics curves, I plotted the effect of domperidone, ivermectin and rifampicin across the range of concentrations tested, as shown in Figure 7.3.

The effect of each compound over a range of two-fold concentrations can be represented by a curve, and the unique shape of this curve may bias the likelihood of identifying synergistic combinations by FICI. Standard FICI analyses consider the effect of using one compound at a concentration four-fold below the MIC$_{90}$ ($\frac{1}{4}$ MIC) in combination with another compound at $\frac{1}{4}$ MIC. In Figure 7.3 the $\frac{1}{4}$ MIC for domperidone, rifampicin and ivermectin is indicated by the green point on each graph. Comparing the three graphs, the effect of the $\frac{1}{4}$ MIC for each compound is different, depending on the curve of the compound. Used alone, the effect of applying $\frac{1}{4}$ MIC domperidone reduces growth to 22%, the effect of applying $\frac{1}{4}$ MIC rifampicin reduces growth to 46% and the effect of applying $\frac{1}{4}$ MIC ivermectin reduces growth to 87% (where absorbance represents growth). Therefore, a shallow curve, such as domperidone’s, is the most favourable for identifying synergistic combinations by FICI and a steep curve, such as ivermectin’s, is less favourable for identifying synergistic combinations by FICI. This corre-
tion was evident in the checkerboard analysis, where 38% of domperidone checkerboards were positive for synergy by FICI (Dr. Ramón-García, data not shown), 25% of rifampicin checkerboards were positive for synergy by FICI and 0% of ivermectin checkerboards were positive for synergy by FICI.
Figure 7.3: Absorbance versus concentration for domperidone (A), rifampicin (B) and ivermectin (C). The points are mean absorbance percentages (compared to absorbance of untreated microtiter culture) over 15 trials. Error bars are standard deviation. The red point on each graph indicates the MIC\textsubscript{90} concentration, which is the lowest concentration that inhibits 90% absorbance (or absorbance falls below 10%) where absorbance measures growth. The green points are four-fold below the MIC.
These curves may have contributed to generating false positives in the “Screen Analysis” of the original drug-drug interactions screen by mistaking combinations that are additive as synergistic. Based on their kinetics curves, domperidone and rifampicin would be expected to be less stringent and yield a greater number of leads because the effect of using \( \frac{1}{4} \) MIC of each reduces growth to 22% and 46% of absolute growth (growth with no treatment), so additive combinations with weakly inhibiting 2º compounds are more likely to be identified as leads. To help address this issue the “Screen Analysis” computer program identified partner 2º compounds that reduced the relative growth (at \( \frac{1}{4} \) MIC or \( \frac{1}{8} \) MIC) of the microtiter culture by an additional 90%. So under these circumstances, the 2º partner compound must reduce growth to 2.2% absolute growth for domperidone or 4.6% absolute growth for rifampicin. This means, in absolute terms, a 2º compound that, at sub-MIC levels, further reduces growth by 19.8% would be a lead with domperidone (22.0% − 19.8% = 2.2%). Similarly, a 2º partner compound that, at sub-MIC levels, reduces growth by 41.4%, would be a lead with rifampicin (46.0% − 41.4% = 4.6%). Additionally, the accuracy of absorbance readings at such low values is compromised, generating significant variability in the data and resulting in false positive hits.

In contrast, ivermectin is expected to be more stringent and yield fewer leads because the effect of using \( \frac{1}{4} \) MIC of ivermectin only reduces growth to 87% before the addition of a 2º compound. Here, for a 2º compound to meet the criteria as a lead, at sub-MIC levels it must reduce growth by 78.3% (87.0% − 78.3% = 8.7%). This prediction proves true in what we see from the “Screen Analysis” results: domperidone has 41 leads, rifampicin has 70 leads and ivermectin has 20 leads.

