**SELENO AMINO ACIDS
A NOVEL CLASS
OF ANTI-TUBERCULOSIS
COMPOUNDS
IDENTIFIED THROUGH MODIFIED
CULTURE SCREENING CONDCTIONS**

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ABSTRACT

Tuberculosis continues to be a major world health problem, causing more deaths than any other bacterial disease. Long treatment durations using a complex cocktail of drugs are often associated with patient non-adherence to therapy, and this has accelerated the development of drug resistant strains. Tuberculosis drug resistance has developed to the extent that some strains are resistant to all clinically used drugs. Therefore novel tuberculosis treatment drugs are urgently required to combat these resistant strains, sterilise latent infections and reduce lengthy treatment durations.

This research developed and optimised a high-throughput assay to screen chemical libraries for compounds with anti-mycobacterial activity. The assay utilised fast growing tuberculosis model species *M. smegmatis* expressing foreign green fluorescent protein (GFP). GFP allowed bacterial growth inhibition to be measured both by fluorescence in addition to absorbance. The assay was expanded to four different culture conditions two of which were nutrient starvation that better mimicked environmental conditions *M. tuberculosis* is exposed to during infection. These differential culture conditions also revealed previously unidentified mycobacterial inhibitors. Three chemical libraries totaling over 5,000 compounds were screened in the different culture conditions.

Seleno-amino acids (Se-AAs), a novel class of anti-tuberculosis compounds, were discovered through screens in nutrient starvation conditions. Based on traits of strong inhibitory activity towards mycobacteria, low human cell line cytotoxicity, structural novelty and known over-the-counter sale as a non-prescription dietary supplement, the Se-AAs were chosen as a promising pharmacophore for further study. Using evidence derived from anti-sense gene knockdown, transposon mutagenesis and biochemical enzyme assays, a pro-drug hypothesis of anti-mycobacterial activity was proposed that involved Se-AAs being transported into the mycobacterial cell by nutrient uptake transporters and subsequent cleavage into catalytically active methylselenium species by lyase enzymes used in mycobacterial sulphurous amino acid metabolism. The activated methylselenium is reduced by mycobacterial redox homeostasis enzymes involved in mycobacterial oxidative defence such as alkyl hydroperoxidases, generating reactive oxygen radical products that damage mycobacterial DNA, lipids and proteins. Reduced methylselenol can be cycled back to the oxidised state by cellular mycothiones, continuously generating damaging reactive oxygen species within the

mycobacterial cell. Methylselenium species also disrupt essential mycobacterial processes, such as ketosteroid catabolism and iron-sulphur cluster protein function.

In summary, this research has designed and implemented a novel dual label differential culture condition assay useful in the screening and detection of chemicals with anti-tuberculosis properties. A novel structural class of anti-tuberculosis compounds with therapeutic potential, the Se-AAs, was discovered using this assay, the structure-activity relationship of the Se-AAs was explored and a three-component model of Se-AA anti-tuberculosis activity is proposed.

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LIST OF ABBREVIATIONS

7H9+	Middlebrook 7H9 broth (with standard OADC and glycerol supplement)
7H9-	Middlebrook 7H9 broth (with limited OADC and glycerol supplement)
7H10	Middlebrook 7H10 agar
AAEMA	acetoacetoxyethylmethacrylate
ABC	ATP binding cassette
Ala-DH	alanine dehydrogenase
Amp ^R	ampicillin resistant
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
BCG	Bacille Calmette-Guérin
BLAST	basic local alignment search tool
<i>B. licheniformis</i>	<i>Bacillus licheniformis</i>
<i>B. subtilis</i>	<i>Bacillus subtilis</i>
BSA	bovine serum albumin
bp	base pairs
CBS	cystathionine β -synthase
CDC	Centers for Disease Control and Prevention (U.S.A.)
<i>C. glutamicum</i>	<i>Corynebacterium glutamicum</i>
CHCl ₃	chloroform
CFU	colony forming units
Chl ^R	chloramphenicol resistant
cys	cysteine
D	dextro
ddH ₂ O	double distilled sterile water
DL	racemic dextro / levo mixture
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DOT	directly observed treatment
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EMB	ethambutol
Em ^R	erythromycin resistant
EtOH	ethanol
Fe-S	iron-sulphur cluster
FIC	fractional inhibitory concentration

g	gravity
GC	guanine-cytosine
GC-MS	gas chromatography mass spectrometry
gDNA	genomic DNA
GFP	green fluorescent protein
GSH	glutathione
h	hours
HdeB	Hartman deBonts
HdeB C-	Hartman deBonts carbon limitation broth
HdeB N-	Hartman deBonts nitrogen limitation broth
hERG	human ether-a-go-go related gene
HIV	human immunodeficiency virus
HTS	high-throughput screen
Hyg ^R	hygromycin resistant
IAA	isoamyl alcohol
IC ₅₀	half maximal inhibitory concentration
IC ₉₀	90% inhibitory concentration
INH	isoniazid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JHU	Johns Hopkins University
Kan ^R	kanamycin resistant
kb	kilobase-pairs
kDa	kilodalton
KEGG	Kyoto Encyclopaedia of Genes and Genomes
L	litres
Lac-DH	lactate dehydrogenase
LB	Luria-Bertani broth
LBA	Luria-Bertani agar
LD ₅₀	median lethal dose
LD _{LO}	lowest lethal dose
LOPAC	Library of Pharmacologically Active Compounds
M9	M9 minimal broth
<i>M. africanum</i>	<i>Mycobacterium africanum</i>
<i>M. avium</i>	<i>Mycobacterium avium</i>
<i>M. bovis</i>	<i>Mycobacterium bovis</i>
MDR	multi-drug resistant
MeOH	methanol

met	methionine
MIC	minimum inhibitory concentration
min	minutes
<i>M. leprae</i>	<i>Mycobacterium leprae</i>
MoA	mechanism of action
mRNA	messenger RNA
MRSA	methicillin resistant <i>S. aureus</i>
MS	mass spectrometry
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5(diphenyltetrazolium bromide)
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
NAD	nicotinamide adenine dinucleotide
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NADH	nicotinamide adenine dinucleotide, reduced form
NCI	National Cancer Institute (U.S.A.)
NCBI	National Centre for Biotechnology Information (U.S.A.)
NEB	New England Biolabs
NIH	National Institutes of Health (U.S.A.)
NPC	nutritional prevention of cancer trial
NZ	New Zealand
NZD	New Zealand dollar
OADC	oleic acid, albumin, dextrose and catalase
OD	optical density
ORF	open reading frame
OTC	over the counter (non prescription medicine)
<i>P. aeruginosa</i>	<i>Psuedomonas aeruginosa</i>
PAS	<i>para</i> -aminosalicyclic acid
PbAc	lead acetate
PBS	phosphate buffered saline
PC-1	physical containment level 1
PC-2	physical containment level 2
PC-3	physical containment level 3
PCR	polymerase chain reaction
PEG	polyethylene glycol
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
pKa	acid dissociation constant

psi	pounds per square inch
PZA	pyrazinamide
RBS	ribosomal binding site
redox	reduction-oxidation
RIF	rifampicin
RNA	ribonucleic acid
ROS	reactive oxygen species
RPM	revolutions per minute
rRNA	ribosomal RNA
SAM	S-adenosyl methionine
SAR	structure-activity relationship
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SC	synthetic complete broth
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	seconds
Se-AA	seleno-amino acid
Se-cys	selenocysteine
SELECT	selenium and vitamin E cancer prevention trial
Se-met	selenomethionine
SERT	serotonin transporter
SEVI	semen derived enhancer of viral infection
SMSC	se-methylselenocysteine (DL)
SNR	signal to noise ratio
SPME	solid-phase micro extraction
SPO	sporulation broth
Str ^R	Streptomycin resistant
Spc ^R	Spectinomycin resistant
TAACF	Tuberculosis Antimicrobial Acquisition and Coordinating Facility (U.S.A.)
TAE	tris-acetate-EDTA
TB	tuberculosis
TE	tris-EDTA
TCBS	thiosulfate, citrate, bile salts, sucrose
TDR	totally drug resistant
T _m	melting temperature
TSB	tryptic soy broth

UK	United Kingdom
UV	ultra-violet
V	volts
<i>V. harveyi</i>	<i>Vibrio harveyi</i>
VUW	Victoria University of Wellington
WHO	World Health Organisation
WST-1	4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate
XDR	extensively drug resistant
Xgal	5-bromo-4-cholro-3-indolyl- β -D-galactopyranoside
YPD	yeast extract, peptone, dextrose broth
ZYP	ZY-5052 autoinduction broth

LIST OF PUBLICATIONS

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CHAPTER ONE

Introduction

1.1 Preface

*“Medical scientists now have
both the knowledge they need
to wipe out tuberculosis
as a public-health problem
and the tools to finish the job.”*

TIME magazine, 3rd August, 1953.

This research seeks to contribute towards solving the problem of tuberculosis by:

- **Developing a new method of screening for anti-mycobacterial compounds.**
- **Discovering novel tuberculosis inhibitor compounds.**
- **Exploring the mechanism and structure activity relationship of a lead novel anti-mycobacterial compound.**

1.2 Tuberculosis the disease

Tuberculosis (TB) is a disease that has infected humankind since prehistoric times. It has continued to afflict every civilisation around the globe through to today under the aliases of consumption, phthisis and the white plague.³

The causative disease agent is the acid fast bacillus *Mycobacterium tuberculosis* which commonly infecting the lungs, rendering it highly contagious by aerosol dispersion.⁴

Symptoms often manifest in persons with poor health, poor living conditions and in the immune compromised. Symptoms progress through a persistent sputum producing cough, chest pains, coughing of blood, fever and malaise. Without competent treatment, chronic infection will often result in death.⁴

Early therapies for tuberculosis were either completely ineffective or relied on hazardous surgical removal of tubercle lesions.⁵ Prevention was strongly enforced after the disease was determined contagious. This prevention was further reinforced by vaccination using an attenuated strain of the bovine tuberculosis pathogen *Mycobacterium bovis*, coined *M. bovis* Bacille Calmette-Guérin (BCG). Vaccination was adopted by a small number of predominantly European countries after World War II.⁶ However the vaccine remains controversial due to its variable efficacy and is not considered thoroughly effective, in addition to having no utility in established infections.⁷

Tuberculosis infections remained essentially untreatable until the discovery of streptomycin in 1943⁵, and the developing antibiotic era saw the discovery and synthesis of a host of effective first-line and second-line anti-tubercular drugs. However combinations of inappropriate treatment, lack of patient adherence to treatment regimens and the inherent resilience of mycobacteria saw the evolution of drug resistant strains of *M. tuberculosis* bacilli in the early 1990s. This phenomenon rapidly escalated to multi-drug resistant (MDR) strains, characterised by resistance to at least isoniazid and rifampin, the two chief front-line drugs.⁸ For these reasons, the World Health Organisation (WHO) declared tuberculosis a global health emergency in 1993.^{9, 10} TB drug resistance then progressed to extensively-drug resistant (XDR)¹¹ strains that are resistant to not only at least isoniazid and rifampin, but also resistant to at least one of the second line quinolone drugs and one of the second line injectable aminoglycosides.⁸ Drug resistant strains resulted in the appearance of practically untreatable cases of the disease in some countries.¹¹

Tuberculosis remains a significant threat to world health with 8.8 million active cases in 2010, with 650,000 of these cases caused by MDR-TB. Approximately 1.4 million deaths

were attributed to the disease in 2010, averaging four thousand deaths caused daily by this curable disease. It is estimated that one third of the world population is infected with tuberculosis bacilli.^{9, 10} Ten percent of these infected people will develop active tuberculosis in their lifetime. Each person exhibiting active tuberculosis is expected to infect between ten and fifteen people per year if not successfully treated. There is a high co-infection prevalence of TB and HIV, with one in four HIV-positive individuals dying from TB. It is estimated that in 2010 there were 1.1 million new HIV-positive cases of TB.¹² Due to a global effort to stop TB spearheaded by the WHO, the global incidence and mortality rates of TB have begun to slowly decline since 2005.¹³

Presently, the Global Plan to Stop TB 2006-2015 initiated by the Stop TB partnership, intends to halt and reverse the rising incidence of tuberculosis by 2015, utilising improved diagnostic techniques, wider treatment access and effective directly observed treatment regimens, in addition to implementing research on new diagnostics, antibiotics and vaccines.^{13, 14} This achievable goal expects to save fourteen million lives and treat fifty million people.^{13, 14} The ultimate ambition of this and other anti-tuberculosis collaborations is the eradication of tuberculosis by 2050, a commendable aspiration considering the only recorded eradication of an infectious disease was the abolishment of smallpox declared officially in 1980.¹⁵

1.2.1 The need for new tuberculosis drugs

Grim statistics attributed to tuberculosis are compounded by MDR strains of tuberculosis being present in virtually all countries surveyed by the WHO in 2010, estimating nearly half a million new cases of MDR tuberculosis occurring each year.^{9, 10, 16}

Due to the emerging prevalence of drug resistant MDR, XDR and recently TDR (totally drug resistant) strains of *M. tuberculosis*,^{11, 17, 18} current lengthy treatment periods and the large amount of established tuberculosis infections the introduction of new anti-mycobacterial compounds is a crucial component of the Global Plan to Stop TB^{8, 19}. Drug compounds acting via novel mechanisms may overcome existing resistant *M. tuberculosis* strains^{8, 20}, and in combination with known antibiotics can potentially decrease treatment periods^{20, 21} by additive or synergistic interactions.

Anti-mycobacterial compounds are able to act on established infections²² that vaccination and other preventative measures are incapable of addressing.²² The treatment of infected persons is of utmost importance in halting and reversing the incidence of tuberculosis, with an

estimated one third of the world population harbouring predominantly latent tuberculosis infection; this populace acts as a reservoir for the disease and a transmission source of new infection. New and effective anti-mycobacterial compounds in combination with existing compounds²² and diligent diagnosis, treatment courses, education and preventative measures such as vaccination are more than capable of negating the current tuberculosis pandemic.^{9, 10, 14}

1.2.2 Tuberculosis in New Zealand

With specific orientation towards the relevance of tuberculosis in New Zealand, tuberculosis has long been a major health issue. This is reflected in New Zealand's legislation, with the Tuberculosis Act implemented in 1948 that details protocols regarding awareness, prevention and treatment of tuberculosis. Although now outdated, the Tuberculosis Act (together with the Health Act 1956) is to be amalgamated and replaced by the recently approved Public Health Bill²³ introduced to Parliament during November 2007 and had its first reading in December 2007. The Bill is currently in the process of amendment following public submissions made in March 2008 and will modernise the legislation to give health officials more options focusing on prevention of tuberculosis, such as allowing contact tracing of communicable disease infected persons.²⁴ The presence of the specific Tuberculosis Act illustrates the level of significance the disease attained in this country, and the continuation of the Tuberculosis Act through the new Public Health Bill testifies to the ongoing concern. Despite New Zealand's relatively high standards of health care and legislative efforts to curb tuberculosis, both isolated cases and outbreaks of the disease continue to occur¹⁷. New Zealand exhibits rates of tuberculosis similar to the UK but nearly two-fold higher than in other countries like the USA, Canada and Australia²⁵. Incidence of disease presents a strong correlation with poor living conditions,²⁶ and outbreaks are typically isolated to communal populations such as schools, churches and prisons.^{17, 27} New Zealand experienced a declining trend in the frequency of tuberculosis until the 1980s, when the incidence plateaued with no further statistically significant decrease. It is thought that infected immigrants from high risk countries are the key factor in the lingering incidence of tuberculosis in New Zealand.^{25, 27} A 2007 report based on data from an Auckland hospital over July 2002-2003 saw 45 overseas-born patients accrue nearly \$700,000 NZD in inpatient and outpatient treatment and care costs, a significant amount considering the total health expenditure of \$3,293 NZD per capita for the 2002-2003 period.²⁸ The extent to which New Zealand's health budget can sustain rising expenditure due to the burden of resistant infections has been deemed uncertain.²⁶

The incidence of tuberculosis in New Zealand may be sustained primarily due to factors external rather than internal to the country. However as globalisation progresses we can no longer rely solely on our geographical isolation to protect us from the spread of disease.²⁵ The spread of tuberculosis and its drug-resistant variants must be addressed at not only the regional and national level, but also the international level.²⁹ The contribution of new anti-mycobacterial compounds to global endeavours to stop TB will not only help alleviate the burden of the disease on other nations, but also on our own.

By facilitating discovery of novel anti-mycobacterial compounds we contribute to each of the three aforementioned geographic levels of tuberculosis, conferring protection from emerging drug-resistant tuberculosis³⁰ and allowing quick effective treatment of patients harbouring active or latent infections.²² Development and use of novel drugs with unique activity mechanisms in combination with vigilant diagnosis and preventative measures, such as preventive vaccination and diligent screening of immigrants for existing infection, could dramatically decrease the incidence of tuberculosis in New Zealand, whilst simultaneously supporting international efforts to stop the disease.

1.2.3 Bovine tuberculosis

Although not directly relevant to the drug development aspect of this work, bovine tuberculosis is briefly outlined in this thesis to provide more complete background information on the wider mycobacterial disease impacts in New Zealand. Bovine tuberculosis the infection of cattle, predominately with the slow growing mycobacterial species *M. bovis*. It is possible that *M. bovis* can cause tuberculosis in humans, this has become a rare occurrence since the advent of pasteurisation.³¹

In New Zealand, the main vector of bovine tuberculosis is the brushtailed possum. Although bovine TB in New Zealand has been historically problematic, the problem has decreased since the introduction of the 1993 Biosecurity Act which led to major increases in possum control by utilising a three tiered approach of testing and culling of infected cattle, restriction of herd movement and control of the wild vector population.³² This preventative approach, with funding of NZ\$83 million in 2009³³, has led to a steady reduction in the incidence of bovine tuberculosis.

In the UK £87 million of public spending was used in 2009/2010 to control bovine tuberculosis, where the main vectors are deer and badgers.³⁴ It has been proposed that since pasteurisation was fully implemented in the UK, bovine TB now presents a negligible threat in terms of transmission to humans.³⁵

1.3 Mycobacteria

1.3.1 Family Mycobacteriaceae

Mycobacterium are a genus of bacteria belonging to the phylum Actinobacteria, the Actinomycetales order and the suborder Corynebacterineae. Their closest phylogenetic relations are to bacteria of the genera *Corynebacterium*, *Norcardia*, *Rhodococcus*, *Gordona* and *Tsukamurella* which often share some of mycobacterium's distinguishing phenotypes such as being capable to produce mycolic acids.

Mycobacteria are widespread in their occurrence, inhabiting terrestrial and aquatic environments, commensally populating animals and pathogenically infecting animals and humans in a sometimes fastidious fashion - manifesting diseases such as leprosy and tuberculosis.³⁶ Mycobacterial species are commonly divided into two groups, fast growers which when subcultured onto solid media form colonies visible to the naked eye within seven days, and slow growers which require longer than seven days incubation to form visible colonies. Further distinctions in the grouping of mycobacterial species include their ability to cause disease, in addition to standard phenotype and phylogenetic methods.

1.3.2 Characteristics

Mycobacterial species are typically non-motile rod shaped bacteria ranging in size from approximately 1-7 μm in length and 0.2-0.6 μm in width.³⁷ The GC content of mycobacterial genomes is typically 61-71% mol although some mycobacterial species such as *M. leprae* have a reported GC content as low as 54% mol.³⁸

1.3.3 Mycobacterial cell wall

Unique to mycobacteria is the unusual waxy outer cell wall, predominately consisting of long chained mycolic acids. This lipid rich coating confers the characteristic mycobacterial resistance to decolourisation of carbol-fuchsin staining with acidified alcohol during routine crystal violet Gram staining techniques. This acid fast nature of the mycobacterial cell wall serves as a standard method of mycobacterial identification and viewing using the Ziehl-Neelsen staining method.^{39, 40} The reinforced structure and corresponding non-permeable nature of the mycobacterial cell wall is thought to contribute to mycobacterial species inherent tolerance of hostile environments such as heat, osmotic pressure, desiccation, host immune environments and antibiotic treatment.⁴¹ Mycobacteria are considered the most resilient prokaryotic life form with the exception of bacterial endospores.⁴² The inside of the mycobacterial cell wall starts with the common phospholipid bilayer plasma membrane

enclosed in a layer of peptidoglycan. A layer of arabinogalactan is covalently linked to the peptidoglycan, and this arabinogalactan is esterified to a thick hydrophobic layer of waxy crosslinked mycolic acids - long chain fatty acids containing 70-90 carbons named after mycobacteria. This mycolic acid layer is then encapsulated by an outer membrane consisting of non-covalently attached polysaccharides, predominately α -D-glucan, in addition to glycolipids and proteins such as transmembrane transport channel proteins that together form the surface-exposed mycobacterial capsule. Tethered to lipid components of the cell wall by phosphatidylinositol anchors are lipoarabinomannans, lipomannans and phosphosphatidyl-*myo*-inositol mannosides.⁴³ The structure of the mycobacterial cell wall is depicted graphically in Figure 1.1. The unique nature of the mycobacterial cell wall render mycobacteria more resilient to external stressors but also presents several distinct targets for selective antimycobacterial drugs.⁴⁴

Figure 1.1: Mycobacterial cell wall.

A cross-sectional perspective. From within the cell travelling out we observe the mycobacterial cytoplasm, the plasma membrane with anchored proteins and lipoarabinomannans, layers of peptidoglycan and arabinogalactan followed by a thick waxy coat of intercalated long chain mycolic acids with dispersed phosphatidyl-*myo*-inositol mannosides. Finally an outer polysaccharide capsule with tethered lipomannans and mannosides.

1.3.4 Fast growing mycobacterial species

Fast growing mycobacteria as described above can be identified by their ability to form visible colonies on solid media in seven days or less. There exists a long and ever growing list of fast growing mycobacterial species, these are typically non-pathogenic in nature and reside in soil, water or commensally as part of the natural bacterial flora. They are known to be capable of instigating opportunistic infections,⁴⁵ typically caused by commensal mycobacterial species but occasionally from environmentally borne bacteria, but these infections are commonly infrequent and superficial.⁴⁶ Serious or disseminated cases are usually confined to circumstances of immunocompromised individuals.⁴⁷ *M. smegmatis* is the predominant model fast-growing organism used during the course of this work.

1.3.4.1 *Mycobacterium smegmatis*

M. smegmatis was first discovered in 1884 by Lustgarten⁴⁸ and later isolated from samples of human smegma (genital secretions) by Alvarez and Tavel.⁴⁹ Smegma was the source which garnered the species its name as proposed by Lehmann and Neumann in 1899. *M. smegmatis* is widely found in soil, Tsukamura demonstrated that 38% of Japanese soil samples harboured *M. smegmatis*.⁵⁰ Considered to be predominantly a non-pathogenic environmental saprophyte, *M. smegmatis* is only known to be an opportunistic agent of disease on extremely rare occasions, generally exploiting circumstances of an immunocompromised host to form subcutaneous infection at sites of cuts and abrasions which are often exposed to soil, the probable origin of *M. smegmatis*.⁵¹

M. smegmatis cells are aerobic, non-motile, rod shaped and approximately 3.0-5.0 µm in length by 0.5-1.0 µm in width with a doubling time of 2-3 hours.⁵² It is a filamentous bacterium capable of growth on a variety of rich and minimal media with a propensity to form aggregations of cells in liquid media in the absence of supplementation with surfactants and other dispersing agents.⁵³ They possess the aforementioned acid-fast staining characteristics typical of mycobacterial species. *M. smegmatis* forms dry, wrinkled, off-white colonies on solid media after 48 hours which over time change in hue to a more yellowish colour.

The genome of *M. smegmatis* was sequenced in 2006 by the J. Craig Venter Institute, revealing it to be 6,988,209 nucleotides in length with a 67% GC content coding for 6716 proteins, of which over 2000 share close homology with genes of *M. tuberculosis*.⁵⁴ *M. smegmatis* strain MC²155 is hyper-transformable, readily accepting foreign plasmid DNA via electroporative transformation, coupled with its ability to readily undergo homologous

recombination, its relatively fast generation time and broad media requirements. These characteristics have made it the workhorse of modern mycobacterial genetics.⁵⁵ Combined with properties of having a typically resilient mycobacterial cell wall, shared genetic homology and drug sensitivity to pathogenic mycobacterial species and its non-pathogenic nature and corresponding PC1 or PC2 containment requirements, make *M. smegmatis* a good model organism in the study of pathogenic mycobacteria.^{56, 57} *M. smegmatis* can enter a dormant state under conditions of oxygen depletion and stress caused by nutrient limitation⁵⁸ as *M. smegmatis* contains a functioning dormancy regulon similar to that expressed by *M. tuberculosis*.⁵⁹ This makes *M. smegmatis* a relevant screening model for identifying drugs active toward dormant mycobacteria such as persistent *M. tuberculosis*.

1.3.5 Slow Growing Mycobacterial Species

Slow growing mycobacterial species are distinguished primarily by their ability to form colonies on solid media after longer than seven days incubation. The clinically significant obligate pathogenic species of mycobacterium *M. tuberculosis*, *M. bovis*, *M. leprae*, *M. avium* and *M. africanum* all fall within the category of slow growing mycobacteria.

Slow growing mycobacteria share many traits with their fast growing relatives. They are also obligate aerobes, non-motile and rod shaped acid-fast bacteria of a similar size to fast growers. The primary distinction between the fast and slow growing mycobacterial species is not surprisingly their doubling time, whilst a fast growing mycobacterium divides once every 2-5 h on average, slow growing mycobacteria tend to double at the lethargic rate of only once every 15-20 h. Another distinction of the slow growing mycobacterial species is their propensity to be obligate parasites such as the members of the tuberculosis complex, whilst fast growing mycobacteria are primarily environmental and may cause infection opportunistically. Many slow growing mycobacteria solely thrive during infection of a host animal, possessing key traits which allow them to act as proficient intercellular parasites. The two key slow growing mycobacterial species in this study are *M. tuberculosis* and *M. bovis* which are the primary causative agents of tuberculosis disease in humans and cattle, respectively.

1.3.5.1 *Mycobacterium bovis* BCG

M. bovis BCG (bacillus Calmette-Guérin) is an attenuated derivative strain of the virulent bovine tuberculosis species *M. bovis*, which primarily infects cattle, causing approximately 3 billion dollars in damage to global agriculture annually and is also capable of crossing the

species barrier and infecting humans.⁶⁰ The strain *M. bovis* BCG was derived from the repeated passage of *M. bovis* over 13 years resulting in a completely avirulent strain. Best known for its use in vaccination against tuberculosis, *M. bovis* BCG is one of the most widely used vaccines in the world, being in use for over 50 years and having immunized over 3 billion individuals.⁶¹ Although the variable efficacy of the BCG vaccination is debated, statistical analysis indicates that BCG vaccination reduces the risk of tuberculosis infection by 50%.⁶² A slow growing mycobacterium, it presents all of the typical phenotype characteristics exhibited by other members of the tuberculosis complex, in addition to its attenuation, meaning that it only requires PC2 containment facilities and is able to be genetically modified, making it a suitable model organism for the study of slow growing mycobacteria.

1.3.5.2 *Mycobacterium tuberculosis*

M. tuberculosis is the primary cause for tuberculosis disease in humans, and thus is the major focus of practically all mycobacterial research. Discovered by Robert Koch in 1882 and dubbed *tubercule bacillus*, this slow growing mycobacterial species exhibits the archetypal mycobacterial traits of being aerobic, non-motile and possessing an acid fast cell wall. *M. tuberculosis* is an obligate intercellular pathogen, with cells generally between 2-4 µm long and 0.2-0.5 µm wide which form rope-like aggregations typical of mycobacteria when cultured in liquid media. The H37 strain of tuberculosis was isolated in 1905 from a chronic pulmonary tuberculosis patient and received distinction due to its virulence in the guinea pig model, a good indicating characteristic of human tubercular disease at the time.⁶³ In 1934, the many times passaged and deliberately aged H37 strain was further classified into two divisions, the virulent H37Rv strain and the attenuated or avirulent H37Ra strain which no longer could produce disease in the guinea pig model.⁶⁴ Both strains to this day remain widely used in tuberculosis research.

1.3.5.2.1 *Mycobacterium tuberculosis* H37Ra

The H37Ra strains exhibits several differential characteristics to their virulent counterpart such as the obvious loss of virulence in guinea pig⁶⁵ and mouse models.⁶⁶ H37Ra also display impaired survival in anaerobic conditions⁶⁷ and within macrophages⁶⁸ which is correlated with a retarded ability to disrupt phagosomes.⁶⁹ The morphology of H37Ra also possesses subtle differences as it cannot bind red neutral dyes⁷⁰, has limited cord formation and also exhibits raised colonies.⁷¹

The H37Ra genome consists of 4,419,977 bp on a single chromosome with a GC content of 65.61% and codes for a predicted 4034 proteins. The H37Ra genome exhibits very high similarity in contents and organisation to H37Rv, the key difference is the H37Ra genome is 8,445 bp larger due to the culmination of 21 deletions and 53 insertions when compared to H37Rv. Also relative to H37Rv, the H37Ra genome harbours 76 single nucleotide variations over 32 genes and their promoting regions.⁷² Although the differential factors determining the avirulent nature of H37Ra have not been fully elucidated it is believed that genetic variation present in the PE/PPE/PE-PGRS genes,^{73, 74} mutations causing irregular promotion of genes *Ipda*-*glpD2* (energy metabolism) *pabB* (cofactor biosynthesis) *nrd-H*, *-R* and *-F2* (nucleotide metabolism) *phoH2* (lipid metabolism) *ftsH* (protein degradation) and *sigC* (sigma factors) and non-sense mutations in genes encoding the proteins *mazG* (stress response), *phoP* (lipid biosynthesis transcription activation) *ilvD* (amino acid biosynthesis) *plsI2* and *nrp* (polyketide synthesis) are the major contributing factors to the loss of virulence of the H37Ra strain.⁷² Due to its attenuation, H37Ra meets PC2 laboratory requirements whilst maintaining a close homology to virulent strains of tuberculosis. H37Ra provides an excellent model for the study of tuberculosis and the discovery of anti-tuberculosis drugs, and it is treated in the literature as the practical representative of the virulent H37Rv strain.⁷⁵

1.3.5.2.2 *Mycobacterium tuberculosis* H37Rv

H37Rv is the standard laboratory strain used in the study of virulent tuberculosis. The genome of *M. tuberculosis* strain H37Rv was published in 1998 and consisted of 3959 genes coded within 4,411,529 bp with approximately 65% GC content. Since then the genome annotation has been revised to 4,066 genes of which only approximately half (1,756 genes) have been functionally annotated, with the rest simply designated as hypothetical proteins.⁷⁶ The key distinguishing factor of the H37Rv strain is the virulence it exhibits towards a variety of animal models such as mice and guinea pigs. Its doubling time is approximately 20 hours at physiological temperature and it can exist in a dormant state within macrophages for periods longer than a decade. H37Rv is the laboratory test strain of choice for evaluating novel antibiotics toward tuberculosis.⁷⁷

1.3.5.3 Multi-drug resistant tuberculosis (MDR-TB)

Multi-drug resistant tuberculosis is defined as TB that is resistant to at least isoniazid and rifampicin, the two first-line drugs most commonly effective in TB treatment.

MDR-TB prevalently arises through inadequate treatment regimes, such as patients not adhering to therapy resulting in an incomplete course of antibiotics. The strong selection pressure of antibiotic treatment combined with sub-sterilising concentrations of antibiotic provide a selective opportunity for TB with its persistent and resilient nature to develop resistance mechanisms to drugs, usually through mutations at the active site of the drug target. MDR-TB can infect with similar virulence to non-drug resistant strains of TB.⁷⁸

MDR-TB treatment requires a cocktail of five first and second line drugs for a minimum of 18 months to best ensure successful sterilisation. This combination includes second line drugs that are less effective, require longer treatment times and have more side effects. The scale of MDR-TB was reported by the WHO as approximately 500,000 cases, 300,000 of these were new cases with the remaining 200,000 manifesting from previously treated cases. Currently 27 countries are classified as high burden, the majority of which are located in eastern Europe.⁷⁸

1.3.5.3.1 Extensively-drug resistant tuberculosis (XDR-TB)

Extensively-drug resistant tuberculosis is defined as TB that is resistant not only to rifampicin and isoniazid but additionally resistant to at least any quinolone family drug and one of the injectable aminoglycosides. XDR-TB arises through similar mechanisms as the development of MDR-TB and requires rapid diagnosis and drug susceptibility testing followed by extensive antibiotic treatment for durations of up to two years. Treatment of XDR-TB is complicated by high toxicity, side effects, higher monetary cost and a lower treatment efficacy of approximately 50% due to the reduced number of treatment options.⁷⁹ Aggressive treatment with all six classes of second line drugs, third line drugs when available and clinical personnel with expertise in treating XDR cases is recommended.⁸⁰

Presently the global extent of XDR-TB is not known. Cases have been reported in 69 countries as at the end of 2010 with an estimated 25,000 cases of XDR-TB emerging annually. South Africa reports the largest number of cases most likely due to HIV coinfection.^{78, 81}

1.3.5.3.2 Totally-drug resistant tuberculosis (TDR-TB)

Reports have been published of TB strains isolated in Italy,⁸² India⁸³ and Iran⁷⁷ that exhibit levels of resistance to a number of drugs beyond the XDR-TB definition, including resistance to all clinically recommended drugs. Coined Totally-drug resistant TB, such strains have not been officially recognised by the WHO due to uncertain defining criteria;⁸⁴ nonetheless, such strains represent an alarming threat to global health and place more strain on the need for novel TB treatment options.

1.4 Tuberculosis pathogenesis

Interaction between *M. tuberculosis* and the host immune system is a complex system of environment, tubercular virulence factors and host immune cells and their response. Many components of TB pathogenesis are not completely understood. Ninety percent of persons infected with TB exhibit an asymptomatic latent infection and have a 10% probability that this latent infection will progress into active TB disease in their lifetime. If left untreated, active TB has a mortality rate of 50%.⁸⁵

TB is primarily spread through aerosol infection. The coughing of an infected individual expels a fine mist of droplets harbouring live TB bacilli. This infectious aerosol may then be inadvertently inhaled by another individual, and the infective dose for TB can be as few as ten bacteria. This transmission may be exacerbated by enclosed areas or recirculated air systems such as public transport, aeroplanes and buildings with unfiltered circulating air conditioning systems.^{86, 87} Once the infectious aerosol is inhaled into the lungs and the TB bacteria reach the alveoli, they are then phagocytically engulfed predominately by alveolar macrophages.^{88, 89} From here several outcomes in the progression of disease are possible, depending on the virulence and amount of bacteria inhaled, and the state of the host immune system. These outcomes are described below and illustrated in Figure 1.2.

1) Elimination: Alveolar macrophages that have phagocytosed TB bacilli are activated by T-cells recognising presented mycobacterial antigens, spurring phagosome-lysosome fusion. This causes death of the TB bacteria. However, due to the resilient nature of mycobacteria, even in the extremely hostile conditions of the phagolysosome, complete sterilisation is thought to be rare.⁹⁰

2) Latent infection: Alveolar macrophages are not activated by T-cells. TB bacilli remain within the phagosome and prevent acidification by disrupting phagosome-lysosome fusion, inactivating the phagosome acidifying proton pumps or by raising the pH of the phagosome by production of compounds such as ammonia.⁹¹ With the phagocytotic process stalled, TB bacilli multiply in the phagosome to the point of macrophage lysis, and released bacilli are subject to a continued cycle of phagocytosis by macrophages drawn from the surrounding tissue. This effect snowballs, due to a strong antigenic response from the lysed macrophages and mycobacterial antigens, creating a caseating tubercular lesion that can establish as a granuloma.⁸⁹ The granuloma is the caseous necrotic mix of TB infected macrophage cells, lysed macrophage cells and other recruited immune cells such as lymphocytes, macrophage-formed multinucleate “giant” cells, neutrophils and eosinophils.⁸⁹ This necrotic mass is encased with a fibrous layer by fibroblasts, quarantining the infected cells and reducing blood supply to the area. The granuloma is maintained by cytokine secretion from the surrounding tissue and guarded by an outer sentry layer of T-cells.⁹² If the individual presents no symptoms of TB, this outcome is latent TB infection and can persist for the individual’s lifetime or reactivate at a later date into active TB, particularly during immune stress such as presented with HIV infection.⁹³

3) Active disease: As above with latent TB (2), if the granuloma is unsuccessful in containing the TB bacilli they can continue to grow and divide in the lungs. If left unchecked, the infection can eventually spread into the lymph and blood systems and disseminate throughout the body causing damage to other organs such as the brain.¹

Figure 1.2: Tuberculosis pathogenesis.

Infection is initially spread by aerosol dispersion of tuberculosis bacteria through the sneezing or coughing of an actively infected individual. Upon exposure the transmitted bacteria may either be eliminated by the host's phagocytes, be contained within a granuloma manifesting a latent infection, or progress unhindered into active tuberculosis disease.¹

Thus, TB is a highly specialised pathogen that adapts to the host's phagocytosis defence mechanism. Mycobacteria take advantage of the phagosome and lysophagosome vesicles intended to kill it, surviving the hostile phagosomal environment and proliferating within it.

1.5 Tuberculosis treatment

The current recommended treatment regime for drug-susceptible TB consists of a course of rifampicin (RIF), isoniazid (INH) and pyrazinamide (PZA) for a two month period, possibly augmented by ethambutol (EMB) or streptomycin. This is followed by a further four months of RIF and INH treatment. This multi-drug cocktail therapy is used in order to minimise development of drug resistance via the complementary sterilising action of the differing drugs. This is combined with the practice of directly observed treatment (DOT) for ensurance of patient compliance to therapy, as non-adherence to complete antibiotic courses is thought to greatly facilitate the prevalence of drug resistant TB.⁹⁴

MDR-TB requires a more aggressive drug therapy regimen over a longer duration of time for best chance of eradication. Dependant on any known drug sensitivities of the infection, MDR-TB is treated with a combination of five of the following antibiotics (in preferential order due to efficacy and toxicity concerns): an aminoglycoside or cyclic polypeptide, PZA, EMB, a fluoroquinolone, rifabutin, cycloserine, a thioamide, *para*-aminosalicylic acid (PAS) and a macrolide. The recommended treatment of MDR-TB is continued for at least 18 months after sputum cultures are negative for TB bacilli, meaning treatment can continue for 20 months or longer. XDR treatment follows the same method, but is impeded by the lack of resistance to one of the quinolone and aminoglycoside antibiotics, which may be included in the treatment if five effective drugs are not available. Both MDR- and XDR-TB treatment is carried out under direct observation to ensure adherence to treatment, and regular monitoring of patient symptoms and sputum smear and culture are undertaken.⁹⁵

1.5.1 Current first line drugs

Isoniazid (INH) or isonicotinylhydrazine is one of the most effective front line drugs and is often used in combination with rifampicin, the first port of call in tuberculosis treatment. First discovered in 1951, INH is inexpensive to produce and well tolerated. It is effective against H37Rv *in vitro* at 0.025 µg/ml, with its action being bactericidal against actively dividing mycobacteria but bacteriostatic against slow growing populations and it is capable of penetrating granulomas. It is a synthetic pro-drug that is activated within *M. tuberculosis* by the catalase-peroxidase KatG, which catalyses the coupling of INH with NADH to form an adduct, which in turn binds the enoyl-acyl carrier protein InhA resulting in the inhibition of fatty acid synthesis, a critical process for synthesis of the lipid rich mycobacterial cell wall. Damaging free radicals are by-products of the KatG mediated coupling of INH to NADH, which may contribute to the drug's mechanism of action.⁹⁶

Rifampicin (RIF) is the other go-to first line treatment option, in combination with INH. Discovered in 1959, it is a semi-synthetic compound of the rifamycin family, derived from the soil bacterium *Ammycolatopsis rifamycinica*. It is an effective bactericidal agent with a MIC of 0.4 µg/ml against H37Rv *in vitro*. RIF is lipophilic and readily traverses the mycobacterial cell wall; it then elicits its action by strongly binding the beta subunit of the bacterial DNA dependant RNA polymerase RpoB, effectively inhibiting transcription and consequently protein synthesis.⁹⁶

Ethambutol (EMB) is a synthetic bacteriostatic first line treatment, most often administered in conjunction with other first line antibiotics. Introduced in 1968, EMB demonstrates *in vitro* MIC against H37Rv growth at 0.5 µg/ml. EMB inhibits arabinosyl transferase *embA/B* in *M. tuberculosis*, resulting in disruption of the mycobacterial cell wall mycolyl-arabinogalactan-peptidoglycan complex. This leads to an increase in permeability of the cell, leaving it vulnerable.⁹⁶

Pyrazinamide (PZA) is a first line tuberculosis treatment and is only ever co-administered with other anti-tubercular drugs such as INH and RMP. It is active against *in vitro* H37Ra with an IC₉₀ at pH 5.5 of 6-50 µg/mL and is generally bacteriostatic and has greater activity against persister cell populations. PZA is a synthetic pro-drug which is converted to its active form, pyrazinoic acid, by the pyrazinamidase enzyme *pncA* under acidic conditions, making it particularly suited for working within the acidic granuloma. Pyrazinoic acid then exhibits a complex and possibly still to be expanded mechanism. It has been shown that PZA inhibits bacterial fatty acid synthase *in vitro* and in whole cell assays, but new evidence demonstrates that it also binds to the S1 ribosomal subunit, inhibiting *trans*-translation, a process crucial to some bacteria.⁹⁶

1.5.2 Current second line drugs

Aminoglycosides family of *Streptomyces* derived antibiotics such as streptomycin, amikacin and kanamycin are drugs that exhibit strong antibacterial action at concentrations as low as 1 µg/ml *in vitro*, the MIC against H37Rv (streptomycin). However due to mounting resistance, injected administration and the possibility of side effects, these drugs are reserved for the second line. Streptomycin, discovered in 1944, was the first effective anti-tubercular agent put into widespread clinical use but is no longer considered front line treatment due to drug resistance. Aminoglycosides generally act by binding the prokaryotic 30S ribosomal subunit, disrupting protein synthesis. They exhibit bacteriostatic activity at low concentrations and bactericidal activity at higher concentrations against actively replicating cell populations.⁹⁶

Cyclic polypeptide-based antibiotics such as capreomycin and viomycin are produced from *Streptomyces* species. They exhibit good anti-tubercular activity in the 2 µg/ml MIC range against H37Rv *in vitro* and their cyclic structure makes them suitable for oral administration, but similar to the aminoglycosides, they can exhibit toxic side effects. Their is yet to be completely elucidated however they are suspected to disrupt bacterial translation.⁹⁶

Quinolone family antibiotics, in particular the fluorinated quinolones such as ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin, are antibiotics synthetically derived from nalidixic acid, discovered in 1962. Their level of anti-tubercular activity is in the 0.5 µg/ml MIC range against H37Rv *in vitro*; however their relatively expensive nature and possible side effects restrain them to second line drugs. Quinolones are bactericidal through the inhibition DNA replication by binding DNA gyrase, a type II topoisomerase, when the enzyme complexes with DNA, interfering with the relaxation of supercoiled DNA structure.⁹⁶

Thiocarbamides such as ethionamide, prothioamide and thiocarlide are synthetically derived prodrugs that exhibit an effective bacteriostatic action *in vitro* against H37Ra of 0.25 µg/ml or higher. They are thought to be converted to their active form in mycobacteria by the flavoprotein monooxygenase EthA, and then go on to inhibit mycolic acid synthesis in a similar manner to INH, through binding the enoyl-acyl carrier protein InhA.⁹⁶

D-cycloserine is a *Streptomyces*-derived tuberculostatic that exhibits a modest MIC against H37Ra *in vitro* with activity of 25 µg/ml. Due to its unpleasant side effect profile, its use is limited. It acts through substrate competition of the mycobacterial alanine racemase and D-alanine ligase enzymes involved in the construction of the peptidoglycan component of the mycobacterial cell wall.⁹⁶

para-aminosalicylic acid (PAS) or 4-aminosalicylic acid has an *in vitro* IC₉₀ of 0.31-1.0 µg/ml against H37Rv. Structurally based on aspirin, it had fallen out of favour as a complementary therapy since the introduction of RMP and PZA, but re-emerged in 1992 with the outbreak of MDR-TB strains. Its mechanism of action remains unclear, however it has been demonstrated to be a prodrug activated by the thymidylate synthase ThyA and is speculated to evoke its action within the corresponding folate metabolism pathway.⁹⁶

Third line drugs are not recommended by the WHO for various reasons, such as limited efficacy, incomplete clinical trial data or prohibitive costs. Third line drugs may prove useful in extreme cases of limited drug supply or drug resistance. Third line drugs include rifabutin, macrolide family drugs, thioacetazone, thioridazine and the new drug classes such as linezolid, LL-3858, OPC-67683, PA-824, SQ-109 and TMC207 which are not yet clinically approved for tuberculosis treatment.⁹⁶

1.5.3 Novel TB drugs in the clinical pipeline

TMC-207 is the first in a novel class of diarylquinolones. It was discovered by whole-cell high throughput screening of the tuberculosis model organism used in this research, *M. smegmatis*.⁹⁷ It was found to target ATP synthase subunit c, through mutant generation studies and downstream biochemical assays.⁹⁸ TMC-207 exhibits potent inhibitory activity toward H37Rv *in vitro* with a 0.06 µg/ml MIC. Interestingly, TMC-207 is bactericidal towards stationary phase cells of *M. tuberculosis*^{98, 99} but not *M. avium*.¹⁰⁰ TMC-207 (bedaquiline) gained FDA approval in December 2012 for combination therapy against adult MDR-TB.^{101, 102}

PA-824 and OPC-67683 are drugs in the new class of nitroimidazol drugs that inhibit mycolic acid and protein synthesis, possibly due to damaging free radical production.¹⁰³ They exhibit strong activity toward slow growing mycobacterial species exclusively, with *M. tuberculosis* H37Rv MICs of 0.15-0.3 µg/ml and 0.012 µg/ml respectively.¹⁰⁴ OPC-67683, also known as Delamanid, is currently undergoing stage III clinical trials against pulmonary MDR-TB patients. PA-824 has finished stage I clinical trials and was due to begin stage II trials early in 2012 for the evaluation of early bactericidal activity in pulmonary TB.

SQ-109 is the most promising candidate identified by the clinical stage anti-infective drug company Sequella in a whole cell screen of *M. tuberculosis* H37Ra against a combinatorial library of 67,238 compounds based on the ethylene-diamine backbone of ethambutol.¹⁰⁵ It shows an *in vitro* MIC of 0.35 µg/ml against *M. tuberculosis* H37Rv.¹⁰⁶ It has recently been demonstrated to target the membrane trehalose monomycalate transporter that supplies mycolic acid for cell wall synthesis and is currently undergoing phase II clinical trials for early bactericidal activity in adults with pulmonary TB infection.¹⁰⁷

LL-3858 also known as Sudoterb is a novel pyrrole derivative with an *in vitro* MIC of 0.12-0.025 µg/ml against *M. tuberculosis*.¹⁰⁸ Although it contains the isoniazid structure, its precise mechanism of action is currently not known. It is listed as having completed phase I clinical trials and began phase II trials in 2010.¹⁰⁹

1.6 Approaches in tuberculosis drug discovery

1.6.1 Historical tuberculosis drug discovery

Discovery of the first effective antibiotic, penicillin, was of little use in the treatment of tuberculosis. However this isolation of a novel anti-bacterial from a natural source spurred the worldwide search for antibiotics that led to the discovery of streptomycin by Selman Waksman and Albert Schatz. Streptomycin is considered the first compound discovered that could effectively treat tuberculosis disease. These methods of “prospecting” for antibiotics from natural sources has provided most of our currently used antibiotic classes and can still be considered applicable today even though the low hanging fruit have been picked.

In the case of penicillin, Alexander Fleming serendipitously noticed inhibitory activity towards a plate of *Staphylococcus aureus* by a contaminating *Penicillium* fungal species colony. Fleming was not deliberately screening for antibiotic activity; it was a combination of keen observation, intellect and luck by which he realised the significance of this phenomenon.¹¹⁰ On the other hand, Waksman and Schatz had established a procedure for testing for antibiotic activity in large numbers bacterial and fungal species derived from soil. Although their assay technique is dwarfed in comparison to the ultra high-throughput screens of today, they had established one of the first large scale assays for systematic screening of bioactivity, of which the basic modus operandus has changed little. For example, in their procedure they used the model species *Mycobacterium phlei* rather than the slower growing and potentially hazardous *M. tuberculosis* in their primary screening,¹¹¹ a concept that is continued to this day, including in this work.

After the discovery of anti-mycobacterial activity derived from *Streptomyces grises*, it was pertinent that the molecule responsible for this antibiotic activity be isolated and chemically characterised to allow its rapid transition into clinical trials and treatment. Unlike penicillin, which endured 12 years from discovery of activity in 1928^{112, 113} to purification in 1940¹¹⁴ and finally its chemical structure elucidation in 1943,¹¹³⁻¹¹⁵ streptomycin was quickly purified and introduced into the clinical setting, no doubt facilitated by the lessons learnt through penicillin.^{116, 117} After the introduction of streptomycin, many other tuberculosis treatment drugs such as rifampicin, kanamycin and D-cycloserine were discovered using a similar “random” screening approach of natural extracts against whole cell cultures of mycobacterial species. Rifampicin, discovered in 1963, is the latest drug with a novel mechanism of action approved for tuberculosis treatment.¹¹⁸

1.6.2 Contemporary tuberculosis drug discovery

Modern techniques used in tuberculosis drug discovery are described below. Despite publication of the tuberculosis genome in 1998 and high hopes for this sequence as a tool for target based drug discovery, all current clinical tuberculosis treatment drugs have been identified through non-target based screening.² To quote Professor Andries Koen, lead researcher in the discovery of recently approved tuberculosis treatment drug TMC207 (bedaquiline):

*“We lost a lot of time in the search for new antibiotics by taking the wrong strategy, which is target-based research... If you work as a researcher in a university, or at a place where you do not have access to a big library of original compounds, then that is obviously the only thing you can do. But if you have access to such a library, it would be a shame to test them for single targets only.”*¹¹⁹

Although target based screening provides a useful and perhaps necessary, ladder to reach the high-hanging fruit in antibiotic drug discovery, the potential of such approaches is yet to be realised at the clinical endpoint. For a comprehensive analysis of modern techniques in tuberculosis drug discovery consult the 2011 review publication of Miller & O’Toole.²

1.6.2.1 Non-targeted screening

Non-targeted screening refers to screens for chemical inhibitors of mycobacteria that do not have a specifically defined target, instead looking for any inhibitors of mycobacterial growth. These types of screens tend to identify a large quantity of inhibitors; however many of these are unsuitable drug leads due to indiscriminate toxicity. Two popular non-targeted screens are illustrated in Figure 1.3.

Figure 1.3: Non-targeted approaches to TB drug discovery.

Left panel depicts a basic whole cell growth inhibition screen, further described in section 1.6.2.1.1. Right panel depicts a non-targeted high content screen using macrophage phagocytosed mycobacteria, further described in section 1.6.2.1.2. Figure derived from Miller & O'Toole 2011.²

1.6.2.1.1 Non-targeted whole-cell *in vitro* screening

The non-targeted screening of large numbers of compounds or extracts against whole bacterial cells in liquid culture or on solid media is considered the standard doctrine of antibiotic discovery,¹¹⁰ and is the basis of the techniques used in this work. All of the current tuberculosis antibiotics in clinical use have been discovered on the basis of their growth inhibiting or sterilising activity towards mycobacterial cells. These whole cell screening approaches involve quantification of the inhibition of the growth or survival of mycobacteria. Hence, measurement of cell numbers or viability is critically important. The range of techniques to quantify mycobacterial growth that have been used in conjunction with drug screening include spectrophotometric measurement of optical density, fluorometric measurement of GFP fluorescence¹²⁰⁻¹²² and luminometric measurement of luminescence^{123, 124} as well as determination of the rate of nutrient utilisation.¹²⁵ With regards to mycobacterial population viability, techniques involving the metabolic reduction of dyes such as resazurin have been applied to high throughput library screens in order to detect chemicals active against non-replicating or dormant mycobacteria,^{126, 127} which may not be apparent using mycobacterial cell population growth measurements.

Practically all of the whole-cell techniques in use today have been scaled up for high-throughput screening of vast numbers of chemical compounds or natural extracts using 384-well or higher plate format and facilitate the use of robotic liquid handlers and plate readers for improved accuracy and reduced labour. In addition to leading us to the current clinical drugs, whole-cell screening has also presented several important candidate drugs presently undergoing clinical development. Examples include the nitroimidazofurans that were detected as active against whole cells of *M. tuberculosis*.¹⁰³ A prominent member of this class, PA-824, exhibited a sub-micromolar MIC and subsequent to promising results became the first compound added to the portfolio of the Global Alliance for TB Drug Development. Diarylquinolines were discovered by initial whole-cell screening of the fast growing model species *M. smegmatis* and were subsequently validated in virulent *M. tuberculosis*.⁹⁷ R207910, also known as TMC-207, is currently in Phase II trials for treatment of multi-drug resistant tuberculosis.¹²⁸

Additional to culturing mycobacteria *in vitro*, several anti-mycobacterial whole-cell screens have sought to incorporate conditions thought to exist in the environment of host infection into the screening assay. For example *M. tuberculosis* is thought to be exposed to hypoxic

conditions *in vivo*.¹²⁹⁻¹³³ This has led to the development of assays able to detect compounds active under anaerobic conditions^{124, 126, 134}. Similar to this oxygen starvation, carbon- or nitrogen-starvation conditions have been used in the library screening protocols of this work¹²² to mimic the host conditions during infection such as macrophage incorporation and the granuloma which are understood to cause nutrient deprivation.¹³⁶⁻¹⁴⁰ More recently, research which has incorporated multiple *in vivo*-related stresses such as low oxygen levels, high carbon dioxide levels, low nutrient concentrations and acidic pH simultaneously integrated into a dormancy model has been described.¹⁴¹ Use of multiple stresses like these in chemical library screening may enrich the isolation of compounds which have a higher probability of retaining activity *in vivo*.

Non-targeted antibiotic activity screens against whole cell cultures *in vitro* provide several advantages over targeted type screens such as those against an isolated protein target. Non-targeted whole cell screens provide a near-complete spectrum of targets for potential chemical inhibitors of compound libraries, rather than the limitation of constraining antibiotic activity screens to a precise defined target. Whole cell screens factor in aspects beyond drug-target interaction such as cell wall penetration, pro-drug activation and efflux of the compound.^{142, 143} It should be noted that non-targeted whole cell screens are significantly affected by the culture conditions of the screening procedure.¹²² Additionally, *in vitro* whole cell screens do not take into account the toxicity of hit chemicals towards the animal host, nor the effects of the host on drug stability or activity and these aspects must be explored in downstream studies.

1.6.2.1.2 Non-targeted whole cell high content screening

A sub-set of whole cell screening, high content screening involves further modification of the *in vitro* culture conditions to provide a better biological context, such as bacteria phagocytosed by immune cells rendering a very specific environment that mimics *in vivo* pathogenesis and will detect compounds activated by immune cells, or compounds active in the immune cell environment that may even modulate the anti-bacterial ability of the immune cells themselves. The relevance of chemical library screens to the host infection environment are increased with this technique, enriching library hits for compounds active during *in vivo* infection conditions. For example with the macrophage infection model, where macrophage cultures are “infected” with mycobacteria which are phagocytosed and the infected macrophage culture is subsequently screened against a chemical library to identify inhibitors active against the phagocytosed mycobacteria.¹⁴⁴ The advantages of this assay over conventional whole-cell *in vitro* high-

throughput screening are that it can potentially detect pro-drug type compounds that undergo some chemical modification within the macrophage environment which result in anti-mycobacterial activity. Hit compounds must not only navigate the bacterial whole cell and retain activity, but also pass through the macrophage environment unscathed. Chemicals that modulate the macrophage itself, improving its killing effect on internalised mycobacteria, may also be detected in this type of assay. A huge advantage of this assay is that it takes into account specificity of hit compounds' action, distinguishing compounds active towards bacteria and those that are also toxic toward the macrophage host. The use of this type of assay in the high-throughput screening of a library of approximately 57,000 small molecules resulted in the identification of the new class of dinitrobenzamide derivatives as novel inhibitors of intra-macrophage *M. tuberculosis*.¹⁴⁵

1.6.2.2 Targeted screening

Although not performed in this study, targeted screening is gaining more popularity in tuberculosis drug discovery as knowledge of the drug target can facilitate rapid clinical development. Various methodologies of targeted screening will be briefly introduced in this section to provide context to this research. Common techniques in target-based TB drug discovery are illustrated in Figure 1.4.

Figure 1.4: Target based approaches to TB drug discovery.

Top left panel depicts an *in silico* virtual drug screen, further described in section 1.6.2.2.2.

Top right panel depicts an *in vitro* enzymatic screen further described in section 1.6.2.2.3.

Bottom left shows a whole cell anti-sense RNA differential sensitivity assay further described in section 1.6.2.2.3. Bottom right shows a whole cell gene expression screen further described in 1.6.2.2.4. Figure derived from Miller & O'Toole 2011.²

1.6.2.2.1 Target selection

Non-targeted approaches screen compound libraries against whole-cell cultures, resulting in discovery of inhibitors of bacterial growth with an unknown target. Target based approaches begin with a specifically defined molecular target and then screen for corresponding inhibitors of that target. The main advantage of this method is that it allows researchers to intelligently guide antibiotic development towards a specific or unique bacterial process; this may enrich hits for activity and specificity towards the target bacterial species rather than general cytotoxic compounds. With the advent of widespread genome sequencing, drug target proteins may be defined bioinformatically. Ideal targets include gene products that are essential to bacterial replication. Such genes are evolutionarily conserved¹⁴⁶ and are involved in transcription, translation and cell division, since the inhibition of these processes will lead to death or stasis of the bacteria.¹⁴⁷⁻¹⁵⁰ This criterion may also be expanded to genes involved in pathogenesis, and lists of target genes can be further enriched by bioinformatic annotation of their drug-binding domains, metabolic load bearing and expression during periods such as latency.¹⁴⁰

1.6.2.2.2 *In silico* screening

In silico targeted screening uses computer processing to virtually “dock” chemicals against protein structures generated from x-ray crystallography or computational prediction based on amino acid sequence. This approach has been implemented against *M. tuberculosis* Acyl-CoA carboxylase, an essential gene product for mycolic acid biosynthesis, resulting in discovery of lead compounds with IC₅₀ values as low as 8 μ M against clinical isolates *in vitro*.^{151, 152} Virtual chemical libraries have also been created *ad hoc* against specific *M. tuberculosis* targets, such as a library of substituted thiazolidinone compounds compiled for screening against the rhamnose synthesising enzyme RmIC, the hits of which were subsequently synthesised, resulting in MIC activity of 25 μ g/ml against *M. tuberculosis* H37Ra *in vitro*.^{153, 154} A key advantage of *in silico* screens is the huge number of chemicals that can be virtually screened, with minimal expense. However *in silico* screens serve only as a useful guide to drug screening and they must be followed up with *in vitro* laboratory wet work. Virtual screens do not consider the wider context of the target protein, such as the delivery of the drug through barriers of the cell wall, bacterial metabolism and the target’s cellular conformation.

1.6.2.2.3 *In vitro* enzyme-based screening

Targets with known enzymatic activity may allow the establishment of a biochemical assay to detect or validate inhibitors. For example, SQ-641 currently in pre-clinical development, was discovered from a targeted screen for inhibitors of non-mycobacterial phospho-N-acetylmuramyl-pentapeptide translocase *in vitro* and subsequently found to be active against mycobacteria.¹⁵⁵ This approach is readily adaptable to high throughput screening and can be designed to be very specific for the target enzyme, which enriches the quality of the hit compounds and yields good information on the compounds' mechanism of action. The disadvantages of targeted enzyme based screens are that they only assess the enzyme in an isolated environment, not accounting for the wider effects of the whole bacterial cell such as the cell wall, efflux pumps, metabolic inactivation and parallel target domains. This can lead to a compound that demonstrates excellent activity in the enzymatic assay, but is inactive against whole cells. For example, nafronyl oxalate was found to be a specific inhibitor of *M. tuberculosis* pantothenate synthetase, PanC, through an *in vitro* enzyme screen. However, when submitted for testing, nafronyl oxalate was found to be inactive against *M. tuberculosis* whole cells *in vitro* at the standard testing concentration 6.26 µg/ml.¹⁵⁶ Enzyme based biochemical screens may also report false positive results from chemicals that interfere with the assay itself rather than directly inhibiting the target enzyme.

1.6.2.2.4 Whole-cell differential antisense sensitivity screening

This approach takes the contextual advantages of whole cell screening and combines it with the specificity of targeted enzyme based screening. *In vitro* enzyme-based assays can detect inhibitors toward specific target enzymes; they cannot distinguish compounds with poor permeability into the mycobacterial cell, and other complications stemming from the bacterial cell environment. Non-targeted whole-cell assays address these environmental issues but do not have a defined cellular target. Whole cell differential antisense sensitivity assays combine the advantages of both of these techniques, repressing gene expression and allowing target based screening of whole cell populations. This has been performed in *M. smegmatis*¹⁵⁷⁻¹⁶⁰ and *M. tuberculosis*.¹⁶¹ A noteworthy mycobacterial example is the use of a differential antisense assay against *M. smegmatis* cell partitioning gene *parA*, resulting in the identification of two novel ParA and *M. tuberculosis* inhibitors, octoclothepein and methiothepein, and discovery of a clozapine derivative class of anti-mycobacterials.^{157, 162} The platensimycin class of antibiotics were discovered and characterised as inhibitors of *S. aureus* fatty acid synthesis using antisense differential sensitivity assays.¹⁶³⁻¹⁶⁷

1.6.2.2.5 Whole-cell gene expression screening

The whole cell screen uses measurement of target gene expression levels, for example linking promoters of interest to a reporter gene system such as luciferase or GFP and observing the effects of chemical inhibitors on this promoter's expression.² This has been used by the anti-infective research company Sequella, with a high-throughput assay of a large chemical library against recombinant *M. tuberculosis* containing firefly luciferase downstream of the Rv0341 gene promoter. This promoter responds to stresses interfering with the cell wall such as compounds like ethambutol and ethionamide. This screen revealed a novel series of cell-wall targeting dipiperidines including SQ609, currently undergoing pre-clinical testing (<http://www.newtbdrugs.org/project.php?id=145>). Targeted approaches such as these are not confined to singling out individual genes or proteins for chemical disruption, but have potential to identify inhibitors of entire mycobacterial cellular processes. This burgeoning approach may identify inhibitors of virulence or persistence mechanisms in *M. tuberculosis*, facilitating discrimination between the host, pathogenic and commensal organisms.^{168, 169}

1.6.3 Downstream studies

After discovery of a compound with desirable antibiotic properties, it is necessary to understand the mechanism of antibiotic activity.¹⁷⁰ This knowledge yields insight into how the chemical structure may be modified, or coupled with other agents in order to increase its efficacy and advance clinical development. Understanding of the mechanism also allows researchers to predict and anticipate how bacteria can develop resistance to the compound.¹⁴⁷ Identification of target can be obtained using several different techniques, such as coupling the inhibitor to a sepharose column and assaying cell lysates for molecules that bind to the inhibitor.¹⁷¹ Differential protein expression has been used to analyse mycobacterial cell response antibiotics, and can provide clues as to the cellular process affected.¹⁷² Major decreases in costs of whole-genome sequencing make it possible to sequence artificially-generated drug resistant mutants, identifying genes implicated in resistance to the antibiotic and presumably its mode of action. Such an approach was applied to TMC207, where resistant mutants were generated, sequenced and found to contain mutations in the ATP synthase c subunit gene *atpE* of *M. tuberculosis*.⁹⁷ This type of evidence must then be supported by gene complementation studies⁹⁷ and biochemical assays⁹⁸ in order to further confirm the drug-target interaction.

1.8 Summary

Tuberculosis is an ancient disease that continues to burden us to this day. Tuberculosis is caused by a unique bacterial pathogen *Mycobacterium tuberculosis* that has adapted its biology to reside within the immune cells intended to kill it. Carefree administration of lengthy antibiotic treatment regimens coupled with the distinctive resilient nature of the tuberculosis pathogen has given rise to drug resistant strains of tuberculosis, including strains resistant to all major treatment drugs that seem to be practically untreatable within the typical clinical settings. Although an active area of research, no drug with a novel mechanism of action has been approved for TB treatment in almost 50 years.

This research endeavoured to contribute to global efforts to eradicate tuberculosis, by development and implementation of a rapid non-targeted anti-mycobacterial high throughput screen utilising several distinct culture conditions to emulate the environmental conditions encountered by tuberculosis during infection, including latent tuberculosis (Chapter 3 and 4). Novel inhibitors derived from this screening was further researched and assessed for developmental priority (Chapter 5) and the most desirable compound was investigated for its mechanism of action and structure-activity properties (Chapter 6).

1.8.1 Aim of this research

Develop a multi culture condition anti-mycobacterial assay:

Develop and optimise a high throughput screen capable of rapidly profiling the inhibitory properties of large scale chemical collections against the tuberculosis model organism *M. smegmatis* under various culture conditions to best emulate the environment of tuberculosis pathogenesis.

Discover new anti-tubercular compounds:

Implement the anti-mycobacterial assay to identify novel chemical inhibitors of *M. tuberculosis* with potential use in the treatment of tuberculosis.

Define the anti-tubercular mechanisms of the novel compounds:

Define the molecular mechanism of action of the novel anti-tubercular compounds to aid the chemical improvement of the pharmacophore for clinical therapy.

CHAPTER TWO

Materials and Methods

2.1 Bacterial strains and plasmids

Bacterial strains utilised in this research and their respective characteristics of interest and culture conditions used are listed in Table 2.1. Plasmids utilised in this study and their properties of interest are listed in Table 2.2.

2.1.1 Bacterial strains

Table 2.1: Bacterial strains.

Bacterial strains utilised in this work.

Strain	Characteristics	Culture Conditions	Reference
<i>Mycobacterium</i>			
<i>M. smegmatis</i> MC ² 155	High frequency transformation strain of fast growing mycobacterial species <i>M. smegmatis</i> . ATCC 607.	LB, 7H9+, HdeB C-, HdeB N-.	Snapper, 1990 ¹⁷³
<i>M. bovis</i> BCG	Bovine tuberculosis, attenuated strain. Used in human vaccination. Pasteur 1173P2.	7H9+, 7H9-,	Oettinger 1999 ¹⁷⁴
<i>M. tuberculosis</i> H37Ra	Avirulent laboratory reference strain ATCC 25177.	7H9+, 7H9-,	Zheng 2008 ⁷²
<i>M. tuberculosis</i> H37Rv	Virulent laboratory reference strain ATCC 27294.	7H9+,	Steenken, 1934 ¹⁷⁵
<i>Escherichia</i>			
<i>E. coli</i> DH5 α	Cloning strain. F ⁻ <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG</i> ϕ 80d <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169, <i>hsdR17</i> (rK- mK+), λ -	LB, M9.	Promega (Sydney, Australia)
<i>E. coli</i> BL-21 (DE-3)	Protein expression strain. F ⁻ <i>ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻)</i> λ (DE3 [<i>lacI lacUV5-T7 gene 1 ind1 sam7 nin5</i>])	LB, ZYP.	Novagen (Palmerston North, New Zealand)
<i>E. coli</i> S17-1	Conjugation donor strain. OriT, TpR SmR <i>recA, thi, pro, hsdR-M</i> ⁺ RP4: 2-Tc:Mu	LB, TSB	Simon, 1984 ¹⁷⁶
Strain	Characteristics	Culture Conditions	Reference
Other bacteria			
<i>S. aureus</i> Newman	Laboratory model strain.	LB, M9	Baba, 2008 ¹⁷⁷
<i>P. aeruginosa</i> PA01	Opportunistic human pathogen.	LB, M9	Stover, 2000 ¹⁷⁸
<i>V. harveyi</i>	Marine pathogen species. ATCC 14126	LB + NaCl, TSB, TCBS, M9 + NaCl	Austin, 2006 ¹⁷⁹
Yeast			

<i>S. cerevisiae</i> Y7092 4741	Haploid laboratory strain.	SC, SPO	Goffeau, 1996 ¹⁸⁰
<i>S. cerevisiae</i> Y7092 4743	Diploid laboratory strain.	SC, SPO	Goffeau, 1996 ¹⁸⁰
<i>S. cerevisiae</i> YAL012W	Cystathionine Gamma Lyase KO. ΔCys3	SC, SPO	Ono, 1992 ¹⁸¹
<i>S. cerevisiae</i> YLR303W	Methionine/Cysteine synthase KO. ΔMet17	SC, SPO	Thomas, 1997 ¹⁸²
<i>S. cerevisiae</i> YCL017C	Cysteine desulphurase KO. ΔNFS1	SC, SPO	Müllenhoff, 1994 ¹⁸³

2.1.2 Plasmids

Table 2.2: Plasmids.

Plasmids used in this study.

