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IDENTIFICATION AND CHARACTERISATION OF NEW COMPOUNDS TO TREAT CHRONIC BACTERIAL LUNG INFECTIONS

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Disclaimer

This PhD thesis is based on work carried out at the University of Sydney, under the supervision of Dr. Jamie Triccas, between February 2010 and January 2014. This thesis incorporates original research that has not been previously submitted for a higher degree to any other university. It does not contain any material previously published or written by any other person except where reference has been made in the text.

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Samantha Ellis

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Abbreviations

^{o}C	Degrees Celsius
µ	Micro
µg	Microgram
µL	Microlitre
µM	Micromolar
ADC	Albumin-dextrose catalase
AES	Australian Epidemic Strain
ANOVA	Analysis of variance
BCG	*Mycobacterium bovis* (Bacille Calmette Guérin)
BCG pSMT1	BCG containing plasmid encoding luciferase gene
BH	Benzoyl hydrazide
BSA	Bovine-serum albumin
CF	Cystic Fibrosis
CFU	Colony forming units
CO_2	Carbon dioxide
DETANO	Diethylenetriamine-nitric oxide
DFO	Desferrioxamine
dH_2O	Distilled water

DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment short-course strategy
ESAT-6	Early secreted antigen, 6kDa
FDA	Food and Drug Administration
g	Grams
Hsp60	Heat shock protein 60 (used in plasmids as promoter)
INH	Isoniazid
kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria-Bertani
LPS	Lipopolysaccharide
m	Milli
M	Molar
MDR	Multi-drug resistant
MDRTB	Multi-drug resistance Tuberculosis
MIC	Minimum Inhibitory Concentration
min	Minute
mM	Millimolar

MRSA	Methicillin-resistant *Staphylococcus aureus*
ng	Nanogram
nM	Nanomolar
OADC	Oleic acid-albumin-dextrose-catalase (medium supplement)
PBS	Phosphate buffered saline
PMA	Physical medium attachment
RO	Reverse osmosis
SD	Standard deviation
SEM	Standard error of the mean
TDW	Triple distilled water
v/v	Volume per volume
w/v	Weight per volume
W.H.O	World Health Organisation
XDR	Extensively Drug resistant
XDRTB	Extensively Drug resistant Tuberculosis

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Publications arising from work in this thesis

ELLIS, S., KALINOWSKI, D. S., LEOTTA, L., HUANG, M. L., JELFS, P., SINTCHENKO, V., RICHARDSON, D. R. & TRICCAS, J. A. 2014. Potent antimycobacterial activity of the pyridoxal isonicotinoyl hydrazone analog 2-pyridylcarboxaldehyde isonicotinoyl hydrazone: a lipophilic transport vehicle for isonicotinic Acid hydrazide. *Mol Pharmacol,* 85**,** 269-78.

ELLIS, S., YU, M., TODD, M., RUTLEDGE, P., & TRICCAS, J.A. Metal Cyclam compounds as a novel therapy for the treatment of Mycobacteral species. Manuscript in preparation.

Related publications

YU, M., LIM, N. H., **ELLIS, S.,** NAGASE, H., TRICCAS, J. A., RUTLEDGE, P. J. & TODD, M. H. 2013a. Incorporation of Bulky and Cationic Cyclam-Triazole Moieties into Marimastat Can Generate Potent MMP Inhibitory Activity without Inducing Cytotoxicity. *ChemistryOpen,* 2**,** 99-105.

YU, M., RYAN, T. M., **ELLIS, S**., BUSH, A. I., TRICCAS, J. A., RUTLEDGE, P. J. & TODD, M. H. 2014. Neuroprotective Peptide-Macrocycle Conjugates Reveal Complex Structure-Activity Relationships in their Interactions with Amyloid β. Submitted manuscript.

Abstract

Drug resistance is a major concern in the treatment of numerous diseases caused by infectious agents. The misuse of current therapies has led to the development of almost complete resistance in some cases, creating an urgent need for the development of new drugs. In this study, I assessed the effectiveness of compounds against two medically important lung pathogens, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*, using complementary methods of drug discovery and assessment.

The first approach assessed whether high-affinity iron chelators of the pyridoxal isonicotinoyl hydrazone (PIH) class can restrict the growth of clinically significant mycobacteria. Screening a library of PIH derivatives revealed that one compound derived from the condensation of 2-pyridylcarboxaldehyde with the first-line anti-tuberculosis drug Isoniazid (INH), namely, 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH), exhibited nanomolar *in vitro* activity against *Mycobacterium bovis* Bacille Calmette-Guerin and virulent *M. tuberculosis*. PCIH displayed minimal host cell toxicity and was effective at inhibiting growth of *M. tuberculosis* within cultured macrophages and also *in vivo* in mice. Further, PCIH restricted mycobacterial growth at high bacterial loads in culture, a property not shared with INH. When tested against *Mycobacterium avium*, PCIH was more effective than INH at inhibiting bacterial growth in broth culture and in macrophages, and also reduced bacterial loads *in vivo*. Iron complexation studies indicate a possible role for chelation as a mechanism of action, however this could not totally account for its potent efficacy, with structure-activity relationship studies suggesting that PCIH acts as a lipophilic vehicle for the transport of its intact INH moiety into the mammalian cell and the mycobacterium. These results demonstrate that iron-chelating agents such as PCIH may be of benefit in the treatment and control of mycobacterial infection.

The second study assessed the ability of a novel series of molecules, termed metal-cyclam complexes (MCyC), to inhibit the growth of medically relevant bacteria. Screening of MCyC against a panel of gram-positive, gram-negative and mycobacterial strains identified that a subset of MCyC specifically inhibited the growth of mycobacteria, exhibiting activity in the low micromolar range *in vitro*. However, none the compounds could restrict the viability of mycobacteria in a non-replicating state, suggesting the mode of action of these novel molecules was linked to replication of bacteria. Encouragingly, the most effective compound identified, a copper(II)-cyclam complex incorporating two naphthalimide pendant groups, could markedly restrict intracellular growth of pathogenic mycobacteira but displayed no host-cell cytotoxicity. Finally, the three most effective compounds were able to work synergistically with current anti-tuberculosis drugs to inhibit bacterial growth *in vivo*, indicating this series of inhibitors are promising leads for further preclinical and clinical development.

The final study developed high-throughput screening assays to determine if a library of 1920 compounds, made up of a diverse series of natural and synthetic products, could inhibit the growth of *Pseudomonas* and mycobacterial species. Only a small proportion of compounds were found to inhibit growth of the drug resistant clinical isolate *P. aeruginosa* CJ2009 (0.3%), however two compounds were active in the low micromolar range (3.13 to 6.25 μM). A similarly low number of compounds were active against *M. avium* (0.1%) and two compounds inhibited growth in the mid micromolar range (12.5 to 25 μM). There was no overlap in activity between the two bacterial species tested, suggesting the identified compounds displayed some specificity for the target organism. However, all compounds displayed some level of toxicity against mammalian cells, limiting their potential use in their current form. Decoding of compounds revealed that toxicity of some of the compounds may be related to their proposed function as either nucleotide or steroid analogs, metal binding capacity or

inhibitors of mammalian cellular processes. This study highlights the necessity to identify compounds with high selectivity for the pathogen of interest for future clinical development and use.

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Chapter 1: Literature review

1.1 Mycobacteriology and disease

Mycobacteria are a group of slow and fast growing, acid-fast bacilli of the order *Actinomycetales*, family *Mycobactericeae*. They are non-motile, non-spore forming bacteria with a lipid rich cell wall (Rogall et al., 1990, Puzo, 1990). The mycobacterial cell wall is very complex, made up of long fatty-acid chains and mycolic acids covalently bound to an arabinogalactan-peptidoglycan co-polymer (Song et al., 2008). It is this characteristic lipid-rich cell wall that renders mycobacteria resistant to many disinfectants and antibiotics, as well as contributing to its many attributes including slow growth, antigenicity, propensity to clump in liquid media, and acid-fastness (Davies, 1998).

Mycobacteria are obligate aerobes that are catalase positive and fall into two main categories of growth; slow growing and fast growing, depending on the amount of time it takes for visible colonies to appear on solid media (Shinnick and Good, 1994). The bacilli can either be straight or slightly curved in shape, and occasionally filamentous (Tsukamura, 1981). The filaments formed are different from those of other actinomycetes, in that they are readily able to fragment into rods or coccoid bodies when disturbed (Tsukamura, 1981).

These organisms are classified in the genus *Mycobacterium* depending on their acid-fastness and the presence of mycolic acid containing up to 90 carbons that are cleaved by pyrolysis to methyl esters. A high guanine/cytosine content in their deoxyribonucleic acid (DNA) is also typical of mycobacteria (Shinnick and Good, 1994). To date, more than 150 species of mycobacterium have been identified, most of which are saprophytes found ubiquitously in the environment (Behr, 2013). Whilst these

bacteria occur in diverse environments, *Mycobacteria* are more commonly known as animal pathogens, and humans are readily infected by mycobacterial species (Bose, 2008). The most pathogenic species include *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium leprae*, the causative agent of leprosy.

Of the genus *Mycobacterium, M. tuberculosis* was the first species to be classified. Subsequently in 1959, Earnest Runyon developed a nomenclature protocol known as the Runyon classification. This classification assisted in organising the clinically important mycobacterial species other than *M. tuberculosis*, depending on growth rate and the ability of the bacteria to produce pigment in the presence or absence of light (Rogall et al., 1990, Tsukamura, 1981). For example, *Mycobacterium avium* and *M. bovis* are classified in Runyon group III, which means they are non-pigmented, slow-growing bacteria (Stahl and Urbance, 1990).

1.1.1 *Mycobacterium tuberculosis*

One of the most pathogenic species of mycobacterium, *M. tuberculosis* is a slow-growing, acid-fast bacillus that primarily infects mononuclear phagocytes (Flynn and Chan, 2001, Wagner et al., 2005). In particular, alveolar macrophages are highly permissible to infection with mycobacteria, as the organisms reside within cytoplasmic vacuoles and employ mechanisms to resist acidification and phagolysosomal fusion, thus enhancing intracellular survival (Bermudez and Sangari, 2001, Wagner et al., 2005).

M. tuberculosis was first isolated in 1882 by Robert Koch as the causative agent of tuberculosis (Koch, 1882). It was initially christened tuberculosis by Scholein in 1839 and in 1865 Jean-Antoine Villemin showed that tuberculosis could be transmitted by culturing bacteria from humans or cows and injecting

it into guinea pigs or rabbits to see if they developed tuberculosis-like disease (Sakula, 1983). Following this, Koch also discovered that the bacterium infecting bovine and human populations were different, thus describing *M. bovis* (Taylor et al., 2003). Pathogen transmission occurs primarily via aerosol route, infecting the lung, but also the mesenteric, lymphatic, circulatory and genitourinary systems, as well as the peripheral and central nervous systems (Bonfioli et al., 2005). Infection is initiated upon phagocytosis of bacilli by alveolar macrophages (Russell et al., 2009). Patients with pulmonary tuberculosis are able to transmit bacilli throughout the population, however patients with non-pulmonary tuberculosis are not infectious (Underwood et al., 2003a).

Symptoms of the disease are non-specific, including fever, night sweats, chills, malaise, weight loss and fatigue (Murray et al., 2005). More specific symptoms include bloody and purulent sputum, which is associated with necrosis of the lung. Diagnosis of the disease is by the Mantoux or tuberculin test, which tests for a cellular immune response to *Mycobacterium*, however this is not indicative of active disease. Other diagnosis tools which test for active infection are culturing sputum samples, but this can take up to 3 weeks (Banfi et al., 2003), or performing an acid-fast stain, however bacteria can be difficult to identify in light microscopic examination of a stained smear. More recent diagnostic assays include the QuantFERON®-TB Gold test, which is an ELISA measuring the release of IFN-γ by antigen-reactive T lymphocytes cultured with antigens specific to *M. tuberculosis* (Connell et al., 2006). These antigens include ESAT-6 and CFP-10, which are present in the genome of *M. tuberculosis* but absent in other mycobacterial species such as the *M. bovis* BCG vaccine (Arlehamn et al., 2012).

1.1.2 Tuberculosis epidemiology

Tuberculosis is the leading cause of death from a single bacterial pathogen worldwide (Arlehamn et al., 2012) and is the second largest cause of mortality by an infectious disease after HIV (W.H.O, 2012). Of the estimated 2 billion people infected, 10 % of people will progress to clinical infection. Co-infection with HIV increases this risk by a further 10 % per year (Bezuidenhout et al., 2009). The 2012 report released by the World Health Organisation (WHO) stated that there was an estimated 9 million new cases of tuberculosis in 2011 and 1.4 million deaths (W.H.O, 2012). Infection with multi-drug resistant (MDR) strains account for approximately 3.7 % of new cases each year, with an estimated 20% of MDR cases in previously treated TB patients (W.H.O, 2012). Of these approximately 3% of MDR cases have been classified extensively-drug resistant (XDR) (C.D.C, 2007). Co-infection with HIV accounted for an estimated 1.1 million (13%) of new cases in 2011 (W.H.O, 2012).

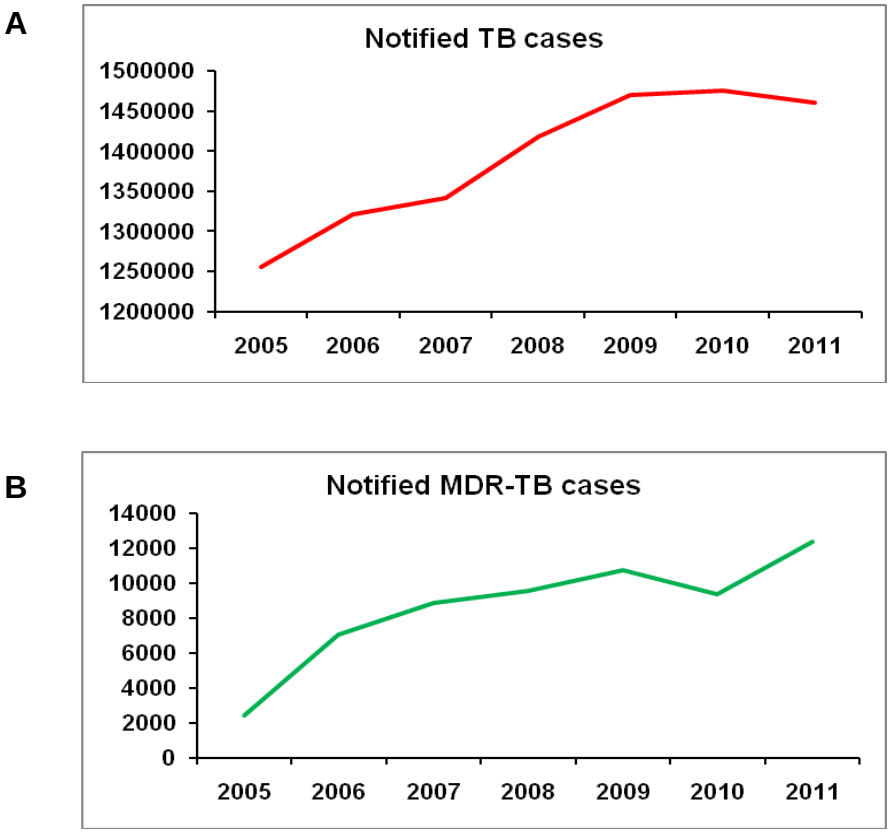


Figure 1.1: Global incidence of susceptible and multiple-drug resistant Tuberculosis. Adapted from www.who.int/tb/data. Absolute number of tuberculosis cases (A) and Multi-drug resistant cases (B) reported globally.

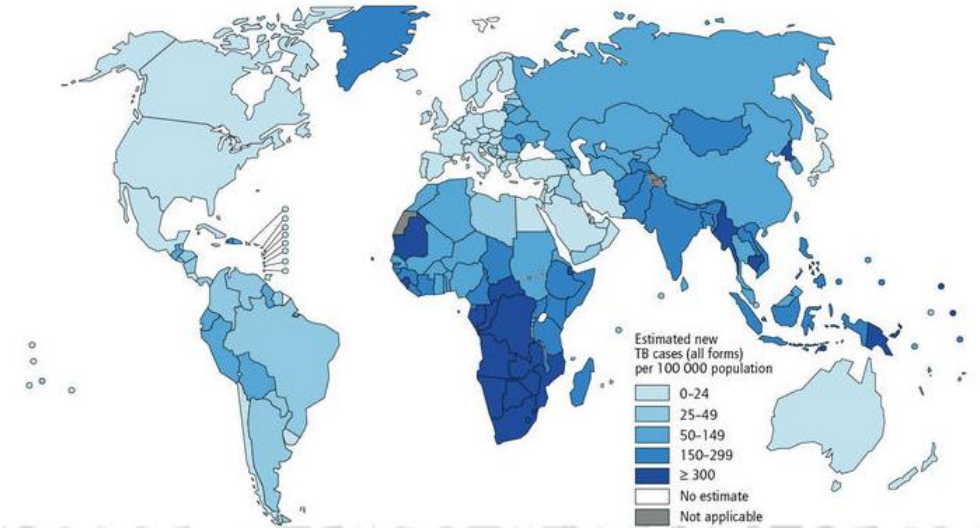


Figure 1.2: Incidence of Tuberculosis cases worldwide (W.H.O, 2012)

Due to the success of the DOTS campaign which commenced in 1995 (see 1.4.4) the number of new cases have started to decline by approximately 2% per year (Figure 1.1a) (W.H.O, 2012). It still remains localised in certain areas (Figure 1.2) with the 22 high burden countries accounting for 89% of the global burden, with Asia and Africa accounting for 59% and 26% respectively (W.H.O, 2012). The number of MDR-TB cases however, has increased annually, as seen in Figure 1.1b, with 4.3% of all new cases being diagnosed as MDR-TB in the 27 high MDR high burden countries (W.H.O, 2012). Whilst considered a low-income country disease, areas considered to be low risk have also seen a rise in MDR-TB incidence such as the United States, (Taylor et al., 1999), the United Kingdom (Abubakar et al., 2008, Kumar et al., 1997) and Australia (Lumb et al., 2008).

1.1.3 *Mycobacterium avium*

M. avium is an opportunistic pathogen that mainly affects the immunocompromised (Sangari et al., 1999). It is a strongly acid-fast, weakly gram-positive aerobic rod that is found ubiquitously in the environment (Davies, 1998). *M. avium* can be transmitted either by aerosols where it can cause pulmonary disease (Yamazaki et al., 2006), or ingested causing disease of the gastrointestinal tract (Sangari et al., 1999). *M. avium* is the only species of mycobacterium that can withstand the high acidic environment of the gastro-intestinal tract (Bermudez and Sangari, 2001). This bacterium couples with *Mycobacterium intracellulare* to form the *M. avium intracellulare* complex (MAC), which predominates in South-eastern USA, Western Australia and Japan (Davies, 1998). It is the most common infection for people co-infected with HIV in the USA (however *M. tuberculosis* is more prominent in African HIV patients). In patients with AIDS, it is commonly a systemic disease, causing infection in almost all organs of the body (Yamazaki et al., 2006).

1.2 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative bacterium from the family *Pseudomonadaceae* that is found ubiquitously in the environment. It is aerobic, non-fermentative, and can withstand higher temperatures than most other *Pseudomonas* species (Stanier et al., 1966). It can adapt to a diverse range of environments, from soil and surface of plants, to hospital equipment and jet fuel (Pitt, 1986). This particular species of *Pseudomonas* has also shown a unique ability to be pathogenic to mammals (Stanier et al., 1966).

P. aeruginosa is an opportunistic pathogen, causing acute infection in burn wounds, the eye through contact lenses and the urinary tract, as well as being the most common lung pathogen in cystic fibrosis (CF) patients (Bielecki et al., 2008). PAO1, a laboratory strain of *P. aeruginosa*, was the 25th genome to be sequenced (Stover et al., 2000a). The genome encodes a range of proteins including approximately 150 outer membrane, 300 cytoplasmic membrane and 521 regulatory proteins (Stover et al., 2000a, Silby et al., 2011) which accounts for its ability to adapt to a wide array of environments. It has a diverse range of virulence factors, including lipopolysaccharide and biofilm formation, and encodes a number of transporters involved in antibiotic resistance that has lead to its ability to cause severe and chronic infection, making it an extremely difficult pathogen to treat.

LPS is a cell wall associated protein that is composed of 3 distinct regions; the O antigen or long-chain O-polysaccaride, lipid A, and the core oligosaccharide (Kocincova and Lam, 2011). Linkages and sugar constituents that make up the O antigen are used to type different strains of *P. aeruginosa* (Kocincova and Lam, 2011). The inner core of the oligosaccharide is highly conserved and is necessary for cell viability where a mutation within this region leads to a lethal phenotype (Lam et al., 2011). The

main functions of LPS are to provide a barrier against the host response to try and evade detection, as a means of targeting and interacting with host receptors and antibiotics, and as a potent signalling molecule assisting in tissue damage (King et al., 2009).

Biofilm formation is a virulence factor involved in persistence. It allows bacteria to survive on generally uninhabitable environments such as taps and pipes (Kerr and Snelling, 2009) which is why it is such a successful nosocomial pathogen (Sanchez et al., 2013). Urinary and lung infections are facilitated by the ability for *Pseudomonas* to exist on catheters and intubation tubes. The presence of a biofilm also results in a greater resistance to antibiotics compared to bacteria in a planktonic state (Kerr and Snelling, 2009), however the mechanism behind this resistance is yet unknown (Sanchez et al., 2013). Lastly, the biofilm is linked to chronic infection (Kerr and Snelling, 2009). Whilst early infection (in CF patients) is able to be readily treated with antibiotics, in late chronic infection *P. aeruginosa* appears to secrete copious amounts of alginate that result in the formation of a matrix which embeds into the lung environment (Eberl and Tummler, 2004). This causes antibiotic resistance and allows the bacteria to persist in the lung environment.

1.3 Cystic fibrosis disease and treatment

1.3.1 Disease state and epidemiology

Cystic fibrosis (CF) is an autosomal recessive genetic disease effecting 1 in 2,500 live births (Vankeerberghen et al., 2002). It is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) protein which acts as a chloride channel in mucosa lined epithelial cells.

This results in the influx of thick mucus secretions into the lung which cannot easily be cleared, creating a niche for opportunistic pathogens. Over 1,800 mutations in the CFTR gene have been discovered, however not all result in CF disease (Foundation, 2012).

The disease is characterised by an exocrine pancreatic insufficiency leading to an inability to absorb fats and proteins, chronic bacterial lung infection and a reduction in weight despite a voracious appetite (Asay, 1965). In both adults and children, signs of liver cirrhosis and diabetes can be present however they are rare (Brusilow, 1970). Sterility is experienced in 98% of males, and puberty can be delayed in some patients due to nutritional deficiencies (Orenstein et al., 2002). Andersen was the first person to categorise CF as an independent disease to celiac, which had a similar symptom profile (Andersen, 1949). She was also the first to suggest that it was a hereditary disease (Andersen Dh, 1946) however it took another 40 years before the gene responsible was discovered (Kerem et al., 1989, Riordan et al., 1989).

The major cause of morbidity and mortality in CF patients is a chronic obstructive lung disease and a largely neutrophil-dominated inflammatory response (Saiman, 2004). The progression of disease is determined by the colonising bacteria within the lung environment, and this in turn determines the morbidity and prognosis of the disease state (Eberl and Tummler, 2004). Diagnosis of the disease is through a sweat test with confirmation by DNA analysis in which a homozygous genetic profile for the mutated CFTR can be found (Orenstein et al., 2002). In 1987-91, the average life expectancy was approximately 28 years of age. Today, if managed appropriately, the median age of survival is 36.8 (Foundation, 2012).

1.3.2 Microbial infection

Due to the mucus-rich environment, the CF lung makes for an adaptable habitat for many bacterial species. In early infection, CF lung pathogens are quite varied, however later infection appears to be more defined. In the majority of cases, by adulthood the lung will be dominated by *P. aeruginosa* (as shown in Figure 1.3). *P. aeruginosa* is one of the best studied pathogens infecting the lung. It has a variety of virulence factors that allow it to reside in the lung without much challenge both from the host immune system and antibiotics (see section 1.2). Studies have shown that *P. aeruginosa* infection causes pronounced obstruction of airways and chronic infection can lead to severe negative clinical effects (Lopes et al., 2012, Goss and Ratjen, 2013). In Australia, approximately 59 per cent of patients were infected with a clonal strain of *P. aeruginosa* (O'Carroll et al., 2004) with more than 30 per cent of CF patients infected with the Australian Epidemic strain 1 (AES-1) (Tingpej et al., 2010). The AES clones have been linked with increased virulence, more hospitalisations and poorer patient outcomes compared to infection with non-epidemic strains. Epidemic clonal *P. aeruginosa* also demonstrate extensive antibiotic resistance, with the AES-1 clone exhibiting 97 per cent resistance to both Amikacin and Gentamicin and more than 40 per cent resistance to most other commonly used antibiotics making this strain incredibly difficult to treat (Tingpej et al., 2010).

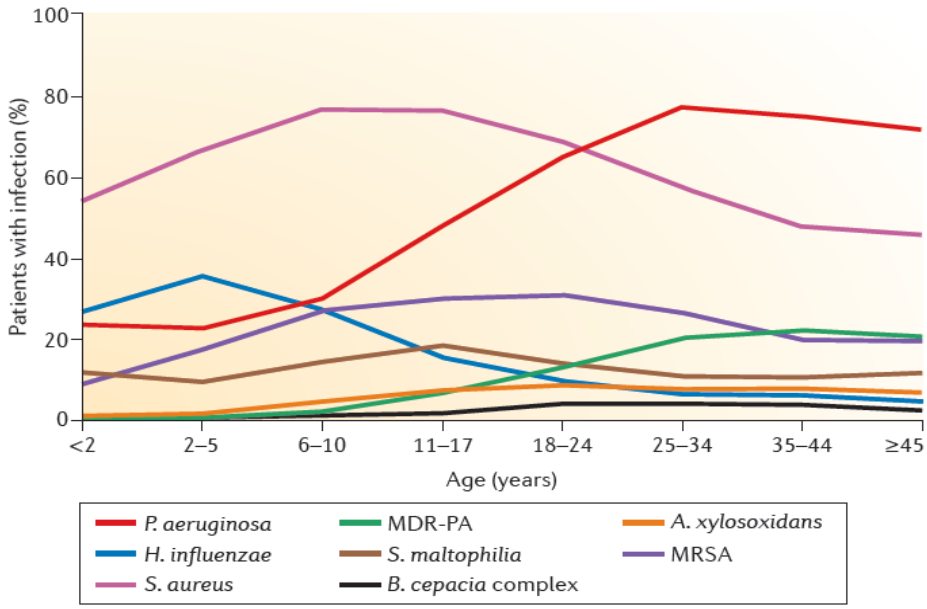


Figure 1.3: The colonisation profiles of the most common pathogens in CF patients. (Foundation, 2012)

Other clinically important bacterial species include *Haemophilus influenzae*, *Staphylococcis aureus*, and *Burkholdaria cepacia*. Viral and fungal infections can be present in the lung, and other bacterial species including *Klebsiella* and *Stenotrophomonas* can contribute to morbidity (Harrison, 2007). *S. aureus* and *H. influenzae* are both common nasal flora of a healthy individual, however they have been found in throat swabs of CF patients. *S. aureus* has been also been found, similarly to *P. aeruginosa*, in the mucus of obstructed airways (Ulrich et al., 1998). Whilst its individual pathogenicity is unknown, it has been suggested that *S. aureas* infection can prime the lung for subsequent chronic *P. aeruginosa* infection by destroying epithelial cells and providing an area for *Pseudomonas* attachment (Lyczak et al., 2002). *B. cepacia* is an emerging CF pathogen that is believed to be spread via patient-to-patient contact or through the environment. It has been associated with severe infection and mortality due to respiratory failure (Whiteford et al., 1995). *B. cepacia* is able to infect epithelial cells of the respiratory tract (Burns et al., 1996), possibly accounting for its ability to cause disseminated disease and be highly

resistant to antibiotics (Lyczak et al., 2002). Co-infection of *B. cepacia* with *P. aeruginosa* has also shown to cause a significant reduction in lung function compared to *P. aeruginosa* infection alone (Whiteford et al., 1995).

Another emerging infection is that of non-tuberculosis mycobacteria (NTM) (Kilby et al., 1992, Torrens et al., 1998, Hjelte et al., 1990) with 4% to 20% of CF patients being infected with NTM (Lyczak et al., 2002). It has been suggested that whilst the clinical importance of such an infection is not well established, it could account for those patients who deteriorate without an obvious cause (Hjelte et al., 1990). As mycobacteria are slow-growing they can be outgrown by other bacterial species within the test culture making it difficult to detect (Kilby et al., 1992). This co-infection with NTM could be more prevalent than currently known.

1.3.3 Current drugs in use

Historically, patients with CF rarely lived to their teenage years due to lack of a proper diagnosis and poorly controlled lung infections. In 1964 a therapeutic regimen was suggested for children suffering from CF which was directed at three factors of disease; the obstructive lung lesion, secondary pulmonary infection and nutritional and pancreatic defects (Matthews et al., 1964). Drugs were given in combination targeting all three factors rather than controlling individual characteristics of the disease. The introduction of this regimen appeared successful and saw a gradual increase in average life-expectancy (Orenstein et al., 2002). As CF is classified by a pancreatic deficiency and weight loss, adequate nutrition is essential for treatment. Improving the nutrition of patients has led to increased weight, healthier BMI levels, and improved lung function (Shoff et al., 2013, White et al., 2013, Beker et al., 2001). This can be achieved in patients through enzyme replacement therapy to assist in the

breakdown of fats (Orenstein et al., 2002) or nocturnal tube feeding for direct nutrient supply (Steinkamp and von der Hardt, 1994, White et al., 2013).

As chronic infection is currently the major dictator of morbidity and mortality, the predominant aim is to treat the infection in the lung. A variety of treatments are available to assist in bacterial removal or the improvement of the lung environment to a more normal state. They include mucus thinners (Reeves et al., 2012, Frederiksen et al., 2006), CFTR modulators (Jih and Hwang, 2013, Wilschanski et al., 2011), anti-inflammatory medication (Lands and Stanojevic, 2013, Noel et al., 2012), and antibiotics (Assael et al., 2012, Fernandez-Olmos et al., 2012). Prophylactic or aggressive antibiotic treatment can lead to a delay or prevention of chronic infection, although not in all cases (Harrison, 2007). Treatments for CF are increasing the life expectancy for patients, however antibiotics to eradicate the chronic lung infection are still needed due to antibiotic failure.

The biggest issue with treatment is the increasing drug resistance of *P. aeruginosa* strains. In hospital settings resistance to commonly used medications such as Piperacillin, Ciprofloxacin, and Imipenem have been increasing from 2 to 4.6 per cent per year (Xu et al., 2013). In 2011, one study showed Gentamicin and Ticarcillin resistance in hospitals was greater than 70 per cent, and resistance to the last line carbapenems were greater than 40 per cent (Xu et al., 2013). Within individual patients, resistance can occur over the lifetime of the infection, making treatment of chronic infection extremely difficult (Mouton et al., 1993). *Pseudomonas* resistance is highly prevalent due to multiple resistance mechanisms intrinsically built into the bacterium and this has resulted in some clinical isolates being resistant to all FDA-approved treatments (Talbot et al., 2006).

There are new drugs coming through the drug 'pipeline', however none have been approved for human use and no new antibiotics possess a novel mode of action (Hurley et al., 2012). Intravenous carbapenems are in clinical trials and have been shown to be effective at restricting the growth of *P. aeruginosa.* One such compound in phase 3 trials, Doripenem, has an MIC_{90} of 4 μg/mL against imipenem-resistant strains and appears to have similar activity to meropenem which is already used in *Pseudomonas* treatment (Alvarez-Lerma et al., 2009).

Other trials have explored the inhalation of already established antibiotics, such as collistin, tobramycin, and gentamicin (Ryan et al., 2011). Tobramycin, collistin and aztreonam are currently used as an aerosolised treatment to manage chronic *Pseudomonas* infection (Lam et al., 2013). Collistin inhalation therapy was the first to be used in chronic infection. It has high levels of activity against *P. aeruginosa* and low levels of resistance, and in one study it appeared to improve patient well-being and prevent lung function deterioration as well as reduce the inflammatory response commonly witnessed upon completion of intravenous therapies (Jensen et al., 1987). Tobramycin is the most widely used inhaled antibiotic being stable under a multitude of conditions, possessing low levels of resistance, having a prolonged post-antibiotic effect and having a high activity level relative to gentamicin (Smith, 2002). The success of these inhaled compounds has meant that more than 60 per cent of chronically infected CF patients are receiving at least one inhaled antibiotic as part of their treatment regimen (Moskowitz et al., 2008).

1.4 Tuberculosis treatment and prevention

1.4.1 BCG and vaccination

Currently, the only available vaccine for tuberculosis is the BCG vaccine (Liu et al., 2009) which is a highly attenuated strain of *M. bovis*, known as *Mycobacterium bovis* Bacille Calmette-Guerin (BCG). It is one of the most widely used vaccines in human history, being both relatively safe and inexpensive (Gerberry, 2009). Historically, after the success of the smallpox vaccine, the initial vaccine trial was to inoculate patients with *M. bovis* directly (Davies, 1998). This proved unsuccessful due to the unforeseen virulence of that particular strain, however the work of two French bacteriologists, Albert Calmette and Camille Guerin, whose work included culturing mycobacterium in various media, showed that by culturing *M. bovis* in a glycerin-bile-potato medium, a less virulent form of *M. bovis* could be created (Davies, 1998, Ritz et al., 2008). In 1924, after 230 passages over a 13 year period, a strain was developed that could be used as an attenuated vaccine (Ritz et al., 2008).

Initially the vaccine was given orally. France was the only European nation that initially took on the BCG vaccine, administering it to infants across the country (Hawgood, 2007). Following this Scandinavian countries developed the vaccine to be administered intradermally, and this was offered in a mass vaccination campaign throughout Europe in the 1950's (Davies, 1998).

The BCG vaccine, despite its wide usage, displays a varying efficacy from 0-80% (Liu et al., 2009). It does prove to be useful in preventing meningitis in children, as well as protecting those infected from severe infection (Ritz et al., 2008). Vaccination with BCG results in varying levels of protection against pulmonary tuberculosis (Brewer, 2000, Honaker et al., 2008) and transmission is not reduced by the vaccine (Maartens and Wilkinson, 2007). It has been suggested that the variation is due to genetic

differences between BCG and *M. tuberculosis*, such as the absence of genes in BCG such as ESAT-6 and CFP10, which are expressed by *M. tuberculosis* (Hoft, 2008, Behr et al., 1999) resulting in differences in antigenicity and protective efficacy. Genetic difference amongst the BCG strains has resulted in further phenotypic differences that affect antigenicity (Honaker et al., 2008).

Currently, research is focussed on the development of new vaccines, however this will not be discussed further in this thesis as it is not a topic of the research described here. It is acknowledged that the most effective way to control tuberculosis is the combination of highly protective vaccines to prevent infection/disease, together with efficacious drugs to treat those already infected. However, there are several factors which necessitate the development of novel, more effective drugs for the control of tuberculosis. These include the emergence of multi-drug resistant strains of *M. tuberculosis*, the dangerous interplay between HIV and tuberculosis co-infection and the shortcomings of current mainstay anti-mycobacterial drugs.

1.4.2 Multi-drug resistance

Three types of drug resistance are defined by the WHO: primary, secondary and initial. Primary resistance is defined as the presence of a resistant strain of *M. tuberculosis* in a patient who denies having had any previous treatment (I.U.T.L.D, 1998). ‘Secondary’ or ‘acquired resistance’ is defined as the presence of resistant strains of *M. tuberculosis* in patients who have undergone treatment for 4 weeks or longer (I.U.T.L.D, 1998). Isolation of resistant strains from patients where previous treatment history cannot be obtained are defined as having initial resistance (I.U.T.L.D, 1998).

Drug resistance, as a subgroup from the above categories, is divided into monoresistance and polyresistance. They are defined as resistance to one of the first-line drugs of tuberculosis

chemotherapy, INH, Rifampicin, Ethambutol and Para-aminosalicylic acid, for monoresistance, or resistance to two or more first line drugs for polyresistance (Loddenkemper et al., 2002). Multi-drug resistance (MDR) is defined as a resistance to at least rifampicin and Isoniazid (W.H.O, 2012), the two most potent anti-tuberculosis drugs, and is classified as polyresistance.

The global spread of MDR tuberculosis is such that it has been declared a pandemic (Farmer and Kim, 1998, Zager and McNerney, 2008). MDR has established due to a combination of factors, including inadequate chemotherapy and treatment regimens, confinement and therefore lack of quarantine measures, population growth and the increased incidence of HIV (Johnson et al., 2006, W.H.O, 2012, I.U.T.L.D, 1998). During the initial stages of drug therapy, when streptomycin was the main-line drug, resistance developed due to the implementation of this mono-therapy (Loddenkemper et al., 2002). Because of this, treatment thereafter was multi-faceted.

Initially treatment was an 18-24 month course, however with the introduction of rifampicin in the 1960's, chemotherapy reduced to a 6-8 month course and there was an overall decline in *M. tuberculosis*. The decline in incidence led to complacency, decreased funding and monitoring of tuberculosis disease for 20 years (Espinal, 2003). Surveillance did not resume until after an increase in HIV/AIDS incidence in the 1980's led to outbreaks of both susceptible and resistant strains of *M. tuberculosis* (Johnson et al., 2006).

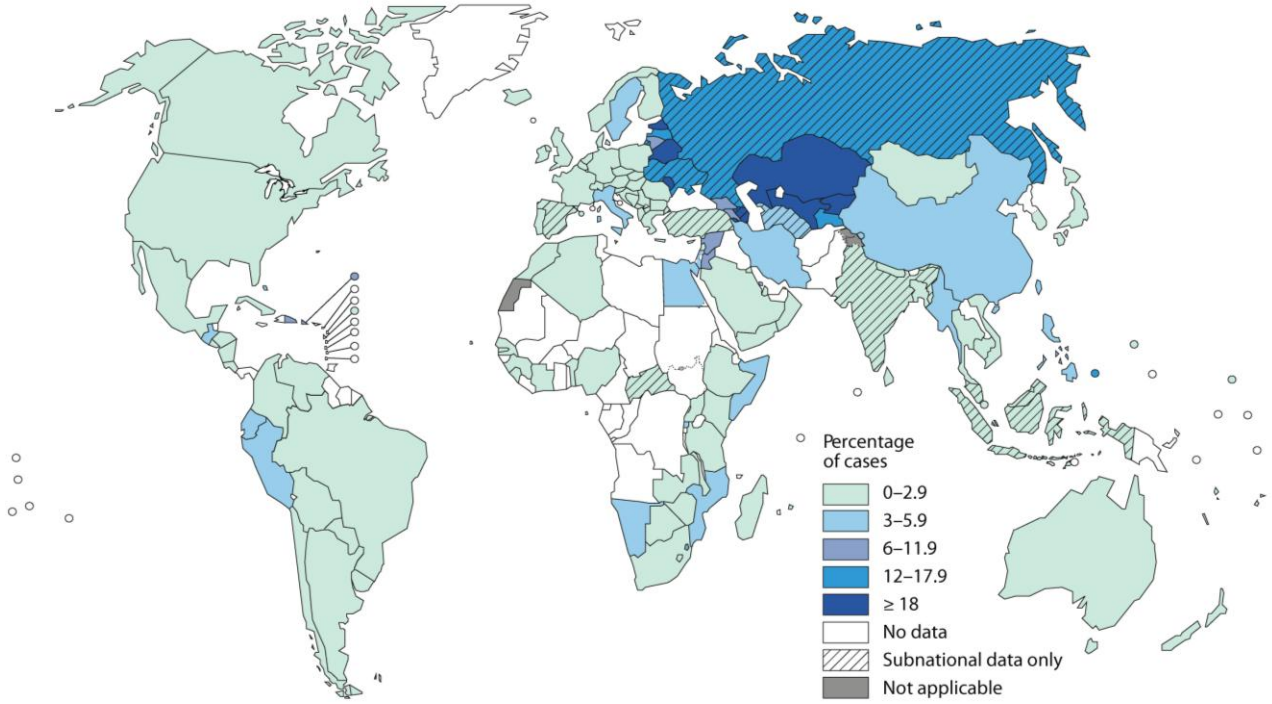


Figure 1.4: Percentage of MDR-TB cases in newly diagnosed patients (W.H.O, 2012)

In 2000, it was estimated that 3.2% of the 8.7 million new tuberculosis cases were MDR (Dye et al., 2002b). That has since increased to 3.1% of 9.27 million new cases of tuberculosis in 2007 (W.H.O, 2009), and currently it is estimated that 3.7% of new cases are MDR (W.H.O, 2012). These 'new' cases were determined as patients who had not received any prior anti-tuberculosis treatment or who had only been on a course of drugs for up to one month (Dye et al., 2002a). The most affected countries with the highest incidence of MDR tuberculosis include India, China, Russian Federation and South Africa, however the highest proportion relative to population is Belarus, Estonia and Kazakhstan (Figure 1.4) (W.H.O, 2012).