To precisely determine the interaction between two compounds, we would first need to establish the concentration to inhibitory activity profile across a linear range of concentrations for each compound alone and then establish the effectiveness of the combination across a linear range of concentrations for both compounds. Then we would need to set a threshold for how much each compound must be reduced (in combination, compared to sole application) to be considered synergistic. These steps are necessary because every compound has a unique kinetic curve, as illustrated by the comparison of domperidone, rifampicin and ivermectin and additionally with the curves of some 2º compounds shown in Figure 7.4. Unfortunately, while precise, this type of experiment is not particularly efficient or practical for testing a large number of combinations.

Fundamentally, the FICI analysis has long been established as a useful tool [14], and it is the current standard for establishing synergy of compound
Figure 7.4: Activity kinetics curves of some $2^o$ compounds across a range of concentrations. The shape of the curve is unique to each compound. The MIC$_{90}$ of each compound is identified as the first point that falls beneath the 10% line.

combinations. However, the FICI is also a limited tool because it relies on the evaluation of the MIC, which is variable, and because it assumes a linear relationship between concentration of a compound and its inhibitory activity. Overall, the FICI is an efficient, standardized method for confirming synergistic combinations but it has limitations which may discount some interesting combinations.

Many of these interesting combinations are the combinations we identified to interact by potentiation. The difference between potentiation and synergy (by FICI) is that in potentiation one of the compounds is used at $\frac{1}{2}$ MIC, rather than $\frac{1}{4}$ MIC (FICI = 0.75). While none of the ivermectin combinations validated as synergy by checkerboard and FICI, there were eight compounds that showed potentiation with ivermectin (see Table 6.2). Considering the steep slope of the kinetics activity curve of ivermectin between growth and no growth (Figure 7.3), any $2^o$ compound that can be used at sub-inhibitory concentrations to make ivermectin more effective at $\frac{1}{2}$ MIC is an interesting partner.
7.3 Data from screens can be mined for new antimycobacterials

The results of the drug-drug interactions screening experiments have so far identified four combinations that are effective in synergy against mycobacteria in liquid 7H9 microtiter culture and one combination that is effective against mycobacteria in an agar lawn. It is exciting to not only have a short-list of combinations that may be effective against *M. tuberculosis*, but to have a large data set that indicates single compounds that are effective against mycobacteria, independent of their effectiveness within combinations. It is especially interesting to find families of compounds that show inhibitory activity against *M. bovis* BCG (*lux*), such as the avermectins (discussed below).

7.3.1 The avermectins may be a future TB treatment

The avermectins, including ivermectin, while known to be potent anthelmintics, have traditionally been regarded as being ineffective against bacteria [5, 12, 16]. This was true for all bacterial species I tested (*E. coli*, *S. lividans*, *R. jostii*, *A. baumannii*, *P. aeruginosa*, *K. rhizophila* and *S. aureus*) with the exception of mycobacteria. The apparent specificity of ivermectin for mycobacteria implies that its target is unique to this taxon of bacteria. The remarkable complexity and uniqueness of the mycobacterial cell wall, not common to other bacteria that are resistant to avermectins (including *S. lividans* and *R. jostii* RHA1, both related actinobacteria), points towards this system as a potential target [11].

There is very little evidence as to what structure or pathway the avermectins may be targeting in mycobacteria. In invertebrate nematodes, ivermectin specifically binds to glutamate-gated chloride channels present in nerve and muscle cells, causing paralysis of movement, paralysis of the pharynx and reduced ability to reproduce [43]. There are no homologues of these channels present in the mycobacterial cell wall, according to BLAST search.

In considering ivermectin as a potential future treatment for TB, current understanding of the drug is promising. Ivermectin is a lipophilic compound that is distributed to most parts of the body [7, 18]. We expect ivermectin would successfully reach the lungs of humans because of its effectiveness against the subcutaneous nematode *Onchocerca volvulus* larvae in humans [40] and because in other mammals such as foals, orally administered ivermectin has been effective in treating *Strongylus vulgaris* larval infection of the lungs [41]. Ivermectin does not bind human ligand-gated
chloride channels in vivo, possibly due to the blood-brain barrier and non-
specific pumps that prevent ivermectin from reaching the central nervous
system [18,43]. These data suggest that ivermectin is likely to reach the site
of TB infection in the lungs and it is unlikely to have toxic effects on the
human nervous system.

