REPURPOSING CHLORPROMAZINE AND ITS METABOLITES FOR ANTITUBERCULOSIS DRUG DISCOVERY

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ABBREVIATIONS

ACT	artemisinin-based combination therapy
ADC	albumin/dextrose/catalase
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
ART	antiretroviral drugs
ATP	adenosine triphosphate
BCG	bacille Calmette-Guérin
BDQ	bedaquiline (TMC207)
BMRC	British Medical Research Council
$CaCl_2$	calcium chloride
$CDCl_3$	deuterated chloroform
cDNA	complementary DNA
CFU	colony forming units
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
$C_5H_{12}O$	isoamyl alcohol
CPZ	chlorpromazine
CTAB	cetyltrimethyl ammonium bromide
CYP450	cytochrome P450
DNA	deoxyribonucleic acid

DOTS	directly observed therapy, short course
DS	drug-susceptible
EDTA	ethylenediaminetetraacetic acid
EMB	ethambutol
ESI	electrospray ionization
$FeCl_3$	ferric chloride
$FeSO_4$	ferrous sulfate
FICI	fractional inhibitory concentration index
gDNA	genomic DNA
H_2O_2	hydrogen peroxide
H3-D	Drug Discovery and Development Center
HCL	hydrochloric acid
HIV/AIDS	human immunodeficiency virus infection/acquired immunodeficiency syndrome
HLM	human liver microsomes
HPLC	high performance liquid chromatography
HTSS	high-throughput synergy screening
INH	isoniazid
IPTG	isopropyl β-d-thiogalactopyranoside
IUATLD	International Union against Tuberculosis and Lung Disease
JNK	c-Jun N-terminal kinase
KANA	kanamycin
LB	Luria-Bertani
LC/MS	Liquid Chromatography - Mass Spectrometry
LC/NMR	Liquid Chromatography - Nuclear Magnetic Resonance

MABA	microplate alamar blue assay
m/z	mass-to-charge ratio
m-CPBA	*meta*-chloroperbenzoic acid
MDGs	millennium development goals
MDR	multi-drug resistant
MeOH	methanol
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MMRU	Molecular Mycobacteriology Research Unit
M. pt.	melting point
MIC	minimum inhibitory concentration
MoA	mechanism of action
Msm	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate-oxidase
NaOH	sodium hydroxide
NH_3	ammonium
NMR	Nuclear Magnetic Resonance
OADC	dubos oleic albumin complex
OD	optical density
PBS	phosphate buffer saline
PZA	pyrazinamide
qPCR	quantitative polymerase chain reaction
QRDR	quinolone resistance-determining region

RIF	rifampicin
RLM	rat liver microsomes
RNA	ribonucleic acid
RT-PCR	real-time polymerase chain reaction
SDS	sodium dodecyl sulfate
SPEC	spectinomycin
STRP	streptomycin
TAE	tris/acetate/EDTA
TE	tris/EDTA
TB	tuberculosis
TDR	totally drug resistant
TIC	total ion chromatograms
UN	United Nations
UNG	uracil-*N* glycosylase
WHO	World Health Organization
XDR	extensively drug resistant
XIC	extracted ion chromatograms
XXDR	extremely drug resistant

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DEDICATION

I lovingly dedicate this dissertation to my entire wonderful family

LIST OF PUBLICATIONS AND CONFERENCES

Publications:

1. **Elizabeth M. Kigondu,** Mathew Njoroge, Kawaljit Singh, Nicholas Njuguna, Digby F. Warner, Kelly Chibale. Synthesis and synergistic antimycobacterial screening of chlorpromazine and its metabolites. Medicinal Chemistry Communication, Vol. 5, pp. 502-506, January 2014.
2. **Elizabeth M. Kigondu,** Antonina Wasuna, Digby F. Warner, Kelly Chibale. Pharmacologically active metabolites, combination screening and target identification-driven drug repositioning in antituberculosis drug discovery. Bioorganic & Medicinal Chemistry. Vol. 22(16), pp. 4453–4461, August 2014.
3. Vinayak Singh, **Elizabeth M. Kigondu**, Kelly Chibale, Digby F. Warner. Chlorpromazine potentiates the activity of spectinomycin against *Mycobacterium tuberculosis* (Submitted).
4. **Elizabeth M. Kigondu,** Vinayak Singh, Atica Moosa, Kelly Chibale, Digby F. Warner. *In vitro* synergistic interactions and possible mechanism of action of chlorpromazine and its metabolites in combination with anti-TB drugs against *Mycobacterium tuberculosis* (Submitted).

Conferences:

1. H3-D Symposium (New Paradigms in Drug Discovery: Challenges and Opportunities in Africa), Vineyard Hotel, Newlands, Cape Town, South Africa, 15th – 18th October 2012. Presented a poster entitled **"Synergistic/Matrix Antimycobacterial Screening of**

Parent Drugs and their Metabolites for Antituberculosis Drug Discovery."

2. Keystone Symposium (Novel Therapeutic Approaches to Tuberculosis), Keystone Resort, Keystone, Colorado, USA, 30th March – 4th April 2014. Presented a poster entitled **"Combination Screening of Chlorpromazine and its Metabolites for Tuberculosis Drug Discovery."**
3. H3-D Symposium (Innovative Approaches to Tuberculosis Drug Discovery: Laboratory to Bedside), Zambezi Sun, Victoria Falls, Zambia, 27th – 29th August 2014. Presented a poster entitled **"*In vitro* Synergistic Interactions and Possible Mechanism of Action of Chlorpromazine and its Metabolites in Combination with Anti-TB Drugs against *Mycobacterium tuberculosis*."**

ABSTRACT

New chemotherapeutics are urgently needed to combat *Mycobacterium tuberculosis (Mtb)*, the causative agent of tuberculosis (TB). The development of compounds that could potentiate the activity of known antimycobacterial drugs is a relatively unexplored approach to new TB drug discovery. This study aimed to generate metabolites of chlorpromazine (CPZ), a phenothiazine with demonstrated *in vitro* activity against *Mtb*, and to investigate their potential utility in combination with anti-TB drugs. 7-HydroxyCPZ **(M2)**, CPZ-*N*-oxide **(M3)**, CPZ sulfoxide **(M1)**, nor-CPZ **(M5)**, nor-CPZ sulfoxide **(M6b)** and CPZ-*N*-*S*-dioxide **(M4b)** were generated from CPZ using various biotransformation systems and identified by Liquid Chromatography - Mass Spectrometry (LC/MS). The identity of **M2** was confirmed with reference to a 7-hydroxyCPZ standard. **M3**, **M1**, **M5**, **M6b** and **M4b** were synthesized *de novo* and used to identify the metabolites generated in the biotransformation samples. Individually, CPZ and its metabolites **(M2, M3, M5)** were weakly active (MIC_{99} >50μM) against *M. smegmatis* (*Msm*) and *Mtb* while **M1, M6b & M4b** did not exhibit a MIC_{99} even at very high concentrations. Generally, an improvement in activity was observed where CPZ or its metabolites were used in combination with known anti-TB drugs. The combinations that exhibited a fractional inhibition concentration index (FICI) of $\leq$ 0.5 were defined as synergistic. A combination of **M2** and spectinomycin (SPEC) exhibited the highest synergism against *Msm* (FICI 0.19) and *Mtb* (FICI 0.13). *In vitro* assays established that CPZ and **M2** are bactericidal against *Mtb* whereas **M3** and **M5** are bacteriostatic on their own. In combination assays, the use of RIF with **M3** and **M5**, bedaquiline (BDQ) with **M2,** and SPEC with **M3** were bactericidal. At 140μM, CPZ and **M1**, **M2**, **M3** treated samples exhibited a 2-fold up-regulation of the *cydA* (Rv1623c) gene which encodes an essential subunit of the cytochrome *bd*-type menaquinol oxidase in *Mtb*. The same observation

was made for RIF/**M2** and RIF/**M5** treated samples. These results suggest that the metabolites retain the mechanism of action (MoA) as the parental CPZ. The *Mtb* 16S rRNA gene, *rrs* (MTB000019) was identified as the biological target for SPEC. This brought into perspective the underlying mechanisms at play when SPEC is used in combination with CPZ, its metabolites or other drugs, against mycobacteria. This study establishes the utility of combination assays in confirming the active metabolite(s) of known drugs and provides proof of concept data to support follow-up investigations of CPZ and its metabolites as potential compounds for novel combination therapies for anti-TB drug development.

CHAPTER 1

INTRODUCTION

1.1 Background

Tuberculosis (TB) remains a global health problem. Since the identification in 1882 of *Mycobacterium tuberculosis* (*Mtb*) as the causative agent of TB, the epidemic continues unabated. It is estimated that every year, approximately 9 million people develop active TB, 30% of which reflect co-infection with HIV.[1–3] According to the World Health Organization (WHO) 2014 report, the majority of TB cases worldwide are in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. The report indicates that India and China alone accounted for 26% and 12% of total cases, respectively (Figure 1.1). The TB incidence rate at country level ranges substantially, with around 1000 or more cases per 100 000 people in South Africa and Swaziland, and fewer than 10 per 100 000 population in parts of America, several countries in western Europe, Japan, Australia and New Zealand.[3–5]

Some shortcomings of the current treatment strategies for TB include limited effectiveness of public health systems, particularly in resource-poor countries where the main TB burden lies. The ease with which TB infection spreads by inhalation of a few droplet nuclei (2-5μm in diameter) containing as few as 1-3 bacilli, has helped to sustain this scourge at current levels.[6]

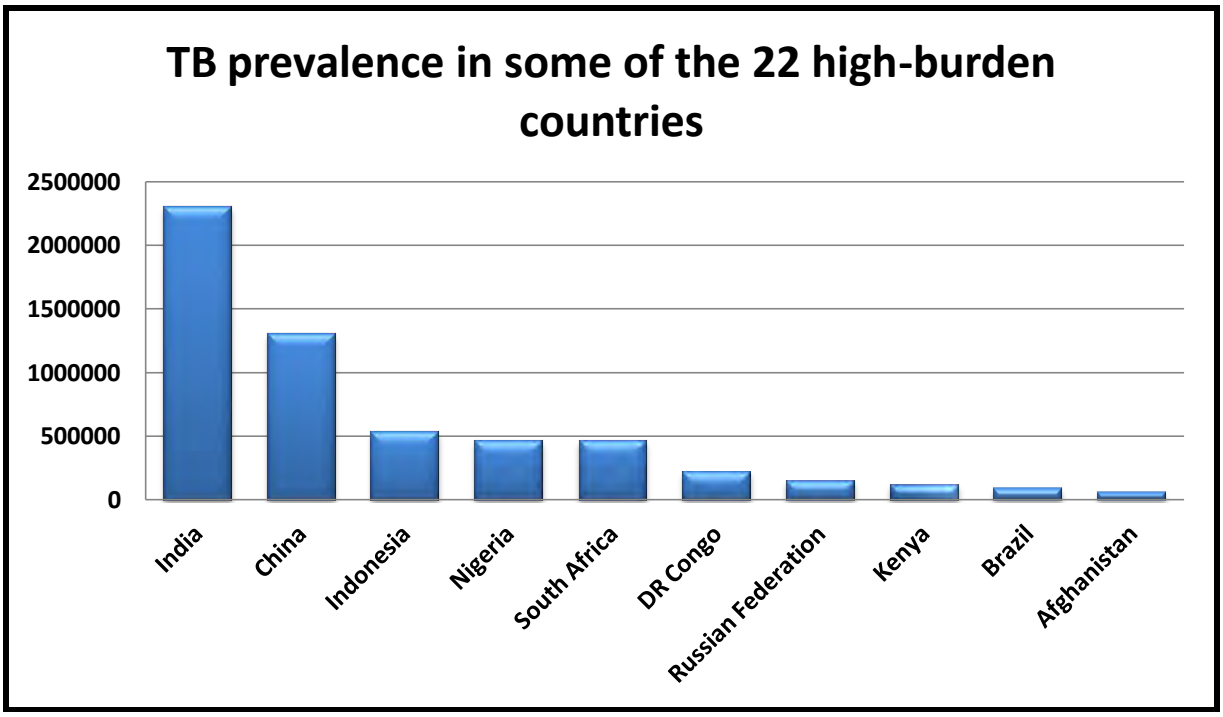

Figure 1.1 Estimated global TB prevalence[3,4,7]

1.2 *Mtb* pathology and physiology

Significant progress has been made in the molecular characterization of the pathogen but more work is still needed to understand how *Mtb* copes with the numerous environments it encounters in the course of a successful infection.

Mtb has a doubling time of ~20h *in vitro* in contrast to most bacteria that divide much more rapidly. Naturally one would assume that, since antibiotics primarily work on cells that are actively dividing, the overall duration of therapy might somehow correlate with the length of the growth cycle. However, this is not the case: antibiotics are able to eliminate actively dividing bacilli *in vitro*, but a number of factors - including drug penetration[8,9] and the ability of the bacillus to enter into states of antibiotic tolerance[10] and extended phases of slow or non-replication[11] ensure that the current combination regimen must be continued for several months to minimize the risk of treatment failure and/or recurrence.[12]

The persistence of *Mtb* within the human population depends on its ability to cycle through repetitive phases of infection, disease, transmission, and clinical latency. *Mtb* affects the lungs (pulmonary TB) but can affect other sites as well (extrapulmonary TB). It uses diverse strategies to survive and replicate within human macrophages, and to evade immune surveillance[1,13] where the initial interaction between host phagocytic cells and microbial pathogens is mediated by specific phagocyte membrane receptors and ligands present on the microbe. *Mtb* replicates intracellularly within human macrophages by preventing phagosomal maturation and modulates other macrophage defenses to promote its survival.[14]

In addition, *Mtb* possesses a sophisticated metabolic system that supports the biosynthesis of all essential amino acids, vitamins, and enzyme co-factors.[15] It is also able to oxidize multiple carbon substrates, and to utilize a variety of alternate electron transport chains during adaptation to variable host environments. Its unusual cell wall - rich in lipids and impermeable to most compounds - ensures inherent resistance to host immune effectors and numerous antibiotic agents, while an array of transcriptional regulators enables a rapid response to environmental cues. The bacillus is also armed with multiple defense and detoxification pathways, as well as DNA repair and maintenance functions.[15,16]

The bacterium is adept at escaping host defense mechanisms in macrophages, and the immune response to infection results in the formation of granulomatous lesions that become caseous and hypoxic, conditions in which the drugs are unable to penetrate or be effective.[12]

1.2.1 Development of drug resistance in *Mtb*

Despite an in-depth understanding of how *Mtb* evolves and overcomes the inhibitory effect of anti-TB drugs,[17,18] development of resistance has been a major impediment to the treatment of TB. Enormous amounts of money have been directed into drug development programs to

discover new anti-TB agents in a bid to overcome this problem.[15] Multidrug-resistant TB (MDR-TB) is defined as resistance to the first-line drugs, isoniazid (INH) and rifampicin (RIF); extensively drug-resistant TB (XDR-TB) is MDR-TB with additional resistance to fluoroquinolones and at least one of the injectable second-line drugs [capreomycin, kanamycin (KANA), and amikacin (Figure 1.2)].[1,19] It is reported that 5.4% of MDR-TB cases are XDR-TB.[1] On the other hand, extremely drug resistant (XXDR-TB) and totally drug-resistant TB (TDR-TB) are terms used to describe resistance to all first-line and second-line anti-TB drugs.[19] These drug resistant infections are treated by a combination of eight to ten drugs with therapies lasting up to 18–24 months that can result in side-effects including nephrotoxicity and ototoxicity with aminoglycosides, hepatotoxicity with ethionamide and dysglycaemia with gatifloxacin.[1]

1.2.1.1 Intrinsic resistance

Intrinsic resistance refers to the mycobacterium's ability to overcome the inhibitory effect of anti-TB agents through its inherent structural or functional characteristics.[20] Intrinsic drug resistance in *Mtb* is thought to be associated primarily with its unique cell wall properties such as the presence of mycolic acids, which are high-molecular-weight *α*-alkyl,*β*-hydroxy fatty acids covalently attached to arabinogalactan, that constitute a very hydrophobic barrier responsible for resistance to certain antibiotics.[21] In addition, *Mtb* possesses *β*-lactamase enzymes, which confer intrinsic resistance to *β*-lactam antibiotics, while efflux mechanisms contribute to resistance to antibiotics such as INH, RIF, tetracycline, linezolid, Bedaquiline (BDQ), fluoroquinolones and aminoglycosides.[19,22,23] Low antibiotic permeabilitiy through the *Mtb* cell wall may also occur in non-replicating bacilli resulting in another form of phenotypic drug resistance that is independent of efflux.[24,25]

1.2.1.2 Acquired drug resistance

Acquired drug resistance occurs when *Mtb* obtains the ability to resist bioactivity of a particular antimicrobial agent to which it was previously susceptible. The resistance is caused mainly by spontaneous mutations in chromosomal genes, and the selective growth of such drug-resistant mutants may be promoted during sub-optimal drug therapy which arises from non-adherence to, or improper use of, medication by TB patients – as well as from the potential for antibiotics to establish concentration gradients across differentially permeable anatomical and pathological loci.[8] Monotherapy will in most cases result in development of resistance as the bacilli undergo genetic mutations to overcome the effectiveness of the drug administered singly. In addition, non-adherence to TB medication prescribed as combination therapy will in most cases result in a relapse of the disease that results from lack of clearance of the bacteria during the first two months of treatment, and presence of lesions in the lung where the tissue has been destroyed and surrounded by a thick fibrotic wall. It may also lead to the emergence of genetic drug resistance, resulting in an even longer treatment duration with less effective and more toxic second-line and third-line drugs.[8,9] Nevertheless, proper use and completion of TB treatment by patients as recommended in the DOTS (Directly Observed Therapy, Short Course) program (section 1.3.1) may reduce the risk for development of acquired drug resistance. However, studies have established that even with all the controls in place TB drug resistance has not been fully contained.[19,26,27]

1.3 TB control

1.3.1 DOTS (Directly Observed Therapy, Short Course)

In response to the global TB epidemic, the WHO developed a control strategy largely based on the pioneering work of the British Medical Research Council (BMRC) and the International Union against Tuberculosis and Lung Disease (IUATLD). This strategy is known as DOTS.[28] The essential elements of DOTS are as follows:

- Strong government commitment to TB control.
- Diagnosis by smear microscopy (or by culture where resources permit).
- Standardized short course chemotherapy with directly observed treatment for at least the first 2 months.
- Secure supply of safe, high-quality drugs.
- Individual reporting of treatment outcome and monitoring of program performance.

A bench-mark was set for this program where the goal was to achieve a significant reduction in the burden and incidence of TB globally by 2015, a target set within the United Nations (UN) Millennium Development Goals (MDGs). Since the global rollout of the DOTS strategy, a cumulative total of 56 million people were successfully treated for TB between 1995 and 2012, saving approximately 22 million lives.[3] However, many concerns have been raised over the effectiveness of the strategy owing to the fact that, despite the successful implementation of the program, the TB burden in Africa and Asia has not been fully contained.[28]

1.3.2 TB vaccine

TB is one of the diseases for which there is still no effective vaccine for the general population. However, the live attenuated vaccine derived from *M. bovis*, which is known as bacille Calmette-Guerin (BCG), has been critical in preventing the disease in children. Regrettably, being the only vaccine in existence it has shown variable efficacy globally especially against pulmonary disease in adolescents and adults. Since the development of BCG, no other vaccine has made it into the market. However, several candidate vaccines are currently in clinical trials. Much is still not understood about BCG which requires further research.[29,30]

1.3.3 Anti-TB drugs, their mechanisms of action (MoAs) and development of resistance

Treatment of drug-susceptible (DS)-TB involves an initial phase of INH, RIF, pyrazinamide (PZA) and ethambutol (EMB) (Figure 1.2) for the first 2 months followed by a continuation phase of INH and RIF for a further 4 months. BDQ, recently discovered after 40 years since the known anti-TB drugs were developed, is currently being used for treatment of MDR-TB. It is normally introduced to the standard TB treatment regimen and administered for a maximum duration of 6 months.[3,31]

Up to 95% of people with DS-TB can be cured in 6 months with the four drug regimen. WHO recommends use of fixed-dose combinations of TB drugs because they are thought to prevent acquisition of drug resistance due to monotherapy, which may occur with individual ("unprotected") drugs.[3] These drugs can be grouped into various classes based on their MoA. Generally, many antibiotics are either bactericidal (kill the bacterium) or bacteriostatic (inhibit growth and replication of the bacterium). The demonstrated efficacy of the oxazolidinone (linezolid), in treating clinical drug-resistant TB indicates that bacteriostatic drugs are as important as those which are bactericidal; however, in the case of the former, the host's immune mechanism must come into play in order to clear the infection.[32]

The future of TB therapy must be one in which treatment time is reduced to only a few months or, better still, a few weeks. The reasons why this is difficult to achieve when other bacterial infections are cured in a matter of days or weeks are probably numerous, certainly very poorly understood, and relate to the biology of the organism, the host response to infection and the properties of the available drugs.

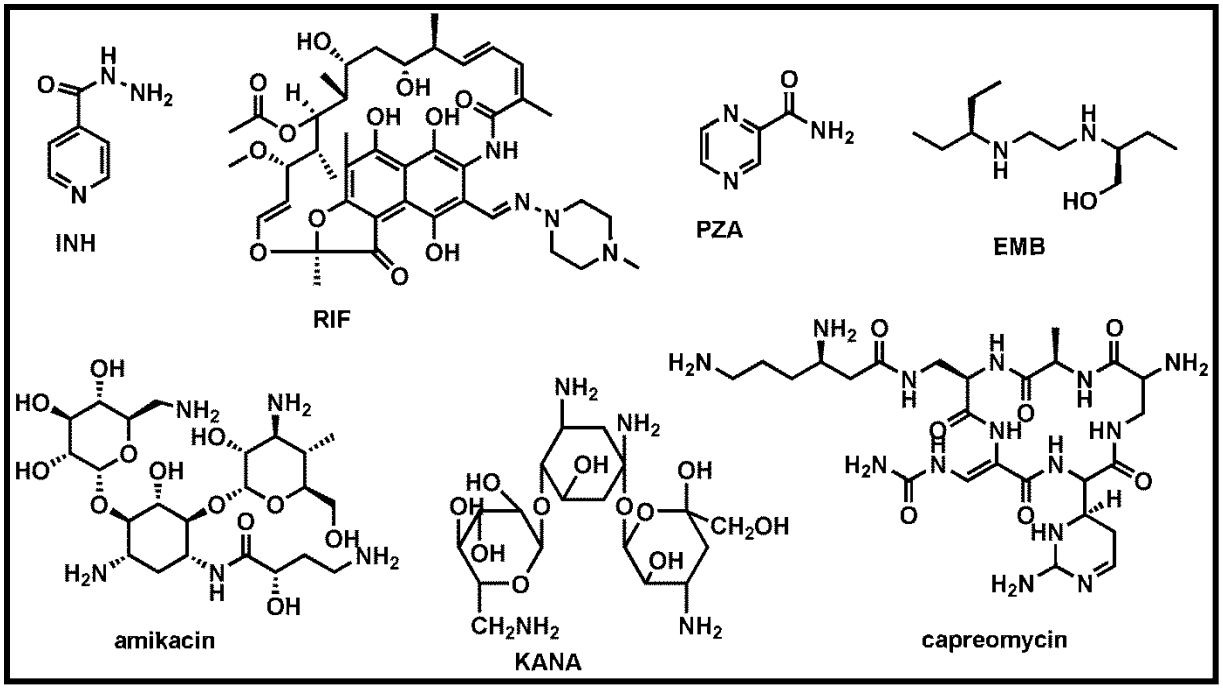

Figure 1.2 First- and second-line drugs for TB treatment

1.3.3.1 Rifamycins

Rifamycins (Figure 1.3) were first discovered in the 1950s through biosynthesis by the bacterium *Amycolatopsis mediterranei.* In the mycobacteria, this group of compounds inhibit RNA synthesis by forming a stable complex with RNA polymerase binding with the *β*-subunit (encoded by *rpoB*) causing an effect on the nucleic acid metabolism and eventually leading to cell death.[33] Amongst all antibiotics used clinically, the MoA is unique to this class of compounds. Examples include RIF, rifabutin, rifalazil, rifapentine among others.[34] It is reported that development of resistance to RIF in *Mtb* occurs as single point mutations in the *rpoB* gene, which encodes the *β*-subunit of RNA polymerase.[19,33,35]

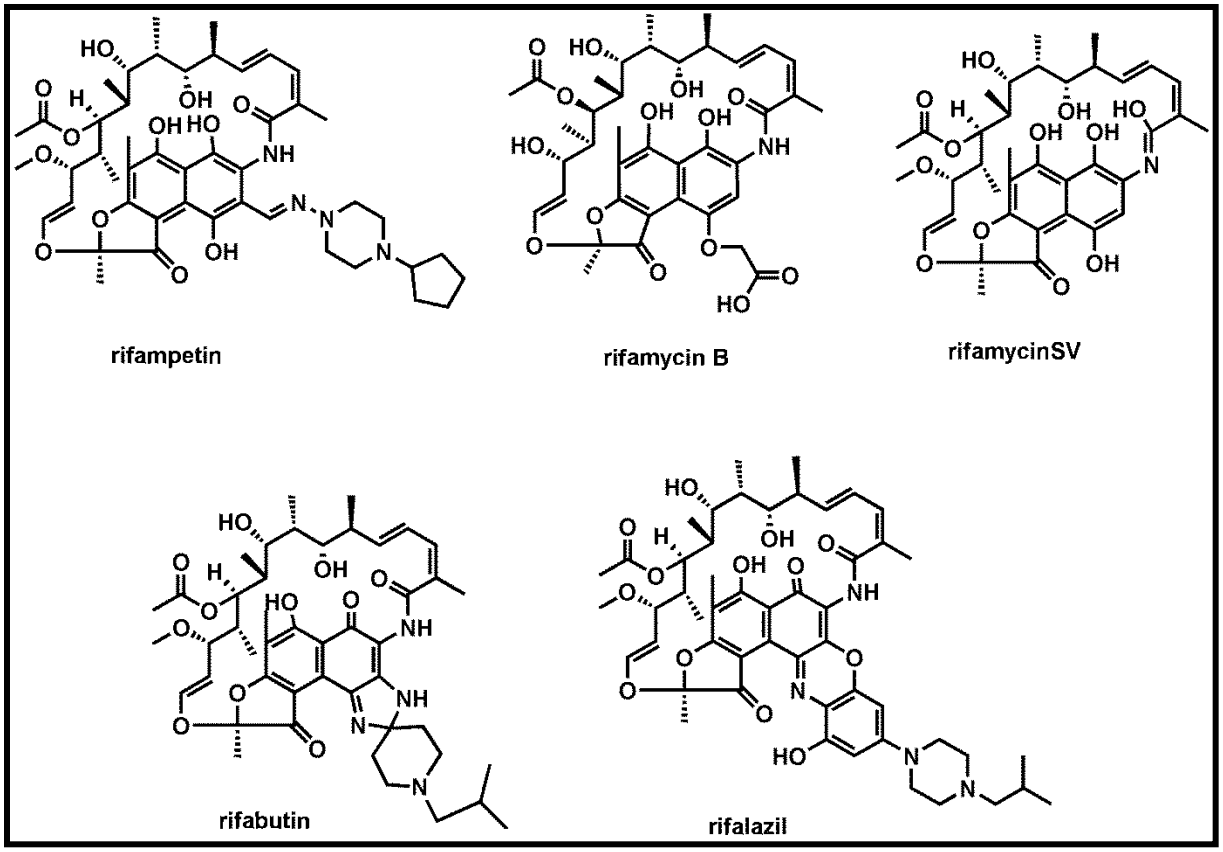

Figure 1.3 Examples of rifamycins (1st line TB drugs)

1.3.3.2 Fluoroquinolones

These drugs (Figure 1.4) are broad-spectrum antibacterials that were discovered by chance during the synthesis of chloroquine. Nalidixic acid was the first generation quinolone to be developed in 1962 and was used for urinary tract infections. Subsequently, other generations of this family of compounds were later developed and examples include ofloxacin, ciprofloxacin, pefloxacin (2nd generation), levofloxacin, sparlofloxacin, balofloxacin (3rd generation) and gatifloxacin, moxifloxacin, prulifloxacin (4th generation).[36,37] The drugs are only used in cases of drug resistance and in combination with other anti-TB drugs. Fluoroquinolones are second-line anti-TB drugs but, in combination with various first-line antituberculosis drugs, have shown greater *ex vivo* antimycobacterial activity in macrophages than the individual drugs alone[38]. Their antibacterial MoA is due to an inhibition of an ATP-dependent DNA gyrase.[39–44] Mutations

in the conserved quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB* leads to development of resistance to fluoroquinolones in *Mtb*. [39,44–46]

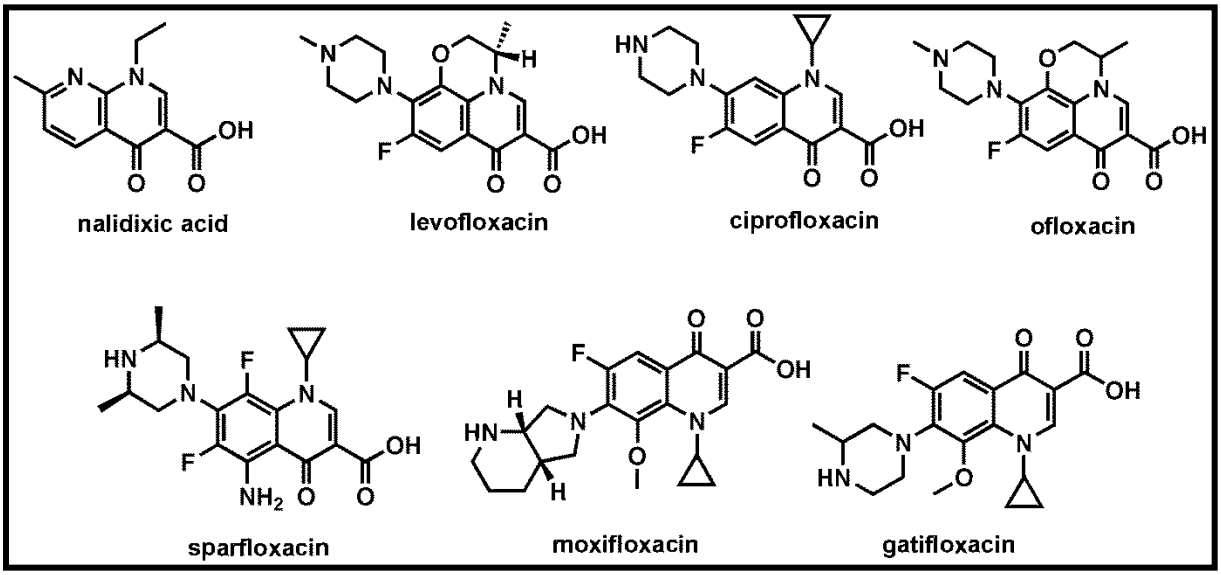

Figure 1.4 Examples of fluoroquinolones (2nd line TB drugs)