These new cases of MDR tuberculosis not only mean that resistance is arising from poor treatment practices or treatment failure but also that the resistant strains have been able to be transmitted within

the population (Dye et al., 2002a). Treatment of MDR tuberculosis requires a prolonged chemotherapeutic course, and the use of second-line drugs which are highly toxic and very expensive (Zager and McNerney, 2008). Standard short course therapy can effectively treat 30-50% of MDR cases if correctly monitored (Bottger and Springer, 2008), however for those patients to whom short course therapy is ineffective, the cost of the drug regime to control MDR tuberculosis is typically hundreds of times greater (Dye et al., 2002b).

1.4.3 DOTS regime

A new treatment regime implemented by the W.H.O was directly-observed treatment and short-course chemotherapy (DOTS). Patients would receive a cocktail of first-line drugs and a medical professional or similar would monitor and record the treatment being taken (Cox et al., 2008). Sputum samples are taken monthly, and patients remained on the DOTS program until they produce two consecutive negative samples (Johnson et al., 2006). This was to prevent patients from prematurely stopping treatment, therefore reducing the risk of acquired resistance.

There are five key elements that form the basis of the DOTS strategy. These elements are commitment by the government, detection of each case by sputum smear microscopy, a standard 6-8 month treatment regime, a constant and regular supply of all necessary tuberculosis drugs and a standard recording and reporting system (DeRiemer et al., 2005). From this framework, four drug regimes were developed. Within these regimes, there is an intensive phase of treatment, where a patient will receive up to four first line drugs, and then a continuation phase using only rifampicin and isoniazid, which are the two most potent anti-tuberculosis drugs (Johnson et al., 2006).

There is evidence that the DOTS regime has a success rate of 90% in countries or areas where correct procedures had been in place prior to the DOTS implementation (Espinal and Dye, 2005). However, in countries where resistance was already prevalent, there was a high level of treatment failure and death (Espinal and Dye, 2005). Overall, DOTS has increased treatment success since its implementation both globally and in high burdened countries such as Africa and Asia (Figure 1.5).

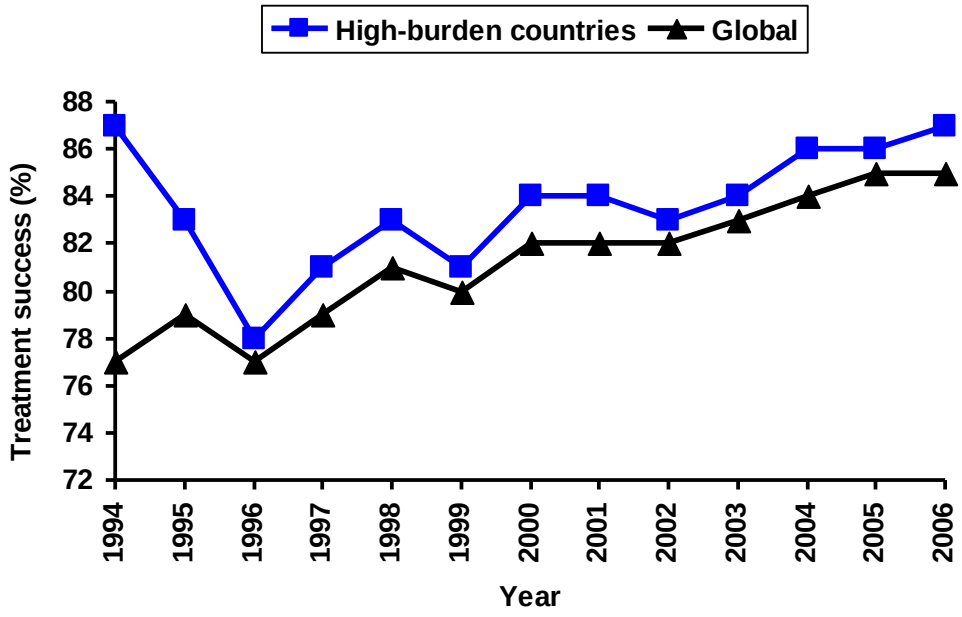


Figure 1.5: Treatment success for new cases of tuberculosis treated under DOTS between 1994 and 2006. Adapted from W.H.O (2009).

DOTS therapy, whilst being effective in controlling cases of susceptible tuberculosis and preventing the increase in MDR incidence, is still not enough to control MDR tuberculosis itself. This is due to the fact that MDR tuberculosis is already resistant to rifampicin and isoniazid, which are the two most effective components of the DOTS regime. This has two implications; firstly, that the treatment will be ineffective and secondly, that this treatment practise could encourage resistance to pyrazinamide and ethambutol in patients with otherwise susceptible strains (Farmer and Kim, 1998).

Whilst the practise of DOTS can be used in MDR cases, it is harder to develop a standardised regimen due to the variation in resistance. This means that patients can be monitored when taking their medication, however success rates are more difficult to judge due to differences in treatment length and efficacy of drug combinations used during treatment.

1.4.4 Extensive-drug resistance

Extensive drug resistance (XDR) is defined as MDR that includes resistance to any fluoroquinolone and one of the second-line injectable drugs; kanamycin, amikacin and capreomycin (Madariaga et al., 2008). It was first described in March, 2005, by the US Centre for Disease Control and Prevention (C.D.C, 2007), and was brought to the public's attention in October of that same year (Jassal and Bishai, 2009).

The first cases of XDR tuberculosis were seen in the KwaZulu-Natal Province of South Africa, in patients suffering from Acquired Immune Deficiency Syndrome (AIDS) (Chan et al., 2009). Of those infected with XDR, 98% of cases resulted in death (Gandhi et al., 2006). A study conducted with the assistance and management of the Stop TB Partnership Green Light Committee (GLC), created in 2000, showed that in a population based assessment, 19% of XDR cases were found in Latvia, which has one of the highest MDR case rates (Jassal and Bishai, 2009). Currently, XDR tuberculosis has been identified in 49 countries worldwide (Sotgiu et al., 2009) and with a high fatality rate and a median survival of 16 days it remains virtually untreatable (Hwang et al., 2009). Thus, there is an urgent need for the development of new treatment for XDR cases.

1.4.5 Current drugs in use

Streptomycin was the first antibiotic used in anti-tuberculosis treatment (Proust et al., 1982). It was discovered in 1943 (Loddenkemper et al., 2002) and first marketed in 1947 (Conti et al., 2004). The drug was initially effective, with bactericidal efficacy against *M. tuberculosis* (Proust et al., 1982) that translated to a marked improvement in prognosis for 51% of patients studied (Davies, 1998). However, the implementation of antibiotic monotherapy resulted in resistance within 2-3 months of its initial use (Jassal and Bishai, 2009, Davies, 1998).

Subsequently, agents such as p-amino salicylic acid (PAS), discovered by Lutz Eric Lehmann in Sweden in 1946, and Isoniazid (INH), which was discovered in 1952, were used in combination therapies against tuberculosis (Ross and Horne, 1976). The British Medical Research Council (MRC) trialled both PAS and isoniazid in mono-therapeutic regimes and also in combination therapy trials using both PAS and isoniazid or with the inclusion of streptomycin (Iseman, 2002). Isoniazid proved to be bactericidal after the first 24 hours, similarly to streptomycin, however PAS was strictly bacteriostatic (Ross and Horne, 1976). Streptomycin, whilst being highly effective was also quite toxic to patients, conversely PAS was relatively non-toxic, however was not as efficacious (Vilcheze and Jacobs, 2007). INH proved to be the most effective, although appeared to be selective for slow-growing strains of mycobacteria. It selectively killed actively replicating bacteria, showing no effect on those in a latent state (Vilcheze and Jacobs, 2007).

Despite the success of these drugs, further research was conducted to discover new anti-microbials, as a precaution to avoid the problems associated with the development of streptomycin resistance. This led to the discovery of pyrazinamide in 1952, cycloserine in 1955, ethambutol and capreomycin in 1961,

and rifampicin in 1966 (Loddenkemper et al., 2002). The introduction of pyrazinamide and rifampicin in particular, resulted in the treatment course being reduced 3-fold, with relapse rates of less than 2% (Iseman, 2002).

In the 1970s, the British MRC implemented a short-course chemotherapy regime, which since has become the recommended protocol for treatment of tuberculosis (Balganesh et al., 2008). It is typically a 6 month treatment program. In the first two months, patients are given a combination of four first-line drugs, including rifampicin, isoniazid, pyrazinamide, and ethambutol or streptomycin. Subsequently, a four month course of rifampicin and isoniazid is implemented and cure rates can be as successful as 90% (Espinal and Dye, 2005).

An increase in drug resistance has led to an urgent need for the discovery of new drugs. Despite this, no new compound has been introduced into the standard treatment program since the 1960s (de Souza, 2006). Current treatment for resistant mycobacteria can last on average 24 months, and rely on second-line drugs that are toxic and costly (Espinal and Farmer, 2009). New compounds need to be bactericidal, have an extended safety profile to accommodate for prolonged treatment duration (Balganesh et al., 2008) and ultimately have a shorter treatment duration to encourage patient adherence (Chan and Iseman, 2002).

1.4.6 New chemotherapies

In December 29th 2012, the FDA approved the first tuberculosis drug in over 40 years. Bedaquiline, an enzyme inhibitor preventing bacterial replication and transport, has been approved for the use in MDR cases along with a standard combination therapy (Goldenberg, 2013). Whilst this was a milestone for TB treatment, the drug has serious safety concerns and the possibility of resistance developing is likely

if not regulated appropriately. It is therefore important that the research and development of new chemotherapies be made a priority.

As shown in Figure 1.6, there are a number of drugs in development, both at the discovery stage and in clinical trials. Delamanid is currently in phase III trials. It was discovered by conducting a screen using nitro-dihydro-imidazooxazole derivatives and targets mycolic acid biosynthesis (Matsumoto et al., 2006). An early bactericidal study concluded that Delamanid showed promising anti-bacterial activity after 14 days, however was not as effective as the current treatment regime (Diacon et al., 2011). Later clinical studies appeared more successful with a 100% increase in sputum culture conversion in Delamanid treated patients compared to placebo treated (~48% and 24% respectively), with 80% of patients classified as having MDR-TB (Gler et al., 2012). Another study showed favourable outcomes and a reduced level of mortality in MDR and XDR patients when treated with Delamanid (Skripconoka et al., 2013). There were numerous side effects experienced, most of which were gastrointestinal issues (vomiting etc), however they were only marginally more prevalent than the placebo treated group (Gler et al., 2012).

Novel regimens are also in phase II trials. In a randomised study conducted in 2012, the combination of PA-824, pyrazinamide and moxifloxacin appeared to be the most effective of those assessed and was just as effective as the current regimen. This is particularly promising as this combination is an INH- and RIF-independent regimen, useful for treatment and prevention of MDR-TB. One negative result from this combination however, was that there were side effects leading to three withdrawals (out of 85) from this trial (Diacon et al., 2012). Other regimes combining bedaquiline (TMC-207), sutezolid (PNU-100480) and PA-824 showed a significant bacterial load reduction in murine models compared to RIF/INH/PZA control, with a lower number of relapses (Williams et al., 2012). Whilst these

combinations are efficacious there are some safety concerns and therefore new compounds that are both safe and effective need to be developed.

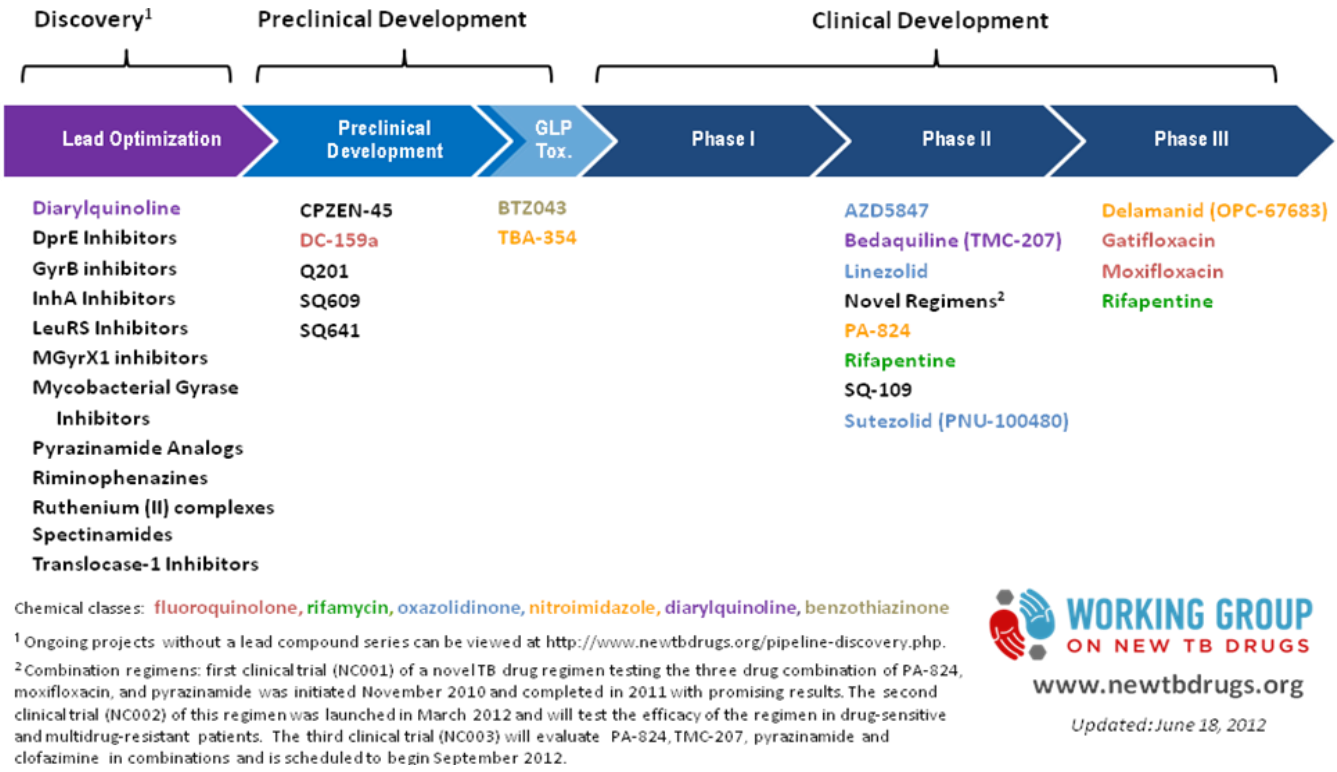


Figure 1.6: New drug pipeline for tuberculosis treatment (www.newtbdrugs.org, 2012)

There are also numerous compounds that are in the developmental phase. Studies into nutritional supplementation have been conducted, looking at whether fat-soluble vitamins are able to restrict mycobacterial growth (Sinclair et al., 2011, Greenstein et al., 2012). This works on the premise that vitamins are involved in immune function, particularly pertaining to macrophages and lymphocytes (Sinclair et al., 2011). Currently, no clinically relevant benefit has been established.

Benzothiazinones are a class of compound that inhibits the enzyme involved in arabinan biosynthesis. Ortho nitro-dialkyldithiocarbamate derivatives were chemically modified to create a variety of

compounds to be tested against mycobacteria (Makarov et al., 2006). These modifications led to a series of compounds, nitrobenzothiazones, which showed promising results with regards to specificity and potency against mycobacteria (Makarov et al., 2009). BTZ043 is in preclinical development (Figure 1.6), having been the most successful. Murine studies have shown that this compound is highly efficacious, being significantly more potent than INH and rifampicin (Makarov et al., 2009). It has also been shown to have a synergistic effect with the current therapies rifampicin, INH and moxifloxacin, as well as with the novel compounds bedaquiline and PA-824 (Lechartier et al., 2012).

1.5. Iron and mycobacterium

1.5.1 Iron and the host

Iron is important for many biological processes including DNA synthesis, erythropoiesis, and electron transport (Richardson et al., 2008). In the host immune system, iron is required for the essential functions of macrophages by promoting the production of nitric oxides and the NADPH oxidative burst. It is also required in the production of certain cytokines (Cronje et al., 2005). Iron in excess however, is toxic, requiring the need for iron regulation within the body (Crichton et al., 2002). Iron chelation therapy is currently indicated in thalassemia, myelodysplasia, and other haematological conditions and is being investigated for the treatment of certain cancers.

Iron is transported into a mammalian cell by receptor mediated endocytosis. Typically, iron serum levels are between 3-5 µg/mL which it is predominantly bound to transferrin (Crichton et al., 2002). Iron-bound transferrin binds to the transferrin receptor 1 (TfR1), which is upregulated when

intracellular iron levels are low (Yu et al., 2006). Within the intracellular space, a change in pH from neutral to acidic results in the iron being released from transferrin, and it can then be used by the cell for various functions (Richardson et al., 2008).

When a macrophage is stimulated by interferon-γ (IFN-γ), this induces the downregulation of the transferrin receptor, which is required to move intracellular iron out of the cell (Collins, 2003, Sow et al., 2007). This results in iron being kept intracellularly, thus allowing the macrophage to function appropriately. Paradoxically, the accumulation of iron interferes with macrophage function, particularly with respect to NFκB activation and nitric oxide production required for phagocytosis (Crichton et al., 2002). Iron is also used by *M. tuberculosis* as a co-factor for a number of enzymatic processes, a fact that has facilitated the exploration of iron chelation therapy as a method of treatment against tuberculosis.

1.5.2 The use of iron in *Mycobacterium tuberculosis* infection

Mycobacteria use iron as a cofactor for over 40 different enzymes encoded within the genome (Cronje and Bornman, 2005, Yellaboina et al., 2006). There are many iron-regulated genes that are conserved within the mycobacterial species, including fatty acid biosynthesis, iron storage, and amino acid biosynthesis (Yellaboina et al., 2006). One of the virulence factors possessed by mycobacteria are siderophores, which are low-molecular weight compounds with a high affinity for iron (Winkelmann, 2002). The siderophore acts by being released into the extrabacillary space within the macrophage. They then bind to the iron molecules available and are reinternalised by the bacteria (De Voss et al., 2000). There are two types of siderophore in Mycobacteria; exochelins and mycobactins (Winkelmann, 2002). Pathogenic mycobacteria, particularly *M. tuberculosis*, only produce mycobactins containing a

salicylic acid derived moity (Byers and Arceneaux, 1998). When *M. tuberculosis* H37Rv was mutated causing a deletion of the *mbtB* gene encoding a siderophore, there was a reduction in the growth of *M. tuberculosis* compared to wild-type mycobacteria under iron limitation, but this effect could be reversed when cultured in a high iron medium (De Voss et al., 2000). This study highlighted the importance of siderophore production under conditions of iron limitation.

To further demonstrate the importance of iron on mycobacterial growth, one study found that the number of *M. tuberculosis* in mice supplemented with iron were higher in both the spleen and lung compared to the control mice (Lounis et al., 2001). In HIV positive patients, *M. tuberculosis* infection (as well as *M. avium* infection) was more prominent in patients with high iron grades (de Monye et al., 1999). Together, these studies suggest that limiting iron availability may be an effective strategy to treat mycobacteria.

The effect of iron on mycobacterial growth has led to the study of iron chelation therapy as a treatment for mycobacterial infection. Chelation therapy inhibited the growth of extracellular *M. tuberculosis* bacteria, but displayed limited efficacy against internalised bacteria (Cronje et al., 2005). *In vitro* studies showed iron chelators were able to inhibit growth of *M. avium* in macrophages to a moderate extent. However, effects were limited in mice (Gomes et al., 2001). Although the studies were independent, both acknowledged that whilst iron limitation could reduce mycobacterial numbers, more effective iron chelators are required.

1.6 The current study

Drug resistance is an increasing problem throughout the world. Numerous bacterial strains, particularly those that are hospital borne, are developing almost complete resistance and the development of new compounds is not meeting the need of the patients. In the case of *Pseudomonas* infection in CF patients, some strains exhibit complete resistance to the main chemotherapies, causing a resurgence in use of potentially toxic compounds such as Polymyxin B, or lung transplants being the only viable therapy. In comparison, for drug susceptible strains of *M. tuberculosis*, the current therapies are very effective, cheap and relatively non-toxic. However, there is a 6-8 month treatment program and the incidence of drug resistant strains is on the increase. This has led to a need not only for new drugs to combat drug resistance but also a requirement to identify compounds that can reduce the treatment time whilst still being as affordable, effective and non-toxic.

The central aim of this project was to discover new compounds that could potentially be used in the treatment of chronic lung diseases, particularly those due to *P. aeruginosa* and *M. tuberculosis*. This was approached through the use of complimentary methods for drug discovery, including targeted discovery using the knowledge of bacterial growth requirements or whole cell screening with libraries comprised of novel natural and synthetic compounds.

Hypothesis: That novel chemical classes can be identified that effectively restrict the growth of bacterial lung pathogens.

Specific Aims:

1. To determine if highly effective iron chelators can inhibit mycobacterial growth both *in vitro* and *in vivo*.

2. To determine if a defined set of novel cyclam-containing compounds display anti-bacterial properties

3. To screen a diverse library of natural and synthetic compounds to identify new structures against *Pseudomonas* and Mycobacterial pathogens.

Chapter 2: Material and Methods

Materials

2.1 Media and supplements

2.1.1 Bacterial culture media

From Sigma Aldrich Australia, unless otherwise stated.

Albumin-Dextrose-Catalase (ADC) - 5 % w/v BSA + 2 % w/v dextrose + 0.003 % w/v catalase in triple distilled water (TDW)**.** Store at 4 ^{o}C.

Filter sterilised at room temperature.

Luria agar - 30.5 g Luria agar powder (Bacto laboratories Pty Ltd, Pritchard, Australia) in 1 L reverse osmosis (RO) water, final concentrations 1 % w/v Bacot-tryptone + 0.05 % w/v Bacto-yeast extract + 1 % w/v NaCl + 1.5 % w/v agar.

Autoclaved at 121 ^{o}C for 20 minutes.

Luria broth (LB) - 15.5 g Luria broth powder (Bacto laboratories Pty Ltd) in 1 L RO water, final concentrations 1 % w/v Bacto-tryptone + 0.05 % w/v Bacto-yeast extract + 1 % w/v NaCl.

Autoclaved at 121 ^{o}C for 20 minutes.

Difco Middlebrook 7H9 medium - 4.7 g Middlebrook 7H9 powder (Bacto laboratories Pty Ltd) in 900 mL dH_2O + 100 mL ADC + 5 mL 50 % v/v glycerol + 4 mL 10 % v/v tween 80.

Autoclave at 121 ^{o}C for 15 minutes. Store in dark conditions at room temperature.

Difco Middlebrook 7H10 agar - 19 g Middlebrook 7H9 powder (Bacto laboratories Pty Ltd) in 900 mL dH_2O + 100 mL Oleic ADC + 5 mL 50 % v/v glycerol + 4 mL 10 % v/v tween

Autoclave at 121 ^{o}C for 15 minutes.

Oleic ADC (OADC) - 5 % w/v BSA + 2 % w/v dextrose + 0.004 % w/v catalase + 0.85 % w/v NaCl + 0.05 % w/v Oleic acid in TDW**.** Store at 4 ^{o}C.

10 % Tween 80 - 10% v/v Tween in distilled water (dH_2O), heated then filter sterilised.

50 % Glycerol - 50 % v/v glycerol (Fluka, BioChemika) in dH_2O, heated then filter sterilised.

2.1.2 Cell culture

From Sigma Aldrich Australia, unless otherwise stated.

Complete DMEM - DMEM (GIBCO) + 10 % foetal bovine serum (FBS) + 200 µM L-glutamine + 1 mM HEPES buffer solution. Store at 4 ^{o}C.

Complete RPMI - RPMI (GIBCO) + 10 % FBS + 200 µM L-glutamine + 1 mM HEPES buffer solution + 1 % v/v β-mercaptoethanol.

Foetal bovine serum (FBS) - Heat inactivated at 65 ^{o}C**.** Store at -20 ^{o}C in 25 mL aliquots.

HEPES buffer solution - 1 M stock solution (GIBCO)**.** Store at 4 ^{o}C.

L- Glutamine - 200 mM stock solution (GIBCO)**.** Store at 4 ^{o}C.

β-mercaptoethanol - 14.3 M stock solution, >99% (SIGMA). Store at 4 ^{o}C.

2 % FBS in Phosphate buffered saline (PBS) - 2 mL heat inactivated foetal bovine serum in 98 mL 1x PBS.

Phorbol-Myristate-Acetate (PMA) - 2 mg/mL in dH_2O.

2.2 Reagents

2.2.1 Restriction digestion and electrophoresis

All enzymes and buffers from New England Biolabs Inc.

NEBuffer 3 - 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM Dithiothreitol, pH 7.9 at 25 °C.

NEBuffer 4 - 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol, pH 7.9 at 25 °C.

TAE buffer - 40 mM Tris-acetate, 1 mM EDTA, pH 8.3 (Sigma Aldrich, Australia).

TSS buffer - 10% w/v PEG 8000, 5% v/v DMSO, 30 mM $MgCl_2$, Luria broth (Bacto laboratories Pty Ltd).

2.2.2 Miscellaneous

From Sigma Aldrich Australia.

Decanal (n-decyl aldehyde) - diluted 1 in 100 in 100 % ethanol. Use immediately.

Diethylenetriamine nitric oxide (DETANO) – 100 mM stock concentration.

Dimethyl Sulphoxide (DMSO) - 100%.

Resazurin - 0.05 % w/v Resazurin in dH_2O. Store at 4 ^oC.

2.3 Compounds

From Sigma Aldrich, unless otherwise stated.

Isoniazid (INH) – Stored at room temperature in powder form.

Rifampicin - Stored at room temperature in powder form.

Pyridoxal Isonicotinic Hydrazone (PIH) derivatives - Provided by Prof. Des Richardson, School of Pathology, University of Sydney**.** Stored at room temperature in powder form. Compound structures are shown in chapter 3. PIH and its analogues were synthesized by established procedures by a simple 1-step synthesis between commercially available aromatic aldehydes and hydrazides (Sigma-Aldrich) (Kalinowski et al., 2008). The following PIH analogues were examined in the current study: 2-pyridylcarboxaldehyde isonicotinoyl hydrazone (PCIH); 2-pyridylcarboxaldehyde benzoyl hydrazone (PCBH); 2-pyridylcarboxaldehyde 3-bromobenzoyl hydrazone (PC3BBH); 2-pyridylcarboxaldehyde 4-bromobenzoyl hydrazone (PC4BBH); 2-pyridylcarboxaldehyde *p*-hydroxybenzoyl hydrazone (PCHH); 2-pyridylcarboxaldehyde *p*-aminobenzoyl hydrazone (PCAH); 2-pyridylcarboxaldehyde 2-thiophenecarboxyl hydrazine (PCTH); 2-pyridylcarboxaldehyde *p*-nitrobenzoyl hydrazone (PCNH);

and 2-pyridylcarboxaldehyde *p*-trifluoromethylbenzoyl hydrazone (PCTFH). Log P_{calc} values of the ligands were the average log *P* values calculated in ChemDraw v4.5 using Crippen's fragmentation (Ghose and Crippen, 1987), Viswanadhan's fragmentation (Viswanadhan et al., 1989), and Broto's methods (Broto, 1984).

Methyl Pyridoxal Isonicotinic Hydrazone (MPIH) derivatives - Provided by Prof. Des Richardson, School of Pathology, University of Sydney**.** Stored at room temperature in powder form. The following MPIH analogues were examined in the current study: HMPAH, methyl pyrazinylketone *p*-aminobenzoyl hydrazone; HMPBH, methyl pyrazinylketone benzoyl hydrazone; HMP3BBH, methyl pyrazinylketone *m*-bromobenzoyl hydrazone; HMP4BBH, methyl pyrazinylketone *p*-bromobenzoyl hydrazone; HMPHH, methyl pyrazinylketone*p*-hydroxybenzoyl hydrazone; HMPIH, methyl pyrazinylketone Isonicotinoyl hydrazone; HMPNH, methyl pyrazinylketone *p*-nitrobenzoyl hydrazone; HMPTH, methyl pyrazinylketone thiophenecarboxyl hydrazone; HMPTFH, methyl pyrazinylketone *p*-trifluoromethylbenzoyl hydrazone (Kalinowski et al., 2008).

Metal-cyclam complex compounds - Provided by Associate Professor Peter Rutledge and Associate Professor Matthew Todd from the School of Chemistry, University of Sydney. Some compounds previously described in Yu *et al* (2011), Yu *et al* (2013a) and Yu *et al* (2013b). Compound structures shown in Appendix 1.

All above drugs were supplied in powder form. Primary stock solutions were made at the concentration of 50 mM or 10 mM in 100% DMSO, depending on the experiment. Typically, secondary stock solutions were prepared from the primary stock by diluting the solution 1 in 100 in a separate 1.5 mL

eppendorf tube, and 10 μL of this solution used for inhibition assays (1 in 1000 of primary stock, 0.1 % DMSO).

Compound Library - 1920 compounds obtained from the Queensland Compound Library. Details of the compounds described in Chapter 5 (section 5.2.3). Compounds supplied in 5 μL liquid droplets in 100% DMSO in 96 well plates.

Methods

2.4 Mycobacterial culture

All bacterial experiments were conducted in a PC2 or PC3 laboratory, using Class II cabinets. OGTR approval was sought and held for any bacterial strain used in experiments.

2.4.1 *Mycobacterium bovis* BCG Pasteur and *Mycobacterium avium* 104

Stocks of each bacterium stored at -80 ^{o}C were grown in 25 mL falcon tubes in complete Middlebrook 7H9 medium. Cultures were incubated rolling at 37 ^{o}C within a dry incubator, maintaining growth through passaging at log phase (OD_{600} of 0.5-1.0). Cultures in log phase were either used for experiments or to make stocks (in 25% glycerol) in 1.5 mL eppendorf tubes and were stored at -80 ^{o}C until needed. Plasmid transformed BCG and *M. avium* were cultured using the same methodology as above, but Middlebrook 7H9 medium or 7H10 solid medium were supplemented with 25 μg/mL hygromycin.

To enumerate the number of bacteria within the stock solutions or for experimental samples, 1 in 10 serial dilutions were made and selected dilutions were plated onto 3 replicate Middlebrook 7H10 quadrant plates. Plates were incubated at 37 ^{o}C in a CO_2 incubator for 3 weeks, and bacterial counts determined and expressed as colony forming units per mL of culture (CFU/mL).

2.4.2 *Mycobacterium tuberculosis* MT103

Stocks of *M. tuberculosis* MT103 (Pérez et al., 2001) stored at -80 ^{o}C were grown in cell culture flasks in complete Middlebrook 7H9 medium. Cultures were incubated at 37 ^{o}C without agitation in a 5 % CO_2 incubator, maintaining growth at log phase (OD_{600} of 0.5-1.0). Cultures in log phase were either used for experiments or to make stocks (in 25% glycerol) in 1.5 mL eppendorf tubes and were stored at -80 ^{o}C until needed.

2.4.3 *Pseudomonas aeruginosa*

Stocks stored at -80 ^{o}C were grown in 15 mL falcon tubes in LB medium. Cultures were incubated at 37 ^{o}C in a shaker, until log phase (OD_{600} of 0.6-1.2) was reached (16-24 hours).

2.5 Construction of M. avium luciferase strain

2.5.1 Plasmid extraction from mycobacteria

BCG transformed with plasmid pSMT1 (Figure 2.1- (Snewin et al., 1999)) was grown to log phase (OD_{600} of 0.45-1.0) and the culture pelleted by centrifugation at 6000 g for 3 minutes. The supernatant was discarded and the pellet resuspended in 125 µL of solution 1 (Qiagen Plasmid Mini kit, Qiagen

Australia) + lysozyme (500 µg/mL), followed by an overnight incubation at 37 ^{o}C. A further 60 minute incubation was conducted at 55 ^{o}C followed by addition of 125 µL of solution 2. BL buffer was then added, mixed by inverting, and allowed to incubate for 1-5 minutes until it became clear. Immediately afterwards, NE buffer was added and mixed by inverting 3 times, and the entire suspension was micro-centrifuged for 10 minutes at 10,000 g. The clear lysate was transferred to a DNA binding column, spun at 13,000 rpm for 1 minute and the flow-through discarded. WP buffer was added to the DNA binding column and centrifuged again at 10,000 g for 1 minute, and flow-through discarded. An additional centrifugation was completed to dry the plasmid, and this was then eluted into an 1.5 mL eppendorf tube using 50 µL of triple distilled water by centrifuging for 2 minutes at 10,000 g.

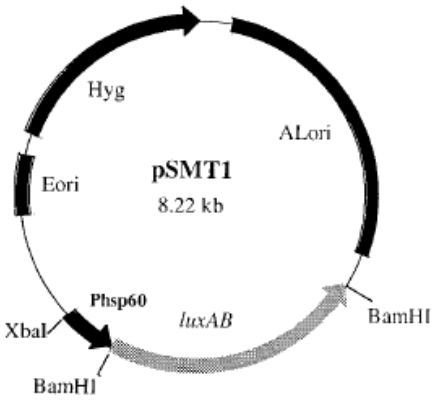


Figure 2.1. Map of the pSMT1 plasmid. The *lux*AB site encodes the luciferase gene, which is controlled by the Hsp60 promoter. Bam*HI* and Xba*I* restriction sites surrounding the luciferase gene can be utilized to verify plasmid integrity. Contains hygromycin resistance gene for selection (reproduced from Snewin et al., 1999)

2.5.2 Transformation of plasmid into *E. coli* via heat-shock

2.5.2.1 Preparation of competent *E. coli* cells

Stocks of *E. coli* DH5α were grown to log phase (OD_{600} of 0.5-1.0) in LB broth. The cells were centrifuged at 6000 g for 10 minutes at 4 ^{o}C. The supernatant was decanted, and the pellet resuspended gently in 5 mL TSS buffer. Ten percent v/v glycerol was added to the suspension, and cells were frozen in 100 µL aliquots at -80 ^{o}C.

2.5.2.2 Heat-shock

E. coli competent cells were removed from -80 ^{o}C storage and thawed slowly on ice. Two µL of the extracted pSMTI plasmid and 100 µL of the cell stock were added to a cold eppendorf tube and incubated on ice for 30 minutes. The eppendorf tube was then incubated at 42 ^{o}C for 45 seconds and immediately returned to ice with the addition of 400 µL sterile LB broth supplemented with 0.04 % glucose. The mixture was shaken for 4 hours at 37 ^{o}C to facilitate expression of the hygromycin resistance gene. Finally, the mixture was plated out onto LB plates supplemented with 50 µg/mL hygromycin and incubated overnight at 37 ^{o}C.

2.5.2.3 Plasmid extraction from *E. coli*

To determine whether the colonies that grew on the LB plates were true transformants, 6 colonies were selected from the plate and each single colony was cultured in 10 mL LB medium supplemented with hygromycin (100 µg/mL) and incubated overnight at 37 ^{o}C. The *E. coli* cells were pelleted for 10 minutes at 6000 g, the supernatant discarded and the pellet resuspended in 250 µL of RS buffer. Plasmid was extracted using a Plasmid Mini kit (Qiagen) according to manufacturer's instructions and

DNA was eluted in 50 µL dH_2O. Two µL of plasmid was digested with restriction enzymes Bam*HI* and Xba*I* (Figure 2.1) and appropriate buffer (NEBuffer3 and NEBuffer4) for 1 hour at 37^oC. For electrophoresis, a 50 mL 1 % agarose gel in TAE buffer was prepared and 1 mg/L Ethidium bromide added. Loading dye was added to the restriction digestion mix, in a 1 in 6 dilution, and loaded into the wells of the gel. The gel was run for 1 hour at 100 V, and visualised using a UV transilluminator (Novex Australia Pty Ltd).

2.5.3 Electroporation of plasmid pSMT1 into *M. avium* competent cells

2.5.3.1 Preparation of electrocompetant *M. avium* cells

Stocks of *M. avium* 104 strain were grown to log phase (OD_{600} of 0.5-1.0) in complete Middlebrook 7H9 broth. The culture was then centrifuged for 5 minutes at 1465 g and the supernatant removed. The pellet was resuspended in 30 mL of 10 % (v/v) glycerol and this process was repeated for a total of three times. After the final spin, the pellet was resuspended in 1.5 mL of 10 % (v/v) glycerol. One hundred and fifty µL volumes of the suspension was aliquoted into eppendorf tubes and stored at -80 ^oC until needed.

2.5.3.2 Electrotransformation

Electrocompetent cells of *M. avium*, taken from -80 ^oC, were thawed slowly on ice. In a cold 1.5 mL eppendorf tube, 150 µL of the cell suspension was mixed with 5 µL of plasmid DNA (pSMT1) and this mixture was allowed to sit on ice for 1 minute. Concurrently, the Gene pulser apparatus (BioRad) was set at 25 µF and 2.5 kV, and the pulse controller was set to 200 Ω. The mixture of cells and DNA was transferred to a cold electroporation cuvette and pulsed twice (producing a time constant of 4-5

milliseconds each pulse). Immediately after pulsing, 400 µL of Middlebrook 7H9 medium was added to the electroporation cuvette, and the total volume was then transferred to a M^{c}Cartney bottle containing 2 mL 7H9 medium. The culture was then allowed to incubate rolling overnight at 37 ^{o}C.

2.5.3.3 Verifying plasmid transformation

The overnight transformation culture was centrifuged for 5 minutes at 2000 rpm. The pellet was resuspended in 500 µL of 7H9 medium and plated onto three 7H10 agar plates supplemented with 25 µg/mL hygromycin, and incubated for 10 days at 37 ^{o}C in 5 % CO_2. Colonies were then picked and cultured in Middlebrook 7H9 complete medium supplemented with 25 µg/mL hygromycin, and luminescence was measured after 3 seconds shaking using 1 % Decanal (Sigma Australia) substrate and the FLUOstar Omega microplate reader (BMG LABTECH Australia). Cultures showing highest luminescent readings were used for subsequent experiments.

2.6 Resazurin Reduction Assay

The Alamar blue assay is based on the reduction of the colourmetric reporter resazurin. In the presence of viable cells Resazurin undergoes an enzymatic reaction where NADH is reduced to NAD, converting the blue Resazurin to a pink coloured derivative, Resorufin (Figure 2.2, (Promega, 2013)). This colour change can be read using a microplate reader due to Resorufin emitting fluorescence at 590 nm after excitation at 544 nm. The advantage of using this technique is that it is fast and simple to use, and the presence of live or dead cells can easily be distinguished.

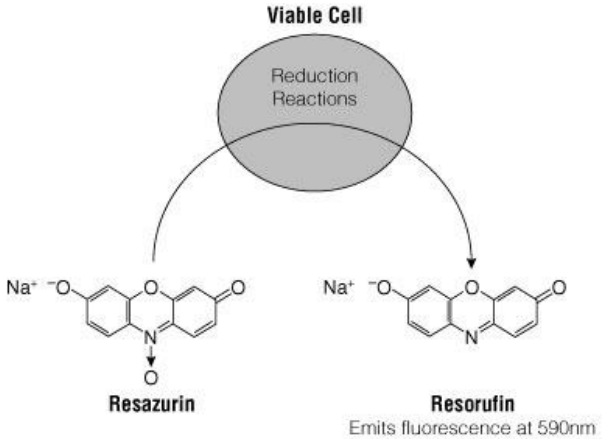


Figure 2.2. Alamar blue assay reaction. In the presence of viable cells a reduction reaction converts a blue coloured Resazurin, to a pink coloured resorufin which emits fluorescence at 590 nm. This allows for both a qualitative and quantitative assessment of cell viability (Promega, 2013).

2.6.1 Optimisation of screening assays

2.6.1.1 Starting concentration of bacteria

384-well microtitre plates were divided into three sections containing bacterial suspensions at an OD_{600} 0.001, 0.005 or 0.01. In each section for mycobacterial experiments, 5 µL of dH_2O or 1 mM rifampicin (complete inhibition control) was added to each well, and 45 µL of bacterial suspension was added at the appropriate concentrations. For *Pseudomonas* experiments, 50 µL dH_2O was used as the complete inhibition control. After a period of incubation (see section 2.6.1.2) 5 µL 0.05 % resazurin was added to each well, incubated for 4 hours and fluorescence measured at 590 nm using the FLUOstar Omega microplate reader. Bacterial growth inhibition was calculated as the percentage fluorescence relative to that of the untreated (complete survival) control.