To my knowledge this is the first report demonstrating the antimycobac-
terial activity of avermectins. The data provided here recommend future
investigation of ivermectin for TB treatment. Its apparent specificity for
mycobacteria and its safety profile in humans make it an attractive thera-
peutic option.

7.4 Future work

To carry this work forward, one direction to explore is the cellular target
and the specific mode of action of the avermectins which is seemingly unique
to mycobacteria. This is likely to point to or elucidate unique essential
functions of mycobacteria as compared to other bacteria.

I propose that an *M. smegmatis* mutant library would begin to address
these questions and could be a first-step in furthering this work.

To continue exploring the potential therapeutic usefulness of ivermectin,
future studies should include ex vivo infection studies and in vivo infection
studies. It is imperative to determine whether ivermectin is, as hypothesized,
able to reach the site of *M. tuberculosis* infection and inhibit bacterial growth
under those conditions and whether the dose is tolerated by mammalian
systems and host cells.

It is also of great interest to consider ivermectin as a potential MDR-TB
treatment. The first steps toward exploring this possibility have already
been taken: experiments in collaboration with Dr. Bill Jacobs’ Lab are
underway to test whether ivermectin is effective against strains of MDR-
and XDR-TB, in vitro.

In another vein of future work, the large data bank from the screens can
be mined for other interesting compounds or classes of compounds that may
show promise for therapeutic use in TB treatment.

With the information presented here and the rich data bank that these
studies have provided, there are many options to carry forward. An investi-
gation into the target and interaction of the synergistic compounds may
be a study that is similar to the pure science, finely-focused approach that
was adopted for the work described in Part I. Further data mining of the
screen results may be a study that is similar to the clinically-relevant, widely-
focused approach that was adopted to do the drug-drug interaction screens in the first place, as described in Part II.

Ultimately, any work that launches from the information presented here has a great capacity to further our understanding of antibiotic susceptibility and intrinsic antibiotic resistance in *Mycobacterium*. It is our aim that this information will lead to a timely introduction of new TB treatments for the world.
References


Appendices
Appendix A

Ethambutol and compound Sweet-1F4 liquid NE checkerboard results

Ethambutol and compound Sweet-1F4 were tested for synergy using a liquid NE checkerboard in microtiter culture (to compare to an NE agar checkerboard). Viability was determined using the MTT assay. The absorbance values of the checkerboard assay are shown in Table A.1.
Table A.1: Photometric values for NE liquid checkerboard of ethambutol (serial $\frac{1}{2}$ dilutions high to low from rows G through H) and compound Sweet-1F4 (serial $\frac{1}{2}$ dilutions high to low from columns 2 through 11). The highest concentration of ethambutol was 2 µg/mL and the highest concentration of compound Sweet-1F4 was 32 µg/mL. The MIC$_{\text{ethambutol}} = D1 = 0.25$ µg/mL and the MIC$_{\text{compound Sweet-1F4}} = H5 = 4$ µg/mL. Strictly from growth versus no-growth, the combination of ethambutol + compound Sweet-1F4 is no more effective than either compound alone, however, the photometric value at C6 is lower than either ethambutol or compound Sweet-1F4 alone, which suggests the compounds may interact with slight potentiation.