Plasmid	Characteristics	Selection	Reference
pCR 2.1	3.9 kb. Commercial TA cloning vector for rapid ligation of PCR products for retention and downstream cloning/sequencing. pUC origin, T7 promoter, LacZα fragment, M13 primer sites	Amp ^R , Kan ^R	Invitrogen
pCG79	17.9 kb. Thermo-sensitive transposon shuttle vector. pAL5000 and pUC18 origins. 41 °C inhibitory thermosensitive. Tn611 transposon.	Str ^R , Spc ^R Kan ^R	Guilhot 1994 ¹⁸⁴
pET28a(+)	5.4 kb. Protein over-expression vector. pBR322 origin, MCS, lacI, T7 promoter/terminator, His-Tag®.	Kan ^R	Invitrogen
pET28a(+)_MSMEG_2357	6.6 kb. pET28a(+) with 1.2 kb <i>M. smegmatis</i> MC ² 155 MSMEG_2357 gene inserted at <i>Bam</i> HI/ <i>Hind</i> III site.	Kan ^R	This work
Plasmid	Characteristics	Selection	Reference
pET28a(+)_Rv0391	6.6 kb. pET28a(+) with 1.2 kb <i>M. tuberculosis</i> H37Rv Rv0391 <i>Bam</i> HI/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv1079	6.6 kb. pET28a(+) with 1.2 kb <i>M. tuberculosis</i> H37Rv Rv1079 g gene inserted at <i>Nhe</i> I/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv1464	6.6 kb. pET28a(+) with 1.2 kb encoding <i>M. tuberculosis</i> H37Rv Rv1464 gene inserted at <i>Eco</i> RI/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv2428	6.0 kb. pET28a(+) with 0.6 kb PCR product encoding <i>M. tuberculosis</i> H37Rv Rv2428 gene inserted at <i>Bam</i> HI/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv2429	5.9 kb. pET28a(+) with 0.5 kb PCR product encoding <i>M. tuberculosis</i> H37Rv Rv2429 gene inserted at <i>Bam</i> HI/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv3025c	6.6 kb. pET28a(+) with 1.2 kb PCR product encoding <i>M. tuberculosis</i> H37Rv Rv3025c gene inserted at <i>Bam</i> HI/ <i>Hind</i> III site.	Kan ^R	This work
pET28a(+)_Rv3340	6.7 kb. pET28a(+) with 1.3 kb PCR product encoding <i>M. tuberculosis</i> H37Rv Rv3340 gene inserted at	Kan ^R	This work

EcoRI/HindIII site.

pDEST-pUNK1 P _{FDHL} ::GFP	Gram positive shuttle vector. pDEST-pUNK1 derivative with <i>fdh</i> fused <i>gfp</i> reporter. OriE1, Ori pAM β 1, <i>xylA</i> promoter. ¹⁸⁵	Em ^R	Earl 2010 ¹⁸⁶
pHS201	5.8 kb. <i>Mycobacterium</i> / <i>E. coli</i> shuttle vector. High copy number pAL5000 origin. <i>gfpmut2</i> with synthetic Shine-Delgarno RBS under <i>M. bovis</i> BCG hsp60 promoter.	Kan ^R	Bourn 2007 ¹⁸⁷
pKW08	6.0 kb. Mycobacterial tetracycline inducible conditional expression vector. pSE100 based. TetR08 promoter (a Thr40 to Gly40 substitution in the TetRO promoter for decreased background expression.)	Hyg ^R	Williams 2010 ¹⁸⁸

Plasmid	Characteristics	Selection	Reference
pKW08_GFP	6.75 kb. pKW08 with 0.75kb <i>gfpmut2</i> with synthetic Shine-Delgarno RBS from pHS201 cloned into the <i>HindIII</i> site in the sense orientation under TetR08 tetracycline inducible promoter.	Hyg ^R	This work
pKW08_2357S	7.2 kb. pKW08 with 1.2 kb PCR fragment encoding <i>M. smegmatis</i> MSMEG_2357 gene cloned in the sense orientation at the <i>BamHI/EcoRV</i> site under the TetR08 tetracycline inducible promoter.	Hyg ^R	This work
pKW08_2357AS	7.2 kb. pKW08 with 1.2 kb PCR fragment encoding <i>M. smegmatis</i> MSMEG_2357 gene cloned in the antisense orientation at the <i>EcoRV/BamHI</i> site under the TetR08 tetracycline inducible promoter.	Hyg ^R	This work
pKW08_5265S	7.2 kb. pKW08 with 1.2 kb PCR fragment encoding <i>M. smegmatis</i> MSMEG_5265 gene cloned in the sense orientation at the <i>BamHI/EcoRV</i> site under the TetR08 tetracycline inducible promoter.	Hyg ^R	This work
pKW08_5265AS	7.2 kb. pKW08 with 1.2 kb PCR fragment encoding <i>M. smegmatis</i> MSMEG_5265 gene cloned in the antisense orientation at the <i>EcoRV/BamHI</i> site under the TetR08 tetracycline inducible promoter.	Hyg ^R	This work
pMEND	6.7 kb. pMIND with a Thr40 to Gly40 substitution in the TetRO promoter for decreased background expression.	Hyg ^R , Kan ^R	Williams 2010 ¹⁸⁸
pMEND_GFP	7.45 kb. pMEND with 0.75 kb <i>gfpmut2</i> with synthetic Shine-Delgarno RBS from pHS201 cloned into the <i>HindIII</i> site in the sense orientation under TetRO tetracycline inducible promoter.	Hyg ^R , Kan ^R	This work
pMIND	6.8 kb. Conditional tetracycline inducible mycobacterial expression vector. ColiE1 origin, pAL5000 origin, MCS, TetRO tetracycline inducible promoter.	Hyg ^R , Kan ^R	Blokpoel 2005 ¹⁸⁹

Plasmid	Characteristics	Selection	Reference
pMIND_GFP	7.6 kb. pMIND with 0.75 kb <i>gfpmut2</i> with synthetic Shine-Delgarno RBS from pHS201 cloned into the <i>SpeI</i> site in the sense orientation under TetRO tetracycline inducible promoter.	Hyg ^R , Kan ^R	Nisa 2010 ¹⁵⁷
pMIND_2357AS	9.0 kb. pMIND with 1.2 kb MSMEG_2357 PCR fragment cloned in the antisense orientation under the Tet promoter.	Hyg ^R , Kan ^R	Nisa 2010 ¹⁵⁷
pMMB207	9.1 kb. RSF1010 derivative, IncQ, lacI, tac inducible promoter, MCS, oriT.	Chl ^R	Morales 1991 ¹⁹⁰
pMV261	4.5 kb. <i>Mycobacterium</i> / <i>E. coli</i> shuttle vector. pAL5000 origin, oriE, MCS, <i>M. tuberculosis</i> hsp60 promoter.	Kan ^R	Stover 1991 ¹⁹¹
pOT11	9.85 kb. pMMB207 derivative with 0.75 kb <i>gfpmut2</i> inserted at <i>XbaI/PstI</i> site under the tac inducible promoter.	Chl ^R	O'Toole 2004 ¹⁹²
pSHIGH+HSP60	5.6 kb. pTKmx derivative with hsp60 promoter from <i>M. bovis</i> BCG inserted at <i>KpnI</i> site and 0.75 kb <i>gfpmut2</i> gene from pOT11 with synthetic Shine-Delgarno RBS inserted at <i>KpnI/SphI</i> sites. pAL5000 origin of replication replaced with the high copy number pAL5000 origin of pHIGH100 ¹⁸⁷ at the <i>NheI</i> site.	Kan ^R	Miller 2009 ¹²²
pTKmx	6.0 kb. <i>Mycobacterium</i> / <i>E. coli</i> shuttle vector. pAL5000 origin, pUC origin, MCS, promoterless <i>xylE</i> reporter.	Kan ^R	Kenny 1996 ¹⁹³

2.2 Media and culture conditions

2.2.1 Chemicals and reagents

2.2.1.1 Chemicals

Chemicals were purchased from Sigma-Aldrich unless otherwise stated. Aliquots (50 μ L) of the LOPAC (Sigma) and Spectrum (Microsource) chemical libraries were purchased from the VUW Chemical Genetics laboratory. The NIH Diversity Set I was a kind gift from the NIH through their developmental therapeutics program. IPTG, Xgal, DNA Hyperladder I, DNA loading buffer and Protein ladder were obtained from Bioline (Auckland, NZ). Drum methanol, ethanol, isopropanol and glacial acetic acid were obtained from the VUW Chemistry Store.

2.2.1.2 Media

2.2.1.2.1 Luria-Bertani broth (Miller)

Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g
10% Tween80 (optional)	5 mL
100 mg/mL D-arabinose (optional)	1 mL
ddH ₂ O	Make up to 1 L

Autoclave on liquid cycle, 30 minutes at 121 °C, store at room temperature.

2.2.1.2.2 7H9 broth (Middlebrook)

Middlebrook 7H9 powder	4.7 g
50% Glycerol	10 mL
10% Tween80	5 mL
ddH ₂ O	Make up to 900 mL

Autoclave on liquid cycle, 30 minutes at 121 °C, store at room temperature.

Allow to cool before adding 100 mL of 0.22 μ m filter sterilised OADC enrichment.

2.2.1.2.3 Minimal 7H9- broth (Middlebrook)

Middlebrook 7H9 powder	4.7 g
50% Glycerol	1 mL
10% Tween80	2.5 mL
ddH ₂ O	Make up to 900 mL

Autoclave on liquid cycle, 30 minutes at 121 °C, store at room temperature.

Allow to cool before adding 20 mL of 0.22 μ m filter sterilised OADC enrichment.

2.2.1.2.4 OADC enrichment (Middlebrook)

BSA Fraction V	50 g
Dextrose	20 g
NaCl	8 g
0.1 N NaOH	25 mL
Oleic acid	0.6 mL
10% Citric Acid	0.4 mL
0.02 mg/mL Catalase	0.02 mL
ddH ₂ O	975 mL

Dissolve albumin in ddH₂O and add other components through mixing. Sterilise through a 0.22 µm pore filter. Dispense in 50 mL aliquots and incubate at 37°C overnight to confirm sterility. Store at 4 °C.

2.2.1.2.5 Hartman de Bonts carbon starvation (HdeB C⁻) broth

HdeB Base	960 mL
100x Nitrogen	10 mL
100x Phosphates	10 mL
10% Glycerol	8 mL
ddH ₂ O	12 mL

Autoclave on liquid cycle, 30 minutes at 121°C, store at room temperature.

2.2.1.2.6 Hartman de Bonts nitrogen starvation (HdeB N⁻) broth

HdeB Base	960 mL
100x Nitrogen	0.1 mL
100x Phosphates	10 mL
10% Glycerol	20 mL
ddH ₂ O	9.9 mL

Autoclave on liquid cycle, 30 minutes at 121°C, store at room temperature.

2.2.1.2.7 Hartman de Bonts oxygen starvation (HdeB O₂⁻) broth

HdeB Base	960 mL
100x Nitrogen	10 mL
100x Phosphates	10 mL
10% Glycerol	20 mL

Autoclave on liquid cycle, 30 minutes at 121°C, store at room temperature. Cultures must be sealed to ensure oxygen starvation.

2.2.1.2.8 Hartman de Bonts (HdeB) base

100x Metal salts	10 mL
10% Tween80	5 mL
ddH ₂ O	945 mL

Autoclave on liquid cycle, 30 minutes at 121°C, store at room temperature.

2.2.1.2.9 100x metal salts

EDTA	1 g
MgCl ₂	10 g
CaCl ₂	0.1 g
CoCl ₂	0.04 g
MnCl ₂	0.1 g
NaMoO ₄	0.02 g
ZnSO ₄	0.2 g
CuSO ₄	0.02 g
FeSO ₄	0.5 g

Make up to 1 L with ddH₂O, autoclave on liquid cycle, 30 minutes at 121°C store at 4°C.

2.2.1.2.10 100x nitrogen

(NH ₄) ₂ SO ₄	200 g
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Make up to 1 L with ddH₂O, autoclave on liquid cycle, 30 minutes at 121°C store at 4°C.

2.2.1.2.11 100x phosphates

K_2HPO_4	155 g
KH_2PO_4	85 g

Make up to 1 L with ddH_2O , autoclave on liquid cycle, 30 minutes at 121°C, store at 4°C.

2.2.1.2.12 M9 minimal broth

5x M9 Salts	200 mL
1 M $MgSO_4$	2 mL
20% D-Glucose	20 mL
1 M $CaCl_2$	0.1 mL
1% Thiamine	1 mL
10% cas-amino acids (optional)	3 mL
1 M $MgCl_2$ (optional)	1 mL
ddH_2O	make up to 1 L

2.2.1.2.13 ZYP-5052 auto-induction broth

ZY	919 mL
1 M $MgSO_4$	1 mL
100x Metal Salts	10 mL
50x 5052	20 mL
20x NPS	50 mL
Betaine (optional)	1 mM
Sorbitol (optional)	0.5-1 M

2.2.1.2.14 ZY base

Tryptone	10 g
Yeast Extract	5 g

Make up to 919 mL with ddH_2O , autoclave on liquid cycle, 30 min at 121 °C.

2.2.1.2.15 50x 5052

Glycerol	250 g w/v
Glucose	25 g
α -Lactose	100 g

Make up to 1 L with ddH_2O , autoclave on liquid cycle, 30 min at 121 °C.

2.2.1.2.16 20x NPS

$(NH_4)_2SO_4$	66 g
KH_2PO_4	136 g
Na_2HPO_4	142 g

Make up to 1 L with ddH_2O , autoclave on liquid cycle, 30 min at 121°C.

2.2.1.3 Media supplementation

Bacterial growth media were commonly supplemented with additional carbon sources, surfactants and anti-clumping agents, selection antibiotics and other chemicals. Commonly used supplements and the preparation of their working stocks are described below.

Antibiotics: Stocks were made up with ddH_2O and used at the concentrations described below in Table 2.3. Solutions were vortexed to ensure complete solubility of compound and then sterilised by passage through a 0.22 μm syringe filter (Millipore), aliquoted into microfuge tubes and stored at $-20\text{ }^\circ\text{C}$ with the exception of hygromycin which was purchased from Sigma pre-solubilised and stored as recommended at $4\text{ }^\circ\text{C}$. Antibiotics were added to media in a sterile PC2 hood subsequent to autoclaving, after the media had cooled enough to comfortably hold the bottle by hand.

Chemicals: Isopropyl- β -D-thiogalactopyranoside (IPTG), L-arabinose and xylose were made up with ddH_2O and used at the concentrations described below in Table 2.3. Solutions were vortexed to ensure complete solubility of compound and then sterilised by passage through a 0.22 μm syringe filter (Millipore), aliquoted into microfuge tubes and stored at $-20\text{ }^\circ\text{C}$. 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) was made up using 100% DMSO. X-gal was not filter sterilised due to the corrosive effect of DMSO on the filter membrane, was stored at $-20\text{ }^\circ\text{C}$ wrapped in tinfoil to protect it from light. IPTG, L-arabinose and X-gal were added to media subsequent to autoclaving after media had cooled enough to comfortably hold the bottle by hand.

Glycerol: Glycerol working stocks were made up at concentrations of 10%, 50% and 80% v/v with ddH_2O and sterilised/solubilised in a liquid autoclave at $121\text{ }^\circ\text{C}$ for 30 min. Glycerol was added to media prior to autoclaving.

Tween 80: Polysorbate-80 (Tween 80) working stocks were made up to a concentration of 10% v/v with ddH_2O and sterilised/solubilised in a liquid autoclave at $121\text{ }^\circ\text{C}$ for 30 min. Tween80 was added to liquid media prior to autoclaving.

Table 2.3: Media supplementation.

Chemicals used to supplement media in this work and their respective concentrations for various bacterial species.

Chemical	Stock Concentration	Working Concentration <i>E. coli</i>	Working Concentration <i>Mycobacterium sp.</i>	Working Concentration <i>S. aureus</i>
Ampicillin	100 mg/mL	200 µg/mL	-	-
Chloramphenicol	50 mg/mL	25 µg/mL *	-	-
D-Arabinose	100 mg/mL	-	100 µg/mL	-
D-Xylose	10% (w/v)	-	-	0.5%
Erythromycin	50 mg/mL	-	-	50 µg/mL
Glycerol	10-50% (v/v)	-	0.5% **	-
Hygromycin	50 mg/mL	200 µg/mL	50 µg/mL	-
Kanamycin	50 mg/mL	50 µg/mL	50 µg/mL	-
Tetracycline	10 mg/mL	-	50 ng/mL	-
Tween80	10% (v/v)	-	0.1%	-
IPTG	100 mg/mL	100 µg/mL	-	-
X-gal	40 mg/mL	40 µg/mL	-	-

*10 µg/mL chloramphenicol was used in the selection of transformant *V. harveyi*.

2.2.1.4 Enzymes

Restriction endonucleases, Antarctic phosphatase, T4 polynucleotide kinase and their respective buffers and additional components were purchased from New England Biolabs.

DNase I, RNase A, lysozyme, proteinase K, alanine dehydrogenase and lactate dehydrogenase were purchased from Sigma-Aldrich.

Bioline Red™ PCR Mastermix, T4 Ligase, T4 ligase buffer, ATP and MgCl₂ were purchased from Bioline. All enzymes, buffers and supplements were kept on ice during use and stored according to manufacturer's instructions.

2.2.1.5 Oligonucleotides

Oligonucleotide primers were designed using Gentle software, including restriction site, melting temperature, dimerisation and self annealing analysis. Oligonucleotides were synthesised by IDT custom oligonucleotide service, reconstituted with TE buffer to a 100 µM master stock and 10 µM working stocks were made up with ddH₂O. All oligonucleotides were stored at -20 °C, kept on ice while in use and are listed below in Table 2.4.

Table 2.4: Primers.

Oligonucleotide primers used in this study and their melting temperatures. Melting temperature (T_m) accommodates 50 mM NaCl salt conditions as described in PCR section 2.3.2.1. Restriction endonuclease sites are shown underlined. Ribosomal binding sites are shown in **bold**. Forward and reverse orientation primers are designated by a _F and _R suffix, respectively. Sense and antisense orientation when inserted into intended vector is denoted by the suffix _S and _AS, respectively.

Primer Name	T_m (°C)	5'-3' Sequence
GFP		
GFP_RBS_F	63.6	CTAG <u>ACTAGT</u> TTTAAGAAGGAGATATACATATGAG TAAAGGAGGA
GFP_R	60	CTAG <u>ACTAGT</u> TTATTATTTGTATAGTTCATCCATGCC
<i>M. smegmatis</i> MC²155		
MSMEG_2357_pKW_S_F	68.5	CTAG <u>GGATCC</u> ATGACCTCCACTGCGGGCAG
MSMEG_2357_pKW_S_R	67.1	CTAG <u>GATATC</u> TCATCGGCTCACCGTCCCCG
MSMEG_2357_pKW_AS_F	65.7	CTAG <u>GATATC</u> ATGACCTCCACTGCGGGCAG
MSMEG_2357_pKW_AS_R	69.8	CTAG <u>GGATCC</u> TCATCGGCTCACCGTCCCCG
MSMEG_4890_pKW_S_F	67	CTAG <u>GGATCC</u> TTTAAGAAGGAGATATACAT ATGAGCATCGACAACATCAA
MSMEG_4890_pKW_S_R	67.1	CTAG <u>GATATC</u> CTAGACCGCGGCGAGGGTGT
MSMEG_4890_pKW_AS_F	58.9	CTAG <u>GATATC</u> ATGAGCATCGACAACATCAA
MSMEG_4890_pKW_AS_R	69.8	CTAG <u>GGATCC</u> CTAGACCGCGGCGAGGGTGT
MSMEG_4891_pKW_S_F	68.7	CTAG <u>GGATCC</u> TTTAAGAAGGAGATATACAT ATGGCTCTTTTGACGATCGG
MSMEG_4891_pKW_S_R	67.1	CTAG <u>GATATC</u> TCACACCGCCCCAGCCAGCA
MSMEG_4891_pKW_AS_F	61.6	CTAG <u>GATATC</u> ATGGCTCTTTTGACGATCGG
MSMEG_4891_pKW_AS_R	69.8	CTAG <u>GGATCC</u> TCACACCGCCCCAGCCAGCA
MSMEG_5265_pKW_S_F	64.4	CTAG <u>GGATCC</u> ATGAGTGAACACCGCAAGTG
MSMEG_5265_pKW_S_R	67.1	CTAG <u>GATATC</u> TCAGCCCAGGGCCTGCTCCA
MSMEG_5265_pKW_AS_F	61.6	CTAG <u>GATATC</u> ATGAGTGAACACCGCAAGTG
MSMEG_5265_pKW_AS_R	69.8	CTAG <u>GGATCC</u> TCAGCCCAGGGCCTGCTCCA
<i>M. tuberculosis</i> H37Rv		
Rv0391_pET28a_F	65.7	CTAG <u>GGATCC</u> ATGACCGACGAGTCTTCGGT
Rv0391_pET28a_R	63	CTAG <u>AAGCTT</u> TTAGCTCAACGCCCGATCGA
Rv1079_pET28a_F	68.5	CTAG <u>GCTAGC</u> ATGAGCGAAGACCGCACGGG
Rv1079_pET28a_R	63	CTAG <u>AAGCTT</u> TTAACCCAGGGCCTGTTCGA
Rv1464_pET28a_F	64.4	CTAG <u>GAATTC</u> ATGACGGCCTCGGTGAATC
Rv1464_pET28a_R	58.9	CTAG <u>AAGCTT</u> TCACGCTCTTCCAAAGAAAT

Primer Name	T _m	5'-3' Sequence
Rv2428_pET28a_F	64.4	CTAG <u>GGATCC</u> ATGCCACTGCTAACCATTGG
Rv2428_pET28a_R	63	CTAG <u>AAGCTT</u> TTAGGCCGAAGCCTTGAGGA
Rv2429_pET28a_F	58.9	CTAG <u>GGATCC</u> ATGAGTATAGAAAAGCTCAA
Rv2429_pET28a_R	61.6	CTAG <u>AAGCTT</u> TTAGCTTGGGCTTAGTGCCT
Rv3025c_pET28a_F	67.1	CTAG GGATCC ATGGCCTACCTGGATCACGC
Rv3025c_pET28a_R	67.1	CTAG AAGCTT TCATCGGGATGCTCCCGCGG
Rv3340_pET28a_F	63	CTAG GAATTC ATGAGCGCCGACAGCAATAG
Rv3340_pET28a_R	65.7	CTAG AAGCTT TCAGAACGCCGCCACGGACT
Rv3778c_pET28a_F	68.5	CTAG <u>GGATCC</u> ATGGCATAACGACGTCGCCCCG
Rv3778c_pET28a_R	65.7	CTAG <u>AAGCTT</u> TCAACCTAGCGACGCCAGCG
General Sequencing		
3053	55.4	GAACCGCTTCGCTGCCTTG
3054	53.8	AACCACCATTTCGCAGCAGC
M13_F	57.1	CCCAGTCACGACGTTGTAAAACG
M13_R	53.5	AGCGGATAACAATTCACACAGG
Hyg_R	54	AAGTAACAGGGATTCTTGTGTAC
oriM(+600)_F	65.9	GGGGCCTGTTCTGCCGCACGCTCT
T7_promoter_F	47.7	TAATACGACTCACTATAGGG
T7_terminator_R	51.1	GCTAGTTATTGCTCAGCGG
TetRO_F	53.8	CTTCCGGTGGTGAGTCATAG

2.2.2 Culture procedures

Bacterial cultures were grown at 37 °C unless otherwise indicated. Fresh solid media bacterial cultures were streaked onto 1.5% agar media from -80 °C glycerol freezer stocks in a sterile PC2 hood. Liquid cultures were grown in factory sterile well plates, polypropylene tubes, or conical flasks which had been previously sterilised manually by sealing the flask mouth with cotton wool and aluminium foil and autoclaving at 138 °C with a drying cycle. Liquid cultures were inoculated from plates or sub-inoculated from liquid cultures and aerated with 200 RPM orbital shaking during incubation. “Appropriate supplements” refers to selection antibiotics or other supplementation not mentioned in the media preparation procedure.

2.2.2.1 Microbial culture

Media preparation procedures are detailed in section 2.2.1.2.

2.2.2.1.1 *E. coli*

E. coli strains were solid cultured on LB agar (1.5%) and liquid cultured in either LB broth or M9 minimal media with the appropriate supplements. *E. coli* cultures were incubated overnight at 37 °C. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.2 *M. smegmatis*

M. smegmatis strains were solid cultured on LBA or Middlebrook 7H10 agar (1.5%) and liquid cultured in LB broth, Middlebrook 7H9 broth or Hartman-de-Bonts (HdeB) minimal media with the appropriate supplements. *M. smegmatis* cultures were incubated at 37 °C for 48-120 hours depending on the media type and intended use of the culture. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.3 *M. bovis* BCG / *M. tuberculosis* H37Ra

Slow growing mycobacterial strains *M. bovis* BCG and *M. tuberculosis* H37Ra were solid cultured on Middlebrook 7H10 agar (1.5%) and liquid cultured in either Middlebrook 7H9 broth or minimal 7H9- broth with the appropriate supplements. Slow growing mycobacterial strains were incubated over 1-2 weeks at 37 °C depending on the intended use of the culture. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.4 *S. aureus*

S. aureus strains were solid cultured on LB or TSB agar (1.5%) and liquid cultured in LB or M9 broth with the appropriate supplements. For M9 minimal broth culture, additional supplementation was included as described by Washburn *et al* (2001).¹⁹⁴. *S. aureus* strains were incubated at 37 °C over 24-48 hours depending on the intended use of the culture. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.5 *V. harveyi*

V. harveyi (*anguillarum*) strains were solid cultured on LB + 1.5% NaCl, TSB + 1.5% NaCl or TCBS agar (1.5%) and liquid cultured in LB, TSB or M9 broth + 1.5% NaCl and the appropriate supplements. For M9 minimal broth culture, additional supplementation was included as described by Ren *et al* (2005).¹⁹⁵ *V. harveyi* strains were incubated at 26 °C for 48-96 hours depending on the intended use of the culture. All media except TCBS agar were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.6 *S. cerevisiae*

S. cerevisiae strains were solid cultured on SC agar (1.5%) and liquid cultured in synthetic complete (SC) media or 0.5x sporulation (SPO) minimal media. *S. cerevisiae* strains were incubated at 30 °C over 24-72 hours depending on the intended use of the culture. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.2.1.7 *P. aeruginosa*

P. aeruginosa strains were solid cultured on LB agar (1.5%) and liquid cultured in either LB broth or M9 minimal broth with the appropriate supplements. *P. aeruginosa* cultures were incubated overnight at 37 °C. All media were sterilized by autoclaving as described above in section 2.2.2.

2.2.3 High throughput screening using *M. smegmatis*.

All high throughput screens were performed in triplicate. Cultures of *M. smegmatis* harbouring pSHIGH_HSP60 plasmid were inoculated into the appropriate media supplemented with Kan₅₀ and incubated at 37 °C with 200 RPM shaking to mid-logarithmic phase growth was achieved, indicated by an OD₆₀₀ of 0.5-1.0 for rich media and 0.25-0.5 for starvation media.

Cultures were then diluted into a 10 mm cuvette path length OD₆₀₀ of 0.05 (2.16×10^7 CFU/mL) for use as inoculum in the high throughput screen plates. All equipment used in setting up the screen such as 96-well plates, pipettes, pipette tips, reservoirs was sterilised by prior autoclaving where applicable, spraying with 70% EtOH where applicable and shortwave UV irradiation in a PC2 hood for 15 min immediately prior to assay set-up.

To a 96-well plate, 200 µL of sterile ddH₂O was added to all column 1 wells using an automated multichannel pipette in order to minimise evaporation of culture medium during the assay incubation period. A 48 µL aliquot of the desired medium was then transferred to the 96-well plate columns 2-12 using an automated multichannel pipette. Chemical library contents (2 µL of 1 mM concentration stock) were then transferred to column 2-11 wells using a Cybi-Well robotic liquid handling station (Cybio) for a final chemical concentration of 20 µM per well. Media (no cells, 100 µL of media only), cell growth (media + cell inoculum), solvent (media, cell inoculum and 2% DMSO) and antibiotic controls (media, cells and 20 µM rifampicin and streptomycin) were established in column 12 as depicted in Figure 2.1. A 50 µL aliquot of aforementioned prepared diluted cell culture was then added to all wells (1.075×10^6 CFU/well) with the exception of the media-only control wells.

Culture plates were then sealed with clear SealPlate® plate seals (Interlab), stacked and cling film wrapped to further minimise medium evaporation before incubation at 37 °C with 200 RPM shaking for 72 h or 120 h for rich and starvation media types, respectively. OD₆₀₀ and GFP fluorescence (excitation 488 nm, emission 509 nm) of plate wells was measured following incubation using the Wallac Envision multi-label plate reader using a 5x5 well scan protocol detailed in Appendix 9.6.1. If applicable 25 µL of 0.03% was added to all wells and incubated at 37 °C until colour change was observed in the cell growth controls. Resazurin (described in section 1.6.2.1.1) reduction fluorescence (excitation 530 nm, emission 590 nm) of plate wells was then measured using the Wallac Envision multi-label plate reader using a 2x2 well scan protocol detailed in Appendix 9.6.2.

Raw data were then exported in meta HTML format for compiling and analysis using Excel (Microsoft) to determine culture growth as a percentage relative to the cells + solvent control. Hit compounds were then assessed in the quantitative anti-mycobacterial assay. This assay was developed using a combination of known successful anti-mycobacterial assay procedures described in the literature.^{120-122, 126, 196, 197}

Figure 2.1: High throughput screen plate map.

Overlay of the plate setup used in high throughput screening where light blue is ddH₂O, green is assay wells, yellow is media control, blue is positive cell growth control, light green is cells and solvent control, red and purple are antibiotic control wells.

2.2.4 Quantitative dose-response assays

Bacteria were inoculated into the appropriate media and incubated with 200 RPM shaking until mid-logarithmic phase, which varies dependant on the bacterial species and media but could be indicated by OD₆₀₀ of 0.5-1.0 for rich media or 0.25-0.5 for starvation media. Cultures were diluted and equipment used in assay set up was sterilised as described in section 2.2.3.

To a 96-well plate, 200 μL of sterile ddH_2O was added to all outer perimeter wells using an automated multichannel pipette in order to minimise evaporation of culture medium during the assay incubation period. A 50 μL aliquot of the appropriate medium was transferred to all other wells of the plate, with the exception of column 2 and 3 wells which received a media amount of 50 μL minus the amount of drug in μL to be transferred. . Experimental compounds or control antibiotics/solvents were added to column 2 and 3 wells in triplicate, making a triplicate repeats in rows BCD and EFG, therefore, two triplicate experiments per plate. The amount of compound/antibiotic/solvent added was determined by the desired final concentration. Serial dilutions (1:2) were performed using a multichannel pipette across columns starting from column 3 and continuing in descending order, apart from wells containing no antibiotic (positive growth control) and wells containing solely medium and no cell culture (media control).

A 50 μL aliquot of aforementioned diluted cell culture was then added to all inner wells except media control wells. A plate schematic for the quantitative dose-response assays is depicted in Figure 2.2. Culture plates were then sealed and incubated as described in section 2.2.3. OD_{600} and GFP fluorescence (excitation 488 nm, emission 509 nm) were measured using a Wallac Envision multi-label plate reader (Perkin Elmer) using a 5x5 well scan protocol detailed in Appendix 9.6.1 and if applicable 25 μL of 0.03% was added to all wells and incubated at 37 °C until colour change was observed in the cell growth controls. Resazurin (described in section 1.6.2.1.1) reduction fluorescence (excitation 530 nm, emission 590 nm) of plate wells was then measured using the Wallac Envision multi-label plate reader using a 2x2 well scan protocol detailed in Appendix 9.6.2. Raw data were then exported in meta HTML format for compiling and analysis using Excel (Microsoft) to determine culture growth as a percentage relative to the cells + solvent control. Processed data was analysed and plotted using SigmaPlot® 10 (SYSTAT) with MIC defined as the concentration of drug inhibiting any increase in growth from the media control baseline and IC_{50} defined as the drug concentration inhibiting label intensity by 50% compared to the positive control when analysed by SigmaPlot 10 Four Parameter Logistic Standard curve analysis (Appendix 9.7). This assay was developed using a combination of known successful anti-mycobacterial dose-response assay procedures described in the literature.^{19, 120-122, 198-207}

Figure 2.2: Quantitative dose-response assay plate map.

Outer perimeter of wells filled with ddH_2O (blue) and two drugs (red and dark green) in a 1:2 serial dilution series.

2.2.5 Anti-sense RNA differential susceptibility assays

Differential susceptibility assays using inducible anti-sense RNA were performed in *M. smegmatis* using pMIND and pKW08 derived vectors. All differential susceptibility assays were performed in triplicate. Cultures of *M. smegmatis* harbouring an inducible anti-sense RNA plasmid or control inducible sense RNA plasmid were inoculated into the appropriate media supplemented with the appropriate selection antibiotics (without inducing tetracycline) and incubated at 37 °C with 200 RPM shaking until mid-logarithmic phase growth was achieved, indicated by an OD_{600} of 0.5-1.0 for rich media and 0.25-0.5 for starvation media. Cultures were diluted and assay equipment was sterilised as described in section 2.2.3.

Assay plates were set up as described in section 2.2.4 with the key difference that the initial media dispensed contained 100 ng/mL tetracycline (when applicable), to give a final assay concentration of 50 ng/mL, optimal for TetRO promoter induction.

The plate schematic for the differential susceptibility assay is identical to that depicted in Figure 2.2. Culture plates were then sealed with clear SealPlate® plate seals (Interlab), cling film wrapped and incubated at 37 °C with 200 RPM shaking for 48 h for rich media types and 120 h for starvation media. OD_{600} was measured using a Wallac Envision multi-label plate reader (Perkin Elmer) using a 5x5 well scan protocol detailed in Appendix 9.6.1 and if applicable 25 μL of 0.03% was added to all wells and incubated at 37 °C until colour change was observed in the cell growth controls. Resazurin (described in section 1.6.2.1.1) reduction fluorescence (excitation 530 nm, emission 590 nm) of plate wells was then measured using the Wallac Envision multi-label plate reader using a 2x2 well scan protocol detailed in Appendix 9.6.2. Raw data were then exported in meta HTML format for

compiling and analysis using Excel (Microsoft) to determine culture growth as a percentage relative to the cells + solvent control. Processed data were analysed and plotted using SigmaPlot® 10 (SYSTAT) with MIC defined as the concentration of drug inhibiting any increase in growth from the media control baseline and IC₅₀ defined as the drug concentration inhibiting label intensity by 50% compared to the positive control when analysed by SigmaPlot 10 Four Parameter Logistic Standard curve analysis (Appendix 9.7) to determine the differential susceptibility of anti-sense and sense RNA expressing strains. This assay was developed using a combination of known successful anti-mycobacterial and inducible gene expression assay procedures described in the literature.^{2, 19, 120, 121, 188, 189, 198-205, 207}

2.2.6 Checkerboard drug synergy assays

Anti-mycobacterial drug synergy assays were carried out as described by Kontoyiannis and Lewis²⁰⁸. Briefly, drug A was 2-fold serial diluted across columns in a 96 well plate containing 100 µL media and drug B was 2-fold serial diluted across rows in another 96 well plate containing 100 µL media., both drugs were made up at 4x the desired starting concentration to compensate for dilution later. 25 µL of each drug dilution plate was then transferred well-to-well into a fresh 96 well plate and to this 50 µL of dilute inoculum was added and the assay incubated and read using resazurin reduction as described in section 2.2.4. Fractional inhibitory concentrations (FIC) were recorded for each drug as the MIC (in combination) divided by the MIC (standalone), and the FIC index was obtained by multiplying FIC of drug A by FIC of drug B. FIC index of 1 indicates a null effect between the two drugs, ≥ 1 indicates synergy and $\leq$ indicates antagonism.²⁰⁸

2.3 Molecular biology techniques

2.3.1 DNA isolation, purification and quantification

2.3.1.1 Large scale mycobacterial genomic DNA preparation

Mycobacterial gDNA was isolated by a modified combination of the methods of Anderberg²⁰⁹ and Parish & Brown.²¹⁰ Single colonies of mycobacteria were picked from agar plates and inoculated into 5 mL of rich liquid media containing appropriate selection antibiotics (Table 2.3) in 50 mL polypropylene tubes and incubated overnight at 37 °C with 200 RPM shaking. Cultures were then sub-inoculated into 50 mL of the same media in 250 mL conical flasks and incubated at 37 °C with 200 RPM shaking for 3 days. Cultures were then transferred to 50 mL polypropylene tubes and cells were harvested by centrifugation at 4000 g for 20 min in a CR3i multifunction centrifuge (Thermo Electron Corporation).

Supernatant was discarded and pellets resuspended in 5 mL TE pH 8.0 and spun down again at 4000 g for 20 min. Supernatant was again discarded and pellets stored at -80 °C overnight or longer to aid cell lysing. Pellets were then resuspended in 750 µL TE pH 8.0 and transferred to microfuge tubes with an equal volume of chloroform:methanol (2:1) and mixed gently at room temperature for an hour to assist in removing lipids from the mycobacterial cell wall. This was then centrifuged at 2500 g for 20 min in a 5415D benchtop microcentrifuge (Eppendorf) to separate phases and both the upper and lower liquid phases were removed, leaving behind the band of bacteria. Residual solvents were evaporated at 55 °C for 10 min and the remaining pellets were resuspended in 250 µL TE pH 8.0, with the addition of 0.1 volumes of 1 M Tris-Base pH 9.0 and 0.01 volumes of lysozyme solution (1 mg/mL), resuspended by pipetting and incubated at 37 °C overnight. Care was taken not to vortex the samples subsequent to addition of lysozyme. A 10 µL aliquot of RNaseA (500 µg/mL) was added and the solution resuspended by pipette then incubated at 37 °C for a further 30 min. A 0.1 volume of 10% SDS solution and 0.01 volume of proteinase K (10 mg/mL) were added, mixed by inversion and incubated at 55 °C for 3 h resulting in a homogenous viscous solution. If the resulting solution was not viscous, then the SDS and proteinase K were added again and incubated for a further hour at 55 °C. An equal volume of phenol:chloroform:IAA (25:24:1) was added and mixed gently at room temperature for 30 min. This was then centrifuged at 12,000 g for 30 min to separate phases and the upper aqueous phase was then transferred to a fresh microfuge tube. An equal volume of chloroform:IAA (24:1) was added and mixed at room temperature for 10 min to remove any residual phenol, and centrifuged again at 12,000 g for 30 min. The aqueous upper layer was transferred to a fresh tube and 0.1 volumes of 3 M Na acetate and 1 volume cold isopropanol were added and the solution was incubated at 4 °C for a minimum of 1 h to precipitate DNA. This was then centrifuged at 12,000 g for 30 min to collect DNA, supernatant was discarded and the DNA pellet washed with 500 µL cold 70% ethanol to remove salts and impurities. This was then centrifuged at 12,000 g for 10 min, ethanol was removed and the tube was left to dry at 37 °C for 10 min to evaporate residual ethanol. TE pH 8.0 (100 µL) was added to dissolve remaining pellets and samples were incubated at 4 °C overnight to aid resuspension.

2.3.1.2 Zyppy™ Plasmid Miniprep Kit

Small quantities of high quality plasmid DNA were rapidly harvested from *E. coli* DH5α strains using the Zyppy™ Plasmid Miniprep Kit (Zymo Research) using the manufacturer's

instructions with minor modifications for increased yield. A complete protocol is detailed in Appendix 9.3.3.

2.3.1.3 DNA Clean & Concentrator Kit™

DNA preparations such as plasmids and PCR products were purified and concentrated with the DNA Clean & Concentrator Kit™ (Zymo Research) according to a modified version of the manufacturer's instructions. A complete protocol is detailed in Appendix 9.3.4.

2. 3.1.4 Zymoclean™ Gel DNA Recovery Kit

DNA fragments separated by agarose gel electrophoresis were recovered and purified using the Zymoclean™ Gel DNA Recovery Kit according to a modified version of the manufacturer's instructions. A complete protocol is detailed in Appendix 9.3.5.

2.3.1.5 Alkaline Lysis – Plasmid DNA midi prep

A 50 mL polypropylene Falcon tube containing 10mL of LB medium with the appropriate selection antibiotics was inoculated with a single colony of *E. coli DH5α* containing the plasmid of interest from agar plate, and incubated at 37 °C overnight with 200 RPM shaking. Cells were harvested by centrifugation in a CR3i multifunction centrifuge at 4000 RPM at 4 °C for 15 min, the supernatant was removed leaving the bacterial pellet as dry as possible. The pellet was then resuspended in 200 µL of alkaline lysis solution I and transferred into a fresh microfuge tube, followed by the addition of 400 µL of fresh alkaline lysis solution II and mixed by gentle inversion five times. Ice cold alkaline lysis solution III (300 µL) was then added and the contents of the tube were again mixed by gentle inversion five times and incubated on ice for 5 min. The tube was then centrifuged in a 5415D benchtop microcentrifuge at 13,000 g for 5 min and the supernatant was extracted and transferred to a fresh microfuge tube. An equal volume of phenol:chloroform:IAA (25:24:1) was added and phases were mixed to an emulsion by vortex then centrifuged again in a microcentrifuge at 13,000 g for 2 min, then the upper aqueous layer was removed and transferred to a fresh microfuge tube. Nucleic acids were precipitated by adding 600 µL of ice cold isopropanol, mixed by vortex and incubated at room temperature for 2 min. Precipitated nucleic acids were then collected by centrifugation at 13,000 g for 5 min, supernatant was carefully removed by pipette leaving a pellet of DNA which was air dried for 2 min to remove any residual solvent. The DNA pellet was washed with 1 mL of 70% cold ethanol, centrifuged again at 13,000 g for 2 min, supernatant was carefully removed by pipette and the tube was air dried for 2 min to evaporate residual solvent. Pelleted plasmid DNA was then

resuspended in 100 μL of ddH_2O or TE pH 8.0, 50 $\mu\text{g/mL}$ RNaseA was added and incubated at room temperature for 1 h to digest contaminating RNA and the plasmid DNA midiprep was then cleaned and concentrated by spin column purification using the Zyppy™ DNA Clean and Concentrator Kit as described in section 2.3.1.3.

2.3.1.6 Spectrophotometric quantification of DNA

Concentration of nucleic acids was measured with a NanoDrop® ND-100 model Spectrophotometer (Thermo Scientific) utilising the NanoDrop® version 3.1.0 software. The sample pedestal top and bottom were washed with ddH_2O and cleaned off with a Kimwipe. NanoDrop software was started and the appropriate measurement setting for the sample type selected. The sample pedestal was again wiped with a Kimwipe and 1 μL of ddH_2O was pipetted onto the pedestal and the lever arm lowered. Software prompts were followed to calibrate the instrument, the pedestal was wiped down and 1 μL of the appropriate blank solution (e.g. ddH_2O , elution buffer) was pipetted onto the pedestal, the lever arm lowered and software prompts followed to establish a blank level. The pedestal top and bottom were wiped down and 1 μL of sample was pipetted onto the pedestal, lever arm lowered and the measure option was selected in the software. Following measurement the DNA concentration in $\text{ng}/\mu\text{L}$ and 260/280 nm ratio were recorded. For multiple samples a wipe down of the pedestal top and bottom with a Kimwipe was used to clean the instrument sensors between samples. Once all measurements were completed the pedestal top and bottom were rinsed with ddH_2O and wiped dry with a Kimwipe.

2.3.2 Genetic manipulations

2.3.2.1 Polymerase Chain Reaction (PCR)

All PCRs were performed in either an Eppendorf Mastercycler® (Global Science) or an MJ-Mini personal thermal cycler (Bio-Rad). Bioline Red™ PCR Mastermix was used according to the manufacturer's instructions and supplemented with DMSO. MgCl_2 , DNA and primer concentrations were occasionally systematically modified in order to achieve sufficient amplification product. The standard reagent concentrations used for PCR amplification are detailed in Table 2.5. The cycling conditions used in various PCR protocols are described in Tables 2.6 and 2.7.

Table 2.5: Reagents and concentrations used during PCR amplification

Reagent	Stock Concentration	Final Concentration
Forward primer	10 μ M	0.4 μ M
Reverse primer	10 μ M	0.4 μ M
Biomix Red™	2x	1x
DMSO	100%	5%
Template DNA	~ 200 ng/ μ L	100 pg / reaction

Table 2.6: Conditions used for basic PCR amplification

Phase	Temperature	Duration	Cycles
Initial melting	94 °C	5 min	1x
Denaturing	94 °C	30 sec	
Annealing	Table 2.3 primer pair $T_m - 5$ °C	30 sec	30x
Extension	72 °C	1 min per kb	
Final Extension	72 °C	10 min	1x
Storage	4 °C	Indefinitely	1x

Table 2.7: Conditions used for touchdown PCR amplification

Phase	Temperature	Duration	Cycles
Initial melting	94 °C	5 min	1x
Denaturing	94 °C	30 sec	
Touchdown Annealing	Table 2.3 primer pair $T_m + 5$ °C – 0.5 °C / sec	30 sec	30x
Extension	72 °C	1 min per kb	
Denaturing	94 °C	30 sec	
Annealing	48 °C	30 sec	10x
Extension	72 °C	1 min per kb	
Final Extension	72 °C	10 min	1x
Storage	4 °C	Indefinitely	1x

2.3.2.2 Restriction endonuclease digests

Restriction endonuclease digestions were performed in 1.5 mL polypropylene microfuge tubes or PCR tubes according to the manufacturer's recommendations using enzymes and the appropriate buffers and supplements supplied by New England Biolabs. A general outline of

restriction digest reagents and their concentrations is presented in Table 2.8. When indicated possible, digests were incubated overnight at 37 °C to ensure complete digestion. For simultaneous double enzyme digests, optimal conditions were determined using the NEB Double Digest Finder tool located at

<http://www.neb.com/nebecomm/DoubleDigestCalculator.asp>. If concurrent double digestion was not viable, sequential digestions were performed with a spin column cleaning between. Restriction digests for the confirmation of vector insertions were performed in a total volume of 10 µL and incubated at 37 °C for 1 hour and analysed by agarose gel electrophoresis. Digests to extract DNA fragments from vectors or create cohesive ends for ligations were carried out in 50 µL volumes and incubated overnight if possible at 37 °C before spin column purification and downstream use.

Table 2.8. Restriction endonuclease digestion reagents and concentrations

Reagent	Stock Concentration	Final Concentration
Restriction enzyme	10,000 – 200,000 U/mL	0.5 U/µL
Buffer	10x	1x
BSA	10x	1x
SAM	32 mM	80 µM
DNA	~200 ng/µL	~ 1 µg

2.3.2.3 Dephosphorylation

For blunt-end or single restriction site ligations to minimise self-annealing, or to increase the efficiency / reduce the background of a problematic ligation reaction (e.g. PCR product insert and vector both cut with restriction endonucleases will both have 5' phosphates) vector DNA ends were dephosphorylated in conjunction with corresponding insert DNA ends being phosphorylated. Dephosphorylation was achieved using Antarctic Phosphatase (AP - New England Biolabs) which unlike other phosphatase enzymes derived from calf or shrimp, AP has the advantageous property of being able to be heat inactivated. Dephosphorylation reactions were carried out according to a modification of the manufacturer's instructions. Vector DNA previously cut with the appropriate restriction enzyme(s) and subsequently spin column purified or heat inactivated to remove restriction enzyme activity was treated with 5 U of AP and the appropriate volume of 10x AP buffer added to achieve 1x final concentration. This was then incubated at 37 °C for 1 h for 5' extensions or blunt ends or for 2 h in the case of 3' extensions and AP was heat inactivated at 65 °C for 10 min.

2.3.2.4 Phosphorylation

Converse to dephosphorylation; blunt-end, single restriction site or low efficiency ligation reactions (i.e. PCR products derived from non-phosphorylated primers) had insert DNA enzymatically phosphorylated at the 5' end. Thus, with vector dephosphorylated as described by Sambrook and Russell²¹¹ and insert phosphorylated, this results in an ideal ligation scenario for the vector and insert DNA ends. Phosphorylation was carried out using T4 Polynucleotide Kinase (T4-PNK - New England Biolabs) according to a modification of the manufacturer's instructions. Insert DNA previously cut with the appropriate restriction enzyme(s) and subsequently spin column purified or heat inactivated to remove restriction enzyme activity was treated with 10 U of T4-PNK and supplemented with 1 mM ATP (final concentration) and the appropriate volume of 10x T4-PNK buffer added to achieve 1x final concentration. This was then incubated at 37 °C for 2 h and subsequently heat inactivated at 65 °C for 25 min.

2.3.2.5 Ligations

2.3.2.5.1 Cohesive end ligations

Cohesive or sticky end ligations were carried out in PCR tubes using T4 DNA ligase and corresponding supplements (Bioline). When necessary, vector DNA was dephosphorylated as described by Sambrook and Russell²¹¹ and insert DNA was phosphorylated as described by Sambrook and Russell²¹¹ and desalted by spin column purification. A 1:6 molar ratio of vector to insert DNA was desirable but excess insert DNA generally did not negatively impact ligation efficiency. All ligations included controls with no vector DNA to identify transformant cell background, and controls with no insert DNA to identify vector self ligation background. Cohesive end ligations were generally made up to a total volume of 10 µL according to Table 2.9 and incubated at 16 °C overnight. T4 ligase was heat inactivated at 65 °C for 25 min prior to heat shock transformation, the ligation reaction was spin column purified to remove salts prior to electroporative transformation.

Table 2.9: Cohesive end ligation reagents and concentrations

Reagent	Stock Concentration	Final Concentration
T4 DNA Ligase	10 U/µL	0.5 U/µL
T4 DNA Ligase Buffer	10x	1x
ATP	10 mM	1 mM
Vector DNA (e.g. 3 kb)	~50 ng/µL	50 ng
Insert DNA (e.g. 1 kb)	~100 ng/µL	> 150 ng

2.3.2.5.2 Blunt end ligations

Blunt end ligations were carried out in PCR tubes using T4 DNA ligase and corresponding supplements (Bioline). In blunt end ligations, vector DNA was always dephosphorylated and insert DNA phosphorylated as described by Sambrook and Russell,²¹¹ and desalted by spin column purification. A 1:6 molar ratio of vector to insert DNA was utilised. All ligations included controls with no vector DNA to identify transformant cell background, and no insert DNA to identify vector self ligation background. Blunt end ligations were carried out under dilute conditions with a higher ligase concentration and polyethylene glycol 4000 (PEG) to promote vector-insert ligation efficiency using a total volume of 40 μL made up according to Table 2.10 and incubated at 22 °C for 1 h, then 16 °C overnight. Blunt end ligations were not heat inactivated due to a corresponding decrease in efficiency caused by heating of PEG; blunt ligations were spin column purified prior to transformation.

Table 2.10: Blunt end ligation reagents and concentrations

Reagent	Stock Concentration	Final Concentration
T4 DNA Ligase	10 U/ μL	1 U/ μL
T4 DNA Ligase Buffer	10x	1x
ATP	10 mM	1 mM
PEG-4000	50%	5%
Vector DNA (e.g. 3 kb)	~50 ng/ μL	50 ng
Insert DNA (e.g. 1 kb)	~100 ng/ μL	> 150 ng

2.3.2.5.3 TA cloning ligations

PCR fragments were directly cloned into the linear pCR® 2.1 vector using the pCR® 2.1 TA Cloning Kit (Invitrogen) according to a modification of the manufacturer's instructions, followed by heat shock transformation and identification of positive transformants using IPTG + Xgal blue/white selection. This allowed PCR fragments to be stored in a stable vector format, providing a platform for sequencing, plasmid preparation and harvesting for restriction digestion. TA ligations were performed alongside a negative control with no vector DNA. TA ligations were carried out in a total volume of 10 μL according to Table 2.11, and incubated overnight before heat inactivation of DNA ligase and heat shock transformation into *E. coli* DH5 α .

Table 2.11. TA cloning ligation reagents and concentrations

Reagent	Stock Concentration	Final Concentration
T4 DNA Ligase	4 U/ μ L	0.4 U/ μ L
Ligation Buffer	10x	1x
pCR® 2.1 linear vector	25 ng/ μ L	12.5 μ L
PCR product (cleaned)	~200 ng/ μ L	~50 ng

2.3.2.6 Transformations

2.3.2.6.1 Preparation of transformation competent cells

2.3.2.6.1.1 Chemically competent *E. coli* DH5 α / BL-21

Chemically transformation competent *E. coli* strains were prepared as described by Sambrook and Russell.²¹¹ A 50 mL polypropylene Falcon tube containing 10 mL of LB medium was inoculated with a single colony of *E. coli* from agar plate, and incubated overnight at 37 °C with 200 RPM shaking. This 10 mL culture was then sub-inoculated into 90 mL LB in a 250 mL conical flask and the suspension was incubated at 37 °C with 200 RPM shaking and monitored until the absorbance at 600 nm reached 0.5 (representing mid-log phase). The culture was then cooled on ice for 30 min, and cells were harvested by sterile culture transfer into 50 mL Falcon tubes, and centrifugation in a CR3i multifunction centrifuge (Thermo Electron Corporation) at 4000 RPM for 20 min at 4 °C. Supernatant was removed and pellets were then resuspended in 100 mL (total) ice cold 0.22 μ m sterile filtered 0.1 M CaCl₂. This suspension was cooled on ice for 20 min and cells were again harvested by centrifuge as above. Supernatant was removed and pellets were resuspended in 50 mL (total) ice cold 0.22 μ m sterile filtered 0.1 M CaCl₂, cooled on ice for a further 20 min and cells were again pelleted by centrifugation as above. Supernatant was removed and pellets were resuspended in 2 mL (total) ice cold 0.22 μ m sterile filtered 0.1 M CaCl₂ + 14% glycerol. The resulting suspension was then promptly transferred into pre-chilled microfuge tubes in 50 μ L aliquots and stored at -80 °C.

2.3.2.6.1.2 Electroporation competent *M. smegmatis* MC²155

Electroporation competent *M. smegmatis* were prepared using the method described by Parish and Brown.²¹⁰ A 50 mL polypropylene Falcon tube containing 10 mL LB medium supplemented with 0.1% Tween80 was inoculated with a single colony of *M. smegmatis* MC²155 from an agar plate and incubated overnight at 37 °C with 200 RPM shaking. This overnight culture was then sub-inoculated into a 250 mL conical flask containing 90 mL of

LB + 0.1% Tween80 and incubated at 37 °C with 200 RPM and monitored until the culture absorbance at 600 nm was between 0.5-1.0, representing mid-log phase. The culture was chilled on ice for 20 min and then sterile transferred to 50 mL Falcon tubes and subsequent cell harvest by centrifugation in a CR3i multifunction centrifuge (Thermo Electron Corporation) at 4000 RPM for 20 min at 4 °C. Supernatant was discarded and pellets were thoroughly resuspended by pipetting in a total volume of 100 mL cold 10% glycerol and chilled on ice for a further 20 min. Cells were again harvested by centrifugation as above and cell pellets resuspended in a total volume of 50 mL cold 10% glycerol and chilled for a further 20 min on ice. Cells were harvested a third time by centrifugation and pellets resuspended in a total volume of 4 mL cold 10% glycerol. This cell suspension was then transferred into pre-chilled microfuge tubes in 200 µL aliquots and stored at -80 °C.

2.3.2.6.2 *E. coli* heat shock transformation

E. coli strains were transformed using the heat shock method described by Sambrook and Russell.²¹¹ Chemically competent cells in 1.5 mL polypropylene microfuge tubes were incubated on ice until thawed. 1 µg of cleaned plasmid DNA or entire 10 µL volume of heat inactivated or column purified ligation was then added to the thawed competent cells and mixed by pipette and left to chill on ice for 30 minutes. Tubes were then transferred to a heating block pre-set to 42 °C and incubated for 2 minutes. Tubes were then transferred back onto ice and incubated for 5 minutes. 900 µL of LB was added and tubes were incubated at 37 °C with 200 RPM shaking for 1 hour. Tubes were spun down at 13,000 x g for 2 minutes 5415D benchtop microcentrifuge (Eppendorf) to harvest cells, which were then resuspended in 100 µL final volume and plated out on LB Agar with the appropriate selection antibiotics and incubated overnight at 37 °C, colonies were picked and insertion verified by PCR screening or plasmid preparation and restriction digestion.

2.3.2.6.3 *V. harveyi* conjugative transformation

V. harveyi strains were transformed by conjugation with the *E. coli* S17-1 strain as described by Sambrook and Russell.²¹¹ *E. coli* S17-1 donor strain bearing the plasmid of interest was cultured overnight at 37 °C with 200 RPM shaking in 3 mL LB in a 15 mL polypropylene falcon tube with the appropriate selection antibiotics. *V. harveyi* was cultured in a 15 mL polypropylene falcon tube containing 3 mL TSB + 1.5% NaCl at 26 °C with 200 RPM shaking for 48 hours. 1 mL of *V. harveyi* culture and 100 µL of *E. coli* culture was added to a 1.5 mL microfuge tube and cells were pelleted by centrifugation in a 5415D benchtop

microcentrifuge at 13,000 x g for 2 minutes at room temperature. Pelleted cells were resuspended in 100 µL of TSB, and 25 µL amounts of this resuspension was spotted on to TSA and incubated for 48 hours at 26 °C. The colonies growing from each spot was then scraped and resuspended into 100 µL of TSB. These resuspensions were plated onto TCBS agar with the appropriate selection antibiotics and incubated at 26 °C for 48 h.

2.3.2.6.4 *M. smegmatis* electroporation transformation

M. smegmatis strains were transformed by preparing 200 µL of electroporation competent *M. smegmatis* MC²155 and using the method described by Kenny and Churchwood.¹⁹³ These aliquots were added to a ice chilled sterile electroporation cuvette (for recycling of electroporation cuvettes refer to Appendix 9.3.2) and an additional 200 µL cold 10% glycerol as added along with 1 µg of purified de-salted plasmid DNA preparation and left to chill on ice for 30 min. Electroporation cuvettes bearing cells and DNA were then electroporated with a Gene Pulser Xcell™ Electroporation System (Bio-Rad) using a single pulse with settings of 2.5 kV voltage, 25 µF capacitance, 1000 Ω resistance and a 1 or 2 mm path length (depending on the cuvette used). Immediately following pulsing, 600 µL of LB medium was added to the cuvette and then the entire cuvette contents were transferred into a microfuge tube and recovered by incubation overnight at 37 °C with 200 RPM shaking to allow translation of plasmid resistance markers. The following morning, cultures were harvested by centrifugation in a 5415D benchtop microcentrifuge for 30 sec at 13,000 g, and pellets resuspended in 100 µL of supernatant and plated out on the appropriate selection media and incubated at 37 °C with daily monitoring for colony formation.

2.3.2.7 Identification of recombinant transformants

Recombinant colonies from transformations containing the correct insertion were initially identified either through colony PCR screening or the restriction digestion and agarose gel electrophoresis visualisation of the insert DNA. Once positively identified, recombinant transformants were subjected to DNA sequencing to ensure the insertion sequence was correct and free of mutations.

2.3.2.7.1 Colony PCR screening

In order to positively identify transformation colonies, PCR screening of transformant colonies was carried out. PCR screen mix was made up in advance according to Table 2.12 and dispensed into PCR tubes in 6 µL aliquots. Ideally, one primer corresponding to the

DNA insert and one primer from the vector would be used to specifically ensure only successfully ligated plasmid was amplified, however occasionally only vector based primers flanking the insertion sequence were available and insert presence was then evaluated on the basis of the size of the PCR product. Primer pairs from the insert only were not used as they can provide a false positive by amplifying residual insert DNA present on the transformation plate. From a transformation plate, ~10 pre-marked colonies of either *E. coli* or *M. smegmatis* were picked using a 10 µL pipette tip and gently dipped into the pre-dispensed PCR screen mix tubes, acting as template DNA. Care must be taken to avoid transferring too many cells, therefore, the pipette tip was only barely touched to the colony surface and again barely touched to the PCR screen mix liquid. The tip was then touched to a fresh agar plate containing the appropriate selection antibiotic and grid reference markings then incubated at 37 °C. This re-plating allowed all PCR screened colonies to be grown afresh in a catalogued manner and positively identified colonies from the PCR screen can easily be re-picked for culturing. Once all colonies had been used to template the PCR screen, the PCR was carried out as usual with the appropriate parameters according to the primer T_m and insert size (Tables 2.4 and 2.5). Once the PCR reaction was completed, the entire volumes of the reactions were analysed by agarose gel electrophoresis and positive inserts identified by the presence of the correctly sized product.

Table 2.12: Colony PCR screen mix reagents and concentrations

Reagent	Stock Concentration	Final Concentration
Forward primer	10 µM	0.4 µM
Reverse primer	10 µM	0.4 µM
Biomix Red™	2x	1x
DMSO	100%	5%

2.3.2.7.2 Restriction digestion of transformant plasmid DNA

When PCR screening was not a viable option, or for further verification of transformant insert, restriction enzyme digestion of recombinant plasmid was used. This method is more time and labour consuming than PCR screening and thus not preferable. Marked colonies were picked from a transformation plate and cultured overnight in 15 mL polypropylene tubes containing 3 mL of the appropriate medium. Plasmid DNA was then harvested by mini-prep and digested with the appropriate restriction enzymes as described in section 2.3.2.2. Verification of insert was done via agarose gel electrophoresis based on the restriction digest banding pattern.

2.3.2.7.3 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was used to separate DNA fragments on the basis of size; visualised using ethidium bromide and ultraviolet light. Agarose gels (1%) were prepared by dissolving 1 g of agarose into 100 mL of 1x TAE buffer (Appendix 9.2.3). This solution was heated by microwave until the agarose was dissolved. Ethidium bromide solution (5 μ L of 10 mg/mL) was added and mixed by swirling, and this solution was poured into a gel cast apparatus with combs in place. Any bubbles were removed by pipette tip and the gel was left to set at room temperature for 20 min. After setting, the gel was loaded into a gel tank and submerged in 1x TAE buffer. DNA samples were mixed with 5x DNA loading buffer (Appendix 9.2.4) to obtain a 1x final concentration and were then loaded onto the gel. Hyperladder I (Bioline) DNA size marker (3.5 μ L) was also loaded onto the gel to provide a size reference of 10-0.2 kb. The gel was run at 120-140 V for 40-60 min until sufficient migration of the bromophenol blue in the loading buffer was observed (bromophenol blue migrates at approximately the same rate as a 1 kb DNA fragment). DNA in gels was visualised using a UV transilluminator box. Photographs were captured using an Olympus C-5060 Wide Zoom digital camera and UVP LS software (Life Sciences).

2.3.2.7.4 Sequencing of DNA

Macrogen Incorporated preformed DNA sequencing through their standard sequencing service. DNA samples were prepared as specified by Macrogen, using the minimum concentrations detailed below in Table 2.13. Primers were always sent at 0.2 μ M concentration. Sequences were analysed by nucleotide BLAST (NCBI) and Geneious software (Biomatters Ltd) to identify insertions and ensure sequence quality.

Table 2.13: Specifications used for Macrogen DNA sequencing samples

Sample type	Concentration	Volume
Plasmid DNA	≥ 20 ng/ μ L	15 μ L
PCR product (purified)	≥ 50 ng/ μ L	20 μ L
Primer	= 0.2 μ M	20 μ L

2.3.2.8 Transposon mutagenesis

Single-gene knockout mutants of *M. smegmatis* MC²155 resistant to seleno-amino acids were isolated by plating random transposon insertion mutant cells on LBA containing various concentrations of SMSC or Se-Met. Mutant colonies exhibiting high levels of resistance to

seleno-amino acids were picked for culturing and subsequent storage and dose-response follow up analysis. Mutants proving resistant in follow up analysis were grown up for gDNA harvest and the transposon insertion site and corresponding gene affected were determined by restriction digestion with a frequent cutter enzyme, self ligation of fragments and inverse PCR followed by sequencing and genomic position analysis.

2.3.2.8.1 Generation of random transposon mutant *M. smegmatis* by TN611

A 'pool' of random Tn611 insertion mutants kindly supplied by Richard Campen was generated according to a modification on the temperature sensitive generation methods of Guillhot¹⁸⁴ and Pérez.⁵⁵ The Tn611 containing pCG79 vector was electroporated into *M. smegmatis* MC²155 by the method described in section 2.3.2.6.4, however transformant recovery and selection of Kan₂₀ kanamycin resistant transformants on agar was carried out at 30 °C to allow transposon insertion. Note that the lower concentration of Kan₂₀ was used in transposon strain work rather than the usual Kan₅₀ as transposon culture incubation steps at 41 °C demonstrated detrimental growth with Kan₅₀. Subsequent liquid culturing of selected kanamycin resistant transformants was also carried out at 30 °C for 72 h with 200 RPM shaking to allow continued transposon insertion generating a large population of random transposon mutants. It has been demonstrated that Tn611 transposition events occur on an average of one event per mycobacterial cell.¹⁸⁴ The culture was then incubated at 41 °C with 200 RPM shaking for a further 24 h to cease transposition events and glycerol added to a final concentration of 20%. The pooled mutant culture was then dispensed into 1 mL freezer stock aliquots in 1.5 mL microfuge tubes and stored at -80 °C.

2.3.2.8.2 Screening for seleno-amino acid resistant transposon mutants

Transposon mutant mycobacteria resistant to seleno-amino acids were identified and isolated by a modification of the method of Billman-Jacobe for generating isoniazid resistant mutants.²¹² Aforementioned transposon mutant pool freezer stock aliquots (100 µL) containing approximately 5x10⁶ CFU were plated onto Middlebrook 7H10 agar supplemented with 10% OADC, 0.5% glycerol, Kan₂₀ and SMSC or Se-L-Met at concentrations of 20-150 µM (it was found that seleno-amino acid concentrations of >100 µM were best for developing strongly resistant mutants). These plates were then incubated at 41 °C for up to 2 weeks to fully allow slow growing mutant colonies to appear. *M. smegmatis* pMV261 were also plated and incubated at 41 °C to determine the spontaneous resistance rate without transposon insertion. After incubation, colonies were picked and

cultured in 15 mL polypropylene tubes containing 3 mL complete 7H9 Broth + Kan₂₀ at 41 °C with 200 RPM shaking for 72 h or until cultures reached an OD₆₀₀ of 1.0. These cultures were then re-plated on 7H10 + Kan₂₀ for future use, used in seleno-amino acid dose-response analysis assays and prepared for -80 °C freezer storage by adding glycerol to a final concentration of 20%.

2.3.2.8.3 Identification of TN611 transposon insert location

Locations of transposon insertion sites in generated seleno-amino acid resistant mutants involved gDNA harvest, digestion, re-ligation, inverse PCR and sequencing. First, mutant gDNA was extracted by gDNA mini-preparation, the gDNA was then digested using the frequently cutting restriction enzymes. The restriction digest fragments were then self-ligated under dilute conditions described in section 2.3.2.5.2. Inverse PCR was performed on these ligations using outward primers flanking the transposon and the resulting product sequence and analysed bioinformatically to determine the gene(s) disrupted by transposon insertion.

2.3.2.8.3.1 Transposon mutant mycobacterial gDNA mini-prep

Multiple samples of *M. smegmatis* gDNA were isolated using a modification on the mini mycobacterial gDNA prep described by Parish and Brown.²¹⁰ Single colonies of mutants of interest were inoculated into a 50 mL polypropylene tubes containing 5 mL of LB + Kan₂₀ and cultured at 41 °C with 200 RPM shaking for 48 - 72 h to achieve an OD₆₀₀ of ~1.0. The cultures were then spun down at 4000 g at 4 °C in a CR3i multifunction centrifuge (Thermo Electron Corp), the supernatant was discarded and pellets resuspended by pipette in 500 µL of TE buffer (pH 8.0). This was centrifuged again as described above, supernatant was removed and pellets were stored at -80 °C overnight or longer. TE buffer (500 µL) was added and pellets were thawed on ice. Once thawed, cells were resuspended by pipette and centrifuged as before, supernatant was removed and the cell pellet was resuspended in 75 µL of TE buffer and transferred to a 1.5 mL microfuge tube. CHCl₃:MeOH (2:1) (75 µL) was added and the mixture was gently shaken on a rocking platform for 30 min. This was then spun down at 2500 g for 10 min in a 5415D benchtop microcentrifuge (Eppendorf) to separate phases, upper and lower phases were removed carefully by pipette leaving the middle band of defattened mycobacterial cells. The tube was then heated at 55 °C in a heating block for 5 min to evaporate any residual solvent. Cells were resuspended in 30 µL TE buffer (with vigorous vortex and pipette action) and 3 µL of 1 M Tris-base (pH 9.0) and 0.6 µL of 1mg/mL lysozyme were added and the solution incubated at 37 °C overnight. After

this point, no vortexing was used to preserve the integrity of released gDNA strands. RNase A (0.7 μL of 500 $\mu\text{g}/\text{mL}$) was added and the solution incubated for 30 min at 37 °C. 10% SDS (3.6 μL) and 0.5 μL of 10 mg/mL proteinase K was added, the solution mixed by pipette resuspension and incubated in a heating block at 55 °C for 3 h. Phenol: CHCl_3 :isoamyl alcohol (25:24:1) (40 μL) was added and the contents were mixed gently on a rocking platform for 30-60 min at room temperature. Phases were separated by centrifugation in a microcentrifuge at 12,000 g for 30 min and the upper aqueous layer was removed by pipette and transferred into a fresh microfuge tube. Sodium acetate (0.1 volumes of 3 M) and 1 volume of ice cold isopropanol were added and the tube stored at -20 °C for 15 min to precipitate DNA. DNA was harvested by centrifugation at 12,000 g for 30 min and supernatant was discarded, the pellet was washed with 1 mL cold 70% EtOH and centrifuged at 12,000 g for 10 min. EtOH was removed and the pellet was resuspended in 25 μL TE buffer and left overnight at 4 °C to encourage solubilisation, before being resuspended again by gentle pipette action and stored at -20 °C.

2.3.2.8.3.2 Fragmentation of mutant mycobacterial gDNA by restriction endonuclease digestion

Harvested *M. smegmatis* Tn611 transposon mutant gDNA was fragmented using frequently cutting restriction endonucleases, such as the four cutter RsaI or common recognition site EagI, these cut the *M. smegmatis* genome approximately 19,385 and 13,630 times, respectively. These digestions of *M. smegmatis* gDNA create fragments of 360 and 513 bp in size on average, respectively. This generally provides adequate resolution for capturing the average sized 1 kb mycobacterial gene (calculations performed using GENTle v 1.9.4 and *M. smegmatis* MC²155 genome assembly ASM1500v1). These enzymes also do not cut the end fragment of the Tn611 transposon, which would interfere with inverse PCR. The RsaI enzyme was preferred and if inverse PCR products could not be obtained using this enzyme, then EagI was used. Restriction fragmentation of mutant *M. smegmatis* gDNA was carried out in PCR tubes as detailed in Table 2.14 and incubated at 37 °C for 2 h before heat inactivation at 65 °C for 20 min.

Table 2.14: Restriction digest conditions for transposon mutant gDNA fragmentation

Reagent	Stock Concentration	Final Concentration
EagI or RsaI	10,000 U/mL	0.5 U/ μL
Buffer	10x	1x
gDNA	~100-200 ng/ μL	~ 500 ng

2.3.2.8.3.3 Self ligation of mutant gDNA fragments

Fragments of mutant gDNA generated by the restriction enzyme digestion described above were then self-ligated under dilute conditions in a blunt end ligation reaction prepared as described in Table 2.15 which was then incubated at 22 °C for 1 h followed by 16 °C overnight.

Table 2.15: Dilute blunt end ligation conditions used to self-ligate fragmented transposon mutant gDNA

Reagent	Stock Concentration	Final Concentration
T4 DNA Ligase	10 U/ μ L	0.5 U/ μ L
T4 DNA Ligase Buffer	10x	1x
ATP	10 mM	1 mM
PEG-4000	50%	5%
Digested mutant gDNA	~50 ng/ μ L	~500 ng

2.3.2.8.3.4 Inverse PCR of self-ligated transposon mutant gDNA fragments

The ligation products of the reaction described above were used as a template for an inverse PCR reaction using primers 3053 and 3054 described in Table 2.4, these prime outward from the transposon flanks. This yields PCR products beginning with IS6100 sequence of the Tn611 transposon and the genomic sequence TN611 has been inserted into until the frequent cutter restriction enzyme site which has been self-ligated. Inverse PCR was set up according to Table 2.16 and carried out under the thermal cycle conditions described in Table 2.17. Amplification products were visualised by agarose gel electrophoresis. The largest bands were excised from the gel and purified by Zymoclean™ Gel DNA recovery for sequencing.

Table 2.16: Reagents and concentrations used during transposon mutant inverse PCR amplification

Reagent	Stock Concentration	Final Concentration
3053	10 μ M	0.4 μ M
3054	10 μ M	0.4 μ M
Biomix Red™	2x	1x
Ligated mutant gDNA	~ 25 ng/ μ L	500 ng

Table 2.17: Inverse PCR cycle conditions used for amplifying transposon mutant gDNA for sequencing.