2.6.1.2 Incubation time

Plates were set up as in 2.6.1.1, and incubated for 4, 5, 6 or 7 days for BCG and *M. avium*, and 4, 7, 24 or 48 hours for *Pseudomonas*. Five µL 0.05 % Resazurin was added to each well, incubated for 4 hours and fluorescence measured at 590 nm using the FLUOstar Omega microplate reader. The percentage inhibition was calculated as described in 2.6.1.1. Once fluorescence was measured, the 'z' factor was calculated using the following equation:

$$Z = 1 - \frac{3SD_{sample} + 3SD_{control}}{mean_{sample} - mean_{control}}$$

The 'z' factor is an indicator of reproducibility. A value between 1 and -1 is the most accurate (-1 being below the limit of detection) and the higher the 'z' value the higher the data quality (Zhang et al., 1999).

2.7 Assays of in vitro growth inhibition

2.7.1 Screening of compounds

For initial screening of compounds, 5 µL of individual test and control compounds (5-20 µM final concentration) were added to 384 well plates in two replicate plates. Forty-five µL of bacterial suspension was added to each well and incubated for 1, 5 or 7 days for *Pseudomonas*, *M. avium*, and *M. tuberculosis*/BCG respectively. Five µL 0.05 % Resazurin was then added, incubated for 4 hours and fluorescence was measured at 590 nm. Inhibition was calculated as a percentage of growth relative

to an untreated control, and compounds exhibiting a greater than 50% inhibition underwent further testing.

2.7.2 Minimum inhibitory concentration (MIC) calculation

Using 96-well plates, compounds were serially diluted by 8 or 12 halving concentrations in dH_2O in triplicate, with an initial concentration of 100 µM, 50 µM or 10 µM depending on the experiment. Ninety µL of bacterial suspension at an OD_{600} of 0.001 was added to the wells, and incubated for 1, 5, or 7 days for *Pseudomonas*, *M. avium* and BCG/*M. tuberculosis* respectively. Ten µL 0.05 % Resazurin was then added, incubated for 4 hours and fluorescence measured at 590 nm. Bacterial survival was determined as percentage fluorescence compared to non-treated controls. Further experiments were conducted at varying bacterial concentrations, where bacteria were grown to log phase, and diluted into 4 concentrations of OD_{600} 0.001, 0.01, 0.1, and 1.0. Assays were performed as described above.

To determine the synergistic effect of compounds in-combination assays adapted from Lechartier *et al*, (2012) were performed by combining serial dilutions of sub-inhibitory concentrations of test compounds and standard compounds (Rifampicin and INH). The compounds were added to a 96-well plate in a checkerboard pattern and incubated for five and seven days for *M. avium* and BCG respectively. Ten µL 0.05 % Resazurin was then added, incubated for 4 hours and fluorescence measured at 590 nm. Bacterial survival was determined as percentage fluorescence compared to non-treated controls.

2.7.3 Iron bound chelators

Iron chelators were bound in a 2:1 ratio with $FeSO_4$, serially diluted in halving concentrations, and 90 µL of bacterial suspension at OD_{600} of 0.001 was added to each appropriate well. The plate was then incubated for 7 days and 5 days for BCG and *M. avium* respectively, 0.05 % Resazurin added, and fluorescence measured after 4 hours as described in 2.6.1.

2.7.4 Non-replicating system for mycobacteria

The method used was an adaption of that described by Bryk et al (2008). DETANO, a nitric oxide adduct, was used to render the bacteria non-replicating by encouraging anaerobic conditions. To determine the optimal DETA-NO concentration, bacteria were grown to log phase, diluted to an OD_{600} of 0.1, and added to a 96-well plate. Differing concentrations of DETA-NO ranging from 0-100 µM (final concentrations) was added over a period of seven days. Bacteria from each well were serially diluted and plated onto 7H11 quad plates at days 0, 1, 4, and 7 post-treatment. Plates were incubated at 37 ^{o}C in 5 % CO_2 for 3 weeks, and counts determined and expressed as CFU/mL. The concentration which resulted in similar bacterial numbers at each timepoint was considered optimal for subsequent assays.

For assessment of inhibitors, compounds at a set concentration (see relevant chapter for concentrations) were added to 96-well plates. Bacteria were grown to log phase, diluted to an OD_{600} of 0.1, and added to the wells. To enumerate the number of bacteria, 1 in 10 serial dilutions were made and selected dilutions were plated onto Middlebrook 7H10 quadrant plates. Plates were incubated at 37 ^{o}C in a CO_2 incubator for 3 weeks, and bacterial counts determined and expressed as colony forming units per mL of culture (CFU/mL).

2.8 Cell culture

2.8.1 RAW267.4 cells (macrophage cell line)

Growing and passaging cells: Cells were taken from stocks in liquid nitrogen and grown to approximately 80 % confluence in cell culture flasks with 25-30 mL complete DMEM. To passage the cells, medium was replaced with 10 mL PBS. The flask was incubated at 37 ^{o}C in 5 % CO_2 for 5 minutes and the side of the flask was tapped repeatedly to ensure most cells had detached. The cell suspension was transferred to a 50 mL falcon tube, 20 mL complete DMEM added and centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and the cells were resuspended in 5 mL complete DMEM, and half the volume transferred to another culture flask containing 25 mL fresh complete DMEM.

Counting cells: To count the cells, the same procedure was undertaken as in passaging to recover the cells, however the cell pellet was resuspended in 10 mL complete DMEM. Ten µL of this suspension was added to an 1.5 mL eppendorf tube containing 90 µL trypan blue, and a haemocytometer was used to count the cells in the mixture.

Once counted the following equation was used to calculate the number of cells.

$$\text{Average \# cells in each quadrant} \times 10^4\,(\text{mL}) \times 10^1\,(\text{dilution factor}) = \text{\# cells/mL}$$

2.8.2 THP1 cells

THP-1 cells are a human leukemic cell line originally cultured from the blood of a boy with acute monocytic leukemia (Tsuchiya et al., 1980). Cells were taken from liquid nitrogen stores and grown to

approximately 80 % confluence in cell culture flasks. To passage the cells, the entire volume within the flask was transferred to a falcon tube and centrifuged for 5 minutes at 1200 rpm. The supernatant was poured off, and the pellet was resuspended in 5 mL complete RPMI. Half this volume was transferred to another cell culture flask containing 25 mL complete RPMI. Cell numbers were determined as in 2.8.1.

2.8.3 THP1 cells infection

THP1 cells were grown to 80 % confluence, harvested, resuspended and counted as described in section 2.8.2. Cells were then seeded at 1×10^5 cells per well of a 96 well microtitre plate in 100 µL of culture medium containing 50 ng/mL phorbol myristate acetate (PMA) and allowed to adhere for 48 hours under standard cell culture conditions (5% CO2, 37 °C). Cells were then infected for 4 hours with 10^6 BCG (± pSMT1), *M. avium* (± pSMT1), or *M. tuberculosis*, prepared in RPMI. The media was removed and cells washed 3 times with 2 % FBS in PBS. One hundred and eighty µL of complete RPMI was then added to the cells.

2.8.4 Detection of luminescence for pSMT1 strains of bacteria

After plating and infecting cells as described in section 2.8.3, various compounds were prepared in halving dilutions, with an initial starting concentration of 10 µM. The 96 well plates were then incubated for up to 7 days at 37^{o}C in 5% CO_2. Using the FLUOstar Omega microplate reader an injection of Decanal substrate was made into each well separately, and a luminescent reading was taken after 7 seconds of shaking. To establish the effect of certain compounds over time, experiments were conducted over a 7 day period and readings were taken at various points throughout. Survival was calculated as percentage luminescence compared to a non-treated control.

2.8.5 Determination of intracellular bacterial survival for *M. avium*, BCG and *M. tuberculosis*

After plating and infecting cells as described in 2.8.3, compounds were added to the wells in 10 fold dilutions in triplicate, using rifampicin-treated and cell-only suspensions as controls. The 96 well plates were incubated for up to 7 days (37 °C, 5% CO_2), cells were lysed with dH_2O, serially diluted and lysates plated onto 7H11 agar plates. Counts were obtained after 3 weeks incubation (37 °C, 5% CO_2).

2.8.6 Assay of cell viability

RAW264.7 and THP1 cells were grown to 80 % confluence, centrifuged, resuspended and counted as described in 2.8.1 and 2.8.2 respectively. Cells were then seeded at 1×10^5 cells per well of a 96 well microtitre plate in 100 μL of culture medium and allowed to adhere under standard cell culture conditions (5% CO2, 37 °C). Compounds were added to the wells in 3 or 5 fold dilutions, and cells and media only were used as controls. The plates were incubated for up to 7 days (37 °C, 5% CO2). Resazurin was added at a final concentration of 0.05% (w/v) per well and incubated for 4 h before a measurement of fluorescence intensity was determined as described in section 2.6.1. The percentage cell viability was calculated in relation to the maximum and minimum measurement of fluorescence caused by cells only (100% survival) or medium alone (100% inhibition).

2.9 In vivo assessment of compound efficacy

C57BL/6 mice, 6-8 weeks of age were purchased from the Animal Resources Centre (Perth, Australia) and maintained in specific-pathogen-free Biosafety Level 2 and 3 facilities. All experiments were approved by the University of Sydney Animal Ethics Committee.

For *M. avium* and BCG infected mice, stocks were diluted to 5×10^6 CFU and 20 μL administered intra-nasally for a final concentration of 1×10^5 CFU per mouse. For *M. tuberculosis* infected mice, a Middlebrook inhalation exposure apparatus (Glas-col) was used. Bacterial stocks were diluted to 1×10^6 CFU/mL in PBS and added to a nebuliser for an infection of 100 bacilli per mouse lung (Pinto et al., 2004, Bean et al., 1999).

At four weeks post-infection, INH (10 mg/kg or 100 mg/kg) or PCIH (10 mg/kg or 100 mg/kg) or vehicle only (50 % DMSO in PBS) were administered daily *via* the intra-peritoneal route for 21 days (*M. avium*, BCG) or 28 days (*M. tuberculosis*). Harvested lungs and spleen were homogenised in dH_2O, serially diluted and plated onto 7H11 agar plates. Bacterial counts were obtained after 3-4 weeks incubation (5% CO2, 37 °C).

2.10 Statistics

The significance of differences for linear and log-transformed assays was evaluated by one-way ANOVA with pair-wise comparison of multi-grouped data sets achieved using the Bonferroni post hoc test. Statistics were calculated using Prism software.

The MIC_{90} was determined by calculating the percentage fluorescence relative to a non-treated control. This was then graphed, and the MIC_{90} was the concentration of compound that resulted in less than 10 % fluorescence.

Chapter 3: Activity of Pyridoxal Isonicotinoyl Hydrazone iron chelators against pathogenic mycobacteria

3.1 INTRODUCTION

Treatment for tuberculosis consists of a 4-6 drug regime, including rifampicin and isoniazid, that requires a minimum six month course (W.H.O, 2012). The increase in prevalence of MDR and XDR strains has highlighted the urgent need for new therapies to treat *M. tuberculosis* infection. Furthermore, frontline drugs such as INH are less effective against non-replicating forms of mycobacteria, which may limit their efficacy against latent TB (Vilcheze and Jacobs, 2007). Strategies to improve TB drug therapy include the modification of existing drugs or the development of novel antibiotics and a number of promising candidates have been described (Skripconoka et al., 2012, Diacon et al., 2012, Gler et al., 2012). There has also been interest in the implementation of nutrient supplementation to control TB, either as a stand-alone therapy or as an adjunct treatment for use with current drug regimens (Sinclair et al., 2011, Greenstein et al., 2012).

Iron is an obligate co-factor for over forty enzymes encoded within the mycobacterial genome and is required for the function of many proteins that are involved in processes such as fatty acid biosynthesis, amino acid biosynthesis, energy generation and DNA synthesis (Yellaboina et al., 2006). The importance of iron for mycobacterial growth suggests a potential role for chelation therapy as a treatment option. This therapeutic strategy would prevent iron utilisation by bacteria and have anti-microbial effects due to the requirement of this nutrient for essential metabolic processes (Kalinowski et al., 2007). Indeed, the use of iron chelation as a potential therapy for infectious diseases has been

well documented, including the *in vitro* use of chelators to inhibit viral replication and reverse transcriptase activity in HIV treatment (Debebe et al., 2007, Traore and Meyer, 2007, Traore and Meyer, 2004). Furthermore, in malarial infection, chelation therapy increased parasite clearance rate (Pradines et al., 2002, Walcourt et al., 2004, Hershko, 1994). Use of current iron chelators against mycobacteria have shown limited effect against mycobacteria (described in section 1.5). For example, the first chelator that was clinically approved for iron overload treatment, desferrioxamine (DFO) (Bernhardt, 2007) has been assessed for efficacy against mycobacteria. Although a low-iron environment has been shown to inhibit *M. avium* growth, DFO was not efficacious in this context, possibly due to the lack of effective iron chelation efficiency (Gomes et al., 2001). DFO, as a therapy for *M. tuberculosis* infection, was able to prevent effects of excess iron on growth, however had little influence on intracellular survival (Cronje et al., 2005), most likely due to its poor membrane permeability (Yu et al., 2006, Bernhardt, 2007). In addition, DFO has short plasma half-life of approximately 20 minutes, is orally inactive and expensive to produce (Yu et al., 2006, Bernhardt, 2007), making it unsuitable to treat intracellular, chronic infections with mycobacteria.

There has been significant progress in the development of new generations of iron chelators, both for use in iron overload disease and for their anti-proliferative activity in the inhibition of tumour growth (Yu et al., 2006, Richardson et al., 2009, Yu et al., 2009). Compounds that have been developed for such purposes include tridentate ligands such as those of the pyridoxal isonicotinoyl hydrazone (PIH) class that possess very high affinity for iron and much lower affinity for other essential metals *e.g.*, Zn(II), Mg(II), Ca(II) (Richardson and Ponka, 1998a). Notably, the advantages of PIH (Figure 1A) and its analogues include: **(1)** it can be simply prepared by a 1-step synthesis; **(2)** the compounds possesses high membrane permeability; **(3)** it can be orally administered (Richardson and Ponka, 1998a); and **(4)**

it is neutral at biological pH (Richardson et al., 1990), allowing for penetration through cell membranes to reach intracellular iron stores (Richardson and Ponka, 1998b). Analogues of PIH have been designed for the treatment of iron overload disease or cancer (Richardson et al., 1995, Sookvanichsilp et al., 1991). Some of these ligands have shown marked iron chelation activity *in vitro* and *in vivo* (Baker et al., 1992, Link et al., 2003), while others have been developed into ligands that demonstrate potent anti-proliferative effects with minimal toxicity *in vivo* (Whitnall et al., 2006, Lovejoy et al., 2012, Kovacevic et al., 2011).

PIH is the result of a simple condensation reaction between pyridoxal and the potent anti-mycobacterial agent, INH, in which the latter structure is well conserved (Hermes-Lima et al., 2000, Kang et al., 2006). Considering the structural identity between PIH and INH, in addition to the ability of PIH to bind the essential nutrient iron (Richardson and Ponka, 1998a), in this chapter I examined the ability of a range of PIH analogues to inhibit the growth of pathogenic mycobacterial strains.

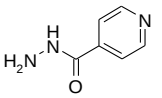
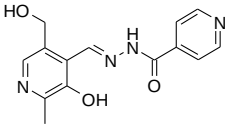
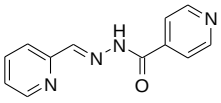
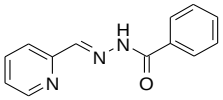
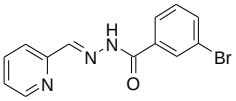
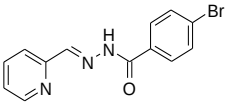
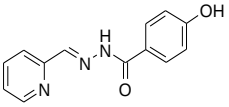
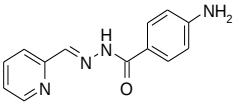
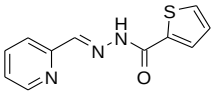
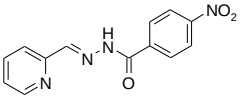
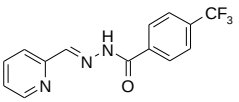
3.2 RESULTS

3.2.1 In vitro inhibition of mycobacterial growth by tridentate iron chelators

Initially, a screen was conducted on 10 ligands derived from (and including) the compound, PIH (Table 3.1), to determine if they could effectively reduce mycobacterial growth in culture. The 9 PIH analogues shown in Table 3.1 all demonstrate marked structural similarity, being members of the PCIH series of chelators (Kalinowski et al., 2008, Becker and Richardson, 1999). Each of the latter have a conserved tridentate iron-binding site (aromatic nitrogen, imine nitrogen and carbonyl oxygen) with substitutions distal to the coordination sphere (Kalinowski et al., 2008, Becker and Richardson, 1999). This group of analogues constitute a series of compounds that all are effective ligands with varying lipophilicity, as reflected by their calculated partition coefficient (log P_{calc}; Table 3.1). Examination of this series enables an assessment of the structure-activity relationships in terms of the role of lipophilicity and chelation efficacy in the anti-bacterial efficacy observed. INH was included as a positive control due to its potent anti-mycobacterial activity (Vilcheze and Jacobs, 2007) and similarity to the isonicotinoyl hydrazone moiety of PIH (Richardson and Ponka, 1998b).

BCG was exposed to a single chelator dose of 10 μM for 7 days and survival was determined in comparison to vehicle-treated bacterial cells (control). Significantly, INH, PIH and PCIH were able to markedly and significantly (p<0.01) restrict the growth of BCG at 10 μM, with the two chelators leading to almost complete (PIH) and complete growth inhibition (PCIH) (Table 3.1). Calculation of the MIC_{90} for PCIH (0.39 μM) and INH (0.39 μM) indicated a similar level of inhibitory activity of the two compounds (Figure 3.1A). The analogue PC4BBH showed inhibitory activity that was comparable to PIH, but less than INH or PCIH. Despite their very similar structures, the other 7 compounds

Table 3.1. Structure and activity of PIH derivatives against mycobacteria.

Compound	Structure	Log P_{calc}	BCG Survival at 10 μM (% of control)[a]
INH		-0.59	0.39 ± 0.13*
PIH		0.30	15.44± 0.04*
PCIH		0.96	0.02 ± 0.11*
PCBH		2.21	95.44 ± 2.36
PC3BBH		3.05	40.98 ± 3.07*
PC4BBH		3.05	13.04 ± 8.04*
PCHH		1.86	89.67 ± 3.19
PCAH		1.41	99.43 ± 3.09
PCTH		2.49	39.30 ± 10.65*
PCNH		1.64	87.15 ± 9.92
PCTFH		3.14	39.68 ± 11.20*

[a] Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between non-treated and treated bacteria were determined by ANOVA (*p<0.01)

screened showed limited efficacy, with PCBH, PCHH, PCAH, and PCNH causing less than a 20% reduction in BCG growth at 10 μM in comparison to the control (Table 3.1). Remarkably, the slight structural change between PCIH and PCBH due to the alteration of the isonicotinoyl nitrogen to a C-H led to an almost total loss of anti-mycobacterial activity. This observation indicated the critical importance of this structural feature for efficacy. This dramatic alteration was unanticipated, as the replacement of the N with the phenyl C-H increased lipophilicity (log P_{calc} increases from 0.96 to 2.21; Table 3.1), which could potentially enhance membrane permeability relative to PCIH. Indeed, previous studies using a human cell line have demonstrated the increased permeability and iron chelation efficacy of PCBH over PCIH (Becker and Richardson, 1999).

Increasing the lipophilicity of PCBH by the synthesis of PCTH, PC3BBH, PC4BBH, and PCTFH (log P_{calc}: 2.49, 3.05, 3.05 and 3.14, respectively) significantly (p<0.01) improved the anti-mycobacterial efficacy relative to PCBH. However, despite the greater lipophilicity of these analogues than PCBH and especially PCIH, the anti-mycobacterial activity was considerably less than PCIH (Table 3.1). Moreover, there was little correlation between the log P_{calc} of these analogues and efficacy at inhibiting bacterial growth (r^2 = 0.46; data not shown), indicating that lipophilicity did not appear to be the primary criterion for efficacy.

Considering the high activity of PCIH against BCG, this agent was also tested against *M. avium* to determine if it displays broad anti-mycobacterial activity (Fig. 3.1B). Interestingly, PCIH also effectively restricted the growth of *M. avium* in culture, being significantly (p<0.01) more effective than INH at concentrations less than 1 μM (namely, 0.31 and 0.63 μM), although the MIC_{90} of both compounds was similar (PCIH: 1.25 μM; INH: 2.5 μM; Figure 3.1B). Therefore, PCIH showed potent inhibitory activity against both BCG and *M. avium.*

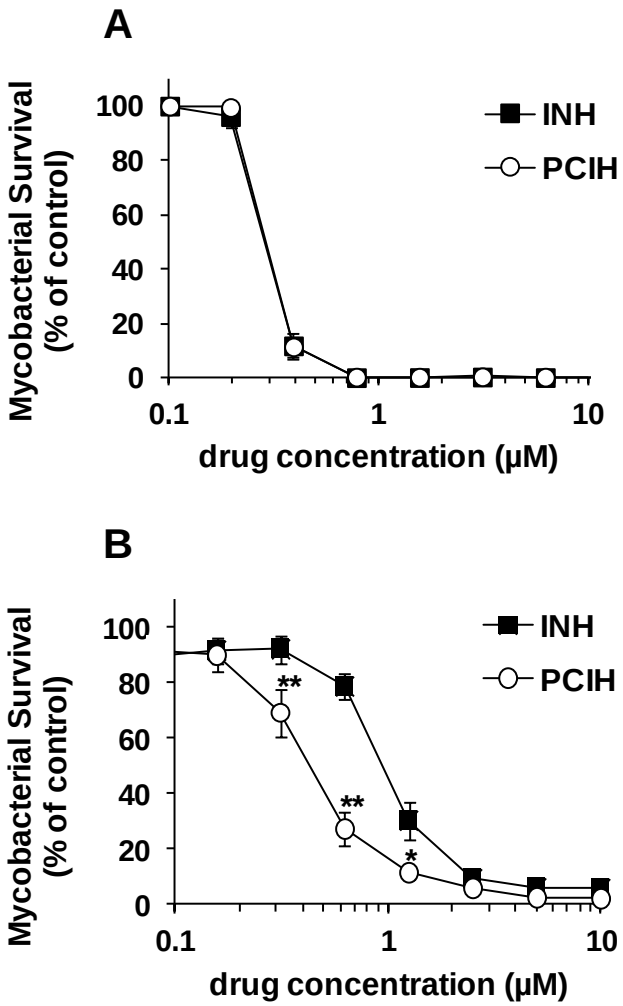


Figure 3.1. Concentration-dependent inhibition of mycobacterial growth by PCIH. BCG **(A)** and *M. avium* **(B)** (OD_{600nm} 0.001) were incubated with varying doses of Isoniazid (INH) (black squares) or pyridoxylcarboxaldehyde isonicotinoyl hydrazone (PCIH) (white circles) and after a 7 and 5 day incubation, respectively, resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by ANOVA (*p<0.01; **p<0.001).

These findings were substantiated through the testing of the individual components. Against both BCG (Figure 3.2A) and *M. avium* (Figure 3.2A) the IH molecule that forms the core structure for INH and PCIH showed similar levels of activity to INH. The substitution of N for a C atom in Benzoyl hydrazide (BH) had a substantial effect with a complete loss of efficacy against both mycobacterial strains. This could explain why PCBH and its more lipophilic derivatives were not as efficacious as PCIH.

3.2.2 Activity of PCIH against varying bacterial load

We next determined if PCIH was able to inhibit growth at varying bacterial concentrations, as this is a factor that may influence drug efficacy in a clinical setting. PCIH showed no significant difference in effectiveness against BCG relative to INH at the lower starting OD_{600nm} of 0.001, 0.01, 0.1; Figure 3.3A-C). However, notably, at an OD_{600nm} of 1.0, PCIH was able to significantly ($p<0.001$) inhibit BCG growth compared to INH at 10 µM and 1 µM (Figure 3.3D). Testing the effect of PCIH against varying bacterial loads of *M. avium* (Figure 3.3E-H) resulted in a significantly ($p<0.01$) greater reduction in bacterial survival compared to INH, which was most apparent at lower bacterial loads (Figure 3.3E-G) and higher concentrations of the agents when bacterial load was increased (Figure 3.3). However, none of the compounds showed growth inhibition at an OD_{600nm} of 1.0, indicating a higher intrinsic resistance to both PCIH and INH by *M. avium* (Figure 3.3H). Taken together, these results indicate that PCIH can restrict the growth of both BCG and *M. avium* over the full concentration ranges, an important property not shared by INH.

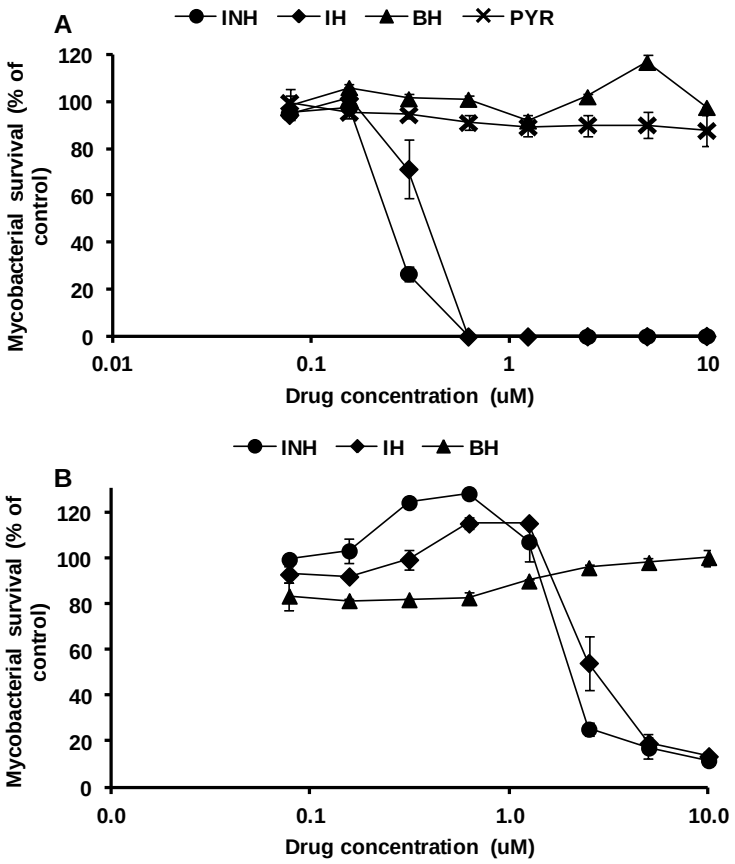


Figure 3.2. Establishing drug activity through testing of components against mycobacteria. BCG (a) and *M. avium* (b) were exposed to varying doses of compound and after seven and five day incubation respectively, 0.05% resazurin added and fluorescence measured. Survival curve was calculated as percentage survival compared to non-treated bacteria. Data is represented as mean survival ± SEM of triplicate cultures.

nb. Isonicotinoyl hydrazide (IH), Benzoyl hydrazide (BH) , Pyridoxyl (PYR)

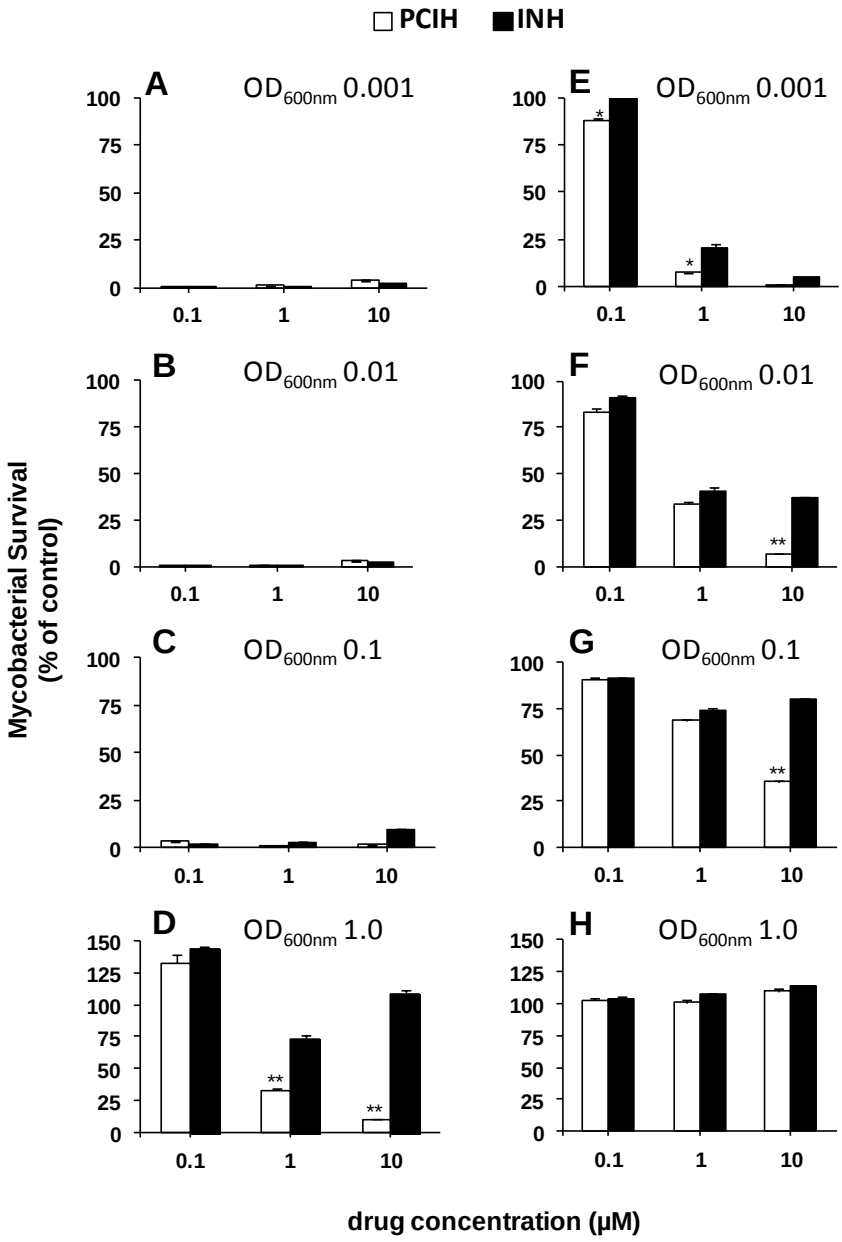


Figure 3.3. Concentration-dependent inhibition of mycobacterial growth by PCIH. BCG **(A-D)** and *M. avium* **(E-H)** (OD_{600nm} 0.001, 0.01, 0.1 or 1.0) were incubated with varying doses of INH (black squares) or PCIH (white circles) and after a 7 and 5 day incubation, respectively, resazurin (0.05%) added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria were determined by ANOVA (*p<0.01; **p<0.001).

3.2.3 Iron-dependent activity of PCIH

Despite PCIH showing effectiveness at restricting mycobacterial growth, it was unknown whether PCIH was functioning primarily by iron chelation or by direct antimycobacterial activity due to its isonicotinoyl hydrazone substructure (Table 3.1). Notably, PCIH has been demonstrated to be an effective tridentate iron chelator (Bernhardt et al., 2001), showing marked activity at mobilizing iron from mammalian cells (Becker and Richardson, 1999, Richardson et al., 2001). To test the role of iron chelation in its activity, PCIH was pre-complexed in a 2:1 ratio with iron to saturate its iron-binding site and thus prevent further iron chelation from cells. BCG was then treated with either the free ligand or the iron complex, and the MIC_{90} was calculated. The iron complex of PCIH resulted in a 2-fold change of the MIC_{90} in these experiments (0.31 to 0.63 μM) relative to PCIH alone (data not shown), suggesting that the ability of PCIH to complex iron had some effect on its capacity to restrict BCG growth. When screened against *M. avium*, formation of the PCIH–iron complex affected its antimycobacterial activity, resulting in an ~8-fold increase in MIC_{90} (1.25 to >10 μM; data not shown). This observation indicates that iron chelation may play a limited role in the activity of PCIH against mycobacteria. Previous studies have demonstrated that PCIH and its iron complex have similar partition coefficients (i.e., log *P*: 1.98 and 1.89, respectively) (Bernhardt et al., 2007), and this suggests that they possess comparable ability to permeate the cell membrane. Thus, it is unlikely that the small change in the partition coefficient of PCIH relative to its iron complex would contribute to the increase in the MIC_{90} observed upon complexation. This finding supports the suggestion that iron chelation, rather than changes in membrane permeability may play some role in the antimycobacterial activity of PCIH.

3.2.4 Restriction of bacterial growth by PCIH within host cells

Considering the efficacy of PCIH in mycobacterial culture, we determined if PCIH could restrict mycobacterial growth within host THP-1 cells. Both INH and PCIH resulted in a similar trend of dose-dependent inhibition of BCG growth by day 7 post-infection of THP-1 cells, as determined by examination of luminescence emitted by luciferase-expressing BCG within THP-1 cells (Figure 3.4A). PCIH was more effective than INH at inhibiting BCG growth at 0.3, 1, and 10 μM, although this did not reach statistical significance (Figure 3.4A). Both INH and PCIH displayed a similar level of effectiveness when tested at 3 and 5 days postinfection (data not shown).

When tested against *M. avium*–infected THP-1 cells, PCIH exhibited a trend of antimycobacterial activity similar to that of INH at high drug concentrations (Figure 3.4B). However, PCIH was significantly ($P < 0.05$) more effective than INH at a concentration of 0.3 μM (Figure 3.4B). The inhibitory effect of PCIH was independent of any toxic effect on THP-1 cells, as the compound had no significant effect on THP-1 viability, irrespective of the inhibitor concentration tested (Figure 3.4C). This result was in good correlation with the low antiproliferative activity of PCIH observed in previous studies using human cells in vitro (Becker and Richardson, 1999).

3.2.5 Effect of other iron chelators on mycobacterial growth

Due to the effectiveness of PCIH *in vitro*, other classes of chelator were studied to determine if they too had a similar efficacy. Methyl pyrazinylketone isonicotinoyl hydrazone (MPIH) and its derivatives, as described in Kalinowski *et al*, (2008) were tested. These compounds are more lipophilic and form iron

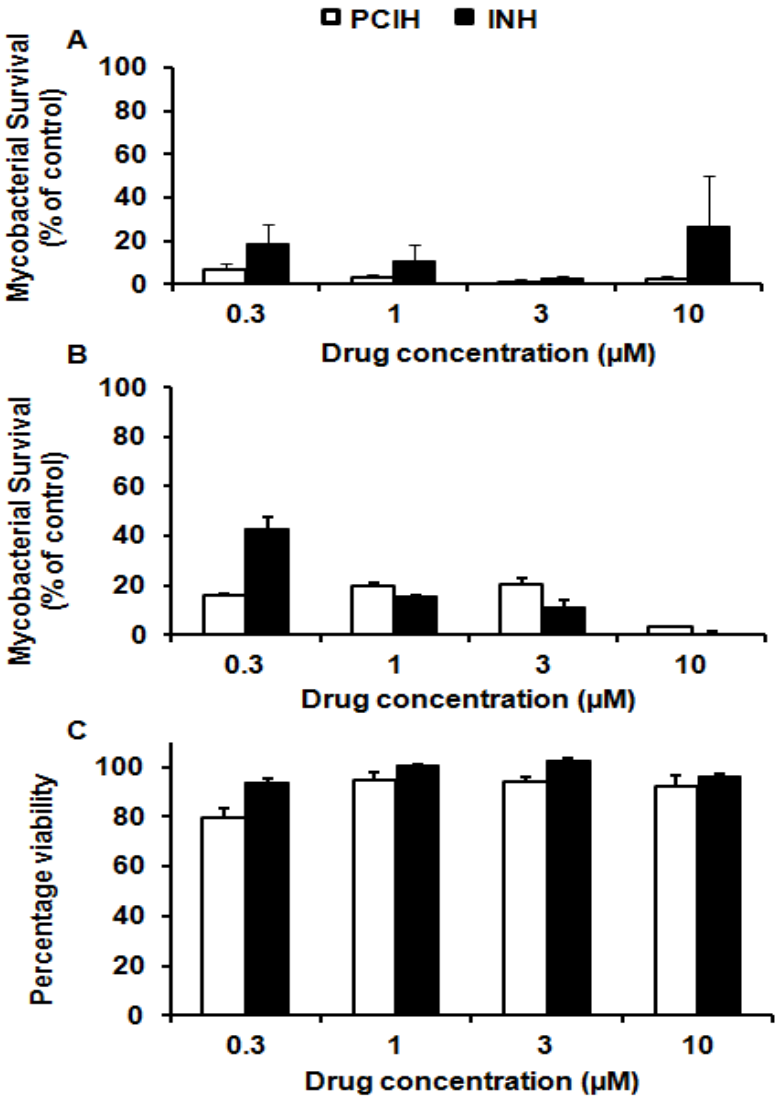


Figure 3.4. Effect of PCIH on intracellular bacteria and host cell viability. THP-1 cells ($5x10^5$) cells infected with BCG pSMT1 **(A)** or *M. avium* pSMT1 **(B)** at a MOI of 10:1 or un-infected control THP-1 cells **(C)** were treated with INH or PCIH (0.3-10 μM). Luminescence was measured 7 days post-infection and bacterial survival was determined relative to non-treated cells. Viability of THP-1 cells was determined relative to untreated cells by assessment of resazurin reduction. Black bars represent INH-treated bacteria while white bars represent PCIH-treated bacteria. Data are mean survival ± SEM of triplicate cultures and represent two independent experiments. Differences between INH- and PCIH-treated bacteria/cells were determined by ANOVA (*p<0.05).

complexes more readily than PCIH (Kalinowski et al., 2008). The most effective compound was MPIH which exhibited an MIC_{90} of 1.3 µM (Figure 3.5), with the remaining compounds displaying an MIC_{90} of 2.5 µM or greater. Figure 3.6 shows a study conducted using other types of aroylhydrazones, 311 and SIH, using N4mT as a non-chelator control (Richardson et al., 1995). Similarly to the MPIH series, none of the compounds exhibited a similar level of efficacy to PCIH, however SIH did have an MIC_{90} of 2.5 µM against BCG (Figure 3.6A). When tested against *M. avium* no aroylhydrazones tested exhibited bacterial growth inhibition (Figure 3.6B). Due to this fact, none of these compounds were tested further.

3.2.6 PCIH effectively restricts mycobacterial growth *in vivo*

Having determined that PCIH effectively restricted bacterial growth *in vitro*, we determined if the compound also displayed activity *in vivo*. Mice were infected with *M. avium* and treated daily with 100 mg/kg PCIH or INH for 21 days. Although PCIH did appear to decrease the bacterial load in the lung, neither INH or PCIH resulted in a significant decrease in *M. avium* load compared to the vehicle-only treated animals (Figure 3.7). However, in the spleen, PCIH showed an approximate 1.5-log reduction in *M. avium* CFU compared to untreated mice ($p<0.05$) (Figure 3.7). This was similar to the reduction observed with INH treatment. Therefore, PCIH retained its activity in an *in vivo* model of mycobacterial infection.