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Appendix B

“Screen Analysis” program code

This is the raw code for the computer program “Screen Analysis”, which was developed and used to analyse the interactions screen results.

```python
import xlrd
import glob
import codecs

#compound 1 is the primary compound, compound 2 is the secondary compound, from the Sweet Library

def read_control(sheet, startrow, endrow, startcolumn, endcolumn):
    values = []  #list of values to be used later
    for column in range(startcolumn, endcolumn+1):
        for row in range(startrow, endrow+1):
            values.append(sheet.cell(row,column).value)
    return values

def read_assay(sheet, startrow, endrow, startcolumn, endcolumn,
               start_concn2, diln_factor2, ID_prefix):
    values = {}  #table of tables of values. ID keys, indexed to tables of concn2 keys, indexed to photometric values
    current_concn2=start_concn2
    for row in range(startrow, endrow+1):
        assay_col_counter = 1
```
for column in range(startcolumn, endcolumn+1):
    ID_current = ID_prefix + str(assay_col_counter)
    assay_col_counter = assay_col_counter + 1
    if ID_current not in values:
        values[ID_current] = {}
    values[ID_current][current_concn2] = sheet.cell(row, column).value
    current_concn2 = current_concn2 * (1/float(diln_factor2))
return values

def MICvalue(alive, dead, MICxx):
    a_average = sum(alive, 0.0)/len(alive)
    d_average = sum(dead, 0.0)/len(dead)
    return (a_average - d_average) * (1-(float(MICxx)/100.0)) + d_average

def make_da_allkill_tables(MICvalue, assay_directory):
    datable = {}
    allkill_table = {}
    cmpd1_diln_list = assay_directory.keys()
    cmpd1_diln_list.sort()
    for cmpd1_diln in cmpd1_diln_list:
        table = assay_directory[cmpd1_diln]
        for ID_current in table.keys():
            concn2_alive = 400.0 #400 is a placeholder for an MIC "off the charts",
            or higher than the highest concentration2 tested
            allkill_flag = True
            current_concn2_list = table[ID_current].keys()
            current_concn2_list.sort(reverse=True)
            for current_concn2 in current_concn2_list:
                if table[ID_current][current_concn2] > MICvalue:
                    allkill_flag = False
                    break
            concn2_alive = current_concn2
            if allkill_flag:
                if ID_current not in allkill_table:
                    allkill_table[ID_current] = []
                allkill_table[ID_current].append(cmpd1_diln)
            if ID_current not in datable:
                datable[ID_current] = []
            datable[ID_current].append(concn2_alive)
    return (datable, allkill_table)

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def find_synergyhits(datable):
    hits_table = {}
    for ID_current in datable.keys():
        concns2 = datable[ID_current]
        ctrl_concn2 = concns2[0]
        hit = False
        for current_concn2 in concns2[1:]:
            hit = hit or (ctrl_concn2 > current_concn2)
        if hit:
            hits_table[ID_current] = concns2
    return hits_table

def check_synergy(datable, screening_table, cmpd1):
    # screening table has all pairs listed
    for ID_current in datable.keys():
        if ID_current not in screening_table:
            screening_table[ID_current] = {}
        concns2 = datable[ID_current]
        prev_concn2 = concns2[0]
        all_equal_flag = True
        hit_flag = True
        for current_concn2 in concns2[1:]:
            if current_concn2 < prev_concn2:
                all_equal_flag = False
            if current_concn2 >= prev_concn2:
                # Both the 1/8 and 1/4 value must show synergy
                all_equal_flag = False
                hit_flag = False
            screening_table[ID_current][cmpd1] = (False, concns2)
            break
            if all_equal_flag:
                screening_table[ID_current][cmpd1] = (False, concns2)
            elif hit_flag:
                screening_table[ID_current][cmpd1] = (True, concns2)

def read_librarydb(sheet): # link ID to cmpd2 name and properties
    librarydb = {}
    column = 0
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row = 0
begin = False
cellvalue = sheet.cell(row, column).value
while cellvalue != 'END':
    if cellvalue == 'BEGIN':
        begin = True
    if begin == True:
        if cellvalue != '':
            cmpd2_name = sheet.cell(row, column+1).value
            cmpd2_class = sheet.cell(row, column+13).value
            cmpd2_target = sheet.cell(row, column+14).value
            cmpd2_mechanism = sheet.cell(row, column+15).value
            librarydb[cellvalue] = (cmpd2_name, cmpd2_class, cmpd2_target, cmpd2_mechanism)
    row = row + 1
    cellvalue = sheet.cell(row, column).value
return librarydb