1.3.3.3 Aminoglycosides

In this class of compounds (Figure 1.5), streptomycin (STRP, an amino cyclitol) was the first to be discovered in 1944[47] leading to further discoveries of other aminoglycosides from nature. *Mtb* mutants resistant to STRP were identified 2 years after the discovery of the drug, however, it is still used in TB combination therapy as a 2nd line drug. Through semi-synthesis, medicinal chemists have been working towards making analogues that are less prone to development of resistance and with superior biological activity. The MoA of aminoglycosides is through inhibition of either of the two ribosome subunits 50S and 30S, which are responsible for protein synthesis in mycobacteria. 50S ribosome inhibitors include macrolides (erythromycin), lincosamides (clindamycin), amphenicols (chloramphenicol) and oxazolidinones (linezolid) while examples of the 30S inhibitors include tetracyclines and amino cyclitols (KANA and gentamicin).[31,40,48,49]

It has been shown that resistance to aminoglycosides in *Mtb* occurs by mutation of the ribosome target binding sites specifically, mutations in the *rpsL* gene, which encodes the ribosomal protein S12, account for approximately half of all STRP-resistant clinical isolates with the K43R mutation predominating. In about 20% of STRP-resistant *Mtb* clinical isolates, such resistance is associated with mutations in the *rrs* gene.[50–52] *Mtb* Eis (enhanced intracellular survival) protein reported to suppress host immune defenses by negatively modulating autophagy, inflammation, and cell death through c-Jun N-terminal kinase (JNK)-dependent inhibition of reactive oxygen species (ROS) generation, has also been demonstrated to contribute to drug resistance by acetylating multiple amines of aminoglycosides, rendering them inactive.[53,54]

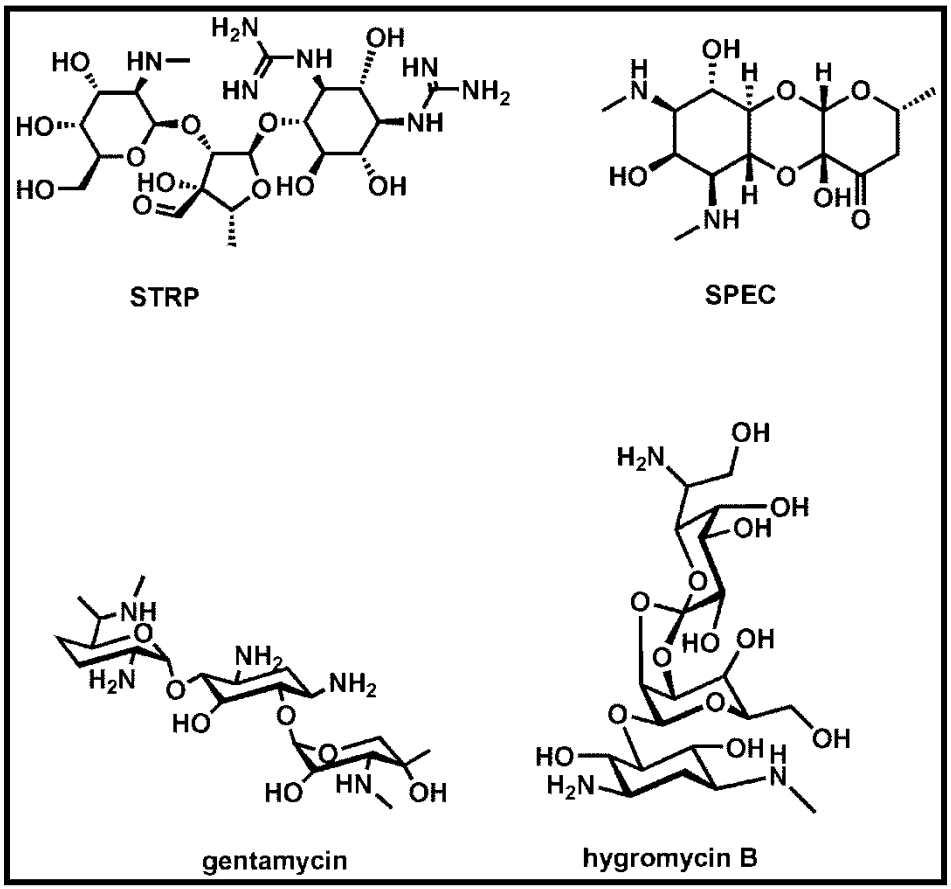

Figure 1.5 Examples of aminoglycosides (2nd line TB drugs)

1.3.3.4 Isoniazid (isonicotinylhydrazine, INH)

INH (Figure 1.2) is a 1st line anti-TB drug that has been very effective since its development. INH inhibits the synthesis of the mycolic acids that form the long chain fatty acids which contribute to the structural composition of the cell wall.[55–57] The biological activity of INH requires activation from a pro-drug state by the mycobacterial catalase-peroxidase enzyme, KatG, which couples the isonicotinic acyl with NADH to form isonicotinic acyl-NADH complex leading to antimycobacterial effect. Activation of INH is also reported to form other derivatives such as reactive nitrogen intermediates, which are also associated with the antimycobacterial activity of the drug.[40,55,58–60] Mutations in INH resistant clinical isolates are most commonly detected in the KatG gene, occurring in 50–80% of cases, thus reducing the ability of the catalase-peroxidase to activate the INH pro-drug.[60,61] It is also reported that, in a few cases, INH resistance may also arise from mutations in *inhA,* resulting in reduced affinity of the enzyme for NADH without affecting its enoyl reductase activity, or in the promoter region of the *mabA-inhA* operon, resulting in over expression of the wild-type enzyme.[62]

1.3.3.5 Pyrazinamide (PZA)

PZA (Figure 1.2) is a 1st line TB drug which was developed in the mid-1980s. Structurally, it is similar to INH. It is a pro-drug, hydrolyzed intracellularly to pyrazinoic acid by pyrazinamidase (Pzase) encoded by *pncA*.[63,64] Interestingly, PZA is bactericidal against non-replicating bacilli but bacteriostatic against fast replicating organisms.[31,40] It does not exhibit activity *in vitro* on many mycobacterium strains but shows very high efficacy *in vivo*. PZA was found to be active against *Mtb* only at an acidic pH (e.g. 5.5), which is thought to occur during active inflammation due to production of lactic acid by inflammatory cells as a key defence strategy in macrophages. This makes PZA a unique drug considering that its development has not been in accordance with the conventional drug discovery process: it wouldn't be identified in most current drug screening algorithms. When added to the current TB regimen, it reduces the duration of

treatment significantly from 9 to 6 months.[40,65] Though its MoA has been elusive, Shi *et al*.[63] identified the ribosomal protein S1 (*rpsA*) as a target for pyrazinoic acid. The protein is vital for protein translation and the ribosome-sparing process of *trans*-translation that is responsible for execution of large changes in the genetic programs, including responding to stress, pathogenesis and differentiation. Development of resistance to this drug is associated with mutations in the *pncA* gene in *Mtb*. Other possible points of mutations include deficient uptake, enhanced efflux, or altered *pncA* regulation.[64,66]

1.3.3.6 Ethionamide

Structurally, ethionamide (Figure 1.6) is also related to INH. It is a pro-drug that was discovered in 1956. It requires activation to form sulfinic acid by *ethA*, a monooxygenase in *Mtb*, and binds NAD to form an adduct which inhibits *inhA* in the same way as INH leading to inhibition of mycolic acid synthesis.[67–69] Development of resistance to ethionamide is attributed to mutations in *ethA* or *inhA*. Other likely mutations of *mshA* a gene encoding the first enzyme involved in the biosynthesis of mycothiol, a major low-molecular-weight thiol in *Mtb*, and thought to also be involved in activation of ethionamide, could occur leading to development of resistance to ethionamide.[67,68,70]

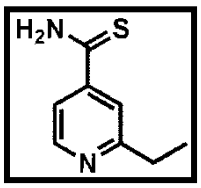

Figure 1.6 Ethionamide

1.3.3.7 Ethambutol (EMB)

EMB (Figure 1.2) was discovered in 1961 and kills actively multiplying bacilli by inhibiting arabinosyl transferases involved in cell-wall biosynthesis in mycobacteria.[71] It is included

amongst the four drugs in the current TB regimen and this is due to the fact that, in combination with some anti-TB drugs, it results in synergy, has low toxicity and is effective in arresting development of resistance. Development of resistance of EMB in *Mtb* is associated with point mutations in the *embBC*. Commonly observed multistep mutations in *embB* codon 306 followed by mutations in pathways for the biosynthesis (Rv3806c) or use (Rv3792) of the cell wall precursor decaprenylphosphoryl-*β*-D-arabinose (DPA), which, finally, are followed by mutations in *embC*, have been reported to be associated with variable degrees of EMB resistance. [72–75]

SQ109 (Figure 1.7) as well as other analogues of EMB have been reported to exhibit similar activity to EMB but with a different MoA that involves inhibition of the enzymes involved in menaquinone synthesis, respiration, and, hence, ATP synthesis.[76] It is also reported that, SQ109 acts as an uncoupler, collapsing the proton motive force (PMF). This inhibition is thought to indirectly impact on a particularly vulnerable and druggable target *mmpL3* gene, which encodes a trans-membrane trehalose monomycolate (TMM) transporter, MmpL3 involved in exporting mycolic acids to the cell membrane. The latter was initially thought to be the sole target for SQ109.[77,78]

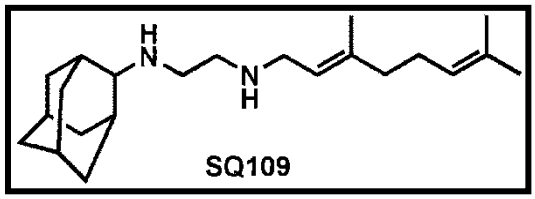

Figure 1.7 Ethambutol (EMB) analogue

1.3.3.8 Bedaquiline (BDQ)

The diarylquinoline, BDQ (also known as TMC207, Figure 1.8), is the first new anti-TB drug to be developed in 40 years.[3] It is highly active on non-replicating bacilli of drug-susceptible, MDR and XDR *Mtb* strains, with no cross-resistance to current first-line drugs. It is therefore added to

the TB treatment regimen only for drug resistant infections.[3,31,79] BDQ inhibits ATP synthase, blocking ATP generation in *Mtb*.[80] When tested alone, BDQ was reported to have similar antimycobacterial activity to a combination of three members of the standard anti-TB regimen (RIF, INH and PZA) and it was more effective than RIF alone in mouse models.[19] Resistance to BDQ is mediated by mutations in the *atpE* gene encoding the trans-membrane and oligomeric C subunit of ATP synthase. However, recent reports indicate that a majority of *in vitro*-generated mutants, resistant to BDQ lack mutations in *atpE*, suggesting that there are other mechanisms of drug resistance.[81,82]

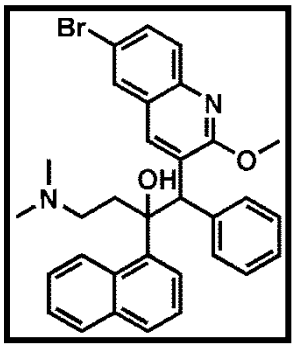

Figure 1.8 Bedaquiline (BDQ)

1.3.3.9 *para*-Aminosalicylic acid

This anti-TB drug (Figure 1.9) is widely used clinically, and was discovered in the 1940s. It is a pro-drug that is activated by dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS) to a hydroxyl dihydrofolate.[83] The active form of the drug is reported to inhibit dihydrofolate reductase (DHFR) involved with folic acid biosynthesis.[84] Initially, resistance to this drug in *Mtb* was reported in clinical isolates where mutations in the *thyA* gene encoding the enzyme thymidylate synthase of the folate biosynthesis pathway were established, signifying that MoA of the drug may be as speculated[84] but more recent studies established that inhibition of DHPS or mutation in DHFS prevents the formation of the hydroxyl metabolite, hence conferring resistance to *para*-aminosalicylic acid.[83,85]

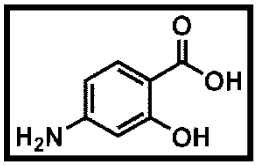

Figure 1.9 *para*-Aminosalicylic acid

1.3.4 Repurposing and repositioning of old drugs for anti-TB drug development

Finding new uses for old drugs is referred to as repurposing. For example, a drug known to treat fungal infections can also exhibit antibacterial properties and can hence be developed as an antibiotic. On the other hand, repositioning refers to the process of carrying out structure modifications on an old drug to acquire different biological properties.[86] Lately, these strategies have been integrated into the drug discovery process, for the following reasons: rescuing stalled pharmaceutical projects, finding treatments for neglected diseases and to reduce the risk, enormous amounts of money, manpower and time that are required to develop a novel drug.[87] This section discusses some classic examples of drugs that have the potential or are in the process of either being repositioned or repurposed for use as anti-TB agents.

Macrolides such as clarithromycin (Figure 1.10) are broad spectrum antibiotics consisting of a macrolide ring, to which one or more deoxy sugars may be attached. The ring is associated with the compounds' bioactivity specifically binding to the bacterial 50S ribosomal subunit and inhibiting RNA-dependent protein synthesis.[88–90] The antimycobacterial activity of this class of compounds against *Mtb* is reported to be weak.[91,92] These drugs are normally faced with intrinsic resistance in *Mtb* attributed to low cell wall permeability, a property that is not found in non-tuberculous mycobacteria.[19] Clarithromycin is a good example of a macrolide that is reported to exhibit *in vitro* antimycobacterial activity but not *in vivo*.[92]

Capreomycin (Figure 1.10) is a macrocyclic polypeptide antibiotic isolated from *Streptomyces capreolus* that has also been shown to have antimycbacterial activity against MDR-TB. The drug interferes with several ribosomal functions by binding to the 16S rRNA helix 44. Resistance to this drug in *Mtb* is associated with mutations in the *rrs* gene encoding 16S rRNA.[19,49,93]

Another antibiotic, **cycloserine** (Figure 1.10) is a D-alanine analogue that has antimycobacterial activity against *Mtb*. Interestingly, the drug has an effect on the central nervous system making it suitable as an anti-TB drug for psychiatric patients.[94] The MoA in *Mtb* involves interruption of peptidoglycan synthesis by inhibiting the enzymes D-alanine racemase (*alr*) and D-alanine:D-alanine ligase (*ddl*) necessary for the synthesis of UDP-muramyl-pentapeptide.[95] More recent studies have established that *ddl* is the primary target of cycloserine, as cell growth is inhibited when the production of D-alanyl-D-alanine is halted. It was shown that inhibition of *alr* may contribute indirectly by lowering the levels of D-alanine, thus allowing cycloserine to outcompete D-alanine for *ddl* binding.[96] Development of resistance was reported in *Msm* and *M. bovis* mainly due to over expression of *alrA* and *ddl* although whether similar mechanisms are responsible for cycloserine resistance in *Mtb* remain to be investigated.[95]

An interesting class of compounds that have been discovered to combat anaerobic bacterial and parasitic infections is the **nitroimidazoles** (Figure 1.10). In *Mtb*, metronidazole is reported to be active under anaerobic conditions, contrary to the effect observed for INH and RIF.[97,98] *In vivo* studies have only revealed the antimycobacterial activity of metronidazole in *Mtb*-infected rabbits.[99] Econazole and clotrimazole are thought to inhibit the P450 mono-oxygenase homologue to the eukaryotic 14α-sterol demethylases and that the imidazole moiety binds the iron of these haem-containing enzymes. [100]

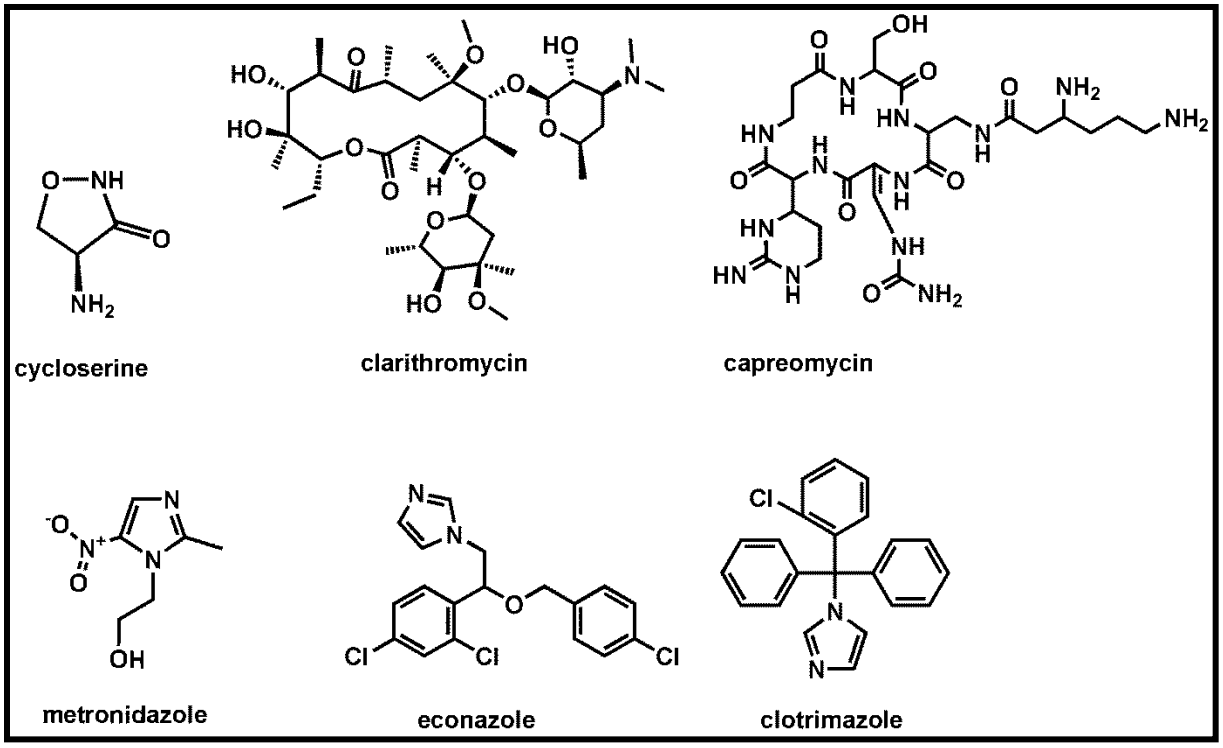

Figure 1.10 Examples of drugs with potential anti-TB properties

PA-824 (Figure 1.11) is a bicyclic nitroimidazopyran related to metronidazole, exhibiting bactericidal activity against replicating and hypoxic *Mtb*.[101] It is highly active against MDR clinical isolates of *Mtb*, suggesting no cross-resistance with current anti-TB drugs; better still, PA-824 inhibits a new target in tubercle bacilli. It is also reported to have antimycobacterial activity against *Mtb* in murine and guinea pig models. The compound is a pro-drug that is activated by a cofactor F_{420}-dependent glucose-6-phosphate dehydrogenase (Fgd) and deazaflavin-dependent nitroreductase (Ddn). PA-824 rapidly disrupts the oxidation of hydroxymycolates to ketomycolates with concomitant accumulation of hydroxymycolates, a class of mycolic acids that are major constituents of the cell envelope of *Mtb*. Resistance to PA-824 is most commonly mediated by mutations that lead to loss of pro-drug activation, including those in the genes Rv0407 (*fgd*) and Rv3547 (*ddn*) which encode the activating enzymes. [101,102]

Delamanid (OPC-67683) (Figure 1.11) is derived from the nitro-dihydro-imidazooxazole class of compounds. It exhibits high *in vitro* activity against drug-susceptible and resistant *Mtb* strains and does not show cross-resistance to any current first-line drugs. It is also reported to have antimycobacterial activity with evidence that infrequent and low dosing may be effective. OPC-67683 has a long half-life, is not metabolized by cytochrome (CYP) enzymes and its efficacy in immune-compromised mice suggest that this drug may be useful for the treatment of co-infected TB/HIV patients. Similar to PA-824, the pro-drug OPC-67683 is converted to its active form by deazaflavin (cofactor F_{420}) dependent nitroreductase (*ddn*) resulting in multiple highly reactive species that inhibit the synthesis of mycolic acids. Development of resistance to delamanid is attributed to mutations in the Rv3547 (*ddn*) gene, indicating defective drug activation.[19,79,86,101,103,104]

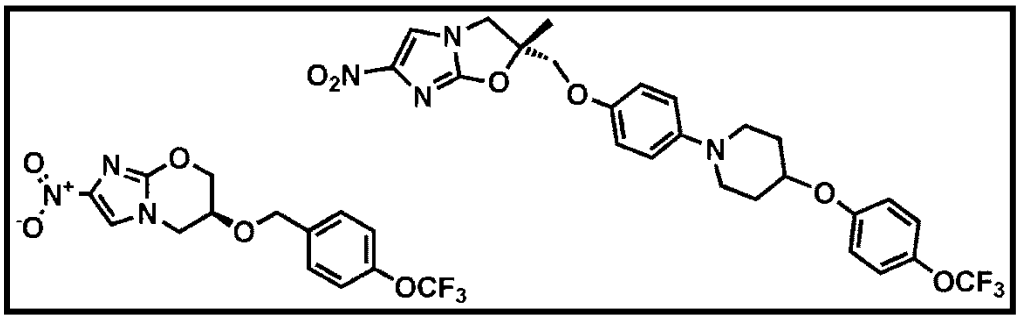

Figure 1.11 PA-824 and delamanid

Spectinomycin (**SPEC**, Figure 1.12) is an aminoglycoside produced by the bacterium *S. spectabilis* and used to treat gonorrhea.[105,106] A recent structure activity relationship (SAR) study of SPEC resulted in derivatives known as **spectinamides** (Figure 1.12) with 50-fold higher antimycobacterial activity against sensitive and resistant strains of *Mtb* (MIC 0.8-1.6 & 1.6-3.1 µg/ml respectively). Absence of cross-resistance with anti-TB drugs was reported for this new class of compounds and most importantly the compounds were said to overcome efflux pumps in *Mtb*.[107]

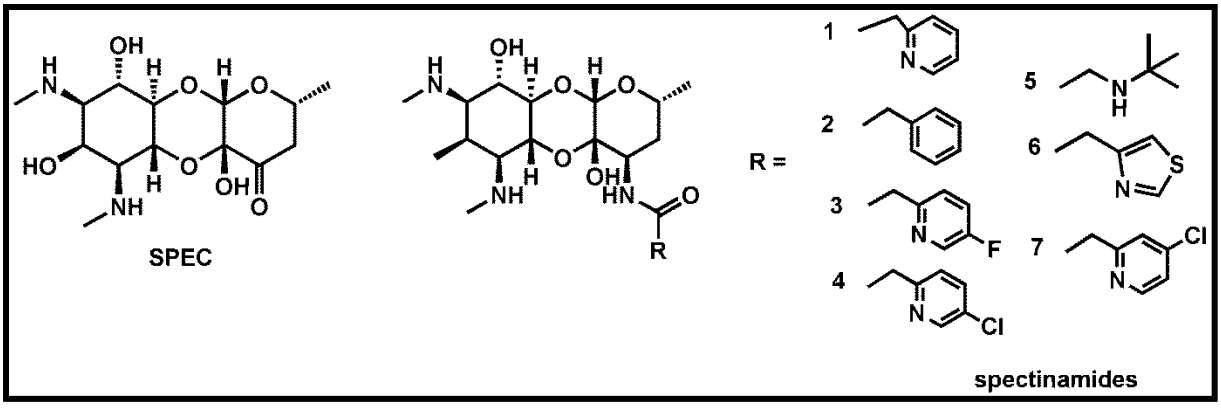

Figure 1.12 Examples of spectinamides

Phenothiazines (Figure 1.13) are antipsychotic drugs that were later discovered to have antimycobacterial activity. Methylene blue was the first compound to be identified with antibacterial and antiparasitic activity though it caused lethargy in humans and further development was channeled towards treatment of psychosis. Interestingly, it was noted that in hospital wards, patients treated with chlorpromazine (CPZ) exhibited lower bacterial infections than other normal patients.[108–110] This observation was not explored for many years until recently when a paradigm shift occurred in drug development resulting in repurposing and repositioning of old drugs for new uses.[86,108,111] Thioridazine and CPZ have been reported to be active against actively replicating or latent, drug-susceptible and drug-resistant *Mtb*.[112] However, due to side effects in humans,[113–115] serum concentrations above the MIC for *Mtb* cannot be safely attained. Nevertheless, against phagocytosed *Mtb* in macrophages, the killing concentration of thioridazine was reported to be as low as 0.1mg/L, 10-20% lower than the concentration used clinically in the treatment of psychosis indicating that clinical use of the drug is possible. This was attributed to the drug concentrating effect of the macrophages.[110,113,114,116,117] Thioridazine has also been demonstrated to have MDR and XDR antimycobacterial activity in mice.[108,110,112,118–120] Moreover, in a study carried out by Abbat *et al*.[121] on TB infected patients, addition of thioridazine to a regimen of anti-TB therapy led to faster culture conversion of sputum and lack of relapse.

The MoA of phenothiazines is reported to be involved in inhibition of the electron transport pathway,[122,123] specifically targeting NADH:menaquinone oxidoreductase activity, which is responsible for aerobic respiration of mycobacteria.[124] It is also thought that the compounds act on enzymes involved in fatty acid metabolism and membrane proteins, particularly efflux pumps causing synergism when combined with other drugs such as anti-TB drugs and other antibiotics. As of now, no mechanisms of resistance for this class of compounds has been reported.[19,111,113,117,124–126]

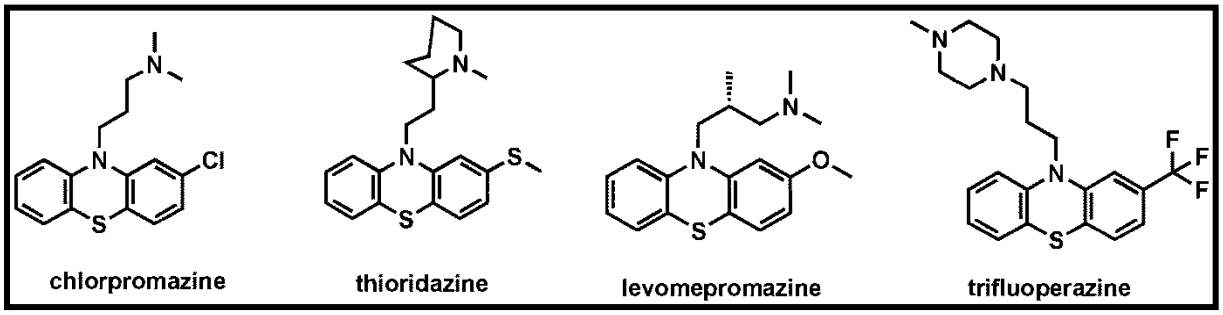

Figure 1.13 Examples of phenothiazines

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These studies are a clear demonstration of how drugs can be repurposed and/or repositioned for use for treatment of TB.