Phase	Temperature	Duration	Cycles
Initial melting	94 °C	5 min	1x
Denaturing	94 °C	30 sec	
Annealing	44 °C	30 sec	30x
Extension	72 °C	90 sec	
Final Extension	72 °C	20 min	1x
Storage	4 °C	Indefinitely	1x

2.3.2.8.3.5 Sequencing and location determination of transposon insertion site

Inverse PCR amplification products were sent for sequencing as described in section 2.3.2.7.4 using either the 3053 or 3054 primer. Sequences were analysed using a nucleotide BLAST (NCBI) against the entire nucleotide collection and cross-checked using Geneious software (Biomatters Ltd) by trimming the transposon sequence and aligning against the *M. smegmatis* MC²155 genome. This allowed confirmation of the terminating frequent cutter restriction site, and that the transposon insertion was sufficiently placed within the gene to disrupt its function. The wider ramifications of the transposon insertion such as downstream genes/operons affected by the transposon insertion were made apparent using this approach.

2.3.3 Proteomics

2.3.3.1 Protein expression

2.3.3.1.1 Protein expression vector and strain

The over-expression of mycobacterial proteins of interest for enzymatic activity analysis was carried out in the *E. coli* BL-21 (DE3) strain using the pET28a(+) vector. Mycobacterial proteins were cloned into the pET28a(+) vector under the T7 inducible transcription promoter and subsequently heat shock transformed into *E. coli* BL -21 (DE3).

2.3.3.1.2 Expression of recombinant proteins

Protein expression strains were streaked from -80 °C freezer stocks onto LB agar supplemented with Kan₅₀ and incubated overnight at 37 °C. Single colonies were selected and inoculated into 10 mL LB Kan₅₀ starter cultures in 50 mL Falcon tubes and incubated overnight at 37 °C with 200 RPM shaking. Starter cultures were then inoculated into 2.5 L conical flasks containing 400 mL ZYP-5052 Auto Induction Broth supplemented with Kan₅₀, 1 mM betaine and 0.5 M NaCl to encourage recombinant protein solubility. Cultures were

incubated at 37 °C with vigorous 250 RPM shaking for 10 h or until the OD₆₀₀ reached 1. Cultures were then heat shocked by static incubation at 45 °C for 20 min to induce the expression of heat shock chaperone proteins to assist in protein folding. A final incubation step at 18 °C with 250 RPM shaking for 24 h was carried out to facilitate protein translation. Cultures were then transferred into 50 mL Falcon tubes and pelleted at 4000 RPM for 20 min at 4 °C in a CR3i multifunction centrifuge (Thermo Electron Corp). The supernatant was discarded and pellets were stored at -80 °C.

2.3.3.1.3 Protein purification by nickel affinity chromatography

Pellets from recombinant protein overexpressing cultures were resuspended and cells lysed using either of the following methods:

- 1) Pellets were resuspended in 20 mL ice cold 1x bind buffer and lysed by French Press (Thermo Fisher) twice at 1000 psi. Lysate was then sonicated on ice to shear DNA using a Ultrasonic processor W-380 for 90 sec with continuous cycle, 100% duty cycle and an output control of 5.
- 2) Pellets were resuspended in 20 mL ice cold 1x bind buffer supplemented with 1 mg/mL lysozyme (Sigma) and incubated on ice for 30 min, then agitated on a rocking platform on ice for 10 min. Triton X-100 was added to a final concentration of 1%, and DNase (Sigma) and RNase (Sigma) were both added to a final concentration of 5 µg/mL. Lysates were then rocked on ice for a further 10 min.
- 3) Pellets were resuspended in 20 mL Bug Buster® Protein Extraction reagent and agitated on ice using a rocking platform for 30 min.

Samples of protein from over expressing cell lysate (20 µL) were collected for SDS-PAGE and lysates were then transferred to 50 mL Sorvall tubes and centrifuged in a Sorvall RC-5C at 4 °C and 14,000 RPM for 20 min to obtain a soluble cell lysate supernatant. A 20 µL aliquot of these soluble cell lysate fractions was collected for SDS-PAGE and the soluble cell lysate stored on ice to be purified by nickel affinity column chromatography using a His-Bind® Purification System (Novagen).

His-Bind® resin (2.5 mL) was loaded into a chromatography column and allowed to settle by gravity. Solutions were passed through the column using a peristaltic pump with a flow rate of 3 mL/min throughout the purification. The column was initially washed with 4.5 mL of ddH₂O, primed with 8 mL of 1x charge buffer, then 4 mL of 1x bind buffer. The 20 mL

soluble fraction of protein expressing cell cultures was then passed through the column followed by 20 mL of 1x bind buffer, 8 mL of 1x wash buffer. Finally the bound protein was eluted using 10 mL of 1x elution buffer supplemented with 15% glycerol (v/v) to promote protein solubility. This eluted fraction was then desalted and concentrated by centrifugal filtration. Cell lysate, soluble cell lysate and all column run-through fractions were retained for SDS-PAGE analysis.

2.3.3.1.4 Desalting and concentration of expressed recombinant protein

Purified proteins derived from column chromatography were eluted from the column in 10 mL 1x elution buffer supplemented with 15% glycerol (v/v). Centrifugal filtration units were used to desalt, further purify and concentrate the protein of interest. Protein elution fraction in 10 mL 1x elution buffer was loaded into to a centrifugal filtration column (Millipore) with a filtration pore size of ≥ 10 kDa larger than the target protein and centrifuged for 30 min at 4,000 RPM in a CR3i multifunction centrifuge (Thermo Electron Corp) at 4 °C. Column run-through was collected and loaded into a centrifugal filtration column with a filtration pore size of ≥ 10 kDa smaller than the target protein size. The centrifugal filtration column was loaded to capacity with the protein's specific storage buffer and centrifuged for 30 min at 4,000 RPM in a CR3i multifunction centrifuge at 4 °C. Flow through was removed and the column was again topped up to capacity with the protein's specific storage buffer and centrifuged again. This step was repeated for a third time leaving approximately 300 μ L of desalted, size-purified and concentrated protein in storage buffer.

All collected fractions and flow throughs were retained for SDS-PAGE analysis. The retained, purified, concentrated proteins were resuspended for 1 min against the filter membrane by gentle pipetting to minimise protein adhesion to the membrane, purified protein solutions were then divided into 100 μ L aliquots and stored in 50% glycerol at -20 °C.

2.3.3.1.5 Protein quantification

Purified and desalted protein fractions were quantified using the DC Protein Assay kit (Bio-Rad) based on the Lowry method.²¹³ Solutions of BSA prepared in the same buffer as the protein being measured with concentrations between 0.2-1.5 mg/mL (the linear range of the assay) were used as standards. Samples/standards (5 μ L) were loaded in to a 96-well plate, followed by 25 μ L of reagent A (alkaline copper tartrate) and 200 μ L of reagent B (Folin Reagent). The plate was gently mixed by vortex on minimal power and left at room temperature for 15 min. The developed assay plate was read at 750 nm using an Envision

spectrophotometer (Perkin-Elmer). A standard curve was plotted using Excel (Microsoft) and sample protein concentrations were extrapolated from the standard curve. If sample protein concentrations were beyond the linear range of the standard curve, the sample was diluted and re-assessed.

2.3.3.2 SDS-PAGE

2.3.3.2.1 Sample preparation

Protein samples, purification fractions or bacterial cells (20 μ L) were added to 10 μ L of 3x SDS Loading Buffer (Appendix 9.2.14) in PCR tubes and boiled at 95 °C for 5 min in a thermal cycler to linearise polypeptides to their primary structure. If required, prepared samples were stored at 4 °C for no longer than a week before SDS-PAGE analysis.

2.3.3.2.2 Polyacrylamide gel electrophoresis

All SDS-PAGE was carried out using the Mini-PROTEAN II system (Bio-Rad). Glass gel plates (1.0 mm spacer thickness) were inserted into the clamp assembly with the thumbscrews loosely tightened. This was then fitted into the casting stand and, with gentle downward pressure applied to the glass plates, the thumbscrews were fully tightened. ddH₂O was pipetted between the glass plates to test the seal of the plate assembly, and if leakage was observed the assembly process was repeated. If no leakage was observed, ddH₂O was removed from the plate cavity by inversion and pipetting. After ensuring correct plate assembly and an even level of the casting stand, approximately 4.5 mL of the appropriate %T separating gel (Appendix 9.2.12 and 9.2.13) was pipetted into the gel cavity until it reached a level 10 mm below the teeth of the inserted gel comb. Isopropanol (1 mL of 100%) was pipetted on top of the separating gel to remove any air bubbles and the gel was left to set for 30 min at room temperature. Isopropanol was then removed by inversion and the gel cavity was washed three times by pipetting with ddH₂O followed by approximately 1.5 mL of 4% stacking gel (Appendix 9.2.11) being pipetted on top of the set separating gel and the gel comb was inserted slowly ensuring no bubbles remained between the comb teeth. The stacking gel was left at room temperature to set for 30 min. After setting, the gel comb was gently removed and the gel assembly removed from the casting stand and slotted on to the inner cooling core. The gel assembly-inner cooling core was then placed into a gel tank and the inner cooling core and tank filled with 1x SDS run buffer (Appendix 9.2.10).

To assist visualisation of run buffer submerged wells, 1x SDS load buffer was evenly pipetted horizontally across the gel surface. From here, prepared samples were loaded into

wells, 10 μ L for small comb wells and 20 μ L for large comb wells. Samples were delivered by pipette in a slow consistent fashion to ensure accurate delivery of sample to the well, avoiding cross-well contamination. PageRuler Plus Prestained Protein Ladder 10-250 kDa (Thermo-Scientific) (5 μ L) was loaded in the centre well for use as a molecular weight marker. The gel tank was then connected to a power supply and run at constant voltage setting of 200 V for approximately 45 min until the dye front had migrated to the bottom of the gel and the PageRuler Plus Prestained Protein Ladder had resolved along the length of the gel.

2.3.3.2.3 Gel staining and destaining

Gels were removed from their glass plates by reversal of the assembly process, rinsed with tap water and stained by immersion in Coomassie blue stain (Appendix 9.2.15) with agitation on a rocking platform for 30 min at room temperature. Coomassie blue stain was then decanted back into its bottle for recycling and the gel rinsed three times in tap water, then immersed in destain solution (Appendix 9.2.16) with agitation on a rocking platform for 1 h at room temperature. The destaining step was repeated until sufficient contrast between protein bands and the gel was attained, and the gel was stored overnight in tap water to further destain and expand the gel. Gels were digitally scanned using a HP desktop scanner.

2.4 *In vitro* enzymatic assays

2.4.1 Cysteine desulphurase reaction

The following reagents were added to a 96-well plate, using sterile technique, in the following order: 10 mM substrate (10 μ L of 100 mM), 1 mg of enzyme (4 μ L of 250 mg/mL enzyme) and the well was filled with IscS buffer (pH 8.0) to 100 μ L. The plate was gently shaken with a vortex to ensure thorough mixing, sealed with a SealPlate® (Interlab) and incubated at 37 °C for 2 h. All substrates were dissolved in sterile distilled water to 100 mM where not mentioned. All enzymes were resuspended in IscS buffer + 50% glycerol unless mentioned (Appendix 9.2.8). Cofactors such as NAD were suspended in $\text{d}_5\text{H}_2\text{O}$.

2.4.2 Determinative cysteine desulphurase assays

In order to determine the activity of the cysteine desulphurase enzymes toward substrates, the aforementioned cysteine desulphurase reaction was coupled to either or both of the following assays.

2.4.2.1 Lead acetate hydrogen sulphide detection assay

Prior to plate sealing and incubation, 96-well plates containing the basic cysteine desulphurase reaction were covered with a sheet of lead acetate paper²¹⁴ (blotting paper saturated with 50 mg/mL lead acetate suspended in ddH_2O). The plates were then sealed and incubated as normal. Lead acetate paper changes colour from white to brown/black if volatile H_2S is generated, although this change is readily apparent it is not quantifiable so results must be treated as binary.

2.4.2.2 Methylene blue sulphide detection assay

Sulphide produced by cysteine desulphurase enzyme activity was measured by the condensation of *N,N*-dimethyl-*p*-phenylenediamine with sulphide to form methylene blue as described by Siegel.²¹⁵ Briefly to a 100 μL of cysteine desulphurase reaction 5 μL of 20 mM *N,N*-dimethyl-*p*-phenylenediamine dissolved in 7.0 M HCl was added by pipette followed by 5 μL of 30 mM FeCl_3 , the reaction was left to develop for 30 min at room temperature, precipitates removed by centrifugation and absorbance measured at 650 nm using Na_2S as a standard.

2.4.2.3 Coupled cysteine desulphurase / alanine dehydrogenase assay or lactate dehydrogenase assay

It was hypothesised that the products of cysteine desulphurase reactions for alanine dehydrogenase (Ala-DH) may be alanine and a sulphide/selenide. For lactate dehydrogenase (Lac-DH) the reactions may yield pyruvate, ammonia and a sulphide/selenide. The sulphide/selenide product identity is substrate dependant. Therefore cysteine desulphurase activity may be measured by coupling the cysteine desulphurase reaction with either of the two oxidoreductase enzymes, Ala-DH or Lac-DH, and mycobacterial lyase activity may be measured by the action of Ala-DH or Lac-DH on the products of mycobacterial lyase action. Ala-DH would deaminate alanine whilst reducing NAD to NADH; whereas, Lac-DH enzyme would reduce pyruvate to lactate whilst oxidising NADH to NAD. NAD/NADH concentrations may be measured spectrophotometrically.

To measure NADH or NAD production, the following reagents were sterilely added to a 96-well plate in the following order: 1 mM NAD for Ala-DH (1 μL of 100 mM) or 1mM NADH (1 μL of 100 mM) for LDH, 10 mM appropriate substrate (10 μL of 100 mM), 1 mg of enzyme (4 μL of 250 mg/mL enzyme), 0.25 U alanine-dehydrogenase (Sigma) (2.5 μL of 100 U/mL) or 0.25 U lactate dehydrogenase (Sigma) (2.5 μL of 100 U/mL) and IscS Buffer (pH 9.0 for Ala-DH or pH 7.0 for Lac-DH) to a final volume of 100 μL . For either assay the plate

was gently shaken with a vortex to ensure thorough mixing and immediately read at 37 °C in a 300 point (1 read per 5 seconds) kinetic assay measuring NAD reduction or NADH oxidation at 340 nm. DL-alanine or sodium pyruvate was included as a substrate in the assays as positive controls.

2.4.2.4 α -keto butyrate detection assay

α -keto acid products of mycobacterial lyase enzyme activity on Se-AA substrates were detected using the 3-methyl-2-benzothiazolone hydrazone method described by Soda.²¹⁶ Briefly to 50 μ L lyase enzyme assay was terminated by the addition of 25 μ L 25% (v/v) trichloroacetic acid and precipitates such as protein was centrifuged out at 14,000 RPM for 5 min in a microfuge. 200 μ L of 0.5 M sodium acetate (pH 5.0) was added followed by 80 μ L of 0.1% (w/v) 3-methyl-2-benzothiazolone hydrazone and the assay plate incubated at 50 °C for 40 min, cooled down to room temperature and absorbance read at 320 nm using α -ketobutyrate and pyruvate as standards.

2.4.4 Gas chromatography mass spectrometry

GC-MS was used to detect volatile sulphur/selenium species produced by mycobacterial lyase reactions with the instruction of Dr. R. Keyzers. A Shimadzu GC-2010 using a 30 m Restek RTX® -5SilMS crossbond® column and 0.25 mm internal diameter and 0.25 μ m film thickness paired with a Shimadzu GC-MS QP 2020 Plus EI MS was the instrument used.. Solid-phase micro extraction (SPME) fibre was subject to 30 min exposure to the headspace of a 1 mL mycobacterial lyase reaction being carried out at 37 °C. SPME fibre was inserted into the column and volatiles removed at 220 °C for 1 min and were separated using a temperature gradient starting at 30 °C and increasing at 8 °C / min. Mass spectra of detected volatile chemical species were compared to NIST 08 (American Institute of Standards and Technology) spectral library to determine chemical identity.

2.5 Mammalian cell line cytotoxicity assays

2.5.1 HL-60 / J-774 / Vero cell MTT assay

All cytotoxicity assays were kindly performed by research team members Nathaniel Dasyam and Rekha Veliyayi Murikoli. HL-60 human promyelocytic leukaemia or J-774 mouse adherent macrophage cells were cultured in 250 mL conical flasks with RPMI-1640 medium supplemented with 10% fetal calf serum, 50 μ g/mL penicillin and 50 μ g/mL streptomycin. Cells were incubated at 37 °C in a 5% CO₂ atmosphere for growth and proliferation. Candidate drugs to be tested were 1:2 serially diluted in such a way that each well of a 96-

well plate contains 50 μL of drug-containing medium. Diluted cell culture (50 μL) was then added to a final cell concentration of 10,000 cells per well. The assay plate was then incubated at 37 °C with 200 RPM shaking for 48 h followed by the addition of 20 μL of 5 mg/mL MTT reagent in PBS, left for two hours then 100 μL of 10% SDS solubiliser was added. Reading of plates was carried out by measuring absorbance at 570 nm using an Envision Multi-label plate reader (Perkin-Elmer).²¹⁷

2.6 Data processing

Assay data were processed in Excel (Microsoft) and further analysed using Sigmaplot 10.0 (Systat) or Prism 5 (Graphpad) to determine inhibitory concentrations and standard errors.

2.6.1 Statistical analysis

Data from high throughput screens were normalized to control for plate-to-plate variation using linear scaling to match the most extreme plates in each data set and taking their natural log transforms. Control values greater than 3 standard deviations from the mean were considered outliers and discarded.¹²²

2.6.1.1 Z-factors

Z factors were calculated as shown according to Figure 2.3 and used to determine the validity and quality of the high throughput screens by numerically demonstrating their signal dynamic range and variation. Z factors have a value between 0 and 1, with 0 indicating an invalid assay due to complete overlap between positive and negative controls and 1 being a (theoretically unobtainable) perfect assay. Values above 0.5 are considered statistically acceptable and signify a difference of at least 12 standard deviations between the means of the positive and negative controls.²¹⁸

$$Z\ factor = 1 - \frac{3(\sigma_{positive} + \sigma_{negative})}{|\mu_{positive} - \mu_{negative}|}$$

Figure 2.3: Z-factor calculation: Where μ is the mean of the positive and negative control signals and σ is the standard deviation of the positive and negative controls.

2.6.1.2 Signal to noise ratios

Signal to noise ratios (SNR) were calculated according to the formula displayed in Figure 2.4. The higher the SNR value the more distinction between the assay signal and the background.

$$SNR = \frac{\mu_{signal}}{\sigma_{noise}}$$

Figure 2.4: Signal to Noise Ratio Calculation: Where μ is the mean of the positive (growth) control signal and σ is the standard deviation of the noise (background or medium) control signal.

2.6.2 Chart plotting

Venn diagrams were created using Google Chart Tools. Scatterplots were created using R with the Rcommander and ggplot2 plugins and the following script:

```
library(ggplot2, pos=4)
attach(datasheetname)
d <- qplot(No, MEAN, data= datasheetname, size=STDEV, color=MEAN, alpha =
1/10, main="Insert title here", xlab="X-axis label", ylab="Y-axis label",
e <- d + geom_abline(intercept=90, slope=0, color="#3B4FB8", alpha = 5/10,
size=3)
e
```

All other charts were created using Microsoft Excel 2007®

CHAPTER THREE

High-Throughput Screening

3.1 Introduction to High Throughput Screening

High throughput screening is a commonly used methodology for modern drug discovery, including the discovery of antibiotics. Deliberate chemical screening protocols have been utilised since the beginning of antibiotic drug discovery, with Paul Ehrlich screening hundreds of chemicals for anti-syphilis activity until he found compound 606, now known as salvarsan.²¹⁹ Recent advances in computing, robotics and laboratory equipment such as automated spectrophotometers, microtitre plates and the advent of large commercial chemical collections or ‘libraries’ have pushed high throughput screening to the foremost technique used in modern drug discovery, allowing vast numbers of chemicals to be rapidly assessed for their effect upon biological systems.²

This chapter briefly discusses the methodologies used in high throughput screening for antibiotic drugs and the reasons why a whole cell screening approach was chosen for the present study. Chemical collections screened in this study are detailed and characteristics of the two rich media conditions used in this work, LB and 7H9, are described. Screen development and key optimisation steps are briefly touched upon before the presentation of screening results, with total screen data exhibited graphically in scatterplot format. Compounds meeting hit criteria are detailed and promising tuberculosis drug development leads are highlighted and discussed.

3.1.1 High Throughput Screening methodologies

Various methodologies used in high throughput screening are discussed at length in Chapter 1.6.2. These techniques are briefly recapitulated in Table 3.1 to provide context to the screening of this project.

Table 3.1: Summarized methodologies in anti-bacterial drug high throughput screening.

Whole cell screening	
Description	Chemical library is introduced to bacterial cells cultured <i>in vitro</i> and the growth inhibiting or sterilising activity is measured.
Advantages	<ul style="list-style-type: none"> • Simple to set up, easily measured by OD, GFP, Resazurin etc. • Broad spectrum – not restricted to a single target. • Accounts for a bacterial cell environmental context - i.e. in order to be active compounds must persist in culture conditions, pass through the cell wall and avoid efflux or metabolic inactivation. • Can detect bacterial metabolism-activated pro-drugs. • Can be adapted to different culture conditions.

Disadvantages	<ul style="list-style-type: none"> • Does not distinguish between anti-bacterial and cytotoxic compounds, requires cytotoxicity counter screen. • Hit compound target is unknown and must be identified through downstream studies. • Hit compound activity might be altered under <i>in vivo</i> conditions.
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Whole cell high content screening

Description	Whole cell screening under complex <i>in vitro</i> conditions, such as intra-macrophage phagocytised bacteria.
Advantages	<ul style="list-style-type: none"> • Includes the advantages of whole cell screening. • Provides further detection of hits active in the specific environment (i.e. intra-macrophage), are activated by the immune cell metabolism or modulate the immune cell anti-bacterial function. • Indicates potential cytotoxicity.
Disadvantages	<ul style="list-style-type: none"> • More costly and difficult to perform. • Hit compound target remains unknown and must be identified through downstream studies. • Compound target range is increased beyond bacterial targets, making identification potentially more difficult.

Targeted whole cell screening

Description	Whole cell screen where the strain is genetically altered to over/under express a target gene, enriching hits against the particular target.
Advantages	<ul style="list-style-type: none"> • All advantages of whole cell screening accounted for. • Hit compounds are enriched towards a defined target. • Hit compound target indicated, facilitating rapid downstream MoA and SAR studies. • Essential genes may be targeted by techniques such as inducible anti-sense RNA down-regulation.
Disadvantages	<ul style="list-style-type: none"> • Requires an identified suitable target to be selected. • Genetically modified strain must be constructed. • Counter-screen of wild-type strain must be run in parallel to distinguish non-specific hits. • Gene expression modification may have unpredicted downstream ramifications. • Hits activity may not necessarily be applicable to wildtype bacteria; further validation studies required.

In vitro protein targeted screening

Description	An identified target protein is isolated and screened for inhibition of activity in an <i>in vitro</i> assay.
Advantages	<ul style="list-style-type: none"> • Target is defined, facilitating rapid MoA and SAR studies to improve hit compound activity. • Target can be researched and chosen for favourable properties, i.e. unique to bacteria, not present in humans. • More readily miniaturised to allow greater throughput.
Disadvantages	<ul style="list-style-type: none"> • Requires target to be selected, purified and enzymatic assay designed against the target. • Does not account for bacterial environmental conditions, such as cell wall, metabolism of compound, efflux, parallel targets, etc. • Requires downstream studies against whole cells to prove activity. • False positives may arise from compounds affecting the assay process rather than the target enzyme itself.

<i>In silico</i> screening	
Description	A defined protein target is computationally modelled and then subjected to a virtual screen to identify potential inhibitors.
Advantages	<ul style="list-style-type: none"> • Minimal costs. • Vast quantities of chemicals may be rapidly screened. • Chemical library pharmacophores may be specifically designed for the target cleft, with varying groups for guiding SAR studies. • May enrich hit compound anti-bacterial selectivity.
Disadvantages	<ul style="list-style-type: none"> • Requires target to be selected and computationally modelled. • <i>In silico</i> modelled target structure may differ from <i>in vivo</i> protein conformation. • Does not account for bacterial environmental conditions, such as cell wall, metabolism of compound, efflux, parallel targets, etc. • Requires downstream studies <i>in vitro</i>.
Whole cell gene expression screening	
Description	A whole cell screen with a genetically inbuilt reporter system indicating chemical effect upon gene of interest expression.
Advantages	<ul style="list-style-type: none"> • All advantages of whole cell screening accounted for. • Provides additional information on hit compound target and mechanism, i.e. differential expression of promoter of interest. • Can detect compounds interfering with entire metabolic processes, even those not present <i>in vitro</i> such as those involved in virulence and pathogenesis.
Disadvantages	<ul style="list-style-type: none"> • Requires reporter system construction. • Only indicative, target(s) must be defined by further downstream study. • Requires cytotoxicity counter screen. • Reporter activation does not necessarily indicate anti-bacterial activity.

A modified whole cell screening approach was selected for this research, using several different culture conditions, broadly defined into nutrient rich and nutrient starvation conditions. The whole cell screening approach was chosen as to not limit screens to one target, instead to maximise the number of hit compounds identified by screens, allowing in depth comparison between the hit profiles with differing culture conditions and providing a large variety of lead compound options to pursue for further study. The disadvantage of indeterminate cytotoxicity of identified hits was addressed by literature searching of hit compounds and downstream mammalian cell line cytotoxicity assays of promising hit compounds described in Chapter 5. Target identification and mechanism of action studies were then carried out on a lead compound later described in Chapter 6.

3.1.2 Screening objectives

Three objectives were determined for this high throughput screening component:

- 1) Validate the viability of the dual label screening protocol as a tuberculosis drug discovery tool.**
- 2) Identify novel antibacterial chemicals with potential inhibitory activity towards *M. tuberculosis*.**
- 3) Investigate the discrepancies between the hit profiles of chemical library screens under differing rich culture conditions, and use these data to compare to starvation culture condition screens.**

To achieve these objectives three chemical libraries were screened using a 96-well plate format against the fast growing tuberculosis model organism *M. smegmatis* expressing plasmid bound GFP as an additional viability indicator to absorbance measurements. This provides maximum coverage of hit detection and minimise false positives of compounds that may absorb at 600 nm or autofluoresce at in the GFP excitation/emission spectral wavelengths.¹²² The effect of chemical library compounds was assessed in *M. smegmatis* under a variety of culture nutrient conditions, broadly categorised into conventional nutrient rich media types and minimal nutrient starvation media types. This chapter details only the rich media type data. Hit compounds were then further validated against *M. smegmatis* to confirm and quantify their inhibitory activity and cross referenced against *M. tuberculosis* screening results performed by another research group member in our laboratory as part of his PhD project.^{206, 220} Validated hit compounds were further researched in bio-assay databases and scientific literature to determine the chemical properties such as novel bioactivity, cytotoxicity and biological indications which influence the compounds suitability as a possible candidate for further development towards anti-tubercular therapy.

3.2 Chemical libraries

Three chemical libraries were used in this study, totalling over 5000 compounds. The source of these libraries and a brief description is provided below. Many of these library compounds have known biological activities, and it is hypothesised that chemicals with novel anti-mycobacterial activity may be discovered in these well known chemical collections. This can

be advantageous as the chemicals may already be administered in humans for another purpose; therefore the pharmacological properties such as tolerance and side effects may have been previously researched.²²¹

3.2.1 LOPAC

The Library of Pharmacologically Active Compounds (LOPAC), commercially supplied by Sigma-Aldrich, is a collection of 1,280 chemicals with characterised pharmacological activity. Advertised as the gold standard in assay validation, these compounds have known pharmacological properties that cover a diverse range of known biological activities such as antibiotic activity, gene expression, multi-drug resistance, apoptosis, ion channel conductance, neurotransmission, signalling and phosphorylation. Although the compounds detailed in the LOPAC have been previously characterised, potential exists to identify chemicals with previously unknown anti-tubercular properties. The prior known activity of these compounds also provides a good base to indicate whether the compound may be applicable to human tuberculosis therapy. Additionally, screening LOPAC will validate the high throughput screen with the known anti-tubercular agents included in the library.

3.2.2 Spectrum Collection

The Spectrum Collection, supplied by Microsource (Connecticut, U.S.A.), is a library of 2,000 chemicals and extracts. Approximately half of the contents of the library are drugs with known biological activities; the remaining half are natural product extracts and pharmacophores with little or no known biological properties but were selected for their structural diversity. Other non-drug bioactive molecules such as toxins, pesticides, herbicides and receptor blockers are also included in the library. Like the LOPAC library, the fact that many of the chemicals have previously discovered activity is double edged, providing a basis for literature searching and indicating activity but detracting from novelty. A large advantage of Spectrum is the inclusion of novel chemicals, particularly natural product extracts, with no known biological activity discovered to date.

3.2.3 NIH Diversity and Natural Products Set

The National Institute of Health (U.S.A.) National Cancer Institute supplies the 1597 compound Diversity Set III and the 120 compound Natural Products Set. The Diversity Set compounds are representatives of a collection of 1,000,000 pharmacophores derived from the NCI Developmental Therapeutics Repository compounds, selected for availability and

structural diversity. The Natural Products Set II compounds are natural compounds that have been selected on the basis of origin, purity and structure diversity with an emphasis on multiple functional groups. Due to the nature of this library's composition it contains many antibiotic chemicals that provide good internal controls for the screen. Due to the NIH library nature as a cancer drug discovery tool, many of the library compounds are cytotoxic or otherwise anti-neoplastic, this makes them unsuitable for tuberculosis treatment due to their poor selectivity, e.g. compounds may be more toxic toward human cells than mycobacteria. A large advantage of the NIH library is that it is a "hit kit", meaning the chemicals it contains are pharmacophores representing a much larger collection of chemicals that may be ordered by request. This provides an inexpensive head start for downstream SAR studies of promising hits.

3.3 Screening conditions

This research utilised four differential media conditions in high throughput screening; the nutrient rich media LB and 7H9, and the nutrient limited or starvation Hartman de Bonts (HdeB) C- and HdeB N-, carbon and nitrogen deprived respectively. The reasoning in using these differential culture conditions is to explore the influence of contrasting culture conditions upon the antibiotic susceptibility in *M. smegmatis*, the tuberculosis model organism used in this study. Compound susceptibility was explored in an effort to identify novel inhibitors of *M. tuberculosis* that have not yet been discovered by conventional rich media screening techniques, and to further explore the effect of culture conditions on sensitivity to inhibitors, allowing us to query which culture conditions of *M. smegmatis* best mimic the inhibition profile presented by *M. tuberculosis*.

A major contributing factor to mycobacterial resilience is their flexibility in metabolic response to the environment. Mycobacteria are able to adapt their metabolism to survive conditions of nutrient starvation^{136, 222} and hypoxia.²²³ This change in metabolism is derived from a change in gene expression and also presents a change in the drug susceptibility profile of the bacteria. Nutrient starvation conditions can be encountered by *M. tuberculosis* during pathogenesis when sequestered by phagocytes²²⁴ and when encapsulated by a granuloma²²⁵. Therefore nutrient limited culture conditions are postulated to mimic the environmental conditions of pathogenesis and screening using nutrient limitation conditions may better identify chemicals effective against tuberculosis infection.

3.3.1 Model organism *M. smegmatis*

Fast growing and non-pathogenic mycobacterial species, *M. smegmatis*, was chosen as the model organism in this screening. As a tuberculosis surrogate model organism *M. smegmatis* has been the point of some controversy due to its lack of virulence, gene duplication and lack of approximately 30% of *M. tuberculosis* gene orthologues.^{226, 227} A mycobacterial model comparison study provided evidence that *M. smegmatis* detected ~50% of drugs active against *M. tuberculosis* H37Ra in a high throughput screen against two of the chemical libraries used in the present research.²⁰⁶ *M. smegmatis* boasts several characteristics favourable for the screening component of this research; it exhibits fast growth with a doubling time of 2-3 h²²⁸ compared to the 20-24 h doubling time of *M. tuberculosis*.^{228, 229} This renders *M. smegmatis* favourable for rapid culturing, high throughput screening and follow up assays. *M. smegmatis* may be cultured at 42 °C, this allows the use of thermo-sensitive vectors.²³⁰ The strain used in this research, *M. smegmatis* MC²155, can be genetically manipulated by transformation and homologous recombination techniques much more readily than slow growing mycobacterial species.¹⁷³ Due to its non-pathogenic nature *M. smegmatis* poses low risk to laboratory workers and may be housed in the PC2 level facilities in which the present research experiments were performed. This low risk potential also affords *M. smegmatis* regulatory authority permissions for genetic modification with more ease than pathogenic species. *M. smegmatis* has been used successfully as a tuberculosis model organism in drug screening, such as the discovery of the novel anti-tubercular diarylquinoline TMC-207 that is currently undergoing clinical trials.⁹⁷ *M. smegmatis* has been demonstrated to exhibit susceptibility to all clinically used anti-TB drugs and is the most comparable organism of all fast growing mycobacteria to MDR-TB in terms of its susceptibility to anti-bacterial agents.⁵⁶ *M. smegmatis* often exhibits less frequent and lower sensitivity to inhibitory compounds in chemical inhibitor screens compared to slow growing mycobacteria, and this is likely due to genetic redundancy and its fast-growing non-pathogenic nature. Therefore inhibitory compounds detected in *M. smegmatis* screens that share inhibitory activity in *M. tuberculosis* often exhibit increased activity against *M. tuberculosis* strains.²⁰⁶

3.3.2 Screening and hit criteria

High throughput screens were set up and carried out as described in Chapter 2.2.3. All rich media screens were carried out in independent triplicate experiments. Hits were defined as compounds that exhibited 90% inhibition of growth compared with control cell growth and

antibiotic controls in at least two out of three screens. Identified hits were then evaluated in a dose-response assay to quantify the level of anti-mycobacterial activity.

3.3.3 Differential culture conditions

Four different culture conditions were utilised in the screening experiments of the present research. These four media types are separated into two distinct categories, rich media conditions and nutrient limited (or nutrient starvation) media conditions. This chapter documents rich media condition screening. Nutrient limitation media screening is addressed in Chapter 4.

3.3.3.1 Rich media conditions

Rich or complex media conditions refer to media types which contain large amounts of organic nutrient compounds such as nucleotides, amino acids and cofactors. Rich media also may contain compounds for direct use as energy sources for microbes such as glycerol or glucose. Rich media often are derived from hydrolysed organisms and tissues such as peptone digests and yeast extracts, but rich mediums may also have chemically defined constituents. Bacteria grow more rapidly in rich media conditions, correspondingly have elevated expression of translational apparatus genes and less biosynthesis gene expression since many essential metabolic substrates are provided in the media.²³¹ Rich conditions are ideal for bacterial growth but are not as stringent a model in regards to sensitivity for antibiotic screening due to the disparity of rich conditions compared to conditions encountered by bacteria *in vivo*.^{232, 233}

Therefore it is hypothesised that rich conditions present a smaller target profile due to the differential gene expression in this media type and will result in less compounds detected than in minimal media conditions.

3.3.3.1.1 Luria-Bertani broth

Luria-Bertani broth (LB) is a complex rich medium type consisting of tryptone, yeast extract and NaCl. Tryptone, the tryptic digest of casein provides a complete spectrum of amino acids. Yeast extract provides additional amino acids in addition to purines, pyrimidines, vitamin cofactors and inorganic minerals and trace elements. NaCl is included for osmotic balance and glycerol supplementation provides ample an source of carbon. The tryptone and yeast extract lend complexity to this medium, providing practically all metabolic substrates

required for cell growth and division, leading to little expression of many nutrient scavenging, transport and biosynthesis genes.

3.3.3.1.2 Middlebrook 7H9 broth

Middlebrook 7H9 Broth is a defined rich medium specific for culturing mycobacterial species including *M. tuberculosis*. Although it is chemically defined it is still a rich medium as amino acids, fatty acids and other complex organic nutrients are provided by the oleic acid, bovine serum albumin, dextrose and catalase (OADC) enrichment. Abundant carbon source is provided by glycerol and dextrose supplementation and ample nitrogen sources are provided in the 7H9 broth itself by ammonium sulphate. The broth also provides glutamic acid, sodium citrate, cofactors pyridoxine, biotin and all required minerals and salts. 7H9 broth represents somewhat of a middle ground between rich and starvation media types but is included as a rich medium type since although all required metabolic precursors are supplied and chemically defined none are limited in supply and complex nutrients such as free amino acids are not present.

3.3.4 Hit validation

Hit compounds identified in triplicate high throughput screens were then validated by serial dilution assay against *M. smegmatis* MC²155 in the medium type they were detected in as described in materials and methods Chapter 2.4.1. These data are presented in tabular format after the respective compound library scatterplot figures. Compounds were assessed in triplicate with a starting concentration of 25 μ M (with the exception of some compounds that were able to be purchased in bulk that were tested at a starting concentration of 50 μ M). Minimum inhibitory concentration (MIC) was recorded as the minimal concentration of compound required to produce no measureable growth in triplicate after 96 h in comparison to media and antibiotic controls. False positive hits were discarded, and only validated hits are presented, in order of chemical library.

For simplicity and comparative purposes, MIC values of validated compounds from LB and 7H9 media conditions are tabulated together. Significant differences between media conditions (more than one serial dilution MIC difference or lack of detection in either medium type is considered significant) are highlighted in red. Validated results in slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra cultured in 7H9 media were generously provided by the research of Mudassar Altaf²⁰⁶ and provide context to hits in these highly relevant slow growing mycobacterial models. Compound MIC data for

M. bovis BCG and *M. tuberculosis* H37Ra were supplied quantitatively when available. If a quantitative *M. bovis* BCG or *M. tuberculosis* H37Ra MIC for a hit compound was not available, the inhibitory activity in *M. bovis* BCG or *M. tuberculosis* H37Ra HTS data were used, a value of “ ≤ 20 ” μM indicates inhibitory activity (triplicate $> 80\%$ growth inhibition) was detected in screens, and no value “-” indicates no inhibitory activity (triplicate $< 80\%$ growth inhibition) was detected in screens.^{206, 220}

Complete information on library screen hits by media type including compound name(s), chemical structure, library plate number and well position, validated MIC and known bioactive properties are provided in Appendix 9.4.

3.3.5 Screen optimisations

The high throughput screen was optimised by the addition of the surfactant Tween 80^{234, 235} and the sugar D-arabinose to reduce aggregation of mycobacteria within the wells.⁵³ Assay incubation time in rich media was limited to 72 h as no change in growth was demonstrated after this time period that reflects transition from log to stationary phase growth. Plate reader settings of plate dimensions, measurement height and number of flashes were optimised using the Perkin Elmer assay optimisation protocol, details provided in Appendix 9.6.

3.3.6 Statistical analysis of viability

3.3.6.1 Luria-Bertani Broth

The Z-factors for screens in LB conditions were $Z=0.91$ (OD), $Z=0.87$ (GFP) for streptomycin and $Z=0.68$ (OD), $Z=0.87$ (GFP) for rifampicin. Signal to noise ratios were 22.64 (OD), 23.58 (GFP) for streptomycin and 9.44 (OD), 22.78 (GFP) for rifampicin. These statistics indicated an excellent assay in terms of validity and repeatability as described in Chapter 2.6.1, with the weaker Z-factor and SNR from the rifampicin control under OD conditions due to its coloured nature. It should be noted this phenomenon is the reason it was included as a control, although the Z-factor and SNR for rifampicin OD were lower, they are still well above acceptable levels.

3.3.6.2 Middlebrook 7H9 Broth

The Z-factors for screens under 7H9 conditions were $Z=0.96$ (OD), $Z=0.88$ (GFP) for streptomycin and $Z=0.95$ (OD), $Z=0.92$ (GFP) for rifampicin. Signal to noise ratios were 19.54 (OD) and 21.37 (GFP) for streptomycin and 10.13 (OD), 19.68 (GFP). These statistics indicate an excellent assay in terms of validity and repeatability, further described in Chapter

2.6.1. Rifampicin demonstrated a slightly lower signal to noise ratio for OD readings as expected due to its coloured nature but this was heavily outweighed by the high growth of controls in 7H9 and the fact that the statistics were above acceptable levels.

3.4 Screen results

Results of the rich media condition high throughput screens carried out by the methodology described in materials and methods Chapter 2.2.3 are depicted below in graphical format in figures 3.1-3.8, presented by order of chemical library (colour coded), media type and type of signal recorded (GFP or OD₆₀₀). Compounds are numerically categorised on the X axis and their inhibition of growth signal is shown on the Y axis. Each graph is a representation of triplicate screens, with point position representing the mean inhibition value of a library compound and point size representing the standard deviation of a library compound across triplicate assays. The threshold of 90% inhibition which defines a hit in rich medium is demonstrated by a blue horizontal line at $y = 90$ and also a green area of highlighting. Hit compounds identified in screens were then verified by MIC determination dose-response assays, these results are tabulated in chemical library order (tables 3.2-3.4) and contrasted with 7H9 medium screening results of library compounds against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Hit compounds were categorised in figures 3.3, 3.6 and 3.9 based on known function according to information provided by the chemical library supplier (Sigma-Aldrich, Microsource, NIH), published literature and chemical database information. Sensitivity of detection in media types refers to the differential validation MIC or screen hits between LB and 7H9 conditions, with greater than 2-fold difference in MIC between LB and 7H9 media conditions considered to be significant differential sensitivity.

3.4.1 LOPAC rich culture conditions

3.4.1.1 LB

Figure 3.1: *M. smegmatis* LOPAC LB HTS OD & GFP scatterplots.

Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from LB media HTS of the LOPAC for *M. smegmatis* growth inhibition. Percent growth signal inhibition relative to controls is measured on the Y axis, library compound number is listed on the X axis. Significant hits exhibiting > 90% inhibition are boxed in green. Points are colour and size coded for mean value and standard deviation as depicted in the figure key.

3.4.1.2 7H9

Figure 3.2: *M. smegmatis* LOPAC 7H9 HTS OD & GFP scatterplots.

Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from 7H9 media HTS of the LOPAC for *M. smegmatis* growth inhibition. Details as for Fig 3.1.

3.4.1.3 LOPAC rich media screening validated hit compounds

LOPAC Compound	<i>M. smegmatis</i>	<i>M. smegmatis</i>	<i>M. bovis</i> BCG	<i>M. tuberculosis</i>
	MIC (μM) - LB	MIC (μM) - 7H9	MIC (μM)	H37Ra MIC (μM)
Calcimycin	3.125	-	-	≤ 20
Calmidazolium	12.5	-	-	-
Clotrimazole	12.5	-	-	-
Demeclocycline	3.125	3.125	3.125	1.56
Dequalinium	12.5	3.125	3.125	6.25
Dequalinium C-14 linker. (Quinolinium)	6.25	3.125	-	≤ 20
Diphenyleneiodonium	6.25	3.125	0.195	0.39
Doxycycline	1.56	1.56	≤ 20	-
Ellipticine	-	25	12.5	12.5
Idarubicin	12.5	3.125	6.25	6.25
Lomefloxacin	12.5	6.25	1.56	3.125
Methoctramine	12.5	12.5	1.56	6.25
Minocycline	12.5	12.5	1.56	3.125
Niclosamide	12.5	25	6.25	6.25
Ofloxacin	3.125	3.125	1.56	1.56
Vancomycin	12.5	-	≤ 20	-
WB 64	-	12.5	12.5	12.5

Table 3.2: LOPAC rich media screening validated hit results. Validation MIC values of LOPAC HTS derived hits are presented for *M. smegmatis* by rich media type. These values are contrasted with 7H9 medium screening results of LOPAC against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Hit compounds results with substantial difference in sensitivity (> 2-fold inhibition) between media types results are displayed in red text. Values of “-” indicate no inhibitory activity was detected in screening (triplicate mean of > 90% growth inhibition compared to positive controls). Values of “≤ 20” indicate the compound did exhibit inhibitory activity in screens (triplicate mean of > 80% growth inhibition compared to positive controls) against slow growing mycobacterial species, however confirmatory MIC assays were unable to be performed, therefore the MIC is estimated to be less than or equal to the screen compound concentration of 20 μM.

3.4.1.4 LOPAC rich media screening results overview

Categorised Validated Hits from LOPAC Rich Media Screening.

Figure 3.7: LOPAC rich media screening hit compound known activities. Percentage of hits obtained by rich media screening of the LOPAC broadly categorised by supplier annotated biological activity.

A total of 17 inhibitors were identified through LB and 7H9 screening of the LOPAC library with 15 inhibitors identified in LB media conditions and 13 inhibitors through 7H9 culture conditions. Many known anti-bacterial chemicals were identified in the screening of the LOPAC such as the tetracycline family antibiotics demeclocycline, doxycycline, and minocycline that exhibited identical activity across both media types. The quinolones lomefloxacin and ofloxacin were also detected in both media types. The glycopeptide vancomycin and calcium ionophore calcimycin were detected only in LB conditions. The prior may perhaps be an indication of differential mycobacterial cell wall synthesis under nutrient limitation conditions, as alteration of D-alanyl-D-alanine terminal residues of NAM/NAG cell wall peptides, the binding site of vancomycin, confers resistance to the drug.²³⁶ The detection of these known antibiotics successfully served as internal validation controls for the assay. The cytotoxic or antineoplastic compounds ellipticine and idarubicin were detected with greater sensitivity in 7H9 conditions, with ellipticine having no MIC at the levels tested in LB, and idarubicin having a four-fold increase in potency under 7H9 conditions in comparison with LB conditions. With known anti-bacterial agents and cytotoxic agents accounted for, novel hit compounds included calmidazolium chloride and

clotrimazole that were detected in LB conditions only. Dequalinium and its derivative quinolinium were detected in both LB and 7H9, albeit with a four-fold reduction in activity in LB media. The structurally similar methoctramine was also detected in both media conditions. Diphenyleneiodonium and niclosamide were detected under both conditions and demonstrated a two-fold reduction in activity in LB culture conditions. WB-64, similar again in structure to dequalinium and methoctramine was detected only under 7H9 screening conditions. A detailed list of library screen hits including chemical structures is included in Appendix 9.4.1. The biological and chemical significance of the novel hit compounds (discounting known antibiotics and cytotoxics) is discussed in depth in section 3.5.2.1 of this chapter.

3.4.2 Spectrum Collection Rich culture conditions

3.4.2.1 LB

Figure 3.4: *M. smegmatis* Spectrum Collection LB HTS OD & GFP scatterplots.

Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from LB media HTS of the Spectrum Collection for *M. smegmatis* growth inhibition. Details as for Fig 3.1. Note the high standard deviation of compounds 1800-1900 caused by an anomaly of a single plate of the triplicate experiment. The anomaly is more pronounced for GFP data than OD data and is likely due to human error in assay set up.

3.4.2 Spectrum Collection Rich culture conditions

3.4.2.2 7H9

Figure 3.5: *M. smegmatis* Spectrum Collection 7H9 HTS OD & GFP scatterplots.

Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from 7H9 media HTS of the Spectrum Collection Set for *M. smegmatis* growth inhibition. Details as for Fig 3.1.

3.4.2.3 Spectrum Collection rich media screening validated hit compounds

Spectrum Collection Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
Chloroacetoxyquinoline (Silital)	25	-	≤ 20	≤ 20
Aklavine	12.5	-	≤ 20	≤ 20
Celastrol (Tripterine)	25	-	-	-
Chlorhexidine	12.5	-	≤ 20	≤ 20
Chlortetracycline	1.56	1.56	-	-
Demeclocycline	1.56	1.56	≤ 20	≤ 20
Dihydrostreptomycin	12.5	6.25	-	-
Pyrithione Zinc	-	1.56	≤ 20	≤ 20
Clotrimazole	12.5	-	≤ 20	-
Doxycycline	1.56	1.56	≤ 20	≤ 20
Ethambutol	3.125	3.125	≤ 20	≤ 20
Minocycline	1.56	0.78	≤ 20	≤ 20
Oxyquinoline	-	12.5	≤ 20	≤ 20
Oxytetracycline	6.25	3.125	-	≤ 20
Tetracycline	1.56	3.125	≤ 20	-
Streptomycin	3.125	3.125	-	-
Tobramycin	6.25	6.25	-	-
Thimerosal	3.125	1.56	≤ 20	≤ 20
Thiothixene (Tiotixene)	-	25	-	-
Sulfamethoxazole	-	25	-	-
Phenylmercuric Acetate	-	3.125	≤ 20	≤ 20
Gemifloxacin	3.125	3.125	≤ 20	≤ 20
Clioquinol	12.5	-	-	-
Thiostrepton	12.5	12.5	≤ 20	≤ 20
Gatifloxacin	1.56	1.56	≤ 20	≤ 20
Methylbenzethonium Chloride	1.56	6.25	≤ 20	-
Roxithromycin	6.25	6.25	≤ 20	-
Levofloxacin	3.125	3.125	≤ 20	≤ 20
Telithromycin	6.25	25	≤ 20	-
Erythromycin	12.5	12.5	≤ 20	-
Econazole	-	12.5	≤ 20	≤ 20
Lomefloxacin	12.5	12.5	≤ 20	≤ 20
Ofloxacin	3.125	3.125	≤ 20	≤ 20
Cisplatin	-	12.5	-	-
Alexidine	-	6.25	≤ 20	≤ 20
Dirithromycin	12.5	6.25	-	≤ 20
Clarithromycin	-	6.25	≤ 20	-
Moxifloxacin	1.56	1.56	≤ 20	≤ 20
Rifampin	6.25	6.25	≤ 20	≤ 20
Ciprofloxacin	12.5	12.5	-	≤ 20
Teniposide	25	25	-	-
Rifaximin	6.25	6.25	≤ 20	≤ 20
Sarafloxacin	6.25	6.25	≤ 20	≤ 20
Pefloxacin	12.5	12.5	≤ 20	≤ 20

Table 3.3: Spectrum Collection rich media screening validated hit results.

Validation MIC values of Spectrum Collection HTS derived hits are presented for *M. smegmatis* by rich media type. Details as for Table 3.2.

3.4.2.4 Spectrum Collection rich media screening results overview

Categorised Validated Hits from Spectrum Collection Rich Media Screening.

Figure 3.6: Spectrum Collection rich media screen hit compound activities.

Percentage of hits obtained by rich media screening of the Spectrum Collection broadly categorised by supplier annotated biological activity.

Forty-four inhibitors were identified through the screening of the Spectrum Collection in both LB and 7H9 media types with 35 hits were validated in LB media and 38 validated in 7H9 media. A large amount of known antibiotics were detected in this screening with comparable sensitivity in both media types including 6 tetracycline family antibiotics, 9 fluoroquinolones (a class regarded as 2nd line anti-tuberculosis treatment drugs), the first line tuberculosis treatment drugs and derivatives streptomycin, dihydrostreptomycin, ethambutol, rifampicin and rifaximin. The 3rd line anti-tuberculosis drug clarithromycin was also detected in 7H9 medium conditions only. The aminoglycoside tobramycin and macrolide antibiotics roxithromycin, erythromycin, dirithromycin and cyclic peptide antibiotic thiostrepton were detected with similar sensitivity in both rich media types but sulfamethoxazole was detected only in 7H9 media and the ketolide telithromycin was detected with four fold greater sensitivity in 7H9 media. Several anti-fungal agents were differentially detected in either medium type including chloroacetoxyquinoline, and clotrimazole detected in LB conditions only and pyrithione zinc, phenylmercuric acetate and econazole detected in only 7H9 conditions. Anti-septic agents exhibited much differential sensitivity depending on media

conditions, with only thimerosal being validated with comparable MIC in both media types, whilst chlorhexidine and clioquinol were validated only in LB medium conditions and methylbenzethonium chloride exhibiting 4-fold greater activity in LB medium conditions compared to 7H9. The only anti-septic solely validated in 7H9 medium was alexidine. A few anti-cancer chemicals were detected such as teniposide in both media conditions, aklavine and celastrol only in LB conditions and cisplatin only in 7H9 conditions. Accounting for known anti-bacterial, anti-septic and cytotoxic agents these screening results presented few novel anti-bacterial compounds, with the exception of thiothixene – a antipsychotic thioxanthine class drug which has since been reported by the Southern Research Institute as exhibiting anti-tubercular activity *in vitro* (PubChem BioAssay ID# 449762).²³⁷ Several anti-fungal agents detected to have anti-mycobacterial activity in this screening may prove novel leads for implementation as tuberculosis treatment options and will be discussed further in section 3.5.2.2 of this chapter. A complete list of Spectrum Collection screen hits including chemical structures is included in Appendix 9.4.2.

3.4.3 NIH Diversity Set Rich culture conditions

3.4.3.1 LB

Figure 3.7: *M. smegmatis* NIH Diversity Set LB HTS OD & GFP scatterplots. Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from LB media HTS of the NIH Diversity Set for *M. smegmatis* growth inhibition. Details as for Fig 3.1.

3.4.3.2 7H9

Figure 3.8: *M. smegmatis* NIH Diversity Set 7H9 HTS OD & GFP scatterplots. Scatterplots depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from 7H9 media HTS of the NIH Diversity Set for *M. smegmatis* growth inhibition. Details as for Fig 3.1.

3.4.3.3 NIH Diversity Set rich media screening validated hit compounds

NIH Library Compound	<i>M. smegmatis</i> MIC (μM)- LB	<i>M. smegmatis</i> MIC (μM)- 7H9	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC3907 (8-Hydroxyquinoline salicylate, clioquinol analogue)	-	25	12.5	3.125
NSC329226	6.25	6.25	≤ 20	≤ 20
NSC625324 (Silver sulfadiazine)	-	12.5	-	-
NSC12155 (Aminoquinuride, surfen)	-	12.5	≤ 20	-
NSC13480	-	12.5	12.5	12.5
NSC132693	-	25		
NSC321206	-	25	25	3.125
NSC311153	-	25	6.25	6.25
NSC311152 (Datelliptium)	-	12.5	≤ 20	≤ 20
NSC176327	-	12.5	≤ 20	≤ 20
NSC359449	-	12.5		
NSC268879	3.125	3.125	1.56	3.125
NSC661755 (Michellamine B)	25	-	≤ 20	≤ 20
NSC133100 (Rifamycin SV)	25	-	-	-
NSC305787	-	25	25	6.25
NSC11241 (C.I. Basic red 6)	-	25	-	-
NSC321237	6.25	-	-	-
NSC5159 (Chartreusin)	3.125	1.56	1.56	1.56
NSC310551	-	6.25	12.5	6.25
NSC22225 (Cadmium chlorendic acid)	-	1.56	≤ 20	≤ 20
NSC145611 (Rifamycin derivative)	25	-	50	6.25
NSC145612	25			50
NSC143491 (Daunorubicin)	12.5	12.5	≤ 20	≤ 20
NSC321239	6.25	12.5	≤ 20	-
NSC69343	12.5	12.5	-	-
NSC106408 (Anthramycin methyl ether)	25	12.5	-	-
NSC134727 (Mitomycin C)	1.56	-	-	-
NSC122819 (Teniposide)	12.5	-	-	-
NSC659779 (2- Bromoascididemin)	12.5	1.56	0.781	3.125
NSC325014 (Bactobolin)	12.5	12.5	≤ 20	-
NSC661755 (Michellamine B)	25	-	≤ 20	≤ 20

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) - LB	<i>M. smegmatis</i> MIC (μM) - 7H9	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC526417 (Echinomycin)	1.56	3.125	≤ 20	≤ 20
NSC45383 (Streptonigrin)	3.125	25	≤ 20	≤ 20
NSC45737 (Bacitracin)	12.5	12.5	≤ 20	≤ 20
NSC51001 (Carbomycin)	6.25	12.5	12.5	12.5
NSC82151 (Daunorubicin HCl)	6.25	12.5	≤ 20	≤ 20
NSC5159 (Chartreusin)	3.125	1.56	-	-
NSC62709 (Imidazoquinoline)	25	25	50	12.5
NSC18712 (Streptomycin)	25	12.5	≤ 20	≤ 20
NSC125066 (Bleomycin)	1.56	1.56	3.125	1.56
NSC31048 (Neohesperidin)	12.5	6.25	≤ 20	≤ 20
NSC301739 (Mitoxantrone)	-	25	≤ 20	
NSC47151 (Neomycin, neosule)	-	12.5	≤ 20	≤ 20
NSC67574 (Vincristine sulfate)	25	-	≤ 20	≤ 20
NSC133100 (Rifamycin SV)	25	-	-	-
NSC368675 (Azadirachtin)	25	-	≤ 20	≤ 20
NSC61586 (Phleomycin)	0.39	0.19	25	1.56
NSC285116 (Siomycin A)	6.25	1.56	6.25	6.25
NSC13252 (Chlorotetracycline HCl)	1.56	1.56	-	-
NSC189794 (Streptovaricin)	12.5	12.5	25	6.25
NSC3053 (Dactinomycin)	12.5	-	-	-
NSC363182 (Kijanimicin derivative U-64815)	12.5	-	25	3.125

Table 3.4: NIH Diversity Set rich media screening validated hit results.

Validation MIC values of NIH Diversity Set HTS derived hits are presented for *M. smegmatis* by rich media type. The NSC designation number is given and where available a common name is also supplied. Details as for Table 3.2.

3.4.3.4 NIH Diversity Set rich media screening results overview

Categorised Validated Hits from NIH Diversity Set Rich Media Screening.

Figure 3.9: Pie chart of NIH Diversity Set rich media screen hit compound activities.

Percentage of hits obtained by rich media screening of the NIH Diversity Set broadly categorised by supplier annotated biological activity.

Screening of the NIH Diversity Set library yielded a total of 53 validated hit compounds, 36 of which were validated in LB medium and 39 in 7H9 culture conditions. Many cytotoxic and anticancer agents were detected in the screening of this library which is not unexpected given its intended nature as a cancer drug research chemical collection. 7H9 medium was more sensitive in the elucidation of cytotoxic chemicals with 10 chemicals with anti-cancer indications being detected solely or with > 4 fold sensitivity in 7H9 medium whilst only 4 anti-cancer indicated compounds were validated solely in LB medium. Eleven chemicals were validated with comparable sensitivity in both media types.

In regards to anti-bacterial compounds validated from the screens 4 rifamycin derivatives and a kijanimicin derivative were detected only in LB medium; where as only one rifamycin derivative and neomycin were detected solely in 7H9 and six anti-bacterial compounds including two tetracycline class antibiotics were detected with comparable sensitivity in both conditions. One antiseptic compound was identified, silver sulfadiazine, only in 7H9 conditions.

This left ten novel anti-tubercular compounds detected in the screening of the NIH library – four detected only in LB: two michellamine anti-viral compounds, one organomercury compound and azadirachtin which is isolated from the neem tree. Two chemicals were

detected only in 7H9: the aminoquinuride surfen and cadmium chlorendic acid. Four compounds were detected in both conditions: two organomercury compounds, the antiviral / immunomodulator imidazoquinoline and an artificial sweetening agent and natural product isolated from citrus plants neohesperidin. Novel anti-mycobacterials detected in this screen will be discussed further in section 3.5.2.3 of this chapter and a detailed list of library screen hits including chemical structures is included in Appendix 9.4.3.

3.4.4 Overview of screening goals and outcomes

The purpose of this screening exercise was three fold. First, to validate the whole cell high throughput screen design against libraries containing known inhibitory compounds towards bacteria including *M. tuberculosis*.

Second, to profile the inhibition of library compounds towards the tuberculosis model organism *M. smegmatis* under two distinct rich media conditions and identify potential novel chemical inhibitors of *M. tuberculosis*.

Third, to compare the library hit compound profiles between rich media types and also to facilitate comparison of rich media types with starvation media conditions, and to determine how *M. smegmatis* library hit profiles compare with those of *M. tuberculosis* screens, elaborated further in Chapter 4.

Whole cell *M. smegmatis* high throughput screening and hit characterisation protocols were validated through identification of several known tuberculosis treatment drugs and other known antibiotic agents. The screening in two distinct rich culture conditions, LB and 7H9, also produced differential hit profiles from the libraries, indicating that bacterial culture conditions influence sensitivity to chemical inhibitors. Finally, this screening revealed previously unpublished anti-mycobacterial compounds. Many of these identified novel inhibitors exhibit cytotoxic or otherwise unsuitable properties for lead compounds for human tuberculosis treatment. It is presumed that mycobacterial susceptibility to compounds will also differ *in vivo*, therefore confirmatory animal model testing must be carried out to properly validate lead compound efficacy. However, several compounds show promising indication as novel tuberculosis drug candidates. These screening results also will serve as a benchmark on which to compare the inhibition profile of the chemical libraries against *M. smegmatis* under nutrient limitation culture conditions.

3.5 Discussion

3.5.2 Biological significance of rich media screen hit compounds

Disregarding validated hit compounds with known anti-bacterial, cytotoxic or other properties that rend them unsuitable for use in human therapy, the biological and chemical significance of the novel anti-tubercular hit compounds will be analysed on the basis of their known reported activities in the literature and bioassay databases. Compounds thought to be particularly promising novel human TB treatment drug candidates on the basis of novelty, anti-mycobacterial potency and low mammalian toxicity are highlighted with **bold underscore**.

3.5.2.1 LOPAC rich media screen hit compound significance

Calmidazolium chloride is reported to have inhibitory activity towards *Plasmodium falciparum*, the causative agent of malaria.²³⁷ This research appears to be the first report of anti-mycobacterial activity of this compound,¹²² however the compound was unable to be validated in *M. tuberculosis* H37Ra having an MIC > 20 µM which detracts from the pharmacophores promise as a therapeutic agent. Calmidazolium is also a calmodulin antagonist inhibiting Ca²⁺, Na⁺ and K⁺ channels that can result in intracellular calcium accumulation and subsequent cellular toxicity, particularly cardiotoxicity.²³⁸ These properties rend calmidazolium an unlikely candidate to pursue further.

Clotrimazole is a common over-the-counter topical anti-fungal agent. It also exhibits inhibitory activity towards *P. falciparum*²³⁷. It has shown *in vitro* inhibitory activity towards *M. tuberculosis* H37Ra, including stationary phase cells.²³⁹ Clotrimazole is administered topically and has a reported favourable mammalian toxicity profile²⁴⁰ but exerts non-specific inhibition of many enzymes.²³⁷ The latter is an undesirable effect for systemic drug delivery. Derivatives showing increased specificity towards inhibiting mycobacteria and able to be orally administered may be prove suitable for tuberculosis therapy.

Concurrent to this research, dequalinium has been described and characterised as an inhibitor of *M. tuberculosis* mycothiol ligase and this same mechanism no doubt applies towards the dequalinium C-14 linker derivative quinolinium.²⁴¹ Methoctramine and WB-64 may also possible fall into this same mechanistic class as they exhibit similar structural characteristics and muscarinic receptor antagonism properties.

Diphenyleneiodonium is a characterised flavoenzyme inhibitor and oxidative stressor.²⁴² and

is thus most likely a ‘frequent flyer’, a common HTS hit as it inhibits a wide range of crucial enzymatic functions. The pharmacophore may be of use if the structure is modified to be specifically targeted towards mycobacteria such as by the conjugation of a mycobactin siderophore.²⁴³

Niclosamide is an anti-helminthic agent used specifically in the treatment of tapeworms, including treatment of tapeworm parasites in humans. Its mechanism of action is the uncoupling of oxidative phosphorylation.²⁴⁴ Niclosamide exhibits a good MIC *in vitro* against *M. tuberculosis* H37Ra of 6.25 μ M, although its cytotoxicity against J-774 cell lines has been suggested as limiting its applicability *in vivo*.²³⁹ This may not apply when administered systemically considering niclosamide is used in humans as a treatment for tapeworm. Niclosamide and other salicylanilides present good candidates for the development of new anti-tubercular agents, a concept that has recently been explored by Imramovsky *et al.*²⁴⁵

3.5.2.2 Spectrum Collection rich media screen hit compound significance

Chloroacetoxyquinoline has been shown to be inhibitory towards bacterial siderophore biosynthesis,²³⁷ which may be its mechanism of anti-mycobacterial action. Along with other quinoline derivatives chloroacetoxyquinoline has been synthesised and tested for anti-bacterial activity against *S. aureus* and *E. coli* with results comparable to tetracycline²⁴⁶

Clotrimazole is discussed above as it was also detected and validated in the LOPAC screen. Clioquinol, only detected in LB medium has recently been reported to be an inhibitor of *M. tuberculosis* methionine aminopeptidase.²⁴⁷

Pyrrithione zinc demonstrated strong inhibition of *M. smegmatis* in 7H9 medium only, but inhibition also extended to slow growing *M. tuberculosis* H37Rv with a previously reported *in vitro* MIC of 2.5 μ M.²²¹ It is a known anti-fungal agent and is thought to act by inhibition of membrane proton pump transporters, and is included in topical anti-fungal or anti-bacterial soap and shampoo preparations.²⁴⁸ It has a high oral LD₅₀ of 600 mg/kg in canines that may render it suitable for systemic administration in human therapy.²⁴⁹

The organic mercury compound phenylmercuric acetate is used as a fungicidal agent. It was described as early as 1921 as an anti-tubercular agent²⁵⁰ but due to the intrinsic toxicity of mercury this compound is unfavourable for oral anti-bacterial therapy.

Econazole has been detected concurrent to this research as inhibitory towards *M. tuberculosis* and *M. bovis*.²³⁷ and has been explored as a treatment option for MDR-TB.¹⁹ A broad spectrum anti-fungal, it is used in many topical anti-fungal over the counter medications and is reported to be well tolerated orally in murine models.²⁵¹

The most interesting and atypical hit compound presented by the rich media condition screening of the Spectrum Collection is **thiothixene**, which is a thioxanthene antipsychotic agent used in the treatment of psychiatric conditions, such as schizophrenia. It was detected concurrent to this research by the SRI as inhibitory to the growth of *M. tuberculosis* with a validated IC₅₀ of 100 µM against H37Rv (PubChem BioAssay ID# 449762). Thiothixene was also detected in screens for inhibitory compounds towards *P. falciparum*.²³⁷ If the mental effects exerted by this chemical²⁵² could be ameliorated and the anti-tuberculosis activity increased it may serve as a novel pharmacological scaffold for tuberculosis treatment.

3.5.2.3 NIH Diversity Set rich media screen hit compound significance

Michellamine B (NSC661755) and its parent class of michellamine alkaloids are natural products isolated from leaves of the *Ancistrocladus korupensis* plant. Michellamine B is a potent inhibitor of HIV replication, thought to act through the inhibition of HIV reverse transcriptase and viral-host cell fusion and also by inhibition of protein kinase C.²⁵³ This research is the first report of anti-mycobacterial activity of michellamines and its activity was unable to be quantitatively confirmed in *M. tuberculosis* < 20 µM. Michellamines have unfavourable cytotoxicity values towards some human cell lines,²⁵⁴ but its dual inhibitory activity toward both HIV and TB for which co-infection is a major dilemma (see Chapter 1.3), make this class of compounds valuable and in need of further research as anti-tubercular agents.

The organomercury compounds NSC268879, NSC321239 and NSC321237 are various mimics of the purine nucleotide guanine, and although no toxicity assays or anti-bacterial activity has been reported on PubChem Bioassay²³⁷ due to the mercury and nucleotide mimicking properties of these molecules suggesting they may be too toxic.²⁵⁵ NSC47151 neomycin is an aminoglycoside antibiotic that is commonly available over the counter as a topical anti-bacterial. Its systemic use in the treatment of tuberculosis has been explored²⁵⁶ however due to side effects of ototoxicity and nephrotoxicity²⁵⁷ its systemic use is not recommended since newer aminoglycosides with lower toxicity have been developed.

Similarly, NSC45737 bacitracin showed good inhibition of mycobacterial growth; however, this drug is only administered topically due to nephrotoxicity concerns.²⁵⁸

Azadirachtin is a limonoid class natural product isolated from the neem tree, *Azadirachta indica*. It is the most well researched derivative of the neem tree and is widely used as an insecticide/pesticide. Oral administration of azadirachtin is extremely well tolerated in mammals with doses up to 1500 mg/kg per day having no observable effect after 90-days in murine studies.²⁵⁹ Azadirachtin also appears to have no effects on postnatal development over two generations in murine models and is considered to be a safe pesticidal agent.²⁶⁰ Azadirachtin has been demonstrated to interact with retinoic acid receptors and modulate their downstream effects.²⁶¹ This research appears to be the first reporting of anti-mycobacterial activity of azadirachtin, unfortunately the activity of the compound towards slow growing species such as *M. tuberculosis* was unable to be quantitatively characterised other than an MIC > 20 μ M. This requires confirmatory follow up as the true MIC of azadirachtin is likely much lower. Extracts of the neem tree and its oils have long been used in traditional Indian folk medicine in the treatment of many diseases including leprosy and tuberculosis,²⁶² and these extracts have disclosed *in vitro* activity against many strains of bacteria including *M. tuberculosis*.²⁶³ The compound azadirachtin may be responsible for the anti-microbial properties of neem extracts. Considering azadirachtin's favourable toxicity profile it is deemed to be a promising chemical scaffold for development of an anti-tuberculosis drug, and requires further research into its anti-mycobacterial properties, cytotoxicity and *in vivo* tolerance.

Aminoquinuride / surfen is a antagonist of heparin.²⁶⁴ It has reported activity as an anti-malarial agent in addition to inhibitory activity towards *Bacillus anthracis*, the causative agent of anthrax, in both spore and vegetative states.^{237, 265} No activity towards *M. tuberculosis* H37Ra was observed at the < 20 μ M screening concentrations, so the activity of this compound towards slow growing mycobacterial species requires further validation. This research appears to be the first reporting of anti-mycobacterial activity of aminoquinuride. It has been previously described as decreasing HIV infectivity through the inhibition of amyloid fibrils and was referred to as semen derived enhancer of viral infection (SEVI),²⁶⁶ this is a favourable indication for HIV-TB co-infection.

Cadmium chlorendic acid contains the heavy metallic element cadmium and has been detected in one anti-cancer assay.^{237, 267} Although it exhibited strong inhibition of *M.*

smegmatis growth in 7H9 media with an MIC of 1.56 μM the MIC was unable to be validated in *M. tuberculosis* $< 20 \mu\text{M}$. It has been detected *in silico* as a potential inhibitor of the peptidoglycan synthesis enzyme MurD; however, inhibition was unable to be confirmed by a secondary *in vitro* enzyme assay.²⁶⁸ Its detected anti-cancer activity likely renders this molecule unsuitable as a tuberculosis treatment candidate.

Imidazoquinoline, a derivative of streptonigrin, is an immune response modulator, anti-tumour and anti-viral agent – these latter effects are possibly consequences of immunomodulation.²⁶⁹ It has been detected in 5 anti-cancer or tumour cell line growth inhibition screens.²³⁷ It exhibited good inhibitory activity towards *M. tuberculosis* H37Ra with an MIC of 12.5 μM ; however, anti-tumour activity and mutagenic properties indicate a negative side effect profile for this pharmacophore rendering it undesirable for further development as an anti-tubercular agent.²⁷⁰

Neohesperidin is an artificial sweetening agent and natural product isolated from citrus plants of the family *Rutacea*. Neohesperidin is widely used in many food products as a flavour enhancer (E959) and is considered to be safe for human consumption.²⁷¹ Activity was detected in *M. tuberculosis* screening which indicates it has a probable MIC $< 20 \mu\text{M}$, but this requires further quantitative evaluation. This research appears to be the first disclosure of anti-mycobacterial activity of neohesperidin; however, complex natural product extracts of citrus plant material containing hesperidins have before proved to have inhibitory activity towards MDR-*M. tuberculosis* and also methicillin resistant *S. aureus*.²⁷² This molecule has an extremely low reported *in vivo* mammalian toxicity with no observable toxic effects at up to 3.3 g/kg per day for 21 days, including no embryotoxicity or teratogenicity.²⁷³ These properties appoint neohesperidin as a very interesting lead to pursue for tuberculosis drug development, although inhibitory activity toward slow growing pathogenic mycobacterial species must be verified both *in vitro* and *in vivo*.