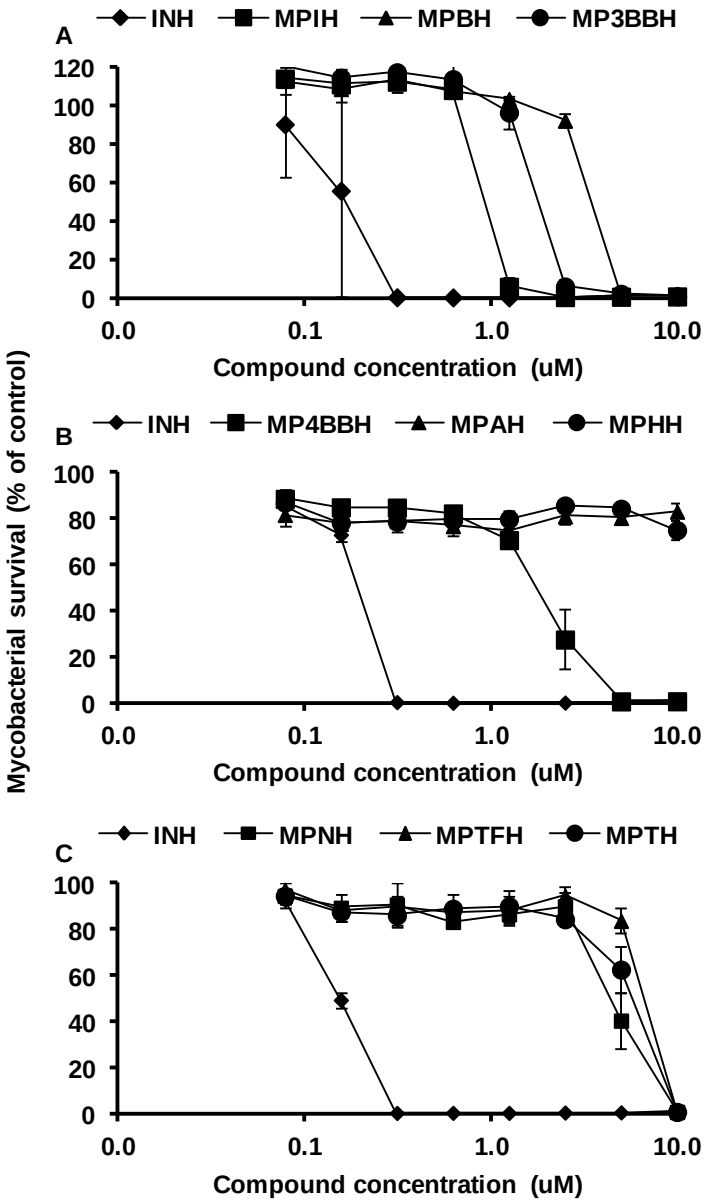


Figure 3.5. Inhibition of mycobacterial growth by modified class of chelators. BCG was exposed to varying doses of compound and after seven and five day incubation respectively, 0.05% resazurin added and fluorescence measured. Survival curve was calculated as percentage survival compared to non-treated bacteria..Data is represented as mean survival ± SEM of triplicate cultures.

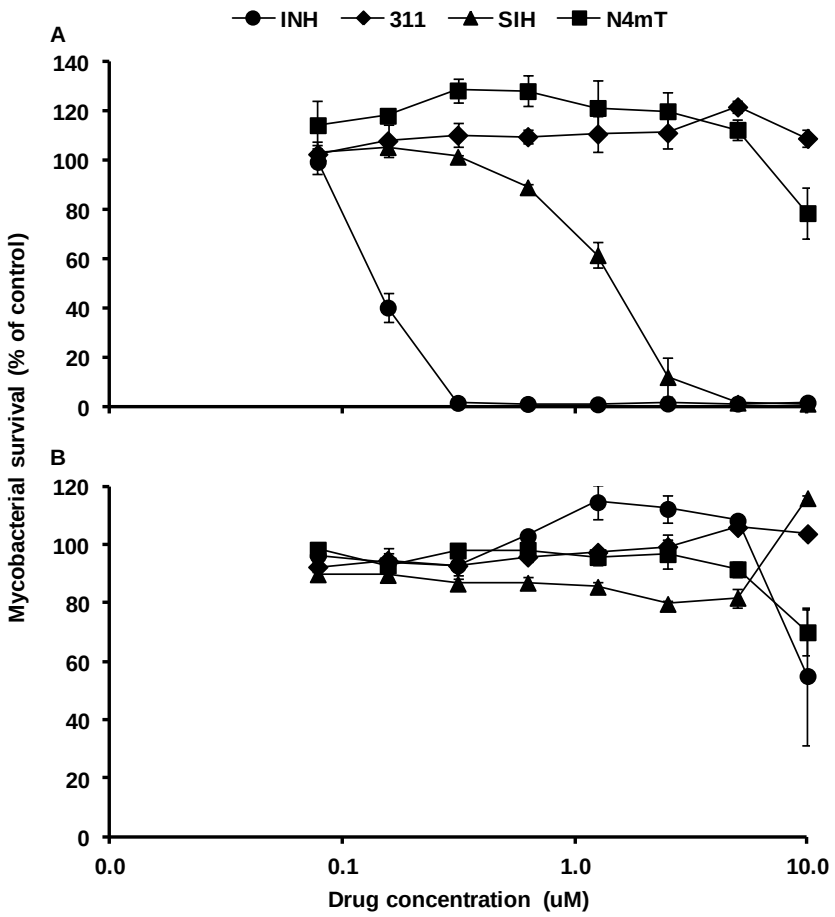


Figure 3.6. Bacterial growth inhibition by high iron affinity chelators. BCG (a) and *M. avium* (b) were exposed to varying doses of compound and after seven and five days of incubation respectively, 0.05% resazurin added and fluorescence measured. The survival curve was calculated as percentage survival compared to non-treated bacteria. Data is represented as mean survival ± SEM of duplicate samples, and is representative of two independent experiments.

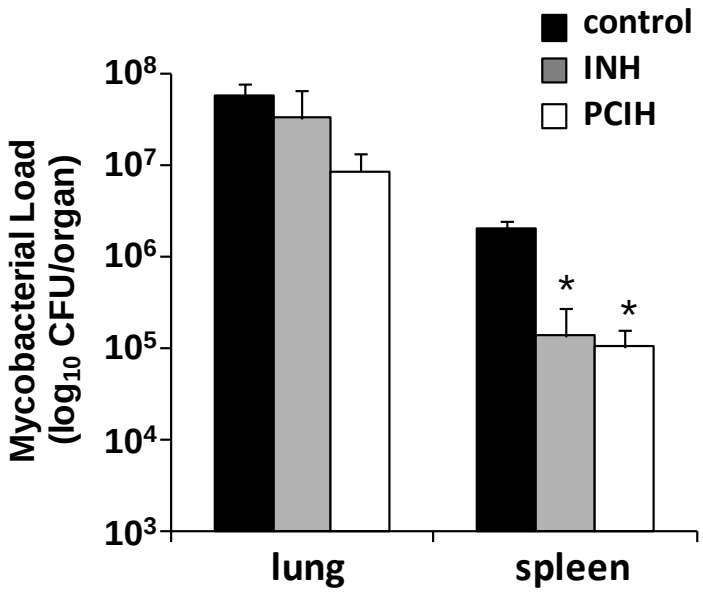


Figure 3.7. ***In vivo*** **efficacy against mycobacterial infection.** C57BL/6 mice (*n*=5) were infected intra-nasally with $1x10^5$ CFU *M. avium.* After 28 days of infection, mice were treated daily for a further 21 days with 100 mg/kg of INH or PCIH. The lung and spleen were harvested and bacterial load determined. Vehicle-treated, black bars; INH-treated, grey bars; PCIH-treated, white bars. Data are mean bacterial survival ± SEM and represent two independent experiments. Differences between INH- and PCIH-infected mice compared to untreated animals were determined by ANOVA (*p<0.05).

3.2.7 PCIH displays anti-tuberculosis activity

As PCIH was able to restrict the growth of both BCG and *M. avium*, we determined if it also displayed activity against *M. tuberculosis.* Similar to that seen in BCG, PCIH showed the same trend of growth inhibition compared to INH when tested against mycobacteria grown in culture medium (Figure 3.8A) with an MIC_{90} of 310 nM and 630 nM for INH and PCIH, respectively. PCIH was able to significantly ($p<0.05$) reduce the number of bacteria recovered from *M. tuberculosis*-infected THP-1 cells compared to untreated cells by more than 3-log units (Figure 3.8B). When PCIH was assessed for potency in *M. tuberculosis*-infected mice, a reduction of CFU was observed in the lung and a significant ($p<0.05$) decrease was found in the spleen compared to control mice (Figure 3.8C-D). Further, treatment with PCIH caused a similar level of reduction of bacterial load as that found for INH (Figure 3.8C-D). Collectively, these data indicate that PCIH displays broad activity against mycobacterial pathogens.

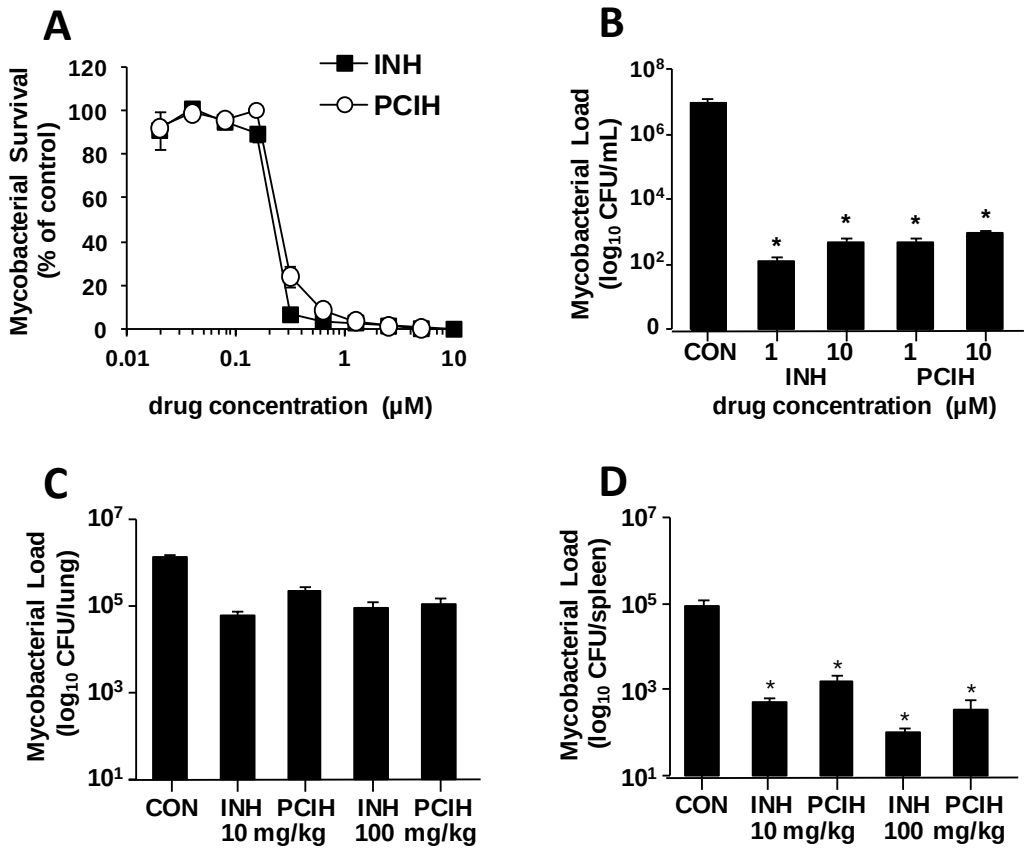


Figure 3.8. Efficacy of PCIH against *M. tuberculosis.* (A) *M. tuberculosis* was incubated with varying doses of compound and after 7 days, resazurin (0.05%) was added and fluorescence measured. Graphs represent percentage survival of bacteria compared to non-treated cells. **(B)** Compounds (10 μM or 1 μM) or vehicle alone control (CON) were added to 1 x 10^5 THP-1 cells previously infected with *M. tuberculosis* at an MOI of 1:1. Cells were lysed 7 days post-infection and bacterial counts obtained. **(C-D)** C57BL/6 mice were aerosol infected with *M. tuberculosis* and 28 days post infection mice were treated daily for 28 days with 100 mg/kg or 10 mg/kg of INH or PCIH, or treated with vehicle control (CON). The lung **(C)** and spleen **(D)** were harvested and bacterial load determined. Data are mean bacterial load ± SEM and represent two independent experiments. Differences between INH- and PCIH-infected groups compared to untreated controls were determined by ANOVA (*p<0.05).

3.3 DISCUSSION

Despite the marked increase in drug resistance, no new drug has been added to the standard treatment regimen for tuberculosis for over 40 years (Ma et al., 2010). This has created an urgent need for effective new agents to be developed. Due to the stringent dependence of mycobacteria on iron (Yellaboina et al., 2006), this study assessed the ability of iron chelating agents to inhibit mycobaterial growth. Of the 9 PIH analogues tested in this study (Table 3.1), PCIH proved to be the most effective ligand at inhibiting mycobacterial growth in both *in vitro* and *in vivo* models. Hence, despite previous studies showing a limited effect of other types of iron chelators in controlling mycobacterial growth (Cronje et al., 2005, Gomes et al., 2001), this study has demonstrated that several PIH analogues are highly potent against mycobacteria.

It has been shown that *M. avium* is highly dependent on iron, with iron supplementation resulting in a 12-fold increase in bacterial load *in vivo* (Dhople et al., 1996). This observation could indicate that a relatively small reduction in iron levels may have a greater effect on the growth of *M. avium* compared to BCG, and indeed, such a result was observed in this study. PCIH is known to effectively chelate iron levels within cells (Richardson et al., 2001, Becker and Richardson, 1999), while its precursor INH is not an effective ligand, lacking the tridentate binding sites necessary for marked iron chelation (Ponka et al., 1979). Hence, considering the important role of Fe in a variety of essential metabolic processes, including DNA synthesis and energy generation (Anderson et al., 2012), depletion of iron levels by PCIH would have a profound impact on mycobacterial growth.

Notably, PCIH has greater membrane permeability when compared to the “gold standard” chelator in clinical use, desferrioxamine (DFO) (Becker and Richardson, 1999, Richardson et al., 2001). This is

due to several factors, including that PCIH is: **(1)** more lipophilic than DFO: **(2)** it is predominantly neutral at physiological pH; **(3)** it is less than half the molecular weight of DFO; and **(4)** it mainly forms neutral iron complexes that can diffuse readily from cells (Becker and Richardson, 1999, Bernhardt et al., 2001, Richardson et al., 2001). In fact, these characteristics of PCIH could contribute to its improved microbiocidal efficacy compared to other iron chelators tested against mycobacteria, such as DFO and silybin (Cronje et al., 2005). High membrane permeability would permit greater access to intracellular bacteria that reside within phagolysosomes (Ehrt and Schnappinger, 2009), thereby reducing the effective dose required for bacterial inhibition within host cells. For example, previous studies have demonstrated that while DFO could reduce the overall concentration of iron available to bacteria grown in culture, it was unable to reduce intracellular bacterial growth, possibly due to the limited ability of DFO to permeate cells (Cronje et al., 2005). This is a problem overcome by PCIH, which is known to rapidly permeate cells and organelles to bind iron (Becker and Richardson, 1999, Richardson et al., 2001), and thus, may be a more viable option as a therapy to treat mycobacterial infection. The tridentate nature of PCIH (Richardson et al., 1999) may also be a key determinant of its anti-mycobacterial activity, as bidentate iron chelators appear to have limited efficacy against mycobacteria.

The enhanced intracellular activity of PCIH may also be due to its greater lipophilicity compared to the parent molecule, PIH, due to the absence of the alcohol side chains present in the former ligand (Becker and Richardson, 1999). This further suggests that PCIH may be more effective because it possesses an appropriate lipophilicity that can more readily enter both host cells and mycobacteria to impart its anti-mycobacterial action. However, an argument relying on merely the greater lipophilicity of PCIH as the sole determinant of its efficacy does not explain the total lack of activity that was

observed with PCBH. Notably, PCBH only differs very slightly in structure to PCIH and due to the slightly greater lipophilicity of PCBH relative to PCIH (Table 3.1), it demonstrates greater activity at mobilizing cellular iron from human tumor cells *in vitro* (Becker and Richardson, 1999). Increasing the lipophilicity of PCBH by preparing the analogues, PC3BBH, PC4BBH, PCTH and PCTFH (Table 3.1), improved the efficacy of these agents over PCBH, but did not lead to an agent as effective as PCIH (Table 3.1). This observation indicated that other factors, in addition to appropriate lipophilicity, played a role in the activity observed.

In the current study, the greatest anti-mycobacterial activity was observed with the chelators, PIH and PCIH, both of which have the INH moiety conserved within the ligand. Taking into account the pronounced loss of activity observed with PCBH (Table 3.1), it is notable that relative to INH, benzoic acid hydrazide (which is the analogous hydrazide used to synthesize PCBH) displays little anti-mycobacterial activity (Quemard et al., 1991). This was validated by testing the components of PIH analogs which showed that benzoic acid hydrazide was unable to restrict mycobacterial growth (Figure 3.2). Thus, hydrolysis of PCBH intracellularly to its components, namely 2-pyridylcarboxaldehyde (2-PC) and benzoic acid hydrazide would not be expected to lead to an effective anti-tuberculosis agent. Considering this, it can be speculated that the efficacy of PCIH relative to INH could be related to the ligand acting not only as an iron-depleting agent that blocks growth, but also a lipophilic vehicle for INH transport into cells and the mycobacterium. In this case, hydrolysis of PCIH within the cell or the mycobacterium may lead to the generation of its components 2-PC and the potent INH moiety (Figure 3.9). The later agent would lead to bacteriocidal activity *via* its well described action on mycobacterial wall synthesis (Figure 3.9). Hence, by this mechanism, PCIH acts as a lipophilic vehicle which facilitates INH entrance into the cell and bacterium, after which INH is liberated. Subsequently,

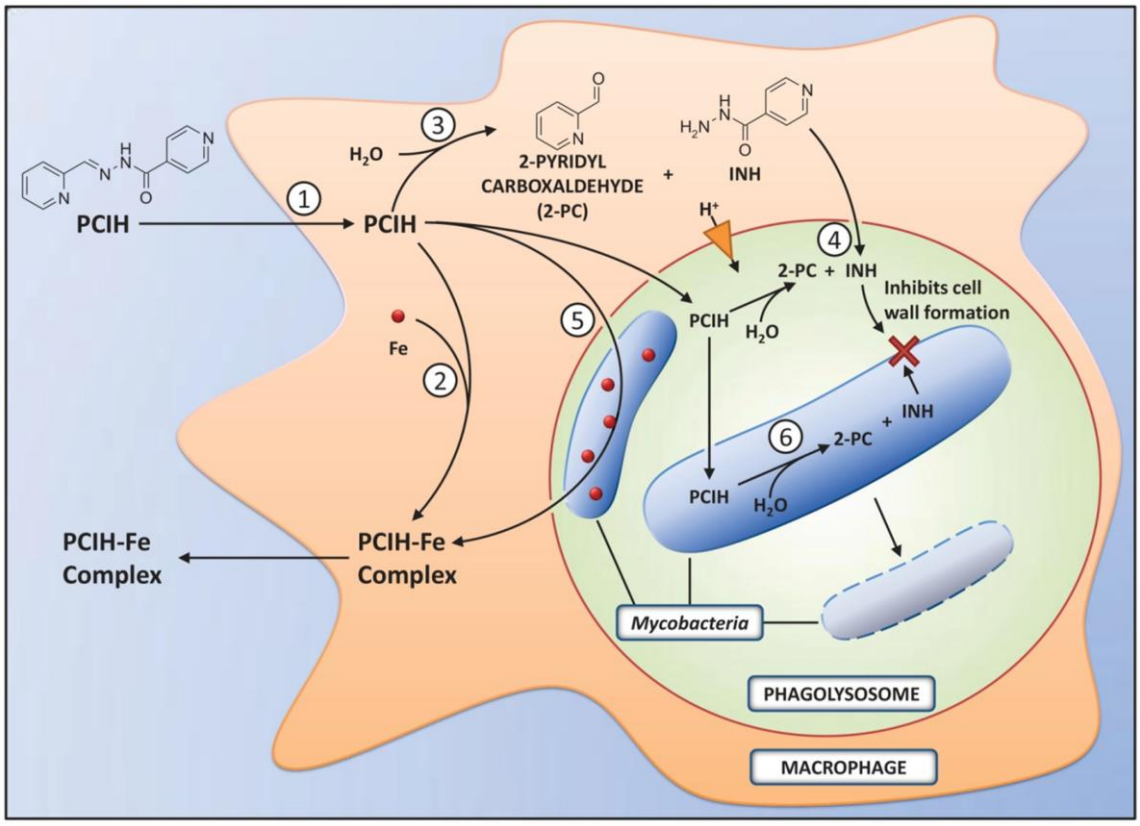


Figure 3.9. Schematic of the possible mechanisms involved in the anti-mycobacterial activity of PCIH. (1) PCIH can enter the cell readily due to it being relatively lipophilic; **(2)** PCIH can bind macrophage iron pools depleting them of iron, and in turn, depriving mycobacteria of iron which is essential for growth and replication; **(3)** PCIH may be hydrolyzed in the macrophage liberating the starting materials, 2-pyridylcarboxaldehyde (2-PC) and isonicotinic acid hydrazide (INH); **(4)** INH can then diffuse into the phagolysosome to inhibit the mycobacterium synthetic machinery needed for cell wall biosynthesis; **(5)** PCIH may enter the phagolysosome to deplete iron directly from the mycobacterium, leading to its iron deprivation; and **(6)** PCIH may enter the phagolysosome and/or mycobacterium itself, and then be hydrolyzed to 2-PC and INH and subsequently the INH then inhibits mycobacterial cell wall synthesis. Notably, the phagolysosome is acidic which could favor hydrolysis of PCIH. All, or some of these mechanisms may be occurring simultaneously, to effectively prevent mycobacterial growth (see text for further details).

through well described classical mechanisms, in the presence of the product of the *katG* gene (Kang et al., 2006), INH is activated leading to the inhibition of mycolic acid biosynthesis (Vilcheze and Jacobs, 2007). Hydrolysis of PIH and its analogues *via* an acid-catalysed reaction is well described (Richardson et al., 1989). Furthermore, it is notable that conditions in the phagolysosome where mycobacteria reside are acidic (Geisow et al., 1981) and could facilitate this process (Figure 3.9).

Examination of the intracellular localisation of effective hexadentate iron chelators (rhodamine B-conjugated, tripodal 3-hydroxy-4-pyridinone molecules) showed that differential distribution of compounds may account for differences in the activity (Nunes et al., 2010). These chelators displayed moderate intracellular anti-mycobacterial activity and this may be due to their inability to enter compartments within host cells that contain bacteria (Fernandes et al., 2010). Similar factors could also be responsible, in part, for the varying activity of the analogues described in the current study. Indeed, the fact that lipophilicity was at least one criterion for activity, particularly considering PCBH and its more lipophilic derivatives PC3BBH, PC4BBH, PCTH and PCTFH, indicates that cellular compartmentalisation may be important to consider.

Administration of PCIH to mice resulted in a significant reduction in *M. tuberculosis* growth compared to that of untreated controls both in host cells and within mouse spleens (Figure 3.8). While the reduction in lung bacterial growth was not significant in this series of experiments for either INH or PCIH, there was an approximate a 1-log reduction in CFU numbers compared to control (Figure 3.8). A similar result was seen with *M. avium*, with INH and PCIH displaying the greatest activity in bacterial clearance in the spleen (Figure 3.7). This may be due to the relatively short treatment time used in our model (21 days) and the difficulty in treating established *M. tuberculosis* infection in the lungs of infected mice. Notably, the changes in bacterial growth observed are similar to that found with

other candidate drugs tested against these bacteria (Kanyok et al., 1994). However, other studies have shown a more significant lung CFU reduction by INH when administered at similar doses to that utilized in the current study (Almeida et al., 2009, Nikonenko et al., 2004). One possible explanation may be the extended infection time before treatment used in our study (28 days) compared to that of previous studies (14 days) (Fernandes et al., 2010). It is possible that after only 14 days of infection bacteria are in a more 'active' state, and thus more susceptible to the action of INH (Vilcheze and Jacobs, 2007).

In summary, this chapter demonstrated that PCIH displays potent activity against a range of mycobacterial strains, in both *in vitro* and *in vivo* models. The pronounced efficacy of PCIH is mediated by a novel mechanism involving iron chelation. Moreover, structure-activity relationship studies suggest PCIH acts as a lipophilic vehicle for the transport of the intact INH moiety into cells and the mycobacterium. These data indicate PCIH should be further evaluated for its potential use to control mycobacterial infection in humans.

Chapter 4: Novel Metal-Cyclam complexes as a treatment option for mycobacterial infection

4.1 INTRODUCTION

Drug resistance is an increasing burden for mankind globally. Tuberculosis in particular has shown a gradual rise in drug resistance resulting in 3.7 per cent of new cases each year being MDR; of these approximately 3 % are classified as XDR (W.H.O, 2012). Despite this, the only drug that has been approved for tuberculosis treatment within the last 40 years is Bedaquiline, and even this drug has serious safety concerns (Goldenberg, 2013). Therefore there is an urgent need to discover new drugs that improve treatment of drug-resistant *M. tuberculosis*.

Identifying novel drug scaffolds is a major strategy to develop new antibacterial agents. Poor outcomes relating to library screens have been linked to the lack of chemical diversity within these collections (Koul et al., 2011). This lack of diversity is in some cases a result of the inclusion of compounds that follow the "rule-of-five" (Lipinski, 2004, Koul et al., 2011). This rule suggests that the most appropriate antibiotics follow a commonality in structure leading to properties important for a useful antibiotic to be created; generally small and lipophilic molecules (Lipinski, 2004). However, an issue with this rule is it does not predict if a drug is pharmacologically active, and the bias towards this structural formulation restricts the potential scope of chemical structures that are tested for drug discovery. Further, most antibiotics do not conform to Lipinski's rule-of-five (Payne et al., 2007) suggesting a broader approach must be taken to identify novel antibacterials.

Metal cyclam complexes (MCyCs) are in a new chemical class that has been created on the basis that it can potentially function as a target activated compound (Yu et al., 2013b). The premise is that when the appended ligand and its biological target meet, it exposes the metal binding site resulting in metal-catalysed antibiotic activity. Some of these molecules have also shown applications as fluorescent sensors to visualise protein binding interactions (Yu et al., 2013b). Interestingly, MCyCs have been developed that are hypothesised to target matrix metalloproteinases (MMPs), zinc-dependant proteases that are involved in the regulation of proteolytic activity (Parks and Shapiro, 2001, Yu et al., 2013a). MMPs have been shown to be upregulated during TB infection and are involved in destruction of the lung matrix (Elkington et al., 2011a). It is this destruction that contributes to the characteristic lung lesions seen during infection, as well as the resulting morbidity and mortality (Elkington et al., 2011b, Hrabec et al., 2002).

In this study a panel of MCyCs were tested for their efficacy against various bacterial strains as well as their toxicity against host cells. Based on these results, the most effective were studied further to determine if they were appropriate for potential use as antibiotics in humans, particularly for the treatment of tuberculosis. It was hypothesised that these novel structures would restrict bacterial, and in particular mycobacterial growth, without impacting host cell viability.

4.2 RESULTS

4.2.1 Screening of Metal-Cylcam Complexes to determine effectiveness against pathogenic bacteria

To explore the biomedical potential of MCyCs, the activity of 63 compounds against medically-relevant bacteria was examined. The structures of all compounds are shown in Appendix 1. To do this, compounds were tested against three major pathogens; *Pseudomonas aeruginosa* (gram-negative); methicillin-resistant *Staphylococcus aureus* (MRSA, gram-positive) and mycobacteria. Bacteria were treated with a single 50 μM dose of each compound and left for their respective incubation times (see Chapter 2). A compound was considered a 'hit' if the same compound on two replicate plates caused more than 50 per cent bacterial growth inhibition. On comparison of both replicate plates, MRSA had 7 hits (Fig. 1A), *P. aeruginosa* CJ2009 had 1 (Figure 4.1B) and *P. aeruginosa* Bb2009 had 6 hits (Figure 4.1C). Against *M. avium* 12 hits appeared in the screen (Figure 4.2A) and 6 against BCG (Figure 4.2B). Despite multiple compounds in each of the screens appearing as potential candidates for further study, the majority did not have relevant hits on their replicate plate.

4.2.2 Determining cytotoxicity of Metal-Cylcam Complexes

Before selecting compounds for further testing, the toxicity of compounds against host cells was determined. Differentiated THP1 macrophage-like cells (Tsuchiya et al., 1980) were treated with 3 doses of compound (50 μM, 5 μM and 0.5 μM) and cell viability measured over 7 days. Only 2 compounds had an effect on cell viability, compounds **C55** and **C59** at 50 μM, however for all other

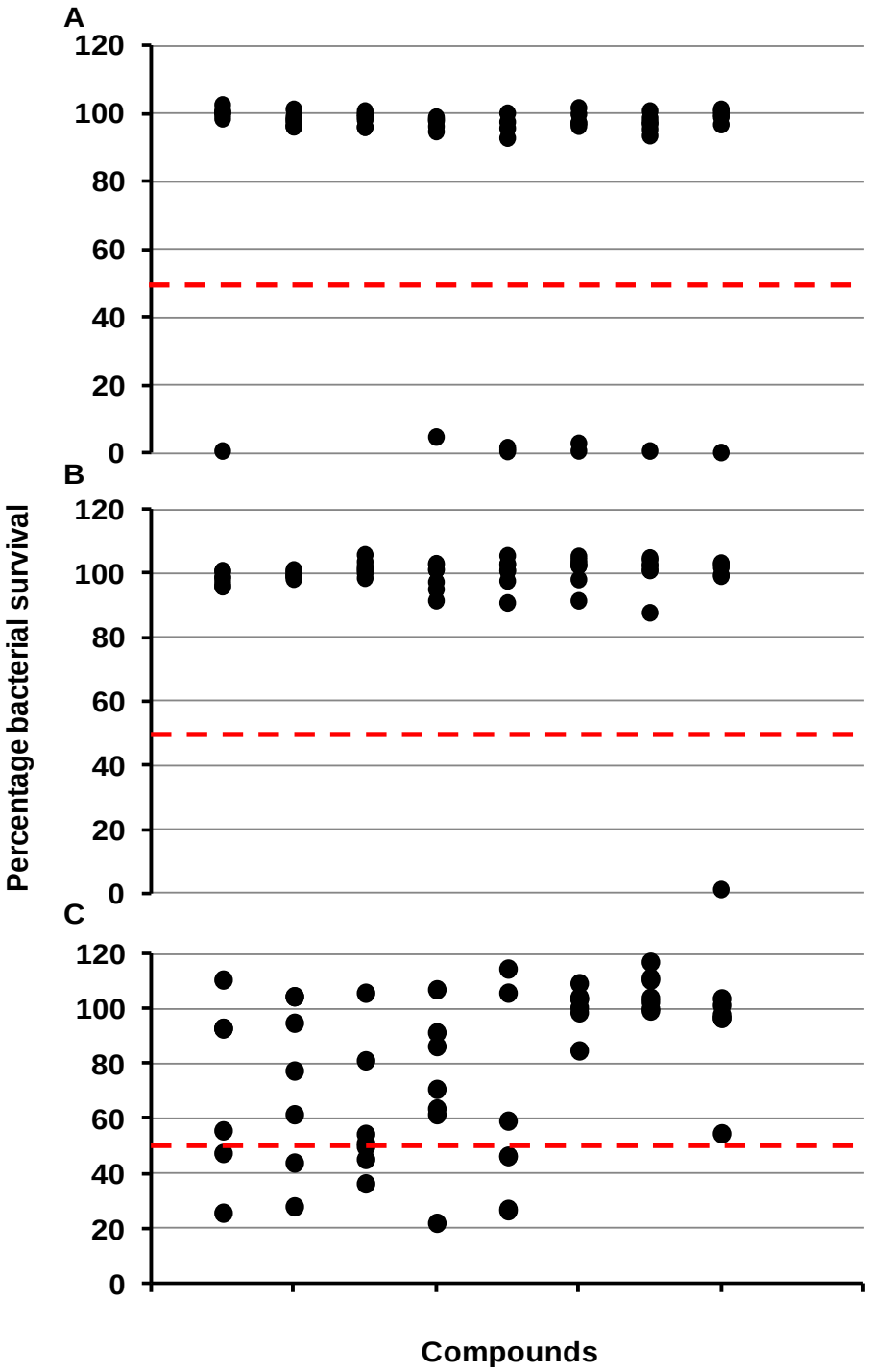


Figure 4.1. Screening of compounds. 63 compounds were added to replicate 96-well plates at a starting concentration of 50µM. Bacterial strains MRSA (A), CJ2009 (B) and Bb2009 (C) were added at an OD_{600} 0.001 and incubated for 24 hours. 0.05% Resazurin was added to each well and fluorescence measured. Percentage survival was calculated as percentage growth compared to an untreated control. A successful "hit" was when both replicate plates showed more than 50% restriction of growth by a single compound (red line). Graphs are representative of 1 replicate plate.

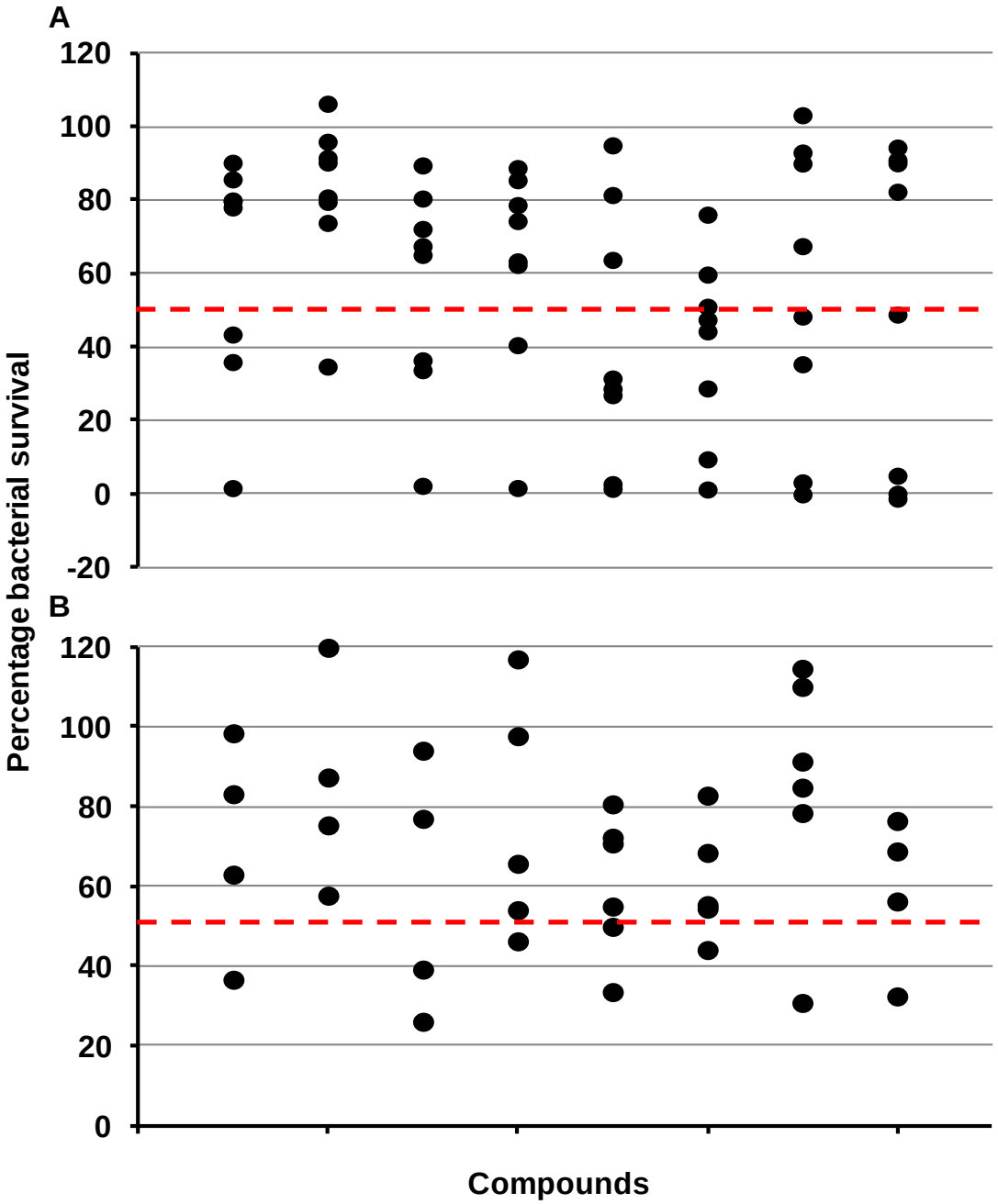


Figure 4.2. Screening of compounds against mycobacteria. 63 compounds were added to replicate 96-well plates at a starting concentration of 50µM. Bacterial strains *M.avium* (A) and BCG (B) were added at an OD_{600} 0.001 and incubated for 5 and 7 days respectively. 0.05% Resazurin was added to each well and fluorescence measured. Percentage survival was calculated as percentage growth compared to an untreated control. A successful "hit" was when both replicate plates showed more than 50% restriction of growth by a single compound (red line). Graphs are representative of 1 replicate plate.

compounds toxicity was not observed (Figure 4.3). **C55** and **C59** were non-toxic at lower tested concentrations (data not shown). All MCyCs that displayed anti-mycobacterial activity did not exhibit toxicity when tested in the mammalian THP-1 cell line, suggesting they were candidates for further analysis.

4.2.3 Determining the MIC_{90} of Metal-Cyclam Complexes

From the screens conducted, 18 compounds were selected for further testing (greater than 50% bacterial growth inhibition in any strain and no cellular toxicity). Bacteria were treated with varying doses of compound and the MIC_{90} calculated as the concentration that inhibited more than 90% bacterial growth relative to an untreated control. The majority of compounds tested against CJ2009, Bb2009 and MRSA were ineffective at the highest concentration measured (50 µM) (Table 4.1). Exceptions included **C47** and **C48** against CJ2009 and **C48** and **C53** against MRSA, which all had an MIC_{90} of 50 µM. The results for Bb2009 were expected as none of the compounds displayed complete inhibition when tested at 50 µM in the initial screen (Figure 4.1C). Further testing on *Pseudomonas* strains was discontinued due to the lack of significant activity observed upon MIC_{90} calculation.

The greatest level of anti-bacterial activity was observed against the mycobacteria. Of the 18 compounds tested, 9 had an MIC_{90} of 25 µM or less, 6 of which had an MIC_{90} of less than 10 µM (Table 4.1). Compounds **C47**, **C48** and **C53** were the most effective at restricting bacterial growth with an MIC_{90} of 1.25 µM against BCG and 1.56-3.13 µM against *M. avium* (Table 4.1). Compounds were also tested against mycobacteria with an OD_{600} 1 to represent a high bacterial load. Bacteria were treated with compound at a concentration of 10 µM and growth inhibition measured. Compared to the

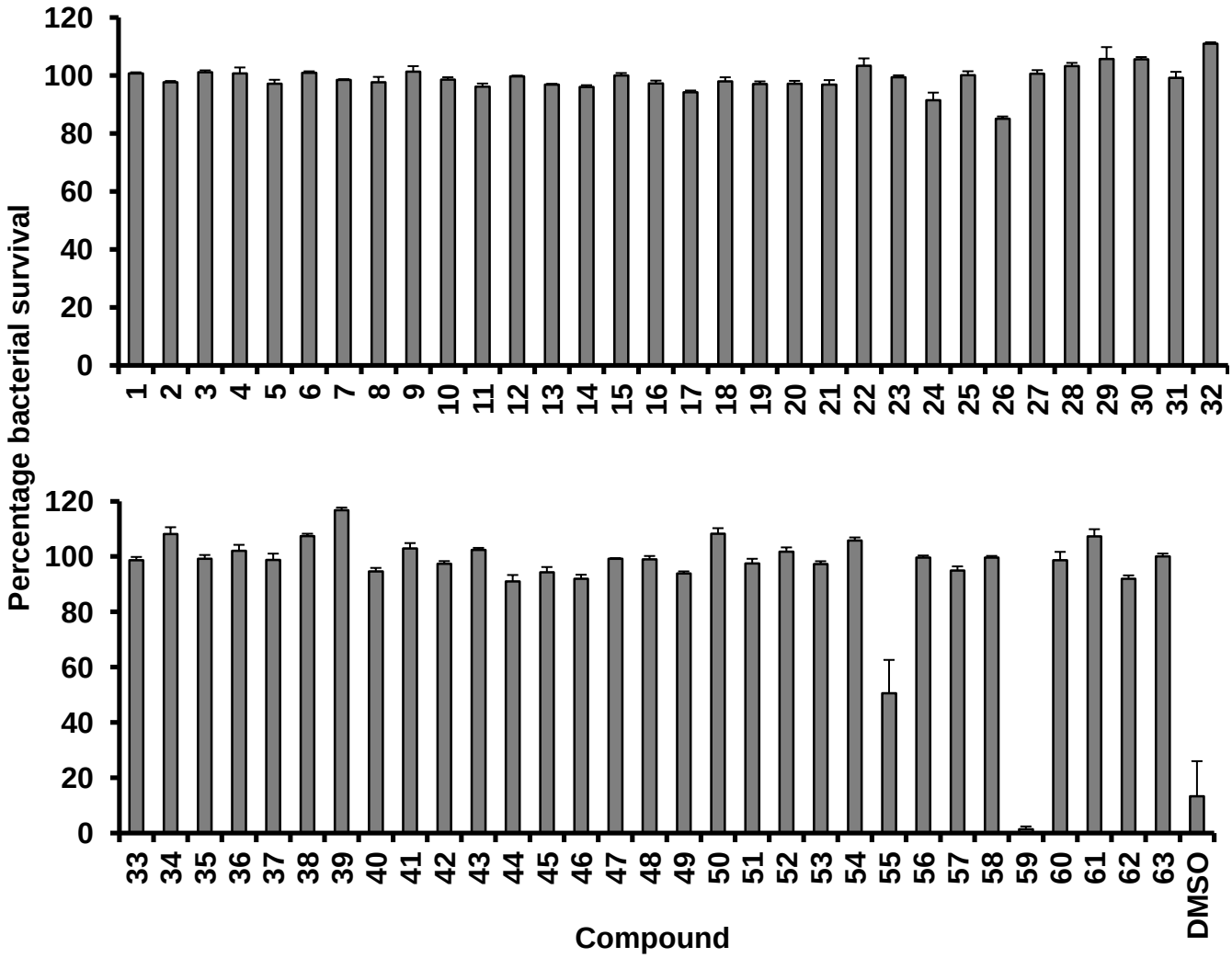


Figure 4.3. Cell viability in human cell line. $1x10^5$ THP1 cells were seeded into 96-well plates and allowed to adhere for 48 hours. Compounds were added at 50 µM in triplicate and incubated in CO_2 for 7 days. 0.05% Resazurin was added to each well and fluorescence measured. Cell viability was calculated as percentage survival compared to an untreated control.