1.4 Drug discovery via biotransformation

To overcome drug-discovery challenges especially in areas such as TB, alternative strategies need to be explored in order to augment and complement the conventional drug-discovery paradigm. The conventional strategies mainly involve the synthesis or isolation of novel chemical entities from natural products. A different approach involves the use of biotransformation in the development of drugs that are based on the active metabolite(s) of parental compounds with demonstrated efficacy. In a drug development program, the use of biotransformation is relevant especially when there exists no chemical synthesis solution. This is

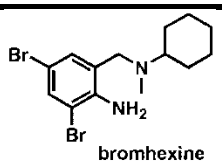
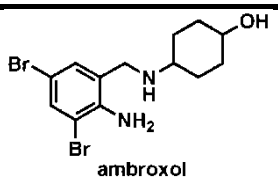
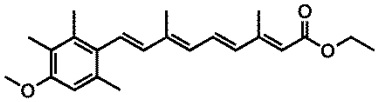
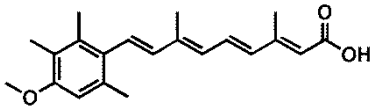
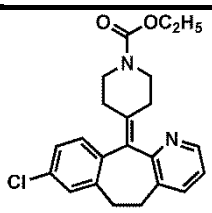
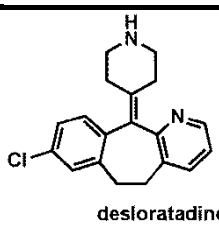
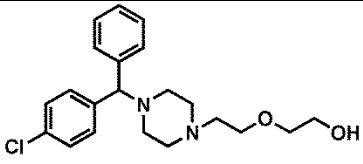
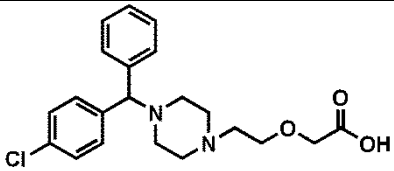
driven by the chemo- regio- and enantioselectivity of enzymes which operate at near neutral pH, ambient temperatures and atmospheric pressures; industrially useful chemistry often requires extremes of these conditions.[127,128]

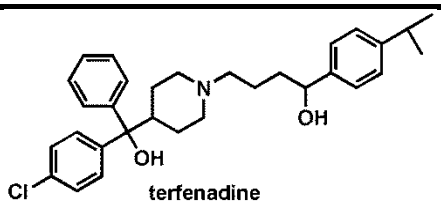
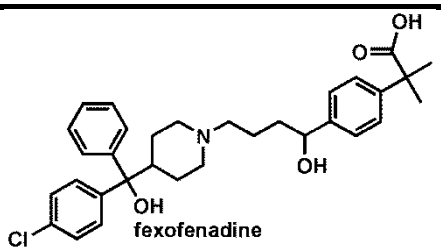
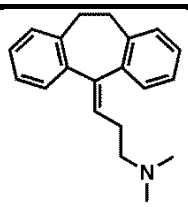
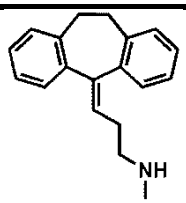
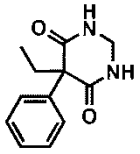
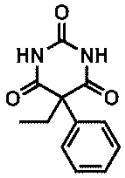
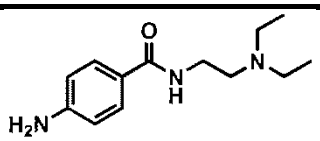
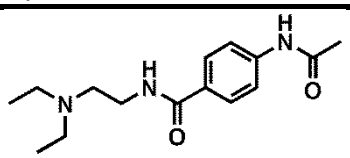
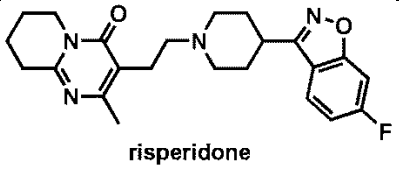
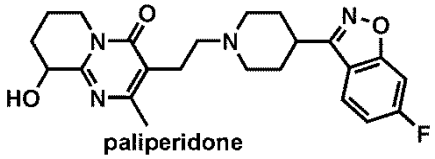
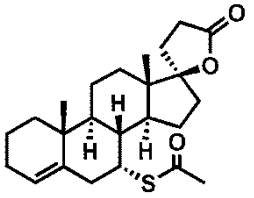
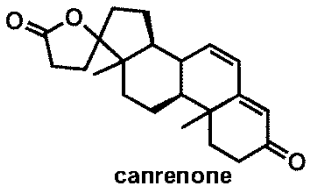
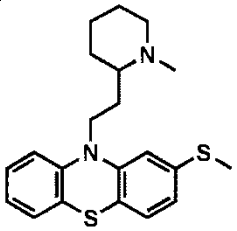
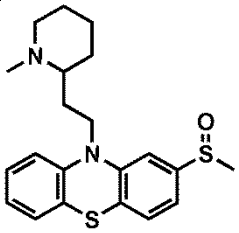
Metabolism is the process that converts compounds into chemicals that can be more readily eliminated from the body.[129] Where the parental compound is a drug, the products of metabolism can include those metabolites which are active against the same pharmacological target as the parental molecule.[128,130–132] The formation of pharmacologically active metabolites is usually mediated by two mechanisms: phase I metabolism, which includes oxidation, reduction, and hydrolysis, and phase II, which occurs via conjugation reactions. Phase I metabolism mediated by CYP450 enzymes is the most common pathway.[129–131] By definition, the metabolism of the active metabolite(s) itself will lead to the formation of fewer total metabolites than will be obtained from the parent compound. Consequently, the use of an active metabolite as a drug may reduce the possibility of off-target toxicity that can be caused by one or more extra metabolites formed from the parent compound.[130–132] That is, the number of metabolites that can be generated from the applied drug should limit the possibility for unwanted effects which can result from the inhibition of essential host functions.[133]

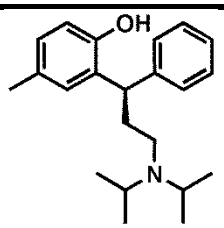
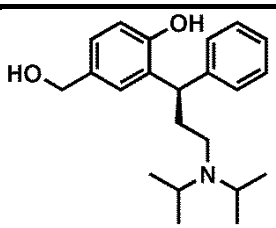
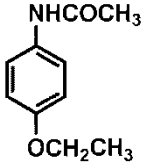
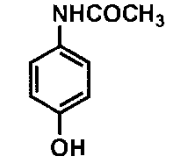
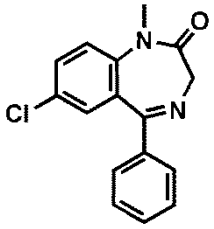
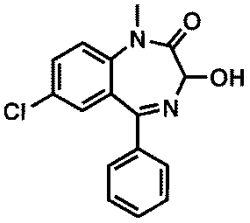
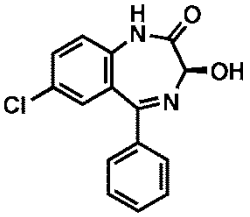
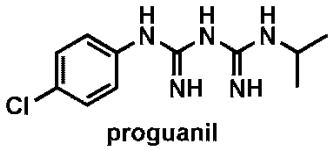
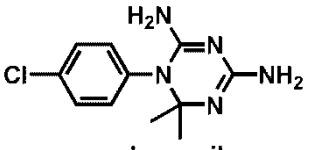
Some years back, the chemical and structural characterization and elucidation of the metabolites derived from novel lead compounds was often not completed until late in the drug development process. It is at this point that the relative contribution of active metabolites to the overall observed therapeutic effect was evaluated. In some cases, a full assessment of the pharmacological significance of metabolites was not made until after drugs had reached the market.[131] Even as technological advances have made early determination of biotransformation profiles feasible, biological transformation as a method of drug design has not been widely exploited.[130,134] Nevertheless, pharmacologically active metabolites have in some instances

been successfully developed as drugs, and these often possess superior physicochemical, pharmacodynamics and pharmacokinetic properties compared to the parent drugs (Table 1.1).[128,130–132] *para*-Acetamidobenzenesulfonamide, a metabolite of prontosil which is a dye and a pro-drug, discovered in mice, was the first sulfonamide antibiotic to be developed.[135] However, it is worth noting that, there are no reports of antibiotics developed from active metabolites of antibacterial compounds. This creates a research opportunity for medicinal chemists especially in the area of TB research.

Table 1.1: Marketed drug metabolites and their parents

Parent	**Metabolite (active, marketed)**	**Use of the marketed metabolite**
 bromhexine	 ambroxol	mucokinetic agent and topical anesthetic
 etretinate	 acitretin	antipsoriatic
 loratadine	 desloratadine	antihistamine
 hydroxyzine	 cetrizine	antihistamine

 terfenadine	 fexofenadine	antihistamine
 amitriptyline	 nortriptyline	antidepressant
 primidone	 phenobarbital	antiseizure
 procainamide	 acecainide	antiarrythmic
 risperidone	 paliperidone	antipsychotic
 spironolactone	 canrenone	diuretic
 thioridazine	 mesoridazine	antipsychotic

 tolterodine	 fesoterodine	urinary incontinence
 phenacetin	 acetaminophen	analgesic
 diazepam	 temazepam	anxiolysis
	 oxazepam	
 proguanil	 cycloguanil	antimalarial

Limitations to drug development from metabolites prepared using CYP450s as biocatalysts on a preparative scale have been reported. They arise from difficulties in preparing large volumes of enzymes, low stability of the enzymes and the high cost of the cofactors.[127,136] However, microbial whole cell systems with the membrane bound cofactors have been used in large scale biotransformation reactions.[136]

1.4.1 *In vitro* metabolite generation

A number of *in vitro* techniques are used for metabolite generation. Methods include use of recombinant CYP450, liver microsomes, and microbial transformation.[137,138] Liver microsomes are the most commonly used *in vitro* method for metabolite generation.[139] Being sub-cellular fractions of the liver, they possess phase I and major phase II enzymes. Either or both of these groups of enzymes can be used by addition of the required enzyme cofactors and activators to the incubation mixture.[140] Liver microsomes are easily prepared by centrifugation of hepatic homogenates,[131] and can be stored for long periods of time without significant loss of activity. However, sample incubation cannot be extended for more than 2h, limiting their use in poorly metabolised samples. In addition, they contain only one major class of phase II enzymes, uridine-5'-diphosphateglucoronyltransferase.[141]

Enzymes are unique in the sense that as catalysts, they have been known to be very efficient with capability to increase reaction rates 10^{17} times causing minimal hazard compared to the conventional transition metal catalyst.[128,142–144] In addition, they are highly chemo-, regio- and enantio-selective and hence advantageous in the synthesis of natural, agrochemical and pharmaceutical products. They work under mild reaction conditions, such as room temperature and neutral or almost neutral pH, resulting in reduced costs that may be incurred, for instance, in traditional energy-consuming chemical processes.[128,142–144] The CYP450 group of enzymes is involved in metabolism of most drugs and is commonly implicated in formation of active metabolites.[131] Recombinant CYP450 enzymes are therefore used to screen drug candidates for metabolite generation and to predict CYP450-based drug-drug interactions. The recombinant enzymes are expressed by bioengineered *Escherichia coli* or insect cells.[137] For optimal catalytic activity, an electron transport protein, which acts as a redox partner to the CYP450 enzyme must either be co-expressed with the CYP450 enzyme or added to the purified enzyme.[145] A variant of this method is the use of the entire cellular system in *E. coli* or insect cell

as a bioreactor expressing one or several CYP450 enzymes.[131] This approach closely models the use of microsomes and these membrane preparations are therefore known as bactosomes[146] when bacterial cells are used, or supersomes[131] when insect cells are used.

There are microbial organisms with enzymes able to produce similar metabolites to those found in humans. Although not all mammalian metabolites can be obtained utilizing the microbial systems, they offer a good option in metabolic studies owing to the relative ease of maintaining cultures. Scale-up to produce higher quantities is also easily accomplished. Fungal strains, especially *Cunninghamella elegans,* and bacterial strains like *Actinomycetes* and *S. griseus* are the most useful.[131]

1.4.2 Structural characterization of metabolites

By coupling liquid chromatography with mass spectrometry (LC/MS), it is possible to quantify and determine the identity of drugs and metabolites of diverse structures in different biological systems with unprecedented speed, sensitivity and specificity. LC/MS, particularly in combination with atmospheric pressure ionization (API) i.e. electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) techniques, can now be used to analyze polar, non-volatile, and thermally labile drugs and metabolites. Less polar molecules can be analyzed with the recently introduced atmospheric pressure photoionization technique. Advances in computer software technology have also greatly facilitated LC/MS data acquisition and interpretation. One-dimensional (1D) proton liquid chromatography with nuclear magnetic resonance (LC/NMR) often provides information about the structure of metabolites. Various 2D NMR experiments can be used to confirm the structure or for identification of more complex structures.[147–152]

1.5 Synergistic/matrix antimycobacterial screening and MoA of drugs in combination

The development of new therapies for bacterial infections has traditionally focused on experimental screening for single compounds that inhibit growth. As the identification of active new compounds using this approach became less productive, pharmaceutical companies diverted their resources to target-based, high-throughput screening campaigns. This approach proved to be a lengthy process, with low yields and profits.[125] In a study by DiMasi *et al.*[153] 68 drugs obtained from a survey of 10 pharmaceutical firms were analyzed, revealing that it takes an average of 14 years and $800 million to bring a single drug to the market. Repurposing or repositioning of existing drugs either alone or in combination based on the concept of simultaneous targeting of more than one signaling pathway or pathway component has been initially attempted and should continue to be explored.[86,154,155]

Drug combinations are a promising strategy to overcome the compensatory mechanisms and unwanted off-target effects that limit the utility of many potential drugs.[156] Synergy occurs when drugs can interact in ways that enhance or magnify one or more effects, or side-effects, of those drugs. Currently, many drugs are being developed in combination to be able to achieve higher efficacy. Good examples include artemisinin-based combination therapy (ACT) for the treatment of malaria, and antiretroviral drugs (ART) for the management of HIV/Aids,.[86,157,158] A number of investigations have been carried out to identify synergistic drug combinations and the desired activity has been observed in many cases.[159–162] In view of the fact that WHO recommends combination therapy for TB treatment (Figure 1.14),[3] it is almost certainly worth subjecting compounds to combination screening at an early stage of drug development in order to select the appropriate partners, which can later be used for development of TB drug regimens. Although there are no reports to date of the successful development of TB drugs based on their properties in combinations *in vitro*, recent results established that some benzothiazinones in

combination with BDQ result in synergistic effects against *Mtb*, suggesting that this might be a useful approach to optimize lead candidates for combination therapy.[163,164]

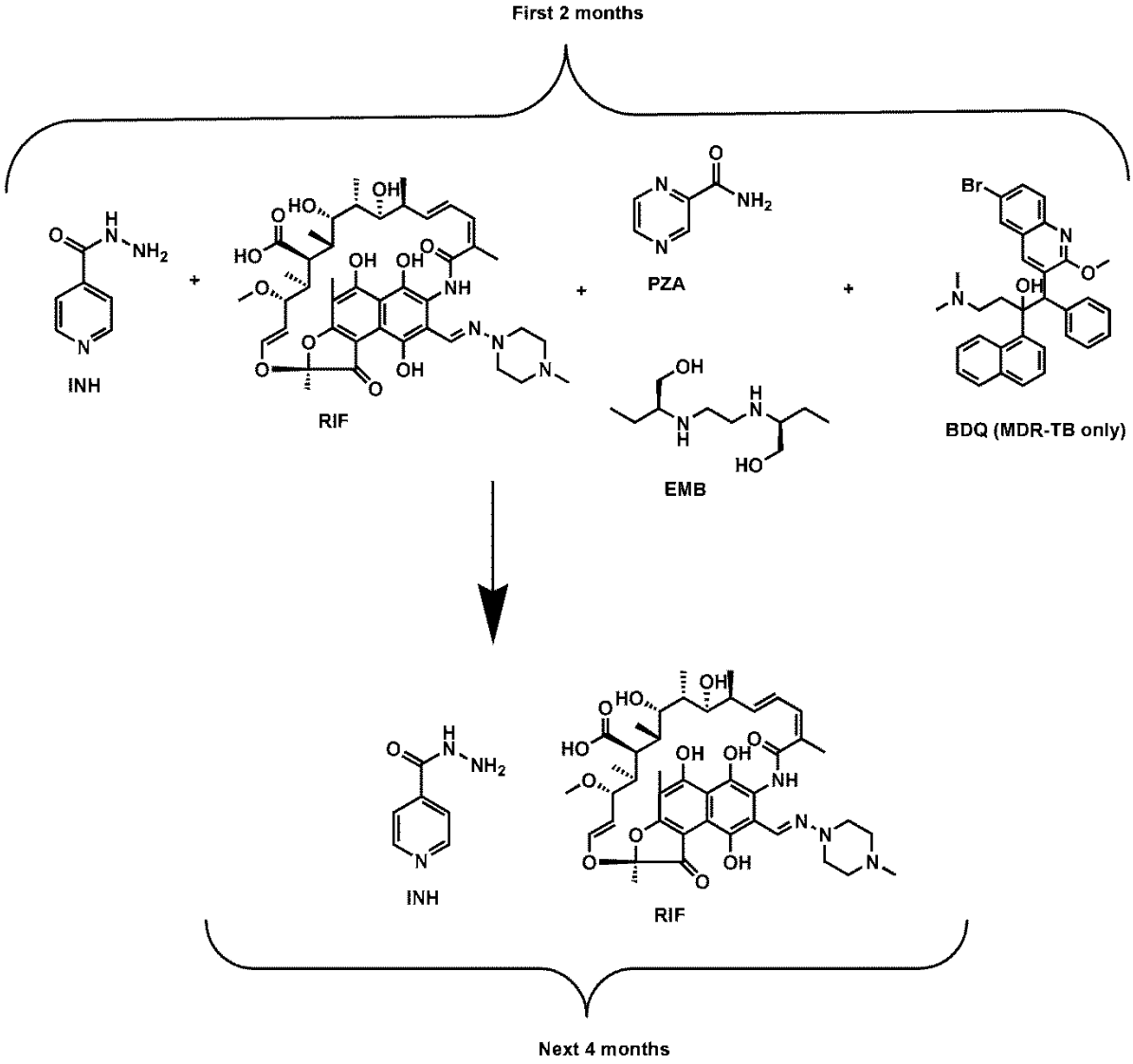


Figure 1.14 Example of TB combination therapy; BDQ is added to the regimen only in case of MDR-TB

Synergistic combinations can result in drugs that show increased potency (equivalent therapeutic effects are seen at lower doses) hence minimal toxicity.[155] The other type of combination is one that leads to enhanced efficacy (whereby the combination has more therapeutic benefit than is attained by the single agents at any dose).[125] Various combination

strategies can be used for synergistic combination studies. Unknown compounds can be tested in combination but this attracts a high risk of failure of identifying an efficacious combination. Known compounds can also be combined with unknown molecules and this would result in a high probability of getting a novel drug combination. The most promising approach would be to screen known compounds in combination with known pharmacological profiles hence increasing chances of hit discovery.[125,165]

The synergistic/matrix combination or "checkerboard" assay is currently the most established tool for testing drugs in combination *in vitro*. The measure of interaction between two or more drugs is known as fractional inhibitory concentration index (FICI). According to this method, synergy is assigned where the FICI $\leq$ 0.5; a FICI $\geq$ 4 is considered an antagonistic interaction, while any value falling in between indicates no interaction.[125,155,166–168] At an early stage of drug discovery, high-throughput checkerboard assays have been used successfully in testing millions of compounds in chemical libraries giving a good sense of possible drug combinations that can be used. Ramon-Garcia *et. al.*[125] identified SPEC (Figure 1.12), an antibiotic with very low antimycobacterial activity that synergizes with other drugs such as bromperidol and butyrophenone (Figure 1.15) used for treatment of psychosis, as well as other family of drugs such as macrolides and azoles.

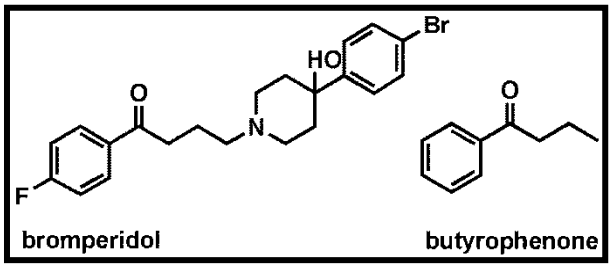


Figure 1.15 Example of drugs synergizing with SPEC

Amongst the many properties to consider while choosing partners for drugs, the MoA is of great importance.[155] The sophisticated nature of *Mtb* requires a well thought out strategy for choosing partners that can be used to clear the infection. A combination of drugs that is active against both replicating and non-replicating forms of *Mtb* should be a good starting point.

Reports indicate that aminoglycosides tend to display synergistic effects when used in combination with other drugs such as cell wall synthesis inhibitors.[48] Various studies have also established that efflux pump inhibitors such as verapamil, reserpine (Figure 1.16) and phenothiazines like thioridazine (Figure 1.16) potentiate antimycobacterial activity of anti-TB drugs with better results being observed with aminoglycosides.[22,23,48,168] These observations can probably be attributed to enhanced accumulation of the partner drug in the pair, within the mycobacterium cells. Although phenothiazines are reported to inhibit NADH:menaquinone oxidoreductase, it is thought that the downstream effect results in efflux pump inhibition hence leading to a significant reduction in the minimum inhibitory concentration (MIC_{99}) of the partner.[113,122,124,155]

A good example of the difficulty in predicting positive interactions is provided by the observation that INH in combination with CPZ is synergistic, whereas INH and EMB are antagonistic.[117,126] Synergism of drugs observed *in vitro* may also not necessarily translate to *in vivo* synergism and in fact, in many cases a linear correlation cannot be achieved.[169] For example, though unrelated to the TB research, lack of correlation between *in vitro* and *in vivo* drug synergy is evident in the various antiaspergillosis combination drug studies carried out and reported in a review authored by Steinbach *et al*.[170] Their review consolidates combination studies carried out between 1966 and 2001 - synergy between various drugs is reported in 36% of the *in vitro* studies but is less frequent *in vivo* at 14%. Lack of correlation may be attributed to the different environments within the *in vitro* and *in vivo* systems. In an *in vivo* environment, physiological

factors come into play such as absorption, distribution, metabolism, transportation, elimination and toxicity of the drugs.[171] Consequently, the information generated in an *in vitro* experiment cannot be used to predict synergy outcome in an *in vivo* environment.

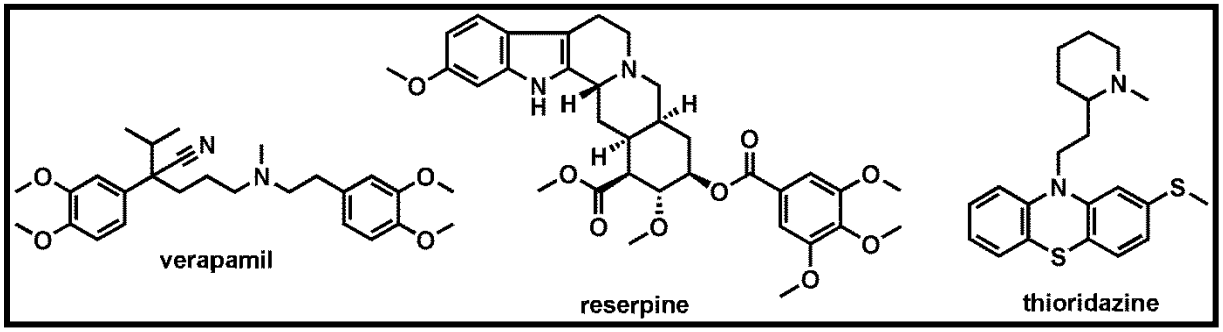


Figure 1.16 Examples of efflux pump inhibitors and a phenothiazine

1.5.1 *In vitro* mycobacterial models

The fast replicating non-pathogenic *Msm* (mc^2155 strain) is an ideal model for the preliminary high-throughput screening of thousands of compounds. Studies have shown that it shares some genetic similarities with *Mtb* and can hence be used in the early stages of drug discovery.[172,173] It provides quick information, which a medicinal chemist can use to select the scaffolds on which to focus. This is consistent with reports which have successfully applied *Msm* for the preliminary identification of hit compounds[174] as well as promising drug combinations.[125]

Since its isolation in 1905, *Mtb* H37Rv has found worldwide application in drug screening and biomedical research. Although recent evidence suggests some genetic variation between different laboratory isolates,[175] most have retained full virulence *in vitro*, *ex vivo* and in animal models of tuberculosis. Critically, H37Rv is also susceptible to all TB drugs and the strain is amenable to genetic manipulation which is key to downstream target identification and validation studies.[46]

1.6 Bactericidal and bacteriostatic effect of drugs tested individually and in combination

It is not only important to establish the MIC_{99} of a drug tested singly or in combination with other partners but also establish whether the drug clears the pathogen (bactericidal) or inhibits its growth (bacteriostatic). In case non-replicating *Mtb* is present, a bacteriostatic agent may not be able to clear the mycobacteria hence resulting in a relapse.[32] The determination of a bactericidal or bacteriostatic effect can be achieved in a 96-well microplate format by plating on Middlebrook agar, the contents of the well with the MIC_{99} concentration of an individual drug or the lowest synergistic concentrations of two drugs in combination. The experiment provides information on the effect of the drug(s) on the mycobacteria at a given concentration. Nonetheless, it is possible to determine a minimal bactericidal concentration (MBC) of a drug through the generation of a time-kill curve.[32]

1.7 Target identification and MoA determination

Target identification is an upstream process of identifying reactions or pathways that are critical for pathogenesis that is, the disease mechanism, and to characterize proteins/enzymes essential for binding of chemical molecules for them to achieve the desired bioactivity. When carrying out target identification, it is important to understand the sophisticated metabolic pathways of *Mtb* before exploring probable drug targets. Of importance, is to identify bacterial genes that are non-homologous to human genes and are essential for the survival of the pathogen, as novel drug targets. Identification of new drug targets has become a major priority mainly due to development of resistant strains of *Mtb* to the old drugs rendering them ineffective.[15,176–180] It is worth noting that genome-derived, target-based drug discovery approaches have had little success in the antibacterial therapeutic area in general. Koul *et al*.[1] suggest that the essentiality of a target for replication may be a prerequisite but it does not ensure its druggability; for many essential targets we are unable to identify specific inhibitors with drug-like properties. However, many studies have been carried out to identify targets for

various anti-TB drugs using this approach.[181] Target identification can be achieved through direct biochemical methods, mutant selection or whole genome mutagenesis.[182] Potential drug targets encoded in microbial genomes include genes involved in translation, transcription, DNA replication and repair, outer membrane proteins, permeases, enzymes of intermediary metabolism, host-interaction factors, among others.[183]

On the other hand, MoA determination is a downstream procedure that is carried out to establish the processes that are involved in causing chemical compounds to exhibit the desired biological effect. This for instance may involve bioconversion of molecules from an inactive form to an active form that readily binds to a given target, by enzymes expressed in the organism (sections 1.3.3.4 – 1.3.3.6, 1.3.3.9). Spontaneous resistant mutant generation is one of the most commonly used methods for determination of the MoA of drugs. The mutations may result in alterations of genes in the organism, that encode the enzymes/proteins that are drug targets or may be involved in bioconversion of the drug into an active form, hence providing an insight on the mode of action of the drug.[178,179] In the case where drug development is carried out via SAR studies or biotransformation of parent compound(s) with a known target, there is a high likelihood that the MoA of the parent will be phenotypically similar to that of its analogue(s) or metabolite(s).[107] This principle may also be applied to a parent drug in combination with various other molecules acting synergistically and with a known MoA, and comparing an unknown MoA for analogue(s)/metabolite(s) of similar drug pairs with similar synergy profiles.[184,185] Since the focus of the work presented in this thesis is mainly on determination of the MoA of selected compounds, some techniques used are discussed in detail in the subsequent sections.

1.7.1 Transcriptional responses

As an adaptation mechanism, organisms respond to changes in the environment by altering the level of expression of some genes. These changes involve co-ordinate regulation of sets of

genes (regulons) often controlled by single transcriptional regulators that function as genetic master switches, committing the bacterium to a major alteration in metabolism.[184] Studies involving establishment of transcriptional responses in drug treated *Mtb* and other bacteria, have previously been carried out extensively to identify potential MoA of new drugs. In their study, Boshoff *et al.*[184] identified transcriptional responses of *Mtb* to various drugs by generating a data set of 430 microarray profiles forming a signature subset of gene clusters, which provided an insight into the unknown MoA of several agents such as pyridoacridones and phenothiazines. Critical information can also be obtained from transcriptional profiling of both crude and purified natural products on both mechanism and detoxification prior to purification that can be used to guide the drug discovery process.[184]

MoA determination can be a daunting task especially during the hit to lead and lead optimization stage in drug discovery programs. Murima *et al.*[186] reported a useful approach based on transcriptional profiling of a miniaturized gene expression assay for efficient MoA deconvolution and discovery chemistry based on a microfluidics platform with integrated fluidic circuits. In their sophisticated approach, they are able to achieve a much higher efficiency of a normal quantitative polymerase chain reaction (qPCR) by 2 orders magnitude in a single assay using nanolitre reaction volumes compared to the standard techniques.

1.7.2 Spontaneous resistant mutant generation

This technique was developed to identify genes in an organism that undergo an alteration as a result of exposure to high concentrations of chemical agents. It starts with a whole-cell screen to identify antimycobacterial compounds followed by *in vitro* selection, isolation and sequencing of mutated genes in *Mtb* that arise from exposure of the mycobacteria to high concentrations of the active drug(s). The contribution of each mutation to the resistant phenotype is then confirmed by

introducing single point mutations into the parental strain by allelic exchange[187] or recombineering.[179]

1.7.3 Biochemical assay

Drug discovery programs were initially based on whole cell screening of drugs followed by determination of activity of the bioactive compounds, on recombineered or purified proteins/enzymes for target identification. Medicinal chemists later decided to adopt a different strategy and have target identification precede activity determination, a strategy that was thought to be more rewarding in pushing forward drugs selected with target specificity. Unfortunately, the target-based screening was not a success with only few compounds making it through the development pipeline.[179] It proved difficult to identify specific inhibitors of essential targets, with drug-like properties.[188,189] Nevertheless, target-based screening remains an important step in drug discovery but should be used as a tool to identify targets for potential lead compounds rather than a criterion for elimination of compounds from the development process before their whole-cell-activity has been determined.[179] A relevant study to the work presented here is the one carried out by Weinstein *et al*.[124] who identified the specific target of phenothiazines as type II NADH:menaquinone oxidoreductase by screening for activity of the compounds on the enzymes expressed in *E. coli*.

1.8 Repurposing of chlorpromazine (CPZ) - selected hit for proof-of-concept

CPZ (Figure 1.17), first synthesized in 1950, belongs to a class of compounds known as phenothiazines. As earlier mentioned (section 1.3.4), for many years, these compounds had been used in the management of psychosis but later it was discovered that the compounds exhibited antimycobacterial activity *in vitro, ex vivo and in vivo*.[111,117,122,190] Martins *et al.*, Crowle *et al.*[117] and Ordway *et al*.[113] demonstrated that CPZ and thioridazine were capable of inhibiting the growth of *Mtb* that had been phagocytosed by human macrophages at concentrations lower

than those used in treatment of pshychosis (0.1 – 3.6mg/L). They attributed this to the drug concentrating ability of the macrophages. These studies demonstrated that as antimycobacterials, or enhancers of antimycobacterial activity of other antibiotics, there is potential of using CPZ or its related compounds in clinical settings.