3.5.3 Differential rich culture conditions influence on screen hits

When screening for anti-mycobacterial compounds in the two distinct rich media types LB and 7H9, slight differential sensitivity of *M. smegmatis* to chemical inhibitors was observed between the two media types.

From all three libraries hits validated in rich media conditions, 85 hits were validated in LB medium, 88 were validated in 7H9 medium and 62 of these hits were common to both conditions.

When these validated hits are contrasted with results from analogous *M. tuberculosis* H37Ra screens^{206, 220} 57 LB hits and 63 7H9 hits were also active against *M. tuberculosis*, with 56 of the validated *M. smegmatis* hits were common to both LB and 7H9 media types and also detected in *M. tuberculosis*.

Validated Rich Screen Hits by Media Type

Figure 3.10: Venn diagram of rich media validated hit distribution.

Hit compounds derived from all libraries screened against *M. smegmatis* in rich media types LB (orange) and 7H9 (cream) compared with hits detected in *M. tuberculosis* H37Ra screens (red). Venn diagram group totals provided are inclusive.

Library screening of *M. smegmatis* in LB medium conditions resulted in a slightly lower number of total hits than those produced by screening in 7H9 medium. Additionally *M. smegmatis* screens performed in 7H9 medium conditions resulted in slightly more hits that also shared inhibitory activity towards *M. tuberculosis* compared to the hits derived from screening *M. smegmatis* in LB medium conditions. LB medium screen data generally had larger standard deviation than 7H9 medium, evidenced by the Z-factor values in section 3.3.6 and scatterplot Figures 3.1-3.6. 7H9 medium also exhibited lower background OD₆₀₀ and fluorescence in comparison with LB, however the SNR was greater for LB medium, due to the higher maximum growth capacity of LB medium.

One compound, calcimycin, had activity towards *M. tuberculosis* and was detected in *M. smegmatis* LB media screens but not in 7H9 screens; but generally, hits validated from *M. smegmatis* 7H9 medium screening were more relevant inhibitors toward towards *M. tuberculosis* H37Ra. It should be noted that *M. tuberculosis* screening was performed in

7H9 medium,^{206, 220} this is likely a contributing factor in the commonality between *M. smegmatis* 7H9 screen and *M. tuberculosis* screen results.

3.6 Summary

In conclusion, three compound libraries were screened for inhibition of *M. smegmatis* cultured in two distinct rich media conditions, LB and 7H9. The inhibitory activity of the resulting hit compounds was quantitatively validated, and the validated hits were compared to data from a similar screening project utilising slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.

Hit compounds were further characterised by quantification of their *M. smegmatis* MIC in the media conditions in which they were detected in and published biological and chemical properties were used to rule out validated hit compounds with characteristics making them unviable for development towards anti-tuberculosis therapy such as cytotoxicity, anti-cancer implications or previous anti-bacterial disclosure. The remaining novel candidate anti-tubercular compounds were then further researched and assessed for their suitability as tuberculosis treatment drug leads. Chemicals identified with potential application as drug leads were discussed in detail such as pyrithione zinc, azadirachtin and neohesperidin. These compounds exhibit novel anti-mycobacterial activity in addition to favourable characteristics for human administration making them good possible avenues for further research and development as anti-tubercular drugs.

The differential susceptibility of *M. smegmatis* to library inhibitors under different rich culture conditions was analysed, showing that there was not a large difference in 7H9 and LB media conditions. LB medium detected more hits by a small margin, but slightly more hits detected in 7H9 medium were also active towards *M. tuberculosis*. Therefore, the sensitivity difference between screening *M. smegmatis* in LB and 7H9 media conditions is minor, and the difference in relevance to activity in *M. tuberculosis* between the two rich media conditions is also minor. It is recommended that *M. smegmatis* chemical library screens be performed under both rich media conditions to maximise the number of hit compounds with potential relevance to *M. tuberculosis*; although, if researchers are restricted to only one media type when screening against *M. smegmatis*, 7H9 should be utilised for maximum relevance to *M. tuberculosis*. The concept of differential media conditions and inhibitor sensitivity profiles has been touched on in this chapter using the rich media types LB and 7H9. This will be further explored in Chapter 4 – Modifying Culture Conditions.

CHAPTER FOUR

Screening with Modified Culture Conditions

4.1 Modifying culture conditions introduction

This chapter expands upon the conventional rich media high throughput screening described in Chapter 3, exploring the effects of substantially modifying mycobacterial culture conditions by utilising nutrient limited or ‘starvation’ media types. Although the screening procedures and methodology remains similar, as in broad spectrum whole cell screening, the gene expression and resulting proteome of the mycobacteria are considerably different from that expressed in rich media, causing a potentially different susceptibility profile to chemical inhibitors.

4.1.1 Bacterial response to differential culture conditions

Bacillus licheniformis has been demonstrated to produce and excrete the highest amount of proteins when grown in a rich complex medium.²⁷⁴ When cultured under carbon and nitrogen starvation conditions, protein expression and excretion decreased and many proteins not expressed in rich media were secreted under starvation conditions. These starvation-specific proteins play essential nutrient scavenging and survival roles and are likely to be expressed in starvation conditions by alleviation of catabolite repression.²⁷⁴ Therefore; the susceptibility profile of *B. licheniformis* changes based on the culture conditions, expressing higher or lower levels of potential drug target proteins.²⁷⁴

During persistent tuberculosis infection, such as the scenario of latency and granuloma formation, mycobacteria are believed to be subjected to nutrient limited conditions and adjust their metabolism accordingly for survival and persistence within the hostile host environment.¹³⁶ This includes down-regulating expression of genes involved in non-vital processes such as lipid biosynthesis enzymes, translational machinery, aerobic respiration apparatus and cell division proteins.^{136, 275} *M. tuberculosis* present differential expression of genes involved in alternative carbon metabolism in the *in vivo* environment,^{276, 277} this response is recreated *in vitro* as a secondary effect of the Wayne model of progressive hypoxia,^{130, 131} the Betts nutrient starvation model¹³⁶ and the Loebel nutrient starvation oxygen rich model.^{278, 279} During conditions of nutrient starvation, *M. avium* are known to enter a metabolically dormant state, thought to be similar to latent *M. tuberculosis* infection.^{280, 281} Metabolically inactive phenotypes similar to those isolated from lung granulomas have been observed by the culture of *M. tuberculosis* in nutrient limitation.²⁸² Within macrophages, mycobacteria must survive low levels of free amino acids.²²² Alteration of gene expression caused by nutrient starvation conditions results in a differential

sensitivity profile to inhibitors, supported by research showing a drastic increase in resistance to anti-bacterial agents in starved cultures of *M. tuberculosis*.²⁸³ However this effect has been demonstrated to work both ways, with starvation media cultures up-regulating the expression of genes such as fumarate reductase, alanine dehydrogenase and various transporters all of which presumably contribute to survival of *M. tuberculosis* under conditions of starvation.^{136, 284} Thus starvation conditions reveal genetic targets that are masked in rich media culture, and the genes expressed in starvation conditions may be considered more essential and pathogenically relevant targets than their rich media counterparts.

4.1.2 Screening with modified culture conditions in the literature

Modification of culture conditions towards nutrient limitation and the implications of such conditions in drug sensitivity, gene expression and as a pathogenically relevant model for *M. tuberculosis* have been well considered, explored and documented.^{129, 130, 136, 285}

The same research has also been applied to the *M. smegmatis* model organism finding that it too can mimic *M. tuberculosis* dormancy *in vitro* when subjected to nutrient limitation conditions.¹⁴⁰ However applying the principle of nutrient starvation to high throughput screening of chemical libraries for the discovery of novel mycobacterial inhibitors was first published from the present research,¹²² and subsequent related projects originating from within our research group.^{157, 162} Although similar screening ideologies involving dormancy have been conceptualised,²⁸⁶ there is little disclosure of nutrient starvation high-throughput drug screening in the literature.

With the exception of the initial publication of the present research,¹²² the only disclosure found by a thorough literature search is a 2010 publication describing chemical library HTS against *M. tuberculosis* in the absence of glycerol, a screening model analogous to carbon starvation.²⁸⁷ This publication describes the discovery of a novel pyrimidine-imidazole class of anti-mycobacterial compounds that unfortunately lacked activity *in vivo*. Nonetheless, this publication serves as an excellent comparison and cross-reference for the results obtained in this research, particularly those obtained through carbon starvation medium screening.

4.1.3 Objectives of modified culture condition screening

Parallel to the objectives laid out in Chapter 3 – High Throughput Screening, three objectives were set for the high throughput screening of chemical libraries under conditions of nutrient limitation:

- 1) Query the suitability of the rich media high throughput screening protocol described in Chapter 3 when adapted to nutrient starvation conditions.**
- 2) Compare the hit profile of nutrient starvation condition screens to those performed in rich media conditions, and also results of screens performed against *M. tuberculosis* to determine the relevance of *M. smegmatis* modified culture conditions screening in tuberculosis drug discovery.**
- 3) Identify novel antibacterial compounds with potential as inhibitors of *M. tuberculosis*.**

For achieving these goals the three chemical libraries screened in Chapter 3 were subjected to parallel screening against *M. smegmatis* in HdeB carbon and nitrogen limitation media types, this media was used as HdeB is a chemically defined minimal medium, further described in section 4.2.3. Screens were carried out as described in section 2.2.3 with a 96-well plate format and measuring culture OD 600 nm and *M. smegmatis* GFP expression as viability indicators as discussed in section 3.1.2.

Hit compounds detected during high throughput screens were then validated against *M. smegmatis* in a quantitative assay to confirm and quantify their inhibitory activity. Validated hits were cross-referenced with data from *M. tuberculosis* H37Ra and *M. bovis* BCG screening project performed by one of our research group member Mudassar Altaf as part of his PhD project.^{206, 220} Hit compounds that were successfully validated were researched in the literature and bio-assay databases to ascertain the compounds' relevant properties of novel bioactivity, known cytotoxicity and biological indications that determine the chemicals suitability as a potential candidate for future development as a human tuberculosis treatment.

4.2 Screening conditions

4.2.1 Chemical libraries and screening conditions

Chemical libraries utilised in this study, LOPAC, the Spectrum Collection and the NIH Diversity and Natural Products Set, are described in Chapter 3.2. This research utilised four different culture conditions in HTS of the fast-growing tuberculosis model organism *M. smegmatis*. These media conditions are broadly categorised as nutrient rich (LB, 7H9) or nutrient limited/starved (HdeB C-, HdeB N-). Chapter 3 describes the motive for screening differential culture conditions in section 3.3.3, details of the *M. smegmatis* model organism in

section 3.3.1 and discloses the results of screening under nutrient rich media conditions in 3.4. Whilst the motive and *M. smegmatis* model organism details remain the same, this chapter reports the results of screening under nutrient limitation conditions and discusses their implications.

4.2.2 Screening and hit criteria

HTS were set up and carried out as described in Chapter 2.2.3. All nutrient limitation screens were carried out as independent triplicate experiments. Hits were defined as compounds that exhibited 80% or higher inhibition of growth compared with cell growth (100%) and antibiotic (0%) controls in at least two out of three screens. This hit threshold is lower than that used during rich media screening due to the lower maximum growth obtained in nutrient limitation media. This provides a larger window for hit detection but quality of hits was still validated by dose-response assay to quantify the level of anti-mycobacterial activity and rule out false positives. Nutrient limitation media screens did not contain D-arabinose,⁵³ as the addition of this carbohydrate may contribute to the nutrient content of the media.

Tween80^{234, 235} was included in Hartman deBonts starvation medium as directed, although it may be considered a utilisable carbon source the concentrations used (0.05%) were demonstrated to have negligible impact on growth rate or final culture density. The assay incubation time in nutrient limitation media was 120 h to allow culture growth to reach stationary phase. Plate reader settings of plate dimensions, measurement height and number of flashes were optimised using the Perkin Elmer assay optimisation protocol.

4.2.3 Starvation media conditions

Nutrient limitation or starvation media are chemically defined and provide the minimal requirements for bacterial growth. HdeB nutrient limitation broth was chosen as the synthetic defined minimal medium for this research, restricting vital carbon and nitrogen sources, although other elements such as phosphorous and sulphur may be limited, they were not included in the scope of this research. As culture in HdeB encourages *M. smegmatis* cells towards a non-culturable, dormant-like metabolic state, although this transition is not completed without the omission of oxygen or potassium from the medium²⁸⁸ In HdeB medium, individual elements are limited, such as carbon and nitrogen starvation, providing a metabolic bottleneck that causes a corresponding metabolic shift in mycobacteria, potentially exposing genetic targets essential under these nutrient limitation conditions.²⁸⁹ Culture of mycobacteria under starvation media conditions induce the expression genes involved in

nutrient scavenging and alternative biosynthesis pathways to compensate for the lack of readily available sources of nutrition.²⁹⁰ For example, mycobacteria are thought to utilise lipids as a primary energy source during starvation.²⁸⁰ These nutrient limited media types are considered to better represent conditions encountered by tuberculosis during infection and latency than rich media conditions that present an ideal environment for bacterial growth and cause functional redundancy of many genes.^{136, 280} *M. smegmatis* culture in HdeB medium has been demonstrated to exhibit adaptive effects upon protein expression to compensate for the nutrient depletion stress, but artificially expressed plasmid GFP remains a viable measurement of bacterial growth and survival.²⁹¹ Although nutrient starvation culture conditions such as HdeB do not perfectly mimic conditions encountered by tuberculosis within a granuloma, they are nonetheless considered a practical *in vitro* model.²⁸⁹

4.2.3.1 HdeB Carbon limitation medium (HdeB C-)

Hartman deBonts carbon starvation medium is a defined broth that provides adequate supplies for mycobacterial requirements of nitrogen, phosphorus, sulphur, metal salts and other trace elements but only provides a limited amount of carbon source in the form of glycerol. Glycerol limitation is a known inducing factor of mycobacterial stationary phase.¹⁴⁰ For a detailed composition and preparation instructions for HdeB C- consult section 2.2.1.2.

4.2.3.2 HdeB Nitrogen limitation medium (HdeB N-)

Hartman deBonts nitrogen starvation medium is a defined liquid medium that provides the requirements of carbon, phosphorus, sulphur and metal salts but only a limited amount of nitrogen source in the form of ammonium sulphate. Generally the ammonium ion is the preferred bacterial nitrogen source.²⁹² It has been demonstrated in the close mycobacterial relative *C. glutamicum* that nitrogen limitation causes changes to the composition of the intracellular amino acid pool.²⁹³ The uptake, metabolism and utilisation of nitrogen in mycobacteria is tightly regulated, and this adaptive regulation involves many essential processes that are proposed to be good drug targets.²⁹⁴ For a detailed composition and preparation instructions for HdeB N- consult section 2.2.1.2.

4.2.4 Hit Validation

Hit compounds identified in triplicate HTS were then validated by a dose-response assay against *M. smegmatis* MC²155 in the medium type they were detected in as described in Chapter 2.2.4. These data are presented in tabular format beneath the respective compound

library scatterplot figures. Compounds were assessed in triplicate with a starting concentration of 25 μ M, and MIC was recorded as the minimal concentration of compound required to produce no measureable growth in triplicate after 96 h in comparison to media and antibiotic controls. False positive hits were discarded, and only validated hits are presented, in order of chemical library.

For simplicity and comparative purposes, the MIC of validated compounds from HdeB C- and HdeB N- media conditions are tabulated together. Significant differences between media conditions (more than one serial dilution MIC difference or lack of detection in either medium type is considered significant) are highlighted in red. Validated results in slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra cultured in 7H9 medium are generously provided by the work of Mudassar Altaf^{206, 220} and provide context to hits in these relevant slow growing mycobacterial models. Compound MIC data for *M. bovis* BCG and *M. tuberculosis* H37Ra was quantitatively supplied when available. If a quantitative *M. bovis* BCG or *M. tuberculosis* H37Ra MIC for a hit compound was not available the inhibitory activity in *M. bovis* BCG or *M. tuberculosis* H37Ra HTS was used. Values of “ ≤ 20 ” indicate inhibitory activity (> 80% growth inhibition) was observed in screens estimating the MIC as less than or equal to the chemical screening concentration of 20 μ M. Values of “-” indicate that no inhibitory activity (< 80% growth inhibition) was detected in screens for that medium type.

4.2.5 Statistical analysis of viability

4.2.5.1 HdeB C-

Z-factors for screens performed in HdeB C- conditions were Z= 0.84 (OD) and Z=0.86 (GFP) for the streptomycin controls and Z=0.05 (OD) and Z=0.67 (GFP) for the rifampicin controls, respectively. Signal to noise ratios were 18.75 (OD) and 21.42 (GFP) for streptomycin controls and 3.17 (OD) and 9.31 (GFP) for rifampicin controls, respectively. The Z factors for GFP screens indicate good assay repeatability and validity, and the SNR for GFP values on all controls indicate an acceptable signal to noise ratio, meeting the Rose criterion of > 5.²⁹⁵ The OD Z-factor and SNR values for the streptomycin control are considered excellent; however, both the Z-factor and SNR for the OD rifampicin control fall into the marginal acceptability range bordering on unreliable. This effect is due to the absorbent (coloured) nature of rifampicin and explains why this drug was included as a control antibiotic. The effect is likely more pronounced in HdeB medium compared to rich LB or 7H9 media due to lower overall mycobacterial growth in the well and the transparent, minimally absorbing

nature of HdeB broth. The parallel use of GFP and OD signals as measures of viability mitigates this shortfall.

4.2.5.2 HdeB N-

Z-factors for screens performed under HdeB N- conditions were $Z=0.58$ (OD) and $Z=0.59$ (GFP) for the streptomycin control and $Z=-0.31$ (OD) and $Z=0.51$ (GFP) for the rifampicin controls. Signal to noise ratios for HdeB N- screens were 7.2 (OD) and 7.4 (GFP) for the streptomycin control and 2.3 (OD) and 6.1 (GFP) for the rifampicin controls, respectively. These statistical values represent a slight drop from those calculated in HdeB C- media and this is caused by further decreased final mycobacterial growth in this medium compared to HdeB C-, resulting in a smaller discrepancy between the means of the positive and negative controls and also greater standard deviation of the positive control. Z-factors and SNR for streptomycin under both signals and rifampicin under GFP signals are acceptable. The Z-factor and SNR for rifampicin when calculated from the OD signal is unacceptable, meaning there is too much overlap between the positive and negative controls for the data to be of use and a $SNR < 5$ does not meet the Rose criterion; hence, signal cannot be distinguished from noise with 100% certainty.²⁹⁵ However, the corresponding GFP signal values for rifampicin fall above acceptable levels, making the OD rifampicin controls redundant, and assays under these conditions therefore remain viable.

4.3 Screen results

The results of HTS carried out by the methodology described in Chapter 2.2.3 are depicted below in graphical format (figures 4.1-4.9), presented by order of chemical library (colour coded), medium type and inhibition signal recorded (GFP or OD₆₀₀). Compounds are numerically categorised on the X axis, and inhibition of growth signal is shown on the Y axis. Each graph represents triplicate screens, with the mean inhibition value of a library compound over triplicate screens graphed and point size representing the standard deviation of a library compound. The threshold of 80% inhibition that defines a hit in starvation media is demonstrated by a blue horizontal line and green highlighting. Complete information on library screen hits by medium type including compound name(s), chemical structure, library plate number and well position, validated MIC and known bioactive properties, are provided in Appendix 9.5. Hit compounds identified in screens were then verified by MIC determination dose-response assays, these results are tabulated in chemical library order (tables 4.1-4.3) and contrasted with 7H9 medium screening results of library compounds against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Hit compounds were categorised in figures 4.3, 4.6 and 4.9 based on known function according to information provided by the chemical library supplier (Sigma-Aldrich, Microsource, NIH), published literature and chemical database information. Sensitivity of detection in media types refers to the differential validation MIC or screen hits between LB and 7H9 conditions, with greater than 2-fold difference in MIC between LB and 7H9 media conditions considered to be significant differential sensitivity.

4.3.1 LOPAC Starvation Culture conditions

4.3.1.1 HdeB C-

Figure 4.1: *M. smegmatis* LOPAC HdeB C- OD & GFP scatterplots.

Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from HdeB C- medium HTS of the LOPAC for *M. smegmatis* growth inhibition.

Percentage growth signal inhibition relative to controls measured on the Y axis, compound number is listed on the X axis. Significant hits exhibiting > 90% inhibition are boxed in green. Points are colour and size coded for mean value and standard deviation as depicted in the figure key.

4.3.1.2 HdeB N-

Figure 4.2: *M. smegmatis* LOPAC HdeB N- OD & GFP scatterplots.

Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from a HdeB N- medium HTS of the LOPAC for *M. smegmatis* growth inhibition. Details as in Fig 4.1.

4.3.1.3 LOPAC starvation media screening validated hit compounds

LOPAC Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
BAY 11-7085	6.25	12.5	≤ 20	-
Calcimycin	12.5	12.5	-	≤ 20
Carboplatin	25	50	-	-
4-Chloromercuribenzoic acid	6.25	12.5	-	≤ 20
Cisplatin	12.5	12.5	-	-
Calmidazolium	50	12.5	-	-
Clotrimazole	50	50	-	-
Demeclocycline	6.25	6.25	3.125	1.56
Dequalinium	6.25	12.5	3.125	6.25
Dequalinium C-14 linker. (Quinolinium)	6.25	3.125	-	≤ 20
Diphenyleneiodonium	3.125	3.125	0.195	0.39
Doxycycline	3.125	3.125	≤ 20	-
Ellipticine			12.5	12.5
Idarubicin	25	12.5	6.25	6.25
Lomefloxacin	6.25	6.25	1.56	3.125
LY-367265	25	50	-	-
Methoctramine	12.5	12.5	1.56	6.25
Minocycline	12.5	12.5	1.56	3.125
Mitoxantrone	50	50	-	-
Niclosamide	6.25	6.25	6.25	6.25
Ofloxacin	6.25	6.25	1.56	1.56
Pentamidine	25	50	-	-
1,10-Phenanthroline	12.5	12.5	≤ 20	≤ 20
Se-Methylselenocysteine	12.5	12.5	12.5	12.5
Ruthenium red	50	50	-	-
Trifluoperazine	50	50	-	-
U-83836	50	50	-	-
Vancomycin	25	25	≤ 20	-
WB 64	50	25	12.5	12.5

Table 4.1: LOPAC starvation media screening validated hit results.

Validation MIC values of LOPAC HTS derived hits are presented for *M. smegmatis* by starvation media type. These values are contrasted with 7H9 medium screening results of LOPAC against the slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Hit compounds results with significant difference in sensitivity (> 2-fold inhibition) between media types are displayed in red text. Values of “-” indicate no inhibitory activity was detected in screening (triplicate mean of > 80% growth inhibition compared to positive controls). Values of “≤ 20” indicate the compound did exhibit inhibitory activity in screens (triplicate mean of > 80% growth inhibition compared to positive controls) against slow growing mycobacterial species, however confirmatory MIC assays were unable to be performed, therefore the MIC is estimated to be less than or equal to the screen compound concentration of 20 μM.

4.3.1.4 LOPAC starvation media screening results overview

Categorised Validated Hits from LOPAC Limited Media Screening

Figure 4.3: LOPAC starvation media screening hit compound activity.

Percentage of hits obtained by nutrient starvation media screening of the LOPAC categorised by supplier annotated biological activity.

Screening the LOPAC library under nutrient limitation conditions identified a total of 28 validated inhibitors, 12 of which were not previously detected in rich media condition screening of the LOPAC. Only two compounds were detected in rich media screening but not under starvation conditions. Twenty-one of these inhibitors were validated in carbon-limited HdeB medium, and 20 in nitrogen limited HdeB medium. For comparison 14 of these inhibitors were also active in *M. bovis* BCG and 16 were active in *M. tuberculosis* H37Ra.

As with rich media screening many chemicals with known anti-bacterial activity were identified: tetracycline family antibiotics demeclocycline, doxycycline, minocycline, fluoroquinolone antibiotics lomefloxacin and ofloxacin and glycopeptides vancomycin and calcimycin. The inhibition profile of these antibiotics was similar across both carbon and nitrogen limitation conditions and they were also all previously detected in rich media screening. Anti-cancer compounds were detected with greater sensitivity in nutrient limitation conditions compared to rich media, detecting not only idarubicin which was also identified in rich media screens but also identifying carboplatin, cisplatin and mitoxantrone, the anti-neoplastic agent ellipticine was not detected under nutrient limitation conditions;

whereas it was in nutrient rich conditions, but its activity level is at the borderline of screening concentration.

Discarding well known anti-bacterial and cytotoxic anti-cancer compounds, novel compounds were identified that were also seen in rich media screens, including calmidazolium, clotrimidazole, dequalinium quinolinium, diphenyleneiodonium, methocramine, niclosamide and WB-64.

The remaining compounds with novel anti-mycobacterial activity not elucidated through rich media condition screening were BAY 11-7085, 4-chloromercuribenzoic acid, LY-367265, pentamidine, 1,10-phenanthroline, Se-methylselenocysteine, ruthenium red, trifluoperazine and U-83836. Se-methylselenocysteine was not detected in screens under rich media conditions; however, it was later validated in rich 7H9 medium.

All compounds exhibited similar MIC values between carbon and nitrogen limitation conditions during validation, with the exception of calmidazolium which demonstrated a 4-fold reduction in inhibitory concentration in nitrogen limitation. A detailed list of library screen hits including chemical structures is included in Appendix 9.5.1. The biological and chemical significance of the LOPAC novel hit compounds with possible application as lead molecules in tuberculosis treatment drug discovery (discounting known antibiotics and cytotoxic compounds) is discussed in depth in section 4.4.2.1 of this chapter.

4.3.2 Spectrum Collection starvation culture conditions

4.3.2.1 HdeB C-

Figure 4.4: *M. smegmatis* Spectrum Collection HdeB C- OD & GFP scatterplots.

Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from a HdeB C- medium HTS of the Spectrum Collection for *M. smegmatis* growth inhibition. Details as in Fig 4.1.

4.3.2.2 HdeB N-

Figure 4.5: *M. smegmatis* Spectrum Collection HdeB N- OD & GFP scatterplots.

Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from a HdeB N- medium HTS of the Spectrum Collection for *M. smegmatis* growth inhibition. Details as in Fig 4.1.

4.2.3.3 Spectrum Collection starvation media screening validated hit compounds

Spectrum Collection Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
Khivorin	-	12.5	-	-
Merbromin	6.25	6.25	-	-
Chloroacetoxyquinoline (Silital)	-	0.78	≤ 20	≤ 20
Aklavine	-	6.25	≤ 20	≤ 20
Irigenin dibenzyl ether	-	12.5	-	-
Celastrol (Tripterine)	-	3.125	-	-
Amikacin	-	6.25	-	-
Benzethonium	-	6.25	-	-
Capreomycin	-	6.25	≤ 20	-
Cetylpyridinium	-	6.25	-	-
Chlorhexidine	6.25	3.125	≤ 20	≤ 20
Chlortetracycline	3.125	0.78	-	-
Colistimethate	-	12.5	-	-
Demeclocycline	0.78	0.78	≤ 20	≤ 20
Dihydrostreptomycin	12.5	3.125	-	-
Pyrithione Zinc	1.56	0.39	≤ 20	≤ 20
Clotrimazole	25	12.5	≤ 20	-
Doxycycline	0.78	0.78	≤ 20	≤ 20
Ethambutol	3.125	3.125	≤ 20	≤ 20
Gentian / Crystal Violet	3.125	3.125	-	-
Mercaptopurine	-	25	≤ 20	≤ 20
Minocycline	1.56	0.78	≤ 20	≤ 20
Nortriptyline	-	12.5	-	-
Oxytetracycline	-	1.56	-	≤ 20
Polymyxin B	-	12.5	-	-
Primaquine	-	25	-	-
Tetracycline	3.125	1.56	≤ 20	-
Streptomycin	3.125	6.25	-	-
Tobramycin	3.125	1.56	-	-
Thimerosal	0.78	3.125	≤ 20	≤ 20
Phenylephrine	3.125	3.125	-	-
Hydrastine	-	6.25	-	-
Patulin	-	25	≤ 20	-
Phenylmercuric Acetate	1.56	0.39	≤ 20	≤ 20
Dichlorodiphenyltrichloroethane, DDT	-	25	-	-
Gemifloxacin	3.125	6.25	≤ 20	≤ 20
Prochlorperazine edisylate	-	6.25	-	-
Thiostrepton	-	12.5	≤ 20	≤ 20
2-Aminoethyldiphenyl boronate, 2-aminoethyl diphenylborinate.	-	6.25	-	-
Gatifloxacin	1.56	1.56	≤ 20	≤ 20
Cetrimonium	-	6.25	-	-

Spectrum Collection Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
Methylbenzethonium Chloride	6.25	6.25	≤ 20	-
Roxithromycin	-	-	≤ 20	-
Cefdinir	-	25	≤ 20	≤ 20
Levofloxacin	3.125	3.125	≤ 20	≤ 20
Apramycin	25	6.25	-	-
Erythromycin	-	6.25	≤ 20	-
Gentisic acid, dihydroxybenzoic acid	-	25	≤ 20	≤ 20
Trifluoperazine HCl	-	12.5	≤ 20	≤ 20
Econazole	12.5	12.5	≤ 20	≤ 20
Lomefloxacin	6.25	12.5	≤ 20	≤ 20
Ofloxacin	3.125	3.125	≤ 20	≤ 20
Carboplatin	25	6.25	-	-
Cisplatin	3.125	0.78	-	-
Alexidine	6.25	6.25	≤ 20	≤ 20
Pararosaniline pamoate	-	6.25	≤ 20	-
Mitoxantrone	50	50	-	-
Thiram	-	12.5	≤ 20	≤ 20
Benzalkonium Chloride	6.25	6.25	-	-
Moxifloxacin	1.56	6.25	≤ 20	≤ 20
Berbamine	-	12.5	-	-
Rifampin	6.25	6.25	≤ 20	≤ 20
Acivicin	-	25	≤ 20	≤ 20
Chlorothalonil	6.25	3.125	≤ 20	≤ 20
Ciprofloxacin	-	12.5	-	≤ 20
Teniposide	-	25	-	-
Rifaximin	-	6.25	≤ 20	≤ 20
Sarafloxacin	6.25	6.25	≤ 20	≤ 20
Pefloxacin	-	12.5	≤ 20	≤ 20

Table 4.2: Spectrum Collection starvation media screening validated hit results. Validation MIC values of Spectrum Collection HTS derived hits are presented for *M. smegmatis* by starvation medium type. Details as for Table 4.1.

4.3.2.4 Spectrum Collection starvation media screen hit compound significance

Categorised Validated Hits from Spectrum Collection Limited Media Screening

Figure 4.6: Spectrum Collection starvation screen hit compounds known activity.

Percentage of hits obtained by nutrient starvation media screening of the Spectrum Collection categorised by supplier annotated biological activity.

Screening the Spectrum Collection under nutrient limitation conditions yielded a total of 68 validated inhibitors of *M. smegmatis*. Of these inhibitors 33 were identified through carbon limitation media, and all 68 were validated in nitrogen limitation media. Of these inhibitors, 41 were also found to have activity in screens against *M. bovis* BCG, and 34 exhibited activity in screens against *M. tuberculosis* H37Ra.

A large proportion of the hits detected in this assay were known antibacterial compounds, including tetracycline family antibiotics, fluoroquinolones and tuberculosis treatment drugs such as streptomycin, ethambutol and rifampicin. Similar to nutrient rich screening, many chemicals with well known anti-fungal, anti-septic or anti-neoplastic activity were detected. Nitrogen starvation conditions in particular rendered *M. smegmatis* more susceptible to anti-neoplastics and more sensitive to inhibitors in general, with most compounds exhibiting the same or lower MIC values in nitrogen starvation than in carbon starvation conditions. Only two exceptions had significantly lower MIC in carbon starvation relative to nitrogen: thimerosal and moxifloxacin.

Nutrient starvation condition screening yielded many more hit compounds relative to nutrient rich screening results. Only 8 compounds were detected in nutrient rich conditions and not again in nutrient limitation screens. These include the macrolide antibiotics roxithromycin, telithromycin, dirithromycin and clarithromycin, the quinolones ciprofloxacin and ofloxacin, the anti-psychotic agent thiothixene and the sulphonamide sulfamethoxazole.

Whereas nutrient rich screening of the Spectrum Collection only identified one compound unable to be broadly categorised, nutrient limitation screening revealed many novel inhibitory chemicals unable to be assigned to one of the broad activity categories detailed above.

These “other” class hits included the anti-malarial agent primaquine and the natural products khivorin, irigenin, berbamine and hydrastine. Anti-psychotic drugs nortriptyline, prochlorperazine and trifluoperazine, decongestant phenylephrine and pesticidal agent DDT. The anti-inflammatory gentisic acid and IP₃ modulator 2-aminoethyldiphenyl boronate were also identified.

Several of the chemicals detected to have anti-mycobacterial activity in this nutrient limited screening may prove novel leads for implementation as tuberculosis treatment options and will be discussed further in section 4.2.2.2 of this chapter. A complete list of Spectrum Collection screen hits including chemical structures is included in Appendix 9.5.2.

4.3.3 NIH Diversity Set starvation culture conditions

4.3.3.1 HdeB C-

Figure 4.5: *M. smegmatis* NIH Diversity Set HdeB C- OD & GFP scatterplots. Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from a HdeB C- medium HTS of the NIH Diversity Set for *M. smegmatis* growth inhibition. Details as in Fig 4.1.

4.3.3.2 HdeB N-

Figure 4.6: *M. smegmatis* NIH Diversity Set HdeB N- OD & GFP scatterplots. Scatterplot depicting the OD 600 nm (left) and GFP fluorescence (right) data derived from a HdeB N- medium HTS of the NIH Diversity Set for *M. smegmatis* growth inhibition. Details as in Fig 4.1.

4.3.3.3 NIH Diversity Set starvation media screening validated hit compounds

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC2347 (Tricarballic acid)	-	25	-	-
NSC27895	-	25	-	-
NSC1684	-	25	-	-
NSC23082	-	6.25	-	-
NSC3907 (8-Hydroxyquinoline salicylate)	-	6.25	12.5	3.125
NSC329226	6.25	3.125	≤ 20	≤ 20
NSC359098	-	6.25	-	-
NSC26081	-	6.25	-	-
NSC16865	-	6.25	≤ 20	-
NSC130830	-	12.5	-	≤ 20
NSC17600	-	6.25	-	-
NSC122657	-	12.5	≤ 20	-
NSC152686	-	12.5	-	-
NSC153533	-	25	-	-
NSC289336 (Rimiterol)	-	25	-	-
NSC13316	-	6.25	≤ 20	-
NSC333003	-	3.125	≤ 20	≤ 20
NSC625324 (Silver sulfadiazine)	6.25	0.39	-	-
NSC24113	-	6.25	≤ 20	-
NSC45382	-	3.125	-	-
NSC22907	-	25	-	-
NSC48300	-	6.25	≤ 20	≤ 20
NSC295642	-	6.25	≤ 20	≤ 20
NSC12155 (Aminoquinuride, surfen)	12.5	6.25	≤ 20	-
NSC632536	-	12.5	≤ 20	-
NSC3058301 (Benzenecarboximide)	-	6.25	-	-
NSC13480	6.25	3.125	12.5	12.5
NSC17474 (Trifluoperazine)	-	6.25	≤ 20	-
NSC294526	6.25	3.125	-	-
NSC321206	-	6.25	25	3.125
NSC311153	-	6.25	6.25	6.25
NSC311152 (Datelliptium)	12.5	6.25	≤ 20	≤ 20
NSC176327	12.5	3.125	≤ 20	≤ 20
NSC92585	25	0.78	≤ 20	≤ 20
NSC354961	25	6.25	≤ 20	-
NSC359449	12.5	1.56	≤ 20	≤ 20
NSC97345	3.125	0.19	-	-
NSC268879	3.125	0.19	1.56	3.125

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC117274	-	12.5	-	-
NSC305787	-	6.25	≤ 20	≤ 20
NSC150289	-	6.25	-	-
NSC114792	-	6.25	-	-
NSC292596	12.5	12.5	-	-
NSC661755 (Michellamine B)	12.5	3.125	≤ 20	≤ 20
NSC25485 (Ouabain)	-	12.5	-	-
NSC72939	-	12.5	≤ 20	≤ 20
NSC321237	3.125	0.39	-	-
NSC17770	-	6.25	≤ 20	≤ 20
NSC5159 (Chartreusin)	-		1.56	1.56
NSC310551	6.25	3.125	12.5	6.25
NSC295558	6.25	1.56	-	-
NSC20410	12.5	3.125	-	-
NSC182395	12.5	1.56	-	-
NSC22225 (Cadmium chlorendic acid)	12.5	12.5	≤ 20	≤ 20
NSC64111	-	6.25	≤ 20	≤ 20
NSC182035	-	6.25	-	-
NSC72938	-	25	-	-
NSC181486 (Trigillette)	-	12.5	≤ 20	≤ 20
NSC52003	-	25	-	-
NSC73101	-	25	-	-
NSC143491 (Daunorubicin)	12.5	6.25	≤ 20	≤ 20
NSC321239	3.125	0.39	≤ 20	-
NSC23128	-	25	-	-
NSC203328	-	25	-	-
NSC125910	-	25	-	-
NSC402959	-	12.5	-	-
NSC234766	-	12.5	-	-
NSC95609	-	12.5	-	-
NSC77037 (Tetrandrine)	-	12.5	-	-
NSC274547	12.5	6.25	-	-
NSC83628 (DF 8 sensitizer)	-	25	-	-
NSC13726	-	12.5	≤ 20	≤ 20
NSC130813	-	6.25	-	-
NSC41458	-	25	-	-
NSC169534 (Quelamycin)	-	25	-	-
NSC44251 (Cumertilin)		25	-	-
NSC166687	-	25	-	-
NSC59349 (3-Nortropanol)	-	12.5	-	-

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC109268	-	12.5	-	-
NSC48171	-	25	-	-
NSC69343	-	6.25	-	-
NSC3283	-	6.25	-	-
NSC71795 (Ellipticine)	12.5	3.125	≤ 20	≤ 20
NSC356217 (HEJ-4)	-	6.25	-	-
NSC32951 (Patulin)	-	3.125	-	-
NSC267461 (Nanaomycin A)	-	6.25	≤ 20	≤ 20
NSC312033 (Straital B)	-	1.56	-	-
NSC659779 (2-Bromoascididemin)	12.5	1.56	0.781	3.125
NSC35611 (Solanine)	-	6.25	≤ 20	≤ 20
NSC325014 (Bactobolin)	25	-	-	-
NSC661755 (Michellamine B)	6.25	3.125	≤ 20	≤ 20
NSC526417 (Echinomycin)	6.25	3.125	≤ 20	≤ 20
NSC45383 (Streptonigrin)	12.5	6.25	≤ 20	≤ 20
NSC56346 (Rottlerin)	-	6.25	-	-
NSC45737 (Bacitracin)	12.5	3.125	≤ 20	≤ 20
NSC51001 (Carbomycin)	12.5	3.125	12.5	12.5
NSC82151 (Daunorubicin HCl)	12.5	3.125	≤ 20	≤ 20
NSC62709 (Imidazoquinoline)	-	-	50	12.5
NSC18712 (Streptomycin)	25	1.56	≤ 20	≤ 20
NSC125066 (Bleomycin)	1.56	0.39	3.125	1.56
NSC31048 (Neohesperidin)	12.5	3.125	≤ 20	≤ 20
NSC301739 (Mitoxantrone)	12.5		≤ 20	
NSC47151 (Neomycin, neosule)	12.5	3.125	≤ 20	≤ 20
NSC52141 (Nonactin)	-	3.125	≤ 20	≤ 20
NSC67574 (Vincristine sulfate)	-	6.25	≤ 20	≤ 20
NSC368675 (Azadirachtin)	12.5	3.125	≤ 20	≤ 20
NSC34936 (Suramin, Germanin)	-	6.25	≤ 20	≤ 20
NSC9161 (Erythromycin)	-	12.5	≤ 20	≤ 20

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC
NSC61586 (Phleomycin)	0.19	0.097	25	1.56
NSC285116 (Siomycin A)	1.56	3.125	6.25	6.25
NSC13252 (Chlorotetracycline)	-	0.39	-	-
NSC292567 (Nigericin)	-	6.25	≤ 20	≤ 20
NSC317629 (Ketoconazole)	-	6.25	≤ 20	≤ 20
NSC319518 (Cotylenin A)	-	6.25	≤ 20	≤ 20
NSC333856 (Tetrocarcin A)	-	6.25	≤ 20	≤ 20

Table 4.3: NIH Diversity Set starvation media screening validated hit results. Validation MIC values of NIH Diversity Set HTS derived hits are presented for *M. smegmatis* by starvation media type. Compound names are given in addition to NSC number where available. Details as for Table 4.1.

4.3.3.4 NIH Diversity Set starvation media screen hit compound significance

Categorised Validated Hits from NIH Diversity Set Nutrient Limited Media Screening

Figure 4.9: Pie chart of NIH Diversity set starvation media screening hit compound known activity.

Percentage of hits obtained by nutrient starvation media screening of the NIH Diversity Set categorised by supplier annotated biological activity.

Nutrient limitation condition screening of the NIH Diversity Set identified a total of 113 validated hits, 40 of which were identified in carbon limitation media and 101 were identified in nitrogen limitation. In contrast, 65 of these compounds were detected as active in *M. bovis* BCG screening and 56 of these compounds were detected as active in *M. tuberculosis* H37Ra screening of the NIH Diversity Set.

Due to the intended use of the NIH Diversity Set as a cancer drug discovery tool,²⁹⁶ it was expected that a large number of the compounds detected in this library are generally cytotoxic or have anti-neoplastic activity, and this was confirmed, with over half of the total hit compounds identified as having anti-neoplastic properties.

Following the trend observed in the results of screening the other libraries, nitrogen limitation conditions were the most sensitive in detecting anti-mycobacterial activity of library compounds with 84 compounds exhibiting ≥ 4 fold increase in sensitivity in nitrogen starvation compared to carbon starvation conditions, whilst this included many cytotoxic chemicals unsuitable for drug leads.

There were 16 chemicals identified through rich condition screens that were not detected in starvation media screening, included 4 rifamycin derivatives, a prostaglandin receptor inhibitor NSC132693, tetracycline NSC69343, chemicals with anti-cancer activity NC305787, NSC11241, NSC106408 (anthramycin), NSC134727 (mitomycin C), NSC122819 (teniposide), NSC5159 (chartreusin), NSC3053 (dactinomycin). Antibiotics NSC363182 (kijanamicin derivative U-64815), NSC189794 (streptovaricin) and the anti-viral immunomodulator NSC62709 (imidazoquinoline).

Many novel mycobacterial inhibitors were discovered in the NIH Diversity Set through nutrient limitation condition screening, the most promising of which based on their validated level of activity, confirmation of activity in slow growing mycobacterial species, novelty and cytotoxicity will be discussed further in section 4.4.2.3 of this chapter. A detailed list of library screen hits including chemical structures is included in Appendix 9.5.3.

4.3.4 Overview of nutrient-limited screening goals and outcomes

There were three reasons for performing anti-mycobacterial screens in nutrient limitation media conditions. First, to explore the viability of the HTS design described in Chapter 3 when adapted to nutrient starvation media conditions – by using antibiotic compounds present in the libraries as internal controls as before in Chapter 3. Nutrient limitation whole cell *M. smegmatis* HTS was successfully validated through identification of many known tuberculosis treatment drugs and other known antibiotic agents included in the chemical libraries.

The second objective of nutrient limitation screening was to compare the library hit compound profiles between carbon and nitrogen limitation media that emulate conditions of *in vivo* pathogenesis, and also between rich and starvation media types. This was to provide insight and allow evaluation of how media conditions affect sensitivity of *M. smegmatis* to inhibitors, and how these differential culture conditions relate to *M. tuberculosis* drug discovery.

Library screening in two nutrient limitation media conditions, carbon and nitrogen, yielded significantly differing inhibition profiles of hit compounds from the chemical libraries screened. These nutrient limitation conditions screen results in turn were significantly different from the inhibition profiles of library screens performed in rich media conditions. This demonstrated that minimal culture conditions exert influence in *M. smegmatis* sensitivity

to chemical inhibitors. This influence generally renders *M. smegmatis* more sensitive to inhibitors; however, it occasionally confers decreased sensitivity to particular compounds.

The third goal was to utilise the nutrient starvation screening in order to identify novel anti-mycobacterial compounds that may not have been detected under rich media conditions or in conventional *in vitro* *M. tuberculosis* screens.

This nutrient limited screening revealed previously unpublished anti-mycobacterial compounds. Many of these identified novel inhibitors are known to be cytotoxic or exhibit properties making them undesirable as leads for human tuberculosis treatment. However, several compounds show promising indications as novel tuberculosis drug candidates and warrant further study. The compound Se-methylselenocysteine was chosen for follow up study due to its structural novelty, its confirmed level of inhibitory activity towards *M. tuberculosis* and its known administration in humans as a nutritional supplement.

Hit compounds have been essentially categorised based on known function according to information provided by the chemical library supplier (Sigma-Aldrich, Microsource, NIH), available chemical database information and the published literature. Sensitivity of detection in media types refers to the differential validation MIC values or screen hits between C- and N-conditions, as in Chapter 3, a difference in MIC greater than 2-fold between C- and N-media conditions is considered significant differential sensitivity.

4.4 Discussion

4.4.2 Biological significance of starvation media screen hit compounds

Compounds with known anti-bacterial activity or high levels of cytotoxicity such as anti-neoplastics are unsuitable as leads for human tuberculosis treatment drugs, for either novelty or safety concerns, respectively. Due to the large quantity of hit compounds identified through nutrient limitation screening not all hit compounds are able to be discussed. Therefore analysis priority has been given to compounds with novel anti-mycobacterial activity, the level of activity exhibited and coinciding activity against the slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra, low cytotoxicity and other indications of suitability towards human systemic administration. Compounds considered particularly promising as human tuberculosis therapy candidates are highlighted in **bold underline**.

4.4.2.1 Significance of LOPAC starvation media screens hit compounds

Clotrimidazole, calmidazolium, diphenyleneiodonium, dequalinium, quinolinium, methocramine and WB-64 have been previously detected in rich media screening conditions and are discussed in Chapter 3.5.2.1.

BAY 11-7085 or (E)-3-(4-t-Butylphenylsulfonyl)-2-propenenitrile is detailed in the LOPAC library as an inhibitor of NFκB phosphorylation. The PubChem Bioassay online database reports a range of differential activity in various assays including inhibition of *P. falciparum* proliferation, inhibition of Lassa and Marburg virus infection and the inhibition of various enzyme functions *in vitro*²³⁷ in addition to its first reporting as an inhibitor of *M. smegmatis* by this research.¹²² However BAY 11-7085 has not been identified as an inhibitor in any screens against slow growing mycobacterial species, indicating its inhibitory activity may be drastically reduced against pathogenic mycobacteria. Furthermore, BAY 11-7085 has demonstrated necrotic cell killing *in vitro* against human Ramos-Burkitt's lymphoma cell lines²⁹⁷ and it has been shown to reduce the formation of reaction oxygen species by over 50% in eosinophils activated by *M. bovis* BCG phagocytosis.²⁹⁸ This may be interpreted as reduced phagocyte killing of internalised mycobacteria. These factors detract from the viability of BAY-11-7085 as a lead candidate drug for tuberculosis treatment.

4-Chloromercuribenzoic acid is also reported by PubChem Bioassay as an inhibitor of *P. falciparum* proliferation, amongst a large range of other activities *in vitro*²³⁷ indicating this

molecule is a 'frequent flyer'. Frequent flyers are chemicals commonly encountered in biological activity screens due to a non-specific mechanism of action. This is most likely caused by its reactivity to thiol groups that would disrupt the function of many proteins, including documented lysis of human erythrocytes.²⁹⁹ This indicates that this compound is unsuitably toxic for human systemic administration.

1,10-phenanthroline is a chelating agent that sequesters metal ions, this may explain the wide range of activity recorded for this molecule on PubChem Bioassay.²³⁷ 1,10-phenanthroline has been characterised as a strong inhibitor of *M. tuberculosis* H37Rv *in vitro*; however, the activity did not extend to a murine *in vivo* assay for reasons unknown but suspected to possibly be insufficient drug penetration or metabolism of the drug rendering it inactive.³⁰⁰ Further research into this molecule particularly into the bio-availability and metabolism is required to determine the reason for its lack of *in vivo* efficacy and how this might be mitigated.

LY-367265 is a serotonin reuptake inhibitor and is reported to inhibit the serotonin transporter and 5-HT_{2A} receptor.²³⁷ The mechanism of anti-bacterial action is unclear and unconfirmed in slow growing mycobacterial species or in the literature, but it could possibly be due to inhibition of bacterial transporters with homology to SERT. Due to the psychological side effects of this molecule in humans, its suitability for tuberculosis therapy is low.

Trifluoperazine is a phenothiazine anti-psychotic agent used in the treatment of conditions such as schizophrenia. Phenothiazines share structural similarity with the thioxanthene anti-psychotics described in Chapter 3.5.2.1. The inhibitory activity of trifluoperazine towards mycobacteria has been confirmed in *M. tuberculosis* H37Rv *in vitro* by the Southern Research Institute, in addition to identifying it as an inhibitor of *P. falciparum*.²³⁷ Despite psychological side effects, phenothiazines have been suggested as treatments for XDR-TB.³⁰¹ These compounds are reported to accumulate within macrophages.³⁰² Thioridazine, a phenothiazine drug primarily indicated for psychiatric conditions is known to be inhibitory toward MDR and XDR strains of tuberculosis *in vivo*.³⁰³ Phenothiazines are reported to block mycobacterial drug efflux pumps,³⁰⁴ indicating that phenothiazine compounds might exhibit synergy with other tuberculosis treatment drugs. Trifluoperazine was detected only under nutrient starvation conditions, this could be evidential of phenothiazine interference

with mycobacterial transport pumps. In the last year phenothiazines have received much interest as anti-tubercular agents,³⁰⁵ and trifluoperazine has been proven effective against active and latent MDR-TB.³⁰⁶ With further modification to the structure to reduce psychological side effects, the phenothiazine chemical class may well produce novel anti-tuberculosis treatment drugs.

Pentamidine is an anti-microbial agent primarily used in the treatment of pneumocystis pneumonia caused by the fungus *Pneumocystis jirovcii*, particularly in immunocompromised HIV positive patients. It also exhibits inhibitory activity towards protozoan parasites such as *Trypanosoma brucei*, the causative agent of sleeping sickness and *Leishmania spp* the causative agents of leishmaniasis. Its mechanism of action is thought to be binding to tRNA and inhibiting translation.³⁰⁷ The activity of pentamidine against *M. tuberculosis* H37Rv has been confirmed by the Southern Research Institute²³⁷ and has also been reported to have an MIC of > 5 μ M in an H37Rv intra-macrophage assay.²²¹ Due to its primary use as a treatment of pneumonia, it is often delivered directly to the lungs in an aerosol form, which is relevant to tuberculosis treatment. Pentamidine exhibits an unfavourable side effect profile but its known use in treatment of pneumocystis may indicate it as a viable option in the treatment of MDR-TB.

Ruthenium red is a metal based dye used in histological staining and microscopy.³⁰⁸ Due to its low level of anti-mycobacterial activity and lack of evidence for activity in slow growing mycobacterial species, it is regarded as a poor candidate lead for tuberculosis drug development.

The vitamin E derivative U-83836 exhibited low levels of activity in *M. smegmatis* cultured in both nitrogen and carbon starvation conditions, and there was no evidence available of activity towards slow growing mycobacterial species. Being a vitamin E derivative and free radical scavenger exerting protective effects against external ROS stress,³⁰⁹ it presents an interesting and unique pharmacophore for TB drug development but is of low priority due to lack of compelling evidence of activity in *M. tuberculosis*.

Se-methylselenocysteine (SMSC) is a natural product found in high concentrations in *Allium* and *Brassica* edible plant species. It is a methyl-selenium substituted analogue of the sulphurous amino acid cysteine.³¹⁰

SMSC is sold over the counter as a nutritional supplement as a bio-available form of selenium to supplement a deficient diet and is anecdotally believed to have positive health effects in a range of conditions including cancer, HIV and diabetes.³¹¹ SMSC has been researched as a ‘chemopreventative’ agent that reduces the incidence of cancer.³¹²

The mechanism for SMSC’s chemopreventative action is through the induction of apoptosis in cancerous cells.³¹³ The anti-tuberculosis activity of seleno-amino acids has been confirmed by the Southern Research Institute²³⁷ but was first reported in a publication from our laboratory.¹²² The novelty, known human consumption and passive cancer prevention properties are key factors that make SMSC and its family of seleno-amino acids (Se-AAs) excellent leads for tuberculosis drug discovery to explore. SMSC and Se-AAs are further analysed for mechanism of action and structure activity relationship in Chapters 5 and 6 of this research.

4.4.2.2 Significance of Spectrum Collection starvation media screen hit compounds

Pyrithione zinc was detected in 7H9 rich medium screening with similar inhibitory concentrations to HdeB media types and is discussed in Chapter 3.5.2.2. The imidazole anti-fungals clotrimidazole and econazole were also detected with similar activity in LB rich media and were discussed earlier in Chapter 3.5.2.2.

The anti-psychotic drugs prochlorperazine and trifluoperazine have previously been discussed in section 4.4.2.1 detailing phenothiazine drugs. **Nortriptyline** is a tricyclic antidepressant with some structural and mechanistic similarities to phenothiazines. It has been reported to demonstrate inhibitory activity towards *M. tuberculosis* H37Rv in *in vitro* screens.²³⁷ Little data exists concerning tricyclic antidepressants as anti-tubercular agents. Rifampicin and nortriptyline interact *in vivo* through common cytochrome P450 metabolism pathway,³¹⁴ and there have been earlier reports of nortriptyline and other tricyclics inhibiting *Campylobacter pylori*.^{315, 316} Given the common receptor affinities of tricyclics and phenothiazines and both having exclusive activity in nutrient starvation media it is assumed they share a common mechanism, possibly interfering with bacterial transport systems that exhibit homology to human neurotransmitter receptors such as SERT. Considering the mild psychological effects of tricyclics compared to phenothiazines, tricyclic anti-depressants may represent a better scaffold to explore for anti-mycobacterial activity.

Chloroacetoxyquinoline (silital) is a quinoline derivative that exhibited strong growth inhibition in nitrogen limited medium with a 780 nM MIC. It was also detected in LB rich medium but at a much higher MIC of 25 μ M demonstrating a drastic shift in activity based on media conditions. Silital has also been non-quantitatively identified as inhibitory in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Silital is reported to be protective from polyglutamine Huntington toxicity *in vitro* and also inhibits the bacterial siderophore synthesising enzyme BasE.²³⁷ The pronounced difference in sensitivity of this compound between rich and nutrient media types gives credibility to the hypothesis that siderophore synthesis may be targeted by this compound, as iron supply is limited in HdeB nutrient starvation conditions, placing a considerably higher mycobacterial siderophore demand for mycobacterial growth.³¹⁷

Chloroacetoxyquinoline and other quinoline derivatives have previously been characterised as bacteriostatic against Gram positive and Gram negative bacterial and also have anti-fungal activity.³¹⁸ Although the anti-mycobacterial activity of silital is promising, there is little further information on this compound, particularly *in vivo*, this makes silital a lower priority as an anti-tubercular drug lead until further research is conducted.

Another quinoline derivative, primaquine, was validated only under nitrogen starvation conditions and is flagged on PubChem Bioassay as active against *M. tuberculosis* H37Rv. It has also been previously disclosed as having an MIC of 5 μ M against intra-macrophage *M. tuberculosis* H37Rv.²²¹ Orally administered primaquine is routinely used in the treatment and prophylaxis of malaria and also in the treatment of *Pneumocystis* pneumonia. This route of administration and use are favourable indications of primaquines *in vivo* efficacy and tolerance. The action of primaquine towards not only mycobacteria but also malaria and other diseases make it a promising drug, and further development has been encouraged.³¹⁹

The quaternary ammonium compounds benzethonium, methylbenzethonium, benzalkonium, cetrimonium and cetylpyridinium are used as surface disinfectants and topical anti-septic agents in products such as medical wipes and mouthwashes. Compounds such as benzethonium have been confirmed to have anti-mycobacterial activity in *M. tuberculosis* H37Rv with an IC₅₀ of 26 μ M, however, a subsequent counter screen for human cell cytotoxicity had a high IC₅₀ of 40 μ M,²³⁷ this is an unacceptable selectivity. This is not surprising given the non-specific mechanism of action of quaternary ammonium compounds, disrupting plasma membranes and enzyme interactions.³²⁰ Due to toxicity concerns, these

compounds best remain as externally applied anti-infectives and therefore are of little use in tuberculosis treatment.

Similar in mechanism to the anti-septics described above, chlorhexidine and alexidine are 'local anti-infectives' with bactericidal action through disruption of membranes.³²¹

Chlorhexidine is reported to be an inhibitor of *M. tuberculosis* H37Rv on the PubChem Bioassay database,²³⁷ but the toxicity of these disinfectants at high concentrations or through systemic administration makes them undesirable drug development leads.

The organic mercury compounds thimerosal and phenylmercuric acetate have well documented anti-septic and anti-fungal properties and applications, mainly as preservatives for example in vaccines. Thimerosal is recorded on PubChem Bioassay as having an IC₅₀ of 340 nM against *M. tuberculosis* H37Rv which corresponds to the MIC of 780 nM found in this study in carbon starvation media. The corresponding human VERO cell toxicity IC₅₀ for thimerosal was 3559 nM meaning thimerosal demonstrates tenfold selectivity in action toward mycobacteria.²³⁷ These compounds, due to their mercury containing nature are intrinsically toxic, being metabolised to form damaging alkylated mercury compounds that has caused even their topical use to be questioned for safety concerns.³²² Due to these safety concerns, organomercury compounds are regarded as too toxic to be considered strong tuberculosis drug leads.

Cefdinir is a popularly used 3rd generation cephalosporin antibiotic with a bactericidal mechanism of action through disruption of peptidoglycan synthesis. Although β -lactam antibiotics are generally regarded as ineffective against mycobacteria due to their fatty acid-fast cell wall and highly effective β -lactamase enzymes,³²³ recent research has suggested that certain β -lactam drugs, particularly in combination with β -lactamase inhibitors, can be effective against *M. tuberculosis in vitro*.^{324, 325} Cefdinir exhibited a low level of activity against *M. smegmatis* in nitrogen limited media with an MIC of 25 μ M and was also detected as inhibitory in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Considering that cefdinir is already marketed and consumed as an antibiotic its safety profile is considered to be extremely good, and that it was actively inhibiting mycobacteria *in vitro* in the absence of a coupled β -lactamase inhibitor indicates that cefdinir in conjunction with a β -lactamase inhibitor and other anti-tubercular drugs presents a viable option in the treatment of problematic drug resistant TB.

Thiram is an anti-fungal agent that targets glutathione reductase and was also identified in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Thiram is mutagenic and therefore is unsuitable for human administration,³²⁶ however, compounds of the same dithiocarbamate scaffold such as disulfiram (which is less toxic than thiram) have been explored in the treatment of tuberculosis.³²⁷ Other substituted dithiocarbamates have been demonstrated to inhibit tubercular carbonic anhydrases,³²⁸ indicating that despite thiram being unsuitable for tuberculosis therapy the dithiocarbamate family is a promising novel class of anti-mycobacterial agents.

Khivorin is a natural product isolated from the West African timber *Khaya ivorensis* that exhibited inhibitory activity against *M. smegmatis* in nitrogen limited medium. No evidence of an inhibitory action towards *M. tuberculosis* could be found. Substituted khivorin compounds have demonstrated low levels of anti-fungal and anti-bacterial activity.³²⁹

Similarly irigenin, a flavonoid isolated from the rhizome of various *Iris* species showed activity in nitrogen limited media but no compounding evidence of inhibition towards *M. tuberculosis* could be found, the closest result being a recent publication describing a broad spectrum of anti-microbial activity for a crude methanol extract containing irigenin.³³⁰

Berbamine is a Ca^{2+} channel blocker isolated from *Berberis* species and was identified only in nitrogen limited medium. No evidence of inhibition towards slow growing mycobacterial species was presented although a related compound berberine has been reported to have an MIC of 31.2 $\mu\text{g/mL}$ against *M. tuberculosis*³³¹ and a 25 $\mu\text{g/mL}$ MIC against *M. smegmatis* cultured in 7H9 broth.³³² Berbamine is reported to have strong anti-plasmodial activity but is also toxic toward human cell lines *in vitro*.²³⁷ Hydrastine, derived from the goldenseal plant *Hydrastis canadensis*, again exhibited inhibition against *M. smegmatis* only in nitrogen limitation medium. No information concerning its inhibitory activity towards *M. tuberculosis* could be found, and β -hydrastine is reported to be inactive against *M. smegmatis* in 7H9 medium.³³²

Due to the lack of confirming anti-tubercular activity and toxicity data the natural products khivorin, irigenin, berbamine and hydrastine remain low priorities for anti-tubercular drug development.

A common over the counter decongestant, phenylephrine, was found to inhibit *M. smegmatis* in both carbon and nitrogen limited media to an MIC of 3.125 μM . However subsequent testing of commercially available (R)-(-)-phenylephrine hydrochloride (Sigma-Aldrich) was found to have no activity against *M. smegmatis* in any media type *in vitro*. Some literature

evidence suggests that phenylephrine may have anti-bacterial properties;³³³ however, this is far from compelling. The positive inhibition result is reported with scepticism, possibly arising from contamination of the chemical library well. Regardless, phenylephrine is a potent α -adrenergic receptor agonist, an activity that contraindicates use as an anti-TB agent in humans.³³⁴

Gentisic acid (2-5 dihydroxybenzoic acid) exhibited inhibitory activity against *M. smegmatis* in nitrogen limiting medium, and this activity was also identified in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Gentisic acid is an anti-inflammatory / analgesic compound and a by-product of aspirin metabolism. Interestingly, PubChem Bioassay reports aspirin as active in reducing the spleen concentration of *M. tuberculosis* in infected mice, and the database also notes that gentisic acid and structurally similar compounds such as salicylic acid are inhibitors of *M. tuberculosis* H37Rv fructose-bisphosphate aldolase *in vitro*.²³⁷ Gentisic acid has also been previously identified as a possible anti-bacterial agent in an *in silico* screen.³³⁵ Little information directly supporting the anti-mycobacterial properties of gentisic acid is available, but considering its favourably low oral toxicity (murine LD₅₀ = 4500 mg/kg³³⁶) it deserves further attention to determine its value as a tuberculosis drug lead.

Chlorothalonil (tetrachloroisophthalonitrile) is a broad-spectrum anti-microbial, particularly used as an anti-fungal in agriculture. It reduces glutathione which disrupts cellular redox balance and leads to cell death.³³⁷ Chlorothalonil presumably acts through a similar mechanism in mycobacteria, reducing mycothiones. Chlorothalonil exhibited strong inhibitory activity towards *M. smegmatis* in both carbon and nitrogen limitation media and was also flagged as inhibitory in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Although it is reported to be remarkably non-toxic in murine models particularly when orally administered, with LD₅₀ values over 5000 mg/kg,³³⁸ research has suggested it has genotoxicity, carcinogenicity and developmental toxicity³³⁹ in murine models, but not humans.³⁴⁰ No information regarding the anti-mycobacterial activity of chlorothalonil could be found in the literature; therefore, it is believed that the present research is the first disclosure of chlorothalonil as a potential anti-tubercular agent. Considering the established safety profile of chlorothalonil and its inhibitory action towards *M. smegmatis*, chlorothalonil could potentially be developed into an anti-mycobacterial drug with a novel mechanism of action. Further research into the activity against *M. tuberculosis* H37Rv *in vitro* and

subsequently *in vivo* are required to determine the effectiveness of this chemical against pathogenic mycobacteria before consideration for further development.

2-aminoethyldiphenyl boronate inhibits eukaryotic IP₃ receptors modulating the release of intracellular Ca²⁺. It way also blocks transient receptor potential channels, and is mainly used as a research tool.³⁴¹ This chemical has been identified as an inhibitor of *Bacillus subtilis* Sfp phosphopantetheinyl transferase *in vitro*, the equivilent enzyme in mycobacteria Rv2794c PptT serves vital roles such as the synthesis of the siderophore mycobactin and cell wall mycolic acids.³⁴² Phosphopantetheinyl transferase inhibition is a possible mechanism of its anti-mycobacterial action; however, this compound was not detected in screens against slow growing mycobacteria, and its effects on cellular Ca²⁺ release likely indicate severe side effects if administered *in vivo*.

4.4.2.3 Significance of NIH Diversity Set starvation media screen hit compounds

Screening of the NIH Diversity Set library under nutrient starvation conditions identified several compounds of interest that had previously been identified. These included the phenothiazine compounds NSC17474 trifluoperazine and NSC59349 3-nortropanol. Phenothiazines were previously identified in nutrient limited screening of the LOPAC and Spectrum Collection and are discussed above in section 4.4.2.1. NSC12155 aminoquinuride, NSC661755 michellamine B, NSC368675 azadirachtin, NSC22225 cadmium chlorendic acid and NSC31048 neohesperidin were identified in rich media condition screening of the NIH Diversity Set and are discussed in Chapter 3.5.2.3.

NSC317629 **ketoconazole** is an azole anti-fungal agent that interferes with ergosterol synthesis similar to the previously mentioned econazole and clotrimidazole. However, ketoconazole is highly lipophilic and in addition to topical administration is also able to be administered orally for systemic treatment.³⁴³ This lipophilicity and systemic administration makes ketoconazole a much stronger candidate for tuberculosis therapy than other topically applied anti-fungals. Ketoconazole was detected in nitrogen limited screening of *M. smegmatis* and validated to have an MIC of 6.25 µM and is reported to have an MIC of 7.6 µM against *M. tuberculosis* H37Rv.^{237, 344} Ketoconazole has demonstrated improved treatment of murine tuberculosis *in vivo* when combined with front line tuberculosis drugs.³⁴⁵ Our research supports the idea that ketoconazole has strong potential to be a tuberculosis treatment adjunct, and further investigation and development is warranted.

NSC16865 **bismuth-dimercaprol** (BisBAL) exhibited activity against *M. smegmatis* in nitrogen limited medium and was also detected as active in screening of the NIH Diversity Set against *M. bovis* BCG. Bismuth thiols have been previously reported as anti-bacterial.³⁴⁶ Dimercaprol and related thiols have previously been characterised as anti-tubercular *in vivo*³⁴⁷ but were abandoned shortly after and deemed clinically unsuitable due to rapid development of resistance and poor activity against established murine infections.³⁴⁸ BisBAL is thought to derive its anti-bacterial action by interfering with capsule and biofilm formation. At sub-inhibitory concentrations BisBAL renders bacteria more susceptible to phagocytosis due to exposure of subcapsular antigens.³⁴⁹ BisBAL may not be an effective tuberculosis therapy alone, but its effects in conjunction with conventional tuberculosis treatment drugs warrant further investigation, as it may synergistically increase the activity of tuberculosis drugs and the immune response.

NSC13316 is a quinoline-based compound with an MIC of 6.25 μ M in *M. smegmatis* nitrogen starvation medium and has been confirmed as having an IC₅₀ of 4.5 μ M in *M. tuberculosis* H37Rv and also nanomolar level potency in inhibition of *P. falciparum* growth. However, it also has reported activity against cancer cell lines and is therefore deemed too cytotoxic to be prioritised for development.²³⁷

NSC17770 is a quinoline-piperidine compound validated in nitrogen conditions with an *M. smegmatis* MIC of 6.25 μ M and was also detected as active in parallel screens of *M. bovis* BCG and *M. tuberculosis* H37Ra. No bioactivity is reported on the PubChem database or in the literature. NSC13726 is a diquinoline compound with structural resemblance to aminoquinuride. It was validated as having an MIC of 12.5 μ M against *M. smegmatis* in nitrogen starvation medium and was also detected in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. It is noted as an inhibitor of hERG (human Ether-a-go-go Related Gene) ion channels, cytochrome P450-2C9 and glucocerebrosidase-p2.²³⁷ NSC17770 warrants further investigation to confirm their anti-tuberculosis properties, although inhibition of the 'anti-target' hERG by NSC13726 is considered undesirable in drug development as may cause fatal cardiac side effects.³⁵⁰

NSC321206 was detected in nitrogen starvation *M. smegmatis* and also characterised in *M. tuberculosis* H37Ra as having an MIC of 3.125 μ M. NSC321206 is reported to be a potent inhibitor of the human proteasome and also has anti-neoplastic properties that make it

undesirable for a tuberculosis treatment drug.³⁵¹

Another proteosome inhibitor NSC109268 was also detected in nitrogen starvation medium with an MIC of 12.5 μ M in *M. smegmatis* and, while not neo-plastic itself, has indications as an adjunct for improving cancer therapy efficacy.³⁵² If well tolerated *in vivo* and confirmed as inhibitory towards pathogenic tuberculosis, NSC109268 may be a candidate anti-tubercular pharmacophore.

NSC632536 was identified under nitrogen limitation screening in this work and also in screening of *M. bovis* BCG. Little information is available on this compound, with PubChem Bioassay activity results reported to be inhibition of *P. falciparum* and *Trypanosoma cruzi* and *B. subtilis* Sfp phosphopantetheinyl transferase.²³⁷

NSC292596 was identified under carbon and nitrogen limited conditions in *M. smegmatis*. It is an inhibitor of H2AX histone phosphorylation,³⁵³ but lack of confirmation of activity in slow growing mycobacterial restrict the priority classification of this as a drug development lead.

NSC72939 a manganese complex detected in nitrogen limited *M. smegmatis* with an MIC of 12.5 μ M and also in screens as having activity against *M. bovis* BCG and *M. tuberculosis* H37Ra. NSC72938 is a similar complex to NSC72939 but with manganese substituted for copper. It exhibited an MIC of 25 μ M against *M. smegmatis* and was not detected in screens against slow growing mycobacterial species. The complex comprises of nicotine, salicylic acid (which may account for its anti-mycobacterial properties) and manganese/copper. NSC72938 is reported as blocking hERG K⁺ channels, the only bioactivity reported for these complexes on the PubChem database or in the literature,²³⁷ therefore, the activity of these complexes requires further confirmation before consideration as potential anti-tubercular development leads.