Table 4.1. MIC_{90} of selected Metal-Cyclam complexes[a]

Drug	CJ2009	Bb2009	MRSA	BCG	*M. avium*
1	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
3	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
13	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
14	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
15	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
24	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
25	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
31	>50 μM	>50 μM	>50 μM	12.5 μM	12.5 μM
35	>50 μM	>50 μM	>50 μM	25 μM	12.5 μM
38	>50 μM	>50 μM	>50 μM	>50 μM	>50 μM
40	>50 μM	>50 μM	>50 μM	>50 μM	12.5 μM
44	>50 μM	>50 μM	>50 μM	6.25 μM	6.25 μM
45	>50 μM	>50 μM	>50 μM	3.13 μM	3.13 μM
46	>50 μM	>50 μM	>50 μM	3.13 μM	6.25 μM
47	50 μM	>50 μM	>50 μM	1.25 μM	3.13 μM
48	50 μM	>50 μM	50 μM	1.25 μM	1.56 μM
49	>50 μM	>50 μM	>50 μM	25 μM	25 μM
53	>50 μM	>50 μM	50 μM	1.25 μM	3.13 μM

[a] Data is representative of at least two independent experiments.

lower bacterial concentration used for the initial screening (OD_{600} 0.001) the majority of the compounds had a markedly reduced efficacy, exhibiting less than 40% growth inhibition at 10 μM (Figure 4.4). **C48** and **C53** however retained some efficacy with 60% and 50% growth inhibition respectively against BCG (Figure 4.4A) and more than 90% inhibition was calculated for **C48** against *M. avium* (Figure 4.4B).

The three most effective compounds against BCG (compounds **C47**, **C48** and **C53**) were tested *in vitro* against an attenuated (H37Ra) and pathogenic clinical (MT103) strain of *M. tuberculosis* (Pérez et al., 2001). The efficacy of the compounds against H37Ra was similar to that seen against BCG, with only a slight decrease in activity for **C47** to an MIC_{90} of 2.5 μM (Table 4.2). There was a 4-fold decrease in activity for all compounds against MT103, however results against this strain were variable (Table 4.2). Therefore the MCyCs appeared to display similar activity against a range of pathogenic mycobacteria.

4.2.4 Activity of Metal-Cyclam Complexes against intracellular mycobacteria.

As mycobacteria are intracellular pathogens, and effective drugs should readily enter host cells, it was necessary to study the effect of the compounds against bacteria within cells. THP1 cells were infected with either BCG or *M. avium* and treated with a 10 μM dose of 9 effective compounds (MCyCs with an MIC_{90} against BCG and *M. avium* of 25 μM or less). Against BCG (Figure 4.5A) only **C48** showed a significant reduction in bacterial survival compared to the untreated control (approximately one log reduction), while all other compounds only marginally reduced bacterial loads. *M. avium* treatment resulted in all compounds significantly reducing bacterial load within THP-1 cells, the most effective

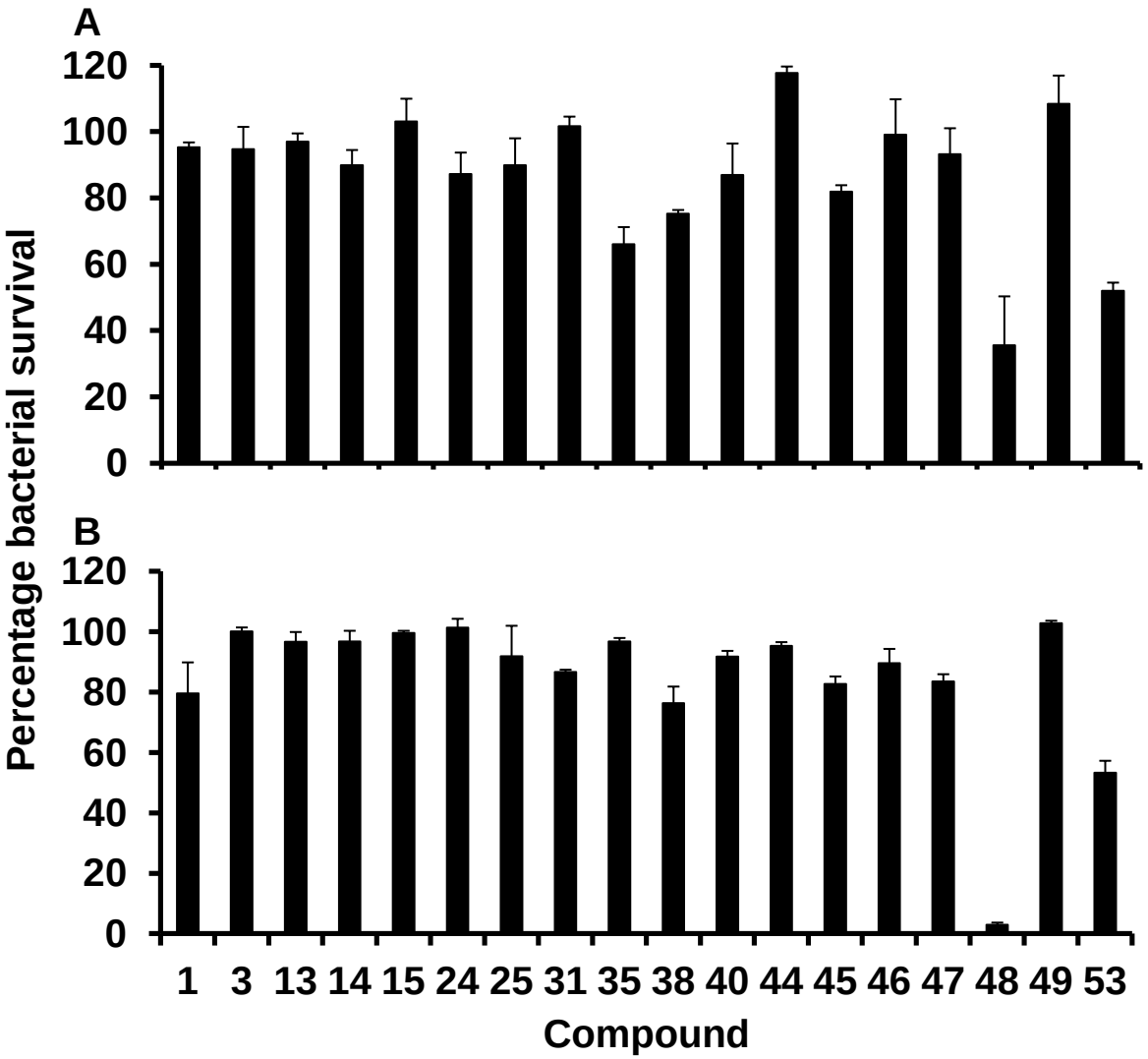


Figure 4.4. Effect of compounds against high bacterial load. Compounds were diluted to 10µM and added to a bacterial suspension of BCG (A) or *M. avium* (B) at OD_{600} 1. After a seven day incubation 0.05% Resazurin was added to each well and fluorescence measured. Data represented as mean survival ± SEM of triplicate samples, and is representative of two independent experiments.

Table 4.2. Comparison of MIC_{90} values between different strains of *M. tuberculosis*[a]

Drug	BCG	H37Ra	MT103
INH	0.3 µM	0.3 µM	0.3 µM
47	1.25 µM	2.5 µM	5-10 µM
48	1.25 µM	1.25 µM	2.5-5 µM
53	1.25 µM	1.25 µM	2.5-5 µM

[a] Data is representative of at least two independent experiments.

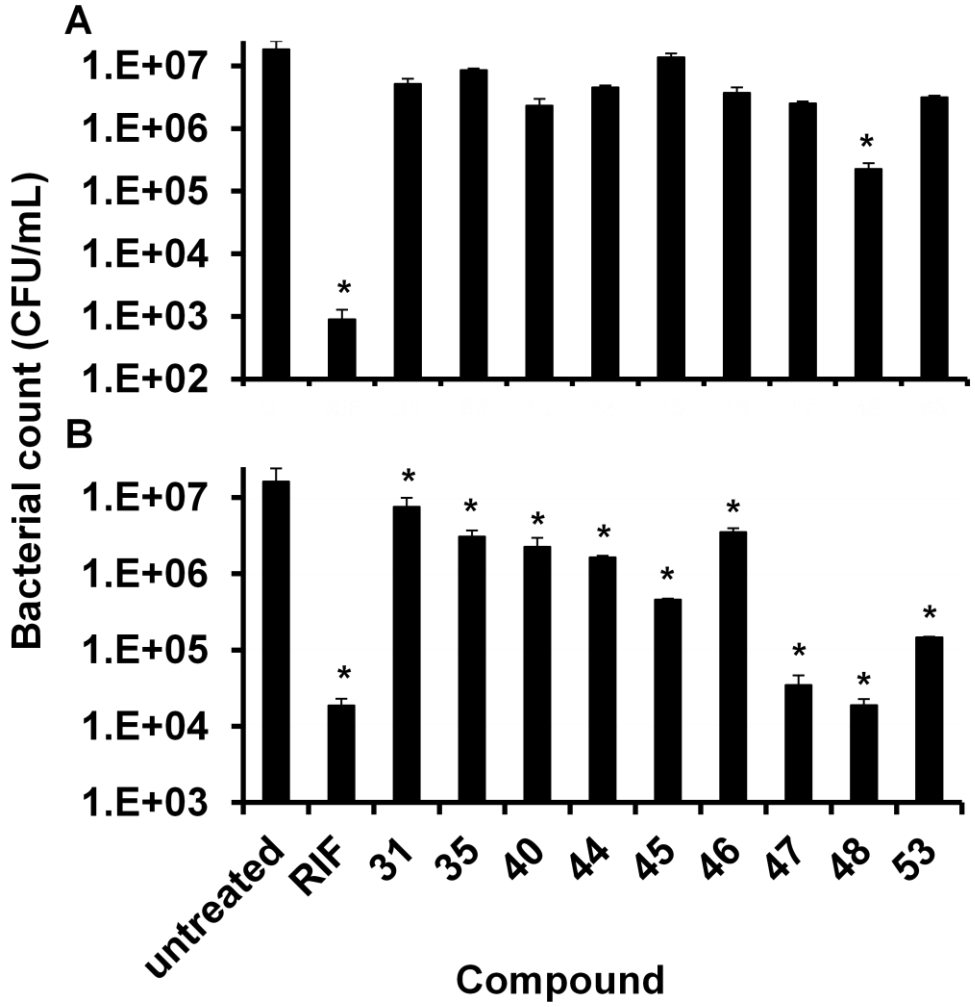


Figure 4.5. Efficacy of compounds in macrophages. THP1 cells were seeded at $5x10^5$ cells/mL into a 96 well plate and left to adhere for 48 hours. Cells were then infected with $1x10^6$ CFU/well of BCG (A) and *M. avium* (B). Compounds were added 4 hours post-infection at a concentration of 10 µM in triplicate and incubated for up to 7 days. Cells were then lysed, serially diluted and plated out onto 7H11 agar plates to obtain bacterial counts. Differences between untreated and compound treated bacterial cells were determined by ANOVA (*=$p<0.05$)

being **C47**, **C48**, and **C53** (Figure 4.5B. Importantly, **C48** resulted in a 3 log decrease in *M. avium* numbers, which was similar to that observed with rifampicin tested at the same concentration.

4.2.5 Synergistic effect of Metal-Cylcam Complexes with currently approved antimycobacterials.

Treatment for tuberculosis, both susceptible and multi-drug resistant, requires a cocktail of antibiotics. Therefore it is necessary to study the effect of any potential compound in combination with other drugs from the regimen, both to test for any drug-drug interactions, but also to see if the compounds work together synergistically. By collating all the data from the previous experiments, it was determined that **C47**, **C48**, and **C53** were the most effective so were selected for this study. By using a checkerboard set up (Lechartier et al., 2012) BCG and *M. avium* were treated with varying doses of selected compounds and either INH or rifampicin, and levels of inhibition measured using the Alamar blue assay.

In combination with INH, **C47** and **C53** appeared to improve efficacy relative to each compound alone against BCG (Figure 4.6a). **C47** combined with INH was the most effective combination restricting BCG growth by 50 per cent at concentrations of 0.12 µM and 0.3 µM respectively. Both compounds at these concentrations independently were unable to restrict growth (Figure 4.6a). No effect was seen in *M. avium* treatment (Figure 4.7a).

When combined with rifampicin, **C47** was the only compound that showed an improved effect against BCG (Figure 4.6b). In particular, combining 0.01 µM Rifampicin with 0.12 µM **C47** saw the greatest

Compound	47			
INH	**0**	**3**	**0.6**	**0.12**
0	102.1312	0.039663	101.4093	109.8654
0.3	108.7311	11.89878	99.14064	53.14789
0.06	125.0245	0.864645	120.8995	118.9402
0.012	104.8124	22.52043	120.1777	117.5996

Compound	48			
INH	**0**	**2**	**0.4**	**0.08**
0	87.38465	0.967768	107.5967	110.0717
0.3	83.67223	9.217589	110.5873	115.0216
0.06	97.28443	33.65769	107.6998	116.0528
0.012	85.73468	0.967768	120.2808	104.0905

Compound	53			
INH	**0**	**3**	**0.6**	**0.12**
0	94.19075	6.536397	120.2808	115.7434
0.3	75.21616	22.62355	116.7746	118.1152
0.06	86.14717	4.061451	75.73178	126.2619
0.012	84.08472	1.380259	113.681	100.8937

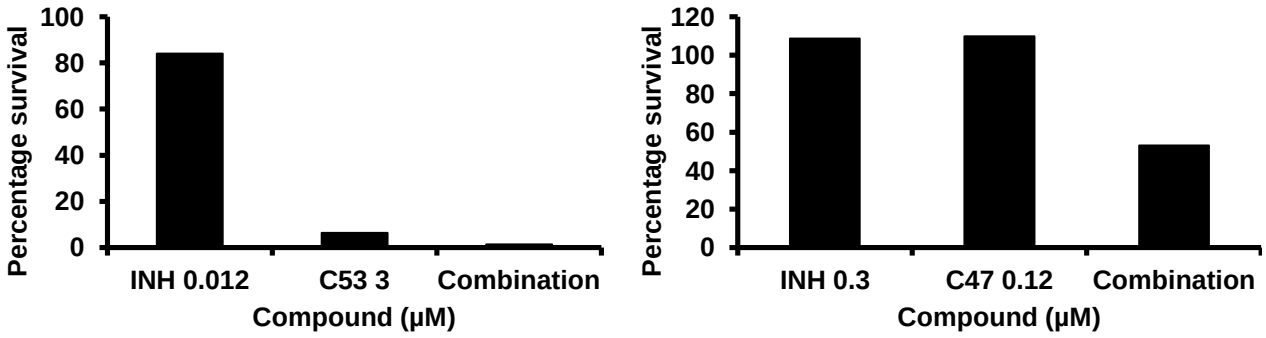


Figure 4.6a. Exposure of BCG to compounds in combination with INH. Bacteria (OD_{600} 0.001) were treated with compounds serially diluted in a checkerboard layout and incubated for 7 days. Resazurin (0.05%) was added and fluorescence measured. Compounds in combination were compared to individual treatment results. Effective combinations are also shown in graphical form. Results are representative of replicate plates.

Compound	47			
RIF	**0**	**3**	**0.6**	**0.12**
0	110.2779	22.21106	122.0339	109.1436
0.05	0.555276	-0.16658	0.761522	0.142785
0.01	118.2184	0.452154	126.3651	16.64243
0.002	113.8872	1.483381	94.60324	116.5684

Compound	48			
RIF	**0**	**2**	**0.4**	**0.08**
0	95.53135	-1.19781	108.7311	93.46889
0.05	-0.78532	-0.99157	1.277136	-1.50718
0.01	20.97358	-0.26971	45.20744	15.81745
0.002	118.4246	-0.78532	120.3839	129.4587

Compound	53			
RIF	**0**	**3**	**0.6**	**0.12**
0	110.4841	-0.06346	122.7558	118.2184
0.05	0.142785	-0.16658	2.617732	0.142785
0.01	131.6243	1.380259	130.3868	143.7928
0.002	108.6279	-0.57907	104.0905	83.25974

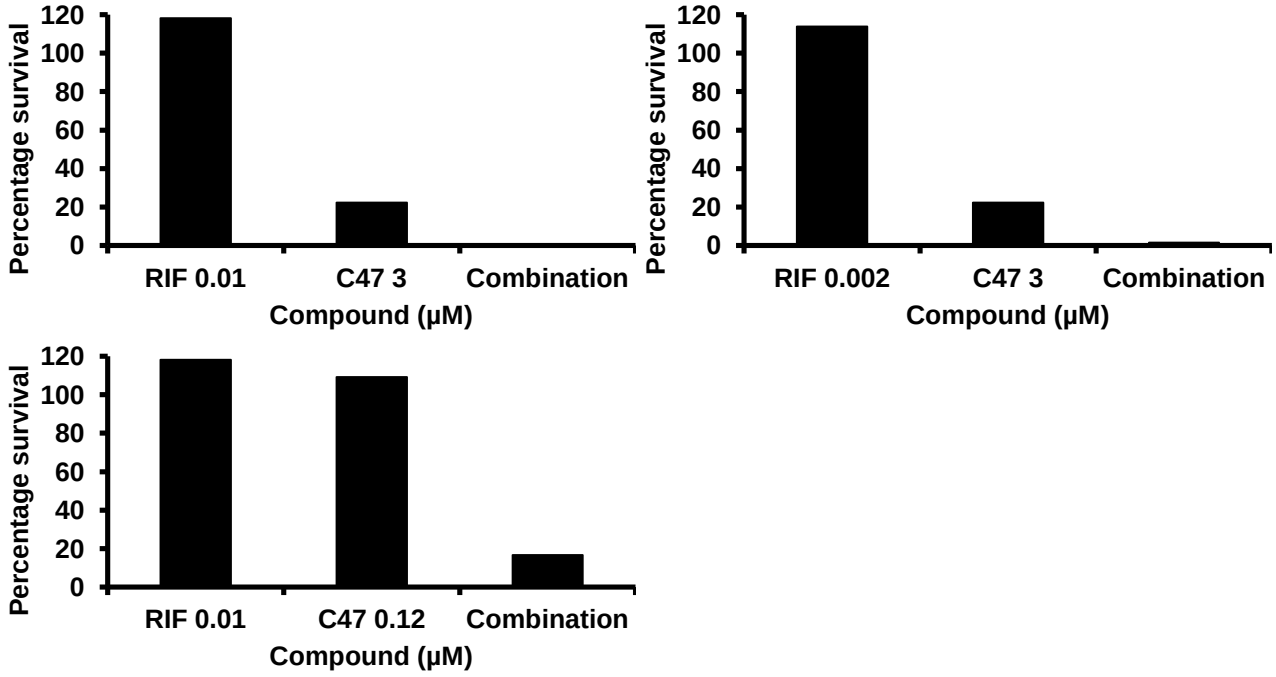


Figure 4.6b. Exposure of BCG to compounds in combination with Rifampicin. Bacteria (OD_{600} 0.001) were treated with compounds serially diluted in a checkerboard layout and incubated for 7 days. Resazurin (0.05%) was added and fluorescence measured. Compounds in combination were compared to individual treatment results. Effective combinations are also shown in graphical form. Results are representative of replicate plates.

effect with less than 20% bacterial survival, which compares to each independent compound showing no growth restriction (Figure 4.6b). Treatment of *M. avium* with all combinations of compound and rifampicin showed some level of synergy (Figure 4.7b-c). The highest concentration of **C48** (2 μM) tested in combination resulted in an improvement in growth inhibition for all concentrations of rifampicin relative to individual levels (Figure 4.7b). **C53** and rifampicin was the most effective combination against *M. avium* resulting in 6 concentration combinations that showed an improvement in efficacy compared to their individual counterparts (Figure 4.7c). Of particular promise was that of the two lowest concentrations tested (0.006 μM Rifampicin and 0.12 μM 48) in combination which resulted in less than 20% bacterial growth. Therefore these two compounds appear to work synergistically at low concentrations which is good evidence to support the use of this compound in further studies and potentially beneficial to future patients if lower concentrations are required.

4.2.6 Effect of Metal-Cylcam Complexes against non-replicating mycobacteria

Over a third of the world's population is latently infected with *M. tuberculosis*. Current chemotherapies are able to efficiently treat active infection with drug-susceptible strains, however a major limitation is the inability of current treatments to kill bacteria in a persistent state (Fattorini et al., 2013). Therefore this section of the chapter examined the ability of selected MCyCs to restrict the growth of bacteria in a non-replicating state.

The model used in this study was one adapted from Byrk *et al*, (2008) which forced the bacteria into a non-replicating state of growth. DETANO, a nitric oxide adduct, was added to render the bacteria non-

Compound	47			
INH	**0**	**3**	**0.6**	**0.12**
0	121.624	-2.40071	128.5084	123.5305
10	111.0327	5.860547	137.6169	126.9197
2	119.188	5.754634	139.5234	129.4616
0.4	109.8676	2.894969	138.2524	122.1536

Compound	48			
INH	**0**	**2**	**0.4**	**0.08**
0	93.45102	-3.8835	109.5499	101.5004
10	112.3036	5.436893	105.8429	107.4316
2	108.7026	8.932039	113.4687	121.7299
0.4	88.89673	20.15887	121.5181	116.8579

Compound	53			
INH	**0**	**3**	**0.6**	**0.12**
0	117.917	0.141218	137.1933	114.7396
10	109.338	9.143866	134.8632	91.7564
2	108.173	3.318623	139.0997	97.05207
0.4	95.6752	9.56752	164.9426	85.61342

Figure 4.7a. Exposure of *M. avium* to compounds in combination with INH. Bacteria (OD_{600} 0.001) were treated with compounds serially diluted in a checkerboard layout and incubated for 5 days. Resazurin (0.05%) was added and fluorescence measured. Compounds in combination were compared to individual treatment results. Results are representative of replicate plates.

Compound	47			
RIF	**0**	**3**	**0.6**	**0.12**
0	66.86673	-3.1421	83.9188	102.6655
0.15	98.32304	10.9444	79.04678	76.50485
0.03	111.88	4.801412	110.3972	123.7423
0.006	125.7546	11.57988	125.4369	116.1165

Compound	48			
RIF	**0**	**2**	**0.4**	**0.08**
0	73.11562	18.46425	124.8014	125.4369
0.15	68.03177	10.62665	83.49515	61.88879
0.03	78.4113	8.508385	110.3972	119.7176
0.006	96.31068	2.789056	112.4095	103.9365

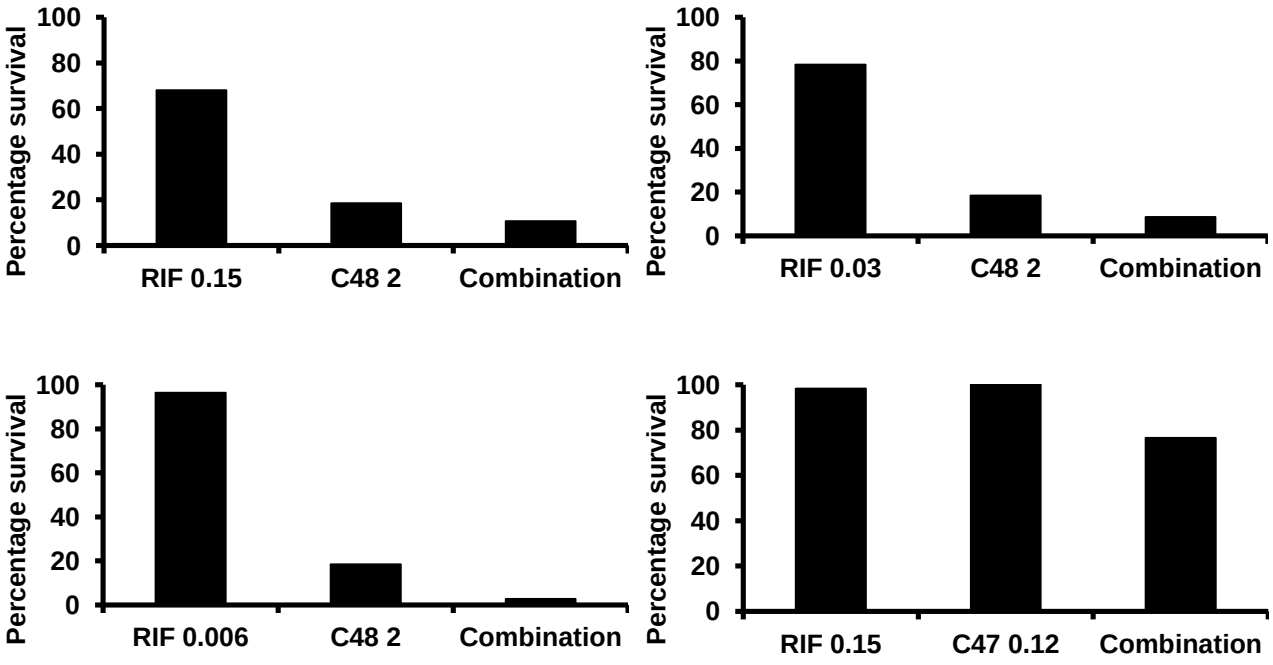


Figure 4.7b. Exposure of *M. avium* to compounds in combination with Rifampicin. Bacteria (OD_{600} 0.001) were treated with compounds serially diluted in a checkerboard layout and incubated for 5 days. Resazurin (0.05%) was added and fluorescence measured. Compounds in combination were compared to individual treatment results. Effective combinations are also shown in graphical form. Results are representative of replicate plates.

Compound	53			
RIF	**0**	**3**	**0.6**	**0.12**
0	127.0256	12.10944	130.4148	108.2789
0.15	73.11562	3.21271	77.77582	47.69638
0.03	114.9515	6.284201	121.9417	120.353
0.006	101.0768	1.200353	39.11739	13.90997

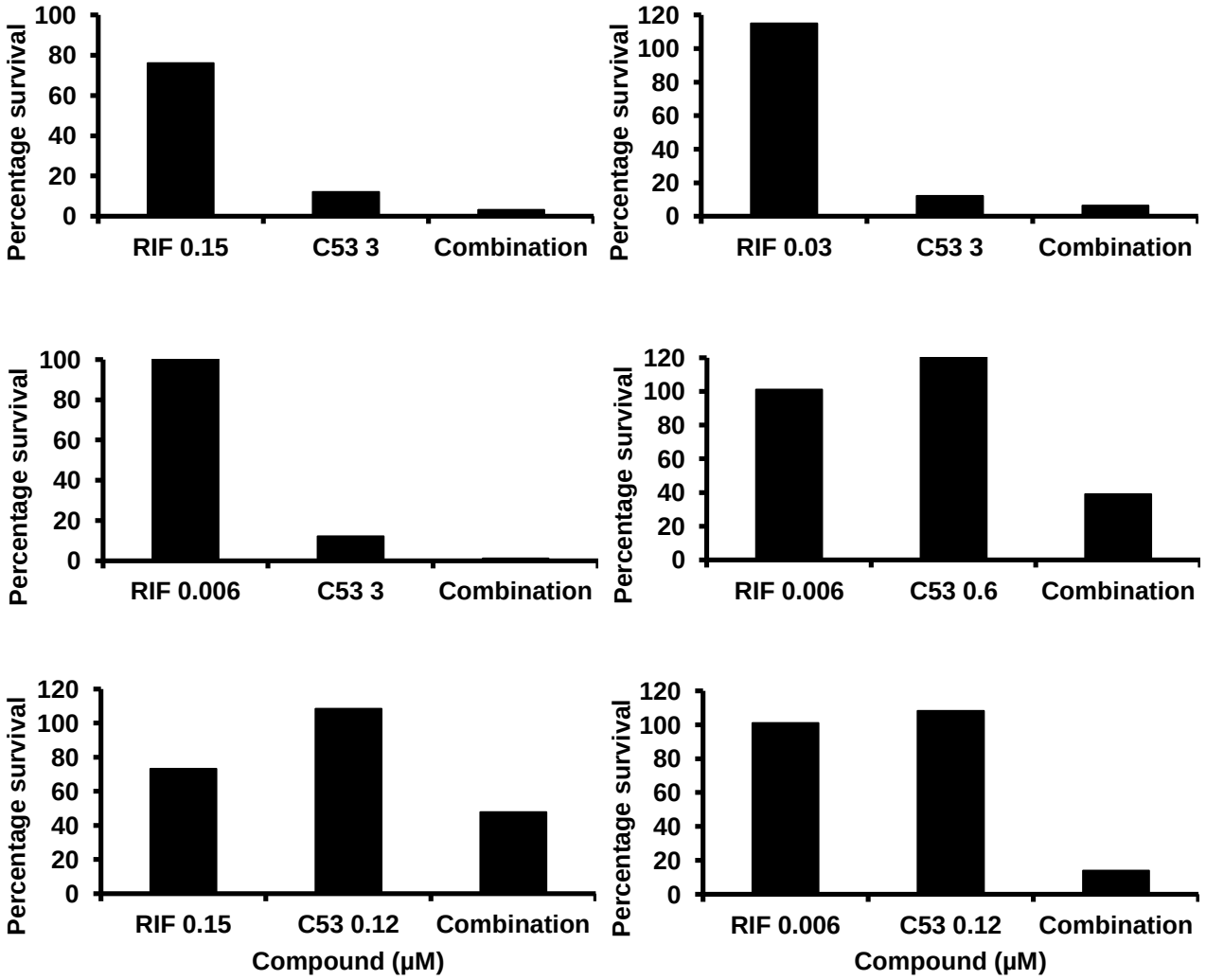


Figure 4.7c. Exposure of *M. avium* to compound 53 in combination with Rifampicin. Bacteria (OD_{600} 0.001) were treated with compounds serially diluted in a checkerboard layout and incubated for 5 days. Resazurin (0.05%) was added and fluorescence measured. Compounds in combination were compared to individual treatment results. Effective combinations are also shown in graphical form. Results are representative of replicate plates.

replicating by encouraging anaerobic conditions. If added to a bacterial population every 24 hours, the bacteria remained in a persistent state of growth (Byrk *et al*, (2008)).

To determine the optimal conditions for the assay, BCG was treated with varying doses of DETANO (0-100 μM) and bacterial CFU were enumerated after 7 days. The three highest concentrations (25 μM, 50 μM and 100 μM) all caused a reduction in bacterial CFU of more than one log by day 7 (Figure 4.8A). Non-replicating conditions however was observed when using 10 μM DETANO, and this concentration was used in subsequent experiments. In order to simplify the assay, a 5-day timepoint was also examined using a DETANO concentration of 10 μM. As seen in Figure 4.8B, over the 5 day time period the bacteria were in a non-replicating state of growth and displayed an approximate 2 log difference compared with the non-treated control. This was similar to that seen with the day 7 timepoint (Figure 4.8A) and thus was considered suitable for the subsequent studies.

To assess killing in this model, 10 μM INH was used as a 'non-killing' control as it does not kill bacteria that are not actively replicating (Wang et al., 1998), and 10 μM rifampicin was used as the 'killing' control, as it can kill bacteria in a non-replicating state (Figueiredo et al., 2009). Results for the rifampicin and INH were as expected against BCG (Figure 4.9A) and *M. avium* (Figure 4.9B). INH did not show any growth inhibition in this model of persistence over the five days (Figure 4.9), whilst rifampicin decreased bacterial growth by 5.5 and 3 logs for BCG (Figure 4.9A) and *M. avium* (Figure 4.9B) respectively.

The only MCyC tested in this study was **C48**. This was due to the fact that it was the most efficacious as a single agent of all compounds from previous experiments. In this model **C48** was shown to reduce bacterial growth of BCG by approximately 3.5 logs after 5 days (Figure 4.9A) and resulted in 0.5 log

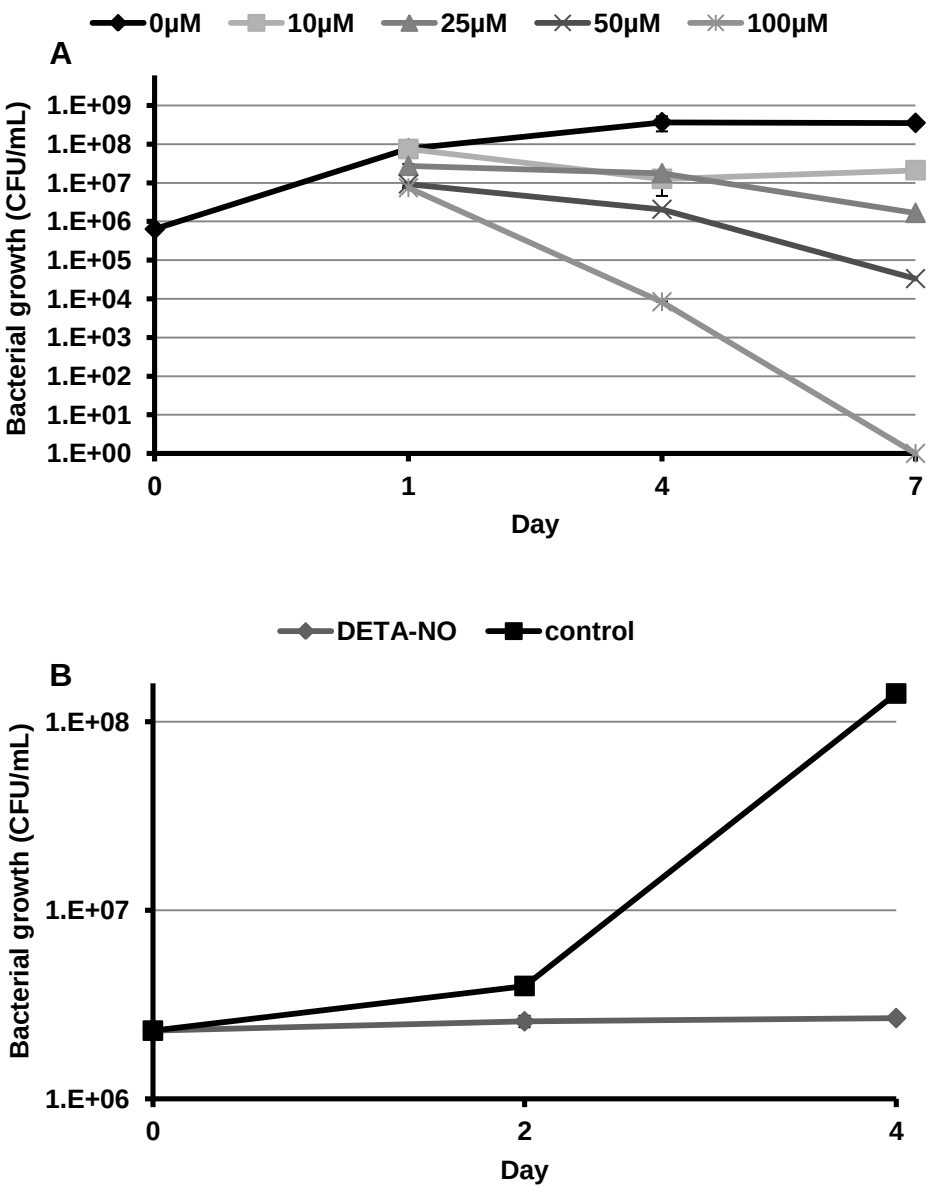


Figure 4.8. Optimisation of dosage and incubation time for a non-replicating model of infection. (A) BCG (OD_{600} 0.01) was treated with varying doses of DETANO (0-100 μM) every 24 hours and incubated for 7 days. Bacterial colonies were enumerated over the 7 day timepoint and persistence determined as the concentration that neither reduced bacterial concentrations nor allowed replication. (B) Experimentation was repeated using 10 μM DETANO over a 5 day timepoint and bacterial colonies enumerated over the time period. All results are CFU/mL ± SEM.

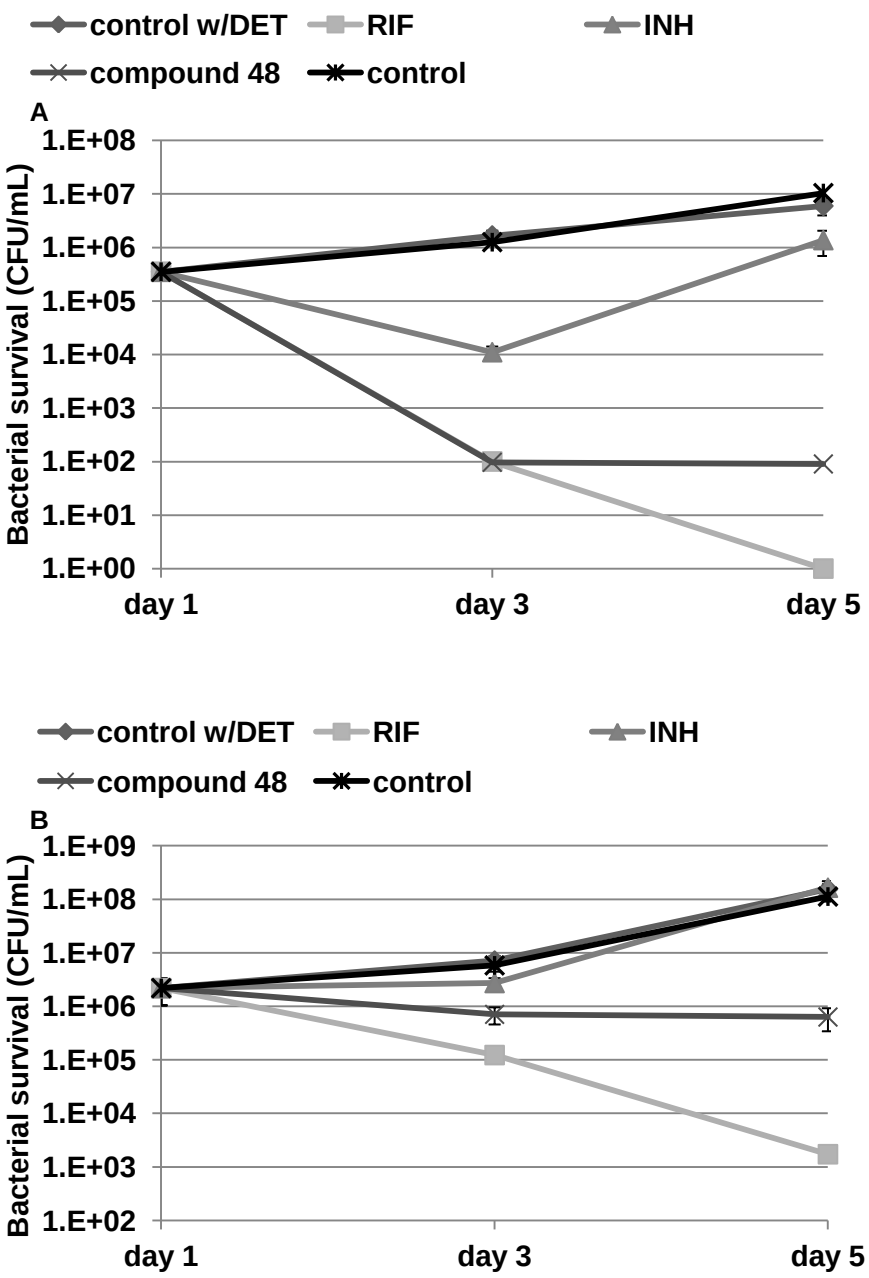


Figure 4.9. Testing compound 48 in a non-replicating model. DETANO treated (10 μM) BCG (A) and *M. avium* (B) (OD_{600} 0.01) was incubated with 10 μM Rifampicin, INH or 48 for 5 days. Bacterial culture was plated out and colonies enumerated to determine absolute bacterial numbers. The compound was effective if bacterial growth was less than the starting concentration. All results are CFU/mL ± SEM.

reduction in *M. avium* (Figure 4.9B) However, DETANO-treated controls for both strains did not enter a non-replicating state, exhibiting a similar pattern of growth to the non-treated bacterial cultures. This was observed after repeated testing. This meant that any effect seen by the MCyCs tested was most likely due to activity against dividing cells, indicating this assay was unreliable using these strains.