Further investigations on these compounds were carried out and in the search for a MoA for phenothiazines, various studies predicted that the compounds inhibit the electron transport pathway,[122,123] specifically inhibiting type II NADH:menaquinone oxidoreductase activity which is responsible for aerobic respiration.[124] The enzyme is a very attractive target due to the fact that it in humans, type I NADH dehydrogenase is utilized in the cells, instead, hence reducing chances of toxicity.[115] Boshoff *et al*.[184] carried out a transcriptional response study of *Mtb* to various drugs including CPZ. Their study indicated that in presence of CPZ, an up-regulation of *cydA* gene that encodes the alternative terminal oxidase (responsible for respiration) in *Mtb* is observed concluding that the gene is an essential target. In a similar approach, Murima *et al.*[186] were able to demonstrate that phenotypic screens can be used to compare transcriptional responses of parent compounds and synthesized derivatives in order to establish the target of the latter. This study provides insights into how target identification can be carried out on metabolites of CPZ. A negative effect of CPZ on the respiration process in *Mtb* has also been reported to reduce intracellular ATP levels consequently leading to a decline in efflux activity. It is therefore not surprising for the drug to exhibit a 4-fold reduction of efflux pump activity in *M. avium.*[190] In addition, CPZ was shown to exhibit synergism in combination with some anti-TB drugs [108,117,122,125,126] and this may be attributed to its efflux inhibition capability. This makes CPZ a potential partner for combination studies with anti-TB drugs.

The major routes of metabolism of CPZ include hydroxylation, *N*-oxidation, sulfoxidation, demethylation, deamination and conjugation.[191] Some studies have indicated that CPZ is

metabolized by CYP2D6 to 7-hydroxyCPZ as the main metabolite and the reaction partially catalyzed by CYP1A2.[192,193] The drug is said to be metabolized to many other products however, a few have been identified in rats, man and dog. They include CPZ-*N*-oxide, CPZ sulfoxide, nor-CPZ, nor-CPZ sulfoxide and CPZ-*N*-*S*-dioxide (Figure 1.17).[194–196] CPZ was therefore selected in this study for synergistic combination studies with anti-TB drugs, generation of metabolites for further combination assays and establishment of the MoA of the metabolites in relation to the parent.

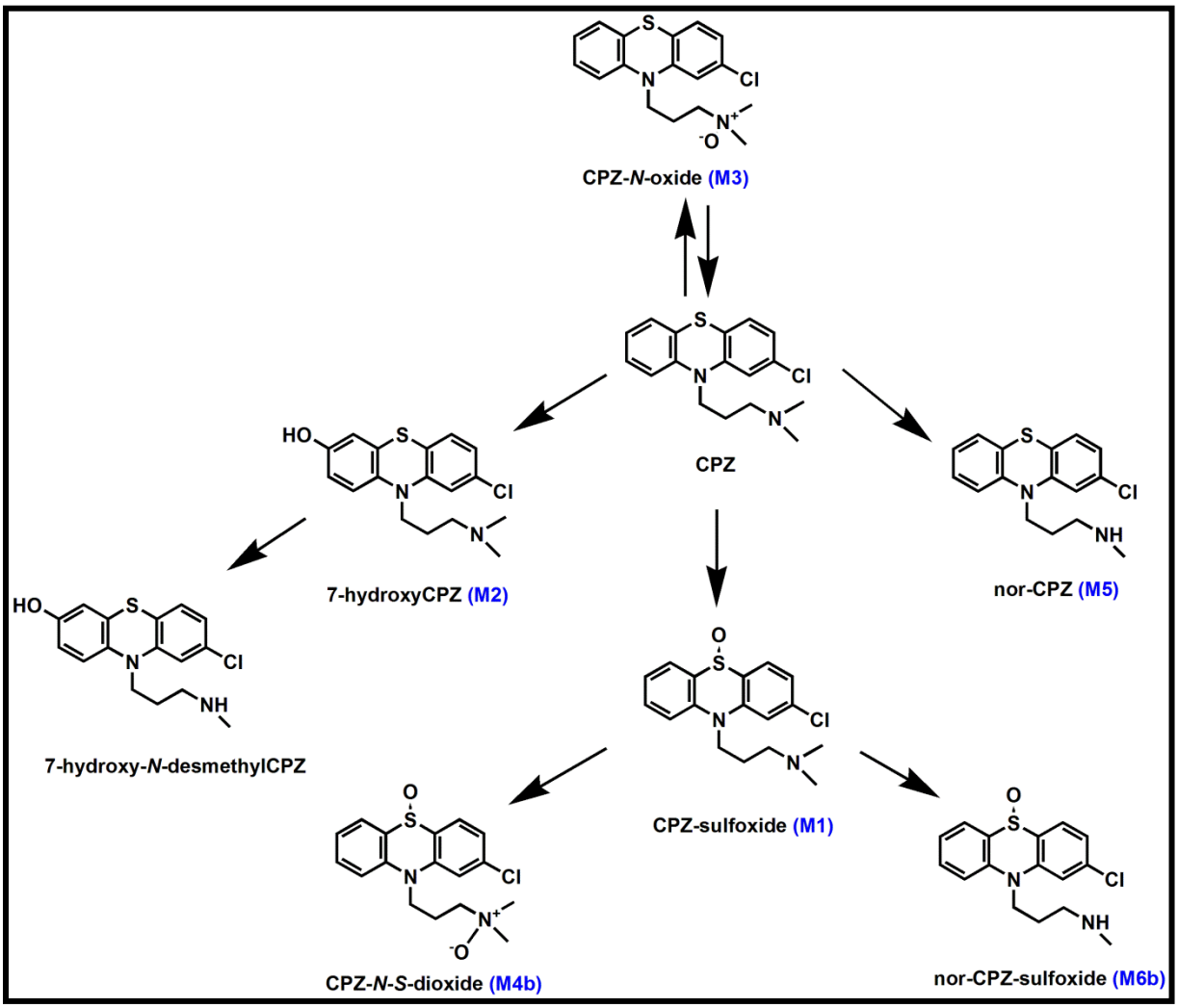


Figure 1.17 Reported CPZ metabolites in man, rat and dog

1.9 Rationale

To summarize what has been discussed in this chapter, new drugs are urgently needed because TB remains a global health priority. An epidemiological synergy exists with HIV/AIDS whereby co-infections represent 15% of the global TB burden and account for almost one quarter of HIV/AIDS-associated deaths. Coupled with the emergence of drug-resitant strains of *Mtb*, inadequate anti-TB treatment and limited effectiveness of public health systems, particularly in resource-poor countries where the main TB burden lies, it has become a real threat to achieve TB control and elimination globally. Drug discovery targeting active metabolites is a viable approach. It is very beneficial to have methods in place to allow screening of compounds for therapeutically useful biotransformation products for several reasons as discussed earlier in this chapter. This approach may lead to drug candidates with superior attributes such as improved pharmacodynamics, improved pharmacokinetics, lower probability for drug–drug interactions, less variable pharmacokinetics and pharmacodynamics, improved overall safety profile and improved physicochemical properties such as solubility. Moreover, early screening for active metabolites includes the potential for modifications of an entire chemical class to improve overall characteristics, and it also provides for more complete patent protection of the parent molecule.

Tracking active metabolites at the drug discovery stage allows for the correct interpretation of the pharmacological effect observed in preclinical species in relation to a predicted effect in humans. Development of drugs from active metabolites generated from parent drugs is an approach that is evident in the various drugs that have been developed and marketed. In addition, synergistic/matrix antimycobacterial screening is a powerful tool for drug discovery because it involves high-throughput synergy screening (HTSS) of chemical libraries having known pharmaceutical properties including thousands that are clinically approved. Hence, the incorporation of biological transformation products into synergy screens suggests the potential

to develop drug combinations with higher efficacy and superior pharmacokinetic properties. On the other hand, determination of the MoA of a drug is important not only to understand how a drug exhibits its biological effect, but also to predict chances of development of resistance. This forms the basis of this study.

1.10 Hypothesis

Anti-TB drug combination therapy can be developed from combinations of anti-TB drugs with CPZ or its metabolites, the latter exhibiting similar antimycobacterial activity and MoA to the parent, but potentially with better pharmacokinetic and safety profiles.

1.11 General objective

The overall objective was 2-fold:

a) To generate metabolites of a selected hit compound partner in the ideal combination for pharmacological evaluation individually and in combination.
b) To conduct synergistic/matrix screening of selected hit compounds with known clinically used antituberculosis agents in order to identify ideal combination partners.

1.11.1 Specific aims

i. Selection of compounds to be tested in combination with clinically used anti-TB drugs.
ii. Selection of parent compounds(s) for metabolite generation based on whole cell screening activity.
iii. Generation of metabolites *in vitro* via various biotransformation systems.
iv. Identification, scale up, purification and characterization of metabolites.
v. Development of combinatorial (matrix) assays for the discovery of synergistic drug combinations.
vi. Pharmacological evaluation of metabolites individually and in combination.

vii. Determination of bacteriostatic/bactericidal properties of identified combinations.

viii. MoA determination of candidate active compounds.

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CHAPTER 2

METABOLITE GENERATION, SCALE-UP AND CHARACTERIZATION

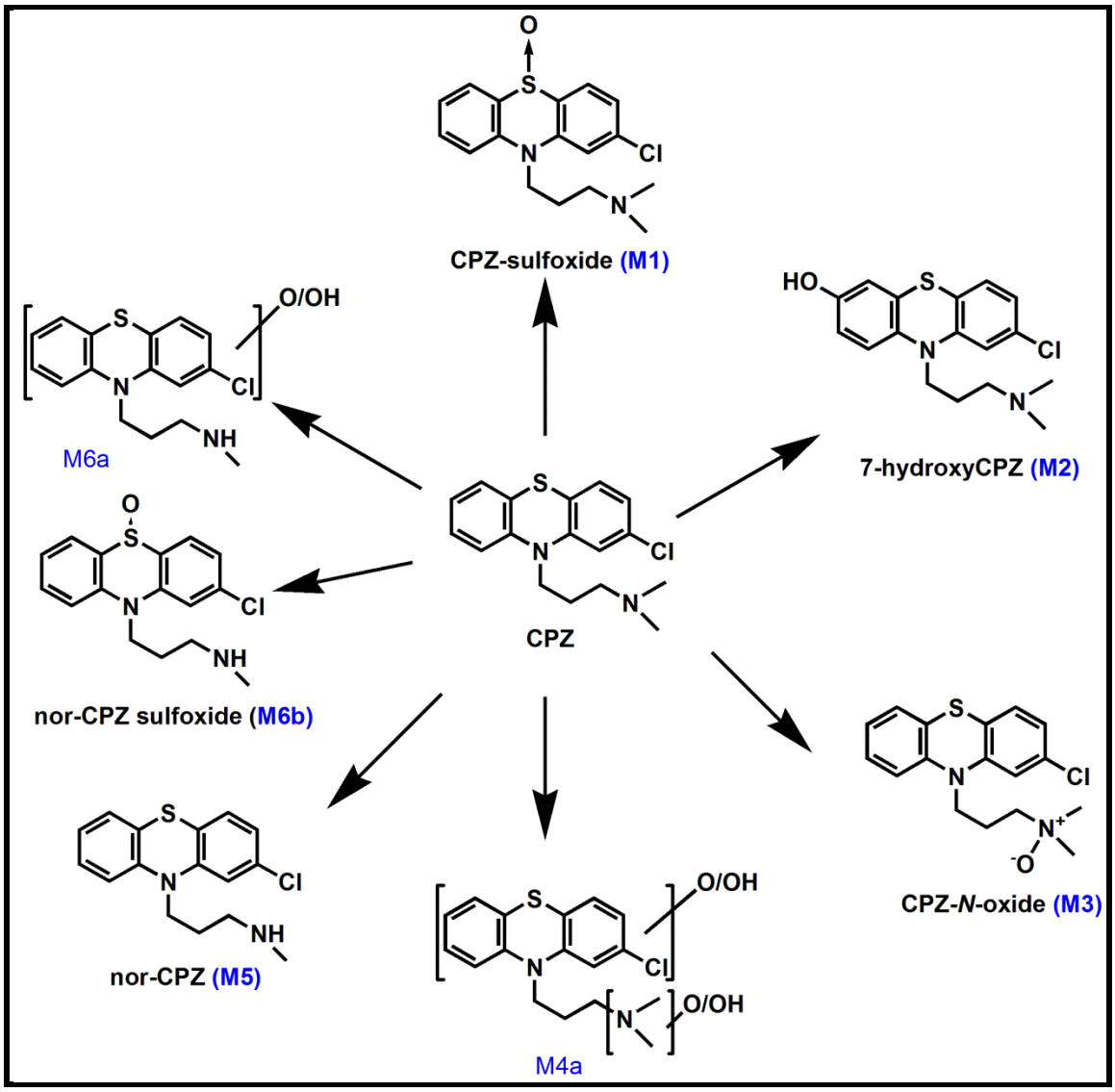
2.1 Introduction

Repurposing or repositioning of existing drugs[1–4] represents an appealing approach to overcome the limited ability of recent screening activities to identify viable hit compounds against *Mycobacterium tuberculosis* (*Mtb*).[5] As discussed in the first chapter, one way to achieve this is through modification of compounds by biotransformation resulting in active metabolites.[3,4,6–8] The use of an active metabolite as a drug is attractive in that it may reduce off-target toxicity: by definition, reducing the number of metabolites that can be generated from the applied drug should limit the possibility for unwanted effects which can result from the inhibition of essential host functions.[3,9] Chlorpromazine (CPZ) was therefore selected for generation of metabolites based on earlier reports indicating that it not only exhibits inhibitory effects on *Mtb* but also synergizes with some antituberculosis (anti-TB) drugs.[10–15] This chapter addresses the first four specific objectives of this study and involves the generation, scale-up, and elucidation of CPZ metabolites.

2.2 Metabolite generation from chlorpromazine (CPZ)

CPZ was initially incubated with several biotransformation systems in order to obtain and identify metabolites that could be scaled-up for antimycobacterial testing. Microsomes from human and rat liver fractions, recombinant and bioengineered cytochrome P450 (CYP450) enzymes, and different actinomycete strains were used in the incubations (section 7.2.1 -7.2.4, Chapter 7). Exposure of CPZ to human liver microsomes (HLM, section 7.2.1, Chapter 7) led to generation of oxidation and demethylation products. Seven metabolites identified and fully

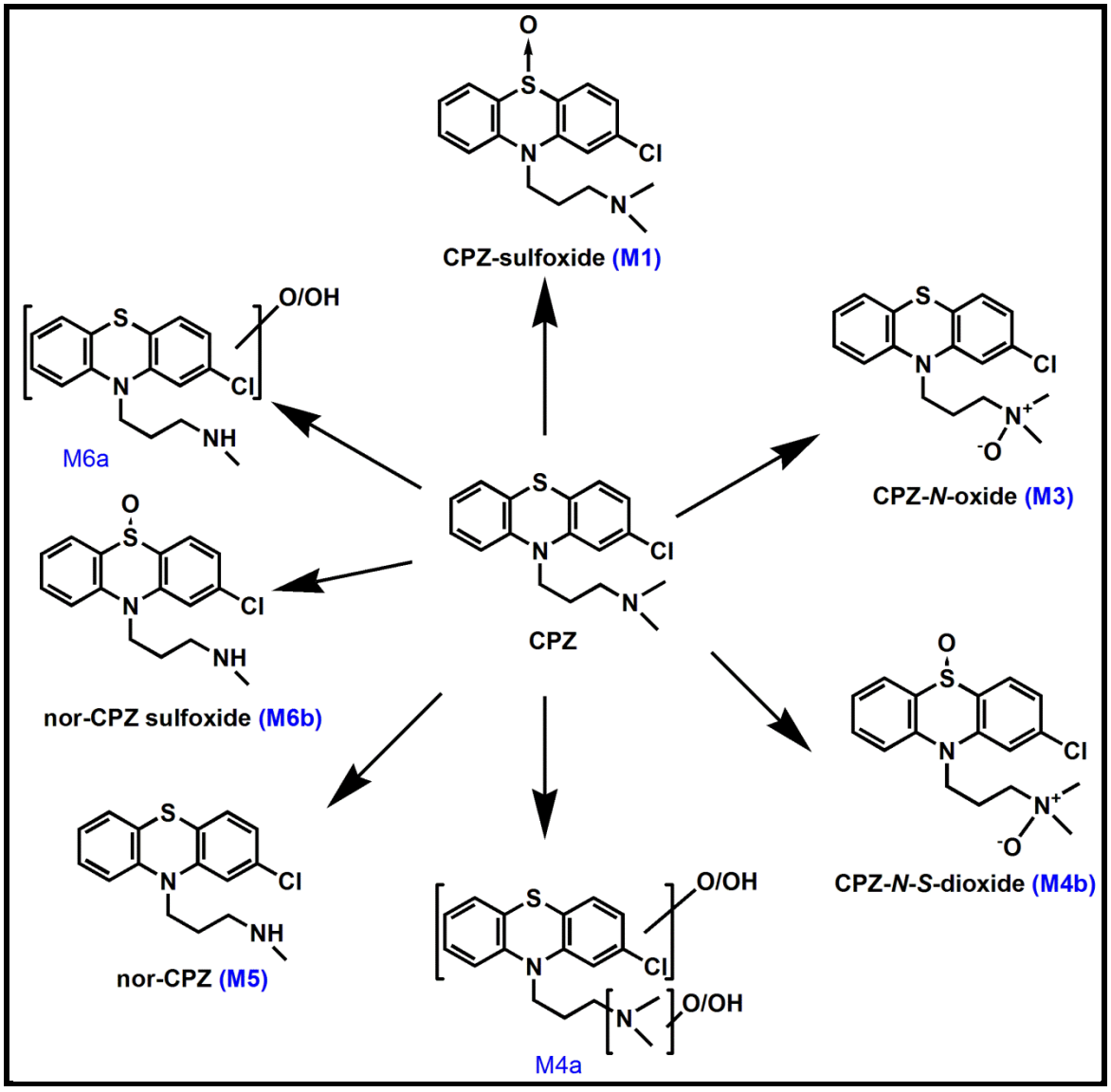
characterized included CPZ sulfoxide **(M1),** 7-hydroxyCPZ **(M2)**, CPZ-*N*-oxide **(M3),** nor-CPZ **(M5)** and nor-CPZ sulfoxide **(M6b)** (Scheme 2.1).



Scheme 2.1 CPZ metabolites generated in HLMs

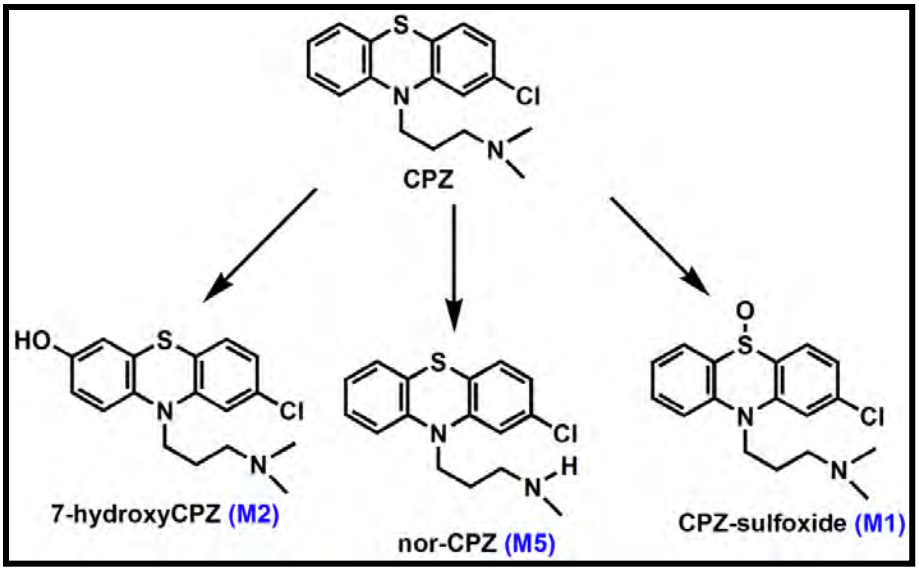
Similarly, exposure of CPZ to rat liver microsomes (RLM, section 7.2.1, Chapter 7) generated 7 metabolites (Scheme 2.2). However, in this case, CPZ-*N*-*S*-dioxide **(M4b)** was present in the RLM incubation but **M2** was not obtained. Full characterization of **M4a** and **M6a** was not achieved due to failure to obtain the respective standards. Identification of CPZ metabolites in

the HLM and RLM incubations in this study is consistent with previous reports in which the same metabolites were identified in the humans, rats and dogs.[16–18]



Scheme 2.2 CPZ metabolites generated in RLMs

Exposure of CPZ to four recombinant CYP450 enzymes - 1A2, 2D6, 3A4 and 2C9 - resulted in few or no metabolites (section 7.2.2, Chapter 7). CYP1A2 with CPZ led to the formation of **M1**, **M2** and **M5** (Scheme 2.3). Other CYP450 isoforms have been reported to be responsible for CPZ metabolism such as CYP1A1 and CYP2B1.[16]



Scheme 2.3 CPZ metabolites generated in CYP1A2

2.3 Identification and confirmation of CPZ metabolites

Summarized in Table 2.1 are the metabolites that were obtained in different systems and the corresponding retention times are shown on Figure 2.1, Figure 2.2 and Figure 2.3 on the extracted ion chromatograms (XIC) of the liquid chromatography/mass spectrometry, electrospray ionization (LC/MS, ESI) experiments (section 7.4, Chapter 7).

Initially, tentative elucidation of the structures of CPZ metabolites was carried out by extracting the known masses of CPZ metabolites from the total ion chromatograms (TIC) of the HLM, RLM and CYP1A2 samples and comparing the fragmentation patterns (Appendices) observed for the metabolites in the incubation samples with those of CPZ, reported previously.[19]

Table 2.1: Summary: comparing LC/MS retention times of CPZ metabolites generated through biotransformation *versus* standards or synthesized molecules

	MH^+ m/z		HLM	RLM	CYP2D6	CYP3A4	CYP1A2	CYP2C9	CYP21B3	Actinomycetes	Synthesized/purchased metabolite
Retention time - Extracted Ion Chromatogram (XIC)											
Oxidation	335	**M1**	5.26	5.17	5.19	5.59	5.21	5.25	-	-	5.20
		M2	6.00	-	-	-	5.95	-	-	-	6.05
		M3	6.92	6.90	-	-	-	-	-	-	6.98
Di-oxidation	351	M4a	5.47	5.41	-	-	-	-	-	-	NS
		M4b	-	6.06	-	-	-	-	-	-	5.98
Demethylation	305	**M5**	6.66	6.63	-	-	6.57	-	-	-	6.61
Demethylation + oxidation	321	M6a	5.92	5.87	-	-	-	-	-	-	NS
		M6b	5.18	5.12	-	-	-	-	-	-	5.26

NS: Not synthesized; - no metabolite detected

Many metabolites tend to ionize in the same mode as the parent drugs. For instance, the structure of the demethylated product **(M5),** that is, nor-CPZ, was confirmed by comparing the reported fragmentation pattern of CPZ (MH^+ peak 319, 274, 246, 214) with that obtained for **M5** (MH^+ peak 305, 274, 246, 214) which clearly indicated that the molecular mass (m/z) of the metabolite was less by 14 mass units due to the absence of one methyl group on the chain. However, the subsequent fragments retained the CPZ fragmentation pattern. All the other metabolites **(M1, M2, M3, M4a, M4b, M6a, M6b)** observed in the biotransformation samples were identified in the same manner (Appendices).

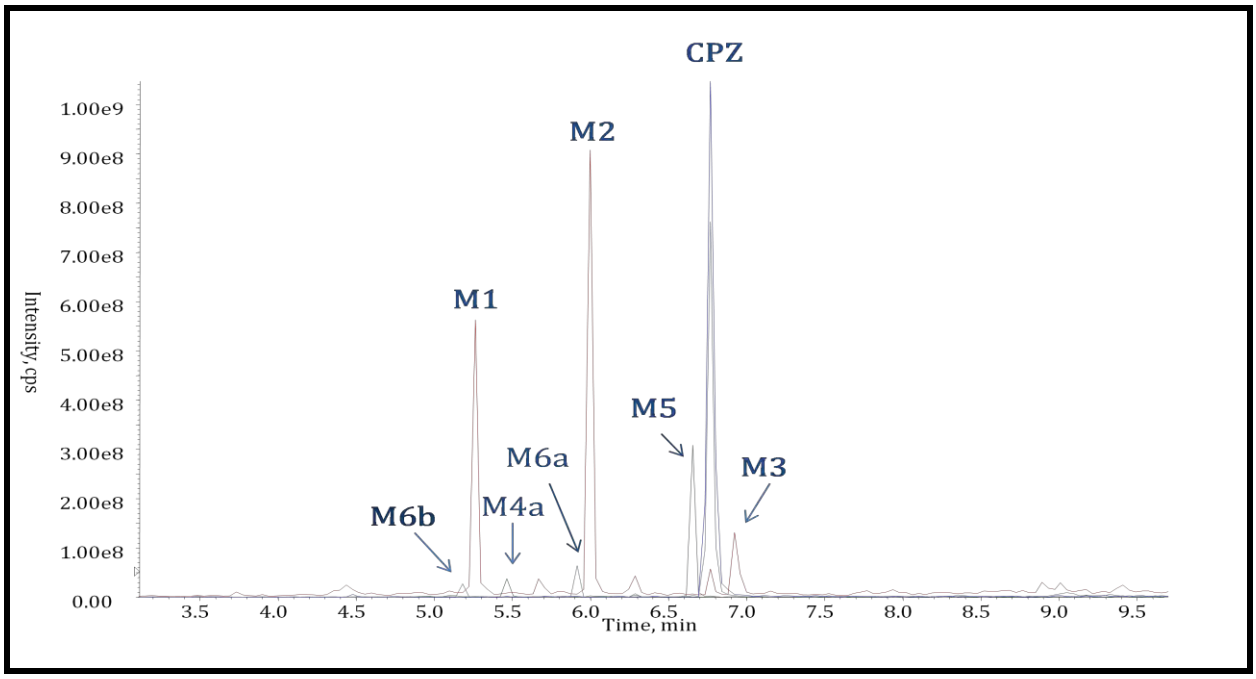


Figure 2.1 XIC of the various metabolites generated by HLM, eluting at different retention times

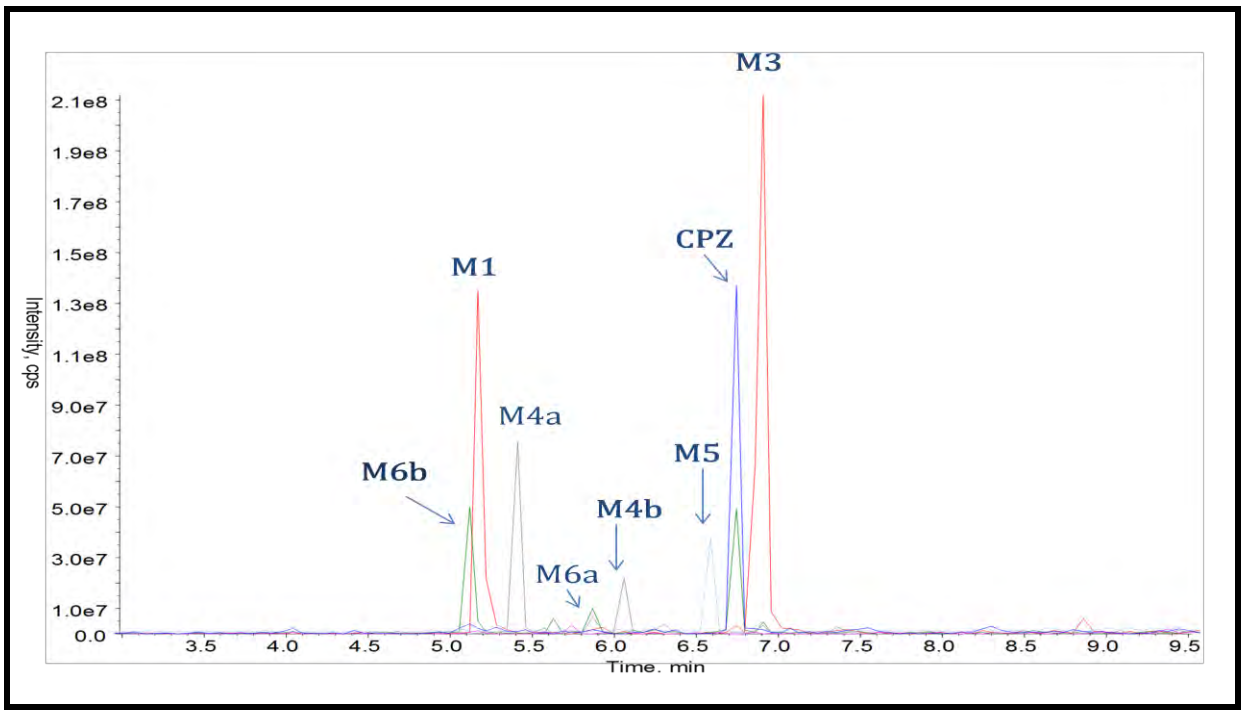


Figure 2.2 XIC of the various metabolites generated by RLM, eluting at different retention times

Relative to other biotransformation systems, microsomal incubations generated most of the CPZ metabolites (Scheme 2.1, Table 2.1). Microsomes are derived from the liver sub-cellular fractions from the respective organism (i.e. human and rat). This observation is not surprising since the liver is the main organ in which xenobiotic metabolism takes place due

to presence of most of the metabolizing enzymes which include CYP450s, esterases, flavin monooxygenase, alcohol dehydrogenase, and aldehyde dehydrogenase among many others. [6,7,20]

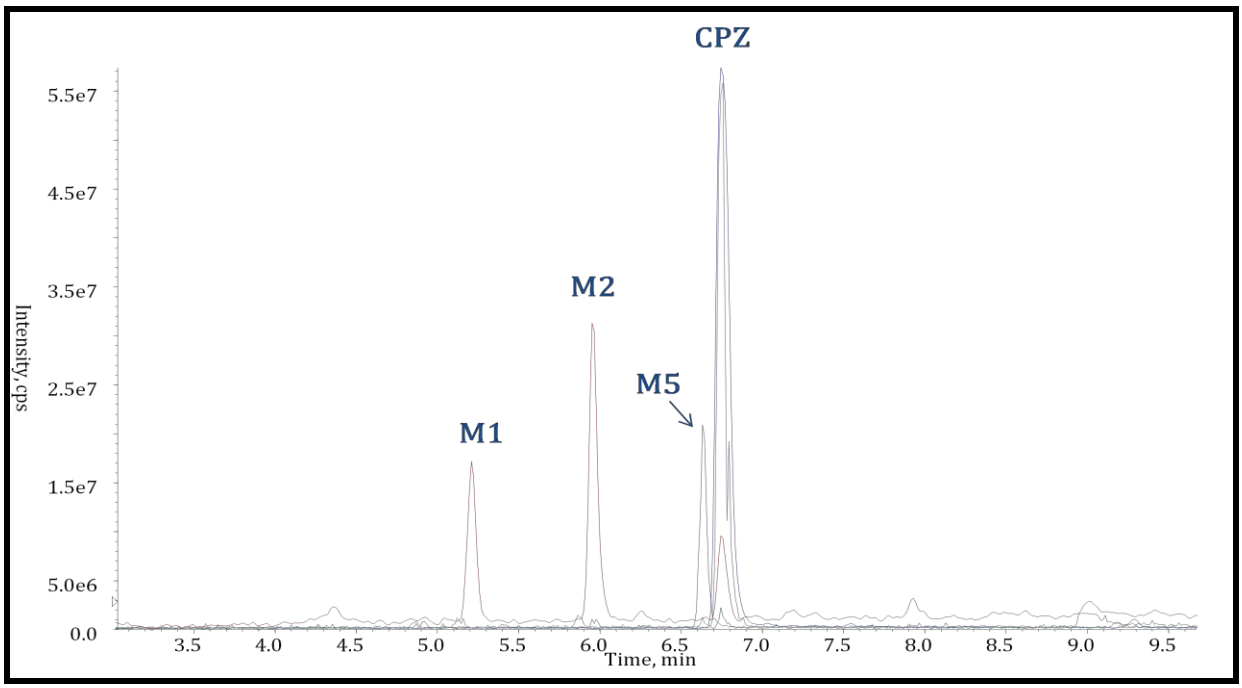


Figure 2.3 XIC of the various metabolites generated by CYP1A2, eluting at different retention times

Although CYP1A2 led to the formation of **M1**, **M2** and **M5**, previous studies have established that 7-hydroxy metabolites are predominantly formed from phenothiazines via catalysis by CPY2D6.[21,22] On the other hand, CYP1A2 is said to be responsible for metabolism of this class of compounds but to a lesser extent.[23] This is contrary to another study which reported that demethylation and sulfoxidation of CPZ is favored by CYP1A2 isoform.[21,23,24] However, in the results presented here, CYP2D6 only generated CPZ sulfoxide **(M1)** which was also formed by catalysis of CPZ by CYP3A4 and CYP2C9. Despite the fact that metabolism of several drugs has been demonstrated using CYP21B3[25] and actinomycetes,[26] these systems did not yield metabolites of CPZ in this study (Table 2.1). Failure to obtain metabolites in the latter systems can be partly explained by the fact that optimization of the incubations was not carried out. On the other hand, the conditions necessary for expression of the recombinant CYP1A2 in *Escherichia coli* and incubation of CPZ with the enzyme were

optimized leading to generation of three metabolites (Table 2.2). This is not surprising because the conditions achieved here for CYP1A2, have been applied in other CYP450 studies.[27] Owing to constraints of time, the same could not be achieved with other systems.