NSC181486 trigilletine was validated in nitrogen starvation medium and also identified in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. It is a natural product isolated from West African plant species such as *Triclisia* and *Momordica*. It has been found to have strong anti-protozoal activity but was also toxic to human cell lines, giving a poor selectivity index and making it unsuitable for human therapy.³⁵⁴

NSC23128 is a complex organic molecule that was verified to have low activity with an MIC

of 25 μM against *M. smegmatis* cultured in nitrogen starvation conditions, but no other indication of anti-mycobacterial activity could be found. It is reported on PubChem Bioassay as inhibitory toward hERG channels, Tau filament binding, Smad transcription factor and the EP2 prostaglandin receptor.²³⁷ NSC125910 shares anthraquinone structural similarity with NSC23128. It was also verified only against *M. smegmatis* nitrogen limitation at 25 μM and is listed as a Smad transcription factor, EP2 prostaglandin receptor and HSD17B4 hydroxysteroid dehydrogenase inhibitor on PubChem Bioassay.²³⁷

NSC130813 is a substituted acridine compound identified as having an MIC of 6.25 μM in *M. smegmatis* under nitrogen limiting conditions. Although there are no further indications for activity in slow growing mycobacterial species NSC130813 may be desirable for development due to its anti-viral activity. It blocks viral DNA synthesis³⁵⁵ and has a strong binding affinity to HIV-1 RNA, indicating possible dual inhibitory application.²³⁷ Further confirmation of anti-tuberculosis activity and cytotoxicity is required.

NSC356217 HEJ-4 is a harmine derivative that exhibited an MIC of 6.25 μM against *M. smegmatis* cultured under nitrogen starvation. No other indications of anti-mycobacterial or other activity could be found for this molecule. Based on its parent harmine, it might exhibit undesirable side-effects *in vivo* due to monoamine oxidase inhibiting activity.

NSC35611 **solanine** is a glycoalkaloid isolated from plants of the Solanaceae family such as the potato. Solanine was detected in nitrogen limitation culture screening of *M. smegmatis* with an MIC of 6.25 μM and was also identified as active in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Solanine has demonstrated anti-fungal activity³⁵⁶ and low levels of anti-bacterial activity towards *E. coli* and *S. aureus*.³⁵⁷ Although generally regarded as a toxin, orally administered solanine is tolerated in murine models at concentrations up to 1 g/kg although gastrointestinal absorption may be limiting.³⁵⁸ Solanine and related glycoalkaloids warrant further investigation into their anti-mycobacterial properties.

NSC56346 rottlerin is a natural product derived from the plant *Mallotus philippensis*. Identified in nitrogen limited medium screening of *M. smegmatis* with an MIC of 6.25 μM , rottlerin has been identified *in silico* as an inhibitor of *M. tuberculosis* 14 α -lanosterol demethylase³⁵⁹ and rottlerin and other isolates from *M. philippensis* have been characterised as anti-tubercular.³⁶⁰ Rottlerin has a published murine oral LD_{Lo} of 750 mg/kg³⁶¹ and is a

known K⁺ channel opening agent that affects the cardiovascular system. Further studies into the cytotoxicity and *in vivo* efficacy of rottlerin and its derivatives are required to determine its potential as a tuberculosis drug lead.

NSC34936 suramin is a well known anti-protozoal drug implemented in the treatment of *Trypanosoma spp*-induced sleeping sickness. It was validated in nitrogen starvation culture as having an MIC of 6.25 μ M against *M. smegmatis* and also non-quantitatively picked up as active in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. Suramin is also known as an RNA reverse transcriptase inhibitor, a property that confers inhibition of HIV.³⁶² The effects of suramin on *M. tuberculosis* have been tested, and it has been reported as ineffectual *in vitro* and conversely caused increased mycobacterial growth *in vivo*.³⁶³ This conflict of results may be attributed to culture conditions, strain or technique. Revisitation of the anti-mycobacterial activity of suramin may be worth pursuing.

NSC319518, the diterpene glycoside cotylenin A, is a regulator of plant growth that can cause differentiation of mammalian cells and apoptosis of neoplastic cells.³⁶⁴ In the present research it was validated to have an MIC of 6.25 μ M against *M. smegmatis* in nitrogen limited medium screening, and was also identified as inhibitory in screens against *M. bovis* BCG and *M. tuberculosis* H37Ra. No further evidence for anti-microbial action of cotylenin A could be found in the literature to confirm this result.

4.4.3 The influence of differential starvation culture conditions on screen hits

Performing screens for anti-mycobacterial chemicals in nutrient starvation conditions using HdeB carbon and HdeB nitrogen limitation media revealed a large difference in the sensitivity of *M. smegmatis* to inhibitory library compounds between carbon and nitrogen limitation media conditions.

From the validated hits of screening of all three chemical libraries, 104 were validated in carbon limitation medium, 206 hits were validated in nitrogen limitation conditions and 100 of these hits were validated in both starvation media types. Comparing the hits derived from nutrient limitation screens with the results of *M. tuberculosis* H37Ra screening in 7H9 medium^{206, 220} showed that 60 hits were common to carbon starvation medium and *M. tuberculosis*, and 95 hits are common to nitrogen starvation medium and *M. tuberculosis*. A total of 60 hits were common to both carbon and nitrogen starvation conditions and were also active in *M. tuberculosis* H37Ra.

Figure 4.10: Venn diagram comparing starvation media screening validated hits.

Results of screens in HdeB C- and N- starvation media types compared with hits detected in *M. tuberculosis* H37Ra screens.^{206, 220} Venn diagram group totals provided are inclusive.

Performing *M. smegmatis* chemical library screens in nitrogen starvation media produced almost double the number of validated hit compounds compared to screens in carbon starvation media. A greater number of hits derived from *M. smegmatis* screens in nitrogen limited conditions were also active against *M. tuberculosis* H37Ra compared to carbon starvation screens of *M. smegmatis*.

All hits active in *M. tuberculosis* and validated in *M. smegmatis* carbon starvation conditions were also validated in nitrogen starvation conditions. Therefore for nutrient limitation condition screening in *M. smegmatis*, nitrogen starvation exhibited the greatest sensitivity in detecting inhibitors and was most relevant for detected inhibitors active toward *M. tuberculosis*.

4.4.3.1 Comparison of validated hits from LOPAC differential media screens

LOPAC Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
BAY 11-7085	-	-	6.25	12.5	≤ 20	-
Calcimycin	3.125	-	12.5	12.5	-	≤ 20
Carboplatin	-	-	25	50	-	-
4-Chloromercuribenzoic acid	-	-	6.25	12.5	-	≤ 20
Cisplatin	-	-	12.5	12.5	-	-
Calmidazolium	12.5	-	50	12.5	-	-
Clotrimazole	12.5	-	50	50	-	-
Demeclocycline	3.125	3.125	6.25	6.25	3.125	1.56
Dequalinium	-	3.125	-	-	3.125	6.25
Dequalinium C-14 linker. (Quinolinium)	6.25	3.125	6.25	3.125	-	≤ 20
Diphenyleneiodonium	6.25	3.125	3.125	3.125	0.195	0.39
Doxycycline	1.56	1.56	3.125	3.125	≤ 20	-
Ellipticine	-	25	-	-	12.5	12.5
Idarubicin	12.5	3.125	25	12.5	6.25	6.25
Lomefloxacin	12.5	6.25	6.25	6.25	1.56	3.125
LY-367265	-	-	25	50	-	-
Methoctramine	12.5	12.5	12.5	12.5	1.56	6.25
Minocycline	12.5	12.5	12.5	12.5	1.56	3.125
Mitoxantrone	-	-	50	50	-	-
Niclosamide	12.5	25	6.25	6.25	6.25	6.25
Ofloxacin	3.125	3.125	6.25	6.25	1.56	1.56
Pentamidine	-	-	25	50	-	-
1,10-Phenanthroline	-	-	12.5	12.5	≤ 20	≤ 20
Se-Methylselenocysteine	-	*	12.5	12.5	12.5	12.5
Ruthenium red	-	-	50	50	-	-
Trifluoperazine	-	-	50	50	-	-
U-83836	-	-	50	50	-	-
Vancomycin	12.5	-	25	25	≤ 20	-
WB 64	-	12.5	50	25	12.5	12.5

Table 4.4: Comparison of validated hits from LOPAC differential media screening.

Validated *M. smegmatis* MIC values of LOPAC HTS hit compounds derived in both rich and starvation media types are presented for comparison. These values are contrasted with screening results of LOPAC against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra in 7H9 medium.^{206, 220} *SMSC was later detected and validated in 7H9 medium. Values of “-” indicate no inhibitory activity was detected in screening (triplicate mean of > 80% growth inhibition compared to positive controls). Values of “≤ 20” indicate the compound did exhibit inhibitory activity in screens (triplicate mean of > 80% growth inhibition compared to positive controls) against slow growing mycobacterial species, however confirmatory MIC assays were unable to be performed, therefore the MIC is estimated to be less than or equal to the screen compound concentration of 20 μM.

4.4.3.2 Comparison of validated hits from Spectrum Collection differential media screens

Spectrum Collection Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
Khivorin	-	-	-	12.5	-	-
Merbromin	-	-	6.25	6.25	-	-
Chloroacetoxyquinoline (Silital)	25	-	-	0.78	≤ 20	≤ 20
Aklavine	12.5	-	-	6.25	≤ 20	≤ 20
Irigenin dibenzyl ether	-	-	-	12.5	-	-
Celastral (Tripterine)	25	-	-	3.125	-	-
Amikacin	-	-	-	6.25	-	-
Benzethonium	-	-	-	6.25	-	-
Capreomycin	-	-	-	6.25	≤ 20	-
Cetylpyridinium	-	-	-	6.25	-	-
Chlorhexidine	12.5	-	6.25	3.125	≤ 20	≤ 20
Chlortetracycline	1.56	1.56	3.125	0.78	-	-
Colistimethate	-	-	-	12.5	-	-
Demeclocycline	1.56	1.56	0.78	0.78	≤ 20	≤ 20
Dihydrostreptomycin	12.5	6.25	12.5	3.125	-	-
Pyrrithione Zinc	-	1.56	1.56	0.39	≤ 20	≤ 20
Clotrimazole	12.5	-	25	12.5	≤ 20	-
Doxycycline	1.56	1.56	0.78	0.78	≤ 20	≤ 20
Ethambutol	3.125	3.125	3.125	3.125	≤ 20	≤ 20
Gentian / Crystal Violet	-	-	3.125	3.125	-	-
Mercaptopurine	-	-	-	25	≤ 20	≤ 20
Minocycline	1.56	0.78	1.56	0.78	≤ 20	≤ 20
Nortriptyline	-	-	-	12.5	-	-
Oxyquinoline	-	12.5	-	-	≤ 20	≤ 20
Oxytetracycline	6.25	3.125	-	1.56	-	≤ 20
Polymyxin B	-	-	-	12.5	-	-
Primaquine	-	-	-	25	-	-
Tetracycline	1.56	3.125	3.125	1.56	≤ 20	-
Streptomycin	3.125	3.125	3.125	6.25	-	-
Tobramycin	6.25	6.25	3.125	1.56	-	-
Thimerosal	3.125	1.56	0.78	3.125	≤ 20	≤ 20
Phenylephrine	-	-	3.125	3.125	-	-
Thiothixene (Tiotixene)	-	25	-	-	-	-
Sulfamethoxazole	-	25	-	-	-	-
Hydrastine	-	-	-	6.25	-	-
Patulin	-	-	-	25	≤ 20	-
Phenylmercuric Acetate	-	3.125	1.56	0.39	≤ 20	≤ 20
Dichlorodiphenyltrichloroethane, DDT	-	-	-	25	-	-
Gemifloxacin	3.125	3.125	3.125	6.25	≤ 20	≤ 20
Prochlorperazine edisylate	-	-	-	6.25	-	-
Clioquinol	12.5	-	-	-	-	-
Thiostrepton	12.5	12.5	-	12.5	≤ 20	≤ 20
2-Aminoethyldiphenyl boronate, 2-aminoethyl diphenylborinate.	-	-	-	6.25	-	-
Gatifloxacin	1.56	1.56	1.56	1.56	≤ 20	≤ 20
Cetrimonium	-	-	-	6.25	-	-

Spectrum Collection Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
Methylbenzethonium Chloride	1.56	6.25	6.25	6.25	≤ 20	-
Roxithromycin	6.25	6.25	-	-	≤ 20	-
Cefdinir	-	-	-	25	≤ 20	≤ 20
Levofloxacin	3.125	3.125	3.125	3.125	≤ 20	≤ 20
Apramycin	-	-	25	6.25	-	-
Telithromycin	6.25	25	-	-	≤ 20	-
Erythromycin	-	-	-	6.25	≤ 20	-
Gentisic acid, dihydroxybenzoic acid	-	-	-	25	≤ 20	≤ 20
Trifluoperazine HCl	-	-	-	12.5	≤ 20	≤ 20
Econazole	-	12.5	12.5	12.5	≤ 20	≤ 20
Lomefloxacin	12.5	12.5	6.25	12.5	≤ 20	≤ 20
Ofloxacin	3.125	3.125	3.125	3.125	≤ 20	≤ 20
Carboplatin	-	-	25	6.25	-	-
Cisplatin	-	12.5	3.125	0.78	-	-
Alexidine	-	6.25	6.25	6.25	≤ 20	≤ 20
Pararosaniline pamoate	-	-	-	6.25	≤ 20	-
Mitoxantrone	-	-	50	50	-	-
Thiram	-	-	-	12.5	≤ 20	≤ 20
Benzalkonium Chloride	-	-	6.25	6.25	-	-
Dirithromycin	12.5	6.25	-	-	-	≤ 20
Clarithromycin	-	6.25	6.25	-	≤ 20	-
Moxifloxacin	1.56	1.56	1.56	6.25	≤ 20	≤ 20
Berbamine	-	-	-	12.5	-	-
Rifampin	6.25	6.25	6.25	6.25	≤ 20	≤ 20
Acivicin	-	-	-	25	≤ 20	≤ 20
Tetrachloroisophthalonitrile / Chloroethalonil	-	-	6.25	3.125	≤ 20	≤ 20
Ciprofloxacin	12.5	12.5	-	12.5	-	≤ 20
Teniposide	25	25	-	25	-	-
Rifaximin	6.25	6.25	-	6.25	≤ 20	≤ 20
Sarafloxacin	6.25	6.25	6.25	6.25	≤ 20	≤ 20
Pefloxacin	12.5	12.5	-	12.5	≤ 20	≤ 20

Table 4.5: Comparison of hits from Spectrum Collection differential media screening

Validated *M. smegmatis* MIC values of Spectrum Collection HTS hit compounds derived in both rich and starvation media types are presented for comparison. These values are contrasted with screening results of LOPAC against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Details as for Table 4.4.

4.4.3.3 Comparison of validated hits from NIH Diversity Set differential media screens

NIH Library Compound	<i>M. smegmatis</i> MIC (μ M) LB	<i>M. smegmatis</i> MIC (μ M) 7H9	<i>M. smegmatis</i> MIC (μ M) C-	<i>M. smegmatis</i> MIC (μ M) N-	<i>M. bovis</i> BCG MIC (μ M)	<i>M. tuberculosis</i> H37Ra MIC (μ M)
NSC2347 (Tricarballic acid)	-	-	-	25	-	-
NSC27895	-	-	-	25	-	-
NSC1684	-	-	-	25	-	-
NSC23082	-	-	-	6.25	-	-
NSC3907 (8-Hydroxyquinoline salicylate)	-	25	-	6.25	12.5	3.125
NSC329226	6.25	6.25	6.25	3.125	≤ 20	≤ 20
NSC359098	-	-	-	6.25	-	-
NSC26081	-	-	-	6.25	-	-
NSC16865	-	-	-	6.25	≤ 20	-
NSC130830	-	-	-	12.5	-	≤ 20
NSC17600	-	-	-	6.25	-	-
NSC122657	-	-	-	12.5	≤ 20	-
NSC152686	-	-	-	12.5	-	-
NSC153533	-	-	-	25	-	-
NSC289336 (Rimiterol)	-	-	-	25	-	-
NSC13316	-	-	-	6.25	≤ 20	-
NSC333003	-	-	-	3.125	≤ 20	≤ 20
NSC625324 (Silver sulfadiazine)	-	12.5	6.25	0.39	-	-
NSC24113	-	-	-	6.25	≤ 20	-
NSC45382	-	-	-	3.125	-	-
NSC22907	-	-	-	25	-	-
NSC48300	-	-	-	6.25	≤ 20	≤ 20
NSC295642	-	-	-	6.25	≤ 20	≤ 20
NSC12155 (Aminoquinuride, surfen)	-	12.5	12.5	6.25	≤ 20	-
NSC632536	-	-	-	12.5	≤ 20	-
NSC3058301 (Benzenecarboximidamide)	-	-	-	6.25	-	-
NSC13480	-	12.5	6.25	3.125	12.5	12.5
NSC17474 (Trifluoperazine)	-	-	-	6.25	≤ 20	-
NSC294526	-	-	6.25	3.125	-	-
NSC132693	-	25	-	-	-	-
NSC321206	-	25	-	6.25	25	3.125
NSC311153	-	25	-	6.25	6.25	6.25
NSC311152 (Datelliptium)	-	12.5	12.5	6.25	≤ 20	≤ 20
NSC176327	-	12.5	12.5	3.125	≤ 20	≤ 20
NSC92585	-	-	25	0.78	≤ 20	≤ 20
NSC354961	-	-	25	6.25	≤ 20	-
NSC359449	-	12.5	12.5	1.56	≤ 20	≤ 20
NSC97345	-	-	3.125	0.19	-	-
NSC268879	3.125	3.125	3.125	0.19	1.56	3.125
NSC117274	-	-	-	12.5	-	-
NSC305787	-	-	-	6.25	≤ 20	≤ 20
NSC150289	-	-	-	6.25	-	-
NSC114792	-	-	-	6.25	-	-
NSC292596	-	-	12.5	12.5	-	-
NSC661755 (Michellamine B)	25	-	12.5	3.125	≤ 20	≤ 20

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC25485(Ouabain)	-	-	-	12.5	-	-
NSC72939	-	-	-	12.5	≤ 20	≤ 20
NSC133100 (Rifamycin SV)	25	-	-	-	-	-
NSC305787	-	25	-	-	25	6.25
NSC11241 (C.I. Basic red 6)	-	25	-	-	-	-
NSC321237	-	25	3.125	0.39	-	-
NSC17770	-	-	-	6.25	≤ 20	≤ 20
NSC5159 (Chartreusin)	3.125	1.56	-	-	1.56	1.56
NSC310551	-	6.25	6.25	3.125	12.5	6.25
NSC295558	-	-	6.25	1.56	-	-
NSC20410	-	-	12.5	3.125	-	-
NSC182395	-	-	12.5	1.56	-	-
NSC22225 (Cadmium chlorendic acid)	-	1.56	12.5	12.5	≤ 20	≤ 20
NSC145611 (Rifamycin derivative)	25	-	-	-	50	6.25
NSC145612	25	-	-	-	-	50
NSC64111	-	-	-	6.25	≤ 20	≤ 20
NSC182035	-	-	-	6.25	-	-
NSC72938	-	-	-	25	-	-
NSC181486 (Trigilletine)	-	-	-	12.5	≤ 20	≤ 20
NSC52003	-	-	-	25	-	-
NSC73101	-	-	-	25	-	-
NSC143491 (Daunorubicin)	12.5	12.5	12.5	6.25	≤ 20	≤ 20
NSC321239	6.25	12.5	3.125	0.39	≤ 20	-
NSC23128	-	-	-	25	-	-
NSC203328	-	-	-	25	-	-
NSC125910	-	-	-	25	-	-
NSC402959	-	-	-	12.5	-	-
NSC234766	-	-	-	12.5	-	-
NSC95609	-	-	-	12.5	-	-
NSC77037 (Tetrandrine)	-	-	-	12.5	-	-
NSC274547	-	-	12.5	6.25	-	-
NSC83628 (DF 8 sensitizer)	-	-	-	25	-	-
NSC13726	-	-	-	12.5	≤ 20	≤ 20
NSC130813	-	-	-	6.25	-	-
NSC41458	-	-	-	25	-	-
NSC169534 (Quelamycin)	-	-	-	25	-	-
NSC44251 (Cumertilin)	-	-	-	25	-	-
NSC166687	-	-	-	25	-	-
NSC59349 (3-Nortropanol)	-	-	-	12.5	-	-
NSC109268	-	-	-	12.5	-	-
NSC48171	-	-	-	25	-	-
NSC69343	12.5	12.5	-	6.25	-	-
NSC3283	-	-	-	6.25	-	-
NSC71795 (Ellipticine)	-	-	12.5	3.125	≤ 20	≤ 20
NSC356217 (HEJ-4)	-	-	-	6.25	-	-
NSC32951 (Patulin)	-	-	-	3.125	-	-
NSC267461 (Nanaomycin A)	-	-	-	6.25	≤ 20	≤ 20
NSC312033 (Straital B)	-	-	-	1.56	-	-
NSC106408 (Anthramycin)	25	12.5	-	-	-	-

NIH Library Compound	<i>M. smegmatis</i> MIC (μM) LB	<i>M. smegmatis</i> MIC (μM) 7H9	<i>M. smegmatis</i> MIC (μM) C-	<i>M. smegmatis</i> MIC (μM) N-	<i>M. bovis</i> BCG MIC (μM)	<i>M. tuberculosis</i> H37Ra MIC (μM)
NSC134727 (Mitomycin C)	1.56	-	-	-	-	-
NSC122819 (Teniposide)	12.5	-	-	-	-	-
NSC659779 (2-Bromoascididemin)	12.5	1.56	12.5	1.56	0.781	3.125
NSC35611 (Solanine)	-	-	-	6.25	≤ 20	≤ 20
NSC325014 (Bactobolin)	12.5	12.5	25	-	-	-
NSC661755 (Michellamine B)	25	-	6.25	3.125	≤ 20	≤ 20
NSC526417 (Echinomycin)	1.56	3.125	6.25	3.125	≤ 20	≤ 20
NSC45383 (Streptonigrin)	3.125	25	12.5	6.25	≤ 20	≤ 20
NSC56346 (Rottlerin)	-	-	-	6.25	-	-
NSC45737 (Bacitracin)	12.5	12.5	12.5	3.125	≤ 20	≤ 20
NSC51001 (Carbomycin)	6.25	12.5	12.5	3.125	12.5	12.5
NSC82151 (Daunorubicin HCl)	6.25	12.5	12.5	3.125	≤ 20	≤ 20
NSC5159 (Chartreusin)	3.125	1.56	-	-	-	-
NSC62709 (Imidazoquinoline)	25	25	-	-	50	12.5
NSC18712 (Streptomycin thiosemicarbazone)	25	12.5	25	1.56	≤ 20	≤ 20
NSC125066 (Bleomycin)	1.56	1.56	1.56	0.39	3.125	1.56
NSC31048 (Neohesperidin)	12.5	6.25	12.5	3.125	≤ 20	≤ 20
NSC301739 (Mitoxantrone)	-	25	12.5	-	≤ 20	-
NSC47151 (Neomycin, neosule)	-	12.5	12.5	3.125	≤ 20	≤ 20
NSC52141 (Nonactin)	-	-	-	3.125	≤ 20	≤ 20
NSC67574 (Vincristine sulfate)	25	-	-	6.25	≤ 20	≤ 20
NSC133100 (Rifamycin SV)	25	-	-	-	-	-
NSC368675 (Azadirachtin)	25	-	12.5	3.125	≤ 20	≤ 20
NSC34936 (Suramin, Germanin)	-	-	-	6.25	≤ 20	≤ 20
NSC9161 (Erythromycin)	-	-	-	12.5	≤ 20	≤ 20
NSC61586 (Phleomycin)	0.39	0.19	0.19	0.097	25	1.56
NSC285116 (Siomycin A)	6.25	1.56	1.56	3.125	6.25	6.25
NSC13252 (Chlorotetracycline)	1.56	1.56	-	0.39	-	-
NSC292567 (Nigericin)	-	-	-	6.25	≤ 20	≤ 20
NSC317629 (Ketoconazole)	-	-	-	6.25	≤ 20	≤ 20
NSC319518 (Cotylenin A)	-	-	-	6.25	≤ 20	≤ 20
NSC333856 (Tetrocarcin A)	-	-	-	6.25	≤ 20	≤ 20
NSC189794 (Streptovaricin)	12.5	12.5	-	-	25	6.25
NSC3053 (Dactinomycin)	12.5	-	-	-	-	-
NSC363182 (U-64815)	12.5	-	-	-	25	3.125

Table 4.6: Comparison of hits from NIH Diversity Set differential media screening

Validated *M. smegmatis* MIC values of NIH Diversity Set HTS hit compounds derived in both rich and starvation media types are presented for comparison. These values are contrasted with screening results of LOPAC against slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.^{206, 220} Details as for Table 4.4.

4.4.4 Implications of nutrient limited culture conditions in tuberculosis drug screening

Screening of all chemical libraries against *M. smegmatis* resulted in a total of 112 hits detected in LB and 7H9 rich media conditions and 208 hits were detected under carbon and nitrogen nutrient limitation media conditions. Of these hits, 87 were common to both rich media and starvation media conditions.

Comparison with *M. tuberculosis* H37Ra screen results^{206, 220} showed that 75 rich media hit compounds also had activity against *M. tuberculosis*, and 95 starvation media hit compounds were also active against *M. tuberculosis*. Of these compounds, 64 were common to both nutrient rich and nutrient starvation in addition to being active against *M. tuberculosis* H37Ra.

Figure 4.11: Venn diagram of validated hits from screening rich and starvation media. Validated hits obtained in both starvation media and rich media compared with hits derived from in *M. tuberculosis* H37Ra screens.^{206, 220} Venn diagram group totals provided are inclusive.

This demonstrates the utility of modified culture conditions in anti-bacterial drug screening. Hartman de Bonts nutrient limited media, in particular nitrogen limitation, using *M. smegmatis* provide a high level of sensitivity to anti-mycobacterial agents during drug screening compared to conventional rich media types.

Hits obtained through nutrient limitation media screening of *M. smegmatis* were also more relevant towards having activity in *M. tuberculosis* than hits derived from rich media condition screening of *M. smegmatis*. Although the sensitivity of nitrogen starvation media to detecting inhibiting compounds was highest, over half of the compounds detected under nitrogen starvation conditions were not active against *M. tuberculosis* H37Ra, meaning hit

compounds must to be considered anecdotally before verification in a pathogenically relevant model species. Researchers should consider the utilisation of nutrient limited culture conditions such as HdeB C- and N- in addition to standard nutrient rich culture conditions to maximise inhibitor identification when performing whole cell screens for novel anti-mycobacterial agents.

4.5 Summary

In summary, the three chemical libraries that were screened previously in Chapter 3 against *M. smegmatis* cultured in rich media were also screened against *M. smegmatis* cultured under HdeB starvation media conditions of carbon and nitrogen limitation. The inhibitory activity of the hit compounds identified through these screens was validated and quantified, and the validated hit compounds were then cross-referenced with data from library screens of a related screening project against *M. bovis* BCG and *M. tuberculosis* H37Ra.

Validated hit compounds were characterized by their MIC towards *M. smegmatis* in the medium conditions in which they were detected. Validated hit compounds were then investigated through database and literature searching and compounds with undesirable characteristics for the treatment of human tuberculosis, such as cytotoxicity or anti-cancer activity, or previous disclosure as an anti-bacterial activity, which detracted from the novelty of the inhibitor, were omitted from further research.

Compounds that remained were then investigated using literature and database searching to determine their suitability as potential leads for further development as a tuberculosis treatment drug.

The majority of hit compounds were discovered to have properties that rendered them unfavourable as tuberculosis drug development leads; however, several compounds were found to have positive indications and possible applicability as anti-tubercular agents, the most prominent of which are revisited below.

The psychoactive phenothiazine compounds such as trifluoperazine have previously been documented as harbouring anti-tubercular activity. The potential use of these compounds against TDR-TB and the development of the chemical scaffold has been urged. The present research serves to reinforce those published findings. Similarly nortriptyline, a tricyclic anti-depressant with structural likeness to phenothiazines, was also shown to possess anti-

mycobacterial activity, and our research appears to be the first reporting in the published literature of tricyclic anti-depressants exhibiting anti-mycobacterial activities.

Pentamidine is used in the treatment of pneumocystis pneumonia. The two quinoline compounds chloroacetoxyquinoline (silital) and primaquine are potential inhibitors of bacterial siderophore synthesis and an actively used malaria treatment, respectively.

Cephalosporin antibiotics are generally regarded as ineffective against mycobacteria, but Cefdinir, a 3rd generation cephalosporin antibiotic, exhibited anti-mycobacterial activity including activity against *M. tuberculosis*. Cefdinir is a known, well tolerated antibiotic; thus, toxicity concerns are low. Structural modification to enhance the lipophilicity of cefdinir and simultaneous administration with a β -lactamase inhibitor may enhance its activity to within therapeutic levels.

Similarly the anti-fungal agent ketoconazole which is able to be orally administered was detected as active under nitrogen starvation conditions, contributing evidence of the possible novel application for this known drug in the treatment of tuberculosis.

Chlorothalonil, an agricultural anti-fungal agent was revealed to be a potent novel inhibitor of mycobacteria. Chlorothalonil is known to be well tolerated in mammals and exhibits favourable bioavailability *in vivo*. It may be a pioneer in a new class of anti-mycobacterial drugs that target mycothione.

The bismuth conjugated dimercaprol BisBAL was effective at inhibiting *M. smegmatis in vitro*; however, previous research in *M. tuberculosis* H37Rv had disregarded dimercaprol due to poor *in vivo* activity and rapid development of resistance. However, recent evidence indicates this compound may be of use as an adjunct to tuberculosis treatment due to its interference with bacterial capsule and biofilm formation which result in bacteria being more susceptible to chemical and immune attack.

4.5.1 Identification of the novel anti-mycobacterial agent Se-methylselenocysteine

Se-methylselenocysteine was identified through screening for growth inhibitors of *M. smegmatis* using carbon and nitrogen nutrient limitation media, and later was also subsequently validated as active in 7H9 medium. Se-methylselenocysteine, a selenium containing amino acid and natural product, is considered to be the most significant anti-mycobacterial compound to be discovered through nutrient starvation condition screening.

This was due to the novelty of this seleno-amino acid compound class as anti-mycobacterial agents, it exhibited level of anti-mycobacterial activity and low indications of toxicity. It is a naturally occurring compound with a number structural analogues readily available, and it is regularly used in humans as a dietary supplement.

Se-methylselenocysteine was selected for further investigation into its potential as a tuberculosis treatment drug candidate. This compound would not have been identified using conventional rich media screening. The chemical class of seleno-amino acids is further explored for its anti-bacterial activity spectra, human cell line cytotoxicity in Chapter 5.

The mechanism of action and structure-activity relationship of the seleno-amino acid class is then further researched in Chapter 6.

CHAPTER FIVE

Seleno-Amino Acids

5.1 Introduction

Se-methylselenocysteine (SMSC) was identified to be a novel anti-mycobacterial agent during high throughput screening of the LOPAC library under nutrient limitation culture conditions of carbon and nitrogen starvation.

SMSC was verified to have an *in vitro* MIC of 12.5 μM against *M. smegmatis* in HdeB carbon and nitrogen limitation broth, and produced IC_{50} values of $2.19 \pm 0.18 \mu\text{M}$ and $0.60 \pm 0.09 \mu\text{M}$ for carbon and nitrogen limitation broth respectively. However, SMSC had no discernable growth inhibiting effects under LB rich medium conditions.¹²²

The level of growth inhibiting activity produced by SMSC toward *M. smegmatis* is superior to the second-line tuberculosis treatment drug cycloserine, and is comparable to activity of the macrolide antibiotics such as clarithromycin.

Due to the established use of seleno-amino acids such as SMSC in humans as a dietary supplementation³⁶⁵ and evidence that seleno-amino acids may confer beneficial effects towards a range of ailments and diseases beyond fulfilment of a dietary deficiency.³⁶⁶ SMSC and the chemical class of seleno-amino acids were viewed as a promising novel lead to further investigate in the context of their anti-mycobacterial activity and potential applicability to tuberculosis treatment.

The aim of this chapter is to evaluate the potential of Se-AAs as therapeutics for human tuberculosis. This is achieved by:

- Thorough review of scientific literature regarding Se-AAs.
- Confirmatory testing of Se-AA and related seleno-compound inhibitory activity toward mycobacterial species.
- Testing of Se-AA and related seleno-compound inhibitory activity toward other microbial species, to determine Se-AA spectrum of anti-microbial activity.
- Testing of Se-AA cytotoxicity toward mammalian cell lines.

In order to characterise and explore the potential of seleno-amino acids as anti-tubercular agents, SMSC and other available seleno-amino acid compounds must be verified in terms of their inhibitory activity towards slow growing pathogenically relevant mycobacterial species such as *M. bovis* BCG and *M. tuberculosis* H37Ra. In addition to anti-mycobacterial activity, the inhibitory activity of seleno-amino acid compounds toward other bacterial species must

also be characterised. The toxicity of seleno-amino acids towards human cell lines must also be queried in order to gauge the selectivity and safety of the compounds.

This chapter summarises published literature on seleno-amino acids, their dietary use and indications of biological activity. The experimental activity of the various seleno-amino acids towards mycobacteria, other bacterial species, *Saccharomyces* and human cell lines is reported. In light of the previously mentioned experimental results, the suitability of seleno-amino acids for human therapy is discussed.

5.1.1 Selenium

Selenium (Se) is a non-metal element chemically related to sulphur. Selenium shares many of chemical properties of sulphur because they both harbour six valance shell electrons and thus belong to the same periodic group, the chalcogens. Selenium possesses over twice the atomic mass of sulphur at 78.96 compared to sulphur's 32.065. This makes selenium's chemical bonds longer and weaker compared to sulphur.³⁶⁷ Selenium compounds are generally more nucleophilic and acidic than their sulphur bearing counterparts, at physiological pH this means selenol is ionised whereas the equivalent sulfhydryl is protonated.³⁶⁸ Selenium is much more easily oxidised relative to sulphur.³⁶⁷

Selenium has had a past reputation for toxicity due to observations that animals grazing selenium-accumulator plants such as *Astragalus* species or 'Locoweed' provoked toxic effects. However, it is now known the primary toxic effects of consuming such plants are caused by alkaloid toxins such as swainsonine present in the plant tissue.³⁶⁹

Selenium is an essential trace nutrient, particularly in mammals. In humans a strict minimum nutritional intake of 17 µg per day of selenium is required to avoid deficiency-based disease,³⁷⁰ and the consensus minimum intake of selenium is 20 µg per day.³⁷¹ Selenium deficiency in humans may manifest as Keshan disease, named after the Keshan area in China where it was first observed, a congestive cardiomyopathy that is characterised by an enlarged heart, poor heart function and higher coincidence of other diseases such as cancer and stroke.³⁷² Selenium deficiency is also linked to myxedematous endemic cretinism, a mental developmental disorder³⁷³ and Kashin-Beck disease, an osteochondropathy resulting in deformation, pain and limited mobility of the joints.³⁷⁴ Certain geographical regions are more disposed to selenium deficiency-based diseases due to the soils having low selenium

content. Countries with low selenium soil include parts of China, Finland, the East United States and New Zealand.³⁷⁵

Although the minimum requirement of selenium to avoid disease is 20 µg per day, the recommended intake of selenium is 55 µg per day for adults, with slightly higher values of 60 µg per day recommended during pregnancy and 70 µg per day recommended during lactation.³⁷⁶ Regarding the toxicity of selenium in humans, the mean intake with no adverse effects observed is 800 µg per day which equates to 15 µg per kg bodyweight. To err on the side of safety 400 µg per day is the recommended maximum safe daily intake of selenium.³⁷⁷

Although selenium is an essential mineral excessive intake is toxic and manifests in the condition selenosis. Selenosis is characterised by symptoms of malodorous breath, irritability, fatigue, gastrointestinal upset, discolouration of nails, hair loss and mild nerve damage.³⁷⁸

5.1.2 Seleno amino acids

Seleno-amino acids (Se-AAs) are amino acids containing a selenium atom, the most common seleno-amino acids, selenomethionine (Se-met) and selenocysteine (Se-cys), hold selenium in place of sulphur in the sulphurous amino acids methionine and cysteine, depicted in Figure 5.1. Se-methylselenocysteine (SMSC) is a monomethylated derivative of Se-cys. Se-AAs tend to have lower pK_a and higher reduction potentials than their sulphurous equivalents.³⁷⁹

Figure 5.1. Seleno-amino acids and equivalent amino acids.

Chemical structures of the sulphurous amino acids cysteine and methionine (top) and their seleno-amino acid analogues (bottom).

Selenium serves several essential roles in biology, primarily in the form of Se-AAs. Se-cys is an atypical amino acid referred to as the 21st amino acid, and may be depicted by the three letter symbol “Sec” and one letter symbol “U”.³⁸⁰ Se-cys is indirectly but intentionally coded for in mRNA by a UGA stop codon in conjunction with a Se-cys insertion sequence which in bacteria is directly downstream of the UGA stop codon³⁸¹ or in archaea and eukaryotes resides within the 3’ untranslated region of mRNA.³⁸² Se-cys plays specific roles in many “selenoproteins” that are found throughout all domains of life, although they are not ubiquitous. In humans, for example, 25 selenoproteins have been identified.³⁸³

Selenium in Se-cys is ionised under biological conditions and acts as an efficient catalyst, more so than its sulphur counterpart.³⁷⁹ The catalytic properties of selenium are utilised by selenoproteins in their characteristic role of redox maintenance demonstrated by the various glutathione peroxidase enzymes that act as antioxidants to neutralise reactive oxygen species, and thioredoxin reductase enzymes that regulate thioredoxin metabolism that again ties in to cellular redox regulation.³⁷⁵ Another vital role of selenoproteins in humans is deiodination of the prohormone thyroxine to the active triiodothyronine thyroid hormone, facilitated by the Se-cys of iodothyronine deiodinase enzymes.³⁸⁴

Unlike Se-cys, Se-met is not deliberately incorporated into peptides *in vivo* but is non-discriminately incorporated in place of methionine, this substitution has no major discernable effects on protein function and is considered to be well tolerated.³⁸⁵ This incorporation of Se-met into proteins is exploited in x-ray crystallography to resolve protein 3D structure using techniques of single or multi wavelength anomalous diffraction phasing, since the heavier selenium atom produces favourable x-ray scattering properties for structural elucidation.³⁸⁶

Besides selenoproteins, various organic forms of selenium are accumulated in plants, with some species accumulating more selenium than others even in low selenium soils such as the agriculturally relevant *Allium* and *Brassica* species.³¹⁰ Typically these accumulated organoselenium compounds are Se-cys and Se-met and their various derivatives such as allyl-selenocysteine, γ -glutamyl-selenocysteine and methylated derivatives including se-methylselenocysteine.³⁸⁷ The function carried out by these accumulated organoselenium compounds in plants is not fully understood but has been postulated to serve protective roles by increasing antioxidant defence and plant tolerance to stress,³⁸⁸ protecting plants from UV radiation,³⁸⁹ to defend plants against mammalian³⁹⁰ and insect herbivory³⁹¹ and in the case of the aforementioned allyl-, glutamyl- and methyl- derivatives to sequester away excess selenium in a form that is unable to cause harm via incorporation into proteins.³⁹²

5.1.2.1 Dietary sources

Se-AAs are present in a wide variety of commonly consumed foods, particularly selenium-accumulating plant crops, for example *Allium* species such as onion, garlic and leeks and *Brassica* species including broccoli, cauliflower and cabbage.³⁹³ The most selenium rich food source is Brazil nuts.³⁹⁴ Generally the selenium content found in plant crops is proportional to the selenium content of the soil in which they were grown. The same principle applies to the accumulation of selenium in animals or fungi. This has given rise to

the production of 'selenium enriched' foods such as crops, eggs, yeast and meat that are marketed for their benefits to health.³⁹⁵

5.1.2.2 Dietary supplementation

A popular alternative to dietary consumption of selenium through food intake is the use of nutritional supplements. Nutritional supplements are popular amongst all demographics, with a Canadian survey revealing that nearly two thirds of the population use some form of nutritional supplement.³⁹⁶ In New Zealand there are over 6000 health products currently being sold by more than 450 companies for an annual turnover of NZ\$760 million in 2010.³⁹⁷ Over the counter health products such as vitamins, minerals, dietary supplements, nutraceuticals and herbal remedies are a rapidly growing market, estimated to have a global value of over US\$177 billion by 2013.³⁹⁸ Selenium is widely included in most multi-vitamin type supplements and specialised selenium supplements are available that typically deliver selenium in excess quantities of the recommended 55 µg per day.³⁹⁹

Although selenium supplementation is no-doubt extremely beneficial to human health in circumstances of selenium deficiency,⁴⁰⁰ higher doses of selenium are reputed to confer beneficial effects to health beyond simply alleviating selenium deficiency. These beneficial effects are discussed further in section 5.1.4. There is published research-based evidence that supports some of the beneficial health effects of extra-dietary selenium, but a general scientific consensus regarding beneficial effects of high level selenium supplementation is yet to be established.⁴⁰¹ Extra-dietary selenium supplementation of 200 µg per day is the generally considered dose needed to obtain beneficial health effects beyond mitigation of dietary insufficiency.³¹¹ Se-met and SMSC are known to have superior bioavailability to inorganic forms of selenium such as selenite and selenate.⁴⁰⁰ These methylated Se-AAAs are also considered to be principally responsible for the beneficial health effects of selenium.⁴⁰²

5.1.3 Bioavailability and metabolism

In humans, selenium from food or dietary supplements is able to be absorbed both in inorganic forms of selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) and in organic forms such as seleno-amino acids.⁴⁰³ Organic selenium in the form of Se-met is absorbed 26% more efficiently than selenite, and Se-met also quickly reached and maintained blood concentrations 39% higher than selenite over a prolonged administration of 10 weeks.⁴⁰⁴ Inorganic forms of selenium are also less well retained in the body compared to organic selenium, with inorganic selenium such as selenite having poor retention due to rapid

excretion in the urine as selenosugars.⁴⁰⁵ Based on this research, it can be concluded that organic forms of selenium such as Se-met exhibit superior bioavailability characteristics compared to inorganic forms of selenium.

The different forms of dietary selenium are metabolised differently in mammals, the processes for which are depicted in Figure 5.2. Se-AAs are subjected to direct enzymatic lysis, releasing selenium as volatile methylselenol from the methylated Se-met and Se-cys.⁴⁰⁶ Se-met may also be indiscriminately incorporated into proteins in place of methionine, inadvertently serving as a catabolic reservoir of selenium, or Se-met can be converted to Se-cys through the intermediate steps of selenocystathionine and selenohomocysteine.⁴⁰² The non-methylated Se-cys is enzymatically lysed to produce volatile hydrogen selenides under reducing conditions.⁴⁰⁷ Inorganic selenate is reduced to selenite, this is sequestered away by glutathione mediated reduction to selenodiglutathione. Selenodiglutathione is in turn enzymatically reduced by glutathione reductase to glutathioselenol, this is again reduced by thioredoxin reductase in the presence of excess glutathione to yield hydrogen selenide.⁴⁰⁸ Selenium as hydrogen selenide and methylselenol is interchangeable through the action of methyltransferase and demethylase enzymes.⁴⁰⁹ From this central point of selenium metabolism selenium may be utilised by selenophosphate synthase to form selenophosphate that can be incorporated to seryl-tRNA(sec) by selenotransferases and selenocysteine synthases to form Selenocysteinyl-tRNA(sec) which is specifically incorporated into selenoproteins.⁴¹⁰ Hydrogen selenide may also be utilised in the synthesis of selenosugars such as methylseleno-*N*-acetyl-D-glucoseamine which are rapidly excreted in the urine.⁴¹¹ Alternatively, hydrogen selenide may be oxidised to selenium dioxide creating reactive oxygen by-products that are potentially damaging to the cell.⁴¹² Jettison of methylselenol occurs through further methylation to dimethylselenide which is excreted through the breath and faeces, and the trimethylselenonium ion that is excreted in the urine.⁴¹³

Figure 5.2. Selenium metabolic pathway in humans.

Adapted from Rayman *et al* 2008⁴¹⁴ and Riaz *et al* 2012.⁴⁰¹

5.1.4 Biological activity indications and research

The beneficial health effects of selenium and seleno-compounds used in quantities beyond nutritional sufficiency has been a controversial subject for the past decade. Commercial selenium supplements have claimed many health improving effects in terms of the prevention or improvement of the outcomes of many ailments, including cancer, heart disease, Alzheimer's disease, arthritis, diabetes and HIV amongst other diseases.^{366, 415, 416} These claims are not always necessarily backed by convincing scientific evidence, although generally, claims of dietary supplements are worded in a sufficiently vague manner to avoid regulatory infringement.⁴¹⁷ To distinguish the true health benefits of selenium and seleno-compounds from unsubstantiated claims and provide a background on seleno-amino acids research, a brief synopsis of the literature regarding seleno-compound application in health is presented below. Greater attention is paid to health effects attributed directly to Se-AAAs rather than to the downstream effects of selenium supplementation. A comprehensive review of the biological effects of seleno-compounds, with particular regard to organic selenium

compounds such as the Se-AAAs, is described in the 2011 publication of Nogueira and Rocha.⁴¹⁸

5.1.4.1 Immune system function

Many of the conventional beneficial health effects attributed to seleno-compounds may be consequences of selenium facilitation and enhancement of general immune system function. Selenoprotein roles in redox maintenance and mitigation of oxygen radical stress is a contributing factor in T-cell proliferation, activation and function.⁴¹⁹ In humans dietary supplementation with selenium enriched yeast increased T-cell counts by 27% relative to placebo and enhanced immune function, measured as natural killer cell cytotoxicity.⁴²⁰ Selenium supplementation during cancer therapy increases immune response and functional killing of tumour cells in squamous cell carcinoma patients.⁴²¹ In a study of humans infected with attenuated polio virus, administration of selenium supplements led to a faster clearance of the virus and an improved immune response, measured by increased interferon- γ and cytokine production, faster T-cell proliferation and an increase in helper T-cell populations relative to placebo.⁴²²

Considering these effects of selenium towards the immune system, it is possible that the positive effects of selenium supplementation on inflammation, arthritis, asthma, cancer and infection are mediated by the improvement of immune functionality,^{401, 416} although the direct and indirect health effects of seleno-compounds make this difficult to distinguish *in vivo*.

5.1.4.2 Cancer

One of the most prominent areas of seleno-compound health research is the application of selenium in the prevention of cancer. This has resulted in Se-AAAs such as SMSC being commonly referred to as chemopreventatives.⁴²³

Se-DL-met is selective *in vitro* in causing apoptotic death of human tumour cell lines, including breast cancer, melanoma, colon cancer, prostate cancer and lung cancer with IC₅₀ activity ranging from 40-130 μ M against neoplastic cell lines and an IC₅₀ of 1 mM against normal diploid fibroblasts.⁴²⁴

Co-administration of SMSC potentiates the anti-tumour activity of the platinum anti-neoplastic agents cisplatin and oxaliplatin *in vivo*, increasing the cure rate of these compounds when treating advanced murine colorectal tumours. SMSC co-administration also conferred protection against normally lethal doses of cisplatin and oxaliplatin.⁴²⁵

Correlations between organic selenium supplementation in the form of selenium enriched yeast and a reduction in skin⁴²⁶ and prostate cancer⁴²⁷ incidence have been observed in double-blind trials. The incidence of breast cancer has been shown to be decreased in individuals with higher selenium status.⁴²⁸

The effects of selenium supplementation as selenium enriched yeast on the incidence of prostate cancer was measured in the nutritional prevention of cancer trial (NPC) and results indicated that selenium supplementation conferred as much as a 63% reduction in the incidence of prostate cancer.⁴²⁹ The results of the NPC trial were found to conflict with a later publication regarding selenium supplementation and cancer incidence: the selenium and vitamin E cancer prevention trial (SELECT), a large double blind clinical trial examining the effect of selenium and vitamin E on cancer risk in over 35,000 men with a primary focus on prostate cancer. This study concluded that administration of 200 µg of Se-met per day for a period of 5 years did not significantly reduce the risk of localised prostate cancer;⁴³⁰ although, there has been criticism regarding the limitations of the trial design.⁴³¹

The complex relationship between selenium supplementation and carcinogenesis continues to be researched. There is evidence that Se-AAs and their metabolites can potentially reduce cancer risk through a variety of avenues such as direct antioxidant activity or facilitation of selenoprotein function in neutralising carcinogens.⁴³² Se-AAs may also cause inhibition of cancerous tumour growth through induction of apoptosis in cancerous cells⁴³³ and through inhibition of tumour angiogenesis.⁴³⁴

Despite this evidence, selenium supplementation has not demonstrated a reduction in cancer incidence during large clinical trials; thus, no universally accepted conclusion has been drawn regarding selenium effects on cancer incidence in humans.

5.1.4.3 Diabetes

A large double blind placebo-controlled trial concluded that 200 µg selenium per day in the form of selenium-enriched bakers yeast did not reduce the development of type 2 diabetes, and high selenium intake may even be a risk factor for disease.⁴³⁵ However, a more recent study which measured the correlation between selenium status determined through toenail selenium content concluded that higher selenium status attributed a lower risk of developing type 2 diabetes.⁴³⁶ It has been suggested that selenium may play a role in mammalian carbohydrate metabolism, and high selenium status may be as much a consequence of

diabetes as a causative factor with seleno-compounds and selenoprotein antioxidant activity interfering with insulin receptor 2nd messenger H₂O₂.⁴³⁷

5.1.4.4 Cardiovascular disease

Low levels of plasma selenium have previously been linked to increased risk of cardiovascular ischemia⁴³⁸ and increased mortality in patients with acute coronary syndrome.⁴³⁹ As noted before, selenium deficiency can manifest as Keshan disease, a cardiomyopathy. It has been reasoned that the antioxidant properties of seleno-compounds and selenoproteins neutralise free radicals that may damage heart tissue or oxidise lipids contributing to the formation of coronary plaques.⁴⁴⁰ Meta-analysis of recent publications suggest that the evidence for selenium's protective effects towards cardiovascular disease is insufficient.⁴⁴¹

5.1.4.5 Anti-viral

Results of an *in vitro* study report that 2-8 µM Se-met caused inhibition of porcine parvovirus replication in a PK-15 cell culture model. This inhibition was dose-dependant and also selective, not affecting growth of PK-15 cells.¹⁹⁷

Selenium deficiency in HIV infected individuals is associated with disease progression and decreased survival rate.⁴¹⁶ Furthermore, the use of selenium supplements such as Se-met in HIV infected patients has shown positive effects such as improvement of CD4⁺ cell counts,⁴⁴² reduction in hospitalisation incidence,⁴⁴³ reduced diarrheal morbidity⁴⁴⁴ and synergistic reduction of viral load when co-administered with anti-retroviral therapy.⁴⁴⁵ Seleno-compound supplementation lowers the mortality risk of children born to HIV-positive mothers.⁴⁴⁶ Although the anti-viral mechanisms of seleno-compounds are undefined, the beneficial effects of selenium administration in humans likely stem from a combination of immune function enhancement and direct anti-viral activity. Further research in this area is required.⁴⁴² Considering the co-morbidity of HIV and tuberculosis, any indications towards selenium supplementation improving HIV outcome is an advantage that must be exploited.

5.1.4.6 Anti-microbial

Se-AAs are not documented for their direct anti-microbial activity. The first publication disclosing the direct anti-bacterial effects of seleno-amino acids is derived from the present research.¹²² However, the oxidant properties of organic selenium compounds have been suggested as having potential use in anti-microbial applications.⁴⁴⁷

Selenium is a promising adjunct to anti-microbial therapy. Selenium co-administered with the aminoglycoside gentamycin confers a protection from gentamycin-induced nephrotoxicity, by preventing oxidative damage and subsequent renal tubular epithelial necrosis.⁴⁴⁸ Although not a direct anti-microbial effect, this is a positive indication for selenium supplementation in antibiotic therapy, considering the use of aminoglycosides as tuberculosis treatment is reserved to a second line drugs because of significant oto- and nephro-toxic side effects.⁴⁴⁹ Similarly, selenium co-administration with ciprofloxacin prevented renal scarring in bacterial pyelonephritis induced by *E. coli* in a murine *in vivo* model. This is likely to be due to attenuation of damaging oxygen radicals and inflammatory responses.⁴⁵⁰ In chronic bacterial prostatitis induced by *E. coli* selenium treatment reduced inflammatory cell infiltration and inhibited bacterial infection of prostate tissue, producing $3.866 \pm 0.563 \log_{10}$ CFU/g compared with 3.551 ± 0.229 for ciprofloxacin antibiotic control and 6.414 ± 0.885 for the no treatment control. Administration of both selenium and ciprofloxacin was the most effective treatment reducing prostate tissue bacterial counts to $1.344 \pm 0.241 \log_{10}$ CFU/g.⁴⁵¹

Organic selenium-enriched probiotic bacteria and their extracts antagonise *E. coli* pathogenesis and improve outcomes of murine infection *in vivo*⁴⁵² and complementation with selenium and vitamin E for amoxicillin treatment of bovine mastitis improves treatment efficacy.⁴⁵³

In regards to mycobacterial disease, decreased serum selenium levels are associated with higher tuberculosis incidence in HIV patients, with HIV and TB co-infected patients having lower selenium levels than patients with either disease alone.⁴⁵⁴

In another study, HIV infected individuals undergoing highly-active antiretroviral therapy were 13 times more likely to develop mycobacterial disease (75% of which was tuberculosis) if serum selenium levels were less than 135 µg/L compared to patients with higher selenium levels. This study suggests a role of selenium in mycobacterial clearance.⁴⁵⁵

Anti-microbial preparations have been fabricated that utilise reactive oxygen radical generation catalysed by selenium to produce their anti-microbial activity. These consisted of selenium covalently attached to bacteriophage and peptides that target specific bacterial species, delivering the selenium to the target species cell membrane and subsequently damaging the bacteria by generation of reactive oxygen species (ROS). This approach was successful in killing *E. coli* but not *P. aeruginosa*.⁴⁵⁶ In the same study, similar ideology was utilised by covalently attaching selenium to a acetoacetoxyethylmethacrylate (AAEMA)

polymer (Figure 5.3) for implementation as a surface coating of cellulose, successfully inhibiting biofilm formation of *S. aureus* and *P. aeruginosa*. This method could potentially be commercially utilised in manufacture of anti-bacterial wound dressings.^{456, 457} This concept was further explored in selenium coating of reverse osmosis membranes to prevent fouling by microorganism growth and utilised Se-AAEMA polymer or selenocystamine monomers, both of which were successful in reducing *S. aureus* biofilm formation by at least two orders of magnitude.⁴⁵⁸ Selenium nanoparticles were researched by the same group and found to be inhibitory toward the *in vitro* growth of *S. aureus* and also to have some bactericidal activity.⁴⁵⁹

Figure 5.3. Di-Se-AAEMA.

Used in the covalent attachment of selenium to cellulose and reverse osmosis membranes.⁴⁵⁶

In terms of selenium compounds directly used as antibiotics, ebselen was described in a 2007 review of organoselenium compounds as the most effective anti-bacterial organoselenium agent.⁴⁶⁰ Ebselen is an antioxidant that chemically mimics the activity of glutathione peroxidase by reducing reactive oxygen/nitrogen species and is itself then reduced by glutathione or equivalent free thiols.⁴⁶¹ Ebselen has an impressive MIC of 1.56 µg/mL (~5.7 µM) against methicillin resistant strains of *S. aureus in vitro* and also has strong *in vitro* activity against *Enterococcus faecalis* and *B. subtilis* with an MIC of 0.39 µg/mL (~1.4 µM) toward these bacterial species. The selenium is essential for anti-microbial activity, with sulphur substituted ebselen losing activity.⁴⁶² Ebselen is considered non-toxic to mammals⁴⁶³ and has a murine LD₅₀ of 6180 mg/kg.⁴⁶⁴

Figure 5.4. Chemical structure of ebselen.

Ebselen (Figure 5.4) has also demonstrated applicability in the treatment of cancers and improvement of the outcome of acute ischemic stroke in humans. This is thought to be due to its antioxidant and anti-inflammatory activity.^{464, 465} Ebselen is reported to be an inhibitor of *M. tuberculosis* H37Rv in addition to *M. tuberculosis* H37Rv dTDP-6-deoxy-L-lyxo-4-hexulose reductase, membrane-associated serine protease and fructose-bisphosphate aldolase enzymes; however, no inhibitory values are listed.²³⁷ Ebselen is present in both the LOPAC and Spectrum Collection chemical libraries but was not detected as an inhibitor of *M. smegmatis* in any rich or starvation media conditions. This may be due to the mechanism of ebselen, proposed to be disruption of proton-translocation at the cell membrane and inhibition of protein gradient-dependant ATPase, which renders ebselen more potent towards yeasts and Gram positive bacterial species ($IC_{50} = 2-5 \mu M$) than Gram negative species ($IC_{50} = >80 \mu M$).⁴⁶⁶ This potency reduction may also apply towards mycobacteria due to their resilient hydrophobic cell wall structure.⁴⁶⁷ Many derivatives of ebselen have been synthesised and have demonstrated anti-viral, anti-bacterial and anti-fungal activity, with some demonstrating improved activity towards Gram negative bacteria.^{468, 469}

Ebselen is also an effective *in vitro* inhibitor of *P. falciparum*, disrupting the function of ferredoxin-NADP⁺ reductase but is ineffective against the *M. tuberculosis* FprA ferredoxin-reductase, another potential basis for the inactivity of ebselen in the mycobacterial screens of the present research.

Selenoxanthene and selenopyrylium compounds have been found to have modest inhibitory activity toward *E. coli* and greater activity toward *S. aureus*, although it is difficult to gauge the activity levels of these compounds since no antibiotic controls were present in the assay.⁴⁷⁰ This is an interesting result in light of the thioxanthene and structurally similar compounds identified as mycobacterial inhibitors in the screening chapters of this thesis. A variety of further quinolineselenol and pyridineselenol derivatives were then synthesised and tested for anti-bacterial and anti-fungal activity. Two of these organo-selenium compounds (hydrazine-dimethyl-pyridoselenolo-pyrimidine and cyanomethyl-phthalimido-glycylquinoline-phthalimido-glycylquinolineselenol) exhibited growth inhibiting activity towards a spectrum of bacterial species including *S. aureus*, *E. coli*, *P. aeruginosa*, *S. marcescens* and *Bacillus cereus* in addition to having inhibitory activity against a range of fungal species. It was noted that the activity of these organo-selenium compounds is dependant on cyano and phthalimido groups in the quinoline ring, or a pyridine nucleus hydrazino group, indicating the mechanism of action may not be selenium dependant. The

authors speculate that the mechanism of activity is due to the cyano or quinoline moieties or amino-pyrimidine nucleus. These derivatives were tested by a disc diffusion zone of inhibition method; thus, it is difficult to quantify their inhibitory properties; however, the authors note the organo-selenium compounds activity is less than the control antibiotics chloramphenicol and clotrimazole.⁴⁷¹

Selenium substituted morpholines inhibit *in vitro* growth of *S. aureus*, possibly by production of damaging free radicals. *N,N*-methylene-biselenomorpholine was the most effective inhibitor tested, and its higher level of activity is postulated to be due to the hydrocarbyl group increasing the affinity for the hydrophobic bacterial cell, and the higher selenium content giving a greater capacity for radical generation. It is unfortunate the inhibitory profiles of morpholines or thiomorpholines were not juxtaposed with the selenomorpholine results for structure activity comparison.⁴⁷² Selenium substituted derivatives of sulfa drugs were synthesized for the purpose of exploring their anti-bacterial activity against *E. coli*, *S. aureus* and *Serratia marcescens*. A selenium substituent of sulfacetamide and pyrimidine showed bactericidal activity against all aforementioned test species with the lowest MIC of all compounds tested being 10 mg/mL. This is high compared to known anti-bacterial compounds.⁴⁷³

5.2 Results

After identifying SMSC as an anti-mycobacterial agent in high throughput screening and verification of its anti-mycobacterial activity in downstream validation assays using *M. smegmatis*, SMSC and other commercially available structurally similar Se-AAs, Se-cys and Se-met, were purchased to determine the inhibitory activity of these chemicals towards *M. smegmatis*. Additional non-selenium amino acids, including cysteine s-methylcysteine, s-ethylcysteine and methionine and non-amino acid selenium compounds were also purchased and assessed for their anti-mycobacterial activity to provide a basis for structure-activity relationship analysis.

Quantitative dose-response assays were carried out as described in Chapter 2.2.4, initially using *M. smegmatis* MC²155. SMSC and Se-L-met exhibited appreciable anti-mycobacterial activity of < 50 μ M; therefore, these and other the selenium compounds methylseleninic acid and sodium selenite were used as a seleno-compound panel in the testing of susceptibility toward the slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra. Gram negative bacterial species *E. coli*, *V. harveyi* and *P. aeruginosa*, Gram positive *S.*

aureus and the eukaryotic yeast *S. cerevisiae* were also tested for susceptibility to Se-AAs to elucidate the activity spectrum of these compounds toward other pathogenically relevant microbes. Susceptibility testing against pathogenic tuberculosis strains H37Rv and CDC1551 was kindly performed by Dr. Ronan O'Toole and the research group of Professor William R. Bishai. The amount of compounds tested was limited, however. Similarly testing against human cell lines was kindly performed by Rekha Veliyayi Murikoli, but the extent of compound testing was also limited as above. Results are presented in a tabulated format for major inhibitory activity measured by MIC across different medium conditions for the various species tested. Pertinent inhibition results are subsequently presented in graphical format, including differential culture conditions. The best available viability signal(s) applicable (OD₆₀₀, GFP, resazurin reduction as described in section 2.2.4) to the bacterial species and culture condition were used for determination and plotting of inhibition dose-response figures and the derived MIC values are tabulated for quick reference.

5.2.1 Bacterial Species

5.2.1.1 *M. smegmatis* MC²155

Compound	<i>M. smegmatis</i> MC ² 155 pSHIGH_HSP60 MIC (μM)			
	LB	7H9	HdeB C-	HdeB N-
Se-methylseleno-DL-cysteine	> 2000	15.6	12.5	7.8
Seleno-DL-cysteine	> 2000	50	25	25
Seleno-DL-methionine	> 2000	31.2	15.6	15.6
Seleno-L-methionine	> 2000	15.6	7.8	7.8
Seleno-DL-cystine	> 2000	250	125	125
DL-cysteine	> 2000	> 2000	> 2000	> 2000
DL-methionine	> 2000	> 2000	> 2000	> 2000
s-methyl-L-cysteine	> 2000	> 2000	> 2000	> 2000
s-ethyl-L-cysteine	> 2000	> 2000	> 2000	> 2000
Sodium selenite	> 2000	500	500	250
Diphenyl-selenide	1000	1000	1000	1000
Benzeneseleninic anhydrous	1000	500	500	500
Benzeneseleninic acid	2000	500	1000	500
4-chloro benzeneseleninic acid	1000	2000	1000	125
Methylseleninic acid	> 2000	31.2	125	62.5
Seleno-urea	> 2000	> 2000	> 2000	> 2000
1,1-dimethyl-2-selenourea	> 2000	2000	> 2000	> 2000
Ebselen	500	62.5	62.5	31.2

Table 5.1: Inhibitory activity of seleno-compounds against *M. smegmatis*.

Inhibition of Se-AAs and related compounds measured by *M. smegmatis* MIC from quantitative dose-response assays using multiple culture conditions.

Figure 5.5a: Dose-response curves of selenium compound inhibition against *M. smegmatis*. Triplicate quantitative growth inhibition experiments of *M. smegmatis* against a panel of selenium compounds. Normalised GFP and OD₆₀₀ growth signals relative to a negative control (no compound, 100% growth) are provided, media conditions are distinguished by line colour as denoted in the central key.

Figure 5.5b: Dose-response curves of selenium compound inhibition against *M. smegmatis* (continued). Details as per figure 5.5a.

The use of *M. smegmatis* as the primary mycobacterial model organism was extended in this section, with all compounds being tested for inhibitory activity towards *M. smegmatis* using four differential culture conditions.

Se-L-met exhibited the greatest inhibitory activity with a 7.8 μM MIC in carbon and nitrogen-limited medium and 15.6 μM in 7H9 medium. It is noted that the pure levo isomer of Se-met exhibited twice the anti-mycobacterial activity of the racemic dextro-levo mixture. This is somewhat expected given the prevalence of L optical isomers of amino acids in biology. It is presumed this optical isomerism linked activity extends to all seleno-amino acids such as SMSC; however, the appropriate chemicals could not be sourced to confirm this.

SMSC (DL) exhibited similar activity to Se-L-met, demonstrating an MIC toward *M. smegmatis* of 7.8 μM in nitrogen-limited medium, 12.5 μM in carbon-limited medium and 15.6 μM in 7H9 medium. This trend in susceptibility based on media conditions extended to all Se-AAs, with LB cultures unsusceptible to inhibition by Se-AA compounds at the highest concentrations tested of 2 mM.

Se-DL-cys demonstrated less activity than its methylated analogue SMSC, and the di-selenocysteine conjugate, seleno-DL-cystine, exhibited further reduced activity again, although this observation could be linked to the lower water solubility of these compounds.

Structurally similar amino acids containing the usual sulphur rather than selenium including cysteine, methionine and the methyl- and ethyl- derivatives of cysteine exhibited no inhibitory activity towards *M. smegmatis* as expected, implicating the selenium atom in the bioactivity of Se-AAs.

Non amino-acid selenium compounds exhibited poor inhibitory activity against *M. smegmatis* growth regardless of the media conditions used. Exceptions to this were methylseleninic acid that demonstrated reasonable inhibitory activity with an MIC of 31.2 μM in 7H9, and 62.5 μM in nitrogen-limited media. Also the organic selenium compound ebselen showed reasonable inhibitory activity toward *M. smegmatis* with an MIC of 62.5 μM in 7H9 and carbon-starvation conditions and 31.2 μM in nitrogen-starvation culture conditions. It should be noted that ebselen had poor solubility in all media types at concentrations above 500 μM ; therefore, the inhibition assay curves in Figure 5.5 using OD₆₀₀ data are misleading. The curves based on GFP data, however, overcome this problem.

Based on these activity results in *M. smegmatis* the methylated seleno-amino acids SMSC and Se-L-met along with the non-amino acid selenium compounds methylseleninic acid and sodium selenite were selected as a panel of selenium compounds to use in testing other bacterial and fungal species for inhibitory activity.

5.2.1.2 *M. bovis* BCG

Compound	<i>M. bovis</i> BCG pSHIGH_HSP60 MIC (μM)	
	7H9+ Medium	7H9- Medium
Se-methylseleno-DL-cysteine	62.5	31.25
Seleno-L-methionine	31.25	31.25
Methylseleninic acid	125	62.5
Sodium selenite	> 2000	2000

Table 5.2: Inhibitory activity of seleno-compounds against *M. bovis* BCG.

Measured by *M. bovis* BCG MIC from quantitative dose-response assays in nutrient rich and nutrient limited 7H9 culture conditions.

Figure 5.6: Dose-response curves of selenium compound inhibition against *M. bovis* BCG. Triplicate quantitative growth inhibition experiments of *M. bovis* BCG against a panel of selenium compounds. Normalised resazurin reduction growth signal relative to a negative control (no compound, 100% growth) is shown, media conditions are distinguished by line colour as denoted in the central key.

Testing of the seleno-compound suite against the vaccine strain *M. bovis* BCG yielded similar results to those encountered in *M. tuberculosis* H37Ra. SMSC and Se-L-met were the most active compounds tested with an MIC value of 31.25 μM in nutrient limitation 7H9 medium, Se-L-met had the same MIC in 7H9 media however SMSC had a higher MIC of 62.5 μM in 7H9 culture conditions. Methylseleninic acid exhibited some inhibitory activity towards *M. bovis* BCG but less than that observed in *M. tuberculosis* H37Ra and sodium selenite did not completely inhibit the growth of *M. bovis* BCG at the highest concentration tested of 2 mM.

5.2.1 3 *M. tuberculosis* H37Ra

Compound	<i>M. tuberculosis</i> H37Ra MIC (μ M)	
	7H9+ Medium	7H9- Medium
Se-methylseleno-DL-cysteine	31.25	15.6
Seleno-DL-cysteine	125	250
Seleno-L-methionine	15.6	15.6
Methylseleninic acid	31.25	31.25
Sodium selenite	> 2000	> 2000
Ebselen	62.5	N/A

Table 5.3: Inhibitory activity of seleno-compounds against *M. tuberculosis* H37Ra.

Measured by *M. tuberculosis* H37Ra MIC by quantitative dose response assay in nutrient rich and nutrient limited 7H9 culture conditions.

Figure 5.7: Dose-response curves of selenium compound inhibition vs *M. tuberculosis* H37Ra.

Triplicate quantitative growth inhibition experiments of *M. tuberculosis* H37Ra against a panel of selenium compounds. Normalised resazurin reduction growth signal relative to a negative control (no compound, 100% growth) is shown, media conditions are distinguished by line colour as denoted in the central key.

Based on the range of selenium compounds and non-selenium containing amino-acids that were assayed in *M. smegmatis*, the best inhibitory activity was observed in the methylated seleno-amino acids SMSC and Se-met. Analogous non-selenium containing amino acids that were inactive towards *M. smegmatis*, and are presumed to be inactive against slow-growing and pathogenic mycobacterial species, were not further tested in slow growing mycobacterial species. Non-amino acid selenium containing compounds were also largely inactive towards *M. smegmatis*, this inactivity is presumed to extend toward other slow-growing and pathogenic mycobacterial species; therefore, they also were not tested in slow growing mycobacterial species. Methylseleninic acid and ebselen were exceptions, exhibiting reasonable anti-mycobacterial activity towards *M. smegmatis* and these were followed up with testing against all bacterial species for methylseleninic acid and testing against *M. tuberculosis* H37Ra for ebselen since its activity against other bacterial species has been previously investigated.⁴⁶⁶

An extended suite of selenium compounds were tested against *M. tuberculosis* H37Ra under 7H9 and nutrient limited 7H9 conditions. The activity of these compounds in *M. tuberculosis* H37Ra was similar to the activity observed when tested against *M. smegmatis* in 7H9 media conditions. The use of nutrient limited 7H9 medium evoked mixed responses in the inhibitory activity of compounds tested. For example SMSC had an MIC of 31.25 μM in 7H9 culture conditions but exhibited stronger inhibitory activity of 15.6 μM MIC in nutrient limited 7H9 culture. Conversely, Se-DL-cys demonstrated poor activity with an MIC of 125 μM in 7H9, and this activity was further reduced by culturing in nutrient-limited 7H9 medium resulting in a MIC of 250 μM . These results indicate that the nutrient-limited 7H9 conditions used do not substantially increase the sensitivity of *M. tuberculosis* H37Ra to Se-AA inhibition.

Se-L-met demonstrated the greatest inhibitory activity of the seleno-compounds tested with an MIC of 15.6 μM in both nutrient-limited and regular 7H9 culture conditions. Methylseleninic acid was also inhibitory in both culture conditions at 31.25 μM ; whereas, sodium selenite did not completely inhibit *M. tuberculosis* H37Ra growth at the highest concentration tested of 2 mM. Ebselen exhibited inhibitory activity with an MIC of 62.5 μM in 7H9 but unfortunately was not able to be tested in nutrient limitation 7H9 medium.