In order to examine the influence of compounds in a more reliable model of non-replicating persistence, *M. tuberculosis* H37Ra was treated with 50 μM DETANO as done in other studies (Hussain et al., 2009, Voskuil et al., 2003) and validated in our laboratory (D. Quan, unpublished observations). A 10 μM dose of each compound was administered and bacterial counts obtained after a five day incubation. When comparing day 0 counts with those obtained on day 4 both via absorbance (Figure 4.10A) and bacterial counts (Figure 4.10B), the DETANO treated control were in a non-replicating state with a consistent absorbance (Figure 4.10A) and significantly less bacterial growth compared to the untreated control (Figure 4.10B). As shown previously (Bryk et al., 2008) rifampicin was effective at reducing bacterial numbers in this model, while INH-treated bacteria did not result in a significant decrease in CFU over the 4-day period (Figure 4.10B). **C48** showed little effect on persistent cells and displayed a similar pattern of growth restriction to INH. The 1-log reduction by both INH and **C48** could be attributed to their killing of actively replicating bacteria that may still be present in the assay. None-the-less, these results indicate that **C48** did not appear to be active against non-replicating *M. tuberculosis*.

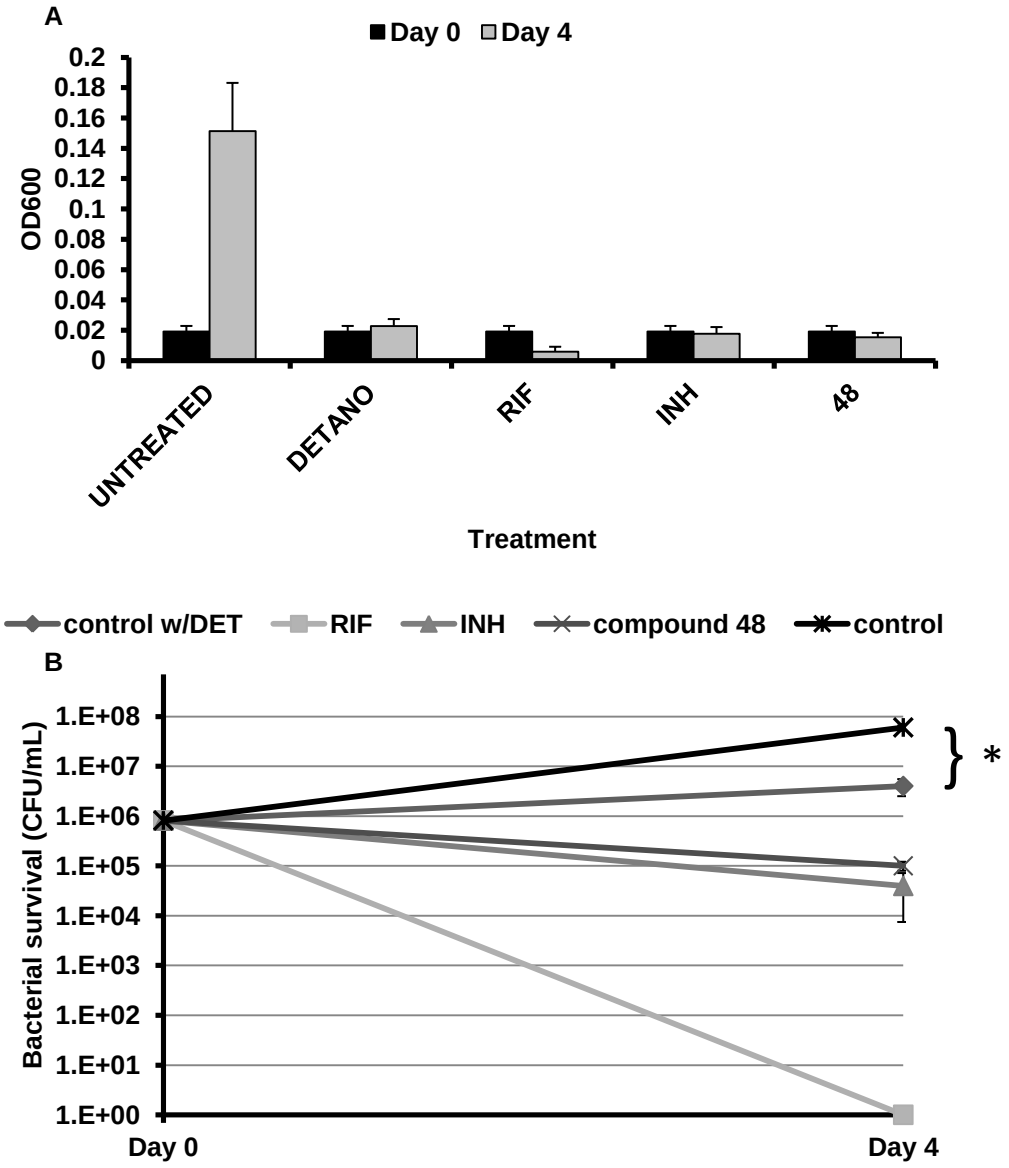


Figure 4.10. Effect of compound 48 against non-replicating bacteria. DETANO treated (50 µM) H37Ra (OD_{600} 0.01) was incubated with 10 µM Rifampicin, INH or 48 for 5 days. (A) Absorbance was measured before and after treatment to determine changes in bacteria growth over time. (B) Bacterial culture was plated out and colonies enumerated to determine absolute bacterial numbers. The compound was effective if bacterial growth was less than the starting concentration. Difference between DETANO treated and untreated cells determine by ANOVA (*=$p<0.05$). All results are CFU/mL ± SEM.

4.3 DISCUSSION

In this chapter a library of cyclam-containing compounds were tested for antibacterial activity (Appendix 1). This compound library was composed of metal-cyclam complexes (Yu et al., 2013b), cyclam-peptide, cyclam-amino acid complexes (Yu et al., 2011) and potential MMP inhibitors (Yu et al., 2013a). Of the 63 compounds tested, there were a number of efficacious and relatively non-toxic individuals that demonstrated specific action towards mycobacteria, as seen by the greatly reduced MIC_{90} of some compounds against mycobacterial strains compared to the same compound against the representative gram positive and gram negative strains tested here (Table 4.1).

One class of compounds in this screen were predicted MMP inhibitors. The major chemical moiety, Marimastat, is an orally active, potent broad spectrum inhibitor of MMPs (Failes and Hambley, 2007, Underwood et al., 2003b). Initial experimentation showed the ability of Marimastat to effectively inhibit multiple MMP subtypes in a range of 3-260 nM and early clinical trials revealed little toxicity and pharmacologically active levels in blood serum after oral treatment (Rasmussen and McCann, 1997). Previous studies have looked at the use of MMP inhibitors as potential treatment options for *M. tuberculosis* infection. One particular inhibitor, BB-94, showed a reduction in bacterial CFU as well as a decrease in leukocyte recruitment, a major source of MMPs, leading to a reduction in disseminated infection (Izzo et al., 2004). Other studies have looked at the inhibitory effects of Vitamin D used as MMP inhibitors (Nursyam et al., 2006, Coussens et al., 2009, Martineau et al., 2007). Results indicated a reduction in time to sputum smear conversion (Nursyam et al., 2006) as well lower levels of lung cavitation (Martineau et al., 2007). These studies suggest that targeting MMPs could be a valid way to improve disease pathology and severity.

The addition of the cyclam molecule, which individually shows limited effect on bacterial growth (as seen in initial screen; Figure 4.1 and 4.2), potentially adds a metal binding site which enhances the effect of Marimastat against bacterial cells. Despite the theory suggesting that MMP inhibitors should be a useful antibacterials, none of the Marimastat derivatives in this study showed effectiveness at the concentrations tested (Figure 4.1 and 4.2) and **C59**, a cyclam-marimistat complex (Appendix 1) was the most toxic to tissue cells of all compounds tested with more than 90 per cent reduction in cell viability (Figure 4.3). Therefore, these compounds were not considered appropriate for use as antibacterials.

Of the 18 compounds that successfully inhibited bacterial growth during the initial screening phase, three contained a cyclen molecule instead of a cyclam (Appendix 1). A cyclen is shorter by two carbon atoms (Appendix 1). Compounds containing this molecule have been associated with peptide cleavage (Chei et al., 2010, Kim et al., 2009) and most importantly, have been looked at with regards to Alzheimer's disease (Wu et al., 2008, Chei et al., 2010). It is selective for peptides and for the treatment of Alzheimer's disease targets Abeta protein found in the brain, and degrades it preventing plaque formation (Wu et al., 2008). As yet there is no evidence of cyclens being used as antibiotic therapies.

C31, C35 and **C40** contained cyclen structures with a metal co-factor (Appendix 1). Whilst they appeared to work to a certain degree against bacterial cultures, their effect within cells was limited. One thing to note was that they were more active against *M. avium* than BCG (Table 4.1 and Figure 4.5). This could be due to these compounds targeting a specific peptide within the cell wall of *M. avium* that is less abundant in BCG. It could also be related to bacterial growth, in that *M. avium* grows at a faster rate than BCG so the effect of the cyclen is more rapid. When toxicity of the compounds was tested

C55 was the only other compound to show a reduction in cell viability at 50 μM (Figure 4.3). This compound was the cyclen molecule which may suggest that some of the effect seen in the MIC_{90} study could be a general toxicity, however his toxicity was not apparent in the cyclen-containing compounds.

The most active compounds from the screen contained a napthalimide structure (Appendix 1). They were created for the purpose of being able to visualise the binding of a biological event such as protein-protein interactions (Yu et al., 2013b). Napthalimides have been primarily used as markers (Grabchev et al., 2012) however one study did look at the use of napthalimide derivatives against gram positive and gram negative bacteria as well as fungi (Lv et al., 2014). Its suggested mode of action was targeting DNA polymerase, and results indicated a somewhat broad effect with some MICs in a range lower than that of the reference drugs. In particular, it seemed effective at restricting the growth of *E. coli* (16 μg/mL) to a much greater extent than for any other strain tested (Lv et al., 2014).

In this study, the compounds containing the napthalimide were far superior to the others tested. Compounds **C44**, **C45**, **C46** and **C49** all contained one napthalimide with the addition of a biotin molecule in all except **C49** (Appendix 1). Interestingly, the compounds that were the most effective contained two napthalimide molecules surrounding a cyclam moiety. These were compound **C47**, **C48** and **C53**, and in all tests these compounds were superior.

C47, **C48** and **C53** did display a level of broader specificity as they were able to inhibit MRSA and *P. aeruginosa* growth at 50 μM (Table 4.1). However activity against mycobacteria was in the low micromolar range, indicating that these compounds may be acting against a specific target in mycobacteria. This could suggest that the compounds are targeting a specific gene or protein within the cell wall. The mycobacterial cell wall is more complex than that of gram positive and negative

bacteria, and is composed of long fatty-acid chains and mycolic acids covalently bound to an arabinogalactan-peptidoglycan co-polymer (Song et al., 2008). These are more complex structures than the lipopolysaccharides found in the cell wall of gram negative bacteria such as *P. aeruginosa*. (Kocincova and Lam, 2011). The result seen in this study could indicate that the compounds are acting in a targeted manner, however further studies would need to be conducted to confirm this. This may include sequencing the genome of spontaneous drug-resistant mutants (Andries et al., 2005, Makarov et al., 2009) or affinity purification of compound-target complexes using mycobacterial cell walls or lysates (Terstappen et al., 2007).

C47, **C48** and **C53** differ solely by the metal iron within a cyclam moiety (Appendix 1). **C48**, the most effective in this study, contained a copper ion and thus this metal may be in part responsible for the activity of the compound. Copper has been shown to be a highly effective molecule against different bacterial strains including *M. tuberculosis* (Mehtar et al., 2008). It appears to encourage the disruption of the cell membrane and subsequent cell death and DNA damage (Grass et al., 2011). In one study, the addition of copper to INH improved efficacy two-fold (Maccari et al., 2004). In another, copper treatment alone was highly ineffective against mycobacteria, however it improved the efficacy of ethambutol when in combination (Kozak et al., 1998).

The likelihood of the copper adding to the mechanism of action is enhanced by the fact that **C53** was not as effective. This compound is not complexed with a metal ion, having only the cyclam shell. It has a 3-fold higher MIC_{90} against *M. avium* (Table 4.1) and is not as effective as **C48** in restricting growth of bacteria at a high concentration (Figure 4.4). It is of interest to note that this compound showed a marked difference to compound **C48** when tested against *M. avium*, with an MIC_{90} of 3.13 µM compared to 1.56 µM for **C48**, and approximately 10-fold reduction in activity against high

bacterial load. When in combination however, compound **C53** was able to improve the activity of rifampicin at all concentrations tested (Figure 4.7c), a property not shared by compound **C48**. This indicates that against *M. avium* compound **C53** may be more beneficial when used in a combination therapy regimen.

C47 differed from the other molecules by the inclusion of a zinc ion. Whist it was highly effective, it had a 3-fold higher MIC_{90} compared to **C48** against *M. avium* (Table 4.1) and was unable to restrict bacterial growth at a high concentration (Figure 4.4). Previous studies using zinc supplementation of current regimens have failed to show any significant impact on morbidity and mortality outcomes relative to placebo or normal treatment (Haider et al., 2011, Das et al., 2012). In a study specifically looking at the use of oral zinc in tuberculosis patients co-infected with HIV, the authors could not recommend zinc as a pharmacological supplement (Green et al., 2005). As zinc appears to add no benefit it is unusual that it is less effective than the parent molecule **C53** against *M. tuberculosis*. It is possible that inclusion of the zinc molecule interferes with target binding in *M. tuberculosis* and thus alters activity relative to **C53**.

One potential benefit of **C47** could be an enhanced activity *in vivo*. Zinc is essential for immunological processes such as the production of cytokines, phagocytosis and intracellular killing (Prasad, 2009). In an *in vivo* setting, the drug could be effective at restricting bacterial growth and assist in 'boosting' the host immune response. We obtained ethics approval for testing these compounds in mice, however we were unable to obtain non-particulate samples at the concentration range to be tested (100mg/kg and 10 mg/kg). This was irrespective of the vehicle used (combinations of PBS, FBS and DMSO) and was also evident at a concentration of 1 mg/kg. Therefore we were unable to deliver these compounds to mice in order to determine their *in vivo* efficacy. However, future studies into these compounds should

examine their activity *in vivo* to determine whether their ability to restrict bacterial growth and affect the immune system could benefit in a clinical setting.

The MCyCs described in this chapter were effective against actively replicating bacteria however did not show efficacy against non-replicating bacteria (Figure 4.10). This suggests that the drug target of **C48** could be a gene that is present or upregulated only during active infection, such as a gene involved in replication, cell wall biosynthesis or energy metabolism. Rifampicin is effective against non-replicating bacteria (Figueiredo et al., 2009) and in this study **C47** improved the effect of rifampicin (Figure 4.6b). Testing this particular compound in combination with rifampicin in vivo could uncover a potentially beneficial partnership.

The inhibitors described in this chapter display a number of properties that are attractive from a medicinal chemistry perspective. They are small and have low lipophilicity, allowing addition of functionality in the optimization process. These are also novel anti-mycobacterials; they bear no resemblance to known drugs and are distinct from anti-TB compounds identified using small molecule libraries (Ballell et al., 2013). Although this chapter explored the biological effects of these compounds, there is scope for further modification of structure. The design of the lead compounds incorporated four key elements: a macrocycle (cyclam), a linker ('click'-derived triazole), the pendant group (naphthalimide) and a metal ion. **C47** and **C48** are the zinc(II) and copper(II) complexes of the free amine form(perchlorate counterion); **C53** is the ammonium salt (trifluoroacetate counterion) (Figure 4.11). To explore and expand the potential of these compounds, each of the four structural components can be varied in turn. Variations in the pendant group in particular could be employed to alter lipophilicity and improve host cell penetration (see Figure 4.5).

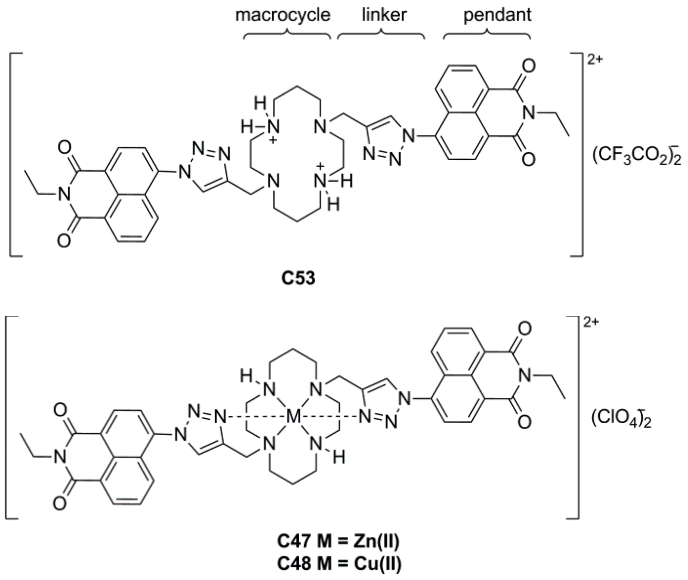


Figure 4.11. Structure of lead MCyCs inhibitors. The zinc(II) (**C47**) and copper(II) (**C48**) MCyCs and the ammonium salt **C53**.

In conclusion this study has uncovered 3 potent compounds against mycobacterial species, both in culture and within host cells. The options to manipulate these compounds to improve their efficacy are numerous, and as they are relatively non-toxic they have potential to be used in future treatment regimens against pathogenic mycobacteria.

Chapter 5: Discovery of new antibacterials against lung pathogens

5.1 INTRODUCTION

In 2011, respiratory disease accounted for approximately 8% of deaths in Australia (ABS, 2013), the third largest cause of mortality after neoplasia and circulatory disease (ABS, 2013). The two most common causes of respiratory disease include influenza and pneumonia, both of which are caused by infectious agents. This suggests that the development of new strategies and treatments to combat lung pathogens could lead to a reduction in mortality nationwide.

Two medically important pathogens of the lung include *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*. Tuberculosis is the leading cause of mortality worldwide by a single bacterial pathogen. Despite the availability of effective medication, drug resistance is on the rise with multiple-drug resistant strains resulting in approximately 4% of newly diagnosed cases globally and as much as 20% in recurring disease states (W.H.O, 2012). *P. aeruginosa* is the main causative agent of chronicity and mortality in Cystic Fibrosis (CF) (Lopes et al., 2012, Folkesson et al., 2012). CF is an autosomal recessive genetic disorder primarily affecting Caucasians. Despite the availability of potent antimicrobials, the use of broad-spectrum antibiotics in hospital settings has led to an emergence of problematic drug resistance in this pathogen (Gorgani et al., 2009). Resistance to commonly used antibiotics has been increasing by 2 to 4.6 per cent each year, and resistance of pathogens to compounds such as the last-line carbapenems is more than 40 per cent (Xu et al., 2013). Both these

pathogens are becoming more difficult to treat with current therapies with slow progress made to develop new treatment options. Therefore the discovery of new anti-bacterials is urgently required.

In recent years, the process of discovering a new antibiotic has been largely target-based and genetics-driven. Whilst the theoretical principle is valid, the strategy has not been very successful due to a lack of correlation between inhibition of enzyme activity and that of whole bacteria (Payne et al., 2007). Large scale screening programs have largely been made up of small molecule 'corporate' chemical libraries that are relatively limited in diversity (Payne et al., 2007). However, the properties of antibiotics are somewhat distinct from those found in such libraries (Payne et al., 2007), and there is a need to develop and/or test novel compounds that may not necessarily fit the typical medicinal chemistry profile.

A number of antibiotics in current circulation have multiple targets within a cell, for example INH is a pro-drug requiring activation by genes within the bacterial cell to function accordingly. It is for this reason that whole-cell screens are being actively pursued within the field of *M. tuberculosis* drug discovery, and most inhibitors in the later stages of development of the drug pipeline have been derived from screens conducted on the whole bacterial cell (Barry et al., 2009, Koul et al., 2011). Delamanid, currently in phase III clinical trials, was discovered through screening a number of nitro-dihydro-imidazooxazole compounds against *M. tuberculosis* and PA824, currently in phase II clinical trials, was discovered by screening a series of bicyclic nitroimidazofurans against whole bacteria (Stover et al., 2000b). This approach allows for the discovery of drugs that directly kill the organism or inhibit its growth (Ymele-Leki et al., 2012), and overcomes the problem of trying to translate specific activity of a gene or enzyme to its effect on the entire organism.

High-throughput screening allows for thousands of compounds to be tested in a relatively short period of time, ultimately speeding up the potential discovery of a new treatment. In this study, a selection of diverse compounds (natural products and synthetic derivatives) were examined for antibacterial properties and toxicity to determine if any were able to successfully restrict growth of selected bacterial pathogens of the lung and had the potential to be future treatment options.

5.2 RESULTS

5.2.1 Selection of appropriate *Pseudomonas aeruginosa* strains for screening

Due to the large number of *P. aeruginosa* strains that infect cystic fibrosis patients, it was necessary to determine the appropriate strain(s) to be used for the most informative antibiotic testing. Epidemic strains AES-1M, AD-2009 and CJ2009, non-epidemic strains 17-2009 and Bb2009, together with the lab strain PAO1, were chosen to represent a more general pathogenic *Pseudomonas* population (Harmer et al., 2012). All strains except the PAO1 were chronic isolates, selected due to having more acquired resistance then non-chronic isolates (Hare and Cordwell, 2010). Bacterial strains were then subjected to varying doses of compounds representing different antibiotic classes in order to determine the MIC_{90}. All clinical strains typically displayed resistance that was greater than that observed for PAO1 (Table 5.1). All epidemic strains displayed greater resistance to norfloxacin, gentamicin, tobramycin and collistin, with a MIC_{90} ranging from 2 to 32 times greater than that for PAO1 (Table 5.1). Rifampicin and tetracycline were the only two antibiotics in which PAO1 was more resistant than the clinical strains (Table 5.1). The non-epidemic strains appeared to have similar levels of resistance to all tested antibiotics with the exception of ciprofloxacin and polymyxin B in which Bb2009 was more resistant by approximately 2 and 8 times respectively (Table 5.1).

While there were some differences between epidemic strains, we selected strains CJ2009 for further analysis, as this strain was determined to be more amenable for genetic manipulation (Dr Gabriella Scandurra, personal correspondence), which would potentially facilitate the identification of targets any of any compounds of interest identified in subsequent screens. Of the non-epidemic strains Bb2009 was comparatively more resistant to all other strains tested and was also selected for additional study.

Table 5.1. MIC_{90} of control antibiotics against *Pseudomonas* to determine the most resistant strain.

Strain	Status	MIC_{90}					
		Norfloxacin	**Polymyxin B**	**Rifampicin**	**Pipericillin**	**Ciprofloxacin**	**Gentamicin**
PAO1	Laboratory	1.6 µM	6.3 µM	50 µM	25 µM	3.13 µM	3.13 µM
AES-1M	Epidemic	50 µM	1.6 µM	12.5 µM	>100 µM	6.25 µM	>100 µM
AD-2009	Epidemic	50 µM	3.1 µM	12.5 µM	3.13 µM	3.13 µM	12.5 µM
CJ2009	Epidemic	25 µM	1.6 µM	3.1 µM	>100 µM	3.13 µM	100 µM
17-2009	Non-epidemic	50 µM	1.6 µM	12.5 µM	6.25 µM	3.13 µM	>100 µM
Bb2009	Non-epidemic	50 µM	12.5 µM	12.5 µM	6.25 µM	6.25 µM	>100 µM

Strain	Status	MIC_{90}					
		Vancomycin	**Tetracycline**	**Tobramycin**	**Collistin**	**Kanamycin**	**Oxacillin**
PAO1	Laboratory	>100 µM	50 µM	3.13 µM	50 µM	>100 µM	>100 µM
AES-1M	Epidemic	>100 µM	6.25 µM	>100 µM	>100 µM	>100 µM	>100 µM
AD-2009	Epidemic	>100 µM	25 µM	25 µM	>100 µM	>100 µM	>100 µM
CJ2009	Epidemic	>100 µM	25 µM	25 µM	1.56 µM	>100 µM	>100 µM
17-2009	Non-epidemic	>100 µM	25 µM	>100 µM	>100 µM	>100 µM	>100 µM
Bb2009	Non-epidemic	>100 µM	25 µM	>100 µM	>100 µM	>100 µM	>100 µM

5.2.2 Optimisation of culture conditions for compound screening

5.2.2.1 Pseudomonas aeruginosa

The goal of this chapter was to identify anti-bacterial agents from novel compound libraries (described in detail on page 86). Initial optimisation sought to determine the ideal conditions for screening in 384-well plates, in order to maximise the number of compounds that could be screened. The lab strain PAO1 and epidemic strain AES-1R were initially used, as these strains are commonly used in the laboratory of our collaborator Dr Jim Manos (Infectious Diseases and Immunology, University of Sydney). Bacteria were added at different concentrations to 384 well plates (50µL), and left for 4, 7, 24 or 48 hours. After each incubation resazurin was added to the wells, fluorescence measured at ex544/em590 and z-factor calculated. As shown in Table 5.2, the most appropriate conditions were a bacterial concentration of OD_{600} 0.001 with one day incubation, which displayed a z-factor of 0.79 and 0.93 for PAO1 and AES-1R respectively. The higher the z-factor value, the higher the quality of data those conditions will produce (Zhang et al., 1999), therefore these conditions were selected on the basis of being the highest z-factor of the conditions tested (see 2.6.1 for further information on 'z' factor).

After initial testing, it was necessary to determine if the conditions were also appropriate for the selected strains from Table 5.1. CJ2009 and Bb2009 were added at OD_{600} 0.001 to the wells of a 384-well plate, incubated for 24 hours, resazurin added and fluorescence measured. As shown in Table 5.2B, CJ2009 had a calculated z factor of 0.68 and Bb2009 had a z factor of 0.81. Therefore these conditions were appropriate for screening.

Table 5.2. Optimisation of *P. aeruginosa* conditions for compound screening. The values shown are Z-values.

A

Strain	Bacterial concentration	Incubation time (hours)			
		4	**7**	**24**	**48**
PAO-1	**0.001**	0.684345	0.587224	**0.792348**	-0.94608
	0.005	0.682513	0.707595	0.776249	-1.63942
	0.01	0.762894	0.5008	0.76305	-0.42682
AES-1R	**0.001**	0.65108	0.661194	**0.932552**	-0.65614
	0.005	0.446265	0.820282	0.812932	0.566818
	0.01	0.719872	0.838072	0.661307	-0.07574

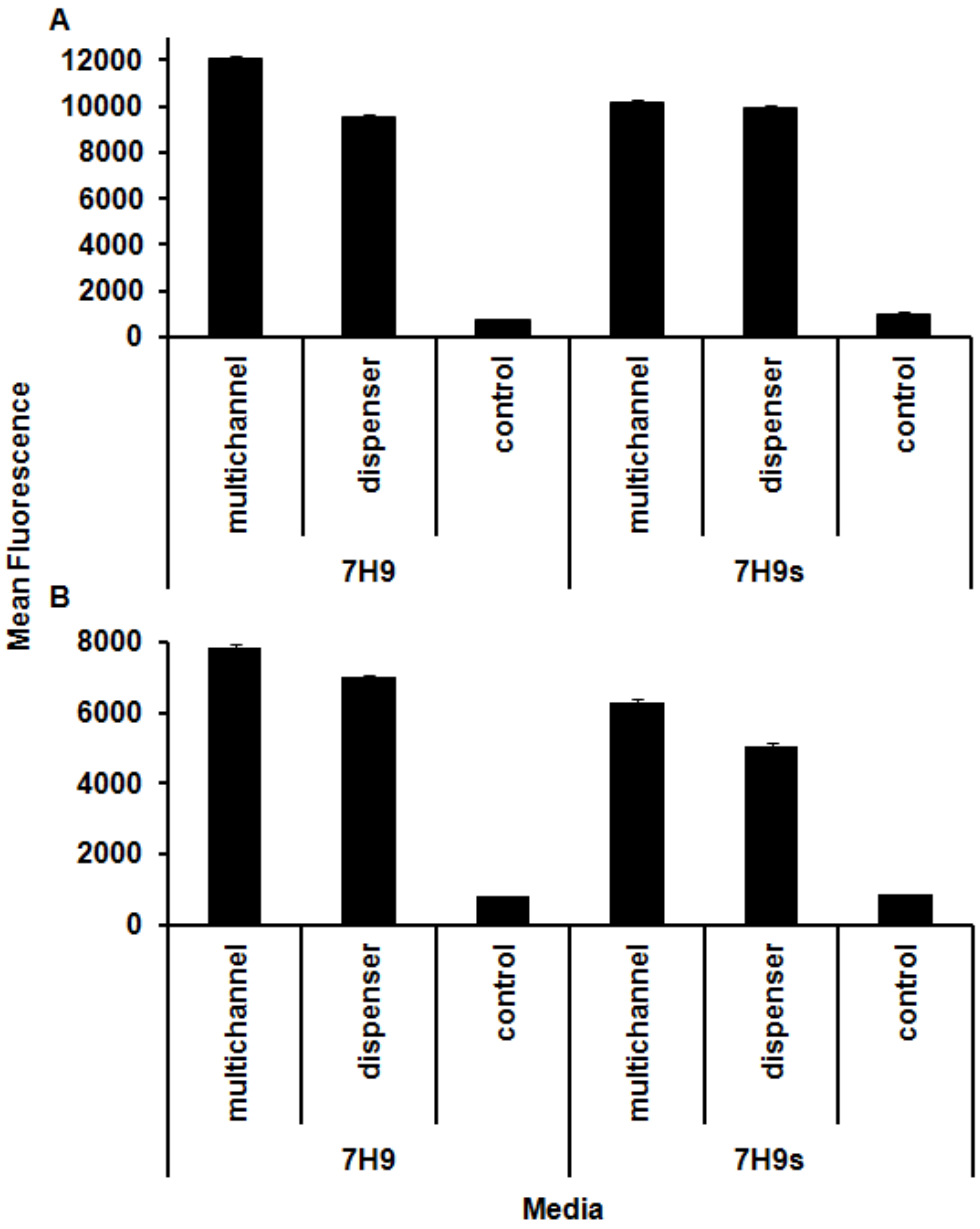
B

Strain	Conditions	Z factor
CJ2009	OD_{600} 0.001	0.686875
Bb2009	24 hours	0.812283

5.2.2.2 Mycobacteria

Mycobacteria can be fastidious when grown in 384 well plates due to the reduced volume of nutrient broth (7H9 medium) and their slow growing time. Therefore the optimum conditions for screening needed to be determined. Firstly, the most appropriate media and method of dispensing was elucidated. 7H9s is a specialised medium containing toloxypol as a detergent in place of tween 80, and assists growth of mycobacteria when growing in plates (correspondence with Dr N. West, University of Queensland). In the initial optimisation experiments *M. bovis* BCG and *M. avium* were diluted to an OD_{600} 0.001 and dispensed into wells either by an automated multichannel pipette or dispenser to determine which method resulted in the least level of variance. Plates containing bacteria were incubated for five days, after which resazurin was added and fluorescence measured. As shown in Figure 5.1, bacteria dispensed via the automated multichannel pipette exhibited the highest fluorescence reading in all conditions. Using 7H9 medium, the difference in fluorescence units between the multichannel and dispenser varied from 800 against *M. avium* to 2500 against BCG (Figure 5.1). The values of fluorescence were less using 7H9s medium, and the control values were higher (965 compared to 739 for BCG; Figure 5.1) indicating this medium was not as useful for screening methods. Subsequent experiments were carried out by diluting bacteria in 7H9 medium and dispensing solutions using an automated multichannel pipette.

The optimal screening conditions for both BCG and *M. avium* were then determined. This was done by incubating various concentrations of bacteria for 4, 5, 6 and 7 days, Resazurin then added to each well, fluorescence measured and the z-factor calculated. As shown in Table 5.3, the optimal condition for both mycobacterial strains was an OD_{600} 0.01 after incubating for 6 days, where the z-factor was 0.97



Strain	Media	Dispensing method	Mean fluorescence ± SEM
(A) BCG	7H9	multichannel	12077 ± 129
		dispenser	9508 ± 122
		control	739 ± 12
	7H9s	multichannel	10177 ± 82
		dispenser	9957 ± 98
		control	965 ± 174
(B) *M. avium*	7H9	multichannel	7856 ± 75
		dispenser	7009 ± 84
		control	793 ± 9
	7H9s	multichannel	6310 ± 97
		dispenser	5059 ± 97
		control	878 ± 7

Figure 5.1. Optimising conditions for use in mycobacterial High-Throughput screen. *M. bovis* BCG (A) and *M avium* (B) was grown and diluted to OD_{600} 0.001 in control media (7H9) or specialised growth media (7H9s). Bacteria were dispensed into 384 well plates either via an automated multichannel pipette or robotic dispenser. Control wells contained TDW only. After five days, resazurin was added and fluorescence measured. Optimal conditions were determined as that exhibiting the greatest fluorescence level compared to control, with the least error.

for *M. avium* and 0.67 for BCG, indicating these conditions allow for an effective comparison between bacterial presence and that in which a drug may effectively restrict growth.

5.2.3 Screening of compound libraries to determine potential pharmaceutical targets

We obtained a library of 1920 compounds from the Queensland Compound Library for screening against the pathogens of interest. Approximately 10% of the compounds were natural products sourced from the Eskitis Institute for Cell and Molecular Therapies, Griffith University, and had been used in previous anti-microbial screens (Professor Ron Quinn, personal correspondence). The compounds spanned a diverse set of compounds including various alkaloids, modified peptides, sesqui- and tri-terpenes and polyketides. The remaining compounds are a structurally diverse set of synthesized small molecules provided by CSIRO Materials Science and Engineering. To reduce any bias in the screen the compounds were coded (provided as sequential numbers) and thus compound identity and structure of compounds was not disclosed. This information was provided once any 'hits' were identified.

Compounds at a final concentration of 50 µM were added to individual wells of a 384-well plate on replicate plates. *P. aeruginosa* was added at OD_{600} 0.001, incubated for 24 hours, resazurin added and fluorescence measured. *Mycobacteria* was added at OD_{600} 0.01 and incubated for 6 days. A compound was considered effective if it showed more than 50 % bacterial growth reduction compared to non–treated controls on both replicate plates.

Table 5.3. Optimisation of mycobacterial strains for high throughput screening. The values shown are Z-values.

strain	bacterial concentration	Incubation time (days)			
		4	**5**	**6**	**7**
M. avium	**0.001**	-0.18295	0.126353	0.506048	
	0.005	0.273328	0.401568	0.733727	
	0.01	0.585773	0.465752	**0.969292**	
BCG	**0.001**		-0.41659	-0.00631	-0.04007
	0.005		0.270119	0.583213	0.556431
	0.01		0.442121	**0.670283**	0.666705

As shown in Figure 5.2, there were six hits in total out of 1920 for the two strains of *P. aeruginosa* tested; four for CJ2009 (Figure 5.2A) and two for Bb2009 (Figure 5.2B). The two compound 'hits' for Bb2009 were also identified in CJ2009 (468620 and 465895), while compounds 465697 and 296405 displayed no activity against Bb2009, further highlighting the high antimicrobial resistance of this strain (see Table 5.1). Due to the overlap, the success rate for this screen was only 0.2 %. Compounds 46820 and 465895 displayed almost complete inhibition of growth at 50 μM, while 465697 and 296405 displayed an intermediate level of inhibition with bacterial survival of 44.9 % for 465697 and 31.9 % for 296405 (Figure 5.2A).

The same compounds against *M. avium* yielded 0.1 % success, with only 2 'hits'. Despite a number of compounds having a survival level below threshold, this was not replicated in both plates, therefore only compounds 467142 and 296385 were considered true anti-*M. avium* compounds (Figure 5.3). Only *M. avium* 104 was screened against the entire library as the mycobacterial representative due to the optimisation results for the 384-well plate format being more reliable in this strain compared to BCG (data not shown).

5.2.4 Determination of activity of anti-bacterial compounds

5.2.4.1 Compounds inhibiting growth of *Pseudomonas*

To determine the effectiveness of the compounds identified in section 5.2.3 the MIC_{90} was calculated. The four compounds selected from the screen in Figure 5.2 were serially diluted with a starting concentration of 50 μM and tested against the relevant strains of *Pseudomonas* CJ2009 and Bb2009.

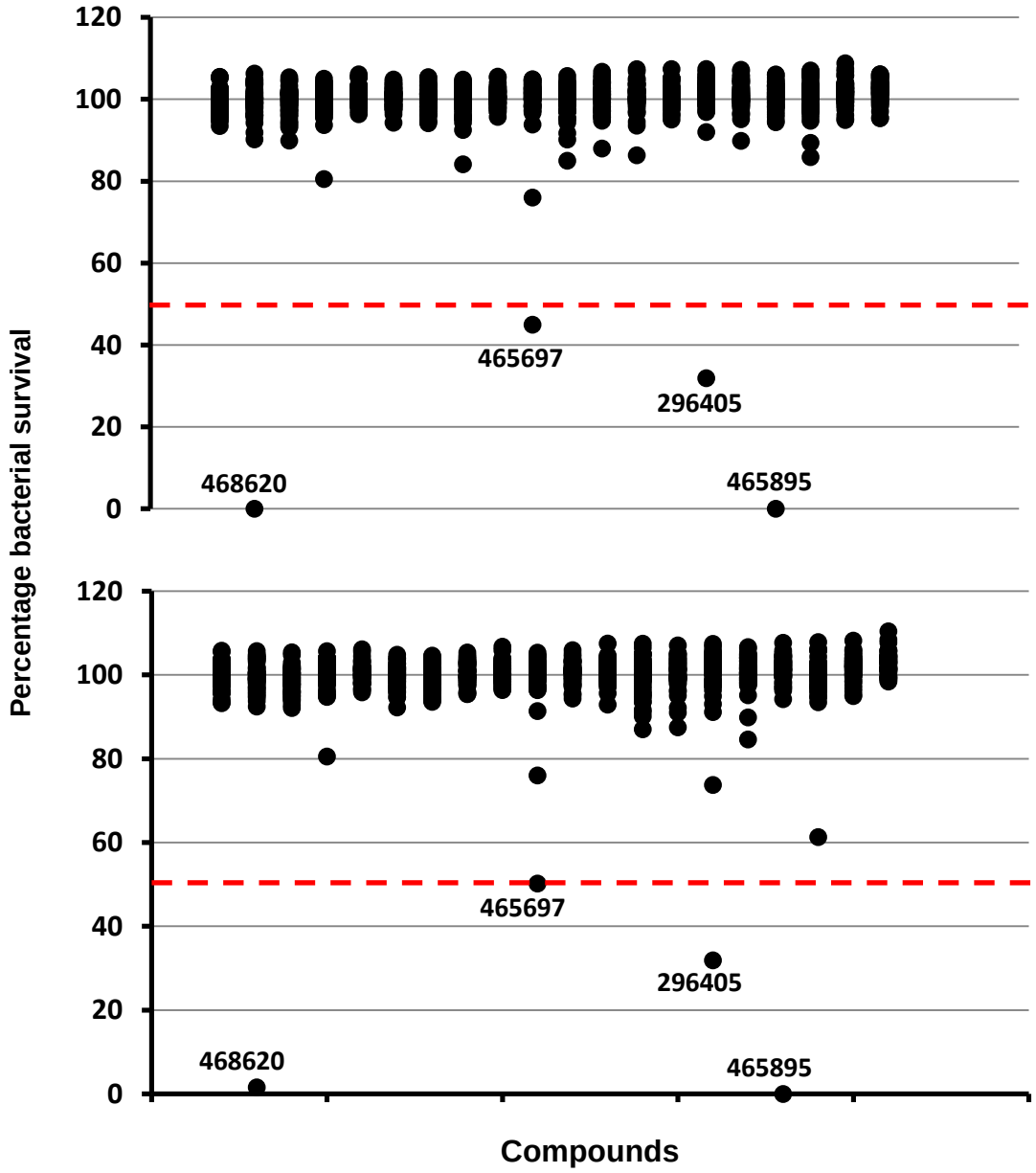


Figure 5.2a. Screening of compounds against *P. aeruginosa* CJ2009. Compounds were aliquoted into single wells on replicate 384 well plates and strain CJ2009 culture added at OD_{600} 0.001. Resazurin (0.05%) was added 24 hours post-treatment and fluorescence measured. Bacterial survival was calculated as a percentage compared to untreated control. A successful "hit" was when both replicate plates showed more than 50% restriction of growth by a single compound (red line). Graphs are replicate plates.