Table 2.2: Conditions optimized for growth of *E. coli*, expression of CYP1A2 and incubation of CPZ

Parameters	Optimal conditions
Induction of CYP450 expression (OD_{600})	0.8 - 1
Duration of growth after induction (h)	48
Growth temperature (^{o}C)	30
Shaking speed (x *g*)	180 - 200
Concentration of drug (µM)	100
Optimal pH of incubation of the drug	7.4
Drug incubation time (h)	48

The purpose of optimizing the conditions for generating metabolites of CPZ with CYP1A2 was to scale-up metabolite yield. However, chemical synthesis of the metabolites became the most viable route due to the large quantities of metabolites required for antimycobacterial screening. Nevertheless, optimizing the conditions given above is an important step for future development of the findings in this study. 7-HydroxyCPZ **(M2)** could in future be obtained through large scale whole cell biotransformation of CPZ via CYP1A2 catalysis, since the metabolite could not be synthesized chemically. It is also worth pointing out that identification of specific enzyme(s) responsible for metabolism of a drug is important in drug discovery programs for the purpose of understanding the pharmacokinetic profiles of a drug, which could potentially interact enzymatically with other drugs, chemicals or food in an *in vivo* environment leading to a positive or a negative outcome.[7]

2.4. Scale-up of CPZ metabolites

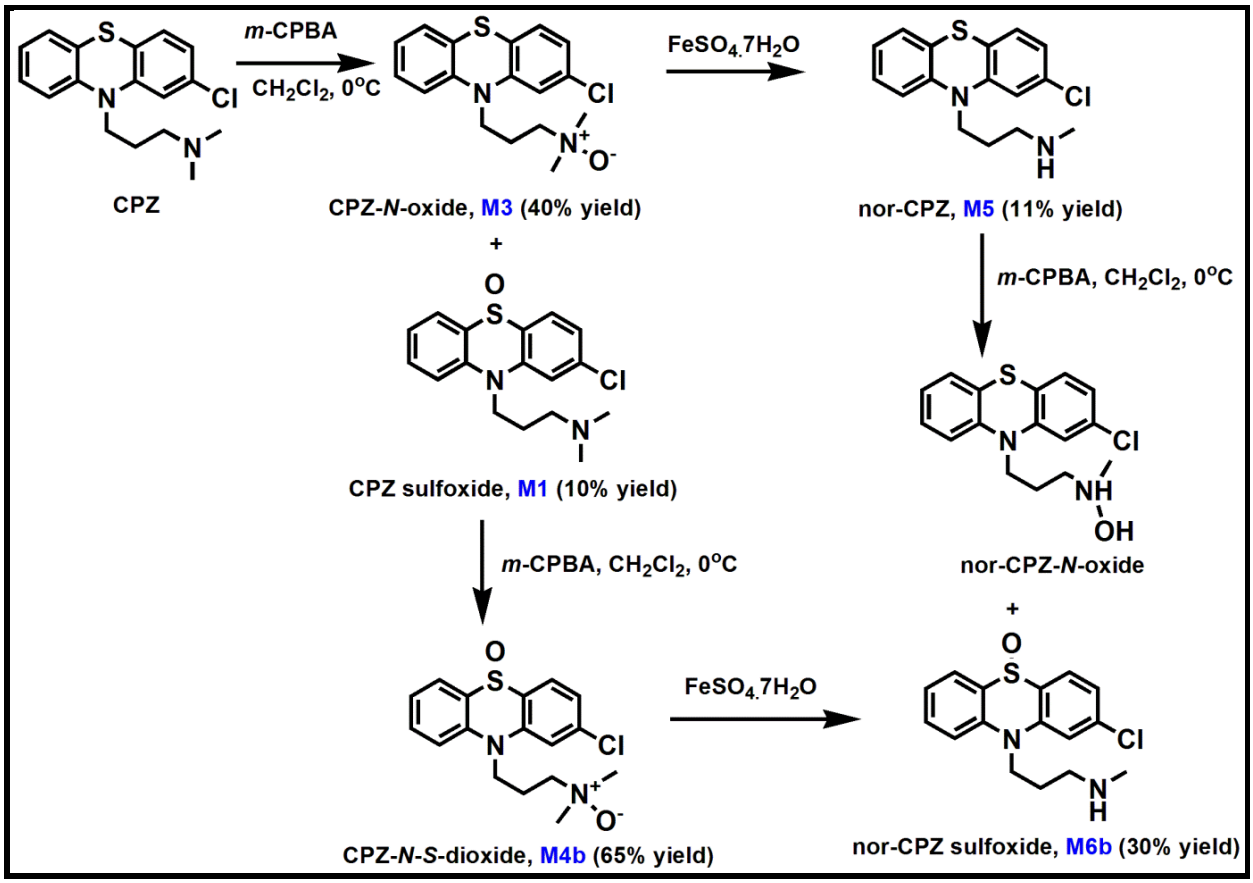
2.4.1 7-HydroxyCPZ (M2)

7-HydroxyCPZ **(M2)** was purchased and used to identify one of the metabolites by comparing the retention times obtained from LC/MS analysis. The retention time of the

standard corresponded to the **M2** metabolite in the HLM incubation of CPZ (section 7.5.1, Chapter 7, Figure 7.1).

2.4.2 Synthesis of other CPZ metabolites

CPZ-*N*-oxide **(M3),** CPZ sulfoxide **(M1),** nor-CPZ **(M5),** nor-CPZ sulfoxide **(M6b)** and CPZ-*N*-*S*-dioxide **(M4b)** were synthesized via a non-classical Polonovski reaction (Scheme 2.4, section 7.5.2 – 7.5.6, Chapter 7).[4]



Scheme 2.4 Synthesis of CPZ metabolites using Polonovski scheme

The central feature of this reaction is that it involves the conversion of the amine to the corresponding *N*-oxide.[28] Depending on the structure of the substrate and the acid anhydride or other activating reagent employed, iminium ion formation can occur through loss of an α-hydrogen, or through fragmentation of a Cα-carbon bond.[28,29] *meta*-Chloroperbenzoic acid (*m*-CPBA) is more selective and easier to handle than other oxidizing agents.[29] The *N*-oxide

formed then reacts with an iron(II) sulfate heptahydrate, which induces elimination and ultimately affords an iminium ion intermediate that reacts further to yield the *N*-demethylated product.[28,29] Singh *et al.*[29] employed a modified form of the scheme to synthesize the *N*-demethylated metabolite of the neuroleptic drug, cyamemazine.

Likewise, in the presence of sodium hydroxide (NaOH), *m*-CPBA in dichloromethane (CH_2Cl_2) was used in this study to oxidize CPZ to form the *N*-oxide **(M3)** and the sulfoxide **(M1)**. CPZ-*N*-oxide **(M3)** was dissolved in methanol (MeOH), the solution was cooled, followed by an addition of a solution of ferrous sulfate ($FeSO_4$) in MeOH. The residue was dissolved in ethylenediaminetetraacetic acid (EDTA) solution at pH 10 adjusted using ammonia (NH_3) solution. Nor-CPZ **(M5)** was then purified using column chromatography.

Further oxidation was carried out on nor-CPZ **(M5)** and CPZ sulfoxide **(M1)** using *m*-CPBA as described for the *N*-oxide **(M3)** to form nor-CPZ sulfoxide **(M6b)** and CPZ-*N*-*S*-dioxide **(M4b)** respectively**.** Interestingly, deviating from the recommended time (4h) of the first step reaction,[29] to 24h, led to the formation of the CPZ-*N*-oxide **(M3)** and not the CPZ sulfoxide **(M1)** hence maximizing on the amount of *N*-oxide **(M3)** that was required for the formation of the demethylated product **(M5)** and the *N*-*S*-dioxide **(M4b)** (Scheme 2.4).

All the synthesized compounds were identified using Nuclear Magnetic Resonance (NMR) and LC/MS (section 7.5, Chapter 7, Appendices). The retention times of the metabolites generated in the microsomal incubations from CPZ were comparable to those observed from the synthesized compounds, thereby confirming the identity and structures of the products in the incubation samples (Table 2.1, section 7.5.2 – 7.5.6, Chapter 7).

2.5. Summary discussion

Generation and scale-up of CPZ metabolites was successfully carried out in this study. Although many biotransformation systems did not generate CPZ metabolites, the major

biotransformation products of the phenothiazine were identified in the microsomal incubations, consistent with what has been reported before.[16–18,21,23,24,30,31] Optimization of CYP1A2 incubations of CPZ was also an important exercise that may guide further work on generation of the hydroxyl metabolite **(M2)** that can potentially be of use for further structure relationship studies (SAR) of this class of compounds for tuberculosis (TB) drug discovery. Synthesis of the metabolites as a way of scaling-up, was also achieved through the Polonovski scheme[4,28,29] resulting in substantial yields of the major metabolites, for antimycobacterial screening experiments.

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CHAPTER 3

SYNERGISTIC/MATRIX ANTIMYCOBACTERIAL COMBINATION SCREENING

3.1 Introduction

Examples from unrelated fields such as cancer drug discovery suggest the potential for simultaneously targeting more than one pathway or pathway component[1] as part of fractional combination therapies designed to achieve synergies in drug action. Drug synergy occurs when the interaction between two or more compounds enhances the individual activities of each. There are a number of examples of drugs that have been developed in combinations designed to increase efficacy while limiting the emergence of drug resistance (discussed in Chapter 1). To accentuate what was mentioned earlier in the first chapter, it is a worthy course to include combination screening in the early stages of drug development in order to identify potential partners for novel antituberculosis (anti-TB) drug regimens, given that combination therapy is standard for tuberculosis (TB).[2] The fifth and sixth specific objectives of this study are discussed in this chapter in which the use of checkerboard assays - which are most commonly applied to test drug combinations *in vitro*, and provide a measure of the nature of the interaction between two drugs in the form of a fractional inhibitory concentration index (FICI) - is demonstrated.[3–5]

3.2 *In vitro* assays against *Mycobacterium smegmatis* (*Msm*)

3.2.1 Microplate Alamar Blue Assay (MABA) for determination of minimum inhibitory concentrations (MIC_{99}) of chlorpromazine (CPZ) or its metabolites

The MIC_{99} values determined (section 7.3.1, Chapter 7) for CPZ and its metabolites are shown in Table 3.1. The antimycobacterial activity of CPZ and its metabolites against *Msm* were generally low. Interestingly, the activity of the CPZ metabolites, 7-hydroxyCPZ **(M2)** and nor-CPZ **(M5)** were comparable to that of CPZ, confirming the two as the active

metabolites of CPZ.[6] This is in agreement with studies where hydroxylation and demethylation of various drugs through biotransformation have led to generation of active metabolites (section 1.4, Chapter 1).[7–9] CPZ-*N*-oxide **(M3)** has been reported to revert to CPZ in solution and this may contribute to some of its activity.[10] In some instances, heteroatom oxidation (*N*, *S*, and *P*) has led to active metabolites.[7] However, in this study, oxidation of *S* and *N* atoms of CPZ **(M1, M4b, M6b)** resulted in metabolites with weak antimycobacterial activity.

Table 3.1: MIC_{99} of CPZ or its metabolites against *Msm*

Drugs/Compound	MIC_{99} (µM) in *Msm*
CPZ	117.26
CPZ sulfoxide (M1)	>1990.89
7-hydroxyCPZ (M2)	124.44±6.01
CPZ-*N*-oxide (M3)	995.43
nor-CPZ (M5)	136.70
nor-CPZ sulfoxide (M6b)	>2077.89
CPZ-*N*-*S*-dioxide (M4b)	>1900.10

3.2.2 Synergistic/matrix screening

Table 3.2 and Table 3.3 summarize the MIC_{99} values determined for the individual compounds, the lowest MIC_{99} achieved in the various combinations of CPZ and its metabolites with known anti-TB drugs and the FICI (section 7.3.2, Chapter 7). According to the standard definition, synergy is assigned where FICI $\leq$ 0.5; an FICI $\geq$4 is antagonistic while any value falling in between indicates no interaction.[3,5,6,11,12] Generally, combinations of CPZ with known anti-TB drugs exhibited improved activity against *Msm*. The combination of CPZ with spectinomycin (SPEC) or 25-*O*-desacetylrifampicin (25-*O*-desacetylRIF) resulted in definite synergy (FICI 0.31, 0.50 respectively, Table 3.2).[6,12] It is worth mentioning that 25-*O*-DesacetylRIF, reported to be the active metabolite of rifampicin (RIF),[9,13] was included in the synergistic matrix screening for further investigation of the contribution of drug metabolites to the biological activity.

Table 3.2: FICI of CPZ or its metabolites in combination with antimycobacterial drugs against *Msm*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction	FIC	FICI
RIF	1.26±0.3	0.63±2		0.500	1.00
25-*O*-DesacetylRIF	2.66±2.17	1.33±1.08		0.500	
RIF	2.53±0.54	0.32 ± 0.19	8	0.126	0.63
CPZ	117.27±6.01	58.62±10.84		0.500	
25-*O*-DesacetylRIF	5.33±1.59	1.33±0.49	4	0.250	0.50
CPZ	117.27	29.32±7.36		0.250	
EMB	0.76±0.12	NC			
CPZ	117.27±6.01				
KANA	7.16±1.1	3.57±0.54	2	0.500	1.00
CPZ	117.27±7.36	58.62±5.52		0.500	
STRP	0.07±0.07	0.02	3.5	0.286	0.79
CPZ	58.62±15.19	29.32±8		0.500	
SPEC	84.12±24.05	5.25	16	0.062	0.31
CPZ	117.27	29.32		0.250	
BDQ	0.05±0.02	0.01	5	0.200	0.70
CPZ	117.27	58.62		0.500	
Nalidixic acid*	1435.28	358.81	4	0.250	
CPZ	117.27	58.62		0.500	
Ciprofloxacin*	0.63	0.30	2	0.476	0.73
CPZ	117.27	29.32		0.250	
Levofloxacin	0.58	NC			
CPZ	117.27				
RIF	2.53±0.6	0.32±0.15	8	0.126	0.63
7-hydroxyCPZ (M2)	62.21±6.01	31.12±3		0.500	
25-*O*-DesacetylRIF	1.33±2.41	0.33±0.6	4	0.248	0.75
7-hydroxyCPZ (M2)	124.44±12.03	62.21±6.01		0.500	
EMB	0.76±0.12	IAE			
7-hydroxyCPZ (M2)	124.44±12.03				
KANA	3.57	0.89	4	0.249	0.50
7-hydroxyCPZ (M2)	124.44	31.12		0.250	
STRP	0.07±0.14	0.02±0.04	3.5	0.286	0.79
7-hydroxyCPZ (M2)	124.44±12.03	62.21±10.33		0.500	
SPEC	84.12±24.05	5.25±10.53	16	0.062	0.19
7-hydroxyCPZ (M2)	124.44±15.91	15.56±2.47		0.125	
CPZ	117.27±12.03	29.32±6.01		0.250	0.75
7-hydroxyCPZ (M2)	124.44±12.03	62.21±6.01		0.500	
RIF	1.26±0.72	0.63±0.18	2	0.500	1.00
CPZ-*N*-oxide (M3)	995.43	497.73		0.500	
25-*O*-DesacetylRIF	2.66±2.41	0.67±0.38	4	0.252	0.75
CPZ-*N*-oxide (M3)	995.43	497.73		0.500	

EMB **CPZ-*N*-oxide (M3)**	0.76 995.43	IAE			
KANA **CPZ-*N*-oxide (M3)**	1.79±1.03 995.43	0.89 497.73	2	0.497 0.500	1.00
STRP **CPZ-*N*-oxide (M3)**	0.29 995.43	0.07 497.73	4	0.241 0.500	0.74
SPEC **CPZ-*N*-oxide (M3)**	168.22±24.05 995.43	84.12 124.44±71.84	2	0.500 0.125	0.63
CPZ **CPZ-*N*-oxide (M3)**	117.27 995.43	58.62 248.85		0.500 0.250	0.75
RIF **nor-CPZ (M5)**	1.26±0.6 136.70	0.32 34.18±6.01	4	0.254 0.250	0.50
25-*O*-DesacetylRIF **nor-CPZ (M5)**	2.66 136.70	0.67±0.15 34.18±6.01	4	0.252 0.250	0.50
EMB **nor-CPZ (M5)**	1.51±1.58 63.33±12.03	IAE			
KANA **nor-CPZ (M5)**	3.57±1.21 136.70±12	0.89±0.23 34.18±6.01	4	0.249 0.250	0.50
STRP **nor-CPZ (M5)**	0.29 136.70±0	0.07±0.01 34.18±6.01	4	0.241 0.250	0.49
SPEC **nor-CPZ (M5)**	84.12 136.70±31.82	5.25±4 34.18±4.51	16	0.062 0.250	0.31
CPZ **nor-CPZ (M5)**	117.27 136.70	29.32 68.33±6.01		0.250 0.500	0.75

IAE – Inconsistent antagonistic effect; NC – No Change; *poor solubility - single experiment

Like the parent (CPZ), 7-hydroxyCPZ **(M2)** resulted in enhanced antimycobacterial activity in combination with anti-TB drugs (Table 3.2). For instance, synergistic interactions were observed for kanamycin (KANA) and SPEC (FICI 0.50, 0.19 respectively). Nor-CPZ **(M5)**, just like CPZ and **M2**, potentiated the antimycobacterial activity of some of the anti-TB drugs selected for this study against *Msm*. Synergy was observed for combinations of **M2** with RIF, 25-*O*-desacetylRIF, KANA, streptomycin (STRP) or SPEC (FICI $\leq$ 0.50, Table 3.2).[6,12] Notably, CPZ and its metabolites reproducibly reduced the MIC_{99} of the antibiotics at least by 2-fold with the highest reduction observed with SPEC at 16-fold followed by RIF at 8-fold.

Table 3.3: Inactive metabolites of CPZ in combination with anti-TB drugs, against *Msm*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction
RIF **CPZ sulfoxide (M1)**	2.53±0.74 >1990.89	NC	
EMB **CPZ sulfoxide (M1)**	0.76 >1990.89	IAE	
KANA **CPZ sulfoxide (M1)**	1.79±1.1 >1990.89	0.89 1990.89	2-fold
STRP **CPZ sulfoxide (M1)**	0.29 >1990.89	0.14 62.21±25.6	2-fold
SPEC **CPZ sulfoxide (M1)**	168.22±48 >1990.89	10.52±3.1 1990.89	16-fold
RIF **CPZ-*N*-*S*-dioxide (M4b)**	2.53±1.46 >1900.10	NC	
EMB **CPZ-*N*-*S*-dioxide (M4b)**	0.76±0.15 >1900.10	IAE	
KANA **CPZ-*N*-*S*-dioxide (M4b)**	1.76 >1900.10	0.89 475.03±44.1	2-fold
STRP **CPZ-*N*-*S*-dioxide (M4b)**	0.29 >1900.10	0.14 237.50±58.93	2-fold
SPEC **CPZ-*N*-*S*-dioxide (M4b)**	84.12 >1900.10	42.05 1900.10	2-fold
RIF **nor-CPZ sulfoxide (M6b)**	1.26±0.3 >2077.89	NC	
EMB **nor-CPZ sulfoxide (M6b)**	0.76±0.12 >2077.89	IAE	
KANA **nor-CPZ sulfoxide (M6b)**	1.76±0.6 >2077.89	NC	
STRP **nor-CPZ sulfoxide (M6b)**	0.14±0.05 >2077.89	0.07±0.07 519.48±37.6	2-fold
SPEC **nor-CPZ sulfoxide (M6b)**	84.12 >2077.89	21.04±5.43 64.92±14.74	4-fold

IAE – Inconsistent antagonistic effect; NC – No Change

CPZ sulfoxide **(M1),** CPZ-*N*-*S*-dioxide **(M4b)** and nor-CPZ sulfoxide **(M6b)** were all inactive. Thus, the respective FICIs could not be calculated for the various combinations involving these metabolites. Interestingly, they were nevertheless able to augment the antimycobacterial activity of some of the anti-TB drugs used in this study. Similar to CPZ and its active metabolites, **M1** and **M6b** reduced the MIC_{99} of SPEC 16-fold and 4-fold

respectively (Table 3.3). A 2-fold drop in MIC_{99} was observed for some of other combinations (Table 3.3).[6,12]

It should be noted that all combinations of CPZ and its metabolites with ethambutol (EMB) against *Msm* resulted in inconsistent antagonistic interactions whereas, on its own, the MIC_{99} value of EMB was rather consistent. Such effects with EMB in combination with other drugs have been reported before.[14,15] Similarly, combinations of isoniazid (INH) with CPZ and its metabolites were also carried out but INH had no activity against *Msm* and consequently a synergistic effect was not observed. The susceptibility of *Msm* to INH is reported to be very low (100 times less than in *Mtb*) hence the observed results.[16,17]

3.3 *In vitro* assays against *Mtb*

3.3.1 MABA for determination of MIC_{99} of CPZ or its metabolites

MIC_{99} values determined (section 7.3.1, Chapter 7) for CPZ and its metabolites against *Mtb* are shown in Table 3.4. Similar to the observations made against *Msm*, the antimycobacterial activity of CPZ and its metabolites against *Mtb* was also generally weak. The activities of CPZ metabolites, 7-hydroxyCPZ **(M2)** and nor-CPZ **(M5)**, were also comparable to that of CPZ, again confirming the two as the active metabolites. The low activity observed against *Msm* was also observed against *Mtb* for **M1, M3, M4b** and **M6b** at the highest concentrations tested.

Table 3.4: MIC_{99} of CPZ or its metabolites against *Mtb*

Drugs/Compound	MIC_{99} (µM) in *Mtb*
CPZ	58.63
CPZ sulfoxide (M1)	>1990.89
7-hydroxyCPZ (M2)	62.21
CPZ-*N*-oxide (M3)	497.73±10.71
nor-CPZ (M5)	68.34
nor-CPZ sulfoxide (M6b)	>2077.89
CPZ-*N*-*S*-dioxide (M4b)	>1900.10

3.3.2 Synergistic/matrix screening

Table 3.5 and Table 3.6 summarize the MIC_{99} values determined for the individual compounds, the lowest MIC_{99} values achieved in the various combinations of CPZ and its metabolites with known anti-TB drugs and the FICI against *Mtb* (section 7.3.2, Chapter 7).

Improved activity was observed in combinations of CPZ or the metabolites with anti-TB drugs against *Mtb*. In fact, interactions obtained for the various pairs tested against *Mtb* resulted in greater synergy compared to those tested against *Msm*. CPZ in combination with RIF and its metabolite, 25-*O*-desacetylRIF, SPEC, INH, bedaquiline (BDQ) or nalidixic acid yielded synergism with FICI $\leq$ 0.5 and a 10 to 500-fold reduction of MIC_{99} value of the antibiotic (Table 3.5). This is in agreement with a previous study where CPZ was reported to augment activity of INH, STRP, pyrazinamide (PZA), RIF, rifabutin and penicillin against *Mtb* and *M. avium* in normal human macrophages, but this was not the case with EMB.[14] Combinations of **M2** with 25-*O*-desacetlylRIF, BDQ and SPEC exhibited the highest synergistic interaction with FICI of 0.36, 0.25 and 0.13 respectively, followed by RIF, INH and STRP (FICI 0.50) against *Mtb*. Nor-CPZ **(M5)** in combination with SPEC and BDQ exhibited synergistic interactions with FICI values of 0.19 and 0.31 respectively (Table 3.5). In addition, **M5** in combination with RIF, 25-*O*-desacetylRIF, and KANA resulted in synergism at FICI of 0.5 (Table 3.5). In addition to the synergistic interactions, at least a 4-fold reduction was observed in MIC_{99} values of most of the antibiotics combined with CPZ and its active metabolites. The highest reduction recorded was for combinations of CPZ with SPEC or BDQ at 128- and 508-fold respectively (Table 3.5).