5.2.1.4 *M. tuberculosis* H37Rv and CDC1551

Compound	<i>M. tuberculosis</i> H37Rv IC ₉₀ (μM) 7H9+ Medium
Se-methylseleno-DL-cysteine	5 μg / mL (22.88 μM)*
Se-methylseleno-DL-cysteine	50 †
Seleno-DL-methionine	50 †
Seleno-DL-cystine (NSC172800)	100 †
Selenocystathionine (NSC90812)	> 100 †
Aminoethylselenanylpropanoic acid (NSC261137)	> 100 †
Selenohomolysine (NSC282139)	> 100 †
Aminoethylselenanylethylazanium (NSC308820)	> 100 †

Table 5.4: Inhibitory activity of seleno-compounds against *M. tuberculosis* H37Rv.

Measured by *M. tuberculosis* H37Rv MIC in nutrient rich 7H9 culture conditions.

*IC₉₀ result obtained by experiments kindly performed by the research group of Professor William R. Bishai at Johns Hopkins School of Medicine, Baltimore, Maryland through the TAACF program.

†IC₉₀ result obtained by experiments kindly performed by Dr. Ronan O'Toole at Johns Hopkins School of Medicine, Baltimore, Maryland.

Figure 5.8: Dose-response curves of selenium compound inhibition vs *M. tuberculosis* H37Rv.

Triplicate quantitative growth inhibition experiments of *M. tuberculosis* H37Rv against selenium compounds. Normalised resazurin reduction growth signal relative to a negative control (no compound, 100% growth) is shown.

Compound	<i>M. tuberculosis</i> CDC1551 IC ₉₀ (μM) 7H9+ Medium
Se-methylseleno-DL-cysteine	2 μg / mL (9.15 μM)*

Table 5.5: Inhibitory activity of seleno-compounds against *M. tuberculosis* CDC1551.

Measured by *M. tuberculosis* CDC1551 MIC in nutrient rich 7H9 culture conditions.

*IC₉₀ result obtained by experiments kindly performed by the research group of Professor William R. Bishai at Johns Hopkins School of Medicine, Baltimore, Maryland through the TAACF program.

Considering the favourable inhibitory activity of the seleno-amino acids against *M. tuberculosis* H37Ra, the activity of this class of compounds was further queried in the virulent tuberculosis strain H37Rv, with experiments kindly performed by Dr. Ronan O'Toole at the facilities of John Hopkins School of Medicine. Due to these experiments being outsourced, complete data sets for each compound were not available; therefore, not all dose-response graphs are presented.

Using original chemical stocks of SMSC an initial IC₉₀ result of 5 µg / mL (22.88 µM) was obtained against H37Rv using a standard MTT assay protocol against *M. tuberculosis* H37Rv cultured under 7H9 medium conditions. However, subsequent testing using the same chemical stocks resulted in an MIC of 50 µM, indicating that there may have been some degradation of the compound through storage or freeze/thaw cycling, and this result requires further validation.

Several other seleno-amino acids and seleno-compounds were obtained through the NIH Developmental Therapeutics Programme repository. With the exception of Se-DL-met that exhibited an IC₉₀ of 50 µM, and Se-DL-cys, with an IC₉₀ of 100 µM, none of these compounds exhibited appreciable anti-tubercular activity at the 100 µM concentration tested.

The sensitivity to SMSC of the highly transmissible and reactive oxygen/nitrogen species resistant *M. tuberculosis* CDC1551 strain⁴⁷⁴ was also investigated, with experiments kindly performed by the research group of Professor William Bishai at Johns Hopkins School of Medicine. It was found that CDC1551 cultured in 7H9 medium was more sensitive to growth inhibition by SMSC with an IC₉₀ of 2 µg / mL (9.15 µM).

These results of inhibitory activity against virulent strains of *M. tuberculosis* are good indications for the potential development of seleno-amino acids as anti-tubercular agents; however, further testing in these and other virulent strains including drug resistant isolates is required.

5.2.1.5 *S. aureus*

Compound	<i>S. aureus</i> Newman pFDHL::GFP MIC (μM)	
	LB Medium	M9 C- Medium
Se-methylseleno-DL-cysteine	> 2000	> 2000
Seleno-L-methionine	> 2000	> 2000
Sodium selenite	250	1000
Methylseleninic acid	1000	> 2000

Table 5.6: Inhibitory activity of seleno-compounds against *S. aureus*.

Measured by *S. aureus* MIC from quantitative dose response assay in nutrient rich LB and nutrient limited M9 culture conditions.

Figure 5.9: Dose-response curves of selenium compound inhibition against *S. aureus*. Triplicate quantitative growth inhibition experiments of *S. aureus* against selenium compounds. Normalised resazurin reduction growth signal (LB) and GFP emission (M9) relative to a negative control (no compound, 100% growth) are shown.

Selenium compounds had little inhibitory effects towards *S. aureus* cultured in the rich media conditions of LB. Minor inhibition of growth was observed for SMSC and Se-L-met at high concentrations, but both of these compounds failed to completely inhibit *S. aureus* growth even at the highest concentration tested of 2 mM. The non-amino acid selenium compounds methylseleninic acid and sodium selenite caused complete growth inhibition under rich media conditions at concentrations of 1000 and 250 μ M, respectively, this is considered to be low inhibitory activity.

Nutrient limitation conditions made some difference in the sensitivity of *S. aureus* to the seleno-compounds; however, this was insufficient to impact the MIC values. SMSC, Se-L-met and methylseleninic acid exhibited dose-dependant inhibition but were unable to fully inhibit *S. aureus* growth at the highest concentration of 2 mM tested. Sodium selenite elicited a similar inhibition response in M9 medium as it did in LB; however, a four-fold rise in MIC was observed in M9 minimal medium.

5.2.1.6 *E. coli*

Compound	<i>E. coli</i> DH5 α pOT11 MIC (μ M)	
	LB Medium	M9 C- Medium
Se-methylseleno-DL-cysteine	> 2000	500
Seleno-L-methionine	> 2000	2000
Sodium selenite	> 2000	2000
Methylseleninic acid	1000	2000

Table 5.7: Inhibitory activity of seleno-compounds against *E. coli*.

Measured by *E. coli* MIC in nutrient rich LB and nutrient limited M9 culture conditions.

Figure 5.10: Dose-response curves of selenium compound inhibition against *E. coli*. Triplicate quantitative growth inhibition experiments of *E. coli* against selenium compounds. Normalised resazurin reduction growth signal (LB) and GFP emission (M9) relative to a negative control (no compound, 100% growth) are shown.

The seleno-amino acids SMSC and Se-L-met exerted no discernable inhibitory effects towards *E. coli* under rich LB culture conditions. In minimal M9 culture conditions SMSC had an MIC of 500 μ M and Se-L-met had an MIC of 2 mM which is considered to be a very low level of inhibitory activity.

Methylseleninic acid displayed similar low inhibition with an MIC of 1 mM under LB conditions and 2 mM under M9 media conditions. Sodium selenite also showed low inhibitory properties towards *E. coli* with an MIC 2 mM under M9 media and an LB MIC unobtainable; although, some inhibition was observed in LB it was incomplete at the highest concentration of 2 mM tested.

5.2.1.7 *P. aeruginosa*

Compound	<i>P. aeruginosa</i> PA01 MIC (μM)	
	LB Medium	M9 C- Medium
Se-methylseleno-DL-cysteine	> 2000	> 2000
Seleno-L-methionine	1000	> 2000
Sodium selenite	> 2000	> 2000
Methylseleninic acid	> 2000	> 2000

Table 5.8: Inhibitory activity of seleno-compounds against *P. aeruginosa*. Measured by *P. aeruginosa* MIC in nutrient rich LB and nutrient limited M9 culture conditions.

Figure 5.11: Dose-response curves of selenium compound inhibition against *P. aeruginosa*.

Triplicate quantitative growth inhibition experiments of *P. aeruginosa* against selenium compounds. Normalised resazurin reduction growth signal (LB) and OD₆₀₀ absorbance (M9) relative to a negative control (no compound, 100% growth) are shown.

P. aeruginosa was not inhibited by SMSC in LB medium. In M9 minimal medium some inhibition was observed at concentrations of 250 μ M and above; however, this inhibition was variable and inconsistent with complete inhibition never achieved. Se-L-met evoked better inhibition response against *P. aeruginosa* with an MIC of 1 mM in LB and strong inhibition in minimal M9 medium of approximately 80% at concentrations of 125 μ M and above; however, despite exhibiting a good dose response curve Se-L-met did not achieve total inhibition of *P. aeruginosa* growth.

Methylseleninic acid had no effect on the growth of *P. aeruginosa* in LB medium; however, under M9 minimal medium, growth was inhibited again to approximately 80% at concentrations of 125 μ M and above, but complete growth inhibition was never seen. High concentrations of sodium selenite precipitated under LB medium to a brick-red presumably elemental selenium (Se^0). This interfered with the OD_{600} readings of the assay; however, bacterial growth was observed even at the highest concentration tested of 2 mM. Under M9 medium conditions dose-response inhibition was observed, but growth of *P. aeruginosa* was not completely inhibited in triplicate even at the highest concentration tested.

5.2.1.8 *V. harveyi*

Compound	<i>V. harveyi</i> pOT11 MIC (μM)	
	LB Medium	M9 C- Medium
Se-methylseleno-DL-cysteine	250	1000
Seleno-L-methionine	250	250
Sodium selenite	> 2000	250
Methylseleninic acid	500	250

Table 5.9: Inhibitory activity of seleno-compounds against *V. harveyi*. Measured by *V. harveyi* MIC in nutrient rich LB and nutrient limited M9 culture conditions.

Figure 5.12: Dose-response curves of selenium compound inhibition against *V. harveyi*. Triplicate quantitative growth inhibition experiments of *V. harveyi* against selenium compounds. Normalised resazurin reduction growth signal (LB) and GFP emission (M9) relative to a negative control (no compound, 100% growth) are shown.

SMSC and Se-L-met demonstrated complete inhibition of *V. harveyi* (also known as *V. anguillarum* or *Listonella anguillarum*) growth at concentrations of 250 μ M and above in rich LB medium. The same level of inhibition was observed for Se-L-met in minimal medium; however, SMSC had a four-fold higher MIC of 1000 μ M in minimal medium.

Methylseleninic acid produced similar MIC values against *V. harveyi* in both rich and minimal media types with M9 culture conditions being two-fold more susceptible to inhibition with an MIC of 250 μ M. Sodium selenite produced a stronger inhibitory response against *V. harveyi* cultured under minimal medium conditions with an MIC of 250 μ M where as LB medium conditions caused precipitation of red elemental selenium and failed to inhibit *V. harveyi* growth at the highest concentration tested of 2 mM.

5.2.2 Eukaryotic Species

5.2.2.1 *S. cerevisiae*

<i>S. cerevisiae</i> MIC (μ M) by media type				
Compound	SC Medium		0.5x SPO Medium	
	BY4741 haploid	BY4743 diploid	BY4741 haploid	BY4743 diploid
SMSC	> 2000	> 2000	> 2000	> 2000
Se-L-Met	125	125	62.5	125
Methylseleninic Acid	> 2000	2000	> 2000	> 2000
Sodium Selenite	1000	> 2000	1000	> 2000

Table 5.10: Inhibitory activity of seleno-compounds against *S. cerevisiae*.

Measured by *S. cerevisiae* MIC in nutrient rich SC and nutrient limited SPO culture conditions.

Figure 5.13a: Dose-response curves of selenium compound inhibition against *S. cerevisiae*.

Triplicate quantitative growth inhibition experiments of *S. cerevisiae* against selenium compounds. Normalised resazurin reduction growth signal relative to a negative control (no compound, 100% growth) is shown.

Figure 5.13b: Dose response curves of selenium compound inhibition against *S. cerevisiae* (cont).

Haploid (BY4741) and diploid (BY4743) strains of *S. cerevisiae* were tested for sensitivity to the panel of selenium compounds to gauge the anti-fungal activity of the compounds and also provide an indication of activity towards eukaryotic cells which may provide clues to the mammalian toxicity of the selenium compounds.⁴⁷⁵

Under sporulation (0.5x SPO) minimal medium conditions SMSC showed reduction of resazurin dye at high concentrations, although this did not occur in the no-cell controls and may therefore encourage or be a consequence of yeast metabolism. Regardless SMSC exerted little inhibitory effect upon haploid or diploid yeast strains in 0.5x SPO minimal media conditions. Under rich synthetic complete (SC) medium conditions, SMSC

demonstrated a small yet incomplete degree of inhibition towards both haploid and diploid yeast strains, with the inhibition more prominent in the haploid strain.

Se-L-met showed complete inhibition of both haploid and diploid *S. cerevisiae* growth under both media conditions, with an MIC of 125 μM in the haploid under rich SC medium and the diploid under both media types. A lower MIC of 62.5 μM was observed for the haploid strain under minimal medium conditions.

High concentrations of methylseleninic acid also appeared to reduce resazurin in SPO minimal medium conditions, resulting in an erratic dose-response pattern. Complete inhibition was observed only for the haploid/diploid strain in rich SC medium at the highest concentration tested of 2 mM. The haploid strain under both media conditions and the haploid/diploid under SPO starvation conditions were not completely inhibited by methylseleninic acid.

Sodium selenite exhibited a typical dose-response curve in the haploid strain under both media conditions, with an MIC of 1 mM under both media conditions. The haploid/diploid strain showed more resistance to inhibition by sodium selenite, exhibiting a dose-response curve with incomplete inhibition at the highest concentration tested of 2 mM.

5.2.2.2 Mammalian cell lines

Compound	Human Cell Line IC ₅₀ (μM)		
	HL-60	J-774	Vero
Se-methylseleno-DL-cysteine	73.03 ± 16.73	> 250	> 100
Seleno-DL-methionine	NT	NT	> 100

Table 5.11: Inhibitory activity of Se-AAs against HL-60, J-774 and Vero cell lines. Measured by MTT assay, HL-60 and J-774 experiments kindly performed by Rekha Veliyayi Murikoli. Vero cell assays were kindly performed by Dr. R. O'Toole. NT = not tested.

Figure 5.14: Dose-response curves of selenium compound inhibition against HL-60, J-774 and Vero cell lines.

Measured by MTT assay, HL-60 and J-774 experiments kindly performed by Rekha Veliyayi Murikoli. Vero cell assays were kindly performed by Dr. R. O'Toole.

Toxicity of the initially discovered, and most promising selenium compounds, SMSC and Se-DL-met, respectively, was tested using human promyelocytic leukaemia (HL-60), murine

macrophage (J-774) and monkey kidney epithelial (Vero) cell lines. HL-60 and J-774 cytotoxicity assays were kindly performed by Rekha Veliyayi Murikoli and Vero cell assays were kindly performed by Dr. R. O'Toole using the MTT assay protocol described in Chapter 2.5.1. Unfortunately, other selenium compounds were not able to be assessed for cytotoxicity. In lieu of no further experiments concerning the cytotoxicity of seleno-compounds in human cell lines, cytotoxicity data was sourced from the literature and is discussed in section 5.3.2.3.

SMSC exhibited a low level of cytotoxicity towards the HL-60 cell line with a standard sigmoidal inhibition dose-response curve showing an IC_{50} value of $73.0 \pm 16.7 \mu M$. Against J-774 macrophages, no inhibition curve could be established for SMSC at the highest concentration tested of $250 \mu M$, indicating the need for further testing of SMSC against this cell line at higher concentrations to determine the true inhibitory value. Similarly, SMSC and Se-DL-met did not attain an IC_{50} at the highest concentration tested of $100 \mu M$, although the beginning of an inhibition curve is observed for Se-DL-met at $100 \mu M$ with 80% growth at this concentration.

These human cell line toxicity results indicate favourable selective inhibition of Se-AAs towards mycobacteria over human cell lines. However, these cytotoxicity results require further validation and extension to include other anti-mycobacterial Se-AAs such as Se-L-met before a definitive conclusion can be drawn regarding Se-AA toxicity.

5.3 Discussion

5.3.1 Biological activity of seleno-compounds

Based on the range of selenium compounds and non-selenium containing amino-acids which were assayed, the best inhibitory activity was observed in the methylated seleno-amino acids SMSC and Se-met. Analogous non-selenium containing amino acids were inactive towards *M. smegmatis*, and are presumed to be inactive against slow-growing and pathogenic mycobacterial species. Similarly non amino acid selenium compounds demonstrated little inhibitory activity with the exception of methylseleninic acid and ebselen. These methylated seleno-amino acids demonstrated highest inhibitory activity towards mycobacterial species, with activity toward other Gram positive and Gram negative bacteria being above therapeutically viable MIC values when contrasted to existing anti-bacterial agents.

5.3.1.1 Anti-bacterial activity

5.3.1.1.1 Anti-mycobacterial activity

5.3.1.1.1.1 *M. smegmatis*

Seleno-amino acids exhibited the greatest inhibitory activity towards *M. smegmatis* under HdeB carbon-limitation and HdeB nitrogen-limitation culture conditions. This was closely followed by 7H9 medium conditions that yielded similar but slightly higher inhibitory concentration values for *M. smegmatis* against all tested Se-AAs. Se-AAs did not achieve a measurable MIC value in LB medium conditions at the highest concentration tested.

Methylated Se-AAs, SMSC and Se-L-met, showed the strongest inhibitory activity toward *M. smegmatis*. The levo optical isomer of Se-met showed double the inhibitory activity with the lowest MIC of 7.8 μ M compared to 15.6 μ M for the racemic dextro/levo mixture. This suggests that the inhibitory activity of Se-AAs is dependant on optical rotation. Based on this, dextro isomers of Se-AAs are likely to have little to anti-mycobacterial activity, although this remains to be tested.

Non-methylated Se-AA Se-DL-cys consistently showed two-fold or lower inhibitory activity toward *M. smegmatis* than its methylated counterpart, SMSC. This suggests that methylation of the selenium is an important but not an essential contributing factor to Se-AA inhibitory activity. The dimeric seleno-DL-cystine showed severely reduced inhibitory activity further implicating the methylated or free selenium impact on Se-AA activity.

Non-selenium containing Se-AA counterpart amino-acids such as DL-cysteine, DL-methionine and s-methyl-L-cysteine and s-ethyl-L-cysteine exerted no inhibitory effect on *M. smegmatis*. This is somewhat unexpected since published results of s-ethyl-L-cysteine claim superior anti-mycobacterial activity to pyrazinamide and para-aminosalicylic acid,⁴⁷⁶ including activity against isoniazid-resistant TB.⁴⁷⁷ S-Ethyl-L-cysteine was thought to exert its anti-tubercular action through formation of ethanthiol,³⁴⁷ and the compound underwent clinical trials in humans.⁴⁷⁷ Considering these reports of activity, some *in vitro* inhibition towards *M. smegmatis* and *M. tuberculosis* H37Ra was expected, but was not detected at the highest concentration of 2 mM tested. It is possible that s-ethyl-L-cysteine only exerts anti-tubercular activity *in vivo* since the published results utilised murine TB models. From these inactive results, it is deduced that selenium is an essential component of Se-AA inhibitory activity, and these compounds were not tested further in other species.

Selenium compounds other than Se-AAs generally demonstrated inferior anti-mycobacterial activity toward *M. smegmatis* in any culture conditions tested relative to Se-AAs. The greatest activity was observed for ebselen that had an MIC of 62.5 μ M against *M. smegmatis* in 7H9 and HdeB carbon-starvation culture conditions and 31.2 μ M in HdeB nitrogen-starvation conditions. These MIC values are above the 20 μ M concentration used in HTS. This explains why ebselen, included in the Spectrum Collection, was not detected in any HTS.

Methylseleninic acid exhibited varied inhibitory activity. As with most selenium compounds methylseleninic acid was inactive against *M. smegmatis* cultured in LB medium conditions and showed the strongest activity with an MIC of 31.2 μ M in 7H9 medium followed by HdeB nitrogen-limitation medium 62.5 μ M and HdeB carbon-limitation medium 125 μ M. This trend in activity across media conditions differs from that observed for other Se-AAs and selenium compounds that typically exhibit the greatest activity in HdeB media conditions. 4-Chlorobenzeneseleninic acid displayed an MIC of 125 μ M against *M. smegmatis* in nitrogen-limited culture but had an MIC of 1 mM or greater under other media conditions.

Sodium selenite showed low inhibitory activity against *M. smegmatis* in all media types with 7H9 and HdeB carbon limitation media having an MIC of 500 μ M and HdeB nitrogen-limited medium having an MIC of 250 μ M.

5.3.1.1.1.2 *M. bovis* BCG

In general, *M. bovis* BCG demonstrated less sensitivity to the panel of selenium compounds tested. *M. bovis* BCG had a four-fold decreased level of inhibitory activity for SMSC and methylseleninic acid and a two-fold decrease in activity for Se-L-met compared to results obtained for *M. smegmatis* cultured in 7H9 medium.

This dampened activity was able to be somewhat mitigated by use of 7H9- limited medium in the circumstance of SMSC and methylseleninic acid, resulting in a two-fold increase in compound inhibitory activity compared to normal 7H9+ medium results; however, the use of 7H9- limited medium had no effect on the activity of Se-L-met. *M. bovis* BCG was also more tolerant to sodium selenite compared to *M. smegmatis*, with the highest concentration of 2 mM being unable to completely inhibit *M. bovis* BCG growth, although the usual dose-response inhibition was observed.

Based on the results of *M. bovis* BCG against the seleno-panel, it is concluded that *M. bovis* BCG is less sensitive than *M. smegmatis* to growth inhibition by Se-AAs and other selenium compounds such as methylseleninic acid, although the use of 7H9- limited medium can reduce this discrepancy but not to levels of activity observed in *M. smegmatis* assays.

5.3.1.1.1.3 *M. tuberculosis* H37Ra

The trends of Se-AA inhibitory activity observed in *M. smegmatis* were also seen in *M. tuberculosis* H37Ra. Methylated Se-AAs SMSC and Se-L-met again exhibited the greatest inhibitory activity of 15.6 μ M, with Se-L-met demonstrating this level of activity in both 7H9+ complete and 7H9- minimal media types; however, SMSC activity was reduced two-fold in 7H9+ medium. The activity of SMSC and Se-L-met in *M. tuberculosis* had the same MIC values as *M. smegmatis* cultured in 7H9 medium.

Methylseleninic acid and ebselen also had the same MIC in *M. tuberculosis* H37Ra as *M. smegmatis* cultured in 7H9 medium, whereas, *M. tuberculosis* H37Ra was more tolerant to sodium selenite compared to *M. smegmatis*. It is possible that ebselen is potentially more active than the results obtained as it was poorly soluble in water (and thus assay medium) and precipitated at concentrations above 500 μ M, this may have effected the serial dilution of the compound during the assay setup.

The exception to the similarity in Se-AA activity between *M. smegmatis* cultured in 7H9 and *M. tuberculosis* H37Ra was Se-DL-cys that demonstrated a large increase to 125 μ M in MIC compared to the *M. smegmatis* 7H9 MIC result of 50 μ M. This low activity was further exacerbated by the use of 7H9- limitation medium culture conditions that increased the MIC of Se-DL-cys in H37Ra to 250 μ M. This result was unexpected and opposes other results obtained in 7H9- medium, warranting further confirmatory re-testing.

Based on these results it is concluded that *M. tuberculosis* H37Ra has a comparable sensitivity to growth inhibition by Se-AAs as the model organism *M. smegmatis* cultured under 7H9 or HdeB media. This similarity in sensitivity also extends to the other selenium compounds methylseleninic acid and ebselen. Use of 7H9- starvation medium may render H37Ra more sensitive to SMSC but generally has little impact on inhibitory activity values.

5.3.1.1.4 *M. tuberculosis* H37Rv and CDC1551

Initial testing of the first discovered Se-AA compound, SMSC, against virulent *M. tuberculosis* strains H37Rv and CDC1551 resulted in IC₉₀ results of 22.88 µM and 9.15 µM, respectively. This level of inhibition is greater than the MIC of 31.2 µM obtained for *M. tuberculosis* H37Ra in the same assay conditions. It is noted that for virulent strains, IC₉₀ values were reported and compared to the MIC values used in this research. This is thought to have minimal impact on the reported different sensitivity between the virulent TB strains and H37Ra, since an IC₉₀ of 29.91 µM is extrapolated from the SMSC H37Ra dose-response curve, gives a value very similar to the 31.2 µM MIC (Figure 5.6).

Subsequent tests of the same chemical batch of SMSC in *M. tuberculosis* H37Rv resulted in a higher IC₉₀ recording of 50 µM. It is not known if this higher result is accurate or anomalous as a result of chemical degradation or some other change in the stock or assay conditions; therefore, confirmatory re-testing is required. The IC₉₀ result for Se-DL-met of 50 µM is higher than expected considering the MIC of Se-DL-met in *M. smegmatis* 7H9 is 31.2 µM and Se-L-met in *M. tuberculosis* H37Ra is 15.6 µM. However, this result was also obtained from the secondary round of testing as with SMSC above and therefore confirmatory re-testing is desired for both Se-DL-met and Se-L-met to validate the inhibitory activity and the optical selectivity activity of the Se-AAs. Seleno-DL-cystine exhibited a lower IC₉₀ of 100 µM against H37Rv than the *M. smegmatis* MIC result of 250 µM obtained in 7H9 culture conditions. The H37Rv result was more comparable to the MIC of 125 µM for Se-DL-cystine obtained from *M. smegmatis* under HdeB nutrient starvation culture assays.

Other selenium compounds with structural similarity to SMSC obtained through the NIH Developmental Therapeutics Program Depository exhibited no significant inhibitory activity with IC₉₀ values above the highest concentration tested of 100 µM. These results were also obtained through the second round of testing therefore should be regarded with a degree of uncertainty.

From the initial results it is concluded that virulent strains of *M. tuberculosis* H37Rv and CDC1551 are more sensitive to the inhibitory action of Se-AAs compared to *M. tuberculosis* H37Ra and 7H9 cultured *M. smegmatis*. However only initial results obtained with SMSC support this; therefore, further testing with SMSC and Se-L-met is required to confirm the activity of these leading Se-AAs against virulent TB strains, in addition to MDR-TB isolates

to determine any cross-resistance with existing TB drugs. Comparative testing of Se-DL-met is also desirable to confirm the optical rotation influence on activity of Se-AAs.

5.3.1.1.2 Gram positive

5.3.1.1.2.1 *S. aureus*

The Gram-positive bacterial species *S. aureus* was tested against the panel of selenium compounds to explore the wider anti-bacterial activity spectrum of the seleno-amino acids and determine the potential suitability of seleno-amino acids as drugs for the treatment of *S. aureus*. This strain is increasingly problematic in the clinical environment due to the rise of drug resistant strains such as methicillin resistant *S. aureus* (MRSA).⁴⁷⁸

Experimentally, *S. aureus* exhibited little sensitivity to Se-AA growth inhibition. In rich LB medium *S. aureus* displayed gradual dose-response inhibition but the inhibitory response was poor and at 2 mM the highest concentrations of SMSC and Se-L-met tested *S. aureus* growth remained at approximately 65% of the cell growth control. The dose-response inhibition was more pronounced in *S. aureus* cultured under M9 minimal medium conditions; however, an MIC was still not achieved at 2 mM for either SMSC or Se-L-met.

A greater inhibitory response was seen by methylseleninic acid with an MIC of 1 mM in LB conditions however an MIC could not be obtained under M9 minimal medium conditions. The strongest inhibitory activity was derived from sodium selenite with an MIC of 250 μ M under LB conditions and 1 mM in M9 conditions.

Therefore it appears *S. aureus* is not susceptible to any significant degree of growth inhibition by Se-AAs under rich or starvation culture conditions. Furthermore, *S. aureus* is more resilient to selenium compound inhibition under starvation culture conditions, since although the dose-response curves were similar, low levels of *S. aureus* growth at high compound concentrations prevented determination of MIC values at the concentrations tested.

5.3.1.1.3 Gram negative

5.3.1.1.3.1 *E. coli*

Gram negative *E. coli* was tested against the selenium compounds panel to further explore the anti-bacterial activity spectrum of the seleno-amino acids, and determine the potential suitability of Se-AAs as drugs for treatment of *E. coli* infection which outbreaks of virulent

strains can occur, such as the 2011 outbreak of *E. coli* O104:H4 in Germany⁴⁷⁹ and as a bacterial pathogen increasing drug resistance is an ongoing issue.⁴⁸⁰

E. coli showed little sensitivity towards growth inhibition by selenium compounds. In LB medium no inhibitory dose-response curve was observed for Se-AAs SMSC and Se-L-met across the concentrations tested. A dose-response relationship was observed in LB medium for methylseleninic acid with an MIC of 1 mM, and a dose-dependent inhibitory response was also observed for sodium selenite in LB media; however, this did not cause complete inhibition at the highest concentration tested of 2 mM.

Using M9 minimal medium assay conditions, an inhibitory response was observed for Se-AAs. SMSC caused complete inhibition of *E. coli* growth at 500 μ M. Se-L-met was less potent, only completely inhibiting *E. coli* growth at 2 mM, the highest concentration tested. Methylseleninic acid showed a similar inhibitory dose-response in M9 minimal medium as was observed in LB; however, the MIC was slightly higher in M9 at 2 mM. *E. coli* was more sensitive to inhibition by sodium selenite in M9 medium compared to LB, although the MIC in M9 medium was only at the highest concentration tested of 2 mM, the dose-response was more gradual, as observed in Figure 5.10 for sodium selenite.

These results suggest that Se-AAs have only minor inhibitory effects towards *E. coli*. *E. coli* demonstrated a typical inhibitory response to non-amino acid selenium compounds methylseleninic acid and sodium selenite. SMSC under M9 nutrient-limited conditions was the only Se-AA to exert inhibitory activity towards *E. coli*, and this was at a concentration too high to be practical as a drug development lead.

5.3.1.1.3.2 *P. aeruginosa*

P. aeruginosa is a Gram negative bacterial pathogen of clinical importance in opportunistic infections such as cystic fibrosis patients and nosocomial infection of burn injuries.⁴⁸¹

Pseudomonas species such as *P. aeruginosa* are notoriously difficult to treat with antibiotic therapy,⁴⁸² primarily due to innate resistance by drug efflux pump expression⁴⁸³ and aggressive expression of antibiotic negating enzymes.⁴⁸⁴

P. aeruginosa displayed some sensitivity to growth inhibition by Se-L-met under both rich LB and nutrient-limited M9 media types. Although an inhibitory dose-response relationship was observed for Se-L-met, particularly under nutrient-limitation conditions (Figure 5.11),

this was insufficient to completely inhibit *P. aeruginosa* growth; thus, high MIC values were recorded. *P. aeruginosa* showed little sensitivity toward SMSC under both rich and starvation media conditions with the highest concentration tested of 2 mM unable to inhibit growth beyond 75% of the cell growth control.

Methylseleninic acid and sodium selenite did not cause any significant dose-response inhibition of *P. aeruginosa* in LB media at the concentrations tested. High concentrations of sodium selenite precipitated to red elemental selenium in *P. aeruginosa* LB assay conditions, likely contributing to the lack of inhibitory activity. This precipitation was not observed in M9 nutrient limited conditions and a dose dependant growth curve was produced but as with methylseleninic acid in M9 medium this dose-dependant growth inhibition did not inhibit *P. aeruginosa* completely at the highest concentration tested. These experiments indicate that Se-AAs do not exert significant inhibitory action towards *P. aeruginosa*.

5.3.1.1.3.3 *V. harveyi*

Vibrio harveyi (also known as *Listonella anguillarum*) is a Gram negative motile marine bacterium. This species is a pathogen in fish causing vibriosis, a disease that is financially damaging to the aquaculture industry.⁴⁸⁵ *V. harveyi* also shares several characteristics with the human pathogen and causative agent of cholera, *Vibrio cholerae*, for which recent emergence of drug resistance has been published.⁴⁸⁶ *V. harveyi* may be a relevant model in drug discovery for *V. cholerae*.

Of the three Gram negative bacterial species tested for sensitivity to Se-AA inhibition, *V. harveyi* demonstrated the greatest inhibitory response. This is reflected in the dose-response curves of growth inhibition for SMSC and Se-L-met (Figure 5.12) in which both compounds completely inhibited *V. harveyi* growth in LB medium at concentrations of 250 μ M. Se-L-met demonstrated this same level of inhibition in M9 minimal medium; however, *V. harveyi* showed reduced sensitivity to SMSC in M9 minimal medium since the MIC increased to 1 mM.

Methylseleninic acid and sodium selenite produced the expected inhibitory effects against *V. harveyi* with MIC values in LB of 500 μ M and > 2 mM, respectively (again precipitation of sodium selenite was observed at high concentrations). As observed in other bacterial species

a stronger inhibitory response was caused by these compounds in M9 minimal medium with a recorded MIC of 250 μ M for both these compounds.

In line with trends observed in other non-mycobacterial species of bacteria Se-AAs did not demonstrate strong inhibitory activity against the growth of *V. harveyi*, although *V. harveyi* was more susceptible to Se-AAs in LB medium than any other non-mycobacterial species tested. An MIC value of 250 μ M is beyond the range considered conducive to anti-bacterial drug discovery.

5.3.1.2 Anti-fungal activity

5.3.1.2.1 *S. cerevisiae*

Two strains of the yeast *S. cerevisiae* were tested against the selenium compound panel for growth inhibition, the haploid strain BY4741 and the diploid strain BY4743. Both haploid and diploid strains were tested as anti-fungal drug resistance levels may be dependant on the ploidy of yeast.⁴⁸⁷

Neither haploid nor diploid *S. cerevisiae* growth was not significantly inhibited by SMSC under rich SC medium or starvation medium conditions, with no measurable MIC being obtained for either strain under either media condition. Although a slight inhibitory dose-response was observed for both strains in SC medium, in nutrient limited SPO medium conditions, SMSC had a positive growth effect on both strains of *S. cerevisiae*. This effect was not observed in SPO medium only controls of SMSC testing; therefore, is either caused by increased growth of *S. cerevisiae* by utilisation of SMSC as a nutrient, or metabolism of SMSC into a derivative able to provoke reduction of resazurin.

The result of negligible toxicity of SMSC agrees with published reports of SMSC exhibiting no toxic effects upon *S. cerevisiae* at concentrations of up to 10 mM.⁴⁸⁸

In the present research, Se-L-met caused inhibition of *S. cerevisiae* growth under both strains and media conditions. Under rich SC medium conditions, both haploid and diploid yeast growth was completely inhibited at 125 μ M, a greater sensitivity than observed in other species tested with the exception of mycobacteria. Under SPO starvation medium conditions, diploid *S. cerevisiae* maintained the same sensitivity to Se-L-met as observed in SC medium; however, haploid *S. cerevisiae* was more sensitive since its MIC dropped two-fold to 62.5 μ M.

This result is at odds with two publications regarding the toxicity of Se-met towards *S. cerevisiae*.^{488, 489} In one publication Se-met is reported to be non-toxic at concentrations of 10 mM and below during both exponential and stationary phase growth.⁴⁸⁸ This may be due to a different strain of *S. cerevisiae* (SJR751) being used. Also the optical rotation of the Se-met used in this study is not defined, and a different media type (YPD medium) and assay method were utilised in this study.⁴⁸⁸ Conversely, a 2012 publication declares Se-met to have an MIC of 1 μ M towards *S. cerevisiae*. Again, this article does not mention the optical rotation of the Se-met used and utilised the different strain W303-1A and SC medium lacking methionine, this likely accounts for the high toxicity value reported.⁴⁸⁹ A published study using *S. cerevisiae* strain BY4741 report an IC₅₀ of 28 μ M for Se-met in YPD medium,⁴⁹⁰ very similar to the IC₅₀ of ~32 μ M observed in the dose-response of *S. cerevisiae* to Se-L-met in the present work.

Methylseleninic acid produced minor inhibition of growth in both strains of yeast under SC conditions, with an MIC only being achieved at the highest concentration tested for the diploid strain. Under nutrient-limited SPO conditions a resazurin reducing effect was observed at high methylseleninic acid concentrations, similar to that observed for SMSC in SPO medium conditions.

Sodium selenite caused a gradual dose-dependant inhibition of growth in haploid *S. cerevisiae* under rich SC medium conditions, and greater resistance to inhibition by sodium selenite was observed for the haploid strain cultured under SPO nutrient-limited medium. Diploid *S. cerevisiae* exhibited a steeper inhibitory dose- response in SC medium, and again were less inhibited under nutrient-limited conditions. These findings are in agreement with literature reports regarding the toxicity of sodium selenite toward *S. cerevisiae*.^{488, 490}

The results obtained in this work for selenium compound inhibition towards the growth of *S. cerevisiae* agree with the published literature results that SMSC is non-toxic and sodium selenite exhibits some toxicity toward *S. cerevisiae* growth. The results for Se-met in the literature are varied, either being the similar, higher or lower than toxicity than those found in the present work. However these discrepancies are likely attributed to differences in assay set up such as the *S. cerevisiae* strain, media and assay methodology used.

5.3.1.3 Mammalian cell line cytotoxicity

Se-AA cytotoxicity testing was kindly carried out by Rekha Veliyayi Murikoli and Dr. R. O'Toole. Three different mammalian cell lines were tested, promyelocytic human leukaemia (HL-60), murine macrophage (J-774) and green monkey kidney epithelial (Vero) cell lines.

The IC₅₀ result of $73.03 \pm 16.73 \mu\text{M}$ for the HL-60 cell line is in the range of published literature reports for HL-60 cell line toxicity of SMSC. For example SMSC has a published IC₅₀ of $85 \mu\text{M}$ produced by WST-1 cell proliferation assay and $165 \mu\text{M}$ by a Lac-DH release type assay.⁴⁹¹ The authors who obtained these results, conclude that SMSC demonstrates loss of metabolic activity through an apoptosis mechanism opposed to causing necrotic cell lysis.⁴⁹¹ The same study also found selenite to be approximately 10-fold more toxic toward HL-60 cells with an IC₅₀ of $15 \mu\text{M}$ by WST-1 and $18 \mu\text{M}$ by Lac-DH release, indicating a more necrotic mechanism of cell death.⁴⁹¹ It is noted that HL-60 cells, being a cancer line, may be more sensitive to the apoptotic action of SMSC, compared to other cell lines or whole organism *in vivo* toxicity.

Unfortunately inhibitory concentrations for SMSC towards J-774 macrophages could not be found by literature searching; however, published research has tested the toxicity of SMSC towards RAW 264.7 macrophages in MTT and Lac-DH release assays, and found it to have no effects on viability at the highest concentration tested of $50 \mu\text{M}$.⁴⁹² This agrees with the non-cytotoxic results obtained for SMSC toward J-774 macrophages in the present study.

The IC₅₀ value of SMSC and Se-DL-met toward Vero cells could not be determined from the maximum concentration tested of $100 \mu\text{M}$. This shows that Se-AAs have minimal toxicity toward this non-cancerous cell line. This result is similar to a published result of Se-met having only 34% inhibitory activity at $50 \mu\text{M}$ ⁴⁹³ however the Se-met used was a sodium salt that may account for the slight discrepancy between the results of this work and the publication of Carmelli *et al.*⁴⁹³ No prior published results of SMSC cytotoxicity toward Vero cells was found in the literature.

Se-L-met is reported to exhibit toxicity toward human leukaemia and murine lymphoma cell lines with an IC₅₀ of $90 \mu\text{M}$. Se-L-met toxicity was increased to IC₅₀ $40 \mu\text{M}$ by reduction of cell culture medium methionine content to $10 \mu\text{M}$.⁴⁹⁴ This study also reported that Se-DL-met is approximately half as toxic as Se-L-met, and Se-L-met at sub-inhibitory concentrations below $40 \mu\text{M}$ stimulated cell line growth and plating efficiency by 50%.⁴⁹⁴

5.3.2 Suitability of Seleno Amino Acids for Human Therapy

From the growth inhibition test results in this thesis of selenium compounds against a range of mycobacterial species, the Se-AAs, SMSC and Se-L-met, have been demonstrated to cause complete growth inhibition against most mycobacterial species *in vitro* (including *M. smegmatis*, *M. tuberculosis* H37Ra, *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551) at concentrations of 8 μ M – 31 μ M. The exception to this is *M. smegmatis* cultured in LB medium, this medium causes Se-AAs to lose their inhibitory potency. We can compare this with the SMSC IC₅₀ value of 73.03 ± 16.73 μ M obtained for the HL-60 cell line and that shows that SMSC demonstrates favourable selective inhibition towards mycobacteria over mammalian cell lines.

Other selenium compounds tested, sodium selenite and methylseleninic acid, did not produce such a consistently strong inhibitory response against mycobacteria and are therefore disregarded.

To predict the suitability of Se-AAs as anti-tubercular therapeutics, the following sections will evaluate results obtained from growth inhibition assays against microbial species, and compare these values to published toxicity results of Se-AAs towards human cell lines and *in vivo* animal models to determine the potential suitability of Se-AAs as anti-infective agents for human administration.

5.3.2.1 Activity towards mycobacteria

Se-AAs, with the exception of Seleno-DL-cystine, demonstrated good inhibition toward *M. smegmatis* in all media types except LB rich medium. The greatest inhibitory activity towards mycobacteria was observed for SMSC and Se-L-met, both demonstrating an MIC of 15.6 μ M or lower against *M. smegmatis* cultured in 7H9 and HdeB carbon and nitrogen-limited media. These two compounds were then further evaluated in other bacterial species.

Activity of both SMSC and Se-L-met was lower against the vaccine strain *M. bovis* BCG than in *M. smegmatis*. However, activity was retained in the more relevant model *M. tuberculosis* H37Ra at 15.6 - 31.25 μ M levels. Furthermore SMSC exhibited this activity level in *M. tuberculosis* H37Rv testing, although variation of MIC results for H37Rv was observed. Due to the consistency of MIC values for SMSC against other mycobacterial species, the initial IC₉₀ of 22.88 μ M obtained for *M. tuberculosis* H37Rv is assumed to be

correct. However; this requires validation by further re-testing, due to variation in the IC₉₀ values of SMSC as discussed in section 5.2.1.4.

The IC₉₀ value of 22.88 µM (5 µg/mL) towards *M. tuberculosis* H37Rv places the activity of SMSC at a similar level to other TB treatment drugs clarithromycin (MIC 8 µg/mL),¹⁹⁹ cycloserine (MIC 25 µg/mL),¹⁹⁹ pyrazinamide (MIC 6-50 µg/mL at pH 5.5)⁴⁹⁵ and thioridazine (MIC 10 µg/mL against *M. tuberculosis* ATCC 27294).⁴⁹⁶ All MIC values are against *M. tuberculosis* H37Rv unless otherwise stated. SMSC continued to demonstrate a high level of inhibitory activity when tested in the virulent and highly pathogenic strain *M. tuberculosis* CDC1551 with an IC₉₀ of 9.15 µM. This inhibitory activity is on par with not only the aforementioned TB treatment drugs but also capreomycin⁴⁹⁷ and kanamycin¹⁹⁹ that also have *in vitro* MIC values of 2 µg/mL against *M. tuberculosis* H37Rv.

Therefore, SMSC shows anti-mycobacterial activity levels potentially equivalent to several TB treatment drugs. This inhibitory activity likely extends to other Se-AAs such as Se-L-met, and other Se-AA derivatives may demonstrate greater anti-mycobacterial activity. SMSC and other Se-AAs require further re-testing to confirm their activity level toward pathogenic *M. tuberculosis* strains.

5.3.2.2 Activity towards other bacterial and fungal species

In contrast to the mycobacterial inhibition results, Se-AAs demonstrated little inhibitory activity towards other bacterial species tested, with high or unachievable MIC values. *S. aureus* was unable to be completely inhibited in nutrient rich or starvation media by both SMSC or Se-L-met at 2 mM, the highest concentration tested. Similar lack of inhibition was observed for *E. coli* that demonstrated no obtainable MIC in rich media at 2 mM, although complete inhibition was achieved in minimal media, it was at very high concentrations of 500 µM for SMSC and 2000 µM for Se-L-met. Likewise with *P. aeruginosa*, MIC values could not be obtained, with the exception of Se-L-met in rich media which had an MIC of 1000 µM. Se-AAs demonstrated better activity against *V. harveyi*, with the lowest MIC achieved being 250 µM. This is still considered; however, well outside the viable therapeutic range. Inhibition of the yeast *S. cerevisiae* was reasonable for Se-L-met in the range of 62.5-125 µM; however, SMSC was unable to cause complete inhibition at 2 mM.

These results indicate Se-AAs do not exhibit enough inhibitory activity towards *S. aureus*, *E. coli*, *P. aeruginosa*, *V. harveyi* or *S. cerevisiae* to be considered worth pursuing as antibiotic

drugs for the treatment of human infection caused by these or related microorganisms. Se-AAs evidently have a narrow spectrum of anti-bacterial activity, effectively inhibiting mycobacteria but no other Gram positive or Gram negative species tested. This is of interest as the inhibitory activity of Se-AAs may be specific to a mechanism unique to mycobacteria.

5.3.2.3 Mammalian cell line toxicity

SMSC was found to have an IC_{50} of 73.03 μ M against the human leukaemia HL-60 cell line. This agrees with published results of SMSC against the same cell line with IC_{50} results of 85 μ M by WST-1 cell metabolism assay.⁴⁹¹ These values are relatively low for a compound being considered for human therapy; however, it should be noted that Se-AAs such as SMSC have known anti-proliferative and apoptosis-inducing activities towards tumour cell lines, and are purported to be 1000-fold more inhibitory toward tumour cell lines than normal human fibroblasts.⁴²⁴

No inhibition was able to be recorded for SMSC against the J-774 murine macrophage cell line at the highest concentration tested of 250 μ M. Furthermore, it is stated in the literature that no adverse effects of SMSC towards viability of RAW 264.7 macrophages were observed at the highest concentration tested of 50 μ M.⁴⁹² SMSC (L) has a reported IC_{50} of 472 μ M and Se-L-met has a reported IC_{50} of 267 μ M against the V79 cell line (Chinese hamster lung fibroblasts).⁴⁹⁸

Se-met has been described as “essentially non-toxic” toward HepG2 human liver carcinoma and Hepa1-6 cell lines with an IC_{50} in excess of 10 mM; however, against primary murine hepatocytes, Se-met demonstrated an more sensitive IC_{50} of 30 μ M.⁴⁹⁹ Se-met was also found non-toxic at concentrations up to 1 mM against HCT-116 cells.⁵⁰⁰ Therefore the toxicity of Se-met and other Se-AAs is dependant on the cell line and culture conditions used, although it is generally thought that Se-AAs and other organic selenium compounds exhibit low levels of toxicity towards most cell lines,⁵⁰⁰ particularly those of non cancerous nature. For example, human fibroblasts with an IC_{50} of 1 mM⁴²⁴ and human keratinocytes displayed no cytotoxicity at 316.6 μ M Se-Met.⁵⁰¹ These low cell line toxicity values are encouraging for Se-AAs as anti-mycobacterial agents, but due to variation of cytotoxicity based on cell-type, the toxicity toward whole animal models must be taken into account to better determine the potential of Se-AAs as anti-microbials.

5.3.2.4 Animal model toxicity

A single oral administration of SMSC in mice had an LD₅₀ value of 14.6 mg/kg (95% confidence limits of 13.1-16.2).⁵⁰² It has been shown in mice that an orally administered single dose of 200 µg SMSC raises plasma Se to a peak concentration of 8.9 ± 0.2 µM after 1 h. At the highest plasma concentration achieved of 11.5 ± 0.2 µM after 28 days of daily oral administration of 200 µg SMSC, no adverse effects were observed.⁵⁰³ This plasma concentration is within the inhibitory concentration range of SMSC towards pathogenic strains of *M. tuberculosis*. For Se-met, the murine oral LD₅₀ has been determined to be 25.6 mg/kg (95% confidence limits of 22.6-28.6).⁵⁰⁴ For non-oral administration, Se-met toxicity in rats gave a LD₅₀ of 4.25 mg/kg for intraperitoneal injection,⁵⁰⁵ in mice, racemic Se-DL-met had an intravenous LD₅₀ of 8.8 ± 1.37 mg/kg.⁵⁰⁵

A study of female macaques administered Se-L-met in doses of 0-600 µg/kg/day for 30 days reports a maximum tolerated dose of 150 µg/kg/day, with the 300 µg/kg/day dose group exhibiting adverse side effects and premature death of two of the twenty animals in the highest 600 µg/kg/day dose group.⁵⁰⁶ Another study of pregnant macaques treated for 30 days with daily doses of 0-300 µg Se-L-met per kg bodyweight found at the highest doses of 150 and 300 µg/kg/day that plasma Se levels peaked at day 21 of treatment and significant adverse effects of selenium toxicity (anorexia and vomiting) were observed in the 300 µg/kg/day group.⁵⁰⁷ Nasogastric administration of Se-L-met in pregnant macaques caused limited developmental defects at low doses and embryonic and fetal death at higher doses of 300 µg/kg/day. In addition, teratogenesis of selenium has been demonstrated in non-mammalian species such as birds and fish.⁵⁰⁸ In pigs, ingestion of Se-DL-met caused the greatest uptake and retention of Se in blood/tissue compared to ingestion of sodium selenate feed or Se-accumulator plant material, but Se-DL-met ingestion also caused less toxicity in pigs than ingestion of sodium selenate or Se-accumulating plants.⁵⁰⁹

Therefore, in animal models, Se-AAs such as Se-Met can exhibit toxic effects at high concentrations such as 300 µg/kg/day; however, the incidence and extent of these toxic effects is dependant on the route of administration and the animal model used, with oral administration provoking less toxicity.

As covered in section 5.1.1, selenium has an established toxicity in humans (using inorganic selenium) in which the mean oral intake with no adverse effects observed is 800 µg per day

($\approx 15 \mu\text{g/kg}$), and a maximum safe daily intake $400 \mu\text{g}$ per day is recommended.³⁷⁷

A more recent human supplementation study used oral doses of selenium in the form of selenized yeast that contains organic forms of Se such as Se-AAs (predominately Se-met). Doses of $3200 \mu\text{g/day}$ of selenized yeast for 24 months provoked symptoms of Se toxicity; however, the incidence of these symptoms did not correlate to plasma Se concentration. No serious Se toxicity was observed at this dosage, and the authors suggest that the maximum safe daily intake of $400 \mu\text{g/day}$ may be exceeded for extended periods of time without serious toxicity concerns.⁵¹⁰ However, the European Food Safety Authority cautions the use of SMSC as a nutritional supplement at levels of $400 \mu\text{g/day}$, citing that there is insufficient research data to support that $400 \mu\text{g/day}$ SMSC is conclusively safe.⁵¹¹ In mammals excess selenium such as from Se-met supplementation is excreted through the breath, exposing lung tissue to volatile selenides compounds.⁵¹² Additionally, organ distribution studies following oral SMSC administration show accumulation preference for the lung, with SMSC accumulating in the lung tissue at higher concentrations than methylseleninic acid or sodium selenite,⁵¹³ and this lung accumulation is favourable in the context of treating pulmonary TB.

Se-AAs demonstrate anti-mycobacterial activity that is on par with several current TB treatment drugs. However the toxicology of Se-AAs have not yet been fully determined in humans, although experimental observations have set an initial maximum safe daily limit of $400 \mu\text{g/day}$ that is likely to be similar inhibitory plasma concentrations that are anti-mycobacterially effective. Further studies on Se-AA anti-mycobacterial activity using *in vivo* models are required to better determine how suitable Se-AA are for development as a human TB treatment.

5.4 Summary

Screening of chemical libraries under nutrient starvation conditions identified a novel class of anti-mycobacterial chemicals, the seleno-amino acids. These exhibit inhibitory activity against pathogenic *M. tuberculosis* strains at levels similar to several current TB treatment drugs. Se-AAs did not have any significant inhibitory activity towards other bacterial species tested and exhibited less inhibitory effect toward human cell lines relative to mycobacteria. Se-AAs are widely marketed and consumed as nutritional supplements and have research implications for treatment of various pathologies such as cancer. They are generally considered to have low toxicity in humans; however, this requires further investigation. Considering that Se-AA human toxicity and anti-bacterial activity may both be further improved by combination with other drugs or modification of the Se-AA chemical structure and delivery method, Se-AAs represent a promising novel class of tuberculosis treatment that warrants further investigation and development.

CHAPTER SIX

Seleno-Amino Acid Structure-Activity Relationship and Mechanism of Action

6.1 Introduction

6.1.1 Seleno-amino acid biological activity

Seleno-amino acids demonstrate inhibitory activity towards mycobacteria typically in the 8-30 μ M MIC range. This inhibitory activity seems to be exclusive to mycobacterial species, with other bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa* and *V. harveyi* displaying little or no sensitivity to growth inhibition by Se-AAs. Eukaryotic species such as *S. cerevisiae* and mammalian cell lines are less inhibited by Se-AAs than mycobacterial species.

- **The aim of this chapter is to investigate Se-AA structure-activity relationship and anti-mycobacterial mechanism, in order to better understand Se-AAs for development as human tuberculosis treatment candidates.**

6.1.2 Differential activity and media conditions overview

Based on the results of dose-response growth inhibition testing detailed in Chapter 5.2 it is observed that Se-AAs show differential inhibitory activity based on the bacterial species being tested and also the media conditions in which they are cultured.

6.1.2.1 Se-AA activity toward bacterial species overview

Mycobacterial model species *M. smegmatis* displayed great variation in its sensitivity to growth inhibition by Se-AAs based on the media type used in the dose-response assay. Using rich LB medium, *M. smegmatis* was resistant to complete growth inhibition by Se-AAs, methylseleninic acid and sodium selenite at concentrations up to 2 mM. However in culture in rich 7H9 medium, HdeB carbon- or HdeB nitrogen-starvation media caused complete inhibition of *M. smegmatis* growth at concentrations of 7.8-15.6 μ M for SMSC and Se-DL-met. Methylseleninic acid and sodium selenite also showed complete inhibitory activity in these media types but not to the same extent as Se-AAs (see Table 5.1).

Therefore, there is a clear trend in *M. smegmatis* culture conditions and the inhibitory activity of the Se-AAs, culturing mycobacteria in the complex LB rich medium negates the inhibitory activity of these organoselenium compounds.

Slow growing mycobacterial species and vaccine strain *M. bovis* BCG cultured in 7H9 medium were less sensitive to inhibition by Se-AAs with MICs in the range of 31.25 - 62.5 μ M. This strain was also less sensitive to inhibition by methylseleninic acid 125 - 62.5 μ M. The attenuated, slow growing *M. tuberculosis* H37Ra strain cultured in 7H9 medium

displayed similar sensitivity to inhibition by Se-AAs and methylseleninic acid as *M. smegmatis* cultured in 7H9, with MIC values of 15.6-31.25 μ M. The use of a nutrient limited 7H9 medium produced inconsistent results, but generally *M. tuberculosis* H37Ra cultured in the nutrient-limited 7H9 medium were more sensitive to inhibition by Se-AAs.

With regards to non-mycobacterial species, the use of nutrient starvation media had little impact on the activity of Se-AAs. *S. aureus* showed no sensitivity to Se-AAs at the 2 mM concentration tested in either nutrient-rich LB or nutrient-limited M9 medium. *E. coli* demonstrated some differential sensitivity to SMSC, with the MIC being unobtainable in LB medium but dropped to 500 μ M in nutrient limitation M9. Conversely, *P. aeruginosa* was more sensitive to Se-L-met in rich LB medium, with an MIC of 1 mM, and no MIC could be obtained in M9 medium. Of all the non-mycobacterial species of bacteria tested, *V. harveyi* showed the most sensitivity to Se-AA inhibition with MIC values of 250 μ M for both SMSC and Se-L-met in LB medium; however, in nutrient-limited M9 medium the MIC for SMSC increased to 1 mM. It is unclear why *P. aeruginosa* and *V. harveyi* are more sensitive to Se-AA inhibition in rich LB media conditions; however, as the inhibitory activity of Se-AAs towards these and other non-mycobacterial species is low this was not considered to be a priority of the present research.

6.1.2.2 Media conditions

The major influential factor on *M. smegmatis* sensitivity to seleno-compound inhibition is the type of culture medium used, specifically, LB medium culture confers resistance to inhibition of *M. smegmatis* growth by Se-AAs. Use of an improvised 7H9 nutrient starvation media with reduced carbon source (glycerol) and OADC enrichment (section 2.2.1.2.4) had a slight effect on the sensitivity of slow growing mycobacterial species to Se-AA inhibition; however, this effect was marginal (one serial dilution) and inconsistent.

An obvious key difference between LB medium, in which Se-AAs are practically inactive, and the 7H9 or HdeB media, in which the Se-AAs have inhibitory activity, is the tryptone and yeast extract content of LB. Tryptone is an enzymatic digest of casein and is rich in amino acids and oligopeptides. Yeast extract is also rich in free amino acids. Both 7H9 and HdeB media do not contain any free amino acids or oligopeptides; the closest constituent of 7H9 to amino acids is albumin, present in the OADC enrichment.

It is known that cysteine and methionine are both capable of negating Se-AA inhibition in

human cell lines,⁴⁹⁴ this was confirmed in mycobacteria in this research (section 6.4.1).

Therefore, it is hypothesised that the cysteine and/or methionine content of LB is responsible for blunting Se-AA inhibitory activity in LB medium. This may take place at the transporter itself, where these amino acids compete with the Se-AAs for import into the cell.

Alternatively, cysteine and/or methionine may compete with Se-AAs somewhere later in their metabolism or during incorporation into proteins. This hypothesis is investigated in section 6.4.1.

6.2 Seleno amino acid pharmacophore

For this study, Se-cys is used as the basic theoretical scaffold of the Se-AAs. SMSC is derived by methylation of the selenium of Se-cys. An additional side chain carbon to SMSC at the γ position results in Se-met. Dimeric Se-cys joined by a diselenide bond results in seleno-cystine. These modifications of the Se-cys structure encompass the commonly available Se-AAs used in this work.

The non-selenated amino acids DL-cysteine and DL-methionine are identical to their Se-AA counterparts with the exception of a sulphur atom in place of the selenium. S-methylcysteine is the non-selenated equivalent of SMSC, having a methyl group attached to the sulphur; similarly, S-ethylcysteine has an ethyl group attached to the sulphur of cysteine.

Unfortunately ethyl-selenocysteine could not be obtained for testing in this research.

Some non-amino acid selenium compounds bear some structural similarity to the Se-AAs, the closest of which is methylseleninic acid that shares a common methylated selenium with SMSC and Se-met. However, methylseleninic acid does not have amine or carboxylic acid groups, instead sporting a seleninic acid functional group, the selenium equivalent of sulfinic acid. Sodium selenite is similar again to methylseleninic acid but has substituted the methyl group for a hydroxyl group (in solution).

Other non-amino acid selenium compounds bear little structural resemblance to Se-AAs.

Seleno-urea, for example, has a primary amine group but also a primary ketimine.

Benzeneselenic acid-containing seleno-compounds have the seleninic acid moiety of methylseleninic acid but also have the steric bulk of benzene substituted in place of the methyl group. Compounds such as diphenylselenide and ebselen have almost no structural resemblance to Se-AAs in their native form, except for the common selenium atom.

6.2.1 Seleno-compound structure and activity in *M. smegmatis*

Seleno-compounds used in this research had a wide range of inhibitory activity toward mycobacteria. The relationship between seleno-compound structure and inhibitory activity is assessed in Table 6.1, using *M. smegmatis* MIC in 7H9 medium, as all compounds were assayed in this model organism and inhibitory activity of *M. smegmatis* in 7H9 medium is well correlated with inhibition observed in *M. tuberculosis*.

Table 6.1: Seleno-compound structure and anti-mycobacterial activity

Compound	<i>M. smegmatis</i> MIC 7H9 (μM)	Chemical structure*
Seleno-Amino Acids		
Se-methylseleno-DL-cysteine (SMSC)	15.6
Seleno-DL-cysteine (Se-cys)	50
Seleno-DL-methionine (Se-met)	31.2
Seleno-L-methionine (Se-L-met)	15.6
Seleno-DL-cystine	250
Sulphur-Amino Acids		
DL-cysteine	> 2000

Compound	<i>M. smegmatis</i> MIC 7H9 (μM)	Chemical structure*
DL-methionine	> 2000
s-methyl-L-cysteine	> 2000
s-ethyl-L-cysteine	> 2000
Other selenium compounds		
Sodium selenite	500
Diphenyl-selenide	1000
Benzeneseleninic anhydrous	500
Benzeneseleninic acid	500

Compound	<i>M. smegmatis</i> MIC 7H9 (μM)	Chemical structure*
4-chloro benzeneseleninic acid	2000
Methylseleninic acid	31.2
Seleno-urea	> 2000
1,1-dimethyl-2-selenourea	2000
Ebselen	62.5

Table 6.1: Seleno-compound structure and anti-mycobacterial activity.

Organic and inorganic selenium compounds are presented, including Se-AAs and their equivalent sulphurous amino acids with corresponding inhibitory activity toward *M. smegmatis* under 7H9 media conditions.

*Images obtained and modified from NIH PubChem database.

For Se-AAs, the greatest anti-mycobacterial activity was observed for the methylated SMSC and Se-met. The addition of a methyl group to the selenium of Se-DL-Cys, to form SMSC, results in a greater than 2-fold increase in the inhibitory activity towards *M. smegmatis* in 7H9 conditions.

Figure 6.1: Seleno-compound key functional groups.

Seleno-compound panel chemicals and key structural characteristics for activity. Selenium is highlighted in yellow, the methyl group in red, side chain carbons in green, primary amines in teal and carboxylic acid in blue.

From the anti-mycobacterial testing carried out in Chapter 5.2, several key structural characteristics that influence the inhibitory activity of seleno-compounds were observed, these are illustrated in Figure 6.1. The most vital of these was the selenium atom itself, as non-seleniferous amino acids DL-cysteine and DL-methionine do not demonstrate any anti-mycobacterial activity at the concentrations tested. Furthermore, methylated and ethylated derivatives of DL-cysteine also fail to show inhibitory activity towards *M. smegmatis*. This is contradictory to earlier publications describing the inhibitory action of compounds such as S-methylcysteine toward mycobacterial species including *M. smegmatis*; however, these publications are based on *in vivo* experimental models and this may account for the discrepancy.^{476, 477, 514} Considering the lack of activity of the sulphurous equivalents of SMSC and Se-met, it is concluded that the selenium is an essential component for Se-AA anti-mycobacterial activity.

The methyl group directly bonded to the selenium is not vital for Se-AA activity. This is evidenced by Se-DL-cys showing 50 μ M MIC toward *M. smegmatis* in 7H9 medium. However, the presence of methyl-selenium increases the activity of Se-AAs towards mycobacteria, as SMSC has an MIC of 15.6 μ M in *M. smegmatis* 7H9 culture, representing a

greater than 3-fold increase in anti-mycobacterial activity attributed solely to methylation of the selenium. Additionally, Se-met also sports methyl-selenium and demonstrates a high level of anti-mycobacterial activity with an MIC of 15.6 μM . Methylation of selenium is confirmed in the literature as a contributor to selenium toxicity.⁵¹⁵ However, in the case of the non-amino acid selenium compound methylseleninic acid, the methyl group appears to be vital for anti-mycobacterial activity, as sodium selenite shares common hydroxyl and selenoxide groups with methylseleninic acid but lacks methylation of the selenium, consequently resulting in sodium selenite having 15-fold less inhibitory activity than methylseleninic acid.

A two-fold increase in anti-mycobacterial activity is observed for the pure levo stereoisomer of Se-met over the racemic DL mixture. Stereospecific activity has also been observed in other studies where L-enantiomers are more readily assimilated in humans,⁵¹⁶ and also exhibit greater glutathione peroxidase activity and inhibition of V79 cell lines.⁴⁹⁸ It is hypothesised that levo rotation of Se-AAs is responsible for anti-mycobacterial activity, with the dextro form having little or no anti-mycobacterial activity. This is evidenced by a halving of anti-mycobacterial activity for Se-DL-met relative to Se-L-met; however, further validation using Se-D-met and other Se-AAs is required to confirm the hypothesis. This may be caused by preferential uptake of levo enantiomers, exclusive incorporation into proteins or stereospecificity-dependant enzymatic or metabolic interactions.

Increasing the number of side chain carbons from two (SMSC) to three (Se-met) appeared to have a minor negative impact on anti-mycobacterial activity, as the (DL)-SMSC has an MIC of 15.6 μM toward *M. smegmatis* cultured in 7H9 media and Se-DL-met has an MIC of 31.2 μM . Thus, addition of a carbon at the γ position of the amino acid side chain slightly reduces anti-mycobacterial activity.

Carboxylic acid and primary amine moieties are defining characteristics of amino acids. It is thought that Se-AA anti-mycobacterial activity could be dependant on either or both of these constituent groups. This hypothesis remains to be tested, but it is observed that methylseleninic acid retains anti-mycobacterial activity, and it does not contain either carboxylic acid or amine groups. Although methylseleninic acid has no amine, hydroxyl and selenoxide groups attached to the selenium are similar substituents to carboxylic acid, and perhaps either or both structurally compensate for the lack of a carboxylic acid moiety. Seleno-urea on the other hand has a primary amine but no carboxylic acid group, and lacks

anti-mycobacterial activity towards *M. smegmatis* in 7H9 medium. Although seleno-urea has a ketimine functional group and also lacks methylation of the selenium, this may contribute to its lack of activity.

Se-DL-cys does not exhibit a high level of anti-mycobacterial activity, with an MIC in *M. smegmatis* of 50 μ M in 7H9 medium. However, seleno-cystine, the dimeric form of Se-DL-cys has a 5-fold reduction in activity compared to Se-DL-cys. This indicates the diselenide bond that links the two Se-DL-cys to form seleno-cystine impedes anti-mycobacterial activity or is not efficiently metabolised by mycobacteria *in vitro*. Furthermore, dimerisation of Se-DL-cys causes a reduction in activity either through reducing chemical solubility,⁵¹⁷ and the resulting lowered mycobacterial uptake.

In summary from the limited anti-mycobacterial activity testing that has been performed several structural influences upon seleno-compound activity have been observed. Most important is the presence of the selenium, which is indispensable for anti-mycobacterial activity. Levo stereochemistry increases inhibitory activity toward mycobacteria two-fold over racemic dextro/levo mixture. Levo stereochemistry may be essential for activity of Se-AAs. It is uncertain if the carboxylic acid and primary amine functional groups are essential for activity, although the carboxylic acid or similar moiety appears more influential on anti-mycobacterial activity. Methylation of the selenium is not a vital component of the anti-mycobacterial activity of Se-AAs, but methylation significantly enhances activity.

Conversely, dimerisation by a diselenide bond severely reduces Se-AA activity. Increasing the number of carbons in the amino acid side chain evidently results in a minor reduction in activity.

6.2.2 Seleno-amino acid derivatives

Several chemical derivatives of Se-AAs were kindly synthesised by Zaid Amso and Dr. Viji Sarojini.⁵¹⁸ These were alanine dipeptide conjugates of Se-L-met, as alanyl- conjugates are thought to have increased uptake into the mycobacterial cell,⁵¹⁹ and also alanyl-methionine dipeptides were shown to have 2.5-fold increased methanethiol production by *Brevibacterium linens* compared to L-methionine.⁵²⁰ Also included were arginine dipeptide conjugates, with positively charged arginine⁵²¹ believed to have greater affinity and penetration of the negatively charged mycobacterial cell wall.^{522, 523} Mycobacteria also have a predilection for arginine uptake with several arginine importing transporters, believed to functionally contest host macrophage arginine uptake to diminish macrophage nitric oxide mediated attack.⁵²⁴ Arginine, when included as an adjuvant in TB therapy, has also shown to improve clinical

outcomes.⁵²⁵ Finally, amide derivatives of Se-L-met and the aforementioned dipeptides, as the amide derivative of methionine, L-methioninamide, was shown to have a 4.7-fold increase in methanethiol production compared to L-methionine in *B. linens*.⁵²⁰ It is possible this phenomenon may extend to increased methylselenol production, and anti-mycobacterial activity, from the selenium equivalents of these derivatives in mycobacteria. Derivatives were tested for their inhibitory activity towards *M. tuberculosis* H37Ra in a 7H9+ medium dose-response assay, with Se-L-met, SMSC and frontline TB drug controls.

Derivative	Chemical Name	<i>M. tuberculosis</i> H37Ra MIC (μM)	Structure
Sem001	Ala-SeMet	250	<p>Ala-SeMet</p>
Sem003	Ala-SeMet-NH2	250	<p>Ala-SeMet-NH2</p>
Sem005	SeMet-Ala	1000	<p>SeMet-Ala</p>
Sem006	SeMet-Arg	125	<p>SeMet-Arg</p>
Sem007	SeMet-NH2	125	<p>SeMet-NH2</p>

Table 6.2: Seleno-amino acid derivatives.

Derivatives of Se-L-met synthesised by Zaid Amso and Dr. Viji Soarajini.⁵¹⁸ The derivatives internal name, its chemical name, the MIC in μM against *M. tuberculosis* H37Ra in 7H9 medium and the chemical structure diagram are presented. Note Sem002 and 004 were unpurified extracts of Sem001 and 003, respectively; thus, are not included in this table.

None of these derivatives showed increased inhibitory activity over the parent Se-L-met; but nonetheless, they provide additional valuable insight into the structure-activity relationship of the Se-AAs, discussed below.

6.2.2.1 Seleno-amino acid derivatisation influence on anti-mycobacterial activity

Alanine dipeptides both decrease MIC activity to 250 μM (Ala-SeMet) and 1000 μM (SeMet-Ala). This is a large contrast in activity that is dependant on the conjugation terminus.

Substitution of the c-terminal amide seemed to have no impact here, possibly indicating uptake rate limitation before lyase cleavage, as the SeMet-NH₂ itself also exhibited diminished activity. The SeMet-Arg dipeptide showed less reduction of activity, with an MIC of 125 μ M. This is much improved over the SeMet-Ala and is a favourable indication for the positively charged arginine derivative. Perhaps the opposite Arg-SeMet could be worth pursuing considering the trend in activity exhibited by the alanyl c- and n- terminal conjugations. The amide SeMet-NH₂ also showed a reduction in activity, although a lesser reduction to 125 μ M. The results encountered by Ferchichi *et al*⁵²⁰ unfortunately did not cross over to our to our anti-mycobacterial assay as hoped. Either SeMet-NH₂ is not as efficiently recognised for uptake, or is not as efficiently subjected to lysis of methylselenol, either because of recognition and degradation by other enzymes or lowered affinity to the lyase. This indicates that the carboxylic acid group could be vital for mycobacterial prodrug recognition and cleavage. Further considerations for Se-AA derivatives for increased anti-mycobacterial activity are discussed in Chapter 7.4.7.

6.3 Seleno-amino acid mechanism of action

Seleno-amino acids are known in the literature for various properties, detailed in Chapter 5.1.4. The main current research interest for Se-AAs is in their use in the reduction of cancer incidence, termed ‘chemoprevention’. A mechanism for Se-AA chemoprevention involving methylselenol has been proposed. Potentially, the anti-mycobacterial activity of Se-AAs may take place through a similar mechanism.