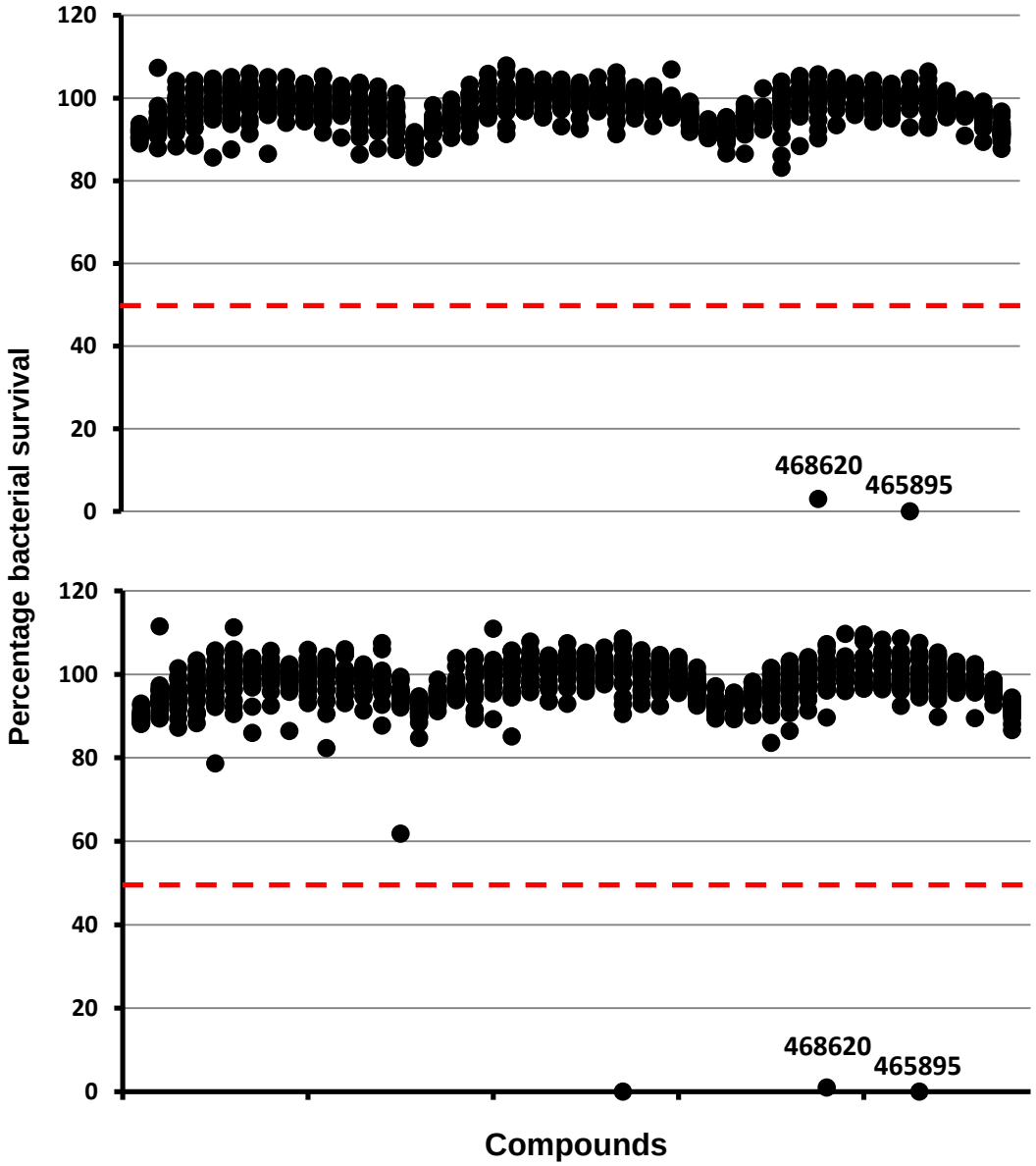


Figure 5.2b. Screening of compounds against *P. aeruginosa* Bb2009. Compounds were aliquoted into single wells on replicate 384 well plates and strain Bb2009 culture added at OD_{600} 0.001. Resazurin (0.05%) was added 24 hours post-treatment and fluorescence measured. Bacterial survival was calculated as a percentage compared to untreated control. A successful "hit" was when both replicate plates showed more than 50% restriction of growth by a single compound (red line). Graphs are replicate plates.

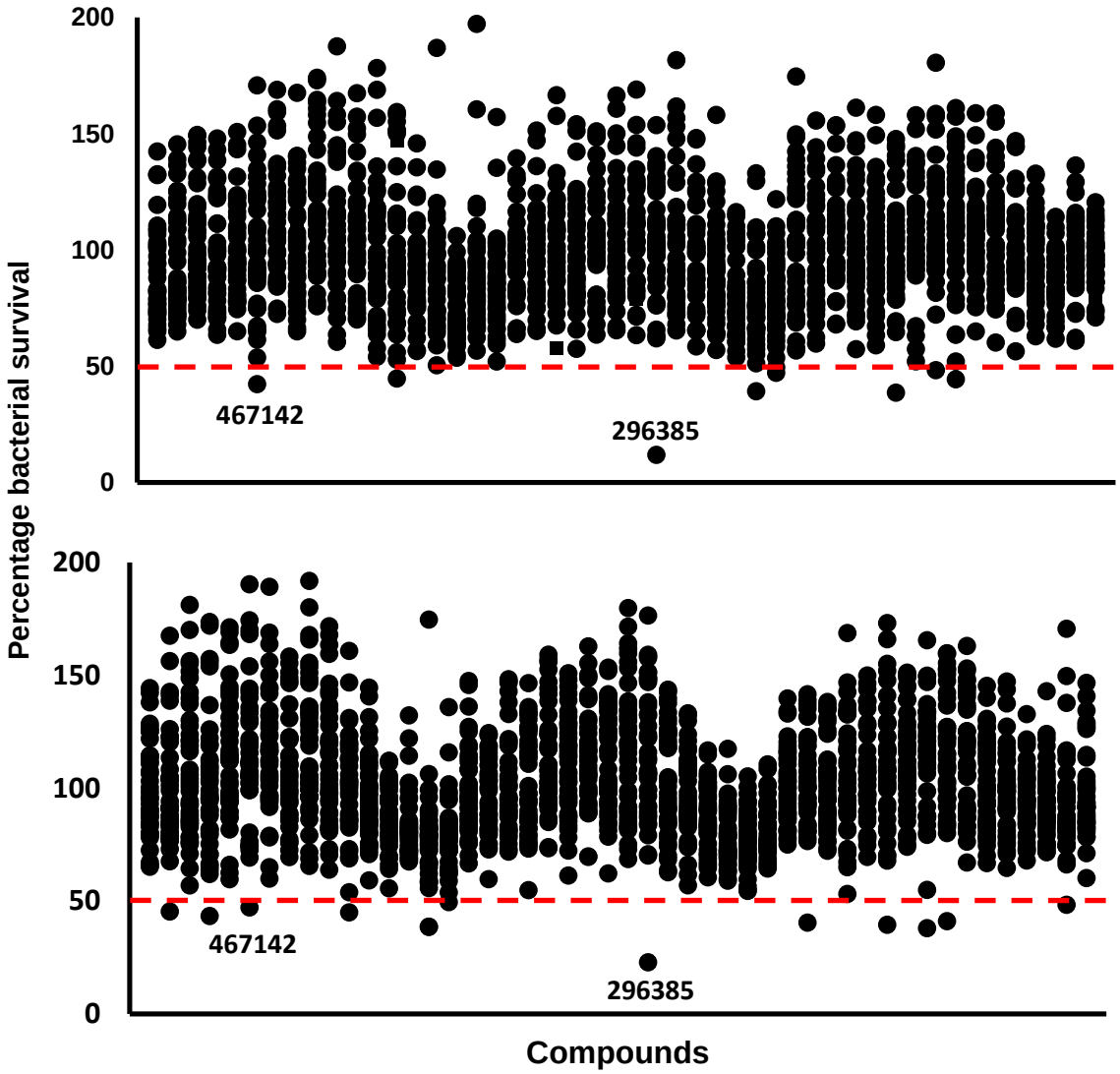


Figure 5.3. Screening of compounds against *M. avium*. Compounds were aliquoted into single wells on replicate 384 well plates and *M. avium* culture added at OD_{600} 0.01. Resazurin (0.05%) was added 24 hours post-treatment and fluorescence measured. Bacterial survival was calculated as a percentage compared to untreated control. A successful "hit" was when both replicate plates showed more than 50% restriction of growth by a single compound (red line). Graphs are replicate plates.

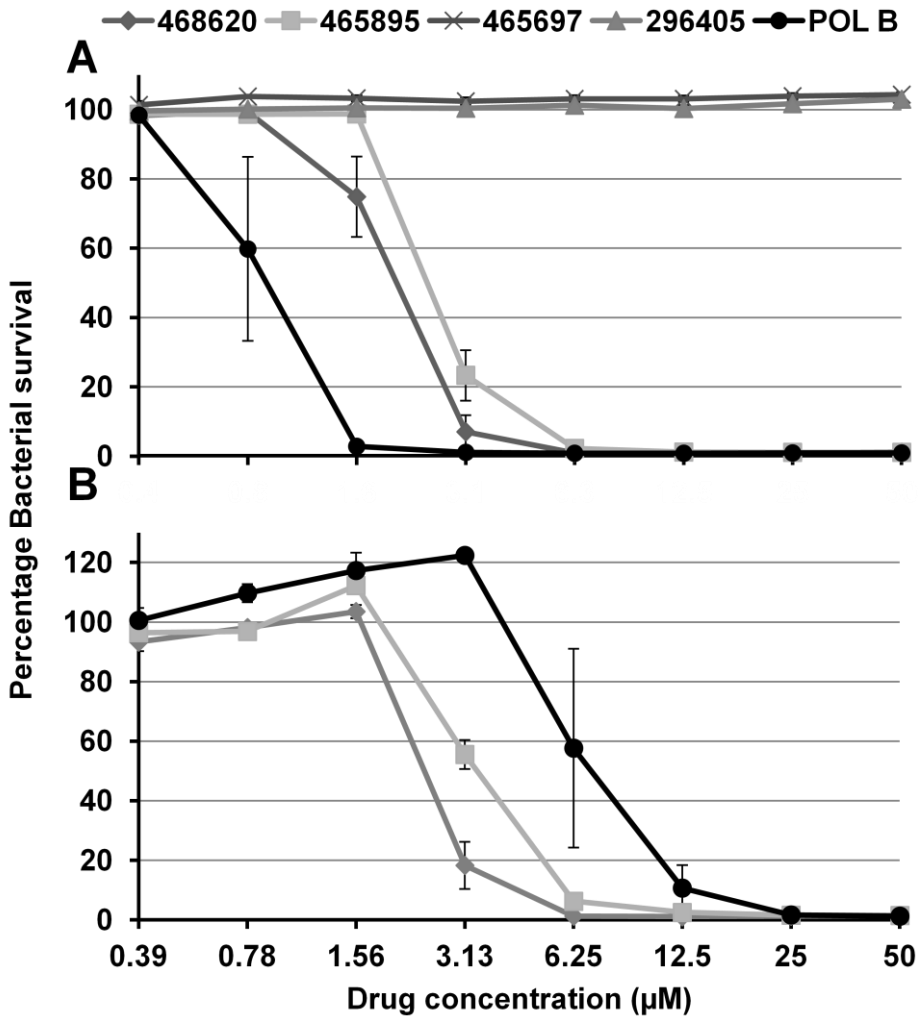
Polymyxin B was used as a control due to the susceptibility of *Pseudomonas* isolates to this antibiotic compared to others tested (Table 5.1). Figure 5.4A shows the survival curves for the four compounds against CJ2009. Both 468620 and 465895 displayed strong anti-*Pseudomonas* activity with MIC_{90} of 3.13 µM and 6.25 µM respectively (Figure 5.4C), while compounds 4655697 and 296405 were not active at the concentrations tested (Figure 5.4A,C). Against Bb2009 (Figure 5.4B) both 468620 and 465895 maintained their activity, both displaying an MIC_{90} of 6.25 µM (Figure 5.4C). Due to their efficacy compound 468620 and 465895 were selected for further analysis.

5.2.4.2 Compounds inhibiting growth of *M. avium*

Active compounds from the *M. avium* screen in Figure 5.3 were serially diluted from a starting concentration of 50 µM to determine the MIC_{90}. Figure 5.5A shows the survival curve of the two compounds against *M. avium*. Compound 296385 had an MIC_{90} of 12.5 µM, and compound 467142 had an MIC_{90} of 25 µM (Figure 5.5B). As these compounds were effective within the range tested indicating some level of activity against *M. avium* they were both selected for further study.

5.2.5 Determining the efficacy of compounds against patient sputum samples

As the CF lung is infected by an array of bacteria, broad spectrum antibiotics would be useful during treatment. Therefore, the effective compounds against *P. aeruginosa* were tested against sputum samples obtained from the RPA Respiratory Clinic and prepared for culturing as previously described (Hu et al., 2013). No bacterial typing was conducted on the samples as they were tested immediately

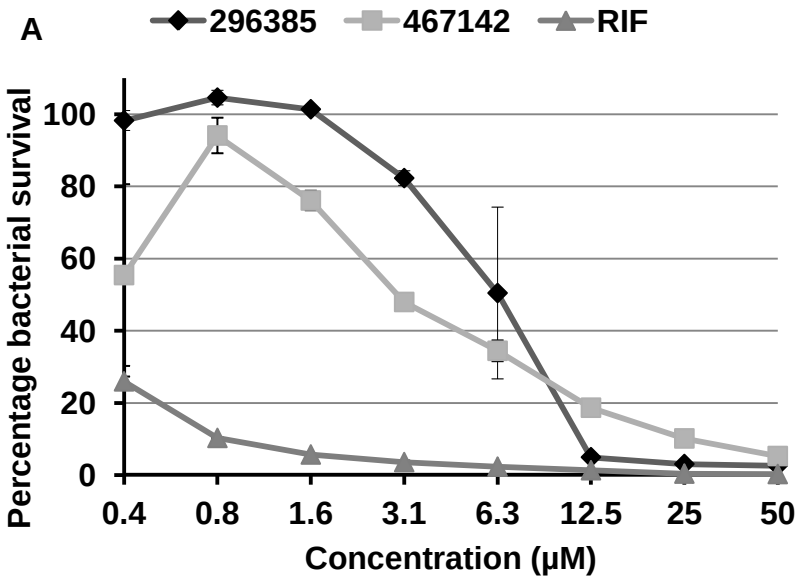


C

Compound	CJ2009	Bb2009
468620	3.13 µM	6.25 µM
465895	6.25 µM	6.25 µM
465697	>50 µM	
296405	>50 µM	
PolB	1.56 µM	12.5 µM

Figure 5.4. MIC_{90} of selected compounds against *P. aeruginosa* strains. Compounds were serially diluted in 96 well plates and CJ2009 (A) and Bb2009 (B) were added at OD_{600} 0.001. Resazurin was added 24 hours post-treatment and fluorescence measured. Survival was calculated as a percentage compared to an untreated control. MIC_{90} (C) was calculated as the lowest concentration that restricted more than 90% bacterial growth. Data are representative of two independent experiments.

A



B

Compound	MIC_{90}
296385	12.5 µM
467142	25 µM
RIF	0.8 µM

Figure 5.5. MIC_{90} of selected compounds against *M.avium*. (A) Compounds were serially diluted in 96 well plates and bacteria were added at OD_{600} 0.001. Resazurin was added 5 days post-treatment and fluorescence measured. Survival was calculated as a percentage compared to an untreated control. MIC_{90} (B) was calculated as the lowest concentration that restricted more than 90% bacterial growth. Data are representative of two independent experiments.

after collection, so the bacterial population was unknown. However it was assumed the bacterial makeup was that of the common CF lung pathogens (Foundation, 2012).

Experimentation was carried out as for other MIC studies. Four patient samples were cultured overnight and added to wells containing serial dilutions of the two most effective compounds, 468620 and 465895 (50 µM starting concentration). Compound 465895 was active against all four samples (MIC_{90} range of 3.13 to 12.5 µM; Figure 5.6) and in 3 of the samples displayed inhibition greater than Polymyxin B (Figure 5.6A, C-D). Compound 468620 was also effective however its inhibitory action was less than compound 465895 (MIC_{90} range of 6.25 to >50µM; Figure 5.6). These results suggest these compounds may have activity as broad spectrum agents against CF pathogens.

5.2.6 Testing the toxicity of compounds to determine viability as a treatment

Many compounds derived from screening programs display generalised toxicity against both microbial and mammalian cells, and are thus unsuitable for further development (Payne et al., 2007). The toxicity of the compounds was next assessed in a murine macrophage-like RAW267.4 cell line (Ralph and Nakoinz, 1977) and the human monocytic THP1 cell line (Tsuchiya et al., 1980). Compounds were serially diluted 1 in 5 from a starting concentration of 25 µM and 7 days post-treatment cell viability determined. Compound 465895 was toxic at all concentrations except 0.2 µM when tested against RAW267.4 cells (Figure 5.7A), and against THP1 cells caused an almost complete reduction in cell viability at the two highest concentrations but was non-toxic at concentrations lower than 5 µM (Figure 5.7B). Compound 468620 exhibited approximately a 20 per cent reduction in cell viability at 25 µM and 10 per cent reduction at 5 µM in both cell types, however was non-toxic at lower concentrations

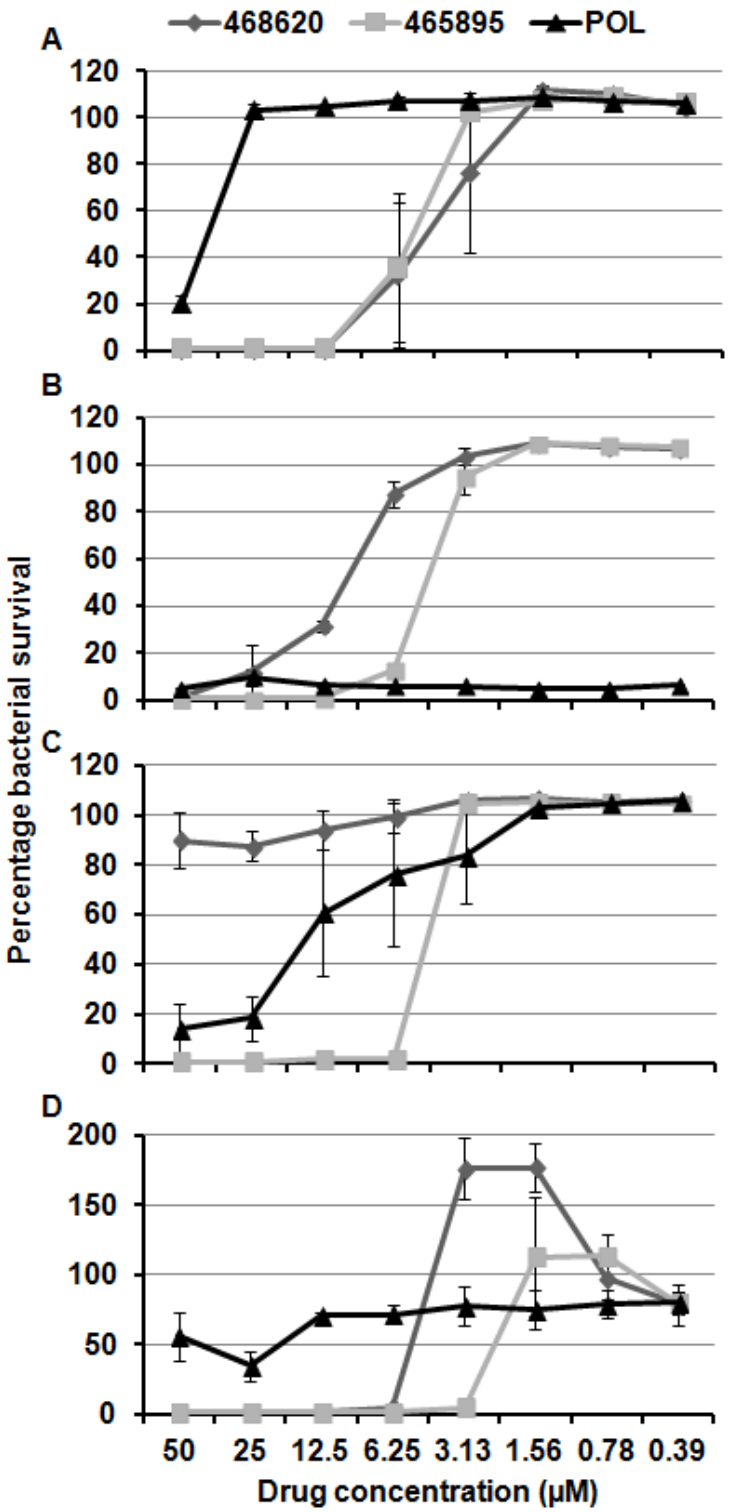


Figure 5.6. Effect of compounds against patient sputum samples. Sputum samples collected from CF patients were cultured overnight, exposed to compound for 24 hours and resazurin (0.05%) added. The legend corresponds to compounds 468620 (620) and 465895 (895). Fluorescence measured and survival calculated as a percentage compared to an untreated control. (A-D) represents different patient samples and is representative of two independent experiments.

(Figure 5.7). Treatment with 296385 caused almost complete cell death at 25 μM in both cell types (Figure 5.8). A 15% of RAW267.4 cell viability (Figure 5.8A) and 30% reduction of THP1 cell viability (Figure 5.8B) was seen for compound 296385, with no toxicity observed at the lowest concentrations for both cell lines. For compound 467142, treatment of RAW267.4 cells at concentrations greater than 1 μM caused almost complete cell death, with no effect seen at the two lowest concentrations (Figure 5.8A). Against THP1 cells, a similar effect was seen (Figure 5.8B), however at 5 μM there was only 40 per cent reduction in cell viability as opposed to >95% in RAW267.4 cells. Therefore these compounds displayed a generalised pattern of toxicity against the mammalian cells tested at concentrations greater than 1 μM.

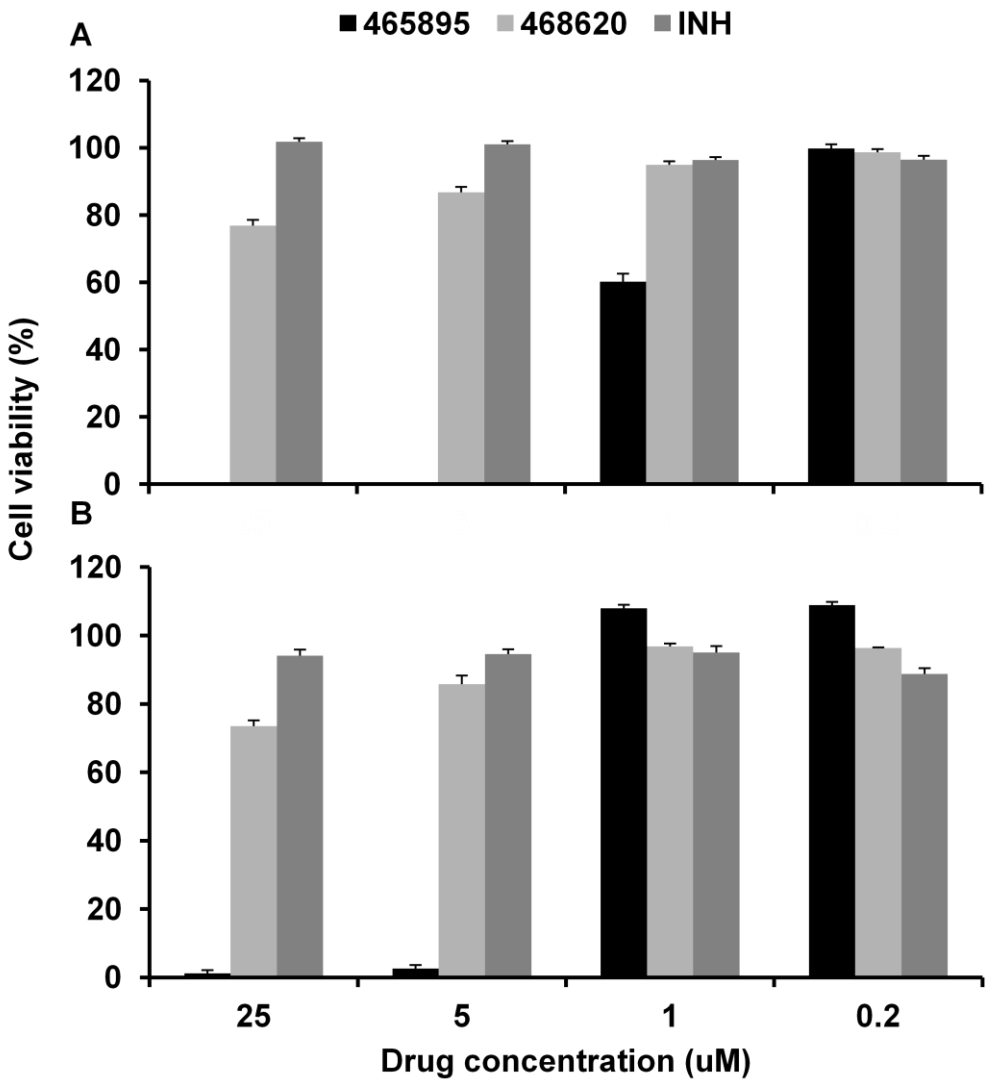


Figure 5.7. Toxicity of compounds effective against *P. aeruginosa*. One x10^5 RAW264.7 cells (A) or THP1 cells (B) were seeded into 96 well plates. Compounds were serially diluted and added to the wells, resazurin was added 7 days post-treatment and fluorescence measured. Toxicity was calculated as percentage cell viability compared to an untreated control.

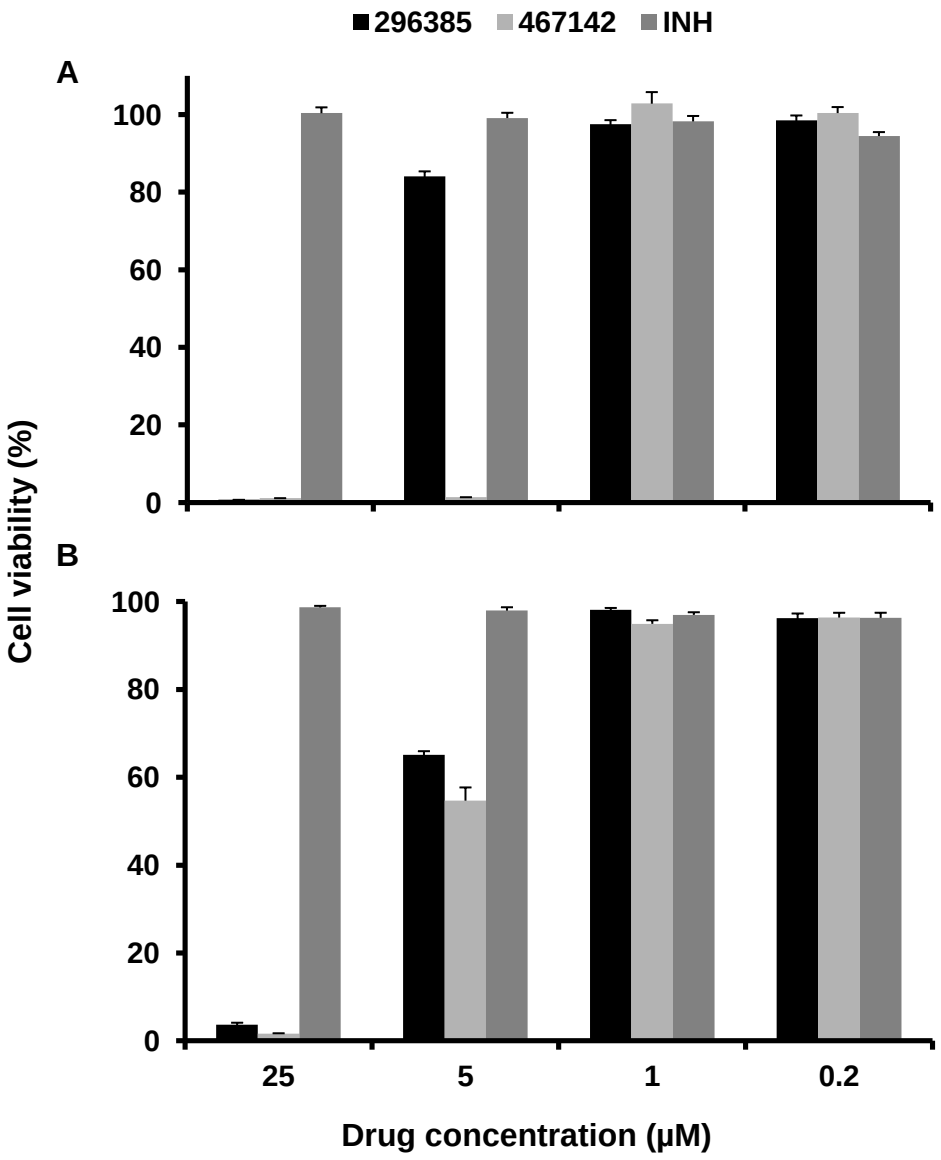


Figure 5.8. Toxicity of compounds effective against *M.avium*. One x10^5 RAW264.7 cells (A) or THP1 cells (B) were seeded into 96 well plates. Compounds were serially diluted and added to the cells, Resazurin was added 7 days post-treatment and fluorescence measured. Toxicity was calculated as percentage cell viability compared to an untreated control.

5.3 DISCUSSION

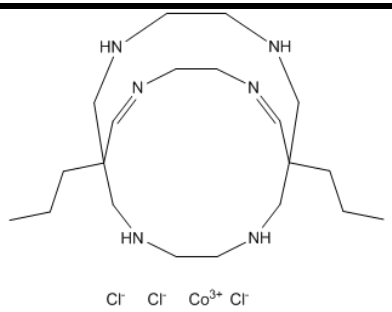
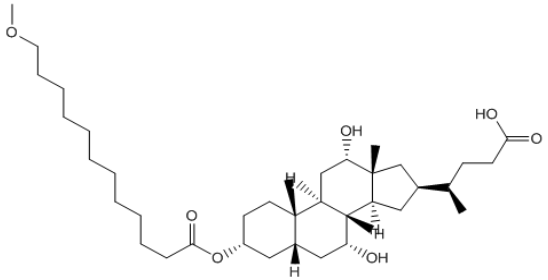
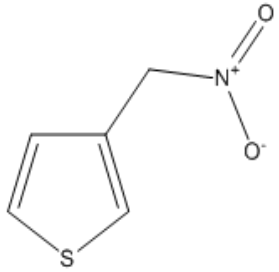
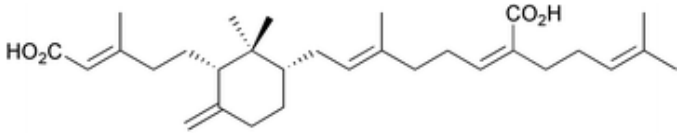
High throughput screening is rapidly becoming a useful and efficient tool in the search for new compounds against various diseases. It allows researchers to test a large number of compounds in a relatively short amount of time increasing the likelihood of finding a new compound for therapeutic use. This study sought to discover new antimicrobial compounds that were effective against highly resistant, difficult to treat bacterial species.

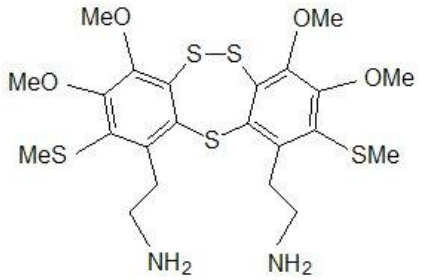
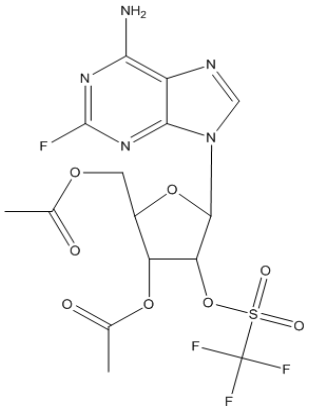
Initial experiments were undertaken to identify strains of resistant *P. aeruginosa*. In early infection, *P. aeruginosa* can be successfully treated with antibiotics (Hare and Cordwell, 2010) however during chronic infection the bacteria are typically unresponsive to chemotherapy (Eberl and Tummler, 2004). Due to an increase in bacterial resistance it was necessary to focus on chronic isolates as compounds that were efficacious against such strains would be more important clinically. Screening of the compound libraries against both *M. avium* and *P. aeruginosa* resulted in 8 potential candidates from an original compound number of 1920. This compares favourably with other studies; for example a review of the screening program at GSK concluded that 14 HTS (approximately 260 000-530 000 compounds per screen) was required to obtain one lead compound, however highly stringent gating criteria were placed on compounds progressing through the GSK screening program (Payne et al., 2007). Against *M. tuberculosis*, a similar success rate to that in this study (0.4 per cent) was achieved from 20 000 novel small molecules when tested at a single concentration of 25 μM (Stanley et al., 2012). Other studies can have higher success rates such as one against *Naegleria* (Debnath et al., 2012), identifying 1.6 per cent from 910 compounds. While the success rate of HTS studies is dependent on the makeup of the compound library used, the current study indicates the results presented in this chapter agree with previous studies identifying anti-bacterial compounds within diverse compound libraries.

In the screens presented in this chapter there were two compounds, 468620 and 465895, that restricted the growth of the two different strains of *P. aeruginosa* studied. This indicates that these particular compounds are broad spectrum or highly active, particularly due to the fact that Bb2009 was the most resistant non-epidemic isolate when tested for antibiotic sensitivity (Table 5.1). Their effect against patient sputum samples indicates they could have broad spectrum activity due to the variety of bacteria in CF sputum that is being restricted in this assay. Whilst the sample populations were not characterised in this study it is known that the lung environment of a CF patient can include bacteria such as *Haemophilus influenzae*, *Staphylococcis aureus*, and *Burkholdaria cepacia* (Foundation, 2012). Patients eventually succumb to the *P. aeruginosa* infection in the chronic stages of disease, however early stages of disease have a wide variety of infecting pathogens. Testing of the compounds in this assay may reflect how these compounds may behave in an *in vivo* setting within the lung. However as the sputum samples were cultured overnight before being treated, there is the possibility that some bacteria are out-competing other species and the compounds may be active against a restrictive set of CF pathogens. Testing the compounds against individual cultures of bacteria found in the CF lung would be a more accurate way of determining whether they are broad spectrum antibiotics. Alternatively, culturing sputum samples and then analysing the sample to determine the bacterial population could also be performed.

Testing of the MIC_{90} showed that compounds 465697 and 296405 that were identified in the initial screen were no longer effective when re-assayed for the determination of the MIC (Figure 5.4). This could be due to the fact that in the screening assays compounds 465697 and 296405 only displayed an intermediate level of inhibition in the initial screen (Figure 5.2a). Despite appearing as ‘hits’ on both replicate plates, the fact that neither compound completely restricted bacterial growth at 50 µM could

Table 5.4. Overview of compound anti-bacterial activity and toxicity.

Compound	Structure	MIC_{90}	Toxicity MIC_{90}	Selective index[c]
468620		3.13 µM[a]	1 µM	0.319488818
465895		6.25 µM[a]	1 µM	0.16
465697		>50 µM[a]	ND[d]	ND
296405		>50 µM[a]	ND	ND

296385		12.5 µM[b]	1 µM	0.08
467142		25 µM[b]	1 µM	0.04

[a] MIC_{90} against Pseudomonas strains CJ2009

[b] MIC_{90} against *M. avium* 104

[c] calculated as MIC_{90} against macrophages (less than 10 per cent reduction in cell viability)/MIC_{90} against bacteria. Compounds with a selective index of >1000 are considered promising candidates for clinical development (Haagsma et al., 2009)

[d] ND, not determined

account for the loss of activity in subsequent experiments, possible due to the compounds being unstable compared to the other hits identified in this study. Decoding of compounds identified 465697 as a small molecule with a molecular weight of 143.16. It has a nitrate group, which when in a cellular environment could potentially be cleaved from the core structure resulting in a compound that is too simple to function as an antimicrobial. Compound 296405 was identified as Mispyric acid which inhibits DNA polymerase β. A DNA polymerase β is found in mammalian cells and is involved in base excision repair (Mizushina et al., 2005). Inhibitors have previously been developed to target this protein as a potential treatment for cancer (Barakat et al., 2012). This compound may have shown limited activity against bacteria due to the lack of a specific target within in a bacterial cell, and instead displaying generalised toxicity. Additionally, there are two carboxylic acid groups which could deprotonate, thus making passage through the cell membrane difficult and preventing the compound from directly acting on the bacteria.

Compound 467142 was effective against *M. avium* at 25 μM yet displayed toxicity at 5 μM against mammalian cells. The compound has a nucleotide-like structure (Table 5.4). Currently, nucleotide analogs have been used in the treatment of herpesvirus and hepatitis B. For example Famciclovir, which is a prodrug for penciclovir, is activated by viral thymadine kinase and inhibits viral DNA polymerase (Mubareka et al., 2010). The prodrug is also able to rapidly diffuse into both viral and host cells (Mubareka et al., 2010). Compound 467142 may work in a similar manner and its relative lack of effectiveness could be due to related compounds displaying activity primarily against viral DNA. As a nucleotide analog it may also readily diffuse into cells and affect host DNA synthesis or repair, thus accounting for the toxicity.

Compound 296385, or Lissoclinotoxin F, is predicted to be an inhibitor of the PI3-K/AKT/mTOR cellular signally pathway (Davis et al., 2003). It has previously been tested for biological activity against tumour cell lines and microbes, showing some effect against fungus and marine bacteria (Liu et al., 2005). The most interesting result was against the tumour cell line HL60, which restricted cell proliferation at 0.23 μM (Oda et al., 2007). If such a low concentration is required to restrict tumour cell growth, this could account for the toxicity levels seen at concentrations greater than 1 μM is this study. As this compound may target cell signalling pathways absent from or poorly conserved from bacterial cells, this may be a reason for the lack of strong activity against *M. avium* (Figure 5.5).

Analysis of compound 465895 indicates it is a steroid analog. It has a long fatty acid chain which, because of the lipophilic nature of this structure, could penetrate cells with no specificity to a particular cell type, mammalian or microbial. The steroid-like structure independent of the fatty acid chain has been studied as an aromatase inhibitor in the treatment of breast cancer, exhibiting an IC_{50} of 0.45-0.75 μM (Varela et al., 2013, Amaral et al., 2013). If such a low dose is required for the inhibition of breast cancer cells, the concentrations tested in this study could account for the toxicity observed against THP1 cells, resulting is a very low selectivity index (Table 5.4). Whilst the fatty acid structure may help with cell penetration, the effect of the steroid moiety could negatively affecting host cells.

The final compound, 468620, contains a metal binding site. Metal complexes have been used in the treatment of cancer, such as the chemotherapy cisplatin, although the metal is generally platinum (Bruijnincx and Sadler, 2008). 468620 contains cobalt which although found naturally in vitamin B can have various toxic effects such as cardiomyopathy, haematological, neurological, reproductive and dermatological effects (Paustenbach et al., 2013). Experimental studies have shown that cobalt can interfere with DNA repair and can cause DNA cross-linking, damage and sister-chromatid exchange

(Jomova and Valko, 2011). The resulting toxicity in this study could be explained by the effect of cobalt. Despite the compound having an MIC_{90} relatively similar to the MIC calculated against mammalian cells, and the selectivity index is low (Table 5.4). The activity of the compound could also be non-specific which would account for the similar level of bacterial and cellular toxicity in this study.

This study showed that whilst high-throughput screening results in a greater number of potential compounds in a short period of time, larger screens may be required to potentially identify compounds of interest (Payne et al., 2007). Despite most of the selected compounds exhibiting useful levels of efficacy in this study, the selectivity index (SI) calculated for all compounds was less than 1. Compounds with a SI of more than 1000 are considered promising candidates for further clinical drug development (Haagsma et al., 2009) therefore through this fact alone no compound was selected for further study. None-the-less, this chapter has identified a subset of novel structures that display some anti-bacterial activity, and future work may involve 'decoupling' toxicity from their anti-microbial effect for potential use in humans.