Table 3.5: FICI of CPZ or its metabolites in combination with anti-TB drugs, against *Mtb*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction	FIC	FICI
RIF	0.0032±0.04	0.00037	8.6	0.116	0.37
CPZ	58.63	14.66		0.250	
25-*O*-DesacetylRIF	0.0017	0.00038	4.5	0.224	0.47
CPZ	29.32±6.01	7.32±1.51		0.250	
Ehthambutol	6.02±3	NC			
CPZ	58.63				
INH	0.38	0.094	4	0.247	0.50
CPZ	58.63	14.66		0.250	
KANA	7.16±3	3.57±0.1	2	0.499	1.00
CPZ	58.63	29.32±6.01		0.500	
STRP	1.15±0.2	0.14	8	0.122	0.62
CPZ	58.63	29.32		0.500	
SPEC	168.22±97.14	1.31±1	128.4	0.0078	0.26
CPZ	29.32±6.01	7.32±4.51		0.250	
BDQ	3.00±0.64	0.0059	508.5	0.002	0.25
CPZ	29.32±6.01	7.32±1.51		0.250	
Nalidixic acid*	1435.28	179.41	8	0.125	0.25
CPZ	58.63	7.32		0.125	
Ciprofloxacin*	2.50	1.27	2	0.508	0.76
CPZ	58.63	14.66		0.250	
Levofloxacin	2.30±3.4	NC			
CPZ	58.63				
RIF	0.0063	0.0016	4	0.254	0.50
7-hydroxyCPZ (M2)	62.21	15.56±1.12		0.250	
25-*O*-DesacetylRIF	0.0016	0.00037	4	0.231	0.36
7-hydroxyCPZ (M2)	31.12±18	3.88±2.24		0.125	
INH	0.73	0.19	4	0.260	0.51
7-hydroxyCPZ (M2)	62.21	15.56		0.250	
KANA	3.57	NC			
7-hydroxyCPZ (M2)	62.21				
STRP	1.15±0.24	0.29±0.06	4	0.252	0.50
7-hydroxyCPZ (M2)	62.21	15.56		0.250	
SPEC	336.47	21.04	16	0.063	0.13
7-hydroxyCPZ (M2)	124.44±14.74	7.76		0.063	
BDQ	0.76	0.094±0.03	8	0.124	0.25
7-hydroxyCPZ (M2)	62.21	7.76		0.125	
Nalidixic acid*	179.41	89.69	2	0.500	0.63
7-hydroxyCPZ (M2)	62.21	7.76		0.125	
RIF	0.0063	0.0016	4	0.254	0.75
CPZ-*N*-oxide (M3)	497.73	248.85±71.83		0.500	
SPEC	336.47	NC			

CPZ-*N*-oxide (M3)	497.73				
BDQ	0.76±0.22	0.047±0.03	16	0.062	0.31
CPZ-*N*-oxide (M3)	497.73	124.44±35.92		0.250	
RIF	0.0063	0.0016	4	0.254	0.50
nor-CPZ (M5)	68.34	17.09		0.250	
25-*O*-DesacetylRIF	0.0067	0.0017	4	0.254	0.50
nor-CPZ (M5)	68.34	17.09		0.250	
INH	0.38	0.095±0.05	4	0.250	0.50
nor-CPZ (M5)	68.34±39.47	17.09±4.93		0.250	
KANA	3.57	0.89	4	0.250	0.50
nor-CPZ (M5)	136.70±39.47	34.18		0.250	
STRP	0.57±0.24	0.036±0.04	16	0.063	0.56
nor-CPZ (M5)	68.34	34.18		0.500	
SPEC	336.47	21.04	16	0.063	0.19
nor-CPZ (M5)	68.34	8.53		0.125	
BDQ	1.49	0.094	16	0.063	0.31
nor-CPZ (M5)	68.34	17.09		0.250	

NC – No Change; *poor solubility - single experiment

Combinations of anti-TB drugs with the inactive CPZ metabolites resulted in improved antimycobacterial activity. CPZ sulfoxide **(M1)** reduced the MIC_{99} value of RIF, BDQ and SPEC by 17-, 4- and 4-fold respectively (Table 3.6). CPZ-*N*-*S*-dioxide **(M4b)** reduced the MIC_{99} of BDQ by 16-fold while nor-CPZ sulfoxide **(M6b)** delivered a reduction in MIC_{99} value of RIF, BDQ and SPEC by 8-, 33- and 4-fold respectively (Table 3.6). This suggests that, in addition to antimycobacterial effect, CPZ and its metabolites exhibit additional mechanism(s) of action (MoA) that result in potentiation of the activity of other drugs.[18–20]

Table 3.6: Inactive metabolites of CPZ in combination with anti-TB drugs against *Mtb*

Drugs/Compound	**MIC_{99} (µM), singly**	**MIC_{99} (µM) in combination**	**MIC_{99} fold reduction**
RIF	0.0063	0.00037	17-fold
CPZ sulfoxide (M1)	>1990.89	1990.89	
SPEC	168.22±48.56	42.05±12.14	4-fold
CPZ sulfoxide (M1)	>1990.89	497.73	
BDQ	0.76	0.18±0.1	4-fold
CPZ sulfoxide (M1)	>1990.89	497.73	
RIF	0.013	NC	
CPZ-*N*-*S*-dioxide (M4b)	>1900.10		
SPEC	336.47	168.22	2-fold

CPZ-*N*-*S*-dioxide (M4b)	>1900.10	950.04	
BDQ	0.76	0.047±0.03	16-fold
CPZ-*N*-*S*-dioxide (M4b)	>1900.10	1900.10	
RIF	0.0063	0.00083	8-fold
nor-CPZ sulfoxide (M6b)	>2077.89	2077.89	
SPEC	336.47	84.12±12.14	4-fold
nor-CPZ sulfoxide (M6b)	>2077.89	519.48	
BDQ	0.76	0.023±0.01	33-fold
nor-CPZ sulfoxide (M6b)	>2077.89	2077.89	

NC – No Change

3.4 Summary discussion

It should be noted that, before the commencement of this PhD project, combinations of CPZ metabolites with anti-TB drugs against *Mtb* had not been previously reported. The first publications in this area arose from this work.[6,12] Furthermore, the results obtained here have established that the checkerboard assay is a powerful tool for measuring interactions between pairs of drugs, as previously reported by others.[3–5,11,21–23]

In summary, synergism was observed in more of the combinations against *Mtb* compared to *Msm*. This may be explained by the fact that *Msm* has inherent resistance due to high metabolic capacity, as an adaptation strategy to the harsh environment that it exists in which is basically in soil, water and plants.[24] This is, however, not the case with *Mtb* that survives in an *in vivo* host environment and hence having less adaptation physiological features compared to *Msm*.

Against *Msm* and *Mtb*, SPEC exhibited the best synergistic interactions with CPZ and its metabolites. This is not unexpected: combinations of SPEC and other drugs were previously reported to be synergistic, though the precise mechanism underlying the ability of SPEC to potentiate multiple different drug classes remains unclear despite the elucidation of its target in this project (discussed in Chapter 5).[5] In general, combinations of CPZ and its metabolites with aminoglycosides resulted in positive interactions, prompting questions on whether or not

the interactions have any correlations with their MoA. It has been reported that aminoglycosides, which are known to target ribosomes leading to inhibition of protein synthesis in the mycobacteria, tend to have synergistic effects with other drugs such as cell wall synthesis inhibitors, resulting in increased accumulation of the drug within the mycobacterial cell.[25] It has been reported that the MoA of CPZ is most likely associated with inhibition of type II NADH:menaquinone oxidoreductase activity which is responsible for aerobic respiration. It is also presumed that down the cascade of the cellular activities related to respiration, inhibition of the oxidoreductase may lead to inhibition of the electron transport pathway.[18,26] Subsequently, in a combination of CPZ or the metabolites with aminoglycosides, higher accumulation of the latter may be achieved in the mycobacterial cell leading to improved activity for this class of compounds. The MoA described here may also be used to explain synergistic interactions demonstrated for combinations of CPZ or its metabolites with the other classes of drugs.

Of importance also is to note that the inactive metabolites of CPZ which are also reported to be inactive in psychosis do potentiate the activity of the antibiotics.[27,28] These metabolites may stand a better chance of not exhibiting the undesired antipsychotic effect hence proving to be better candidates for selection for an anti-TB combination therapy.

Finally, an observation was made whereby improved antimycobacterial activity was not achieved in similar combinations with fluoroquinolones, except for nalidixic acid against *Mtb*; in this case, however, the poor solubility of nalidixic acid is associated with inconsistent MIC_{99} values.

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CHAPTER 4

DETERMINATION OF THE MECHANISM OF ACTION (MoA) OF CHLORPROMAZINE (CPZ) & ITS METABOLITES, AND COMBINATIONS WITH ANTITUBERCULOSIS (ANTI-TB) DRUGS

4.1 Introduction

Determination of the MoA of a drug is normally carried out when its biological activity has been established as discussed in section 1.7, Chapter 1. Drug susceptibility of the mycobacteria is determined by multiple factors, which include growth state, the inherent impermeability of the mycobacterial cell wall, and the ability of a drug to interact with a target.[1] Critically, the recent identification of some "promiscuous" membrane targets, including QcrB, suggests that the respiratory electron transport chain is an essential pathway that drug molecules can be designed to target.[1–3] Interestingly, *Mtb* is able to survive in aerobic and anaerobic environments by switching its respiratory functions from the former state to the latter and vice versa.[3] This chapter explores various possibilities with regard to the MoA of chlorpromazine (CPZ) metabolites and the interactions observed in the combination assays (Chapter 3) in relation to the reported target of CPZ, type II NADH:menaquinone oxidoreductase. The dehydrogenase plays the role of initiating the branched aerobic respiratory chain that terminates in a cytochrome *bd* and a cytochrome *aa3* complexes encoded by *cydABCD* and *ctaBCDE* respectively.[1,3–5] The type II NADH dehydrogenase in *Mtb* is an important antimicrobial target because in humans, the mitochondria use only type I NADH dehydrogenase.[3] This would therefore reduce chances of toxicity. The chapter therefore addresses the seventh and eighth specific objectives of this study.

4.2 Synergistic/matrix combination assay as a preliminary means of determining MoA

Fractional inhibition concentration index (FICI) can be used as a preliminary means to establish whether the MoA of analogues or metabolites compared to the parent, is retained. In this case, this approach was used to determine whether the derived CPZ metabolites retained the MoA of the parental compound. Comparison was made between FICI values determined for CPZ in combination with a panel of known antituberculosis (anti-TB) drugs and the FICI values obtained for the corresponding metabolites in similar combinations (Chapter 3). A close correlation between CPZ and each of the active metabolites was observed, suggesting that the derivative compounds remained "on target." Moreover, combinations of CPZ with the metabolites yielded FICI values close to 1, indicating an additive effect as a result of effective doubling of the concentration (Table 3.2, Chapter 3).[6]

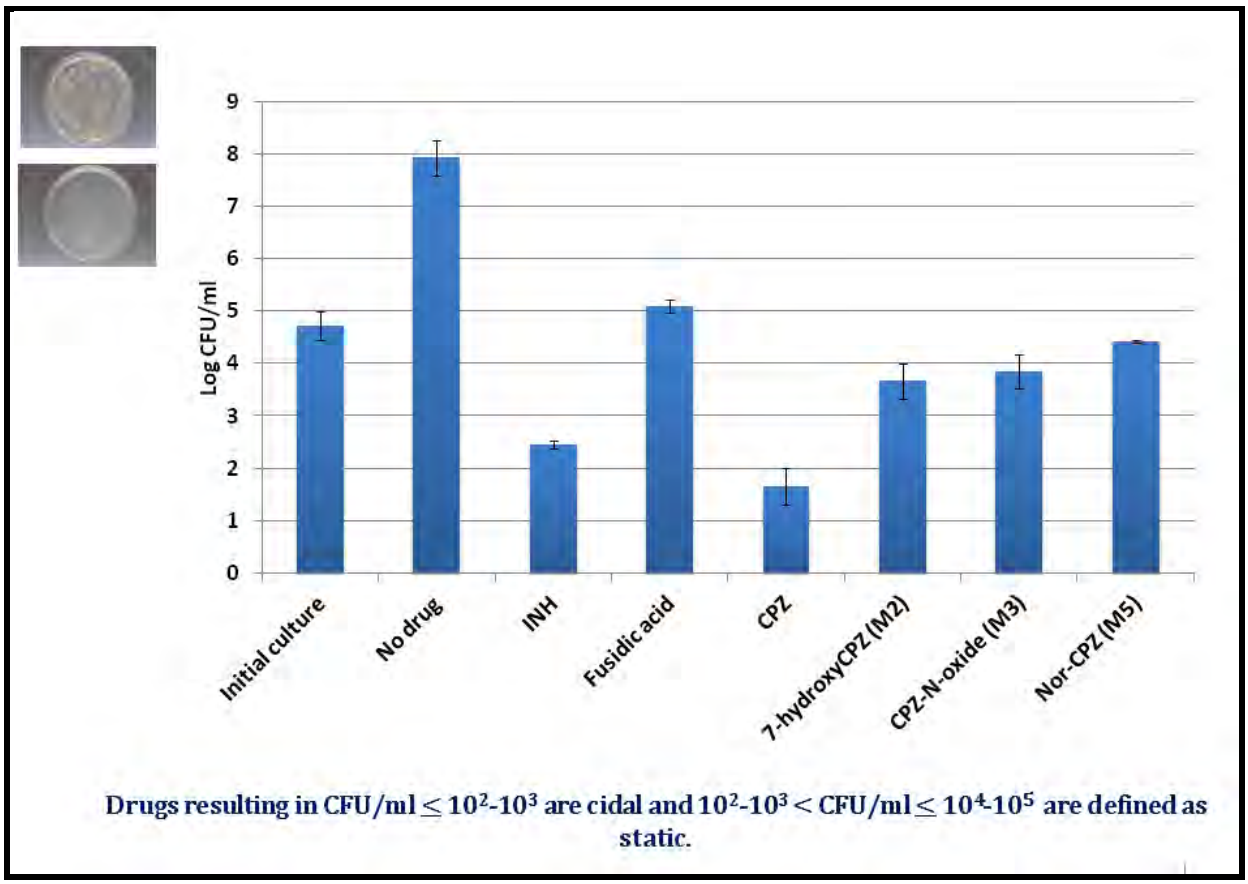
4.3 Bactericidal and bacteriostatic effect

Microplate alamar blue assay (MABA) screening was carried out for CPZ, its metabolites or anti-TB drugs, singly, with low inoculum of *Mtb* (H37RvMa). Likewise, a few interesting drug combinations discussed previously (Chapter 3) were also prepared using the checkerboard assay. The wells with the lowest concentration that exhibited a blue color were plated on 7H10 solid media for determination of bactericidal/bacteriostatic effects. Colony forming units per milliliter (CFU/ml), which is a rough estimate of the number of viable mycobacteria cells present in a culture, and the bactericidal/bacteriostatic effect were determined for CPZ, its metabolites and the combination pairs (section 7.3.3, Chapter 7); the results are summarized in Table 4.1 and Table 4.2; Graph 4.1 and Graph 4.2. The initial culture, added to 96 well plates with the various drugs prepared either singly or in combinations, resulted in $\sim 10^4$-10^5 CFU/ml count. Therefore, individual drugs or combination pairs that exhibited a 99% killing effect relative to the number of bacilli in the initial culture (i.e. those which resulted in fewer than or equal to 10^2-10^3 CFU/ml post exposure) were defined as bactericidal, whereas those with greater than $\sim 10^2$-10^3 but less than or equal to $\sim 10^4$-10^5 CFU/ml were considered bacteriostatic.

Table 4.1: CFU/ml counts after exposure of *Mtb* to CPZ, its metabolites and anti-TB drugs

Compound	Blue (MIC_{99} well)	Pink (well after MIC_{99} well)	Bactericidal/Bacteriostatic
CPZ	7.0×10^1	1×10^5	Bactericidal
7-hydroxyCPZ (M2)	2.7×10^3	1.078×10^7	Bacteriostatic
CPZ-*N*-oxide (M3)	7.0×10^3	9.25×10^6	Bacteriostatic
Nor-CPZ (M5)	2.6×10^4	5.6×10^6	Bacteriostatic
INH	2.8×10^2	1.0025×10^7	Bactericidal
Fusidic acid	1.223×10^5	5.0×10^6	Bacteriostatic
RIF	0	4.68×10^6	Bactericidal
BDQ	8.5×10^2	5.5×10^5	Bactericidal
SPEC	4.667×10^1	0	Bactericidal

INH – isoniazid; RIF – rifampicin; BDQ – bedaquiline; SPEC - spectinomycin; initial culture - 5.2×10^4 CFU/ml; controls with no drug - 8.3×10^7 CFU/ml; the data are representative of at least two independent biological replicates



Graph 4.1 Bactericidal or bacteriostatic effect (CPZ & its metabolites); INH (bactericidal) and fusidic acid (bacteriostatic) are positive controls; initial culture and culture with no treatment are the negative controls; the data are representative of at least two independent biological replicates

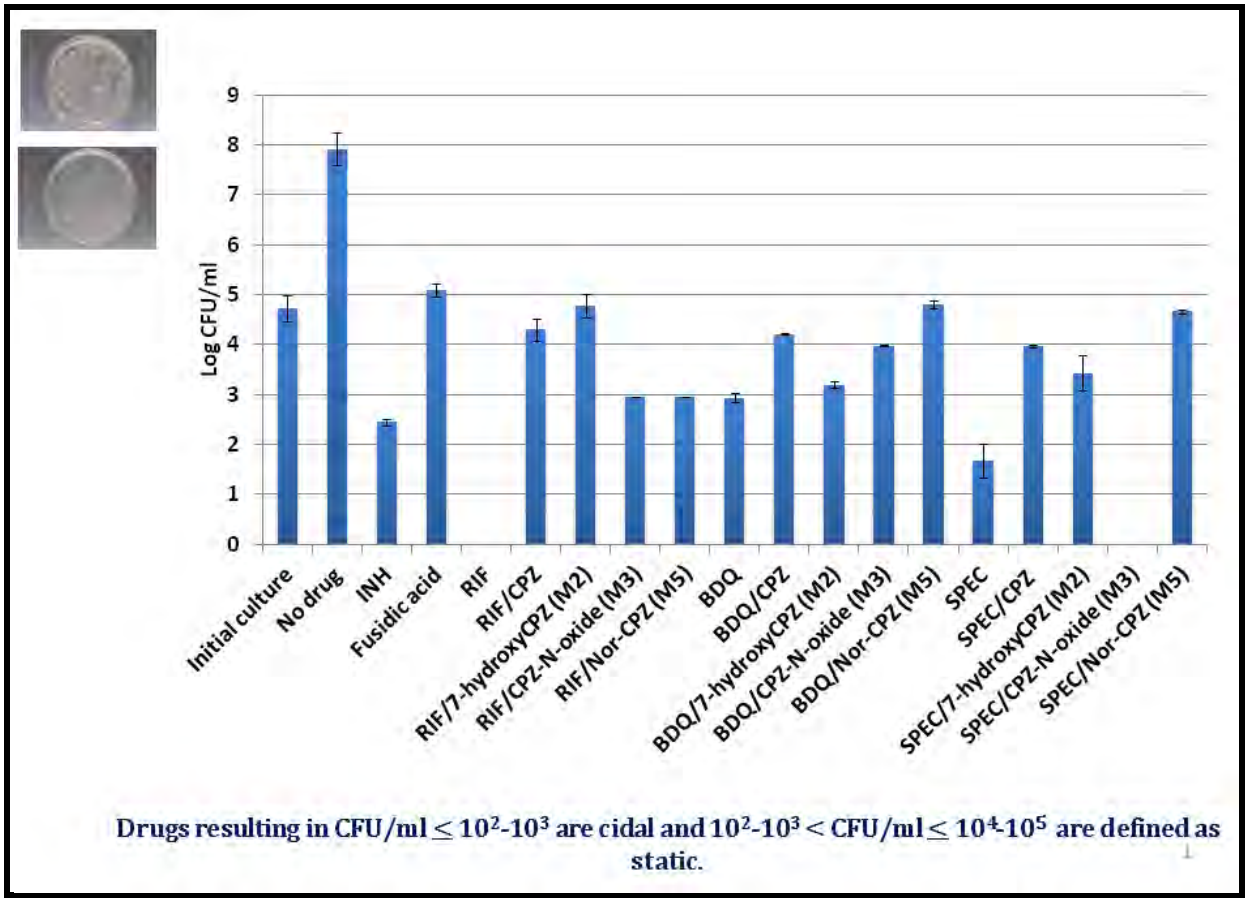
A bactericidal effect was observed for CPZ. However, 7-hydroxyCPZ **(M2)**, nor-CPZ **(M5)** and CPZ-*N*-oxide **(M3)** exhibited a bacteriostatic effect indicating that the killing effect of

CPZ is bactericidal due to the bioactivity contribution of the three metabolites, **M2**, **M5** and **M3**. Nevertheless, it was previously reported that CPZ and 7-hydroxyCPZ **(M2)** are bacteriostatic against gram-positive and gram-negative bacteria.[7,8]

Table 4.2: CFU/ml counts of combinations of CPZ or its metabolites with anti-TB drugs

Combinations	Blue well (MIC_{99})	Pink well (MIC_{99})	Bactericidal/Bacteriostatic effect
RIF **CPZ**	2.0×10^4	4×10^6	Bacteriostatic
RIF **7-hydroxyCPZ (M2)**	6.1×10^4	8.2×10^7	Bacteriostatic
RIF **Nor-CPZ (M5)**	9.0×10^2	1.03×10^7	Bactericidal
RIF **CPZ-*N*-oxide (M3)**	9×10^2	2.2×10^7	Bactericidal
BDQ **CPZ**	1.643×10^4	4.9×10^6	Bacteriostatic
BDQ **7-hydroxyCPZ (M2)**	1.575×10^3	1.1×10^6	Bactericidal
BDQ **Nor-CPZ (M5)**	6.417×10^1	6.4×10^6	Bacteriostatic
BDQ **CPZ-*N*-oxide (M3)**	6.29×10^4	3.6×10^6	Bacteriostatic
SPEC **CPZ**	9.425×10^3	5.0×10^5	Bacteriostatic
SPEC **7-hydroxyCPZ (M2)**	2.7×10^3	1.2×10^6	Bacteriostatic
SPEC **Nor-CPZ (M5)**	4.619×10^4	6.5×10^5	Bacteriostatic
SPEC **CPZ-*N*-oxide (M3)**	0	3.4×10^6	Bactericidal

Initial culture - 5.2×110^4 CFU/ml; controls with no drug - 8.3×10^7 CFU/ml; the data are representative of at least two independent biological replicates



Graph 4.2 Bactericidal & bacteriostatic effect (combinations); INH (bactericidal) and fusidic acid (bacteriostatic) are positive controls; initial culture and culture with no treatment are the negative controls; the data are representative of at least two independent biological replicates

The determination of the bactericidal and bacteriostatic effect of some of the combinations that demonstrated synergistic interactions might provide some insight into the effect experienced by mycobacteria when exposed to the different pairs of drugs. RIF (bactericidal) in combination with CPZ-*N*-oxide **(M3)** (bacteriostatic) and nor-CPZ **(M5)** (bacteriostatic) exhibited a bactericidal effect while in combination with CPZ (bactericidal) and 7-hydroxyCPZ **(M2)** (bacteriostatic), a bacteriostatic effect was observed. SPEC (bactericidal) with **M3** was bactericidal but bacteriostatic with CPZ, **M2** and **M5**. BDQ (bactericidal) was bacteriostatic with CPZ, **M3** and **M5** but bactericidal with **M2**.

No particular pattern can be drawn from the different effects of the combinations used in this experiment. It can only be concluded from these findings that despite RIF, BDQ and SPEC

being bactericidal[9–11] when combined with CPZ and its metabolites, the dominating effect at the lowest synergistic concentrations of the partners becomes bacteriostatic. This difference can be attributed to the reduced concentrations of the partner drugs in the wells in which synergism is observed: that is, inhibition of growth occurs at lower concentrations and can be mediated at a concentration of drug(s) that is bacteriostatic, in contrast to the higher inhibition concentrations recorded for compounds tested individually.

Similar observations were made previously by Bonapace *et al.*,[12] who explained that the inconsistencies exhibited in the two techniques (liquid *versus* solid media assays) may be due to the fact that they measure two different endpoints: bacterial growth inhibition and bacterial killing, respectively. Minimum bactericidal synergistic concentrations should therefore be determined for the best combinations discussed here in order to inform the development of bactericidal drug combinations.[13]

4.4 Spontaneous resistant mutant generation

An attempt to generate spontaneous resistant mutant strains of *Mtb* on 7H10 solid media containing 5 x, 10 x, 15 x and 20 x MIC_{99} concentrations of CPZ, 7-hydroxyCPZ **(M2)** and nor-CPZ **(M5)** was carried out in this study (section 7.3.4, Chapter 7) but was not successful, suggesting that the MoA of the parent and the metabolites is likely pleiotropic and, potentially, similar. This is not surprising because, as of now, there are no reports of generation of spontaneous mutants of *Mtb* that are resistant to CPZ. The MoA of phenothiazines was elusive for some time. It was originally proposed that the compounds function as efflux pump inhibitors.[14–16] However, as mentioned earlier, *in vitro* assays strongly suggested that phenothiazines target the type II NADH:menaquinone oxidoreductase which is essential for respiration in mycobacteria. Subsequently, it is thought that inhibition of the dehydrogenase would presumably result in inhibition of the cytochrome *bd* and cytochrome *aa3* complexes and thereby would prevent the organism from entering a state of non-replicating persistence.[1,3,17] In addition, it is possible that blocking the electron

flow to the menaquinone may result in downstream inhibition effects on energy-dependent functions such as efflux, which would lead to increased accumulation of a drug in combination with a phenothiazine such as CPZ,[18] as reported in the synergistic/matrix combination assay discussed in Chapter 3. Based on these observations, it can be presumed that inhibition of the dehydrogenase causes alterations of various functions of the respiration system of the bacilli, causing the phenothiazines to behave as though they interact with different targets. It is therefore unlikely that the *Mtb* respiration complex would tolerate mutations, hence making it difficult to raise spontaneous mutants.

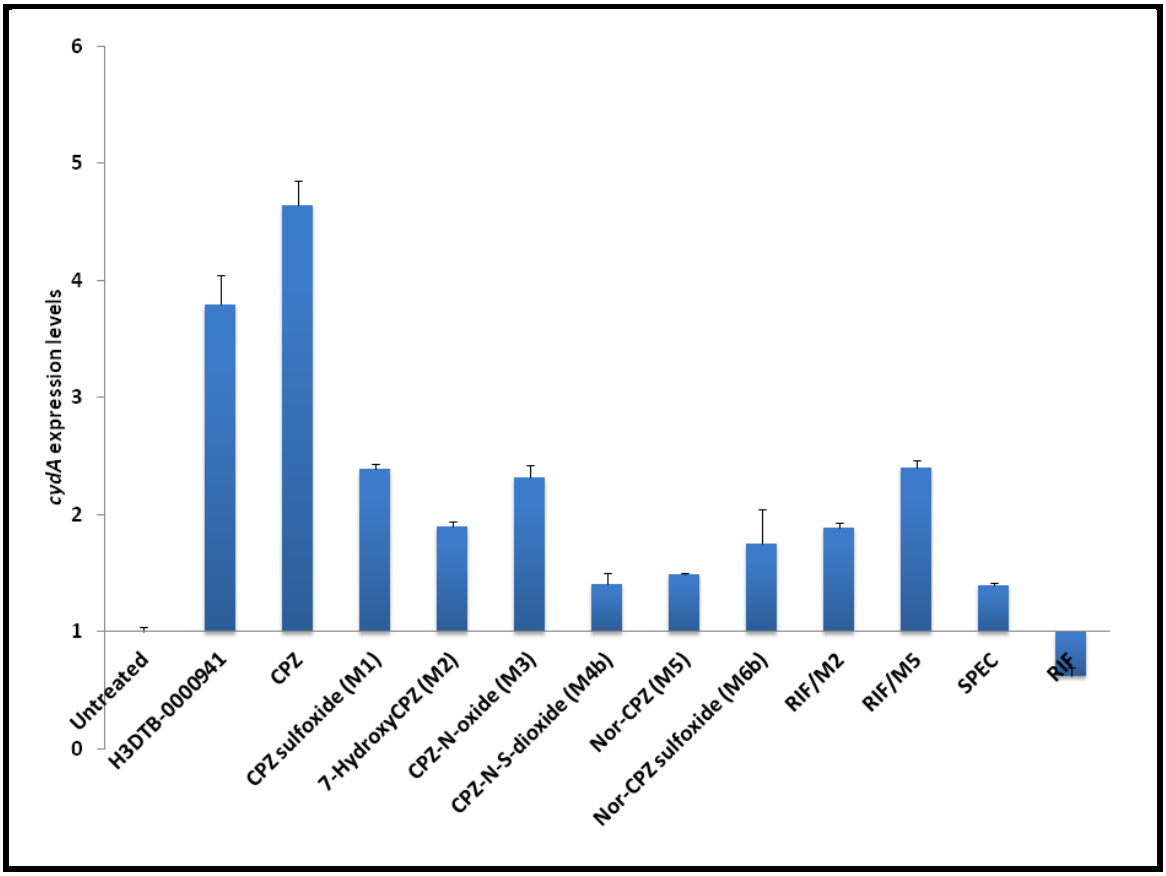
4.5 Transcriptional response of *cydA* gene

Electron transport in mycobacteria is initiated by the activity of various NADH and succinate dehydrogenases (SDH), which transfer electrons to menaquinone. Electrons are then passed to various cytochrome oxidases, whose activity is determined by the prevailing oxygen availability.[1,3,4] Recent work from Berney *et al.*[4] suggests that cytochrome *bd* oxidase, a non-proton-pumping terminal oxidase, is a relevant target for drugs: these authors suggest that cytochrome *bd* oxidase inhibitors might be usefully combined with BDQ, an inhibitor of adenosine triphosphate (ATP) synthase and characterized by a slow killing effect. Specifically, they propose that inhibition of ATP synthase may not be sufficient due to the subsequent induction of cytochrome *bd* oxidase expression, which relieves the back pressure on the electron transport chain and allows the mycobacterium to prolong maintenance of its membrane potential in the absence of ATP synthase activity leading to extrusion of a drug from the bacilli. This claim is supported by the results obtained here, in Chapter 3, Table 3.5 where a 508-fold reduction of the MIC_{99} of BDQ in combination with CPZ was observed.

Arora *et al.*[5] demonstrated that there is a risk in designing drugs that only inhibit the respiratory *bc1* complex, due to the fact that it has affinity for many compounds such as the imidazo[1,2 α]pyridines, hence exhibiting “promiscuity.” Moreover, inhibition of *bc1* leads to

up-regulation of the cytochrome *bd* oxidase and *cydDC* genes, which are part of an alternative respiratory complex. Since CPZ has previously been reported to inhibit the dehydrogenase activity,[3,19–22] and has been shown to induce expression of *cydAB* genes that encode the cytochrome *bd* oxidase,[17] *cydA* (Rv1623c) gene was hence selected for this study (section 7.3.5, Chapter 7).

SPEC, a protein synthesis inhibitor in bacteria[23] was used as a negative control. H3DTB-0000941, an imidazopyridine compound currently in development at the University of Cape Town (UCT), Drug Discovery and Development (H3-D) Center, was found to up-regulate *cydA*, and was hence used as a positive control. An untreated sample was also included as a negative control. Graph 4.3 illustrates the effect on *cydA* expression following exposure of *Mtb* to CPZ, its metabolites and some of their combinations.



Graph 4.3 *cydA* expression in *Mtb* treated with CPZ, its metabolites and some combinations; 6h treated samples

As expected, the untreated and SPEC treated samples exhibited very low *cydA* expression levels whereas the positive control treated sample exhibited a 3.8-fold up-regulation of *cydA* gene. The highest expression of *cydA* gene (4.6-fold up-regulation) was observed in *Mtb* treated with the parent drug CPZ, consistent with previous analyses of the CPZ-induced mycobacterial transcriptome.[17] A 2-fold up-regulation of *cydA* was observed for *Mtb* treated with **M1**, **M2** and **M3**. With **M4b, M5** and **M6b** about 1.5-fold up-regulation of the gene was observed but was not significant since it was comparable to the SPEC treated sample. It is, however, important to note that the initial concentrations that were utilized in the combination assays (Chapter 3) for **M1**, **M4b** and **M6b** (1991, 1900 and 2078μM) were much higher than the concentrations used in the transcriptional response experiment (140μM). At high concentrations, the three inactive metabolites significantly reduced the MIC_{99} of some of the anti-TB drugs used in the combination assays. This suggests that higher *cydA* gene expression may possibly be observed at much higher concentrations of the three metabolites.

To further investigate observations made in the combination assays discussed in Chapter 3, two of the most promising combinations were selected for the transcriptional response experiment. Interestingly, combinations of RIF and two metabolites **M2** and **M5** exhibited at least a 2-fold up-regulation of *cydA* while a down regulation was observed for the RIF treated sample. It is also worth noting here that to test for *cydA* gene expression, MIC_{99} concentrations of the partners used, were 2.5 times lower than the concentrations used for **M2** and **M5** when tested singly. It is possible that higher *cydA* gene expression may perhaps have been observed had the combinations been prepared with 140μM of the metabolites.