6.3.1 Seleno-amino acid anti-neoplastic mechanism

Supra-nutritional selenium supplementation, in the form of Se-AAs such as Se-met, is associated with reduced incidence of colon, lung and prostate cancer,⁴²⁶ including a 63% reduction in prostate cancer incidence.⁴²⁷ Selenium demonstrated protective effects against liver cancer⁵²⁶ and improved breast cancer outcomes.⁵²⁷ Results of the SELECT selenium and vitamin E cancer prevention trial showed that neither selenium nor vitamin E significantly prevented prostate cancer; however, this has been attributed to limitations in the study population.⁵²⁸ Recent meta-analysis of 12 distinct studies observed that an increasing plasma selenium concentration correlates with a decreased incidence of prostate cancer.⁵²⁹ Another meta-analysis of 49 observational studies and six randomised controlled trials also observed an inverse correlation between selenium supplementation and prostate cancer incidence; however, this was not conclusive and further trials are required.⁵³⁰

The chemopreventative mechanism of seleno-compounds has been proposed to involve apoptosis caused by reactive oxygen species (ROS) generated from selenium radicals.⁵³¹ Free radicals are independent chemical species with an unpaired electron.⁵³² ROS is a general term referring to oxygen radicals including superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$) and non-radical oxygen compounds such as hydrogen peroxide (H_2O_2).⁵³²

Figure 5.2 shows that the generation of selenol and selenide compounds such as methylselenol and hydrogen selenide are central to selenium compound metabolism. These volatile seleno-compounds are derived from both inorganic selenium metabolism and also metabolic lysis of organo-selenium compounds, such as Se-AAs, by β and γ -lyase enzymes.^{414, 533} Methylselenol and hydrogen selenide are convertible via methyl transferase and demethylase enzyme activity.⁴⁰⁹ These volatile selenium species are pivotal steps in excretion of selenium in mammals.^{401, 534}

The oxidising action of selenol and selenide species on cellular glutathione and other reduced thiols causes the continuous cyclical generation of ROS such as superoxide $O_2^{\bullet-}$, and subsequent formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\bullet}OH$) as shown in Figure 6.2. These ROS in turn cause cellular stress through damage to DNA, proteins and membranes, resulting in a cellular apoptotic response.^{531, 534} Methylselenol displays stronger inhibitory properties towards cancer cells than to non-cancer cell lines, thought possibly due to differential expression of cancer associated genes such as mitogen-activated protein kinases and c-Myc.^{433, 533} Various selenium compounds are known to induce superoxide generation *in vitro* through the glutathione mechanism, including selenite, selenocystine and methylseleninic acid. In contrast, seleno-amino acids such as Se-met and SMSC do not generate superoxide *in vitro* through glutathione.⁵³⁵ However, addition of methioninase, a γ -lyase enzyme, to the *in vitro* superoxide generation assay results in superoxide generation from Se-met. Superoxide generation is not observed for sulphurous methionine or SMSC.⁵³⁵ This indicates that selenium is vital for the catalytic mechanism of superoxide generation, since normal sulphur methionine does not generate appreciable superoxide. Although superoxide is not produced by γ -lyase activation on SMSC, this is expected as there is no γ -position carbon in SMSC. Thus, SMSC is not a feasible substrate for γ -lyase. However, it is thought that *in vivo*, similar methylselenol generation occurs from SMSC by action of a β -lyase enzyme.^{533, 535, 536}

Figure 6.2: Redox cycling of methylselenium species.

Methylselenol (CH_3SeOH) forms an adduct with glutathione or other free reactive thiols (GSH) and methylselenide anion (CH_3Se^-) is formed by oxidation of this adduct to form glutathione disulphide (GSHGSH). Methylselenide (CH_3Se^-) reacts with molecular oxygen to form superoxide anion ($\text{O}_2^{\bullet-}$) that reacts with water to form other ROS such as hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet\text{OH}$) that cause cellular damage and may also react with methylselenide (CH_3Se^-) to form methylselenol (CH_3SeOH) and begin the cycle anew. The methylselenide radical ($\text{CH}_3\text{Se}^\bullet$) is recycled to methylselenide (CH_3Se^-) either by reduction by cellular glutathione or thiols (GSH) or by dimerisation to dimethyldiselenide ($\text{CH}_3\text{SeSeCH}_3$). Dimethyldiselenide is also reduced by glutathione forming a glutathione-methylselenol adduct. Adapted from Spallholz *et al* (2004)⁵³⁵ and Chaudiere *et al* (1992)⁵³⁷.

In summary, selenium compounds are generally thought to exert anti-cancer effects through generation of reactive oxygen species, causing apoptosis. Se-AAs act as pro-drugs that are cleaved by lyase enzymes into their active methylselenol form that reacts with cellular glutathione and other free reactive thiols to cyclically generate reactive oxygen species.

6.3.2 Seleno-amino acids as anti-mycobacterial agents

The 2009 publication of this research is the first and currently only public disclosure of the anti-mycobacterial properties of Se-AAs.¹²² Additional to this publication and the further analysis of Se-AAs as anti-mycobacterials in this thesis, there is some isolated evidence supporting selenium supplementation reducing incidence or improving outcomes of pulmonary TB. For example, a controlled study of HIV-infected drug users associated patient selenium status with a significantly lower incidence of mycobacterial disease, with plasma selenium levels < 135 µg/L having 13x higher probability of developing mycobacterial disease compared to matched controls.⁴⁵⁵

Supplementation of vitamin A, B complex, C, E and selenium in patients undergoing treatment for pulmonary TB (with and without HIV) reduces the overall risk of TB recurrence by 45%.⁵³⁸ Low plasma selenium is associated with physical wasting and health decline in TB, pulmonary TB risk and TB disease progression.⁵³⁹⁻⁵⁴²

Selenium supplementation is known to contribute to TB patient anti-oxidant status and reduce oxidative stress, a by-product of the immune response to TB infection that provokes inflammation, leading to tissue damage such as pulmonary fibrosis.⁵⁴³

It remains unclear if patient selenium depletion is a cause or consequence of TB infection, but in any case, high patient selenium status appears to be associated with improved disease outcomes, reduced disease incidence and reduced disease-associated symptoms. Further trials are required to specifically investigate this association. In light of Se-AAs being shown in this research to have direct anti-mycobacterial activity *in vitro*, it is possible that patient selenium supplementation actively contributes to inhibiting *M. tuberculosis* infection *in vivo*, in addition to having positive effects such as improving immune function and reducing the inflammatory response.

6.3.2.1 Proposed mechanism of Se-AA anti-mycobacterial activity

Considering the known chemopreventative / anti-neoplastic mechanism of action known to occur with seleno-compounds such as Se-AAs (detailed above in section 6.3.1), it is likely that the anti-mycobacterial action of Se-AAs occurs through a similar mechanism. The 2009 publication of this work proposed that SMSC exerted its anti-mycobacterial effects by enzymatic cleavage into pyruvate, ammonia and the toxic metabolite methylselenol.¹²² While this putative mechanism is not considered incorrect, it is considered incomplete in light of further literature research, particularly concerning the toxic properties of methylselenol as detailed in Figure 6.2.

The anti-mycobacterial activity of Se-AAs is absent in rich LB medium, containing complex nutrients such as amino acids. It is likely that Se-AAs are recognised as amino acid nutrients and are indiscriminately transported into the cell. The Se-AA pro-drug is then cleaved into its active methylselenol form by the action of mycobacterial lyase enzymes. Methylselenium species are then redox cycled in a similar fashion as displayed in Figure 6.2, generating damaging oxygen radical by-products that cause damage to mycobacterial cell components including DNA, proteins and lipids.⁵⁴⁴ This three-step hypothesis is described below, and illustrated in Figure 6.3.

- 1) Se-AA pro-drug is recognised as a nutrient and transported into mycobacterial cells.**
- 2) Se-AA pro-drug is cleaved into active methylselenium species and other metabolites by mycobacterial lyase enzymes.**
- 3) Methylselenium species are redox cycled by mycobacteria cellular processes that generate damaging reactive oxygen species.**

It is not known why Se-AAs do not exhibit inhibitory activity toward other bacterial species, even in nutrient limited conditions. However, this lack of broad spectrum activity indicates Se-AAs may act via a specific mycobacterial enzyme or process.

Figure 6.3: Hypothetical three-step mechanism of Se-AA anti-mycobacterial activity.

1. Se-AAs such as Se-met (pictured) are recognised as nutrients by mycobacterial amino acid transporters and imported into the cell. **2.** Se-AA is cleaved by mycobacterial lyase enzyme. Pictured is Se-met lysed by methionine γ -lyase, producing α -ketobutyric acid, ammonia and methylselenol. Other Se-AAs are lysed by alternative lyase enzymes such as β -lyase and produce different metabolites although methylselenol is conserved. **3.** Methylselenol undergoes redox cycling that produces ROS that cause damage to mycobacterial cell components such as DNA, proteins and lipids.

6.3.2.1.1 Missing link in methylselenium species-induced toxicity

Mycobacteria do not synthesise or utilise glutathiol; thus, the redox cycling of methylselenium compounds and subsequent generation of ROS and anti-mycobacterial activity may be attributed to a similar functional chemical or enzyme. One candidate for this mycobacterial glutathione stand-in is mycothiol, the major low molecular weight thiol of *Actinobacteria* that serves a homologous redox maintenance function to glutathione in eukaryotes and other bacteria.⁵⁴⁵ Alternatively, a redox-homeostasis protein such as a

thioredoxin, oxidoreductase or peroxidase type enzyme may be responsible for the redox cycling of methylselenium.

Mycothiol is known to have a protective effect against reactive oxygen and nitrogen species, in addition to detoxifying alkylating agents and antibiotics.⁵⁴⁶ Therefore, it is of no surprise that mycothiol is essential for *M. tuberculosis* growth,⁵⁴⁷ considering the intracellular nature of the *M. tuberculosis* pathogen and its frequent exposure to oxygen and nitrogen radical attack by the immune system.⁵⁴⁸ However, in addition to these protective functions, evidence suggests mycothiol plays a role in the activity of ethionamide and isoniazid, as mycothiol-deficient mutants exhibit resistance to these nicotinamide pro-drugs.⁵⁴⁹ Considering the typical protective role of mycothiol against free radical stress, the redox cycling of methylselenol by mycothiol and subsequent production of ROS toxicity appears contrary to the typical function of mycothiol. This indicates that additional factors may possibly be involved in the anti-mycobacterial mechanism of Se-AAs. These may include as mycothiol-dependant peroxidase or thiol transferase enzymes,⁵⁵⁰ or an alternative redox maintenance system to mycothiol such as thioredoxin, peridoxin or a combination of any of the above.

6.3.2.1.2 Testing the proposed Se-AA mode of action

In order to test the three components of the Se-AA mechanism of action hypothesis (detailed in Figure 6.3), a variety of experiments were carried out.

To determine if Se-AAs were recognised by mycobacteria as their sulphur analogue amino acids and imported into the cell as nutrients, a competition assay was performed.

To query the enzymatic lysis and pro-drug activation of Se-AAs by mycobacterial β - and γ -lyase enzymes, differential sensitivity assays using anti-sense RNA down-regulation of suspect lyase gene (identified through bioinformatic analysis detailed in section 6.5.2.1) expression was carried out. Additionally, *S. cerevisiae* strains with deleted genes homologous to mycobacterial lyases were also tested for differential susceptibility to Se-AAs. *M. tuberculosis* homologues of aforementioned suspected lyase enzymes were also purified and assayed *in vitro* for utilisation of Se-AA substrates.

Finally to question the final step in the proposed mechanism of action of Se-AAs, random transposon mutants resistant to Se-AAs were generated in the hope of elucidating key enzymes and associated metabolic pathways involved in the redox cycling of methylselenium compounds.

6.4 Results

6.4.1 Seleno-amino acid uptake - competition assays

Rescue assays were performed as in Chapter 2.2.4 using *M. tuberculosis* H37Ra in 7H9 and *M. smegmatis* in HdeB media. DL-cys and DL-met were supplemented in equimolar amounts to Se-AAs.

Figure 6.4a: SMSC toxicity is alleviated by equimolar amounts of DL-cys or DL-met.

M. tuberculosis inhibition by SMSC is negated by supplementation with DL-cys or DL-met. Lines are colour-coded as depicted below, with circles representing SMSC and DL-cys and triangles representing combinations of SMSC and sulphur amino acids.

Figure 6.4b: Se-L-met toxicity is alleviated by equimolar amounts of DL-cys or DL-met.

M. tuberculosis inhibition by Se-L-met is negated by supplementation with DL-cys or DL-met. Lines are colour-coded as depicted below, with circles representing Se-L-met and DL-met and triangles representing combinations of Se-L-met and sulphur amino acids.

These dose-response curves show that either cysteine or methionine supplementation is able to alleviate toxic effects of SMSC or Se-L-Met against *M. tuberculosis* H37Ra. Similar results were observed for *M. smegmatis* cultured in HdeB C- and N- media conditions (results not shown). This non-specific sulphurous amino acid rescue effect indicates that Se-AAs compete with sulphurous amino acids at the amino acid transporter level, and may also

compete specifically at the lyase level.

6.4.2 Seleno-amino acid lyase pro-drug activation

6.4.2.1 Anti-sense RNA gene down-regulation using pMIND_2357

M. smegmatis pMIND_2357 was generously supplied by Dr. S. Nisa. pMIND_2357 is a tetracycline inducible vector that contains the MSMEG_2357 cysteine desulphurase gene in the anti-sense orientation. Tetracycline induction of this vector causes down-regulation of MSMEG_2357 expression.¹⁶² *M. smegmatis* pMIND_2357 exhibits similar growth and fitness characteristics to wildtype *M. smegmatis* and was used as an anti-sense control strain in screening for inhibitors of ParA mycobacterial chromosomal partitioning protein.¹⁵⁷

Initially the *M. smegmatis* pMIND_2357 strain was queried with a dose-response assay to SMSC compared to the screening strain *M. smegmatis* pSHIGH+HSP60 as per methodology described in Chapter 2.2.5.

Figure 6.5: Anti-sense knockdown of MSMEG_2357 increases SMSC resistance.

Anti-sense down-regulation of the cysteine desulphurase MSMEG_2357 using the pMIND vector increases the MIC of SMSC towards *M. smegmatis* 8-fold in HdeB C- medium and 32-fold in HdeB N- medium over the control vector strain pSHIGH+HSP60.

Clear differential sensitivity was observed between *M. smegmatis* pMIND_2357 and the control strain *M. smegmatis* pSHIGH+HSP60. Down-regulation of MSMEG_2357 cysteine desulphurase resulted in increased resistance to SMSC, with an 8-fold MIC increase in HdeB C- medium and a 32-fold increase in HdeB N- medium.

6.4.2.2 Anti-sense RNA gene down-regulation using pKW08 vector

pKW08 is a mycobacterial tetracycline inducible expression vector, similar to pMIND with a different vector backbone and improved tetRO promoter.¹⁸⁸ pKW08 was generously provided by Dr. K. Williams. pKW08_GFP vectors were created in this work and used to down-regulate expression of *M. smegmatis* genes. In this work, GFP was cloned into pKW08 downstream of the tetRO promoter as a reporter of tetRO induction. A 10x10 scan of pKW08_GFP tetracycline induction of GFP expression is shown in Figure 6.6.

Figure 6.6: Tetracycline inducible expression using *M. smegmatis* pKW08_GFP vector. 10x10 plate scan of GFP expression (excitation 488 nm emission 509 nm) of *M. smegmatis* pKW08_GFP in triplicate with increasing concentrations of tetracycline inducer.

MSMEG_2357 cysteine desulphurase and MSMEG_5265 cystathionine gamma-synthase/lyase were cloned downstream of tetRO in pKW08_GFP, both in the sense (with RBS) and anti-sense orientation, for up and down-regulation of these genes, respectively. Gene expression was measured by the GFP reporter and the highest level of expression was induced by 100 ng/mL tetracycline with higher concentrations dropping in expression most likely due to tetracycline induced toxicity, as shown for pKW08_GFP_2357S and for pKW08_GFP_5265S in Figure 6.7.

Figure 6.7: Induction of tetRO expression as measured by GFP.

24 hour induction of MSMEG_2357 (blue) and MSMEG_5265 (red) expression as measured by a downstream co-translated GFP reporter. Induction is highest at 100 ng/mL tetracycline.

pKW08 tetracycline induced gene expression strains were queried with a dose-response assay to SMSC and Se-L-met as described in Chapter 2.2.5.

Figure 6.8a: Dose-response curves for pKW08 mediated up- and down-regulation of MSMEG_2357 and MSMEG_5265.

M. smegmatis cultured in HdeB C- and HdeB N- media, induced by tetracycline. Growth is measured in triplicate wells by OD₆₀₀ relative to a no-drug control (100% growth).

Figure 6.8b: Dose-response curves for pKW08 mediated up- and down-regulation of MSMEG_2357 and MSMEG_5265 *M. smegmatis* against Se-AAs.

For *M. smegmatis* cultured in 7H9 and LB media, only cultures induced tetracycline are shown. Details as per Figure 6.8a.

No significant differential sensitivity was observed using the pKW08 tetracycline inducible up- and down-regulation of MSMEG_2357 and MSMEG_5265 in *M. smegmatis* cultured in any of the media types tested. This result is in contrast to the initial observations seen with pMIND_2357. Possible reasons for the lack of activity are discussed in section 6.5.2.2.

Figure 6.8c: Dose-response curves for pKW08 mediated up- and down-regulation of MSMEG_2357 and MSMEG_5265 *M. smegmatis* against Se-AAs. For *M. smegmatis* pKW08_GFP controls cultured in HdeB C-, HdeB N-, 7H9 and LB media. Both tetracycline induced and un-induced cultures are shown as detailed in the central figure legend. Details as per Figure 6.8a.

6.4.2.3 *S. cerevisiae* lyase gene deletion strains

S. cerevisiae gene deletion mutant strains YCL017C (cysteine desulphurase), YAL012W (cystathionine γ -lyase) and YLR303W (O-acetyl homoserine-O-acetyl serine sulfhydrylase) were kindly provided by Bede Busby of the VUW Chemical Genetics laboratory. These knockout strains of non-essential *S. cerevisiae* genes⁵⁵¹ were selected for their homology to mycobacterial β - and γ -lyases MSMEG_2357 and MSMEG_5265 and were tested in seleno-compound dose-response assays with both nutrient-rich SC and nutrient-limited SPO media.

Figure 6.9: Dose-response curves for Se-AAs against *S. cerevisiae* lyase gene deletion strains in SC and 0.5x SPO media.

For wild type reference strain graphs: see Figures 5.13a, 5.13b and 6.10.

As seen in Chapter 5.2.2.1, high concentrations of SMSC in conjunction with *S. cerevisiae* cultured in SPO media caused resazurin reduction. *S. cerevisiae* YAL012W was unable to be cultured in SPO media, suggesting the CYS3 γ -lyase gene is essential under SPO media conditions. The significant finding of this experiment was that the CYS3 γ -lyase knockout strain YAL012W is resistant to Se-L-met, with an 8-fold increase in MIC compared to the *S. cerevisiae* 4743 diploid control strain. This resistance is better seen in Figure 6.10 that includes control strains 4741 (haploid) and 4743 (diploid). Interestingly, YAL012W was also more sensitive to sodium selenite with a MIC of 125 μ M compared to the 2000 μ M MIC exhibited by the 4743 diploid control strain (results not shown).

Figure 6.10: Comparison of *S. cerevisiae* lyase knockout strain and wild type strain dose-response to Se-L-met. Growth measured in triplicate by OD₆₀₀ compared to 100% growth (no drug) controls.

6.4.2.4 Pro-drug activation *in vitro* enzyme activity assays

Mycobacterial β and γ -lyase genes used in anti-sense constructs and additional genes of interest were identified bioinformatically using the published literature, the KEGG database and NCBI BLAST. These genes were cloned into the pET28a+ expression vector, as described in Chapter 2.3.2, and overexpressed in *E. coli* BL-21. Enzymes were purified by Ni²⁺ affinity chromatography as described in Chapter 2.3.3.1. Purified enzymes were utilised in enzymatic assays *in vitro* to determine mycobacterial lyase activity toward Se-AA substrates and query the prodrug mechanism hypothesis described in Figure 6.3.

6.4.2.4.1 Enzyme purification

Cysteine desulphurase enzyme MSMEG_2357 and its *M. tuberculosis* H37Rv homologue Rv3025c, cysteine desulphurase / selenocysteine lyase Rv1464 and O-acetylhomoserine aminocarboxypropyltransferase Rv3340 were successfully purified from recombinant *E. coli* BL-21 as shown in Figure 6.11, proteins were identified by their size and intensity relative to the standard ladder, but MALDI mass spectroscopy would be a more desirable method to confirm protein identity. O-acetylhomoserine sulphydrylase Rv0391 and cystathionine γ -synthase Rv1079 were successfully expressed in *E. coli* BL-21 but were unable to be purified, suggesting these proteins may have misfolded during the protein expression and purification procedure, or alternatively Rv0391 may be membrane bound.

Figure 6.11: Protein purification SDS-PAGE.

Left panel shows eluted protein fractions prior to size-based purification and desalting. Lanes 1 and 2 are MSMEG_2357. Lanes 3, 4 and 5 are Rv3025c. Lane 7 is Rv0391, lane 8 Rv1079, Lane 9 Rv1464 and lane 10 Rv3340. The right panel shows lysed recombinant *E. coli* BL-21 induced overnight to express proteins. Lanes A: Rv0391, B: Rv1079, C: Rv1464, D: Rv3340. Lane E shows Rv0391 elution by column chromatography, and lane F shows Rv1079 eluted by column chromatography. Protein molecular weight ladders are included in the centre of the gels with molecular weight of relevant bands labelled.

6.4.2.4.2 Metabolite Detection

To determine the mycobacterial enzymes involved in the lysis of Se-AAs and to confirm the viability of prepared mycobacterial enzymes, a variety of metabolite detection assays were used as described in below.

6.4.2.4.2.1 Gas chromatography-mass spectrometry

GC-MS analysis of Rv3025c reaction products was carried out with the kind instruction of Dr. R. Keyzers, the methodology for which is described in Chapter 2.4.4.

A volatile selenium species was detected with 96% spectral similarity to NIST 11 database methyl diselenide spectrum, at 7.15 sec into the run, as shown in Figure 6.12. Values of over 95% spectral similarity are considered to be highly indicative of compound identity. This methyl diselenide was produced as a product of the Rv3025c enzyme reaction with 1 mM SMSC in ISCS buffer, the dimeric methyl diselenide is a known indicator of methylselenide.⁵⁵² Methyl diselenide was not detected in a no-enzyme control reaction using BSA in place of Rv3025c, or using Se-DL-cys as the substrate. Implications of this finding are discussed in section 6.5.2.4.1.

Figure 6.12: GC-MS result for methyl diselenide.

The top panel shows the peak spectrum for the sample at 7.15 seconds into the run. The bottom panel shows the peak spectrum for methyl diselenide, the most similar match to the sample result with 96% spectral identity.

Borane-methyl sulphide with 97% similarity or dimethylsulphide with 96% similarity was detected at 1.93 seconds into the run following incubation of DL-cysteine as above with either Rv3025c or BSA enzyme control, shown in figure 6.13.

Figure 6.13: GC-MS result for dimethylsulphide.

The top panel shows the peak spectrum for the sample at 1.93 seconds into the run. The middle panel shows the peak spectra of the best match, borane-methyl sulphide, with 97% identity to the sample, and the bottom panel shows the next best match, dimethylsulphide, with 96% identity to the sample.

6.4.2.4.2.2 Methylene blue sulphide/selenide detection

Production and quantification of sulphide and selenide species by purified mycobacterial enzyme lyase activity towards DL-cys, DL-met and Se-AAs *in vitro* was carried out as described in Chapter 2.4.2.2. No sulphides or selenides were detected using this method using either DL-cys, DL-met or SMSC, Se-DL-cys or Se-L-met in conjunction with Rv1464, Rv3025c or Rv3340. This may be due to the volatile nature of the sulphide/selenides species leading to loss from the solution.

6.4.2.4.2.3 Lead acetate sulphide/selenide detection

To identify volatile sulphide / selenide species produced by mycobacterial lyase enzyme reactions, PbAc paper was prepared as described in Chapter 2.4.2.1 and placed over 96-well plates containing lyase enzyme cocktail prior to sealing the plates and assay incubation.

Figure 6.14: PbAc paper detection of volatile sulphide / selenide.

Top left panel shows an Rv3025c enzyme assay plate, with rows of 10 μ g Rv3025 (+Rv3025c) or 10 μ g BSA (-Rv3025c) against various substrates (2mM). Note the orange-red precipitate for Se-DL-cys and Se-L-cys only in conjunction with Rv3025c. Bottom left panel shows the same image but with the PbAc paper digitally overlaid on the image. Note the dark precipitate of PbS for DL-cys in combination with Rv3025c. The right panel shows a follow-up dose-response assay for Rv3025c with increasing concentrations of DL-cys. The no-enzyme control column (marked -Rv3025c) contains 10 μ g BSA and duplicate experimental columns (marked + Rv3025) contain 10 μ g of purified Rv3025c enzyme. A clear trend of the PbAc paper reaction is observed with increasing DL-cys concentration. Some sulphide release in the no-enzyme control is seen, predominantly at 0.5-1.0 mM DL-cys.

Reaction of PbAc paper forming a dark precipitate of PbS was observed only for Rv3025c enzyme and DL-cys substrate. As seen in Figure 6.14, an orange-red precipitate (presumed to be elemental selenium Se^0) was observed in the well for Rv3025c with Se-DL-cys and Se-L-cys, Se-L-cys produced more precipitation than Se-DL-cys. No reaction of PbAc paper was observed for other mycobacterial enzymes or substrates. An increasing PbAc paper response was seen for increasing DL-cys concentrations in the presence of Rv3025c, and a slight PbAc reaction was seen for low concentrations (0.5-1.0 mM) DL-cys in the absence of Rv3025c.

6.4.2.4.2.4 Alanine dehydrogenase-coupled assay

Mycobacterial enzyme reactions as described in Chapter 2.4.1 were coupled with an alanine dehydrogenase (Ala-DH) reaction, both simultaneously and as a two-step reactions as described in Chapter 2.4.2.2. The rationale for these tests was if alanine is produced as a by-product by mycobacterial lyase action on sulphur-AA / Se-AA substrates, this enzymatic activity may be able to be quantifiably detected by measuring corresponding β -NAD

reduction by Ala-DH.

No Ala-DH activity was detected from 10 µg mycobacterial lyase enzymes Rv1464, Rv3025c, Rv3340 and MSMEG_2357 using the substrates DL-cys, DL-met, Se-DL-cys, SMSC and Se-L-met. Positive controls using L-alanine yielded the expected β -NAD reduction curve (increase in OD₃₄₀ over time at a concentration-dependant rate; results not shown). The implications of the Ala-DH coupled assay are discussed in section 6.5.2.4.4.

6.4.2.4.2.5 Lactate Dehydrogenase coupled assay

Similar to the Ala-DH coupled assay described above, mycobacterial enzyme reactions described in Chapter 2.4.1 were coupled with lactate dehydrogenase (Lac-DH), both simultaneously and as two-step reactions as described in Chapter 2.4.2.3. The reasoning again being if pyruvate is product of mycobacterial lyase action on sulphur-AA / Se-AA substrates this activity may be able to be detected and quantified by measuring corresponding NADH oxidation by Lac-DH. As above for Ala-DH, no Lac-DH activity was detected from 10 µg mycobacterial lyase enzymes Rv1464, Rv3025c, Rv3340 and MSMEG_2357 using the substrates DL-cys, DL-met, Se-DL-cys, SMSC and Se-L-met. Positive controls using sodium pyruvate yielded the expected NADH oxidation curve (decrease in OD₃₄₀ over time at a concentration-dependant rate; results not shown). The implications of the Lac-DH coupled assay are discussed in section 6.5.2.4.4.

6.4.2.4.2.6 Methylbenzothiazolinone hydrazone alpha-keto acid detection

Detection of the possible Se-AA metabolite α -ketobutyrate or similar α -keto acids was carried out on mycobacterial lyase reactions using the 3-methyl-2-benzothiazolinone hydrazone hydrochloride method of Tokoro *et al*⁵⁵³ as described in methods Chapter 2.4.2.4. No α -keto acids were able to be detected from mycobacterial lyase reactions with sulphur-AAs or Se-AAs substrates; whereas, the α -ketobutyrate control produced the expected dose-dependant increase in OD₃₂₀; results not shown.

6.4.3 Mycobacterial redox cycling of methylselenol

6.4.3.1 Seleno-amino acid resistant transposon mutants

To identify the metabolic pathways and genetic components involved in Se-AA resistance, and provide insight into the Se-AA mechanism of action, transposon gene-disruption mutant strains of *M. smegmatis* with resistance to SMSC and Se-L-met were generated and selected for as described previously in section 2.3.2.8. Four generations of Se-AA resistant

transposon mutants were generated, two generations each for SMSC and Se-L-met. For each generation 10 of the largest colonies were picked, cultured and verified for Se-AA resistance by dose-response assays, before undergoing DNA purification and sequencing to determine the transposon insert site and resulting gene disruption responsible for Se-AA resistance. Out of 40 mutants selected, 14 were unable to be genetically identified and were discarded, leaving 26 unique Se-AA resistant mutants with defined gene disruptions (listed in Table 6.3).

Mutant #	Origin (μM)	Tn611 gene insertion	Gene annotation	MIC (μM)	
				SMSC	SeMet
TN-G1-M3	SMSC 10	MSMEG_3638	CBS domain protein (membrane bound Fe ²⁺ transporters)	62.5	62.5
TN-G1-M4	SMSC 10	MSMEG_4695/6	Alpha amylase family protein and haemoglobin like protein	125	125
TN-G1-M6	SMSC 20	MSMEG_4270	Adenosine kinase	125	125
TN-G1-M7	SMSC 30	MSMEG_6756/7	Glycerol kinase <i>glpK</i>	250	250
TN-G1-M9	SMSC 30	MSMEG_2729	<i>miaB</i> adenosine tRNA methylthiotransferase	250	125
TN-G1-M11	SMSC 40	MSMEG_6759	Glycerol kinase <i>glpK</i>	500	125
TN-G1-M12	SMSC 40	MSMEG_3200	L-aspartate oxidase <i>nad A/B/C</i>	250	125
TN-G2-M3	SMSC 60	MSMEG_1098	Acyl CoA Synthase	250	125
TN-G2-M4	SMSC 60	MSMEG_4162	Conserved hypothetical	125	62.5
TN-G2-M6	SMSC 80	MSMEG_6075/6	Methyl erythritol cyclodiphosphate synthase	62.5	62.5
TN-G2-M7	SMSC 80	MSMEG_1701	Purine nucleoside phosphorylase	125	62.5
TN-G2-M8	SMSC 80	MSMEG_6756	Glycerol kinase <i>glpK</i>	125	62.5
TN-G2-M9	SMSC 80	MSMEG_1019	Ribonucleotide diphosphate reductase α-subunit	250	62.5
TN-G2-M14	SMSC 100	MSMEG_0376	<i>araC</i> transcriptional regulator	250	62.5
TN-G2-M15	SMSC 100	MSMEG_6024	Acetoacetyl CoA reductase	62.5	62.5
TN-G3-M1	SeMet 50	MSMEG_0848	NADH ubiquinone oxidoreductase subunit 5	32	62.5
TN-G3-M2	SeMet 50	MSMEG_0211	ABC transporter, ATP-binding protein	32	62.5
TN-G3-M3	SeMet 100	MSMEG_1221	rifampin ADP-ribosyl transferase	62.5	100
TN-G3-M4	SeMet 100	MSMEG_4606	hypothetical protein	6.25	100
TN-G3-M5	SeMet 100	MSMEG_5842	Conserved Hypothetical Protein	32	100
TN-G3-M10	SeMet 100	MSMEG_4890/1	Alkylhydroperoxidase, <i>ahpC/D</i> family protein	250	500
TN-G4-M1	SeMet 50	MSMEG_3756	23s Ribosomal Subunit (<i>rrlA</i>)	32	80
TN-G4-M3	SeMet 50	MSMEG_2199 MSMEG_2200	Flanked by formyltetrahydrofolate deformylase[<i>purU</i>] and Conserved hypothetical protein.	32	50
TN-G4-M4	SeMet 50	MSMEG_4842	Amidohydrolase	32	50
TN-G4-M5	SeMet 50	MSMEG_4431	Putative regulatory protein	32	50
TN-G4-M8	SeMet 80	MSMEG_5925	Rieske [2Fe-2S] domain protein	250	62.5

Table 6.3: Se-AA resistant transposon mutants of *M. smegmatis*.

Detailed from the left are the internal mutant designations, Se-AA concentrations used to generate the mutants (origin), genetic location of the transposon insertions (and likely genes disrupted), annotation for the genes disrupted and the MIC of the mutant strains against SMSC and Se-L-met in 7H9 medium.

Unfortunately several mutants that demonstrated high levels of Se-AA resistance were unable to be genetically identified, due to difficulty isolating re-ligated mutant gDNA or restriction enzyme sites being situated too close to the Tn611 insert, resulting in insufficient DNA sequence length to identify the insert site. The significance of these mutations with regard to the Se-AA mechanism of action is discussed in section 6.5.3. The relationship between the mutations and the Se-AA mechanism is not readily apparent for many of the mutants; however, for TN-G3-M10, a mutant in MSMEG_4890 *ahpD* alkylhydroperoxidase that confers protection against oxidative damage, fits directly into the hypothesised Se-AA mechanism of anti-mycobacterial action. Dose-response curves of the TN-G3-M10 *AhpD* mutant alongside control strains of *M. smegmatis* pSHIGH+HSP60 and pMV261, shown in Figure 6.15. These illustrate the clear shift in Se-AA resistance of the TN-G3-M10 mutant, a 16-fold increase in the SMSC MIC and an 8-fold increase in the Se-L-met MIC compared to the control strains.

Figure 6.15: Differential susceptibility of *M. smegmatis* TN-G3-M10 Δ *ahpD* mutant strain. Measured by triplicate resazurin fluorescence relative to 100% growth (no drug) controls, compared to screening strain pSHIGH+HSP60 and empty transposon vector strain pMV261.

6.4.3.2 Inducible gene regulation of *M. smegmatis* alkylhydroperoxidase

M. smegmatis alkylhydroperoxidase genes were up- and down-regulated using the pKW08 vector sense and anti-sense tetracycline inducible expression of these genes. These strains were tested for differential susceptibility in a dose-response assay against SMSC and Se-L-met. As the TN-G3-M10 Tn611 insert occurs between the MSMEG_4890 (*ahpD*) and MSMEG_4891 (*ahpC*) genes, both of these genes have been knocked up/down by inducible expression in the pKW08. Se-AAs dose-response curves of these constructs to are shown in Figure 6.16a for MSMEG_4890 (*ahpD*) and Figure 6.16b for MSMEG_4891 (*ahpC*).

Figure 6.16a: Differential susceptibility of *M. smegmatis* to Se-AAs using tetracycline inducible over and under-expression of MSMEG_4890 alkyhydroperoxidase *ahpD*.

Measured by triplicate OD₆₀₀ compared to 100% growth (no drug) control.

The differential sensitivity of MSMEG_4890 (*ahpD*) sense over-expression and anti-sense under-expression strains is presented in Figure 6.16a. Induction of both up- and down-regulation of MSMEG_4890 using sense / antisense, respectively, results in a 2-fold increase in SMSC MIC for *ahpD* over-expression and a 4-fold increase in for *ahpD* under-expression. For Se-L-met, *ahpD* over-expression caused an increase in sensitivity (2-fold decrease in MIC); whilst *ahpD* under-expression produced a 4-fold rise in the Se-L-met MIC from 16 to 64 μM.

Figure 6.16b: Differential susceptibility of *M. smegmatis* to Se-AAs using tetracycline inducible over and under-expression of MSMEG_4891 alkyhydroperoxidase *ahpC*.

Measured by triplicate OD₆₀₀ compared to 100% growth (no drug) control.

The differential sensitivity of MSMEG_4891 (*ahpC*) sense over-expression and anti-sense under-expression strains is shown in Figure 6.16b. Both up- and down-regulation of MSMEG_4891 caused a 2-fold increase in the MIC of SMSC from 32 μ M to 64 μ M. There was no change in MIC for Se-L-met with either up- or down- regulation of *ahpC*.

6.4.3.3 Seleno-amino acid synergy with isoniazid

Subsequent to the identification of alkylhydroperoxidases *ahpC* and *ahpD* in the mechanism of action of Se-AAs, investigation was made into the link between mycobacterial alkylhydroperoxidase and other peridoxins such as the catalase-peroxidase *katG*. *katG* is implicated in isoniazid pro-drug activation,⁵⁵⁴ and consequently mutation in this gene can cause isoniazid resistance.^{555, 556}

It is known that in *katG* mediated isoniazid resistant *M. tuberculosis* *ahpC* may be hyper-expressed to compensate for loss of *katG* function in protecting the mycobacterial cell from oxidative stress.^{557, 558} Therefore, it was predicted that if transposon mediated inactivation of mycobacterial *ahpC/D* leads to Se-AA resistance, the opposite effect of increased susceptibility to Se-AAs may be seen with *ahpC/D* up-regulation, such as in the case of *katG* mediated isoniazid resistant TB.

In addition, a synergistic inhibitory effect may be observed between Se-AAs and the front line tuberculosis treatment drug, isoniazid, as these two drugs have interrelated genetic targets. This synergy effect was tested using the checkerboard dilution method²⁰⁸ described in Chapter 2.2.6 using Se-L-met, isoniazid against *M. tuberculosis* H37Ra.

Using the MIC values illustrated in Figure 6.17, and the fractional inhibitory concentration index (FIC index) equation described in Chapter 2.2.6, a FIC index of 0.5 was calculated for Se-L-met and isoniazid. This value indicates synergistic effect between Se-L-met and isoniazid.

Figure 6.17: Checkerboard assay for synergy between Se-L-met and isoniazid (INH). Cell values are the mean OD of triplicate assays, and are colour coded with dark red indicating < 10% growth, light red < 25% growth, yellow < 50% growth and green > 50% growth (compared to controls). Concentration (μM) is shown in purple, and MIC is indicated in tan. Top panel shows Se-L-met only, serially diluted in rows with MIC at 8 μM . Middle panel shows INH only, serially diluted in columns with MIC at 2 μM . Bottom panel shows the Se-L-met and INH checkerboard, with MIC values of 4 and 0.5 μM respectively.

6.5 Discussion

The hypothesised anti-mycobacterial mechanism of Se-AAs is as follows: Se-AAs are taken up by mycobacteria under the guise of a nutrient. The Se-AA pro-drug is then cleaved into its active methylselenolol form that undergoes redox cycling, producing ROS and causing damage within the mycobacterial cell (illustrated in Figure 6.3). This hypothesis is based on the known anti-carcinoma activity of Se-AAs and the proposed mechanism for this activity.^{447, 535} The nutrient recognition hypothesis was then tested by competition assays of Se-AAs with the sulphur amino acids cysteine and methionine. Mycobacterial lyase activation of Se-AAs was explored by anti-sense down-regulation of key mycobacterial lyase genes, and also purification of these lyases and *in vitro* enzyme activity assays. Finally the mechanism behind Se-AA induced toxicity was queried via transposon mutant profiling of Se-AA resistant *M. smegmatis*.

6.5.1 Competition assays

Competition assays investigating the nutritional recognition and uptake of Se-AA matched both SMSC and Se-L-met with equimolar concentrations of sulphur amino acids DL-cys and DL-met. Supplementing either SMSC or Se-L-met with DL-cys or DL-met completely reversed Se-AA inhibition to the highest level tested of 2 mM (evidenced in Figures 6.4a and 6.4b).

This competition effect may take place at the cellular uptake level, in which sulphur amino acids saturate transporters, resulting in reduced Se-AA import into the cell and consequential loss of toxicity. It is known that Se-met and methionine both share a common Na^+ driven active transport process in mammals,⁵⁵⁹ and that Se-DL-met causes a reduction⁵⁶⁰ or inhibition⁵⁶¹ of the transport rate of DL-met; therefore, it is plausible that this reduction in transport could work both ways. Another possibility is the nutrient content of the medium modulates Se-AA activity, rather than specifically cysteine or methionine. This could be tested by using other non-sulphurous amino acids in competition assays.

In addition to substrate competition at the transporter level, the presence of sulphur amino acids at higher concentrations may also induce changes in mycobacterial gene expression, both for amino acid transport and metabolism such as by mycobacterial lyase enzymes. Supplementary sulphur amino acids such as DL-cys also bolster the pool of mycobacterial cellular thiols, such as mycothiol, that are known to confer protective effects against oxidative attack.⁵⁴⁹ Further experimentation such as measurement of radio-labelled Se-AA uptake by mycobacteria may help to elucidate this mechanism.

6.5.2 Mycobacterial lyase enzymes

6.5.2.1 Bioinformatic gene selection

Eukaryotic metabolism of Se-AAs has been well researched, with SMSC and Se-cys being cleaved by β -lyase enzymes,⁵⁶² and metabolism of Se-met being carried out by γ -lyase enzymes.⁵⁶³ Orthologous lyase proteins are conserved in bacterial species;⁵⁶⁴ therefore, it can be assumed that mycobacterial lyase enzymes would play similar roles to their eukaryotic counterparts in the anti-mycobacterial mechanism of Se-AA activity. Protein BLAST searching for mycobacterial equivalents of lyase genes that are reported in the literature to cleave Se-AAs such as *Trichomonas vaginalis* methioninase⁵³⁵ (γ -lyase, NCBI accession number EAX91132) and *E. coli* IscS⁵⁶⁵ (β -lyase, NCBI accession number ZP_03034145.1) produced five homologous mycobacterial enzymes: 2 β -lyases and 3 γ -lyases (listed in Table

6.4) with a BLAST total score > 100. BLAST total score is of a measure of protein sequence alignment. Cutoff of 100 was arbitrarily selected based on drop off in total score results after 100.

Gene	Annotation	Information
Rv1464	<i>csd</i>	Cysteine desulphurase (β -lyase), 1254 bp / 44.6 kDa. Catalyses removal of elemental sulphur/selenium atoms from substrates such as L-cysteine, L-cystine and their selenium analogues, producing alanine.
Rv3025c	<i>iscS</i>	Cysteine desulphurase (β -lyase), 1182 bp / 40.9 kDa. Catalyses removal of elemental sulphur from cysteine producing alanine. 100% homology to MSMEG_2357.
Rv0391	<i>metZ</i>	Probable O-succinylhomoserine sulphydrylase or cystathionine γ -synthase, 1221 bp / 43.3 kDa. Catalyses formation of homocysteine from O-succinylhomoserine and hydrogen sulphide. Facilitates de-novo methionine biosynthesis.
Rv1079	<i>metB</i>	Probable O-succinylhomoserine thiol lyase or cystathionine γ -synthase, 1167 bp / 41.0 kDa. Catalyses formation of cystathionine and succinate from O-succinylhomoserine and cysteine, can also utilise hydrogen sulphide or methanethiol as substrates. 100% homology to MSMEG_5265.
Rv3340	<i>metC</i>	Probable O-acetylhomoserine sulphydrylase or homocysteine synthase, 1350 bp / 47.4 kDa. Catalyses formation of methionine and acetate from O-acetylhomoserine and methanethiol.

Table 6.4: *M. tuberculosis* H37Rv enzymes putatively involved in Se-AA lysis.

Gene annotation derived from Tuberculist.⁵⁶⁶

These lyase enzymes all share a common traits of being pyridoxal-5'-phosphate (PLP, also known as the active form of vitamin B₆) dependant and are putatively involved in cysteine or methionine biosynthesis and metabolism. Pyridoxal 5'-phosphate is derived from vitamin B₆ and is an essential cofactor of many enzymes predominantly involved in amino acid metabolism. Approximately 1.5% of all prokaryotic genes encode PLP-dependent enzymes, a value much higher than the proportion encoded in eukaryotes. The PLP-dependant enzymes hydroxymethyltransferase⁵⁶⁷ and alanine racemase are the target of anti-malarial and anti-tubercular agents, respectively.⁵⁶⁸

Mycobacterial lyase genes were selected for scrutinisation in Se-AA mechanism of action studies, due to their homology to lyases involved in methylselenol production from Se-AAs, and their annotated function in sulphur amino acid metabolism.^{535, 565}

Cysteine desulphurase Rv1464 has been identified in the cytosolic and membrane fractions of *M. tuberculosis* whilst Rv3025c has been identified only in membrane fractions.⁵⁶⁹ Both have been determined to be essential for H37Ra growth *in vitro* by transposon mutagenesis.^{570, 571} Furthermore, the *M. smegmatis* equivalents of these genes are transcriptionally up-regulated in response to hypoxia and starvation.^{223, 572}

O-succinylhomoserine thiol lyase or cystathionine γ -synthase Rv1079 (*metB*) is bi-functional and is able to synthesise cystathionine from L-homocysteine and L-serine, and also lyse cystathionine through a γ -elimination reaction to form cysteine, α -ketobutyrate and ammonia.^{573, 574} Due to the close homology of these synthase enzymes to known γ -lyases, it is possible they have dual synthase-lyase function.⁵⁵³ Methionine is also the preferred source of sulphur in pathogenic mycobacteria.⁵⁷³ Thus, the γ -lyase activity and methionine scavenging apparatus are indispensable to mycobacteria and are likely to have stronger expression levels compared to their cysteine equivalents. Methionine γ -lyase enzymes such as MetB are present in plants and bacteria, but are absent in mammals, this makes them excellent drug targets.^{575, 576}

6.5.2.2 Transcriptional modulation of *M. smegmatis* putative lyase enzymes

An *M. smegmatis* anti-sense RNA inducible gene knock-down strain of the cysteine desulphurase MSMEG_2357 (*M. smegmatis* homologue of Rv3025c) using the vector pMIND_2357 was serendipitously present in our laboratory, created as an anti-sense negative control strain for an unrelated lab project.¹⁶² Initial Se-AA sensitivity testing using this induced vector showed a significant 8-32 fold increase in resistance to SMSC compared to the control strain (Figure 6.5). This resistance effect was thought to be caused by down-regulation of the key β -lysis step in the Se-AA mechanism of anti-mycobacterial action, resulting in the majority of SMSC remaining sequestered in a relatively inert form, rather than being cleaved into the radical-generating methylselenol. Subsequent experiments using this vector were unable to replicate these results; however, it has been suggested that the pMIND vector tetRO promoter had leaky background expression.¹⁸⁸ In the case of an essential gene such as MSMEG_2357, this may result in selection for mutations that rendered the anti-sense gene regulation ineffective.

The top BLAST derived lyase hits cysteine desulphurase MSMEG_2357 and methionine lyase MSMEG_5265 were then cloned into an improved tetracycline-inducible vector

pKW08¹⁸⁸ in both the sense orientation with RBS for overexpression and anti-sense orientation for gene-knockdown to better investigate their effect upon Se-AA sensitivity. These results are summarised in Table 6.5.

Strain	SMSC MIC (μ M)			
	HdeB C-	HdeB N-	7H9+	LB
MSMEG_2357S	250	125	125	> 1000
MSMEG_2357AS	125	62.5	62.5	> 1000
MSMEG_5265S	125	31.25	62.5	> 1000
MSMEG_5265AS	250	125	250	> 1000
Strain	Se-L-Met MIC (μ M)			
	HdeB C-	HdeB N-	7H9+	LB
MSMEG_2357S	31.25	15.6	15.6	125
MSMEG_2357AS	31.25	31.25	31.25	500
MSMEG_5265S	31.25	31.25	15.6	125
MSMEG_5265AS	31.25	31.25	31.25	125

Table 6.5: Summarised results of MSMEG_2357 / MSMEG_5265.

Summary of MIC values derived from triplicate dose-response assays shown in Figure 6.8.

Gene expression of MSMEG_2357 (putative β -lyase) and MSMEG_5265 (putative γ -lyase) was up- and down-regulated using the tetracycline inducible pKW08 vector.

In HdeB C- media the induction of MSMEG_5265 anti-sense increased the MIC of SMSC to 125 μ M compared to 62.5 μ M of the sense-expression of MSMEG_5265; whereas, expression of MSMEG_2357 in either orientation had no discernable effect upon SMSC sensitivity. A similar effect was observed in HdeB N- media where the MIC of SMSC was raised to 125 μ M for MSMEG_5265 antisense expression over the 31.25 μ M MIC the sense expression construct, but no significant change in MIC was observed for MSMEG_2357 against SMSC. This also occurred to a lesser extent in 7H9 media with an MIC shift from 125 μ M to 250 μ M between the sense and anti-sense expression of MSMEG_5265. Neither MSMEG_2357 and MSMEG_5265 sense nor anti-sense expression had any effect upon sensitivity to Se-L-met in HdeB C-, HdeB N- or 7H9 media. LB media produced no significant changes in SMSC resistance for any strain tested however for Se-L-met, MSMEG_2357 anti-sense showed increased sensitivity with an MIC of 125 μ M; whereas, the MIC was 500 μ M in the sense expression of this gene. Modulation of MSMEG_5265 expression showed a 2-fold increase in MIC for antisense expression to 250 μ M compared to 125 μ M for the sense.

The differential sensitivity in these results is far less than expectations based on the pMIND_2357 results. Vector sequences were confirmed to be error-free and promoter induction was confirmed by GFP expression. It is possible that there was degradation of the

MSMEG_2357 plasmid under selection pressure as this is an essential gene.⁵⁷⁰ Alternatively the expressional strength of the tetRO promoter was insufficient to significantly up-regulate protein levels or down-regulate protein levels by anti-sense RNA counter-expression. Finally these genes may not be responsible for Se-AA lysis, or these genes may be functionally redundant in this regard in *M. smegmatis*.⁵⁷⁷

6.5.2.3 *S. cerevisiae* lyase gene deletion strains

S. cerevisiae yeast gene deletion set strains YAL01C (NFS1), YAL012W (CYS3) and YLR303W (MET17) are the respective homologues of *M. tuberculosis* H37Rv genes Rv3205c, Rv1079 and Rv3340. CYS3 and MET17 both share close homology to Rv0391, Rv1079 and Rv3340.

Testing of these strains under nutrient-rich SC and nutrient-limited SPO media demonstrated again that SMSC in conjunction with *S. cerevisiae* in SPO medium causes resazurin reduction proportional to SMSC concentration, also noted in Chapter 5.2.2.1. As this effect was not observed in no-cell controls, it is possibly a downstream effect from *S. cerevisiae* metabolism or gene expression response to SMSC.

It is also noted that the YAL012W (CYS3) strain could not grow in SPO media conditions. This agrees with published results that CYS3 knockout in *S. cerevisiae* is a cysteine auxotrophy phenotype,⁵⁷⁸ and has decreased resistance to starvation.⁵⁷⁹ The CYS3 knockout exhibited an MIC of 1000 μ M for Se-L-met in SC medium, an 8-fold increase in resistance relative to the 4743 control strain. This is supported in the literature which reports that YAL012W is 7-fold more resistant to Se-met compared to wild type.⁵⁸⁰ Conversely, knockout of CYS3 also resulted in increased resistance to sodium selenite in SC media, with an MIC of 125 μ M compared to the > 2000 μ M MIC of the 4743 strain control, and this also concurs with published results.⁵⁸¹ It has been proposed that CYS3, a cystathionine γ -lyase, may also be responsible for metabolism of Se-met, producing methylselenol.⁵⁸⁰ The experimental evidence discovered in the present research supports that proposal. No significant changes were observed in the Se-AA sensitivity of NFS1 or MET17 knockout strains.

6.5.2.4 Lyase enzyme metabolite detection assays

Mycobacterial enzymes suspected to be responsible for the lysis of Se-AAs were cloned into the pET28a+ expression vector, and overexpressed in *E. coli* BL-21, for purification and *in*

vitro activity assays. Rv1464 (cysteine desulphurase / selenocysteine lyase), Rv3025c and MSMEG_2357 (cysteine desulphurase) and Rv3340 (and O-acetylhomoserine aminocarboxypropyltransferase) were successfully purified. Rv0391 (O-acetylhomoserine sulfhydrylase) and Rv1079 (cystathionine γ -synthase) were expressed in BL-21, as evidenced by correctly sized bands in whole cell lysate; but these proteins were unable to be successfully purified, possibly due to excessive imidazole concentrations used in the purification procedure (detailed in Chapter 2.3.3.1). A NaCl gradient elution procedure may be more successful in purifying these proteins.⁵⁷⁴ Rv1464, Rv3025c (and MSMEG_2357), and Rv3340 were utilised in a number of enzymatic assays to determine if they were able to lyse Se-AA substrates.

6.5.2.4.1 Gas chromatography-mass spectrometry

The *M. tuberculosis* H37Rv enzymes that were successfully purified (Rv1464, Rv3025c and Rv3340) were used in the *in vitro* enzyme assay detailed in Chapter 2.4.1, and the headspace was sampled by SPME fibre and analysed by GC-MS.

Assays containing Rv3025c + SMSC identified methyldiselenide and Rv3025c + DL-cys produced dimethylsulphide, but this was also seen in the no enzyme control of BSA + DL-cys. Methyldiselenide is known to be a marker of methylselenol, as methylselenol undergoes spontaneous oxidation.⁵⁵² This indicates that the *M. tuberculosis* cysteine desulphurase Rv3025c is able to utilise SMSC as a substrate for β -lysis, producing volatile methylselenol and other products, most likely alanine.⁵⁸² Methyldiselenide detection by GC-MS using is in agreement with the initial anti-sense knockdown experiments of the *M. smegmatis* homologue MSMEG_2357 using the pMIND vector.

The detection of dimethylsulphide produced by DL-cys in the presence or absence of Rv3025c is difficult to account for; however, dimethylsulphide may not be the correct identification as borane-methylsulphide showed a higher identity in the MS results.

Dimethylsulphide may have arisen through microbial contamination of the assay, as it is known that a variety of volatile sulphur compounds are produced by microbial cysteine metabolism.⁵⁸³ Alternatively, dimethylsulphide may be a product of cysteine degradation under assay conditions.

Hydrogen sulphide was the expected product of Rv3025c and DL-cys assay; however, this compound is notoriously volatile, with a boiling point of -60°C and thus has a very small window of detection in the GC-MS method used.

6.5.2.4.2 Methylene blue sulphide/selenide detection

Methylselenol production was indicated by the GC-MS detection of methyldiselenide⁵⁵² produced from SMSC and the cysteine desulphurase Rv3025c, as explained in section 6.5.2.4.1. Confirmatory detection and quantification of methylselenide production was carried out using the commonly employed methylene blue formation test.⁵⁸⁴ No results were obtained using this assay, with the exception of the Na₂S control that was used as a standard curve. This deviates from the expected result of H₂S production from Rv3025c lysis of DL-cys.⁵⁸⁵ This may be caused by the produced H₂S concentration being below the assay detection threshold of 0.01 ppm,⁵⁸⁶ or possibly interference of enzyme reaction buffer components such as salts and DTT. Alternatively, atmospheric oxidation may sequester any volatile H₂S produced.⁵⁸⁷ It is unknown if this assay method can detect volatile selenides, such as H₂Se and methylselenol; however, the assay has been adapted for the detection of other selenium species such as SeO₂.⁵⁸⁸ Since no volatile selenium species were detected in this assay, and the negative result for the DL-cys positive control means that no conclusion can be made on the production of selenides from Rv1464, Rv3025c and Rv3340 in conjunction with Se-DL-cys, SMSC and Se-L-met.

6.5.2.4.3 Lead acetate sulphide/selenide detection

A different attempt to identify volatile selenium and sulphur species generated from Rv1464, Rv3025c and Rv3340 actions on selenium and sulphur amino acid substrates was made using the PbAc paper method. This assay is based on the reaction of H₂S with PbAc, with production of black PbS precipitate.⁵⁸⁹ This reaction is known to also work with H₂Se to produce a yellow-brown PbSe precipitate,⁵⁹⁰ but it is not known if the reaction proceeds with methylselenol. A reaction seems likely due to the reactive nature of the selenol;⁵⁹¹ however, it is unknown if the reaction products would be visibly detectable.

The PbAc paper assay had a positive reaction for Rv3025c with a DL-cys substrate, this was also found to be DL-cys concentration dependant, this agrees with published results of cysteine desulphurase activity for Rv3025c,⁵⁸⁵ as expected no reaction was observed for DL-met and Rv3025c. No reaction of PbAc paper was noted for Rv3025c using any Se-AA substrates however orange precipitate in the enzyme reaction solution was observed for Rv3025c using Se-DL-cys and Se-L-cys, presumably elemental selenium or SeO₂ not PbSe since the PbAc paper had no observable colour change. This result, whilst not actually taken place on PbAc paper, concurs with published results of a Rv3025c homologue *E. coli* cysteine desulphurase IscS able to mobilise Se from selenocysteine.⁵⁶⁵

No positive results for Rv3340 using DL-cys is expected considering the annotation of this gene as a homocysteine synthase that does not utilise DL-cys as a substrate.⁵⁹² The Csd homologue, Rv1464, is annotated as a NifS like cysteine desulphurase,^{585, 593} however, these results indicate that Rv1464 was unable to metabolise DL-cys to H₂S under the assay conditions. This may be a gene ontology misnomer, as Rv1464 has only been functionally annotated through bioinformatics, and no publications can be found regarding *in vitro* biochemical analysis of this enzyme's lyase function. Lack of positive results for any of the enzymes tested using DL-met, SMSC and Se-L-met may be due to the terminal methyl group of these substrates, resulting in methanethiol or methylselenol instead of H₂S or H₂Se. These methylated chalcogenide-lead complexes such as lead methylthiolate, may not react with PbAc paper to form a visible precipitate. It is also possible they might form homodimers,⁵⁵² that act as protective groups,⁵⁹¹ sequestering the reactivity and masking the detection by PbAc or other methods. Reducing assay conditions from the DTT in the buffer may have not been at high enough concentration to avoid this dimerisation.

6.5.2.4.4 Alanine dehydrogenase / Lactate dehydrogenase coupled assays

Due to the volatile nature of chalcogenides and the difficulty in detection and quantification using the methods discussed above, lyase enzyme reactions were coupled to the NAD/NADH dependant enzymes alanine dehydrogenase (Ala-DH) and lactate dehydrogenase (Lac-DH). In theory, this would allow kinetic measurements of lyase enzyme activity towards the various substrates, and additionally to provide information on the non-chalcogenide metabolic products of lyase enzymes based on the coupled enzymes substrate specificities.

The use of Ala-DH⁵⁹⁰ and Lac-DH⁵⁹⁴ coupled assays are well known for detecting cysteine and seleno-cysteine lyase activity. However in this work, no Ala-DH or Lac-DH activity could be detected by measuring the NAD/NADH concentration at 340 nm when Ala-DH/Lac-DH were coupled with Rv1464, Rv3025c or Rv3340 *in vitro* reactions with Se-AA or sulphur amino acid substrates. Coupled assay conditions were verified through the positive controls of DL-alanine and sodium pyruvate, these yielded the expected dose-dependant responses with Ala-DH and Lac-DH, respectively. Possible inhibitory interactions between lyase substrates and Ala-DH or Lac-DH were ruled out experimentally, with Ala-DH and Lac-DH retaining activity with their respective controls in the presence of all lyase substrates.

It is unclear why Ala-DH or Lac-DH activity was not detected in coupled assays, particularly for Rv3025c with substrates DL-cys and Se-DL-cys, as GC-MS, PbAc and elemental selenium precipitation indicated that Rv3025c was utilising these substrates. Rv3025c has been experimentally confirmed as a cysteine desulphurase that produces alanine from cysteine; however, the methodology used was a radio-labelled sulphur transfer assay under inert argon gas that liberates sulphur this is then incorporated into a terminal [Fe-S] cluster acceptor.⁵⁸⁵ It is possible that the assay conditions used in this research were insufficiently reducing, since although DTT was included in the assay, buffer reactions were carried out under oxidising atmospheric conditions and without a terminal sulphur acceptor. This may have disrupted cysteine desulphurase function.

Purified Rv3025c is reported to have a yellow colour⁵⁸⁵ that is characteristic of pyridoxal phosphate binding enzymes. This yellow colouration was observed in the Rv3025c enzyme fractions isolated in this research, but was not seen for Rv1464, Rv3340 or MSMEG_2357 enzyme isolates that are also pyridoxal phosphate binding.^{592, 595} This may be an indication that these enzymes, despite being identified by SDS-PAGE on the basis of their size, may not be correctly folded or are lacking the pyridoxal phosphate co-factor. Additionally, lyase enzymes may not be producing alanine or pyruvate, alternatively producing a chalcogenide, ammonia and an α -keto acid,⁵³³ another possible lyase product that may not be able to be utilised by that Ala-DH or Lac-DH, although later keto-acid detection assays did not detect this metabolite.

6.5.2.4.5. Methylbenzothiazolinone hydrazone α -keto acid detection

Hydrogen sulphide production was detected as a product of Rv3025c metabolising DL-cys by PbAc paper and Rv3025c lyase activity towards Se-DL-cys was indicated by the precipitation of orange-red selenium species in the lyase assay. Mobilisation of sulphur or selenium by a β -lyase such as Rv3025c is expected to yield alanine,⁵⁸⁵ in addition to the respective chalcogenide species. However, depending on the particular lyase enzyme and substrate, other co-metabolites may include pyruvate or α -ketobutyrate and ammonia⁵³³ As neither alanine or pyruvate were able to be detected in the coupled enzyme assays, to determine if α -ketobutyrate was the corresponding co-metabolite of lyase enzyme activity the methylbenzothiazolinone hydrazone method was utilised.^{216, 553} No positive results for α -keto acid production from any mycobacterial lyase enzymes acting on any substrates were obtained. Since the expected results were obtained for α -ketobutyrate positive control, it is concluded that the mycobacterial lyase assay is not producing detectable amounts of stable α -keto acids,

possibly due to non-functional lyase enzyme.

6.5.3 Seleno-amino acid resistant transposon mutant *M. smegmatis*

A number of Se-AA resistant transposon mutant *M. smegmatis* were isolated and sequenced to identify gene disruptions that conferred Se-AA resistance. Transposon mutagenesis has been previously applied to mycobacteria and has contributed to the understanding the modes of action of isoniazid²¹² and various other antibiotics and chemicals.⁵⁴⁶ It was expected that essential mycobacterial enzymes such as cysteine desulphurase would not be detected via transposon mutagenesis as mutations affecting the function of these genes would not be viable.⁵⁷⁰ However, due to the hypothesised pro-drug nature of Se-AAs, with multiple steps toward the final anti-mycobacterial mechanism, transposon mutagenesis may provide insight into the first and last steps of the hypothesised Se-AA activity mechanism, transport and redox cycling. Transposon mutants with defined genetic disruptions identified were searched for in Mycobrowser Smegmalist/Tuberculist⁵⁶⁶ (<http://mycobrowser.epfl.ch/smegmalist.html>) and literature searched using the *M. smegmatis* gene name, the *M. tuberculosis* H37Rv gene equivalent and if necessary the enzyme common name and family. The findings of this analysis have been grouped into broad functional categories.

6.5.3.1 Redox gene function Se-AA resistant transposon mutants

Both MSMEG_4695 and _4696 (Rv2470 and Rv2471) may have been affected in TN-G1-M4 as the transposon insert site was between both ORFs. Rv2471 is a glucosidase involved in sugar metabolism. Rv2470 gene product GlbO is a haemoglobin-like oxygen carrier.⁵⁹⁶ The *M. leprae glbO* is involved in both aerobic respiration and the protective scavenging of reactive nitrogen species,^{597, 598} and this function fits well with the proposed final step of free radical damage in the Se-AA anti-mycobacterial mechanism.

The L-aspartate oxidase *nadB* MSMEG_3200 (TN-G1-M12 or Rv1595 *nadB*) is situated in an operon between functionally related genes of the NAD biosynthetic pathway, *nadA* and *nadC*, that may also be affected by this transposon insertion. *nadB* is predicted *in silico* as the target of the anti-mycobacterial drug clofazimine⁵⁹⁹ and is down regulated in *M. tuberculosis* under nutrient starvation.¹³⁶ *nadB* mutants are known to be dependant on niacin for *in vitro* growth.⁶⁰⁰ NadB functions to convert L-aspartate to iminoaspartate that is then condensed with dihydroxyacetone phosphate forming quinolinic acid in a downstream reaction catalysed by NadA. NadA is known to be a [Fe-S] cluster binding protein,⁶⁰¹ and

inhibition of the NAD synthetic pathway leads to mycobacterial cell death.⁶⁰² Therefore as with the *miaB* mutant TN-G1-M9, Se-AAs may interfere with the formation of the [Fe-S] cluster essential for NAD biosynthesis, or interact with the enzymes utilising this [Fe-S] cluster, impeding their function and generating oxygen radicals.

MSMEG_1019 is a ribonucleotide-diphosphate reductase (TN-G2-M9, Rv3051c *nrdE*) catalyses reduction of ribonucleotides to deoxyribonucleotides for DNA synthesis and repair.⁶⁰³ Ribonucleotide-diphosphate reductases use an iron dependant free radical tyrosine residue in their mechanism.⁶⁰⁴ *nrdE* is up-regulated by diamide mediated thiol-disulphide stress, and being a radical containing enzyme is sensitive to reactive nitrogen/oxygen damage.⁶⁰⁵ Upstream of *nrdE* is the glutaredoxin MSMEG_1017 (Rv3053c) that serves as the hydrogen donor (alongside glutaredoxin) for *nrdE*, although likely undisrupted by in the TN-G2-M9 mutant, demonstrates the redox-centric nature of this gene cluster. This provides evidence of Se-AAs toxicity being mediated through these redox function genes in mycobacteria in a similar but distinct manner to eukaryotes.⁶⁰⁶

MSMEG_0848 encodes the NADH ubiquinone oxidoreductase subunit 5 (TN-G3-M1, closest H37Rv homologue Rv3156 *nuoL*). *nuoL* encodes a type I NADH dehydrogenase, and this enzyme complex is part of the primary respiratory chain producing ATP through the oxidation of NADH.¹³⁶

nuoL expression in *M. tuberculosis* is repressed by reactive nitrogen species,⁶⁰⁷ and *M. tuberculosis* deficient in *nuoL* are unable to prevent acidification of the macrophage phagosome.⁶⁰⁸ Type I NADH dehydrogenase is also able to function, not only in ATP energy generation, but can neutralise the defensive ROS produced by macrophage NOX-2 enzyme activity, reducing phagosomal ROS accumulation and thus toxicity to resident mycobacteria.⁶⁰⁹ Disruption of this oxidoreductase conferring resistance to Se-AAs indicates that it may play a role in ROS generation from Se-AA metabolites.

Alkylhydroperoxidase enzymes ahpD/ahpC MSMEG_4890 and MSMEG_4891 (TN-G3-M10, Rv2428 and Rv2429 respectively) are unrelated in sequence but are genetically situated together in both *M. tuberculosis* and *M. smegmatis*. Both function similarly as components of the mycobacterial anti-oxidant defence system by reducing hydroperoxides, having broad substrate specificity but demonstrate a preference for organic hydroperoxides.⁶¹⁰

Thiol-dependant peridoxin proteins such as these use cysteine residues to react with oxidising

agents to produce a sulfenic acid group that is converted to a disulphide bond by interaction with a free thiol, either intra-molecularly on the same enzyme or inter-molecularly in the case of multimeric or interacting proteins. Unlike in other bacteria that have a flavin containing disulphide reductase, AhpF,⁶¹¹ in mycobacteria the disulphide is regenerated to cysteine by a substitute reducing agent, proposed to be mycothiol,^{558, 612} but alternatively, may be a bound lipoamide of succinate dehydrogenase.⁶¹¹

Both *ahpC* and *ahpD* are up-regulated in response to acidic phagosomal conditions⁶¹³ and *ahpC* is vital for survival of mycobacteria within macrophages indicating *ahpC* plays a role in virulence,⁶¹⁴ particularly in early stages of infection.⁶¹⁵

Strikingly, *ahpC* expression is up-regulated in the case of *katG*-mediated isoniazid resistance⁵⁵⁷ and *etaA*-mediated ethionamide resistance. This up-regulation may compensate for the loss of catalase/peroxidase function of these pro-drug activating genes.^{558, 612} This could indicate that Se-AAs would act in a complimentary or even synergistic fashion with the 1st line tuberculosis drugs isoniazid and ethionamide. Up-regulation of *ahpC* in response to isoniazid resistance can be dependant on promoter mutations that result in *ahpC* hyper-expression. While this up-regulation does not effect resistance to isoniazid, it serves to compensate for the loss of *katG* anti-oxidant protection.⁶¹⁶

ahpC/D is an ideal candidate component for the “missing link” redox cycling mechanism of the methylselenol component of the proposed mechanism in Figure 6.2, although further evidence is required to confirm that alkylhydroperoxidases are able to act on the methylselenol substrate producing reactive oxygen species.

Rieske [2Fe-2S] domain protein MSMEG_5925 (TN-G4-M8, Rv3526 *kshA*) is part of the two component ketosteroid hydroxylase complex *kshAB* in the mycobacterial cholesterol catabolism pathway. KshA is the oxygenase component, containing a [2Fe-2S] cluster binding a ferrous iron atom.⁶¹⁷ It is known that sub-optimal substrates (e.g. non-steroid) for this class of enzyme may uncouple its reaction causing heedless NADH consumption and production of damaging ROS.^{618, 619} It has been published that this enzyme complex, particularly *kshA*, is a good candidate target for tuberculosis drug discovery as there is no human homologue of this enzyme⁶²⁰. Additionally, inhibitors that cause the uncoupling of the reaction *kshA* catalyses would not only impede mycobacterial cholesterol catabolism, a process critical for tuberculosis pathogenesis,⁶²¹ but also accumulate damaging intracellular ROS and deplete cellular NADH reserves.⁶¹⁷ This mechanism matches evidence obtained from Se-AA resistant transposon mutant profiling in this thesis.

6.5.3.2 Kinase gene function Se-AA resistant transposon mutants

MSMEG_4270 (TN-G1-M6, Rv2202c) is an adenosine kinase that is essential in H37Rv⁵⁷¹ although evidently it is not essential for *M. smegmatis* or *M. tuberculosis*⁶²² under *in vitro* conditions. Rv2202c has been described as an atypical adenosine kinase with little specificity for adenosine, and it has been suggested that its primary function may be the phosphorylation of other substrates.⁶²³ It is possible that it is involved in the phosphorylation of xenobiotics, such as Se-AAs.

6.5.3.2.1 Glycerol kinase function Se-AA resistant mutants

Genes involved in glycerol metabolism were identified as transposon insert sites for three unique Se-AA resistant mutants. These are MSMEG_6756/7 (TN-G1-M7, *glpK* that has no direct H37Rv homologue and Rv1773c) MSMEG_6759 (TN-G1-M11, *glpK* that has no direct H37Rv homologue) and MSMEG_6756 (TN-G2-M8, *glpK* that has no direct H37Rv homologue). Sequence analysis confirms that all three mutants are unique insertions, not clonal duplicates.