batch_list = ['Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 1', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 2', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 3', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 4', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 5', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 6', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 7', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\Library 8', 'Y:\Leah\Synergy Screening\BCG luc liquid assay\Python Program\IVR with Library']
cmpd1_list = ['ETH', 'RIF', 'CLA', 'CLOF', 'DPD', 'IVR']
library_list = ['1', '2', '3', '4', '5', '6', '7', '8']
plate_list = ['A', 'B', 'C', 'D', 'E', 'F', 'G', 'H']
concn1_list = ['18', '14'] #represents 1/8 and 1/4
sheet_to_read = 1 #excel sheet where data is
MIC_percent_list = [90]
hits_output_fname = 'hits.csv'
effective_cmpd_output_fname = 'effective.csv'
screening_output_fname = 'screen.csv'
librarydb_fname = 'Library Functions.xls'
alldata_fname = 'All Combinations.xls'

hits_output_file = codecs.open(hits_output_fname,'w','utf_16')
effective_cmpd_output_file = codecs.open(effective_cmpd_output_fname,'w','utf_16')
screening_output_file = codecs.open(screening_output_fname, 'w','utf_16')

#hits output file headers: MICxx, CMPD1, ID, 0.0, 0.125, 0.25
where 0.125 is 1/8 and 0.25 is 1/4
hits_output_file.write('MICxx	CMPD1	ID	0.0	0.125	0.25')
hits_output_file.write('
')

#effective cmpd output file headers: MICxx, ID, MIC(cmpd2)
effective_cmpd_output_file.write('MICxx	ID	MIC(CMPD2)')
effective_cmpd_output_file.write('
')

#screening output file headers: ID, NAME, COUNT, CMPD1, HIT?,
0.0, 0.125, 0.25 where 0.125 is 1/8 and 0.25 is 1/4
screening_output_file.write('ID	NAME	COUNT')
for cmpd1 in cmpd1_list:
    screening_output_file.write('	CMPD1	HIT	0.0	0.125	0.25')
screening_output_file.write('
')

wb_librarydb = xlrd.open_workbook(librarydb_fname)
sh_librarydb = wb_librarydb.sheet_by_index(0)
librarydb = read_librarydb(sh_librarydb)

screening_table = {}
hits_histogram = {}
for folder in batch_list:
    print folder
    for MIC_percent in MIC_percent_list:
        for cmpd1 in cmpd1_list:
            cmpd1_fpat = folder + '\' + '*' + cmpd1 + ' controls*' 
            cmpd1_fnames = glob.glob(cmpd1_fpat)
            if len(cmpd1_fnames) > 1:
                print 'Error opening control file for compound ' + cmpd1
                exit()
            elif len(cmpd1_fnames) == 0:

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wb_cmpd1_control = xlrd.open_workbook(cmpd1_fnames[0])
sh_cmpd1_control = wb_cmpd1_control.sheet_by_index(sheet_to_read)

alive = read_control(sh_cmpd1_control, 16, 23, 1, 2)
dead = read_control(sh_cmpd1_control, 16, 17, 7, 8)
MIC_cmpd1 = MICvalue(alive, dead, MIC_percent)

for library in library_list:
    for plate in plate_list:
        # get library/plate control
        
        check_plate_cmpd1_pat = folder + '\\' + library + plate + ' ' + cmpd1 + '*'
        check_plate_cmpd1_list = glob.glob(check_plate_cmpd1_pat)

        if len(check_plate_cmpd1_list) == 0:
            continue

        plate_control_path = folder + '\\' + library + plate + ' control.xls'
        wb_plate_control = xlrd.open_workbook(plate_control_path)
        sh_plate_control = wb_plate_control.sheet_by_index(sheet_to_read)