Murima *et al.*[24] recently described the use of a medium-throughput micro-fluidic (fluidigm) assay to explore the MoA of synthesized analogues in relation to the parent compounds using a “diagnostic” transcriptional response in *Mtb*. Analogous to that study, the findings

here strongly suggest that the metabolites do retain the same MoA as the parental compound, CPZ.

4.6 Determination of MIC_{99} of CPZ and its metabolites against *cytochrome bd* oxidase *Mtb* mutant strains

Having observed an up-regulation of *cydA* (section 4.3), there was need to ascertain whether CPZ or its metabolites retained their activity against an *Mtb* strain lacking a functional cytochrome *bd* oxidase. To this end, CPZ, 7-hydroxyCPZ **(M2)**, nor-CPZ **(M5)** and CPZ-*N*-oxide **(M3)** were tested against the Δ*cydBDC* mutant[5] as well as Δ*cydA* and Δ*cydAB* mutants (Moosa, Mizrahi, Warner unpublished) of *Mtb* in which cytochrome *bd* oxidase function is eliminated (Table 7.1, section 7.3.1, Chapter 7). The results in Table 4.3 indicate that all the four compounds retained their activity relative to H37RvMa (wild-type) confirming further that CPZ metabolites remain "on target." It can also be deduced here that, despite the fact that these compounds cause an up-regulation of the genes in the *cydABCD* operon, deletion effect of the cytochrome *bd* oxidase-encoding genes does not compromise the killing effect observed when the compounds inhibit type II NADH. This result is consistent with the suggestion made by Weinstein *et al.*[3] who postulated that inhibition of the dehydrogenase by trifluoperazine, a phenothiazine, may result in an inhibition effect on both respiratory branches, cytochrome *bd* and a cytochrome *aa3* systems, ultimately leading to the death of the mycobacteria.

Table 4.3: MIC_{99} of CPZ or its metabolites against the cytochrome *bd* oxidase *Mtb* knockout mutant strains (in GAST/Fe minimum media)

Compound	MIC_{99} (μM)			
	H37RvMa	**Δ*cydKO***	**Δ*cydA***	**Δ*cydAB***
CPZ	28.1	28.1	28.1	28.1
7-hydroxyCPZ (M2)	59.7	59.7	119.5±34.53	59.7
Nor-CPZ (M5)	65.6	65.6	65.6	65.6
CPZ-*N*-oxide (M4b)	497.2	497.2	497.2	497.2
CDM143*	0.156±0.18	0.0001	0.0097±0.01	0.0012

. *Positive control – imidazopyridine in development at H3-D Center; targets QcrB

4.7 Summary discussion

Based on prior identification of type II NADH:menaquinone oxidoreductase as the target for CPZ,[1,3,17] several approaches were used in this study to interrogate the MoA of CPZ metabolites. With the use of different approaches discussed, it was established here that metabolites of CPZ retain the same MoA as the parent. The results also suggest that inhibition of NADH dehydrogenase is effective in arresting both respiratory complexes.[1,3] This is contrary to the ATP synthase and the *bc1* complex inhibition effects reported for BDQ and imidazo[1,2 α]pyridines respectively, that result in an up-regulation of the *cyd* genes encoding the alternative cytochrome *bd* complex leading to a delayed killing effect of the mycobacteria.[4,5] In addition, it has been demonstrated here that the use of multiple assays for investigation of the MoA of drug(s), is more beneficial than the use of a single assay. However, with the failure to raise spontaneous *Mtb* mutants, it still remains unclear whether CPZ and its metabolites are able to interact with one or more targets.

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CHAPTER 5

MUTATIONS IN 16S rRNA CONFERRING SPECTINOMYCIN RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* (*Mtb*)

5.1 Introduction

With a renewed interest in the possibility of exploiting alternative strategies to address the well-documented bottlenecks in antibiotic development,[1] the potential to repurpose approved drugs for use as novel anti-infective agents,[2–5] or to use them as leads in drug repositioning strategies, is an attractive approach. Ramon-Garcia *et al.*[6] demonstrated that spectinomycin (SPEC) exhibits very low antimycobacterial activity on its own but can be combined with other drugs to produce a synergistic effect. Based on their observations, as well as the results presented in Chapter 3, SPEC was selected for further investigations of the mechanism of action (MoA). SPEC (Figure 5.1) is an aminoglycoside produced by the bacterium *Streptomyces spectabilis* and used to treat gonorrhea.[7,8]

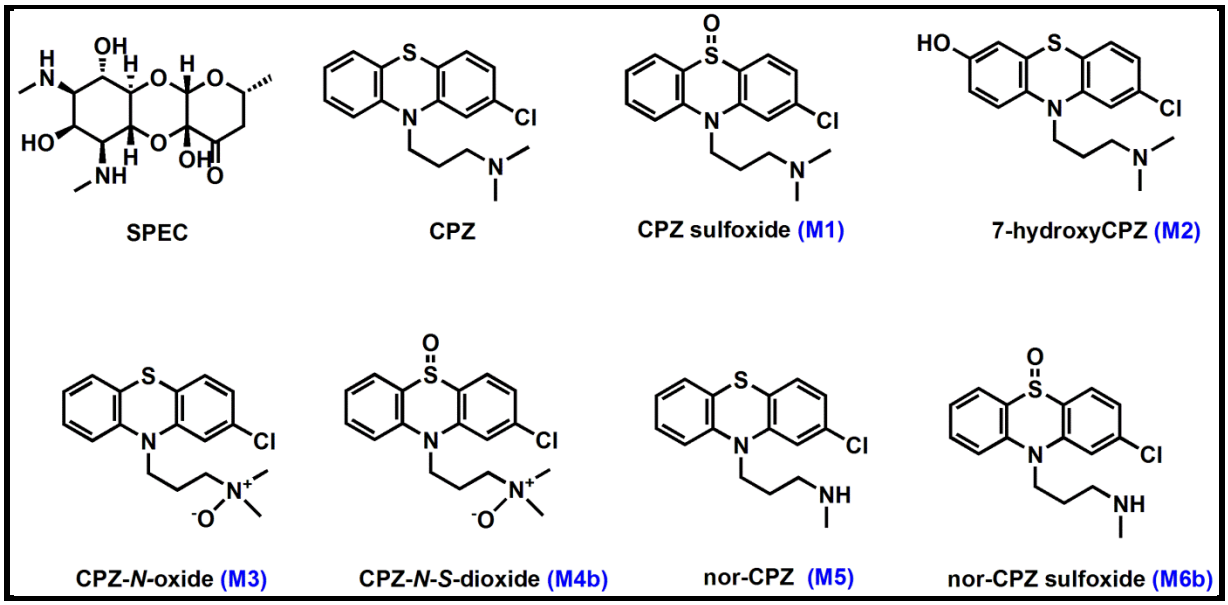


Figure 5.1 SPEC, chlorpromazine (CPZ) and its metabolites

CHAPTER 5

In the results discussed in Chapter 3, SPEC was shown to have synergistic interactions with CPZ or its metabolites (Figure 5.1) against *Mycobacterium smegmatis* (*Msm*) and *M. tuberculosis* (*Mtb*). In fact, in combination with CPZ, a 128-fold reduction of its MIC_{99} value was observed against *Mtb* (Table 5.2). Moreover, Lee *et al.*[9] carried out a structure activity relationship (SAR) study of SPEC leading to the discovery of derivatives known as spectinamides. The analogues exhibited a 50-fold higher antimycobacterial activity compared to the parent, against sensitive and resistant strains of *Mtb* and with an ability to overcome efflux. Based on findings from these studies, SPEC is highly attractive for further development as an anti-TB drug however, it raised questions on whether it is suitable for further development due to its ability to synergize with many classes of compounds, an indication of off-target activity.

It therefore became necessary to explore the MoA of SPEC which, in combination with CPZ and its metabolites, resulted in the highest synergistic effect (Chapter 3). In addition, we wanted to establish why it synergizes with a wide range of drugs. The MoA of SPEC has been extensively investigated in bacteria. 16S ribosomal mutations in *Escherichia coli* and *Neisseria gonorrhoeae* have been reported before.[7,10–15] For instance, in *E. coli*, SPEC has been reported to be an inhibitor of protein translation by binding to the bacterial 30S ribosomal subunit. It specifically interacts directly with 16S rRNA and inhibits the elongation factor G (EF-G)-catalyzed translocation of the peptidyl-tRNA from the A site to the P site during polypeptide elongation.[13,16] Reports also indicate that the interaction of SPEC with 16S rRNA is in the upper stem of helix 34, close to the base-paired C1063G1064U1065 and A1191C1192G1193 where resistance to the drug has been mapped in *E. coli*.[7,10,17] In *N. gonorrhoeae*, only a single nucleotide polymorphism (SNP), specifically a C1192U transition, in 16S rRNA has been verified to result in high-level SPEC resistance.[11,12] Nonetheless, the MoA of SPEC in *Mtb* remains unknown.

5.2 SPEC in combination with CPZ or its metabolites against *Msm* (mc^{2}155) and *Mtb* (H37RvMa)

A compilation of the combination assay results of SPEC with CPZ or its metabolites (discussed in Chapter 3) was carried out to bring into perspective why SPEC was selected for spontaneous mutant generation in *Mtb*. A summary of the MIC_{99} and the lowest MIC_{99} achieved in the various combinations of CPZ or its metabolites with SPEC, and the fractional inhibitory concentration indices (FICI values) against *Msm* (mc^{2}155) and *Mtb* (H37RvMa), are given in Table 5.1 -Table 5.4.

As defined in Chapter 3, it should be noted that synergy is assigned where fractional inhibition concentration index (FICI) $\leq$ 0.5; an FICI $\geq$4 is antagonistic while any value falling in between is additive/no effect.[6,18–20] Evidently, synergism was observed in most of the combinations of SPEC with CPZ or its active metabolites (FICI < 0.5). The highest FICIs observed were with 7-hydroxyCPZ **(M2)** at 0.19 and 0.13 against *Msm* and *Mtb,* respectively (Table 5.1 & Table 5.4). Interestingly, a high reduction of the MIC_{99} values of SPEC was observed for most of the combinations, the highest being with CPZ (128-fold, Table 5.2) against *Mtb*.

Table 5.1: FICI of CPZ or its metabolites in combination with SPEC, against *Msm*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction	FIC	FICI
SPEC	84.12±24.05	5.25	16	0.062	0.31
CPZ	117.27	29.32		0.250	
SPEC	84.12±24.05	5.25±10.53	16	0.062	0.19
7-hydroxyCPZ (M2)	124.44±15.91	15.56±2.47		0.125	
SPEC	168.22±24.05	84.12	2	0.500	0.63
CPZ-*N*-oxide (M3)	995.43	124.44±71.84		0.125	
SPEC	84.12	5.25±4	16	0.062	0.31
nor-CPZ (M5)	136.70±31.82	34.18±4.51		0.250	

Table 5.2: FICI of CPZ or its metabolites in combination with SPEC, against *Mtb*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction	FIC	FICI
SPEC	168.22±97.14	1.31±1	128.4	0.0078	0.26
CPZ	29.32±6.01	7.32±4.51		0.250	
SPEC	336.47	21.04	16	0.063	0.13
7-hydroxyCPZ (M2)	124.44±14.74	7.76		0.063	
SPEC	336.47	NC			
CPZ-*N*-oxide (M3)	497.73				
SPEC	336.47	21.04	16	0.063	0.19
nor-CPZ (M5)	68.34	8.53		0.125	

NC – No Change

In combination with the inactive CPZ metabolites (Table 5.3 and Table 5.4), at least a 2-fold reduction in MIC_{99} of SPEC was observed but, most importantly, the highest recorded at 16-fold against *Msm*. This demonstrates that, despite the absence of a killing effect of **M1**, **M4b** & **M6b**, an additional MoA of the pairs was at play. Hence, generation of spontaneous mutant strains of SPEC followed.

Table 5.3: Inactive metabolites of CPZ in combination with SPEC against *Msm*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction
SPEC	168.22±48	10.52±3.1	16-fold
CPZ sulfoxide (M1)	>1990.89	1990.89	
SPEC	84.12	42.05	2-fold
CPZ-*N*-*S*-dioxide (M4b)	>1900.10	1900.10	
SPEC	84.12	21.04±5.43	4-fold
nor-CPZ sulfoxide (M6b)	>2077.89	64.92±14.74	

Table 5.4: Inactive metabolites of CPZ in combination with SPEC against *Mtb*

Drugs/Compound	MIC_{99} (µM), singly	MIC_{99} (µM) in combination	MIC_{99} fold reduction
SPEC	168.22±48.56	42.05±12.14	4-fold
CPZ sulfoxide (M1)	>1990.89	497.73	
SPEC	336.47	168.22	2-fold
CPZ-*N*-*S*-dioxide (M4b)	>1900.10	950.04	
SPEC	336.47	84.12±12.14	4-fold
nor-CPZ sulfoxide (M6b)	>2077.89	519.48	

5.3 Frequency and identification of SPEC-resistant (SPEC^R) mutations

The frequency of the SPEC^R mutants of *Mtb* was determined by plating 10^8 -10^9 cells of logarithmic phase H37RvMa strain on standard media broth, 7H10 plates containing 1.3 mM of SPEC, a concentration 5-fold higher than the MIC_{99}. The colonies typically appeared and were scored after 4 weeks' incubation at 37°C. The spontaneous mutants arose at a frequency of 2.09 x 10^{-6}, comparable to the value reported for spectinamides mutant strains.[9] Five individual colonies were picked, grown in standard 7H9 ADC media, and tested for SPEC susceptibility (section 7.3.6, Chapter 7). All SPEC^R mutants showed a >64-fold increase in MIC_{99} (section 5.4, Table 5.8).

As discussed above, SPEC^R mutations have been primarily mapped to the 16S rRNA in other bacteria.[11,13–15] According to Lee *et al*.,[9] exposure of *Mtb* to spectinamides led to mutations in the *rpsE* gene, encoding ribosomal protein S5. Consequently, 16S rRNA *rrs* (MTB000019) and *rpsE* genes were sequenced in the five SPEC^R mutants to determine if similar mutations were responsible for resistance in *Mtb* (section 7.3.6.4, Chapter 7). Point mutations as a result of a guanine-to-thymine (G-T) transition at position 1379 in the *rrs* gene generated MtbSR1 strain. Mutant MtbSR3 strain was a result of a replacement of adenine by a guanine in position 1183 in the same gene. The other three mutants, interestingly, had an insertion of cytosine at position 926 (Table 5.5).

Table 5.5: Sequencing results of SPEC^R mutant and H37RvMa strains

Strain	**Mutation in *rrs* gene**
Mtb H37RvMa	No mutation
MtbSR1	G1379T
MtbSR2	Insertion of C (926)
MtbSR3	A1183G
MtbSR4	Insertion of C (926)
MtbSR5	Insertion of C (926)

G – guanine; T – thymine; A – adenine; C - cytosine

To our knowledge, this is the first time that the insertion of cytosine at position 926 has been described as a mechanism for SPEC resistance in bacteria. It is worth noting that targeted sequencing revealed that none of the five SPEC^{R} isolates carried additional *rpsE* polymorphisms. However, in *E. coli,* mutations in the gene, are reported to cause resistance to SPEC.[22]

A closer look at the predicted structures of *rrs* RNA sequences of the SPEC mutants in Figure 5.2 in relation to H37RvMa (wild-type), indicate that a single nucleotide substitution or insertion can cause a dramatic change in folding pattern of *rrs* secondary structure. This is mainly observed for instance in MtbSR1 mutant strain with fewer structural modifications predicted for MtbSR3 and MtbSR4.

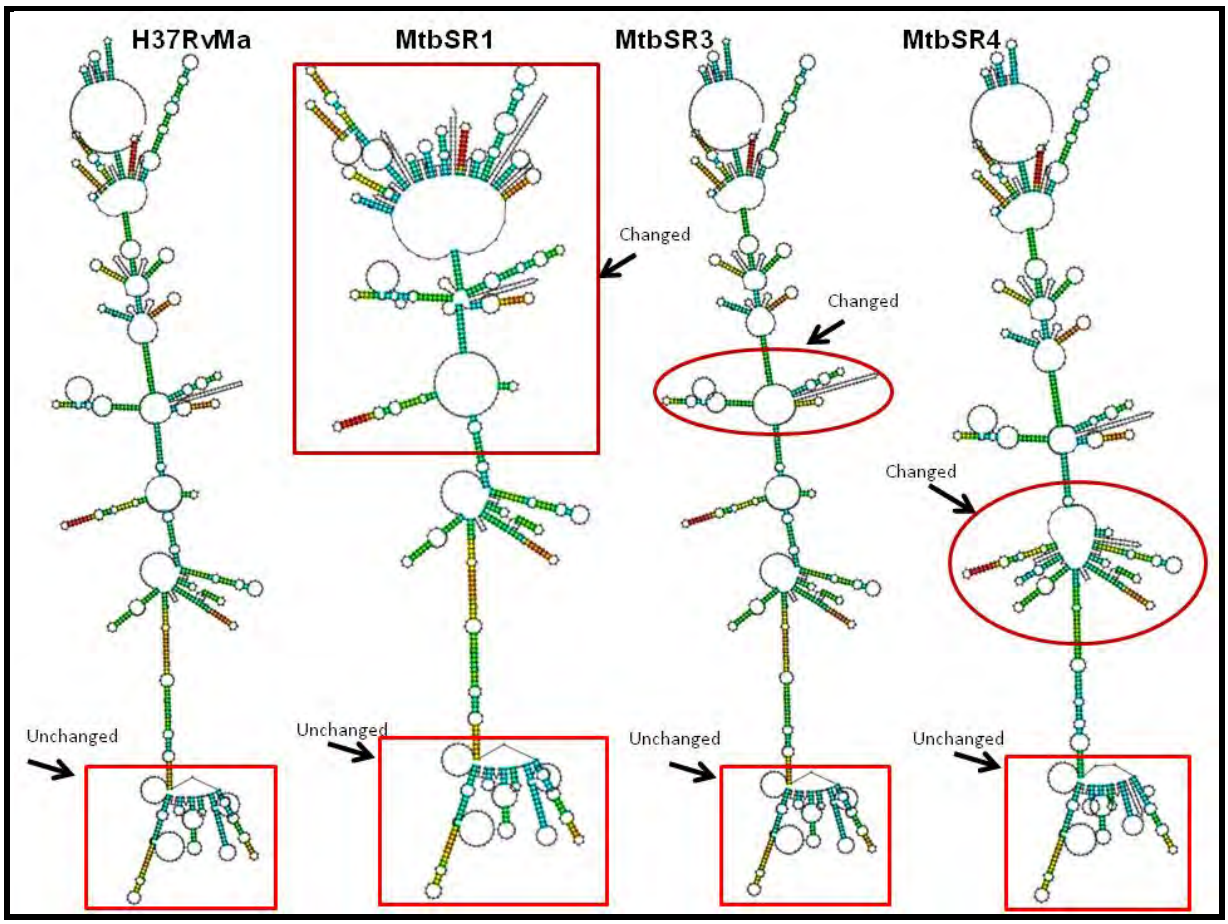
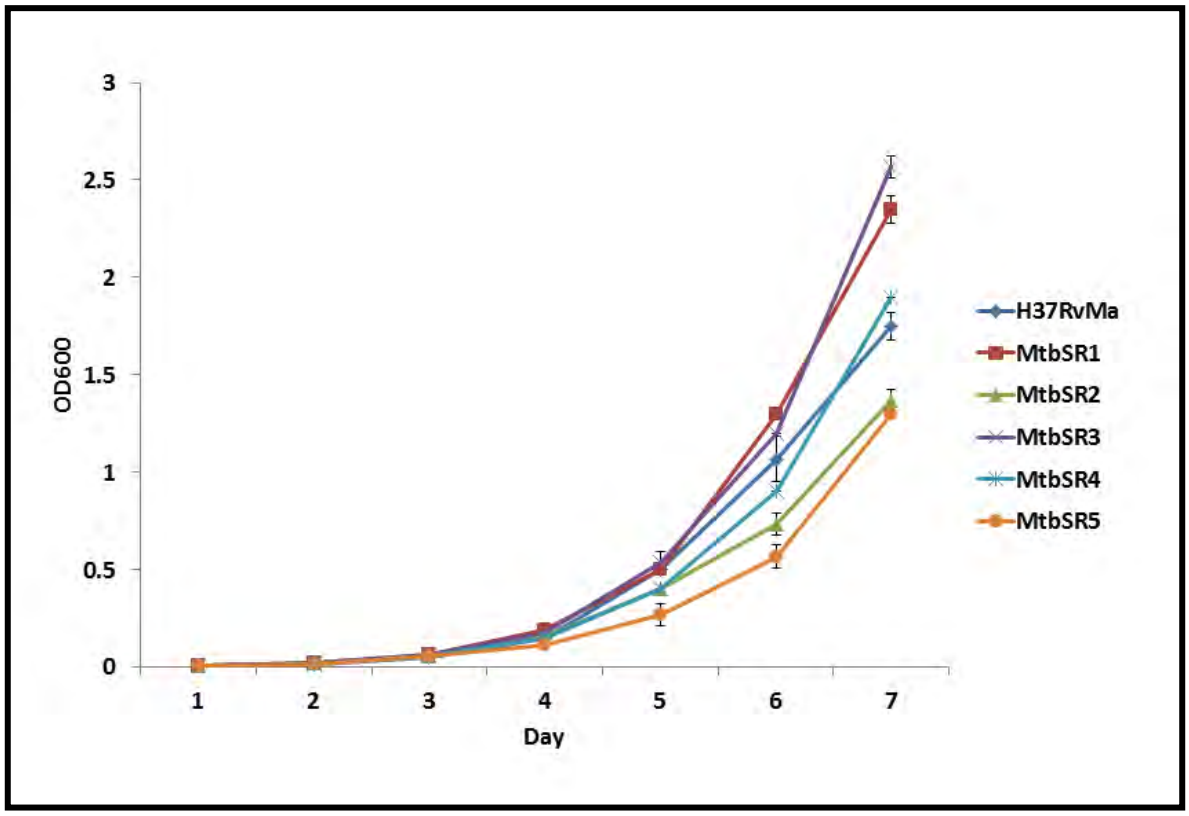


Figure 5.2 Structures of the *rrs* genes of SPEC^{R} mutant *Mtb* strains (drawn using CentroidFold software[21])

SPEC dependence has been reported in *Bacillus subtilis* when double mutations occur in the ribosomal genes.[23] This lack of dependence was confirmed by the ability of all SPEC^R *Mtb* mutants to grow in the absence of SPEC.

5.3.1 Fitness cost of SPEC^R mutant strains

Most bacteria possess more than one copy of 16S rRNA gene however, *Mtb* carries only one copy.[24,25] This prompted the need to look for any fitness cost associated with the mutation in 16S rRNA (section 7.3.6.5, Chapter 7). The *rrs* gene structural changes considerably affected the growth kinetics of the mutant strains (Graph 5.1, Table 5.6) and the morphology of the resistant mutant colonies (Appendix 10A).



Graph 5.1 Growth curve for SPEC^R *Mtb* mutant strains; OD_{600} – optical density at 600nm wavelength; the data are representative of at least two independent biological replicates

Table 5.6: CFU/ml counts of SPEC^R mutant strains

Strain	Days					
	0	**1**	**3**	**4**	**5**	**6**
H37RvMa	3.5×10^5	7.5×10^5	6.8×10^6	4.72×10^7	4.2×10^8	7.0×10^8
MtbSR1	4.2×10^5	7.5×10^5	7.5×10^6	4.15×10^7	1.95×10^8	6.65×10^9
MtbSR2	2.25×10^5	4.55×10^5	7.0×10^5	2.4467×10^7	3.0×10^7	1.0×10^8
MtbSR3	3.5×10^5	9.0×10^5	6.9×10^6	7.16×10^7	7.4×10^8	9.0×10^8
MtbSR4	4.53×10^5	7.0×10^5	5.5×10^6	4.45×10^7	6.0×10^7	2.5×10^9
MtbSR5	2.15×10^5	3.3×10^5	1.0×10^5	7×10^5	2.0×10^7	1.0×10^8

The data are representative of at least two independent biological replicates

Surprisingly, the mutations that occurred in *rrs* genes of MtbSR1, MtbSR3 and MtbSR4 strains resulted in a reproducibly faster growth rate compared to the wild-type (H37RvMa) strain as assessed by optical density (OD_{600}, Graph 5.1). On the other hand, slower growth was observed for MtbSR2 and MtbSR5 strains (Graph 5.1). MtbSR2, MtbSR4 and MtbSR5 colonies appeared to be smaller and fewer compared to the ones of H37RvMa, MtbSR1, and MtbSR3 (Appendix 10A). Interestingly, lower doubling times of MtbSR1, MtbSR3, MtbSR4 and MtbSR5 (20.18, 19.24, 19.79 and 20.47h respectively; Table 5.7) compared to H37RvMa wild-type strain (21.65h), were observed. However, MtbSR2 exhibited longer doubling time.

Table 5.7: Strain doubling times calculated during growth in liquid media

Strain	Doubling time (h)
H37RvMa	21.65
MtbSR1	20.18
MtbSR2	22.80
MtbSR3	19.24
MtbSR4	19.79
MtbSR5	20.47

Doubling times are calculated using changes in OD_{600} value over 24 hour intervals; the data are representative of at least two independent biological replicates

5.4 Susceptibility testing singly and in combination of SPEC with CPZ or its metabolites on SPEC^R mutant strains

Table 5.8 summarizes the MIC_{99} determined for SPEC, CPZ, CPZ metabolites and anti-TB drugs against the resistant mutants (section 7.3.1, Chapter 7). The results here indicate that a >64-fold increase of the MIC_{99} of SPEC against the mutant strains relative to wild-type strain, occurred. On the other hand, the MIC_{99} of CPZ, CPZ metabolites and the anti-TB drugs remained unaffected against the mutant strains relative to the wild-type strain, confirming absence of cross-resistance. However, it was noted that less resistance to SPEC was exhibited by MtbSR4 and MtbSR5 mutant strains (MIC_{99} 16823 µM) compared to the other mutants (MtbSR1, MtbSR2 and MtbSR3) which resulted in SPEC MIC_{99} >16823 µM.

Table 5.8: MIC_{99} of various drugs against different SPEC^R mutant strains

Drugs	MIC_{99} against different resistant colonies (µM)					
	MtbSR1	**MtbSR2**	**MtbSR3**	**MtbSR4**	**MtbSR5**	**H37RvMa**
CPZ	59	29±17.3	59	29	29	59
7-HydroxyCPZ (M2)	62	62	62	31	31	62
Nor-CPZ (M5)	68	68	68	34	34	68
CPZ-*N*-*S*-dioxide (M4b)	>1900	>1900	>1900	>1900	>1900	>1900
SPEC	**>16823**	**>16823**	**>16823**	**16823**	**16823**	**263**
STRP	0.22	0.22	0.11±0.06	0.22	0.22	0.45±0.16
KANA	4.46	4.46	2.23	4.46	4.46	4.46
GENT	6.98	6.98	6.98	6.98	6.98	6.98
HYGR	15.8	15.8	15.8	15.8	15.8	15.8
CLMP	25.79	25.79	25.79	12.9	12.9	25.79
RIF	0.004	0.004	0.008±0.02	0.008±0.02	0.002	0.008±0.02
25-*O*-DesacetylRIF	0.013	0.0067	0.013	0.0067	0.013	0.013
INH	0.2	0.2	0.2	0.2	0.2	0.2
EMB	6.01±1.73	3	3±01.73	3	3	6.01
BDQ	0.47	0.94±0.27	0.47	0.47	0.47	0.47
LEVO	2.31±0.7	1.15	1.15	1.15	1.15	2.31

STRP – streptomycin, KANA – kanamycin, RIF – rifampicin, INH – isoniazid, BDQ – bedaquiline; GENT – gentamycin; HYGR – hygromycin; CLMP – chloramphenical; 25-*O*-desacetylRIF - 25-*O*-desacetylrifampicin; EMB – ethambutol; LEVO – levofloxacin; H37RvMa – wild-type

As shown in Table 5.9, combining SPEC with CPZ or its metabolites did not restore the antimycobacterial activity of the antibiotic against MtbSR1 and MtbSR5 mutant strains.

Antimycobacterial activity was only observed for CPZ or its metabolites, further confirming the absence of cross-resistance. This also suggests that the synergism observed in similar combinations against the wild-type strain is as a result of drug pairs acting on different targets, and is not a function of a non-specific property. That is, SPEC synergizes with other drugs owing to its ability to disrupt 16S rRNA function.

Table 5.9: Combinations of SPEC with CPZ or its metabolites against MtbSR1 and MtbSR5 mutant colonies

Drugs/Compound	MIC_{99} (μM), singly	MIC_{99} (μM) in combination
$SPEC^R$ (MtbSR1)		
SPEC	>673	NC
CPZ	59	NC
SPEC	>673	NC
7-HydroxyCPZ (M2)	62	NC
SPEC	>673	NC
Nor-CPZ (M5)	68	NC
SPEC	>673	NC
CPZ-*N*-*S*-dioxide (M4b)	>1900	NC
$SPEC^R$ (MtbSR5)		
SPEC	>673	NC
CPZ	29	NC
SPEC	>673	NC
7-HydroxyCPZ (M2)	31	NC
SPEC	>673	NC
Nor-CPZ (M5)	34	NC
SPEC	>673	NC
CPZ-*N*-*S*-dioxide (M4b)	>1900	NC

NC – No Change

5.5 Summary discussion

The 16S rRNA *rrs* gene (MTB000019) was identified in this study as the target for SPEC in *Mtb*. The target has also been previously reported in other bacteria that possess more than one copy of *rrs*. However, *Mtb* carries only one copy suggesting that the gene may potentially be essential for survival.[24,25] Mutations due to an insertion of a cytosine in the gene have not previously been reported, making this a unique finding in this study.