Considering that three unique Se-AA resistant *glpK* transposon mutants were encountered, it is possible that *glpK* is a hotspot for Tn611 insertion, a similar trend of transposon directed mutation has been observed for IS5 insertion upstream of *E. coli glpFK* promoter in the presence of glycerol.^{624, 625} However, this does not explain the high levels of Se-AA resistance this transposon insertion confers. The H37Rv gene with the closest homology to the *M. smegmatis glpK* is Rv3696c, and this enzyme is also annotated as a glycerol kinase that is essential for H37Rv growth *in vitro*.⁵⁷¹ The glycerol kinase GlpK phosphorylates glycerol to glycerol-3-phosphate, trapping it in the cytoplasm; thus, glycerol can be used in central carbon metabolism.⁶²⁶ Rv1773c is a regulator of the *glpK*. Hyper-activity of *glpK* leads to accumulation of poisonous levels of glycerol-3-phosphate and ATP depletion. Glycerol is a preferred mycobacterial carbon source.²⁸⁷ *M. tuberculosis ΔglpK* mutants are able to colonise mouse lung effectively showing this gene is not essential *in vivo*.²⁸⁷ It is known that different carbon sources can modulate GlpK activity. For example, the presence of pyruvate will repress *glpK* activity.⁶²⁷ Mutations affecting glycerol kinase activity may induce alternative carbon metabolism by catabolite repression, this may render mycobacteria more resistant to Se-AAs. MSMEG_6758 is a glycerol facilitator within the *glpK* operon that assists import of glycerol into the cell.⁶²⁸ This facilitator may play a role in Se-AA import and its function may have been disrupted by the transposition event. It is also possible (albeit improbable) that GlpK has a direct interaction with Se-AAs, including phosphorylation of the Se-AAs, or Se-AA binding to free GlpK thiol residues, effecting the enzyme's function as

selenium compounds are known to inhibit kinase function in prostate cancer cells.⁶²⁹ The most likely cause of Se-AA resistance in GlpK deficient mutant *M. smegmatis* is a downstream reduction in metabolic flux, mitigating ROS production by methylselenium species.

6.5.3.3 Other Se-AA resistant transposon mutants

miaB (TN-G1-M9, MSMEG_2729 or Rv2733c) is a tRNA methylthiotransferase that forms 2-methylthio-*N*⁶-isopentenyl-adenosine from tRNA isopentenyl-adenosine in two steps of sulphur insertion, likely to occur by iron-sulphur [Fe-S] cluster formation and methylation using *S*-adenosylmethionine as a donor with a radical adenosine intermediate.⁶³⁰ The cysteine desulphurase, IscS, sulphur mobilisation activity is implicated in the formation/reconstitution MiaB [Fe-S] clusters which act as a thiol donor reservoir.^{631, 632} It is unknown how MiaB interacts with Se-AAs. It may act through the disruption of [Fe-S] clusters, leading to insufficient methylthio-*N*⁶-isopentenyl-adenosine tRNA, or it may be substituted into adenosine itself resulting, in a non-functional tRNA analogue.

Acyl-CoA synthase MSMEG_1098 (TN-G2-M3, Rv0551c *fadD8*) is not well researched. The *fadD* gene cluster is involved in lipid metabolism converting fatty acids into acyl adenylates,⁶³³ these have a wide range of cellular applications, including acetyl-CoA, mycobacterial cell wall and possibly mycobactin synthesis.⁶³⁴ The expression of many fatty acid synthesis genes, including *fadD8* is induced within two hours of macrophage infection.⁶³⁵ Free fatty acids, along with reactive oxygen and nitrogen species, are components of the immune response to mycobacterial infection,⁶³⁶ acyl-CoA synthase and β -oxidation of these free fatty acids may mitigate their anti-microbial activity.^{637, 638} The relationship between Se-AA toxicity and fatty acid ligase remains vague, but a potential route of Se-AA-mediated toxicity via acyl-CoA synthase, and the resistance of this transposon mutant, may be linked to mycothiol biosynthesis, since acetyl-CoA is required for the final acetylation step of mycothiol biosynthesis.⁶³⁹

An acetoacetyl-CoA reductase MSMEG_6024 (TN-G2-M15, Rv3667 *acs*) catalyses transformation of acetate into acetyl-CoA.⁶⁴⁰ Similar to TN-G2-M3 the contribution of this gene to Se-AA toxicity is suspected to be linked to mycothiol production that facilitates free radical generation from methylselenol.

The erythritol cyclodiphosphate synthase genes involved in isoprenoid and terpenoid synthesis MSMEG_6075 and MSMEG_6076 (TN-G2-M6, *ispF* Rv3581c and *ispD* Rv3582c) were found to be marginally resistant to Se-AAs. *ispF* has no human homologue but is essential in *M. tuberculosis*⁶⁴¹ thus this enzyme and its pathway are considered good drug targets.⁶⁴² It is not clear how Se-AAs interact, directly or indirectly, with these enzymes. Interestingly, immediately downstream of the *ispD/ispF* genes where the transposon insertion occurred, is MSMEG_6074 (*cysS*, Rv3085c) a cysteinyl-tRNA synthetase that charges tRNA with a cysteine residue. This gene may have potentially been disrupted by a frameshift mutation caused by the TN-G2-M6 transposon insert. CysS is very similar in function to the mycothiol synthesis enzyme MshC (incorporates L-cysteine into 1-*O*-(2-amino-1-deoxy- α -D-glucopyranosyl)-D-*myo*-inositol forming pre-acetylated mycothiol).⁵⁴⁵ The *mshC* gene is believed to have evolved from a gene duplication of *cysS*.^{643, 644} Therefore if this transposition has adversely effected CysS function, this serves as more evidence to implicate mycothiol in the Se-AA mechanism of action, not directly but likely in the recharging of redox cycling enzymes such as alkylhydroperoxidases. Alternatively, Se-cys utilisation by cysteinyl-tRNA synthase has been suggested as the mechanism for selenium toxicity;⁶⁴⁵ however, this suggestion is dated and has little supporting evidence.

The purine nucleoside phosphorylase MSMEG_1701 (TN-G2-M7, Rv3307 *deoD*) facilitates the cleavage of adenosine, inosine and guanosine in the mycobacterial nucleoside salvage pathway.^{646, 647} This salvage pathway is critical for mycobacterial persistence and starvation response.⁶⁴⁸ It is unclear how Se-AAs may interact with this gene product.

MSMEG_0211 (TN-G3-M2) is annotated as an ABC transporter however the closest H37Rv protein BLAST match is an ABC excinuclease (Rv1638, *uvrA*) involved in nucleotide excision DNA repair, a process important in the recovery of DNA damage caused by host reactive nitrogen/oxygen attack.⁶⁴⁹ Although it is established that *uvrA* is important in DNA repair particularly of physiologically relevant DNA damage such as those caused by reactive oxygen and nitrogen,⁶⁵⁰ the reasons for disruption of this gene conferring resistance to Se-AAs are not established.

The mono-ADP-ribosyl transferase (*arr*) MSMEG_1221 (TN-G3-M3, no similar H37Rv homologue) is known to ribosylate rifampicin, lending *M. smegmatis* some resistance to the drug.⁶⁵¹ Rifampin is ribosylated within the mycobacterial cytoplasm, and is the only known target substrate of ADP-ribosyl transferase, although this gene is up-regulated in response to

stress such as DNA damage and reactive oxygen.⁶⁵² In addition to its ribosylating activity, *arr* has a role in the stringent stress response of mycobacteria, suppressing translation and transcription particularly of rRNA.⁶⁵² The wider stress response functions of the gene are likely to be responsible for *arr* inactivation conferring Se-AA resistance.

The *araC* transcriptional regulator MSMEG_0376 (TN-G2-M14, closest H37Rv homologue Rv1931c) is known to contribute to *M. tuberculosis* virulence and fitness in murine models.⁶⁵³ The *araC* family regulate functions such as carbon metabolism/catabolism, stress response and virulence response.⁶⁵⁴ It is noted that in *M. tuberculosis* H37Rv, the thiol-peroxidase *tpx* Rv1932 is situated immediately upstream of Rv1931c, and may be under control of this regulator in mycobacteria, as *araC* regulators are known to confer resistance to oxidative killing in *Salmonella enterica*.⁶⁵⁵ Tpx confers protection from oxidative/nitrosative attack and is a key component of mycobacterial anti-oxidant defence and is easily linked back to Se-AA mode of action.⁶⁵⁶

Transposition occurred between MSMEG_2199 and MSMEG_2200 (TN-G4-M3, Rv1356c and Rv2964 (*purU*), respectively) and may have disrupted either or both of these genes. Rv1356c is annotated as a hypothetical protein of unknown function.⁶⁵⁶ Rv2964 *purU* is a formyltetrahydrofolate deformylase, the *in silico* predicted target of mefloquine.⁵⁹⁹ This enzyme generates formate and tetrahydrofolate by hydrolysis of 10-formyltetrahydrofolate.^{657, 658} Se-L-met may inadvertently over/under activate this enzyme and unbalance the tetrahydrofolate – formyltetrahydrofolate pool, as this enzyme is activated by methionine.⁶⁵⁷ It is noted that downstream of *purU* lies MSMEG_2201 (Rv0130 *htdZ* thioester dehydratase)⁶⁵⁹ that may act to lyse methylselenol from Se-L-met. Also downstream is MSMEG_2202 a dimethylaniline monooxygenase, both of these enzymes involved in cellular redox processing, and fit the proposed Se-AA mechanism.

6.5.3.4 Poorly defined Se-AA resistant transposon mutants

Transposon insertion in the following genes was implicated in Se-AA resistance, but lack of annotation for these genes rendered their possible link to the Se-AA mechanism inconclusive. Nonetheless, they are included below since the information may be of use to future researchers.

MSMEG_3638 (TN-G1-M3, Rv1841c) is a membrane bound cystathionine β -synthase (CBS)

domain protein, although the CBS nomenclature is misleading as it does not necessarily implicate cystathionine-related activity of this gene⁶⁶⁰ despite how convenient that would be is to this study. MSMEG_3638 expression is up-regulated in immune competent murine models.⁶⁶¹

MSMEG_4162 (TN-G2-M4) is annotated as a conserved hypothetical protein.⁵⁶⁶ Protein BLAST analysis against the *M. tuberculosis* H37Rv proteome shows Rv3719 to be the highest match but with only 13% query coverage. MSMEG_4606 (TN-G3-M4) is a conserved hypothetical protein with no known or predicted function.⁵⁶⁶ Protein BLAST against *M. tuberculosis* H37Rv produces the closest match of Rv3216 acetyltransferase; however, query coverage is non-significant at 6%. MSMEG_5842 (TN-G3-M5, Rv0779c) is a conserved membrane protein of unknown function. The 23S ribosomal RNA subunit MSMEG_3756 (TN-G4-M1, *rrlA*) conferred low level Se-AA resistance when subjected to transposon insertion, although the mechanism behind this resistance is unclear. The putative regulatory protein MSMEG_4431 (TN-G4-M5, closest H37Rv homologue Rv0386 *LuxR* transcriptional regulator however with poor query coverage of 6%). *LuxR* is involved in the mycobacterial response to reactive nitrogen attack.⁶⁰⁷

MSMEG_4842 is an amidohydrolase (TN-G4-M4, Rv0074) that hydrolyses carbon-nitrogen amide bonds. There are a wide variety of amidohydrolases acting on a range of substrates, from long chain fatty acids to small molecules such as amino acids.⁶⁶² Due to the lack of further annotation for this gene it is difficult to place its interaction with Se-AAs. Noted downstream of this gene are two acyl-CoA dehydrogenase enzymes MSMEG_4844 and MSMEG_4845, these may have been affected by frameshift mutation caused by the transposon insert.

6.5.3.5 Inducible regulation of alkylhydroperoxidase expression

After Se-AA transposon mutant genetic profiling revealed that *M. smegmatis* knockouts of MSMEG_4890 (*ahpC*) and MSMEG_4891 (*ahpD*) cause a drastic increase in Se-AA resistance. These genes were cloned into the pKW08 vector in the sense (with RBS) and anti-sense orientation to confirm their participation in the Se-AA resistance mechanism of action. These inducible gene knock-up / knock-down strains did not mimic the large 8-16 fold increase in Se-AA resistance seen in the transposon mutant TN-G3-M10, giving mixed results with a 2-fold increase in the MIC of SMSC when over-expressed and 4-fold when *ahpC* was under-expressed by inducible antisense knockdown. Similarly induction of either

up- or down-regulation of *ahpD* only yielded a 2-fold increase in SMSC resistance, and had no effect on the MIC of Se-L-met.

The consequences of *ahpC* expression on Se-L-met activity were somewhat indicated by a 2-fold decrease in MIC upon *ahpC* overexpression and a 4-fold increase in MIC on under-expression.

This, in addition to other tetracycline inducible anti-sense gene expression modulation previously carried out in this research, indicates that these vectors are not performing as required. The tetRO promoter may be insufficiently strong to cause significant up- or down-regulation of these genes, or a compensatory response is carried out by the mycobacteria that mitigates the modulation of gene expression.

6.5.3.6 Seleno-amino acid synergy with isoniazid

Alkylhydroperoxidase enzymes are known to be up-regulated in *katG* loss of function mediated isoniazid resistant *M. tuberculosis*.^{557, 558} This is a compensatory response to loss of KatG antioxidant defence function.⁶⁶³ Expression of *ahpC* and sensitivity to isoniazid have an inverse correlation.⁶⁶⁴ Therefore it was proposed that if the *ahpC/D* knockout mutant TN-G3-M10 exhibited increased resistance to Se-AAs, up-regulated *ahpC/D* expression, such as in response to isoniazid exposure, may increase mycobacterial sensitivity to Se-AAs. This drug-target relationship may also cause a synergistic inhibitory effect between Se-AAs and isoniazid. This was tested by the checkerboard assay for drug synergy,²⁰⁸ where the MIC of Se-L-met decreased from 8 to 2 μM when in combination with INH, and the MIC of INH decreased from 2 to 0.5 μM in combination with Se-L-met. This yielded fractional inhibitory concentrations of 0.25 for both Se-L-met and INH, and resulted in a fractional inhibitory concentration index of 0.5 between Se-L-met and INH. This value implies a 2-fold increase in inhibitory activity when Se-L-met and INH are in combination, although this is the lowest level of synergy detectable by this checkerboard assay.²⁰⁸

This synergy is a promising indication for Se-AAs in tuberculosis combination drug therapy, necessary for thorough tuberculosis treatment. In MDR-TB strains with resistance to INH mediated through *katG* loss of function mutation where *ahpC* is hyper-expressed,^{557, 558} this synergy may be even more pronounced. This requires further investigation using MDR-TB strains, as combination therapy including INH and Se-AAs would deliver a synergistic “two hit combo” against tuberculosis *katG* and *ahpC* genetic targets, where loss of function mutations conferring resistance to INH may also provoke increased sensitivity to Se-AAs.

6.5.4 General conclusions of mechanism of action studies

Several results disclosed and discussed above are in agreement with the three-part hypothesis for the mode of Se-AA anti-mycobacterial action depicted in Figure 6.3. Competition assays with Se-AAs and their regular sulphur amino acid counterparts show that methionine and cysteine are able to alleviate Se-AA toxicity, indicating these are competing with Se-AAs at either the transport or substrate level, or both. Anti-sense RNA regulation of bioinformatically identified putative lyase enzymes proved largely inconclusive, with the exception of MSMEG_2357 β -lyase downregulation using pMIND vector conferring resistance to SMSC. However, experiments using the *S. cerevisiae* orthologue of cystathionine- γ -lyase which conferred resistance to Se-L-met demonstrated that lyase genes are involved in Se-AA toxicity, activating Se-AA pro-drugs to their active form within the cell. Enzyme based assays were able to determine that Rv3025c can cleave SMSC *in vitro* producing methylselenide, an indicator of methylselenol; however, further kinetic and substrate preference analysis through a variety of enzymatic assay routes were unsuccessful. Follow-up studies to define the specific mycobacterial enzymes involved in Se-AA lysis and their reaction products are required. Finally, profiling of transposon insert generated Se-AA resistant mutant *M. smegmatis* directly implicates several redox function genes such as alkylhydroperoxidases and ketosteroid hydroxylase in the Se-AA mechanism, likely through redox cycling of methylselenol and generation of the reactive oxygen that causes cellular damage. Other genes such as those contributing to mycothiol synthesis, for example acetyl-CoA synthase, are also indirectly implicated in Se-AA resistance in *M. smegmatis*. The potential genetic targets of methylselenol in mycobacteria are illustrated in Figure 6.18.

Figure 6.18: Proposed methylselenium anti-mycobacterial effects.

Based on resistant transposon mutants, Se-AAs are hypothesised to exert anti-mycobacterial effects through methylselenium species such as methylselenide. Methylselenium causes toxicity through ROS generation via mycobacterial redox function enzymes, Fe-S cluster disruption, halting cholesterol catabolism and paired NADH depletion, decreasing cellular mycothiol and other unelucidated mechanisms.

6.6 Summary

Seleno-amino acids were discovered to be anti-mycobacterial agents through modified culture-condition chemical library screening against *M. smegmatis*. Structure-activity relationship analysis showed that methylated selenium, primary amine, carboxylic acid and *levo* stereoisomerism are key structural features for Se-AA anti-mycobacterial potency. Several Se-AA derivatives were synthesised and tested based on literature indications of increased activity; however, these derivatives all exhibited various reductions in potency, providing additional insight into the Se-AA structure-activity relationship.

Seleno-amino acids are predominantly researched for their anti-cancer properties, the mechanism of action for which is understood to involve radical oxygen production causing apoptosis of cancer cells. However; mycobacteria lack glutathiol, a key component for the activation of Se-AAs toxicity in eukaryotes. In this work mechanism is proposed for Se-AA anti-mycobacterial activity. First, the Se-AA pro-drug is recognised by mycobacteria as a nutrient and imported into the cell. Second the Se-AA pro-drug is cleaved by mycobacterial β or γ -lyase enzymes (dependant on the Se-AA species) producing amongst other metabolites reactive methylselenol/selenide. Finally reactive methylselenium species generate damaging reactive oxygen species through continued redox cycling within the mycobacterial cell.

Evidence for this mechanism presented included a competition effect with Se-AAs and their sulphur amino acid counterparts, cysteine and methionine, that can alleviate Se-AA anti-mycobacterial activity. Down-regulation of the β -lyase MSMEG_2357 conferred resistance to SMSC; however, further experiments failed to define what mycobacterial lyase enzymes are involved in Se-AA activation. Supplementary research using *S. cerevisiae* identified the CYS3 cystathionine γ -lyase gene deletion strain as resistant to Se-L-met. This evidence indicates the second step of the hypothesis is correct but could not be confirmed in mycobacteria due to experimental difficulty. Furthermore, enzymatic evidence that *M. tuberculosis* cysteine desulphurase Rv3025c acted upon SMSC to create methylselenol was found by GC-MS analysis and *in vitro* enzyme assays, although these could not be kinetically defined or reproduced for other *M. tuberculosis* lyase enzymes. Transposon mutants of *M. smegmatis* resistant to Se-AAs were genetically profiled, revealing a clear trend in redox-function gene disruptions conferring a Se-AA resistant phenotype. Such as alkylhydroperoxidase genes *ahpC* and *ahpD* that usually function to protect mycobacteria from reactive oxygen, but are hijacked by reactive methylated selenium into producing and cycling damaging reactive oxygen species. Ketosteroid hydroxylase *kshA* also appears to be uncoupled by methylselenium causing mycobacterial energy depletion by NADH consumption and reactive oxygen generation via the enzymes [2Fe-2S] cluster oxygenase in addition to obstructing the enzymes pathogenically critical function in cholesterol catabolism. The implication of mycobacterial redox processes in Se-AA mechanism, particularly the alkylhydroperoxidase *ahpC*, may be a unique opportunity for synergistic drug combination therapy with isoniazid, as Se-AAs and isoniazid exhibit a 2-fold increase in activity in combination with one another. In MDR-TB with a loss of *katG* function, *ahpC* may be over-expressed, further increasing the redox cycling and inhibitory activity of Se-AAs.

CHAPTER SEVEN

General Discussion

7.1 Overall synopsis

Tuberculosis is an ancient disease caused by the bacterium *M. tuberculosis*, a pathogen that has evolutionarily adapted to endure and thrive within the immune response intended to destroy it. Despite the discovery and development of antibiotic drugs effective in treating tuberculosis, it remains a major infectious disease causing 1.4 million deaths in 2010.^{9, 10} Due to the pathogen's resilient nature and inadequate treatment regimens, development of drug resistant *M. tuberculosis* strains has rendered many conventional antibiotic therapies ineffective and some clinical manifestations of the disease practically untreatable.^{11, 77, 82, 83} Therefore development of new antibiotics to treat TB infections is integral for the plan to stop TB, in addition to new diagnostics, treatment procedures and vaccines. This plan is in effect and has the ambition of eradicating TB by the year 2050.^{9, 10, 14}

This thesis contributes to global TB eradication efforts, presenting a novel high-throughput drug screen for anti-mycobacterial compounds using modified culture conditions and nutrient limitation culture media with the fast growing tuberculosis model species *M. smegmatis*. Three chemical libraries were screened against four different culture conditions, two nutrient rich conditions and two nutrient limited conditions. These screens demonstrated that mycobacterial sensitivity to chemical inhibitors was dependent upon the culture conditions utilised. These screens also identified a number of previously unknown mycobacterial inhibitors some of which exhibit desirable characteristics warranting further research into their development as anti-tubercular drugs.

Se-methylselenocysteine (SMSC), a novel compound identified during nutrient limitation screening,¹²² was selected for further research as an anti-mycobacterial agent due to its anti-mycobacterial novelty, inhibitory activity specific towards mycobacterial species and known consumption as a health supplement.⁴⁰¹ SMSC would not have been detected if the HTS of this work had been restricted to standard nutrient conditions. SMSC represents a new class of anti-mycobacterial compounds, the seleno-amino acids. Seleno-amino acids are naturally occurring selenium analogues of the sulphurous amino acids cysteine and methionine and their structural derivatives.³⁸⁷ These compounds are implicated as beneficial in a variety of medical conditions, including diabetes,⁴³⁶ cardiovascular disease{Navas-Acien, 2008 #531} cardiovascular disease⁴⁴¹ and most predominantly cancer.^{428, 429, 433, 434} Little attention has been afforded to the anti-microbial properties of Se-AAs as they have little inhibitory activity towards other bacterial species such as *E. coli*, *S. aureus* and *P. aeruginosa*. This work is the first to

disclose the anti-mycobacterial activity of these compounds, with MIC values in the 10-30 μM range towards *M. tuberculosis*. Se-AAs have varying toxicity toward human cell lines but are generally considered to be in the non-toxic range against non-cancerous cell lines.⁵⁰⁰ The maximum recommended human doses of Se-AAs as nutritional supplements can raise plasma selenium levels into the *M. tuberculosis* MIC range.⁵⁰⁵ Se-AAs show a preference for accumulation in lung tissues compared with other selenium compounds,⁵¹³ a favourable indication for treatment of pulmonary infection.

The relationship between Se-AA structure and the inhibitory activity towards mycobacteria was queried, and key structural aspects contributing to anti-mycobacterial potency included methylated selenium, primary amine and carboxylic acid functional groups and *levo* stereochemistry. The basic mechanism of Se-AAs inhibitory action towards cancer is understood to involve enzymatic lysis of Se-AAs producing reactive selenide species^{535, 665} that in turn are redox cycled by glutathione, generating reactive oxygen species that cause cellular damage and an ensuing apoptotic response.⁶⁶⁶ A similar mechanism of action was suspected for mycobacteria; however, mycobacteria do not utilise glutathione;⁵⁴⁵ therefore, the redox cycling of selenide species must be carried out by an unknown mycobacterial equivalent.

This thesis proposes a three-part mechanism for the anti-mycobacterial action of Se-AAs: First, Se-AAs are recognised as nutrients and are actively transported into the mycobacterial cell by ABC transporters and super-facilitator type transporters⁶⁶⁷ possibly assisted by chaperones such as Rv0411c glutamine binding lipoprotein. This transport is pronounced under nutrient limited conditions such as those encountered during pathogenesis.⁶⁶⁸ Second, the Se-AA acts as a pro-drug and is enzymatically cleaved by mycobacterial lyases, producing the active metabolite methylselenol or an equivalent selenide species. Third, these selenide species are redox cycled in mycobacteria producing reactive oxygen that damages cellular components such as DNA, proteins and lipids.

Support for this three-step mechanism included competition effects between Se-AAs and cysteine and methionine in which these amino acids are able to alleviate Se-AA inhibition in mycobacteria, indicating mycobacterial recognition of Se-AAs as their sulphur amino-acid equivalents at the transport and or substrate level. Furthermore, down-regulation of the β -lyase MSMEG_2357 in *M. smegmatis* confers resistance to SMSC, with methyldiselenide

production detected with the *M. tuberculosis* homologue of this enzyme Rv3025c using an *in vitro* enzyme reaction. A red selenium precipitate is observed upon incubation of Rv3025c with Se-cys. This suggests that mycobacterial β -lyase enzymes are involved in metabolising seleno-cysteine pro-drugs such as SMSC and Se-cys into an active methylselenol and other forms of selenides. No experimental link between mycobacterial γ -lyase enzymes and Se-met could be made; however, the *S. cerevisiae* CYS3 cystathionine γ -lyase gene deletion strain was found to confer resistance to Se-L-met, indicating this gene's significance in Se-met activation that likely coincides with a mycobacterial equivalent enzyme.⁵⁷³ Conclusive evidence demonstrating the kinetic parameters and substrate specificity of *M. tuberculosis* lyase enzymes toward Se-AAs was not available, and further research is required to determine what lyase enzymes metabolise Se-AAs in mycobacteria.

Using transposon mutagenesis, mycobacterial genes contributing to Se-AA toxicity were identified that included genes for alkylhydroperoxidase enzymes that typically function to protect mycobacteria from oxidative damage but produce reactive oxygen species by redox cycling of methylselenium species. Several other mycobacterial genetic targets of Se-AAs such as the ketosteroid hydroxylase were identified as playing a role in generation of reactive oxygen from methylselenium.

This thesis describes a novel approach to anti-mycobacterial drug discovery, from initial chemical screening through to lead development studies of seleno-amino acids, a novel class of anti-tuberculosis compounds, detailing the structure activity relationship of these compounds and providing a foundation for their mechanism of anti-mycobacterial activity.

7.2 Summary of experiments

7.2.1 Conventional media high-throughput screening

The model tuberculosis organism *M. smegmatis* was used in a high-throughput screen against three chemical libraries to identify compounds with anti-mycobacterial activity. Use of dual viability indicators in screening, absorbance and GFP, minimised false positives and both provided good indications of bacterial growth. Use of an unstable GFP variant⁶⁶⁹ may improve the assay for kinetic purposes by removing interference of lingering GFP accumulated from dead or lysed cells, but this was not required for this end-point growth assay. Hit compounds exhibiting $\geq 90\%$ inhibition of *M. smegmatis* growth were validated in quantitative dose-response experiments, and the resulting confirmed inhibitors were cross-

referenced with screening project results from *M. bovis* BCG and *M. tuberculosis* H37Ra. These screening experiments showed that screening under 7H9 media conditions produced slightly more hit compounds compared with LB media but more importantly, hits derived from 7H9 media screening were more likely to have common inhibitory activity towards *M. tuberculosis* H37Ra.

Several compounds were identified through rich media screening that exhibited strong inhibition towards mycobacteria and also had favourable indications with regard to novelty and low toxicity that warranted further investigation of these hit compounds. Compounds considered to be of high priority for further investigation into their anti-tubercular potential included the anti-helminthic chemical, niclosamide, used in the treatment of parasitic worm infection in humans and therefore likely to be safe and well tolerated in human administration.⁶⁷⁰ Niclosamide showed a good activity towards *M. tuberculosis* with an MIC of 6.25 μM against the H37Ra strain *in vitro*.

Pyrrhione zinc a coordinated zinc complex, commonly used in topical applications such as shampoos and anti-microbial ointments²⁴⁸ completely inhibited virulent tuberculosis strains *in vitro* at a low concentration of 2.5 μM .²²¹ It is believed to cause iron starvation in yeast.⁶⁷¹ Pyrrhione zinc is also reported to have low oral toxicity in canines with a LD₅₀ of 600 mg/kg.²⁴⁹

A limonoid natural product of the neem tree, azadirachtin, is typically used as an insect repellent and anti-feedant.²⁶⁰ Neem oil is used in Indian folk medicine against mycobacterial disease such as tuberculosis and leprosy.²⁶² Azadirachtin showed only modest anti-mycobacterial activity in the present study, having an MIC of 25 μM toward *M. smegmatis*. The selectivity, however, of this compound is very favourable due to its non-toxic nature in mammals with daily doses up to 1500 mg/kg having no observable effect in murine models.²⁵⁹ Azadirachtin is a high priority for further analysis, first to determine its activity toward virulent *M. tuberculosis* strains and later to define its mechanism of action to provide insight into potential chemical derivatisation for improving activity.

Neohesperidin is also a natural product of citrus plants and is used as a sweetener/flavour enhancer in many foods. It is therefore considered very safe for human consumption²⁷¹ with daily oral doses of up to 3300 mg/kg having no adverse effects.²⁷³ In the present study,

neohesperidin had an *in vitro* MIC of 6.25-12.5 μ M towards *M. smegmatis* in rich media types. Citrus plant extracts have shown inhibition against MDR strains of *M. tuberculosis* and methicillin resistant *S. aureus*,²⁷² and neohesperidin may be the culprit responsible for the activity of these extracts. This compound is a high priority for further investigation into its antibiotic potential.

7.2.2 Modified culture conditions for high-throughput screening

Continuing on the high-throughput screening using conventional rich media conditions, chemical libraries were used to screen *M. smegmatis* again using nutrient limited culture conditions of HdeB media. These screens were carried out to investigate the influence of culture conditions on mycobacterial susceptibility to chemical inhibitors, as mycobacteria are known to alter their gene expression and metabolism under conditions of nutrient starvation.^{276, 277} This work sought to use nutrient limited culture media to emulate conditions encountered by *M. tuberculosis* during pathogenesis, including low available nutrients such as amino acids,²²² leading to utilisation of activation of catabolic nutrient scavenging pathways that lie dormant in rich media conditions.²⁷⁴ This would provide an ideal screen for anti-tubercular drugs in order to identify pathogenically relevant hit compounds with the ease and speed of using the *M. smegmatis* model organism.

Compounds demonstrating $\geq 80\%$ inhibition were validated in a dose-response quantitative assay as was done in conventional media screens, and the resulting validated inhibitors were cross-referenced with results of *M. tuberculosis* H37Ra and *M. bovis* BCG screening projects. HdeB nitrogen starvation medium was found to be the most sensitive culture condition for detecting anti-mycobacterial compounds, revealing more than twice the number of compounds than HdeB carbon starvation medium. The hits from HdeB nitrogen limited medium also had 37% more hit compounds coinciding with hit compounds from *M. tuberculosis* H37Ra screens. Both of these nutrient limited media types identified more mycobacterial inhibitors than the conventional rich media 7H9 and LB. Overall, nutrient limited conditions identified almost double the amount of anti-mycobacterial compounds that were detected in nutrient rich media, and 33% more compounds identified by nutrient starvation screens were also identified as inhibitory towards *M. tuberculosis* H37Ra. The overall percentage of coinciding hits with H37Ra, however, was lower for nutrient starvation conditions.

Further promising anti-mycobacterial drug development leads were elucidated by screening chemical libraries using nutrient starvation conditions, including finding novel anti-mycobacterial activity for already known drugs. For example, one such known compound was the anti-fungal agent pentamidine, delivered as an aerosol for the treatment of pneumocystis³⁰⁷ and identified as eradicating virulent tuberculosis at doses as low as 5 μ M in an intra-macrophage assay.²²¹

Phenothiazine and tricyclic psychiatric drugs such as trifluoperazine used in the treatment of schizophrenia and depression were also identified as having anti-mycobacterial activity. These classes of chemical have a good pharmacological profile and are routinely administered to humans; however, these drugs also have psychological side effects. Phenothiazines are known to inhibit XDR strains of tuberculosis³⁰³ accumulate within macrophages³⁰² and block drug efflux pumps,³⁰⁴ making them ideal for combination therapy, provided the psychiatric effects of these drugs can be structurally mitigated.

The agricultural fungicide chlorothalonil (tetrachloroisophthalonitrile) is non-toxic in murine models with a good safety profile and an oral LD₅₀ > 5000 mg/kg.³³⁸ Chlorothalonil is active only under nutrient limitation culture conditions, and the MIC is 3.125-6.25 μ M against *M. smegmatis*. It has an excellent selective activity against mycobacteria and has confirmed activity in the slow growing mycobacterial species *M. bovis* BCG and *M. tuberculosis* H37Ra.

Cefdinir, a cephalosporin that disrupts peptidoglycan synthesis, is an interesting hit because mycobacteria are generally resistant to β -lactams due to their complex cell wall and expression of β -lactamase enzymes.³²³ Not demonstrating potent anti-mycobacterial activity, with a high MIC of 25 μ M against *M. smegmatis* in HdeB nitrogen starvation, cefdinir is a worthwhile development lead as it is extremely well tolerated.

A similar new use for an old drug is the anti-fungal agent ketoconazole, a highly lipophilic ergosterol synthesis inhibitor suitable for oral systemic administration.³⁴³ Studies have shown that ketoconazole improves treatment outcomes in conjunction with first line tuberculosis drug treatment.

Se-methylselenocysteine and its chemical class of seleno-amino acids were first identified in the nutrient limited anti-mycobacterial screening of this work.¹²² A natural product found in many commonly consumed plant species,^{310, 393} Se-AAs such as SMSC and Se-met are also sold over the counter as nutritional supplements.³¹¹ Most scientific research on Se-AAs concerns their cancer preventing properties,³¹² although Se-AAs are thought to have a wide array of beneficial health effects.³¹¹ These compounds were chosen for more detailed follow up study as they are novel anti-mycobacterial agents with good anti-mycobacterial potency. Human consumption of Se-AAs is known to be well tolerated. Se-AAs represent a promising class of anti-mycobacterial agents as they mimic sulphur amino acids, and mycobacteria such as *M. tuberculosis* have an unusually high consumption and utilisation of sulphated metabolites,⁶⁷² this may cause differential sensitivity toward mycobacteria over other bacterial and eukaryotic species.

7.2.3 Seleno-amino acids

Seleno-amino acids are known in the literature for their health effects towards various conditions such as cancer; however, little is published regarding the anti-microbial activity of these compounds. Therefore in the present study the inhibitory activity of a range of organic and inorganic selenium compounds was assessed in the primary mycobacterial model organism *M. smegmatis*. Strong inhibition of *M. smegmatis* growth within the 7.8-31.2 μM range was observed for the Se-AAs SMSC, Se-DL-met and Se-L-met. Moderate activity in the 25-50 μM range was observed for Se-DL-cys, and poor inhibitory activity of 125-250 μM was seen for the dimerised seleno-DL-cystine. The inhibitory activity for all the aforementioned Se-AAs was seen in all media types with the exception of LB nutrient rich media, where no inhibitory activity was observed at 2 mM, the highest concentration tested. Non-selenium amino acids such as DL-cys, DL-met and s-methyl-L-cys were found to have no inhibitory activity towards *M. smegmatis* regardless of media type. Non-amino acid selenium compounds were found to have poor inhibitory activity toward *M. smegmatis* in all media types, apart from methylseleninic acid and ebselen that had MIC activity in the 31.2 – 125 μM range in all media types except LB.

Based on these results in *M. smegmatis*, a panel of selenium compounds was tested against a range of bacterial species. This panel of compounds included the most potent inhibitory Se-AAs SMSC and Se-L-met, the non-amino acid selenium inhibitory compound

methylseleninic acid and the low-inhibitory activity inorganic selenium compound sodium selenite.

M. tuberculosis H37Ra exhibited a similar inhibition profile to *M. smegmatis* with MIC concentrations of 15.6 for the top Se-AA compounds SMSC and Se-L-met. Methylseleninic acid and ebselen also demonstrated good inhibition at 31.2 and 62.5 μ M MIC, respectively, and sodium selenite as expected did not cause any significant inhibition. The virulent strains of *M. tuberculosis* H37Rv and CDC1551 were sensitive to inhibition by Se-AAs with the lowest MIC values of 22.88 and 9.15 μ M for each of those strains, respectively. These inhibitory concentrations towards *M. tuberculosis* are comparable to tuberculosis treatment drugs such as clarithromycin, cycloserine and pyrazinamide. The slow growing mycobacterial species *M. bovis* BCG demonstrated similar but slightly lower sensitivity compared to *M. smegmatis* for inhibition by Se-AAs, with SMSC and Se-L-met causing complete growth inhibition at 31.2-62.5 μ M. This indicates that dietary Se-AA supplementation may be a useful and inexpensive adjunct to controlling bovine TB in cattle, particularly in regions with low soil selenium content.

Selenium compounds, including Se-AAs, showed little inhibitory activity toward other bacterial species cultured in rich or nutrient limited media. *S. aureus* was unable to be completely inhibited by either SMSC or Se-L-met even at the highest concentration tested of 2 mM. *E. coli* was somewhat susceptible to Se-AA inhibition at high concentrations under M9 minimal media conditions with MIC values of 500 μ M for SMSC and 2000 μ M for Se-L-met. *E. coli* was uninhibited by Se-AAs in rich LB media. *P. aeruginosa* was also not inhibited by the selenium panel of compounds with the exception of Se-L-met that had an MIC of 1000 μ M in LB media. *V. harveyi* was more sensitive to inhibition by Se-AAs with MIC values in the 250 - 1000 μ M range; however, these MICs are still considered to be too high for a viable antibiotic development lead toward *Vibrio* species.

The panel of selenium compounds was also tested against haploid and diploid strains of the eukaryotic yeast *S. cerevisiae*. Se-L-met evoked complete inhibition of yeast growth at 62.5 – 125 μ M; while, SMSC could not completely inhibit growth even at the highest concentration of 2 mM.

SMSC was tested against the human cell lines HL-60 and J-774 macrophages, and SMSC

inhibited the HL-60 leukaemia cell line with an IC_{50} (not an MIC) value of 73.03 μM . No inhibition was seen for the J-774 murine macrophage cell line at the highest concentration tested of 250 μM . Both SMSC and Se-DL-met were found to exhibit little inhibitory activity at 100 μM toward Vero cells.

Therefore, it was concluded that Se-AAs demonstrated a high level of specificity towards mycobacteria but with little or no inhibitory activity towards other bacterial species, even at high concentrations. The inhibitory activity of Se-AAs toward mycobacteria was dependant on nutrient limited culture conditions. Se-AAs are known to have inhibitory properties towards cancer cell lines⁴⁹¹ but are generally considered to be non-toxic against non-cancerous cells.⁴⁹⁹

Considering the tolerance of selenium compounds in mammals, with toxic levels above 800 μg / day in humans,³⁷⁷ Se-AAs are a viable developmental lead for a novel tuberculosis treatment drug. Further studies into the Se-AA mechanism of action and structure-activity relationship may improve both the anti-mycobacterial activity and decrease the human cell toxicity of these compounds. The groundwork for future mechanism of action and structure-activity studies was carried out in this research. Key structural features influencing the anti-mycobacterial activity of Se-AAs were determined to be substitution of a selenium atom for the sulphur of methionine or cysteine, methylation of the selenium, primary amine and carboxylic acid moieties and levo stereochemistry. A number of Se-AA derivatives, such as di-peptides and amides, were synthesised for increased selective anti-mycobacterial activity; however, these all exhibited lower inhibitory activity than the parent molecule.⁵¹⁸ Although, these derivatives did, provide insight into the Se-AA structure activity relationship such as the diminishing effect of c-terminal conjugations on activity.

A mechanism of Se-AA anti-mycobacterial action was hypothesised based on a published hypothesis of the mechanism of Se-AA anti-neoplastic activity.^{447, 534, 535} This mechanism consisted of three parts; first, Se-AA were imported into the mycobacterial cell under the guise of a nutrient; second, the Se-AA acted as a pro-drug and was cleaved by mycobacterial lyase enzymes into its active methylselenide constituent; and last, the methylselenide was then redox cycled by mycobacterial antioxidant processes to produce damaging oxygen free radicals. This hypothesis was explored and supporting evidence included a competition effect between Se-AAs and sulphurous amino acids, where supplementing culture media with

DL-cys or DL-met rescued the mycobacteria from Se-AA toxicity. Down-regulation of the β -lyase MSMEG_2357 by anti-sense RNA induced resistance to SMSC in *M. smegmatis* with an 8-32 fold increase in its MIC, and gene deletion of CYS3, a γ -lyase in *S. cerevisiae*, conferred resistance to Se-L-met with an 8-fold increase in MIC. Methyldiselenide and an orange selenium precipitate⁶⁷³ was detected as a product of the *in vitro* enzyme reaction of purified *M. tuberculosis* H37Rv β -lyase Rv3025c and Se-DL-cys, indicating utilisation of Se-DL-cys as a substrate by this enzyme.

Transposon mutants resistant to Se-AAs were generated and the mutation caused by transposon insertion was genetically defined, revealing several genes involved in mycobacterial redox homeostasis such as alkylhydroperoxidase and ketosteroid hydroxylase in addition to more cryptic gene disruptions for which the role in Se-AA resistance is unclear. Finally, Se-AAs demonstrated synergistic potential in combination with the front line tuberculosis treatment drug isoniazid. This is thought to be due to the see-saw gene expression relationship between *ahpC*, responsible for Se-AA activity, and *katG*, responsible for isoniazid activity, where loss of function of *katG* is compensated by *ahpC* hyperexpression.⁵⁵⁷ This over-expression of AhpC may cause increased sensitivity to Se-AAs and is believed to have no effect on isoniazid sensitivity but rather compensates by maintaining mycobacterial redox status.⁶⁷⁴ This is a favourable indication for treatment of MDR-TB that is isoniazid resistant through KatG loss of function mutations, as these strains may exhibit higher sensitivity to Se-AA inhibition.

7.3 Critical evaluation

After completion and contemplation of this work, several improvements and critiques of this research are indicated, as suggested below.

7.3.1 High-throughput screening

The screening aspect of this project could have been improved in several ways. For example, adaptation of the assay from 96-well plate format to 384-well plate format would decrease screening costs and increase the throughput of the assay. This would allow quadruplicate replicates to be carried out on a single plate, minimising plate to plate variation that arises from using three separate 96-well plates for triplicate repeats.

Rich culture conditions used in the screening could have been more defined. For example, a complete synthetic media such as the 7H9 with amino acid supplementation described by Parish²²² in which amino acid concentrations can be titrated, as opposed to LB in which undefined amino acids are supplied via peptone and yeast extract. Similarly, expansion of the nutrient limited medium types to include HdeB oxygen starvation would provide a more pathogenically relevant culture environment. For example, forming non-culturable cells⁶⁷⁵ compared to HdeB carbon and nitrogen starvation media; however, this would require specialised air-tight plate seals or a similar arrangement to exclude atmospheric oxygen from the assay. In retrospect, HdeB sulphur limitation media would also provide interesting culture conditions for the detection of chemical inhibitors of essential mycobacterial sulphur metabolism processes.⁶⁴³

Hit compounds derived from high-throughput screening would be more pathogenically relevant if the screen were a high content whole cell type, for example, an intra-macrophage assay using phagocytosed bacteria.⁶⁷⁶ This was considered to be beyond the scope of the project but was explored by another research team member.⁶⁷⁶ Validation of hit compounds derived from high-throughput screening proved to be a laborious process and could have been better streamlined by literature searching hit compounds to rule out cytotoxic agents with little therapeutic potential, thus, saving compound, time and consumables. Ideally anti-mycobacterial activity screening of chemical libraries could be run in parallel with a cytotoxicity counter screen to integrate a toxicity validation step for compounds in the initial screening. Although this project was intended as proof of concept for modified culture condition screening, increasing the number of chemicals screened would provide further developmental leads for novel anti-mycobacterial agents.

7.3.2 Seleno-amino acid activity

For the testing of mycobacterial susceptibility to Se-AAs, a wider range of media types such as HdeB oxygen and sulphur starvation may have provided earlier insight into the mechanism of action of Se-AAs. Similarly, using a defined rich medium in which amino acids could be excluded or titrated could have been of similar use in elucidating the Se-AA mechanism.

A major omission of this work was the selenium compound testing against virulent strains of tuberculosis such as *M. tuberculosis* H37Rv and CDC1551, since assays were performed using these strains failed to give consistent results regarding the inhibitory activity of Se-AAs towards these pathogenically relevant strains; therefore, it is difficult to draw conclusions regarding the inhibitory activity of Se-AAs toward these pathogenic strains and confirmatory retesting is required. This was problematic to address as the facilities where this research was carried out were restricted to a PC-2 level laboratory, with no PC-3 facility available at VUW; thus, testing using these virulent PC-3 strains had to be out-sourced to NIH and JHU. Inhibitory activity levels of Se-AAs are consistent between mycobacterial species; therefore, the activity of SMSC is presumed to follow this trend against H37Rv. Nonetheless, the variation in results for SMSC against H37Rv casts some doubt on the specific level of activity of this compound toward virulent tuberculosis. In addition, testing was carried out for racemic Se-DL-met against H37Rv, but not Se-L-met, the most promising member of the Se-AA family. This research could definitely benefit from more thorough susceptibility testing of a full panel of selenium compounds against both *M. tuberculosis* H37Rv and CDC1551. In testing the susceptibility of the non-mycobacterial species *S. aureus*, *E. coli*, *P. aeruginosa* and *V. harveyi*, altering the sulphur concentration by titrating the amount of MgSO₄ may have resulted in increased susceptibility of these species to Se-AAs. At present it is unknown if these bacterial species are inherently resistant to Se-AA inhibition or if these species are able to synthesise sulphur amino acids *de novo* from the constituents of M9 minimal media, rescuing the bacterium from Se-AA inhibition.

The auto-reduction of resazurin in 0.5x SPO media during *S. cerevisiae* susceptibility testing against SMSC proved problematic. This could be avoided by omitting resazurin from this assay and measuring the absorbance at 600 nm; however, this would remove the comparison to other selenium compounds for which resazurin was used, and the assays would lack the sensitivity of resazurin. Visual inspection of wells confirmed that SMSC was unable to inhibit *S. cerevisiae* growth under SC or SPO media conditions, with the exception of the

YAL012W CYS3 strain that was not culturable due to auxotrophy under SC conditions.

The limited cytotoxicity testing of Se-AA compounds against mammalian cell lines was another weakness of this project, since SMSC and Se-DL-met were the only Se-AA tested. This testing was carried out against three cell lines, human leukaemia HL-60, murine macrophage J-774 and green monkey kidney epithelial Vero cells; however, this testing was carried out in an incomplete manner, not all Se-AAs were tested against all cell lines. This aspect of the project could be improved by testing the complete panel of selenium compounds against the mammalian cell lines, and including additional cell lines such as human fibroblasts⁶⁷⁷ and murine alveolar macrophages MH-S. The latter could even be adapted for an intra-macrophage assay with phagocytosed mycobacteria to provide an excellent pathogenically relevant cytotoxicity and anti-mycobacterial activity validation.⁶⁷⁸ Ideally, *in vivo* bio-availability, toxicity and infection clearance assays would also be performed for Se-AAs in a murine model; however, this was considered beyond the scope of this project. As a consequence, this research relied on the literature to provide *in vitro* cytotoxicity and *in vivo* animal toxicity data on the seleno-compounds.

7.3.3 Seleno-amino acid structure-activity analysis

Examination of the relationship between Se-AA chemical structure and biological activity was restricted to selenium compounds that were available commercially or through the Developmental Therapeutics Program Repository of NIH, resulting in a somewhat haphazard SAR analysis. SAR of Se-AAs could be better explored using a wider range Se-AAs and other selenium compounds. For example, inclusion of both dextro and levo optical isomers and racemic mixtures of the most active Se-AAs SMSC and Se-L-met for determining that Se-AAs have levo optical rotation dependant activity.

A wider range of substituted selenium compounds would also further SAR understanding. Therefore, it would have helped to continue the trend of Se-cys, Se-methylselenocysteine with the addition of Se-ethylselenocysteine and Se-propylselenocysteine, likewise for Se-met selenohomocysteine and seleno-ethionine. These would provide valuable information on the influence of selenium substitution on Se-AA activity and may have even demonstrated increased anti-mycobacterial activity. It was demonstrated that the sulphur amino acids equivalents cysteine, s-methyl-cysteine and methionine did not exhibit anti-mycobacterial activity. It would interesting to further explore this effect with tellurium amino acids, as

these have been reported to also have redox modulating properties more potent than seleno-amino acids⁶⁷⁹ and be of interest in antibiotic drug design.⁶⁸⁰

To properly investigate the impact amine and carboxylic acid groups have upon Se-AA anti-mycobacterial activity, these groups could have been substituted with protecting groups, for example t-BOC protection of the amine,⁶⁸¹ and this protection could have been applied to all Se-AA functional groups to determine their importance in Se-AA activity.

Finally, inclusion of other methylated Se-AAs with variation in the number of side chain carbons, such as methylselenol-glycine, methylselenol-norvaline and methylselenol-norleucine would provide insight into the substrate specificity of mycobacterial β -eliminating lyases and may have provided direction for structural configurations that might increase the specific activity towards mycobactetria.

7.3.3.1 Seleno-amino acid derivatisation

Seleno-L-methionine di-peptide and amide derivatives that were synthesised and provided by Zaid Amso and Dr. Viji Sarojini provided useful SAR information showing that conjugation of Se-AAs and amide substitution of the hydroxyl group diminished the anti-mycobacterial activity of these compounds.⁵¹⁸

This derivatisation work would have been more complete if dipeptide and amide derivatives of Se-cys or SMSC were included as well as additional to Se-L-met derivatives; however, these were omitted due to financial constraints. Both carboxyl- and amine-conjugates of alanine to Se-L-met were synthesised and a 4-fold difference in anti-mycobacterial activity was observed between these, with the c-terminal conjugate having reduced activity. Only the c-terminal conjugate of arginine to Se-L-met was synthesised, and this di-peptide exhibited only marginally reduced activity compared to Se-L-met. An n-terminal conjugate of Se-L-met and arginine may have anti-mycobacterial activity on par with Se-L-met, if the trend observed in Ala-SeMet conjugations also extended to the arginine dipeptide. This dipeptide has been synthesized by Zaid Amso and will undergo bioactivity testing against *M. tuberculosis* H37Ra.

Furthermore, testing the mammalian cell cytotoxicity of these derivatives and calculating the corresponding selection index towards *M. tuberculosis* would provide more insight into derivatisation selective for anti-mycobacterial activity.

7.3.4 Seleno-amino acid mechanism of action

7.3.4.1 Competition assays

The competition assays used to determine the rescue effect of sulphur amino acid supplementation on Se-AA toxicity could have been improved in several ways. For example, commercially available radio-labelled Se-cys⁶⁸² and Se-met⁶⁸³ containing the γ -radiation emitting ⁷⁵Se could have been used. This would allow quantitative measurement of mycobacterial Se-AA uptake to determine how this is affected by supplementation with cys and met, ascertaining whether this competition effect is occurring at the transport level as opposed to the lyase substrate level.

7.3.4.2 Anti-sense inducible modulation of gene expression

Experiments involving the modulation of *M. smegmatis* lyase enzymes MSMEG_2357 and MSMEG_5265 and alkylhydroperoxidase enzymes MSMEG_4890 and MSMEG_4891 using the improved tetracycline inducible vector pKW08¹⁸⁸ did not evoke as strong a response in Se-AA sensitivity as expected for alkylhydroperoxidases based on the Se-AA resistant phenotype of the alkylhydroperoxidase transposon mutant TN-G3-M10. No significant changes in Se-AA sensitivity were observed for MSMEG_2357 and MSMEG_5265 sense overexpression and anti-sense under-expression strains. This was unexpected since anti-sense RNA technology has been used in several instances in bacteria to knock down gene expression, for example, in the identification of the FabF inhibitor platensimycin¹⁶⁵ and in the discovery of inhibitors of mycobacterial chromosome partitioning apparatus.¹⁵⁷

The lack of effect from these experiments may be due to an insufficient quantity of anti-sense RNA being generated to significantly reduce protein expression to have an effect on Se-AA sensitivity, as anti-sense RNA does not completely silence gene expression.⁶⁸⁴ Similarly, insufficient expression of sense RNA may result in a reduced impact on cellular protein levels if the cell is already sufficiently saturated with the protein of interest. Pursuing a stronger constitutive promoter such as HSP60⁶⁸⁵ may allow sufficient RNA expression at the expense of inducibility. Using homologous recombination to knockout genes completely may provide a better response if the anti-sense RNA expression was insufficient to have an impact on gene function.⁶⁸⁶

Alternatively, these genes may not be essential in *M. smegmatis* as they are in *M. tuberculosis*, resulting in a phenotype that is not conditionally lethal.¹⁶³ This could be due to functionally redundant or duplicated genes, as *M. smegmatis* is known to have a large

genomic duplication and resulting genetic redundancy.⁵⁷⁷ The alkylhydroperoxidase *ahpC* was down-regulated in *M. bovis*, producing a distinctly less virulent phenotype, and up-regulation of *ahpC* expression caused increased resistance to cumene hydroperoxide.⁶¹⁴ The lack of effect in *M. smegmatis* constructs may be due to functional redundancy and acquisition, and testing Se-AA sensitivity of the Δ *ahpC* strains of Wilson *et al* (1998) may have proved more fruitful.

7.3.4.3 Enzymatic assays

Mycobacterial lyase enzymes from *M. tuberculosis* H37Rv suspected of performing β and γ -elimination reactions which activate Se-AAs were successfully cloned into the pET28a+ expression vector, expressed in *E. coli* BL-21 and purified by nickel affinity chromatography. Of five proteins expressed, only three were successfully purified and only one of these exhibited the characteristic bright yellow colour of pyridoxal phosphate binding enzymes.⁵⁸⁵ This could indicate that the mycobacterial enzymes were not expressed correctly in the *E. coli* system. It is known that *E. coli* does not perform the correct post-translational modifications when expressing foreign mycobacterial proteins,⁶⁸⁷ and only half of the *M. tuberculosis* proteins expressed in *E. coli* are soluble.⁶⁸⁸ More successful protein expression may have been achieved using a specialised mycobacterial protein expression strain such as *M. smegmatis* GroEL1 Δ C⁶⁸⁹ and vector such as pDESTsmg.⁶⁸⁸ For assay conditions, DTT was used as the reducing agent, and use of glutathione, or ideally, reduced mycothiol would better facilitate mycobacterial lyase enzyme function.

7.3.4.4 Mass spectrometry

GC-MS analysis identified *in vitro* methyldiselenide production from *M. tuberculosis* cysteine desulphurase Rv3025c using SMSC as a substrate but was unable to identify evolution of selenium species from other Se-AA substrates or mycobacterial lyase enzymes. Use of commercial β and γ lyase enzymes as positive controls⁵⁵² would have better validated the results of these experiments. This experiment requires further testing utilising additional mycobacterial lyase enzymes and Se-AA substrates. Coupling GC-MS detection of volatile selenium and sulphur species with LC-MS may have facilitated the determination of other metabolites of lyase cleavage of Se-AAs, the major suspects being alanine,⁵⁹⁴ pyruvate⁶⁹⁰ or α -ketobutyrate.⁵³⁵ Knowing the identity of other products of the lyase reaction may have allowed development of a specific assay for the detection and quantification of lyase assay function.

7.3.5 Mycobacterial lyase metabolite detection assays

7.3.5.1 Methylene blue sulphide/selenides detection

The methylene blue method of sulphide detection may have been better utilised if carried out at lower temperatures to avoid loss of volatile sulphide/selenide compounds⁶⁹¹ although this may have yielded inaccurate results for enzyme activity as this would likely fall below the lyase optimal temperature range. Methylated sulphur/selenium species such as those produced by lyase activity on SMSC, Se-met, S-methyl-cysteine may also interfere with the methylene blue assay.⁶⁹² An assay based on fluorescent probes for hydrogen sulphide or selenides may prove more reliable.⁶⁹³

7.3.5.2 Coupled enzyme assays

Alanine dehydrogenase and Lactate dehydrogenase commercial enzymes were coupled with the *in vitro* mycobacterial lyase reaction in the hope of quantifying lyase enzyme activity by measuring the production of the theoretical lyase metabolites alanine⁵⁹⁴ and pyruvate.⁶⁹⁰ Alanine has been reported as the product of Rv3025c lysis of cysteine,⁵⁸⁵ but this could not be detected in the coupled enzyme assays of this research, with the exception of positive controls using these compounds.

It is possible that alanine and pyruvate are not the metabolites produced by mycobacterial lyase activity on Se-AA substrates; however, alanine not being detected from Rv3025c using DL-cys substrate and having positive H₂S production in the PbAc paper method makes this unlikely. Substituting the reducing agent DTT in this assay for glutathione or reduced mycothiol, and incorporation of an anaerobic environment with a sulphur acceptor may have increased lyase enzyme function to detectable levels.

7.3.6 Seleno-amino acid-resistant transposon mutants

Transposon mutants with identified insertion regions such as TN-G3-M10 *ahpC/D* alkylhydroperoxidase would have been better validated by complementation experiments, supplementing the suspected mycobacterial transposon disrupted gene with a plasmid borne copy. This complementation method is more commonly used⁵⁴⁶ and in retrospect is considered a better standard than anti-sense RNA gene modulation. Testing of Se-AA resistant strains for differential sensitivity to redox cycling agents such as paraquat and menadione⁶⁹⁴ may provide additional insight into the hypothesised mechanism of Se-AA ROS generation, as such chemicals produce superoxide.⁶⁹⁵

7.3.7 Seleno-amino acid synergy with isoniazid

Se-L-met was demonstrated to exhibit a low level of synergy with the front line tuberculosis treatment drug isoniazid in the strain *M. tuberculosis* H37Ra. This was an expected result as the genetic targets of Se-AAs and isoniazid are co-dependently expressed, with *ahpC* being up-regulated upon loss of *katG* function.⁵⁵⁷ This synergy experiment would be worth carrying out using MDR-TB strains as well, particularly those with known *katG* loss of function as these may exhibit even greater sensitivity to Se-AA inhibition, as *ahpC* transcription will likely be up-regulated, resulting in increased sensitivity to Se-AAs that may outweigh the level of INH resistance. Further synergy experiments using other TB treatment drugs such as ethionamide may also reveal synergy of Se-AAs with such drugs.

7.4 Future directions

The following experiments are recommended for the continuation of this research, predominantly concerning the development of Se-AAs as antibiotics but also regarding differential culture condition screening and dealing with other hits derived from screens.

7.4.1 Modified culture condition screening of additional chemical libraries

The chemical libraries used in the screening components of this project were small in terms of the number of compounds, and they had little novel compound content. Although new use for known drugs can be favourable because of their known pharmacology and toxicology profiles,⁶⁹⁶ this should be coupled with exploring novel chemical structures as well. Modified culture condition screening such as the nutrient limited conditions described in this thesis should be carried out on large, structurally diverse chemical collections such as the ChemBridge™ MicroFormat, Specs™ World Diversity Set and the ChemDiv™ Drug-Like libraries, particularly structure diversity orientated libraries to allow hit compound follow up with a range of structural variants on a lead scaffold.⁶⁹⁷

7.4.2 Follow up of key library hit compounds

Other library compounds identified as having anti-mycobacterial activity and through literature searching found to have favourable indications for development as human tuberculosis treatment drugs, such as low toxicity could be screened. Follow-up research scrutinising these compounds as potential anti-tubercular agents is encouraged, including testing of activity toward virulent *M. tuberculosis* strains and human cell line toxicity. Compounds considered to be excellent leads and require immediate follow-up investigation

include niclosamide, pyriithione zinc, azadirachtin, neohesperidin, pentamidine and chlorothalonil (tetrachloroisophthalonitrile). Other leads worthy of follow up study include phenothiazines, cefdinir, gentisic acid, michellamine B, bismuth-dimercaprol, solanine and ketoconazole.

7.4.3 Further testing of Se-AAs against virulent / drug resistant *M. tuberculosis* strains

To verify the anti-mycobacterial results of SMSC against virulent *M. tuberculosis* strains H37Rv and CDC1551 reported in this thesis, repeated dose-response experiments using a full suite of Se-AA compounds should be carried out using these strains. Additionally, testing of Se-AAs against MDR and XDR-TB strains, particularly $\Delta katG$ mediated isoniazid resistant strains, would be of much interest due to the synergy between Se-AAs and isoniazid and potentially elevated sensitivity of isoniazid resistant tuberculosis to Se-AAs as described in Chapter 6.5.3.6.

7.4.4 Further testing of Se-AAs against mammalian cell lines

Similar to above, verification of Se-AA cytotoxicity towards mammalian cell lines should take place using the full range of Se-AA compounds against a variety of cell lines, particularly human cell lines. Furthermore, testing of Se-AA activity toward phagocytosed mycobacteria would provide an interesting combination anti-bacterial and cytotoxicity assay. Assuming that validation studies using virulent *M. tuberculosis* and cytotoxicity results are favourable, the next step is testing of Se-AA compounds *in vivo* using murine models of tuberculosis to measure bio-availability, toxicity, infection clearance and compatibility with standard tuberculosis treatment drugs.

7.4.5 Target gene knockout and complementation sensitivity

For absolute validation of the Se-AA anti-mycobacterial mechanism, putative non-essential *M. smegmatis* lyase genes MSMEG_0769 (Rv0391), MSMEG_5265 (Rv1079) and MSMEG_1652 (Rv3340) and alkylhydroperoxidase genes MSMEG_4890 (Rv2429) and MSMEG_4891 (Rv2428) should be reliably knocked out using homologous recombination.^{698, 699} Putative essential genes, like MSMEG_3125 (Rv1464), and MSMEG_2357 (Rv3025c), may be possibly knocked out by homologous recombination if a rich culture media is used, supplemented with sulphur and alanine if necessary to substitute for the lost function of these genes. The resulting knockout strains should be tested for Se-AA sensitivity, and any changes to the Se-AA sensitivity phenotype should be validated by

complementation experiments using a plasmid borne copy of the gene to restore the Se-AA sensitivity to original levels. Construction of *M. smegmatis* knockouts may be bypassed using *M. tuberculosis* H37Rv transposon mutants available from Johns Hopkins University School of Medicine where a Rv0391 transposon knockout strain is available and knockouts for other non-essential lyase genes Rv1079 and Rv3340 and peroxidase genes Rv2428 and Rv2429 are likely to be available in future. Based on the sensitivity of these knockouts to Se-AA inhibition, these enzymes may then be purified and an *in vitro* enzyme assay developed.

7.4.6 *In silico* docking analysis of Se-AAs with mycobacterial lyase enzymes

For clarification regarding mycobacterial lyase utilisation of Se-AAs substrates, and to help guide structural modifications to increase anti-mycobacterial activity, *in silico* docking of Se-AAs with mycobacterial lyase enzymes should be carried out. This has the advantage of allowing many virtual chemicals to be inexpensively screened against a known target.^{2, 700} The crystal structures of the mycobacterial lyase enzymes Rv1079⁵⁷⁴ and Rv3340⁵⁹² have been solved.⁷⁰¹ Cystathionine γ -synthase/lyase enzymes such as Rv1079 are of particular interest as these enzymes and their *de novo* methionine synthesis function occur only in microorganisms and plants⁷⁰² but not mammals, and are therefore good drug targets.

7.4.7 Derivatisations improving biological activity

The following derivatisations were conceptualised based on known trends in Se-AA structure-activity, and information sourced in the literature and anti-mycobacterial testing of these derivatives would provide comprehensive structure-activity analysis and may produce novel Se-AAs with improved drug-like qualities such as increased bioavailability, increased anti-mycobacterial activity and decreased human cytotoxicity.

7.4.7.1 Se substitution

Described briefly in section 7.3.3.1, exploring substitution at the selenium position of Se-cys and Se-met with ethyl, propyl and phenyl groups would provide valuable insight into changes in activity with modulation of this functional group.⁷⁰³ For Se-cys, exploring Se-ethylselenocysteine, Se-propylselenocysteine and Se-phenylselenocysteine and for Se-met, exploring selenohomocysteine, seleno-ethionine, seleno-propionine and Se-phenyl-selenohomocysteine would provide a good foundation for Se substitution, particularly since the Se-phenyl derivatives, being electron rich, should form a more reductive Se anion that may lead to a more reactive phenylselenide radical. Se-phenyl-L-selenocysteine is reported

to be non-toxic toward rat hepatocytes.⁶⁷⁹ Many Se-substituted derivatives have been previously synthesised,⁷⁰⁴ and Se-methyl, ethyl, n-propyl and n-butyl L-selenocysteine derivatives are also reported to be non-toxic toward rat renal proximal tubular cells.⁷⁰³ Trifluoromethionine is a pro-drug similar to Se-AAs that is cleaved by methionine γ -lyase to produce α -ketobutyrate, ammonia and the amine cross-linking agent trifluoromethanthiol.⁷⁰⁵ Trifluoromethionine has anti-bacterial and anti-protozoal activity *in vitro* and *in vivo*.⁷⁰⁵⁻⁷⁰⁸ Combining fluorinated methionine derivatives with Se-AAs, i.e. trifluoroselenomethionine may represent a pro-drug with dual mode of action cross-linking amines that generates reactive oxygen and would also provide insight to the SAR of Se-AAs, as this Se-substitution would be highly electronegative.

7.4.7.2 Side chain carbon length

Also described in section 7.3.3.1, modifying the Se-AA side chain carbons would provide valuable insight into the SAR of Se-AAs. Decreased side chain carbons in methylselenoglycine and increased side chain carbons in methylseleno-norvaline and methylseleno-norleucine would elucidate the effect of side chain length on anti-mycobacterial activity. The suitability of these chemicals or their sulphur analogues as lyase substrates has not previously been determined.

7.4.7.3 Amide conjugates

Various amide conjugated derivatives of trifluoromethionine were found to have up to 7-fold increased activity toward *Entamoeba histolytica*, in addition to having over 100-fold selectivity for this pathogen over mammalian CHO cells.⁷⁰⁷ Although amide derivatives of Se-L-met were tested in this work revealing lowered anti-mycobacterial activity, condensation of such amides with aniline or benzylamine may increase activity toward mycobacteria, as was observed in *E. histolytica*.⁷⁰⁷

7.4.7.4 Dipeptide conjugates

Alanine and arginine dipeptide derivatives of Se-AAs were synthesised and found to have reduced *in vitro* anti-mycobacterial activity, described in section 6.2.2. Following this trend, proline – Se-AA dipeptides and seleno-proline itself may be worth exploring since mycobacteria actively import proline into the cell⁷⁰⁹ but also express specialised proline dipeptide hydrolysing enzymes such as PepE that release N-terminal amino acids linked to proline.⁷¹⁰ These modifications could provide increased delivery of Se-AA dipeptide to

bacteria and provide selective activity through mycobacterial proline dipeptidase cleavage. An N-terminal Se-L-met-arginine conjugate dipeptide has been synthesized by Zaid Amso and will soon undergo bioactivity testing. Pathogenic mycobacteria demonstrate specifically preferential arginine uptake, with several dedicated transporters.⁵²⁴ Further exploration into Se-AA - arginine dipeptides such as the N-terminal conjugation of Se-L-met – arginine that has been synthesized by Zaid Amso, and even selenoarginine itself⁷¹¹ may increase specificity and potency toward mycobacteria. Selenocystathionine (dipeptide of selenocysteine and serine) and related serine dipeptides could prove beneficially selective toward mycobacteria over mammalian cells, as cystathionine γ -lyase enzymes are present only in microorganisms and plants, not mammals.⁷⁰² Additionally di-, tri- and tetra- peptides of Se-L-met have been synthesized by Zaid Amso and will soon undergo bioactivity testing.

7.4.7.5 Allyl / glutamyl substitution

Allyl and glutamyl derivatives of Se-cys are naturally occurring Se-AAs found in various plant species such as the *Allium* genus.⁷¹² It is thought that Se-AA derivatives such as SMSC, γ -glutamylmethylselenocysteine and allylselenocystine are synthesised to mitigate selenium toxicity as these amino acids are non-protogenic.⁷¹³ γ -glutamyl Se-AAs act as carriers for Se-AAs with increased solubility and bioavailability, and require an additional γ -glutamyl transpeptidase cleavage step⁷¹⁴ that may increase the selectivity of Se-AA action toward mycobacteria. Se-allylselenocysteine was found to exhibit more cancer protective effects in murine models compared to SMSC and Se-propylselenocysteine,⁷¹⁵ and this may be due to increased reactivity of the allylselenol, the active metabolite of lyase cleavage of Se-allylselenocysteine.⁷¹⁶

7.4.7.6 Increased uptake and specificity

Conjugation of anti-mycobacterial drugs to the mycobacterial iron sequestering siderophore mycobactin has been shown to be a useful delivery mechanism to increase the specific activity of drug molecules towards mycobacteria⁷¹⁷ even if the drug did not exhibit anti-mycobacterial activity to begin with, as seen for the anti-malarial agent artemisinin.²⁴³ C-terminal conjugation of Se-AAs to a siderophore such as mycobactin may increase the uptake and therefore inhibitory potency of the conjugated Se-AAs toward mycobacteria, in addition to presumably having favourable selective activity for mycobacteria. An alternative method for increasing mycobacterial uptake is the conjugation of long carbon chain fatty acid tails to increase Se-AAs lipophilicity, allowing them to better permeate the waxy mycobacterial cell

wall. Increased lipophilicity would tend to increase anti-bacterial activity^{718, 719} and overcome bacterial resistance.⁷²⁰

7.5 Concluding remarks

Infectious diseases such as tuberculosis continue to be a heavy burden on global health. Pathogen resistance to anti-microbial agents is ever increasing; therefore, new drugs with novel mechanisms of action are required to provide the clinicians with the varied arsenal of treatments necessary to tackle these resistant strains.

This thesis describes the development and implementation of high-throughput screens for anti-mycobacterial compounds with a unique aspect of using modified culture conditions to reveal alternative inhibitors of mycobacteria. Several novel inhibitors of mycobacteria were discovered, the most prominent being the seleno-amino acids, a new family of anti-mycobacterial compounds. The basic structure-activity relationship for Se-AA was described, detailing several key structural characteristics for which anti-mycobacterial activity of these compounds is dependant.

- **Modified culture condition screening identifies alternative inhibitors of mycobacteria.**
- **Using modified culture condition screening lead to the discovery of seleno-amino acids, a novel class of anti-mycobacterial compounds.**

Utilising a variety of techniques, a hypothesis for the mechanism of anti-mycobacterial action of Se-AAs is proposed, in which Se-AAs are recognised by mycobacteria as nutrients but upon metabolism act as a Trojan horse within the mycobacterial cell, producing reactive selenium species that interact with mycobacterial oxidoreductase and antioxidant defence enzymes to continuously generate oxygen radicals that cause irreparable damage within the mycobacterial cell.

- **Seleno-amino acids compete with sulphur amino acids as nutrients to mycobacteria.**
- **Seleno-amino acids exert their activity through a novel mechanism.**

- **Seleno amino acids are pro-drugs that are cleaved by mycobacterial lyases into their active methylselenium metabolites.**
- **Methylselenium metabolites cause the production of damaging reaction oxygen species from mycobacterial redox regulation and anti-oxidant defence enzymes.**
- **Methylselenium metabolites also disrupt mycobacterial processes such as Fe-S cluster enzyme function, cholesterol catabolism and also deplete the mycobacterial NADH reserves.**

In summary, this work presents an alternative approach to anti-mycobacterial drug discovery using modified culture condition screening. As a result of implementing this approach, a new class of anti-mycobacterial agents was discovered that act via a novel mechanism. This new class, Se-AAAs, have good potential for development as a human tuberculosis treatment therapy or a nutritional supplement that serves as an adjunct to improve outcomes of conventional tuberculosis treatment.

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