Chapter 6: General discussion

Of thousands of drug candidates tested every year in research labs and pharmaceutical companies, only a fraction are approved for clinical development (CISCRP, 2013). Of those that do reach late clinical trial stage, on average only 1 in 6 make it to market (DiMasi et al., 2010). In addition to project attrition, time is a key challenge for market entry. It can take approximately 7 years to get a candidate drug through the trial phases, and in the case of cancer and neuropharmacologic drugs, it can take up to nine years from the start of human trials to market approval (Kaitin, 2010).

December 2012 was a milestone for tuberculosis treatment with the first new drug being approved by the FDA in over 40 years (Goldenberg, 2013). Bedaquiline, an ATP-synthase inhibitor, is highly efficacious, with results indicating that culture conversion is more rapid (in 14 days) and more prevalent than the current therapeutic combination of rifampicin, INH, ethambutol and pyrazinamide (Diacon et al., 2013). However, it has serious toxicity concerns, including unexplained mortality and risk of QT prolongation (Chahine et al., 2013), underscoring the need to maintain the pipeline of new compounds.

Drug susceptible tuberculosis generally requires a 6-8 month treatment regimen. During this treatment course, patients can experience acute renal failure, thrombocytopenia, paresthesia, hepatitis, angina, and various gastrointestinal, nervous, cardiovascular and musculoskeletal problems. Drug resistant disease however, requires a 20 month regimen using less efficacious drugs with a higher toxicity profile (Zumla et al., 2013b). With the level of worldwide MDR cases increasing (W.H.O, 2012), it is important to develop new drugs that are highly efficacious, can reduce treatment length and are relatively non-toxic.

Recently, drug discovery has been target driven, which although valid in theory has resulted in less success in finding a lead compound due to the lack of correlation between enzyme inhibition and whole cell bacterial inhibition (Payne et al., 2007). Never-the-less, new approaches have been sought to overcome these problems. There are a number of ways in which drug development can occur. These can include; old compounds being re-synthesised with altered side chains to improve their potency or change their target site to improve target specificity; the development of new compounds with a specific target, for example ones that target a particular gene, protein or pathway within a pathogen; and high-throughput screening of a range of compounds, whether novel, for a different indication, or natural (Payne et al., 2007, Koul et al., 2011). The methods chosen by the researcher are dependent on available materials and the level of specificity desired for each compound tested. In this study all methods of drug discovery described above were utilised.

6.1 Iron chelation and mycobacteria

The use of iron chelators in chapter 3 fit two criteria for drug discovery. Iron is an obligate cofactor for over 40 genes encoded within the *M. tuberculosis* genome (Yellaboina et al., 2006) suggesting that targeting this growth determinant of the pathogen could affect its viability and hence the infective potential. The group of PIH derivatives tested are more potent than the commonly used DFO for clinical treatment; these derivatives have a high affinity for iron (Becker and Richardson, 1999) and are orally active (Yu et al., 2006), making them a desired class of compound to test against a pathogen that so heavily relies on iron for growth. As seen from this study, these compounds were effective and relatively safe when delivered to mammalian cells (Chapter 3). When tested *in vivo* these compounds significantly reduced bacterial CFU for both *M. avium* and *M. tuberculosis*, however there was no

significant difference compared to INH in this model, and thus the clinical potential for these compounds to 'supersede' the use of INH is uncertain, particularly as both ING and PCIH appear to share the same target (Chapter 3). Recently, new compounds have been discovered that target other steps in the biosynthetic pathway for mycolic acid production (Stanley et al., 2013) or directly target *inhA* (Encinas et al., 2014) may be beneficial to treat INH-resistant strains. However, it was shown in Chapter 3 that PCIH could inhibit growth at high bacterial loads, a result not observed with INH treated cells, and was more effective against *M. avium*. This suggests that PCIH is not simply acting in a similar fashion to INH, but may function as a lipophilic vehicle to penetrate both mycobacterial and mammalian cells and release INH intracellularly. Future studies may involve the development of derivatives with improved uptake into mammalian cells, which may improve the clinical benefit of these molecules. Indeed, as INH is a relatively simple compound that is inexpensive and exhibits a tolerable safety profile, derivatives with improved therapeutic potential (i.e. require less drug for efficacy) could become important components of new anti-tuberculosis treatment regimes.

6.2 A novel class of mycobacterial inhibitors

The second study (Chapter 4) looked at the antibacterial effect of metal-cyclam moieties; novel compounds that were varied in both structure and application. Lack of chemical diversity in potential antibiotics has led to poor outcomes in drug development (Koul et al., 2011). This lack of diversity is in part a result of the need for compounds to follow the "rule-of-five" (Lipinski, 2004, Koul et al., 2011). This rule suggests that antibiotics should have structures that lead to known properties in currently effective therapies (Lipinski, 2004), however, this rule does not predict if a drug is pharmacologically active. It also encourages bias towards particular structures which can limit the availability of certain

molecules during testing and may cause potentially effective compounds to be neglected on the basis of structure. This study is an example that compounds not fitting the "rule-of-five" model can be active against bacteria as is commonly seen with other antibiotics (Payne 2007); for example the effective compounds described had a molecular mass around 1000 daltons, where the rule of 5 states compounds should have a molecular mass less than 500 daltons. This further highlights the necessity to 'cast the net wide' to find new targets to treat pathogenic bacteria. As these structures appear to be novel antimicrobials, it is possible they may be active against drug-resistant strains of *M. tuberculosis*; future studies using such strains would further validate the clinical potential of these compounds.

The three most efficacious compounds could not be tested *in vivo* due to low solubility at the concentrations required for dose response testing in mice, irrespective of the delivery vehicle employed. Additional modifications may improve compound solubility and allow *in vivo* assessment of these compounds (see chapter 4.3). Such *in vivo* characterisation is critical for evaluating the potential of these compounds as antibacterials for use in humans. These *in vivo* studies would need to assess the pharmacokinetics, safety and efficacy of compounds against drug-sensitive and drug-resistant strains of *M. tuberculosis*, as has been detailed for other novel candidates (Pethe et al., 2013). Similarly, as a subset of these compounds display some efficacy against drug-resistant clinical *Pseudomonas* isolates, murine models of *P. aeruginosa* infection could also be employed (Ueda et al., 2005) to determine if these compounds display broad-spectrum activity.

6.3 Discovery of new drugs from diverse compound libraries

High-throughput screening allows otherwise unconsidered molecules to be tested rapidly against the pathogen of interest. This could reveal potent compounds against the bacterium of interest or permit a good starting point for future modification of anti-bacterial scaffolds (Cooper, 2013). Chemical modifications on a compound of interest could include the elimination of toxic side-chains, the addition of components to increase substrate range, or the addition of components that could be involved in glycosylation, methylation or phosphorylation (Schmidt, 2004). The modification of compounds to develop new, more effective anti-bacterials is both cost-effective and faster than traditional methods (Schwarz and Kehrenberg, 2006), hence appropriate for use in this study.

In the final study (Chapter 5) various compounds, both synthetic and natural, were screened resulting in 6 successful 'hits'. Unfortunately these yielded disappointing results due to compound toxicity and very low selectivity index scores (SI<1). The MIC_{90} of 4 of the identified compounds were at a level (6.25-25 μM) that modifications to 'decouple' toxicity from antibacterial activity could be investigated; however any resultant activity would need to be in the low μM to nM range to warrant additional assessment (Payne 2007).

One positive outcome of the screen described in Chapter 5 was the identification of compounds that inhibited either *P. aeruginosa* or *M. avium,* however no overlapping activity was identified. As a gram negative bacterium, *P. aeruginosa* has a distinct lipid bilayer characterised by the attachment of lipopolysaccharide to the outer membrane (Lam et al., 2011). *M. avium*, like other mycobacterial species, has a complex cell wall with long fatty acid chains and mycolic acid (Song et al., 2008). As the compounds in this screen did not overlap between species, it is tempting to speculate that these compounds may target diverse structures in the cell wall; identification of the true target of the

compounds would clarify this, however at this stage the relatively weak antibacterial activity and toxicity of these compounds does not warrant further investigation. None the less, this observation has parallels with results observed in Chapter 4, with different metal cyclam complexes having quite distinct activity against different strain of mycobacteria, further highlighting the potential complexity of the mode of action of novel inhibitors.

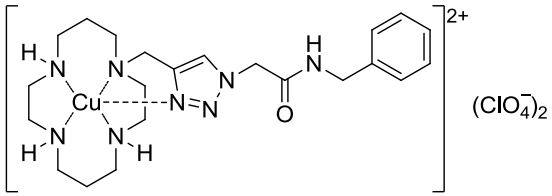
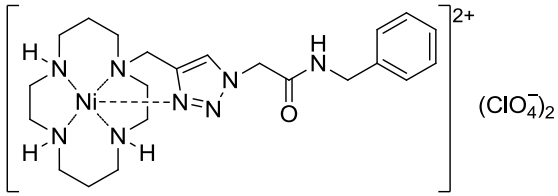
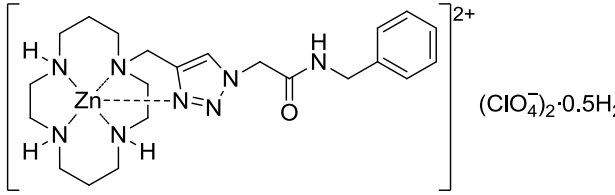
One issue with the screening performed in Chapter 5 was the relatively low success rate of finding active compounds (approximately 0.3 per cent). In a whole-cell assay against *M. tuberculosis* using 20,000 small molecules, the success rate was 0.4 per cent (Stanley et al., 2012), similar to that observed in our study. Cleary the relatively small subset of compounds used in our library may have limited the number of active compounds obtained, however it is possible the conditions used for the screen may not be optimal for identifying compounds of interest. There is growing interest in developing screening models that more accurately 'mimic' the conditions encountered by pathogenic mycobacteria within the host. These include the screening of non-replicating bacteria (Mak et al., 2012) or high-content imaging of *M. tuberculosis*-infected macrophages (Christophe et al., 2010). In the case of *P. aeruginosa*, the development of a specialised medium to mimic the CF lung environment (Fung et al., 2010) has meant that potentially screens could be performed on bacteria that more closely represent those found in a clinical setting. Results therefore would be more reminiscent of actual treatment outcomes. Such strategies may accelerate the identification of compounds for potential use in humans.

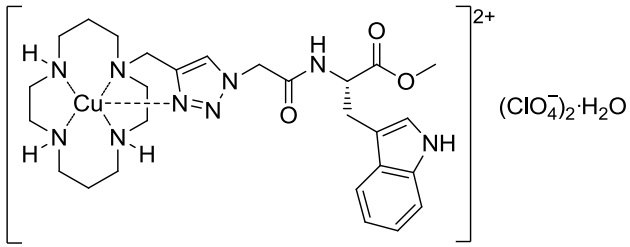
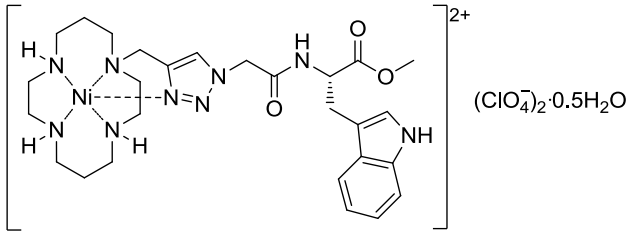
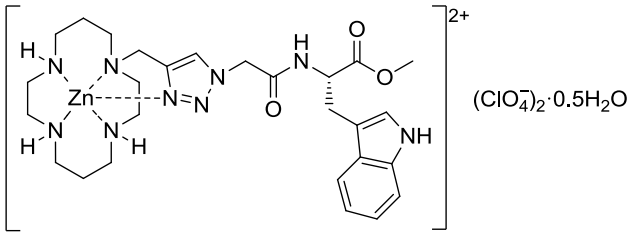
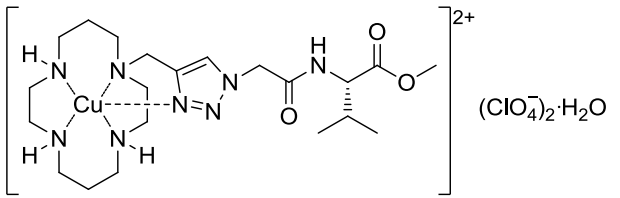
6.4 Concluding remarks

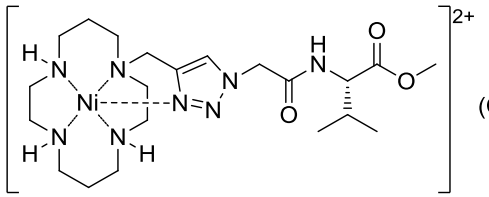
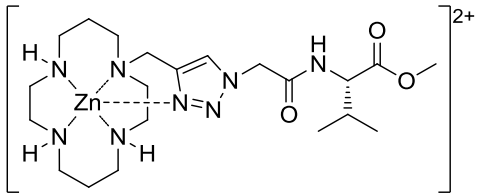
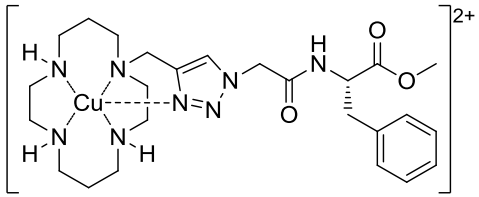
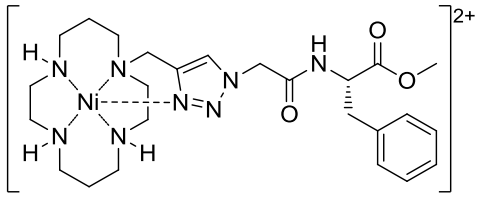
Despite the promise of some of the compounds identified here, this study is only the very first step in the drug development process. The treatment of *P. aeruginosa* is relatively straightforward in that any new compound simply needs to be novel and efficacious in order to combat the increasing resistance seen particularly in hospital settings. *M. tuberculosis* is a more complicated in that there are many criteria that new drugs for tuberculosis need to fulfil, including the ability to treat MDR-TB and XDR-TB cases, shortening treatment duration from the 6-8 months, reducing pill numbers, lowering frequency of pill intake, and drug-drug interactions, particularly in the case of HIV medications (Koul et al., 2011). These criteria have been established as the most important in the drug discovery field. However other factors that must be considered include the potential patients to be treated, the countries in which these drugs are to be implemented and possible future resistance. To combat resistance controls need to be put in place to restrict usage of new drugs so that overprescribing and monotherapeutic treatment is prevented. This requires education and assistance by country leaders, health departments, international organisations and advocate groups. The WHO carefully monitors drug usage however it is unable to regulate individual countries, so must rely on leaders to implement the strategies they put in place without jeopardising the new compound. The private sector is the most difficult to control, as people can afford treatment so this section of the health industry is less likely to regulate usage. To try and combat this, the WHO has started to encourage Public-Private Mix, whereby private companies and individuals are encouraged to send tuberculosis patients to the public sector. In the countries where this has been implemented there has been cooperation between the public and private sector, so there is hope that any future drug can be protected, at least in this case, from inappropriate use (W.H.O, 2007, Yesudian and Raviglione, 2009).

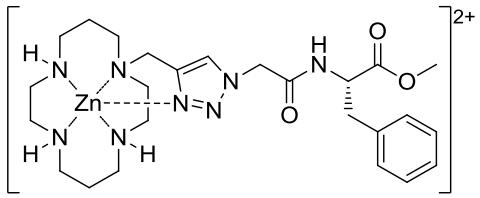
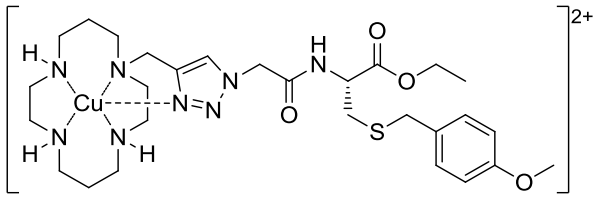
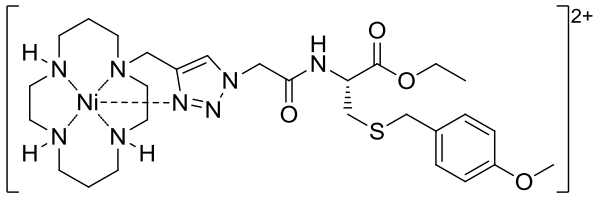
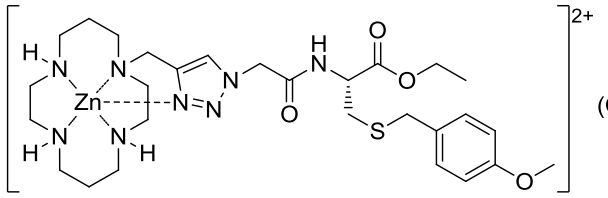
Possible areas that could be utilised for guidance of tuberculosis drug development are those of HIV and malaria. These two prominent infectious agents had reached a critical level of resistance such that there needed to be urgent reforms in their drug development and implementation. Similarly to TB, HIV and malaria have experienced high levels of resistance. In the case of malaria, countries such as Kenya were experiencing over 25% treatment failures in their studies indicating an urgent need for new drugs to be developed (Amin et al., 2007). A new regimen was developed and the first recommendation made by the WHO was in 2001 (W.H.O, 2001). It took a further three years before this new combination therapy was adapted into country policy (Sipilanyambe et al., 2008). Tuberculosis, while a global problem, is not yet at the stage of malaria or HIV in that susceptible tuberculosis, if treated appropriately, has more than a 95 per cent success rate (Zumla et al., 2013a). It is therefore crucial that studies such as those conducted throughout this thesis be consistently undertaken to increase the number of potential candidate drugs that may one day assist in the decline and hopeful eradication of this life threatening disease.

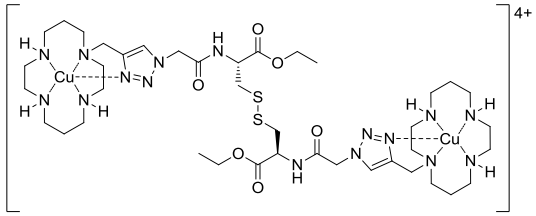
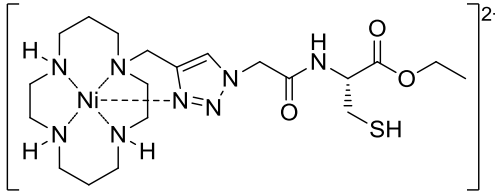
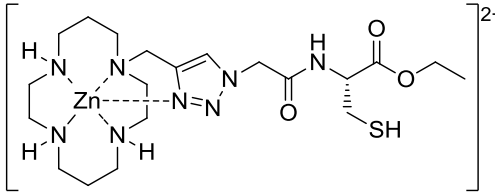
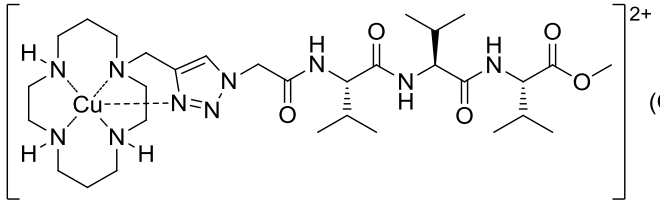
Appendix 1: Compound structures and related data of metal-cyclam compounds.

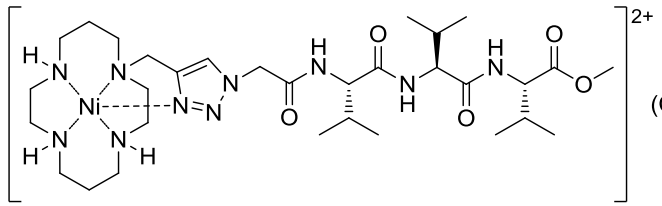
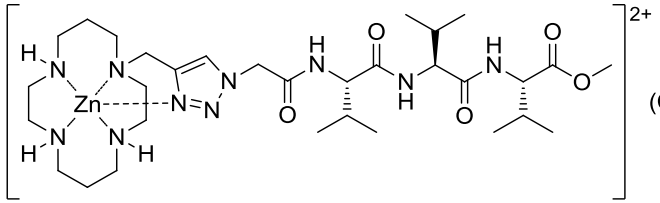
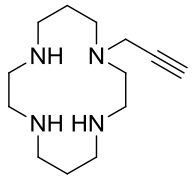
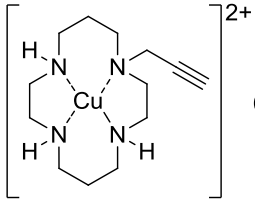
			MIC$_{90}$			
Compound	**Compound Structure**	**Molecular Weight**	***M. avium***	***M. bovis* BCG**	**MRSA**	***P. A*[a] CJ2009**
1		691.02144	>50 μM	>50 μM	>50 μM	>50 μM
2		686.16884	>50 μM	>50 μM	>50 μM	>50 μM
3		701.86308	>50 μM	>50 μM	>50 μM	>50 μM

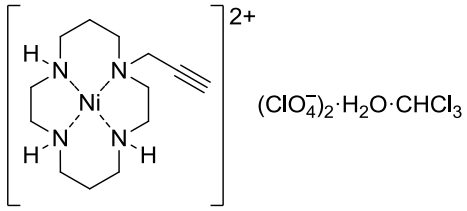
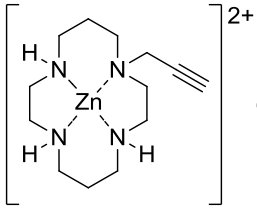
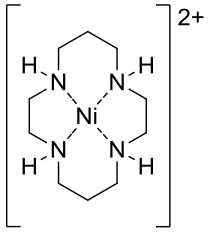
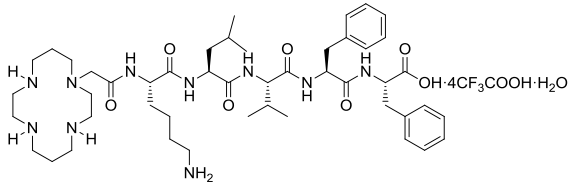
4		820.13542	>50 μM	>50 μM	>50 μM	>50 μM
5		806.27518	>50 μM	>50 μM	>50 μM	>50 μM
6		812.96178	>50 μM	>50 μM	>50 μM	>50 μM
7		733.05658	>50 μM	>50 μM	>50 μM	>50 μM

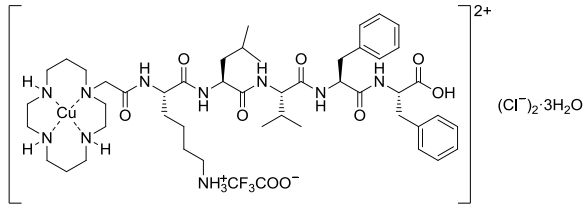
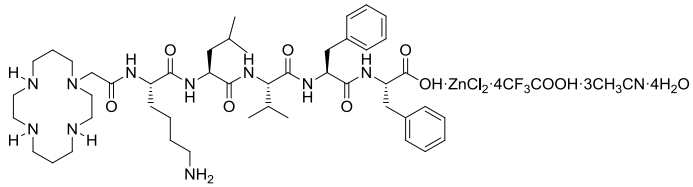
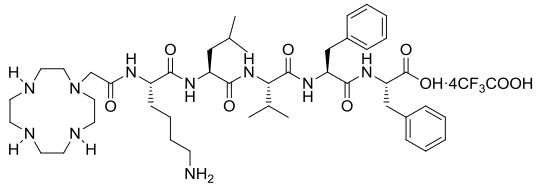
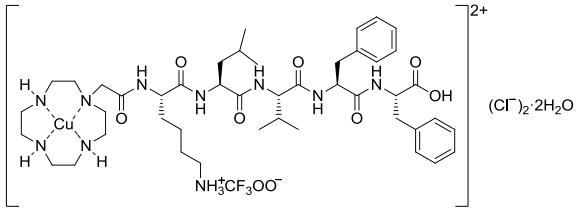
8	 $(ClO_4^-)_2$	710.18870	>50 μM	>50 μM	>50 μM	>50 μM
9	 $(ClO_4^-)_2 \cdot H_2O$	734.89058	>50 μM	>50 μM	>50 μM	>50 μM
10	 $(ClO_4^-)_2 \cdot H_2O$	781.09938	>50 μM	>50 μM	>50 μM	>50 μM
11	 $(ClO_4^-)_2 \cdot 0.5H_2O$	767.23914	>50 μM	>50 μM	>50 μM	>50 μM

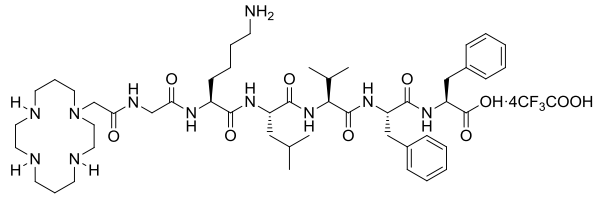
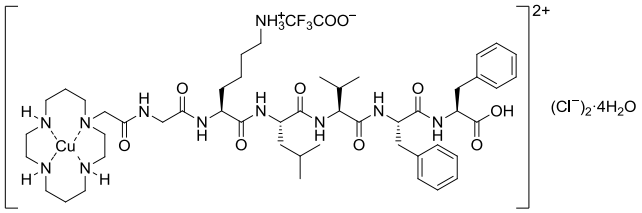
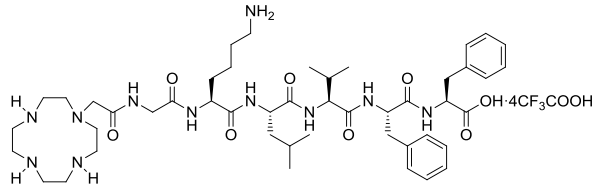
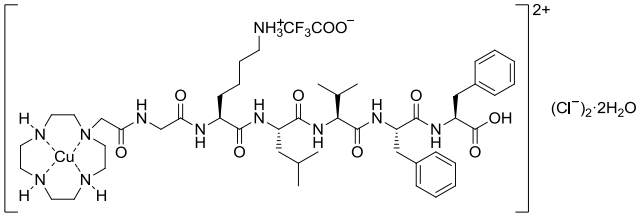
12	 $(ClO_4^-)_2 \cdot 0.5H_2O$	773.92574	>50 μM	>50 μM	>50 μM	>50 μM
13	 $(ClO_4^-)_2 \cdot H_2O$	871.24352	>50 μM	>50 μM	>50 μM	>50 μM
14	 $(ClO_4^-)_2$	848.37564	>50 μM	>50 μM	>50 μM	>50 μM
15	 $(ClO_4^-)_2 \cdot 0.5H_2O$	864.06988	>50 μM	>50 μM	>50 μM	>50 μM

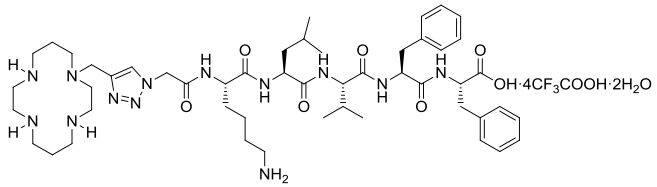
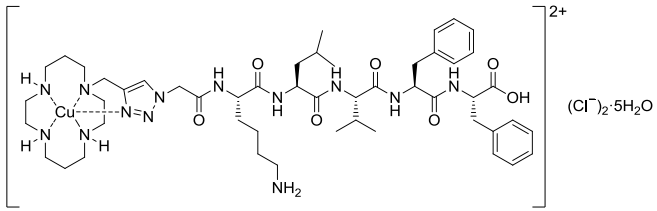
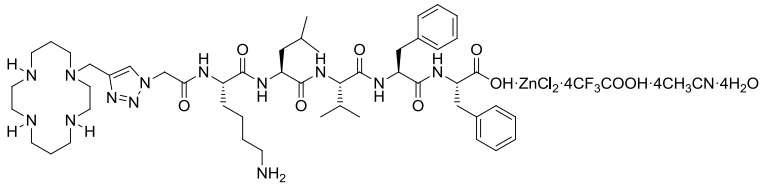
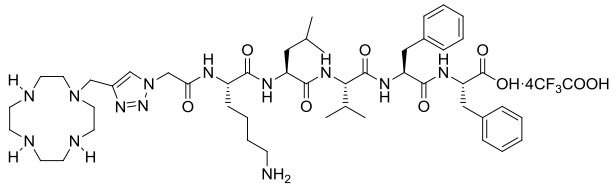
16	 $(ClO_4^-)_4 \cdot 4H_2O$	1536.20468	>50 μM	>50 μM	>50 μM	>50 μM
17	 $(ClO_4^-)_2 \cdot 0.5H_2O$	737.23476	>50 μM	>50 μM	>50 μM	>50 μM
18	 $(ClO_4^-)_2 \cdot 0.5H_2O$	743.92136	>50 μM	>50 μM	>50 μM	>50 μM
19	 $(ClO_4^-)_2 \cdot H_2O$	931.31870	>50 μM	>50 μM	>50 μM	>50 μM

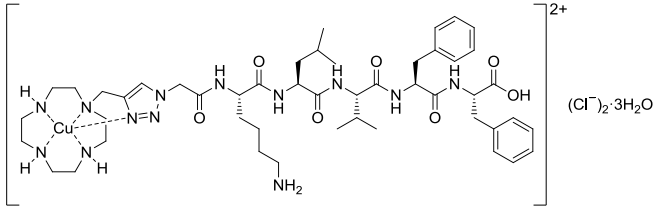
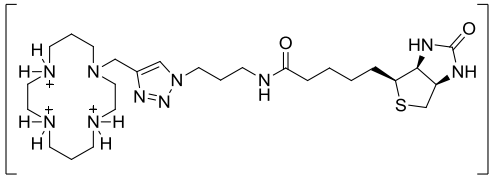
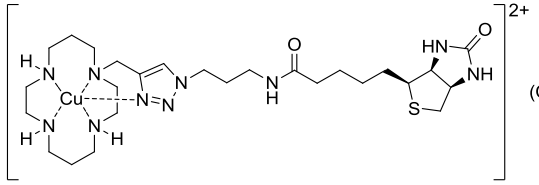
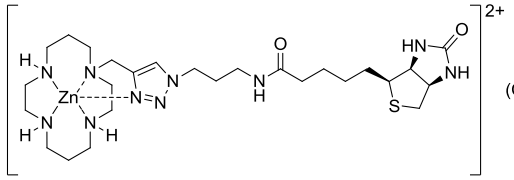
20		908.45082	>50 μM	>50 μM	>50 μM	>50 μM
21		942.16034	>50 μM	>50 μM	>50 μM	>50 μM
22		238.37234	>50 μM	>50 μM	>50 μM	>50 μM
23		532.86140	>50 μM	>50 μM	>50 μM	>50 μM

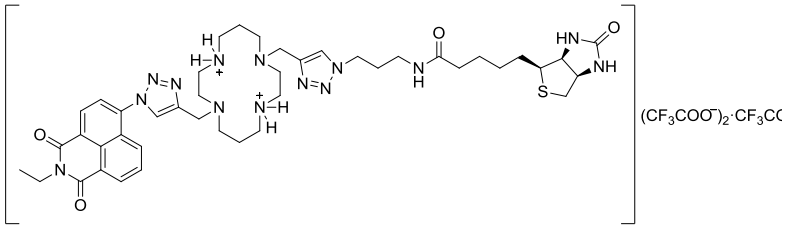
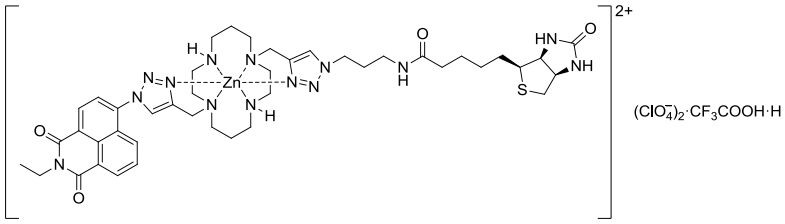
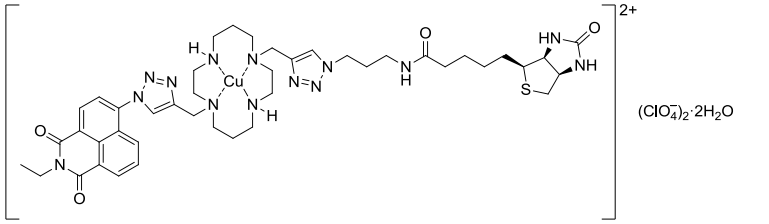
24	 $(ClO_4^-)_2 \cdot H_2O \cdot CHCl_3$	633.35986	>50 μM	>50 μM	>50 μM	>50 μM
25	 $(ClO_4^-)_2$	502.65354	>50 μM	>50 μM	>50 μM	>50 μM
26	 $(ClO_4^-)_2$	457.91896	>50 μM	>50 μM	>50 μM	>50 μM
27	 $OH \cdot 4CF_3COOH \cdot H_2O$	1367.27782	>50 μM	>50 μM	>50 μM	>50 μM

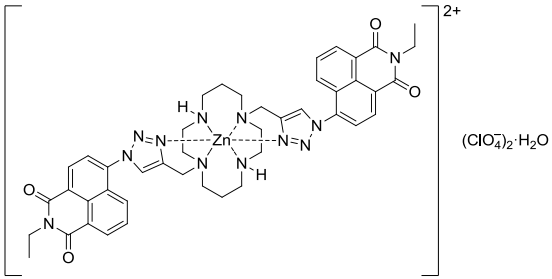
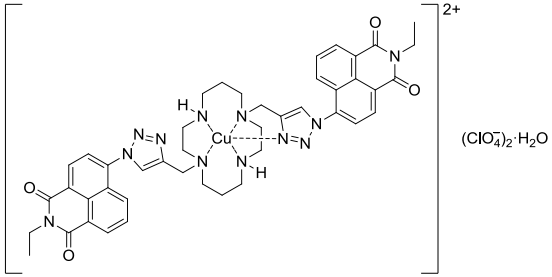
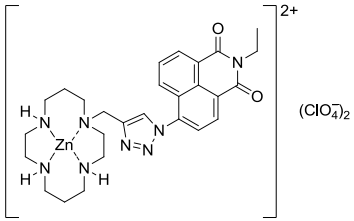
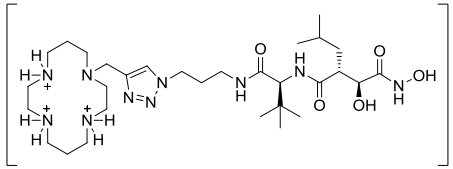
28		1195.69033	>50 μM	>50 μM	>50 μM	>50 μM
29		1680.76542	>50 μM	>50 μM	>50 μM	>50 μM
30		1321.20938	>50 μM	>50 μM	>50 μM	>50 μM
31		1149.62189	12.5 μM	12.5 μM	>50 μM	>50 μM

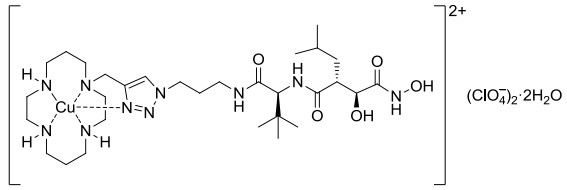
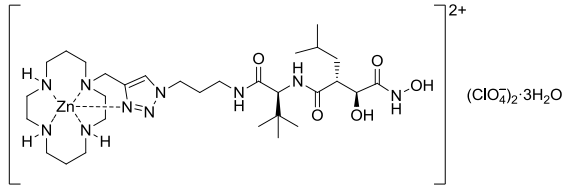
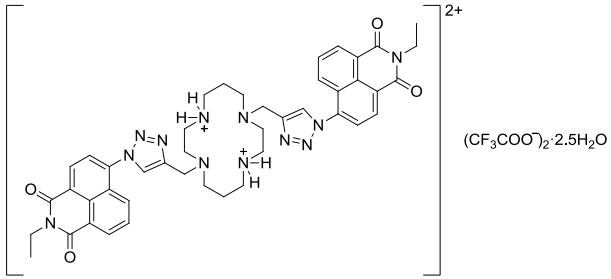
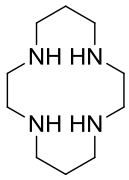
32		1406.31386	>50 μM	>50 μM	>50 μM	>50 μM
33		1270.75693	>50 μM	>50 μM	>50 μM	>50 μM
34		1378.26070	>50 μM	>50 μM	>50 μM	>50 μM
35		1206.67321	12.5 μM	25 μM	>50 μM	>50 μM

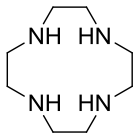
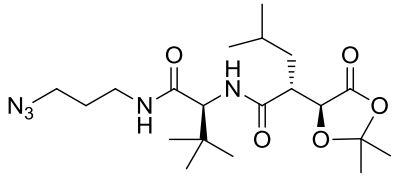
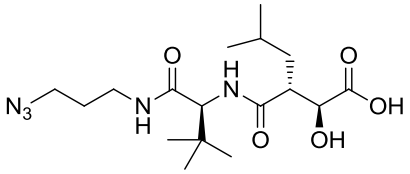
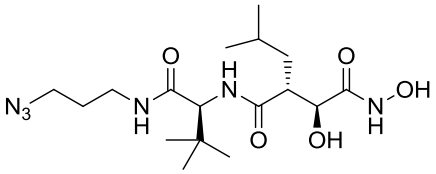
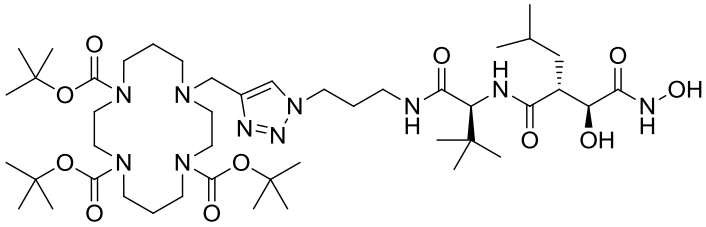
36		1466.36912	>50 μM	>50 μM	>50 μM	>50 μM
37		1198.77356	>50 μM	>50 μM	>50 μM	>50 μM
38		1802.89336	>50 μM	>50 μM	>50 μM	>50 μM
39		1402.28540	>50 μM	>50 μM	>50 μM	>50 μM

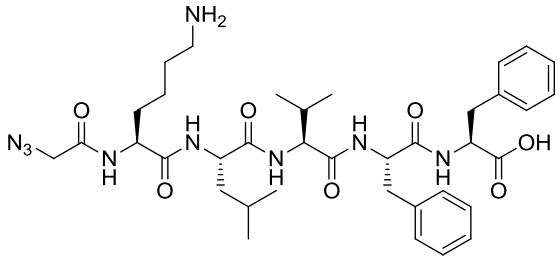
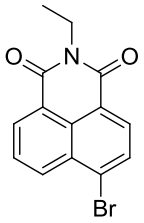
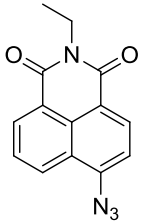
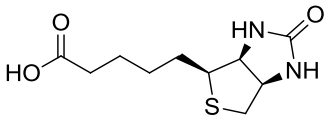
40		1134.68984	12.5 μM	50 μM	>50 μM	>50 μM
41		906.86017	>50 μM	>50 μM	>50 μM	>50 μM
42		881.28316	>50 μM	>50 μM	>50 μM	>50 μM
43		961.10995	>50 μM	>50 μM	>50 μM	>50 μM

44		1247.19351	6.25 μM	6.25 μM	>50 μM	>50 μM
45		1265.41273	6.25 μM	6.25 μM	>50 μM	>50 μM
46		1167.57066	3.13 μM	6.25 μM	>50 μM	>50 μM

47		1091.22640	1.56 μM	3.13 μM	>50 μM	>50 μM
48		1089.39240	0.78 μM	1.56 μM	50 μM	>50 μM
49		768.90834	25 μM	25 μM	>50 μM	>50 μM
50		980.91557	>50 μM	>50 μM	>50 μM	>50 μM

51		937.32328	>50 μM	>50 μM	>50 μM	>50 μM
52		957.17256	>50 μM	>50 μM	>50 μM	>50 μM
53		1082.01482	3.13 μM	3.13 μM	50 μM	>50 μM
54 (cyclam)		200.32436	>50 μM	>50 μM	>50 μM	>50 μM

55 (cyclen)		172.27120	>50 μM	>50 μM	>50 μM	>50 μM
56		425.52240	>50 μM	>50 μM	>50 μM	>50 μM
57		385.45854	>50 μM	>50 μM	>50 μM	>50 μM
58		400.47318	>50 μM	>50 μM	>50 μM	>50 μM
59		939.19298	>50 μM	>50 μM	>50 μM	>50 μM

60		735.87282	>50 μM	>50 μM	>50 μM	>50 μM
61		304.13870	>50 μM	>50 μM	>50 μM	>50 μM
62		266.25480	>50 μM	>50 μM	>50 μM	>50 μM
63 (biotin)		244.31064	>50 μM	>50 μM	>50 μM	>50 μM

[a] ***P. aeruginosa*.**

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