        # add control to assay_list
        assay_list = []
        assay_list.append(read_assay(sh_plate_control, 16, 23, 1, 12, 100, 4, library + plate))

    for concn1 in concn1_list:
        # build assay_list
        
        plate_cmpd1_concn1_path = folder + '\\' + library + plate + ' ' + cmpd1 + ' ' + concn1 + '.xls'
        wb_plate_cmpd1_concn1 = xlrd.open_workbook(plate_cmpd1_concn1_path)
        sh_plate_cmpd1_concn1 = wb_plate_cmpd1_concn1.sheet_by_index(sheet_to_read)
        assay_list.append(read_assay(sh_plate_cmpd1_concn1, 97
assay_directory = {0: assay_list[0]}
start_concn1 = 0.125
current_concn1 = start_concn1
diln_factor1 = 2.0
for assay in assay_list[1:]:
    assay_directory[current_concn1] = assay
    current_concn1 = current_concn1 * diln_factor1

(datable, allkill_table) = make_da_allkill_tables(MIC_cmpd1, assay_directory)
check_synergy(datable, screening_table, cmpd1)
hits_table = find_synergyhits(datable)

for hit_id in hits_table.keys():
    # column headings:
    # 'MICxx,CMPD1,ID,CMPD1=0,CMPD1=1/8,CMPD1=1/4\n'
    cmpd2_concn_list = hits_table[hit_id]
    hits_output_file.write(str(MIC_percent) + '\t' + cmpd1 + '\t' + '[' + hit_id + ']')
    for curr_cmpd2_concn in cmpd2_concn_list:
        hits_output_file.write('\t' + str(curr_cmpd2_concn))
    hits_output_file.write('\n')
    # column headings:
    # 'MICxx,ID,CMPD1=0\n'
    effective_cmpd_output_file.write(str(MIC_percent) + '\t' + '[' + hit_id + ']')
    effective_cmpd_output_file.write('\t' + str(cmpd2_concn_list[0]) + '\n')
    # count the times a cmpd2 is considered a hit
    if hit_id not in hits_histogram.keys():
        hits_histogram[hit_id] = []
    hits_histogram[hit_id].append(cmpd1)

hits_output_file.close()
effective_cmpd_output_file.close()
for cmpd2 in screening_table.keys():
    cmpd2_hit_count = 0
    for cmpd1 in screening_table[cmpd2].keys():
        (hit, cmpd2_concns) = screening_table[cmpd2][cmpd1]
        if hit:
            cmpd2_hit_count = cmpd2_hit_count + 1
    hits_histogram[cmpd2] = cmpd2_hit_count

for cmpd2 in screening_table.keys():
    cmpd2_name = ''
    if cmpd2 not in librarydb:
        print 'no library information for cmpd2 ID: ' + cmpd2
    else:
        # screening output file headers: ID, NAME, COUNT, CMPD1, HIT?, 0.0, 0.125, 0.25
        (name, cmpd2_class, cmpd2_target, cmpd2_mechanism) = librarydb[cmpd2]
        cmpd2_name = name
        screening_output_file.write('['+cmpd2+']' + '	' + cmpd2_name + '
        for cmpd1 in screening_table[cmpd2].keys():
            (hit, cmpd2_concns) = screening_table[cmpd2][cmpd1]
            hit_string = 'No'
            if hit:
                hit_string = 'Yes'
            screening_output_file.write('	' + cmpd1 + '	' + hit_string + '
            for concns in cmpd2_concns:
                screening_output_file.write('	' + str(concns))
        file.write('

screening_output_file.close()

print 'Done!'
Appendix C

Disc diffusion interaction assay

*M. smegmatis* grown on NE agar with 2 μg/mL compound Sweet-1F4. Discs soaked in decreasing concentrations of ethambutol: 64, 32, 16, 8, μg/mL (left to right).

*M. smegmatis* grown on NE agar with 0.125 μg/mL ethambutol. Discs soaked in decreasing concentrations of compound Sweet-1F4: 256, 128, 64 and 32 μg/mL (left to right).

Figure C.1: Disc diffusion interaction assay of ethambutol and compound Sweet-1F4.