Generally, the SNPs resulted in a dramatic change in mycobacterial growth kinetics. The growth rate of the $SPEC^R$ mutant strains was found to either be higher in MtbSR1, MtbSR3 and MtbSR4 mutants or lower in MtbSR2, and MtbSR5 strains relative to H37RvMa strain. This indicated that point mutations due to a replacement of a nucleotide resulted in a positive growth impact on the mycobacteria, while an insertion of an extra base led to a growth-defect except for MtbSR4 that grew fast but with smaller colonies relative to wild-type. In addition, it has been demonstrated here that the spontaneous mutations generated in *Mtb* were selective for SPEC given that cross-resistance was not observed.

It can also be inferred that synergistic interactions exhibited in combinations of SPEC with other drugs against *Msm* and *Mtb* may actually be beneficial. This is because it has been shown here that SPEC does not interact with several targets but specifically binds to the *rrs* gene that encodes the 16S rRNA. Hence, it can be deduced that an off-target activity is unlikely to occur with SPEC, qualifying it as a suitable partner for combination therapy for TB treatment.

A limitation of SPEC is that it is subject to efflux; this motivated the work by Lee *et al*.[9] who showed that engineering the drug to the spectinamide analogues provided a powerful way to circumvent this problem. It would be great if future research could look at combinations of spectinamides with CPZ. If there's no effect, then one can infer that CPZ is potentiating SPEC by inhibiting Rv1258c function that encodes an efflux pump in *Mtb*, and is thought to cause drug tolerance.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

With a growing need to design new chemotherapeutics for tuberculosis (TB) treatment, a renewed interest in repurposing and repositioning old drugs is emerging especially due to the increasing numbers of drug-resistant cases. Phenothiazines represent one of many classes of drugs that have been the focus of research with the aim of modifying their structures or their use for anti-TB drug discovery. In this project, the approach adopted was to generate metabolites of chlorpromazine (CPZ), a phenothiazine, and carryout combination screening with known anti-TB drugs.

The generation and scale-up of CPZ metabolites was successfully carried out and resulted in six metabolites, CPZ sulfoxide **(M1)**, 7-hydroxyCPZ **(M2)**, CPZ-*N*-oxide **(M3)**, CPZ-*N*-*S*-dioxide **(M4b),** nor-CPZ **(M5)** and nor-CPZ sulfoxide **(M6b)**. Exposure of CPZ to human and rat liver microsomes (HLM & RLM) gave rise to the highest number of metabolites compared to the other biotransformation systems used in this study. This further guided the synthesis of the metabolites, with the aim of increasing the yield for antimycobacterial screening. Optimization of cytochrome P4501A2 (CYP1A2) incubations of CPZ, leading to generation of three metabolites **(M1, M2, M5),** was important for isolation of the hydroxyl metabolite **(M2)** that could potentially be utilized in the synthesis of analogues of this class of compounds for TB drug discovery. In future studies, it is recommended that the cytotoxicity, physicochemical as well as pharmacokinetic properties are determined for the metabolites and compared with the ones of the parent (CPZ). It is expected that the metabolites would exhibit superior properties compared to CPZ, and would be better candidates for anti-TB combination drug development. The results presented here further suggest that structure activity relationship (SAR) studies of this class of compounds should be carried out in future

in order to optimize antimycobacterial activity of the compounds for discovery of novel TB combination therapy. This may also result in scaffolds with less neural activity.

Combinations of CPZ or its metabolites with anti-TB drugs exhibited synergistic interactions with some fractional inhibition concentration indices (FICI) observed below 0.5. The highest synergism observed was with spectinomycin (SPEC), a drug used for treatment of gonorrhea but has recently interested research groups working in the TB field for repurposing and repositioning. Based on the findings here, it is recommended that optimization studies of CPZ or its metabolites in combination with SPEC are carried out in order to identify appropriate concentrations of the drugs, to be applied *in vivo* for further investigations. Consideration should focus on the use of human dosing and pharmacokinetic parameters, in future studies. It is also recommended that combination assays are carried out for CPZ or its metabolites with spectinamides in order to establish whether the former compounds have an inhibitory effect on Rv1258c efflux pump, based on the findings by Lee *et al*.

Previously, the mechanism of action (MoA) of CPZ had been established and the target was identified as type II NADH:menaquinone oxidoreductase responsible for initiating the branched aerobic respiratory chain. Here, it has been shown that the MoA of CPZ and its metabolites, is similar based on the findings from the different techniques that were used in this study. It still remains unclear whether there is a possibility that these compounds interact with different targets, given that generation of spontaneous *Mycobacterium tuberculosis* (*Mtb*) mutants was not achieved. However, Weinstein *et al.* shed some light on the matter by demonstrating that the inhibition of the NADH dehydrogenase by trifluoperazine, a phenothiazine, may probably lead to a downstream effect on the cytochrome *bd* and a cytochrome *aa3* respiratory complexes that the mycobacteria uses interchangeably depending on its environmental conditions. Coupled with the fact that the inhibition effect may lead to further downstream effects on the efflux, this is likely to further reduce the

chances of inducing spontaneous mutations in the mycobacteria. This may also suggest that there are less chances of development of resistance to phenothiazines by *Mtb* in a clinical situation. Nevertheless, it would be worthwhile to use less concentrations of CPZ or its metabolites to try and raise *Mtb* spontaneous mutant strains since the targeted genes maybe essential for the survival of the mycobateria. With further optimization of CPZ and its metabolites through structure modifications and identification of optimal pharmacokinetic properties, the compounds could potentially be incorporated into the current TB regimen especially for populations at risk of development of drug resistance.

In addition to the strong synergistic interactions of SPEC in combination with CPZ or its metabolites, the antibiotic was also reported to have similar effects when combined with other drugs. As a follow-up on these observations, this study sought to explore the MoA of the antibiotic. Spontaneous mutant strains of SPEC were raised followed by sequencing of 16S rRNA *rrs* gene (MTB000019) of some of the selected mutant colonies. Three mutations identified, resulted in some structural changes of the gene and subsequently a change in the growth kinetics of the mycobacteria. This finding not only revealed the target for SPEC in mycobacteria, but it also suggests that the *rrs* gene may be essential due to the fact that the organisms consist of only one copy of the gene. This was evident from the different growth patterns observed for the mutant strains relative to wild-type. However, more studies need to be carried out to validate this fact, as well as explore more possible binding sites for SPEC. It hence became clear that SPEC's ability to synergize with many classes of drugs is not due to its interaction with several targets as it was initially perceived, because this could have been a potential source of undesired activities. Using *in vitro* techniques, it has been established that SPEC is a potential drug for repurposing or repositioning for combination therapy TB drug discovery. This is also very evident from the work of Lee *et al.* who successfully designed analogues of SPEC with higher antimycobacterial activity compared to the parent, and able to overcome resistance due to efflux. Likewise, based on the findings of this project, synergistic interactions between SPEC and CPZ or its metabolites may be

due to the ability to overcome efflux. It is therefore recommended that further optimization studies are carried out on combinations of these drugs for further development of a combination regimen for TB treatment.

Some challenges were nonetheless encountered during this study. Large-scale metabolite generation was not viable due to production of few biotransformation products and in low amounts, however, chemical synthesis was achieved successfully. As mentioned earlier, full elucidation of the MoA of CPZ and its metabolites was also not achieved since *Mtb* spontaneous mutant strains were not successfully raised. Nevertheless, the use of various methods discussed in Chapter 4 helped to establish that the MoA of CPZ metabolites is most likely similar to that of the parent. In conclusion, some studies were not exhaustively carried out due to time constraints but the findings presented here form a good basis for further work that could lead to development of an anti-TB combination regimen derived from CPZ or its metabolites.

CHAPTER 7

EXPERIMENTAL

7.1 Chemicals and reagents

All chemicals and solvents were purchased from Sigma Aldrich (SA) or Merck (SA). 7-Hydroxychlorpromazine (7-hydroxyCPZ) **(M2)** was purchased from Sigma Aldrich (SA). 25-*O*-Desacetylrifampicin (25-*O*-DesacetylRIF) was purchased from American Custom Chemicals Corporation (San Diego, USA). Human and rat liver microsomes (HLM & RLM) were purchased from XenoTech, LLC (Lenexa) through AiBST (Zimbabwe). The recombinant cytochrome P450 (CYP450s) were generously donated by Prof. Guengerich (Vanderbilt University, USA), the bioengineered CYP21B3 by Prof. Frances Arnold (Caltech, USA) and the actinomycetes by Dr. Marilize Le Roes-Hill (Cape Peninisula University of Technology, SA). *Mycobacterium tuberculosis* (*Mtb*) H37Rv wild-type (H37RvMa) was donated by Dr. C. Sassetti's laboratory, University of Massachusetts while the cytochrome *bd* oxidase knockout mutant strains Δ*cydKO*, Δ*cydA* and Δ*cydAB* were prepared from H37RvMa strain, in our Molecular Mycobacteriology Research Unit (MMRU) laboratory. The details of the knockout mutant strains are provided in Table 7.1.

Table 7.1: Cytochrome *bd* oxidase mutant strains

Strain	Details	Reference
Δ***cydKO***	Deletion of the 3' end of *cydB*, deletion of the entire *cydD* and deletion of the 5' end of *cydC*.	Arora *et al.*[1]
Δ***cydA***	Deletion of 1210bp of the 1458bp *cydA* gene (in-frame deletion).	Moosa, Mizrahi & Warner (Unpublished)
Δ***cydAB***	Deletion of 2298bp of the 2499bp *cydAB* genes (in-frame deletion).	

7.2 *In vitro* metabolite generation

7.2.1 Human liver microsomes (HLM) and rat liver microsomes (RLM)

Incubations were performed using a modified protocol based on,[2] with human (pooled human mixed gender, Xenotech) and rat (male rat IGS, Xenotech) liver microsomes at a small scale to establish chlorpromazine (CPZ) metabolism and to identify the metabolites. 10μM CPZ was incubated in 0.5mg/ml of microsomes and 100mM phosphate buffer (pH 7.4) with 5mM $MgCl_2$. 1mM NADPH was added and incubation was carried out at 37°C with shaking for 1h. An equal volume of ice-cold acetonitrile was added to stop the reactions and to precipitate the proteins. After centrifuging the mixtures at 14000 x *g* for 30min, the supernatant was transferred to high performance liquid chromatography (HPLC) vials. Control samples with no NADPH, no microsomes and a T0 (time 0) sample were also included and processed in a similar way to the samples.

7.2.2 Recombinant cytochrome P450 (CYP450) whole cells

Whole cell incubations with CYP450s expressed in *Escherichia coli*: 2D6, 3A4, 2C9, 1A2 were carried out as described.[2,3] A colony of the bacteria containing the respective plasmid for expression of the enzymes was inoculated in autoclaved Luria-Bertani (LB) media supplemented with 100mg/ml ampicillin and incubated at 37°C with shaking for 16h. The culture was used to inoculate Terrific Broth media that contained 100mg/ml ampicillin, trace elements (2.5ml/L), 1mM thiamine and 0.5mM $FeCl_3$. Incubation was carried out at 30°C with shaking for 7-8h. To induce the cells to start expressing the enzymes, 0.5mM *δ*-aminolevulinic acid and 1mM isopropyl *β*-D-thiogalactopyranoside (IPTG) was added and further incubation was carried out for 48h. The cells were washed with 1 x phosphate buffer saline (PBS) several times with centrifuging then finally the pellet was used for the incubation of CPZ. As in the case of the microsomes, the same concentration of CPZ was incubated in 0.6g/L of the cells in 100mM phosphate buffer with glucose (10mg/ml) but the cofactor was not added. The advantage of using whole cells is that they do not require an

electron transport protein, nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) which acts as a redox partner to the CYP450 enzyme.

7.2.3 Bioengineered cytochrome P450 (CYP450) 21B3

Bioengineered CYP21B3 (in *E. coli*) was also used to metabolize CPZ as described.[4] A 5ml culture from a freezer stock was prepared and allowed to incubate at 37°C for 16h (overnight) with shaking. The culture was then added to 50ml LB media. Incubation was carried out for 16h to allow growth of *E. coli* cells to an OD_{600} 2. Terrific media (2L) was prepared as described in section 7.2.2 and the pH was adjusted to 7.4, then autoclaved. The 50ml culture was added to the 2L terrific media and the growth of the culture was performed to an OD_{600} 0.35 in the bioreactor. 0.5mM *δ*-aminolevulinic acid was then added. At OD_{600} 0.56 the culture was induced with 1mM IPTG and growth continued for another 18h. The cells were washed in 1 x PBS and the pellet was then re-suspended in lysis buffer (5ml lysis buffer for 500ml culture) then 1mg/ml lysozyme, 2.5mM $MgCl_2$, 500U DNase1 and 0.5mM $CaCl_2$ were added. This mixture was incubated on ice for 30min with gentle shaking after which 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate (CHAPS) and 0.1% Triton X-100 were added. Incubation again was performed on ice for 30min with gentle shaking and the lysate was spinned at 17700 x *g* for 30min at 4°C to remove cell debris.

10mM of CPZ (1.25µl) was added to 1.6µM of cells (25µl) in 0.1mM phosphate buffer (pH = 8.25, 219µl) and 125mM hydrogen peroxide (H_2O_2, 5µl). The mixture was allowed to incubate at 37°C with shaking for 3h with addition of 5µl H_2O_2 after every 30min. The reaction was stopped with an equal amount of acetonitrile. The samples were spun at 14000 x *g* for 15min at 4°C and the supernatant was filtered into HPLC vials. Two controls were included: a negative control that consisted of cells with no 21B3 enzyme (DH5α) and a positive control (drug but no cells).

7.2.4 Actinomycetes

Incubations with actinomycetes were carried out based on a modified protocol as described.[5] Four strains of actinomycetes were used, *Streptomyces griseus griseus* (SGG), *Streptomyces coelicolor* (SC), *Gordonia terrae* (GT) and *Nocardia gamkensis* (NG). Freezer stocks of the actinomycetes were used to inoculate a 500ml flask containing 100ml malt extract medium (2g dextrose, 1g malt extract, 1g yeast extract, 0.1g peptone, pH = 7 adjusted using 1N NaOH /1N HCL). Incubation was carried out for 48h at 37°C with shaking at 250 x *g*. 200µl of the culture was transferred into 96 deep well plates and 0.5µl of 100mM CPZ was added. Two controls were included for each strain, cells with no drug and drug in media with no cells. The plates were then incubated at 30°C, 200 x *g* for 48h and the reactions were quenched using equal amounts of frozen acetonitrile. Centrifugation was carried out (section 7.2.1) and the supernatant was collected, filtered and analyzed on liquid chromatography - mass spectrometry (LC/MS).

7.3 Anti-mycobacterial screening

7.3.1 Broth microdilution assay/microplate alamar blue assay (MABA)

Minimum inhibitory concentration (MIC_{99}) for the various compounds tested, was determined as described.[6] Briefly, a pre-culture of *Mycobacterium smegmatis* (*Msm*) (mc^2155) was prepared from a glycerol stock and grown for 16h followed by a sub-culture grown to an OD_{600} 0.6-0.8 (logarithmic phase) in filter-sterilized 7H9 media supplemented with 10%OADC, 0.2% glycerol and 0.25% Tween 80 (20% in H_2O) at 37°C with no shaking. The media was added to 96 microtitre well plates followed by addition of the drugs which were then serially diluted. Finally the culture (diluted x 1000) was added to the wells. The controls included media only and the solvent used to dissolve the drugs at a concentration corresponding to that of the working solutions of the drugs. Incubation was carried out at 37°C with no shaking for 48h then resazurin dye was added to the plates. Further incubation was carried out for 24h in the same conditions.

In the same manner, *Mtb* (H37RvMa, or cytochrome *bd* oxidase knock-out mutant strains) glycerol stocks were used to prepare pre-cultures that were grown in 7H9 media, supplemented with 10%ADC, 0.2% glycerol and 0.25% Tween 80 (20% in H_2O) (H37RvMa wild-type strain) or GAST/Fe (pH 6.6 glycerol-alanine-salts, with 0.05g of ferric ammonium citrate) minimum media (cytochrome *bd* oxidase mutant strains), for 4 days (OD_{600} 0.8). The sub-culture was grown to log phase (OD_{600} 0.6-0.8). The sub-cultures of H37RvMa wild-type or cytochrome *bd* oxidase mutant strains were diluted x 1000 and x 100 respectively, then added to the wells. The plates were incubated for 14 days. MIC_{99} of the various drugs was then recorded. Drug susceptibility tests with spectinomycin (SPEC) *Mtb* mutants were carried out as described here, in 7H9 ADC media. All assays were repeated three times in duplicate.

7.3.2 Synergistic drug combinations for TB therapy

A two dimensional array of serial dilutions of two test compounds was prepared in 96 well plates and a mycobacterium culture prepared as described in section 7.3.1 was added to the wells. Incubations were carried out under the same conditions as described (section 7.3.1). Relative MIC_{99} concentrations in wells representing various ratios of the two compounds was then used for calculations to determine whether paired combinations exert inhibitory effects that are more than the sum of their effects alone (synergy). Cell viability was then determined and the fractional inhibitory concentration index (FICI) values which correspond to either compound (A or B) were defined as:[7–9]

MIC_{99} of A (combination)/MIC_{99} of A (singly) + MIC_{99} of B (combination)/MIC_{99} of B (singly)

The assays were repeated three times in duplicate.

7.3.3 Determination of bactericidal *versus* bacteriostatic activities

A pre-culture of *Mtb* (H37RvMa) was prepared from a glycerol stock followed by a sub-culture grown to an OD_{600} 0.2 (lag phase) in filter-sterilized 7H9 ADC media at 37°C with no shaking. CPZ and its metabolites were then added to a 96-well plate followed by a 2-fold

serial dilution. Combinations of CPZ or its metabolites with antituberculosis (anti-TB) drugs were prepared on the best synergy achieved against *Mtb*. The culture was diluted (x 1000) and was added to the 96-well plates containing the drugs. Part of the culture was transferred to 7H10 solid media supplemented with 10% OADC and 0.5% glycerol (agar media), for colony forming units per milliliter (CFU/ml) counting (0 time control). The plates were then incubated for 14 days at 37°C. On the 14th day, 100µl of the MIC_{99} wells, the subsequent wells (control) and wells with no drugs (control) were plated in required dilutions on standard 7H10 solid media plates followed by further incubation at 37°C for 4 weeks. CFU/ml counts were then calculated for determination of bactericidal or bacteriostatic effect. All assays were repeated twice in duplicate.

7.3.4 CPZ or its metabolites spontaneous resistant mutant strain generation

A pre-culture of *Mtb* (H37RvMa) was prepared from a glycerol stock followed by a sub-culture grown to an OD_{600} 0.6-0.8 (logarithmic phase) in filter-sterilized 7H9 ADC media at 37°C with no shaking. The culture was centrifuged for 10min at 18°C and 3500 x *g* and the pellet was re-suspended in 1ml 7H9 OADC media. 100µl of the 1ml culture was serially diluted and plated onto standard 7H10 solid media plates with no drug. Similar volumes were plated onto standard 7H10 solid media plates containing 5 x, 10 x, 15 x or 20 x MIC_{99} concentrations of CPZ, 7-hydroxyCPZ **(M2)** and nor-CPZ **(M5)**. The plates were prepared in duplicates and incubation at 37°C for 4 weeks.

7.3.5 qRT-PCR analyses of *cydA* expression

7.3.5.1 Extraction of RNA from treated *Mtb* culture

A preculture of *Mtb* (H37RvMa) was prepared in 7H9 ADC media followed by sub-cultures (25ml) that were grown to logarithmic phase (OD_{600} 0.6) at 37°C. The cultures were then treated with 140µM CPZ or the metabolites.[10] RNA was extracted after 6h of incubation at same conditions, with FastRNA Pro^{TM}blue kit (MP Biomedicals) as per the manufacturer's instructions. Briefly, the cultures were pelleted at 3500 x *g* for 12min at 18°C and the

supernatant discarded. The pellets were re-suspended in 1ml of lysing matrix B and transferred into blue-cap tubes. The samples were processed in the FastPrep® homogenizer instrument (IEPSA Medical Diagnostics) for 40sec at a setting of 6.0 followed by centrifugation at 12000 x *g* for 5min at 4°C. The supernatant was carefully transferred into clean 1ml tubes and the RNA was extracted using chloroform ($CHCl_3$) then centrifuged at 12 000 x *g* for 5min at 4°C. RNA in the upper phase was then transferred into clean 1ml tubes and cold 500µl absolute ethanol was added. The mixture was left to stand for 30mins in -20°C. Centrifugation followed for 15min at 12000 x *g* and the pellet formed was washed again in 70% ethanol. The RNA was dried then re-suspended in 50µl DEPC-treated RNase-free water and stored at -80°C (Appendix 8A).

7.3.5.2 DNase treatment

DNase treatment was carried out on each 3µg RNA sample with 2U of TURBO™ DNase (Ambion®) according to the manufacturer's instructions for 30min initially, followed by addition of 2U of DNase and incubated for further 30min. The RNA was subsequently cleaned up using RNeasy MiniKit (Qiagen). The quantity of RNA was determined using a Nanodrop 2000c Spectrophotometer (Thermo Scientific). All samples were tested for residual DNA by polymerase chain reaction (PCR) using a FastStart Taq DNA polymerase kit (Roche Diagnostics GmbH) and confirmed to be free of DNA when no amplified product could be detected after 34 cycles. The PCR products were visualized on 1% agarose gel prepared in Tris/Acetate/EDTA (TAE) buffer (Appendix 8B).

7.3.5.3 Design of *cydA* gene primers

The *cydA* gene sequence was obtained from TB Genomes Database (TB DB) and used to design the probe and the primers using Primer Express 3.0.1 (Applied Biosystems software). The designed primers/probes for *cydA* and *sigA* (Table 7.2) were obtained from Applied Biosystems Oligonucleiotide® Custom Primers & Probes, Life Technologies.

Table 7.2: *cydA* and *sigA* primers and probes for qRT-PCR

Primers/probes for *cydA* & *sigA*	**Sequence**
***cydA*RTF (forward)**	5'-CGATCTGCAGCAGGAATACCA-3'
***cydA*RTR (reverse)**	5'-AAG AGGTTGGGCCGGTAGTC-3'
***cydA* probe**	5'-CAGCGCTTCGGACCA-3'
***sigA*RTF (forward)**	5'-CGAGCCGATCTCGTTGGA-3'
***sigA*RTR (reverse)**	5'-TTCGATGAAATCGCCAAGCT-3'
***sigA* probe**	5'-ACGAGGGCGACAGC-3'

Conditions for the PCR reaction were set according to the instructions provided in the DNA polymerase kit but with slight modifications, on a C1000^{TM} Thermocyler (Bio-Rad) (Table 7.3).

Table 7.3: PCR reaction conditions

Reaction step	**Temperature (°C)**	**Time**
Initialization	95	3min
Denaturation	95	30s
Annealing	60	30s x 34 cycles
Amplification/Elongation	72	30 s
	72	5min

7.3.5.4 Synthesis of cDNA

100ng of the DNase treated RNA was used to synthesize cDNA for each sample using a MMLV High Performance Reverse Transcriptase kit (Epicentre) as per the manufacturer's protocol. Random hexamer primers were used in this case. A negative control (no reverse transcriptase enzyme) was included for each RNA sample and the conditions for this reaction on a C1000^{TM} Thermocyler (BioRad) were as shown on Table 7.4.

Table 7.4: cDNA synthesis reaction conditions

Reaction step	**Temperature (°C)**	**Time**
Annealing (RNA + the hexamer random primer)	65	2min (then chill on ice for at least 1 min)
Incubation (annealed RNA primer + master mix)	25	10min
First strand reaction	37	1h
	85	5min

7.3.5.5 Real-time PCR (RT-PCR)

cDNA obtained for each sample including the negative controls were used in RT-PCR on a PikoReal 96 Real Time PCR System (Thermo Scientific), to amplify a portion of *cydA* gene in a reaction that was carried out using Taqman Universal MMix II with UNG (Life Technologies) according to the manufacturer's instructions and normalized to levels of *sigA* (Rv2703), a housekeeping gene whose expression is reported to remain constant in various growth phases.[11] The conditions that were used for this reaction are shown on Table 7.5. The average expression levels, normalized to *sigA*, and standard deviations were calculated using data from three technical replicates of two independent experiments.

Table 7.5: RT-PCR reaction conditions

Reaction step	Temperature (^{o}C)	Time
UNG incubation	50	2 min
AmpliTaq gold enzyme activation	95	10 min
PCR	95	15s } x 40 cycles
	60	1min }

7.3.6 Spectinomycin (SPEC) spontaneous mutant generation

An *Mtb* (H37RvMa) culture was prepared as described in section 7.3.4 and plated onto 7H10 solid media prepared with 5 x MIC_{99} concentration of SPEC. After 4 weeks' incubation, the number of mutant colonies was recorded and the frequency of mutation was determined in relation to the colonies obtained in plates with no drug. Colonies were randomly selected and grown in 7H9 ADC media and the MIC_{99} of SPEC and other drugs were determined again using cultures prepared from the mutant colonies.

7.3.6.1 Isolation of genomic DNA from mycobacteria

For isolation of genomic DNA from H37RvMa and SPEC-resistant ($SPEC^{R}$) mutant *Mtb* strains, seed culture was prepared by growing strains in 7H9 OADC media at 37°C with shaking to OD_{600} 0.8. The cells were sub-cultured by diluting 50-fold in fresh 7H9 ADC media

and grown to OD_{600} 0.8. 10ml of culture was harvested and cells were heat killed at 85°C for 2h followed by centrifugation at 3000 x *g* for 10min. The pellets were washed twice with 10ml ice cold PBS and finally re-suspended in 1ml Tris/EDTA (TE) buffer supplemented with 400µl of 50mg/ml lysozyme stock and 10µl of 10mg/ml RNAase. The mixture was then incubated overnight at 37°C. After incubation, 1% SDS and 100µg/ml proteinase K were added to the suspension and incubated at 65°C for 2h. The suspension was centrifuged at 3000 x *g* for 30min to pellet cell debris. The pellet was discarded and clear supernatant was collected in a 15ml tube. An equal volume of buffered phenol was added to the supernatant, mixed gently and the two phases were separated by centrifugation at 12000 x *g* for 10min. The upper aqueous layer was removed carefully and extracted twice with $CHCl_3$:isoamyl alcohol ($C_5H_{12}O$) solution (24:1). DNA in the aqueous phase was precipitated by the addition of 0.1 volume of 3M sodium acetate and equal volume of isopropanol and pelleted by centrifugation at 12000 x *g* for 15min at 4°C. The pellet was washed twice with 70% ethanol, air dried and suspended in 50µl TE buffer (pH 8.0).

7.3.6.2 DNA amplification by PCR

PCR reactions were set in 25µl volume using 50ng of template DNA, 0.5µM of each primer, 200µM of each dNTPs, 1unit of Phusion Taq DNA polymerase in PCR buffer with 1.5-2.0mM $MgCl_2$.

7.3.6.3 Purification of PCR amplified product

The DNA fragment was excised from the gel and extracted using gel extraction kit (Qiagen). Briefly, the excised gel was weighed and 3 volumes of QG buffer was added to one volume of gel, mixed properly and incubated at 50°C for 10min with slight shaking. After the dissolution of gel, one volume of isopropanol was added and mixed properly. The sample was transferred in QIAquick spin column and centrifuged. The flow-through was removed and 500µl of QG buffer was added in column. Centrifugation of the column followed at 12000 x *g* for 1min. The column was washed with 750µl of buffer PE followed by

centrifugation at 10000 x *g* for 1min. The flow-through was discarded and column was extra-centrifuged for 1min for complete removal of ethanol. The column was placed in a fresh tube and 55µl of DEPC-treated RNase-free water was added followed by incubation for 5min. The column was then centrifuged for 1min and eluted DNA was collected in fresh tube.

7.3.6.4 Nucleotide sequencing of cloned insert

The sequencing reaction of 20µl final volume contained: PCR product (100ng), 5 pmol primer (Table 7.6), Big Dye Terminator reaction mix 8µl, milli-Q water to final volume of 20µl.

Table 7.6: Primers for sequencing

16SrRNA	**Primer**
*rrs*_seqF1	5'-CCGAAGCGGGCGGAAACAA-3'
*rrs*_seqF2	5'-GCGAACGGGTGAGTAACAC-3'
*rrs*_seqF3	5'-AATTACTGGGCGTAAAGAG-3'
*rrs*_seqF4	5'-GTTAAGTCCCGCAACGAGC-3'
*rrs*_seqR1	5'-TTACTGCCCAGAGACCCGC-3'
*rrs*_seqR2	5'-TGATCTGCGATTACTAGCG-3'
*rrs*_seqR3	5'-ACCACTATCCAGTTCTCAA-3'
rpSE	**Primer**
rpsE seqF1	5'-GGCGTGCCGGGTGACAAAA-3'
rpsE seqF1	5'-GCTTTGGTCATCGTGGGCG-3'
rpsE seqR1	5'-CCACATTGATCGCGTTGTC-3'
rpsE seqR2	5'-CTATGTCTTCCCTCCGGTC-3'

The reaction mixture was mixed properly and subjected to the PCR cycle as indicated in Table 7.7.

Table 7.7: PCR reaction conditions

Reaction step	**Temperature (°C)**	**Time**
Initialization denturation	95	4 min
Denaturation	94	20s
Annealing	50	20s x 30 cycles
Amplification/Elongation	60	5s

The sequencing reaction product was then submitted to the sequencing facility at Stellenbosch University, South Africa, to run the capillary electrophoresis. The obtained results were analyzed using CLC Main Workbench 6.0.2.

7.3.6.5 Fitness cost of SPEC^R mutant strains

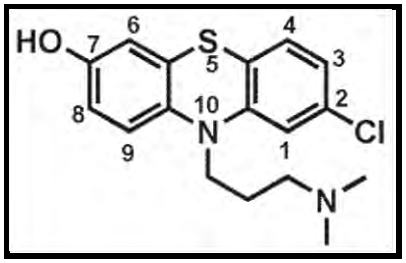
A preculture of H37RvMa and SPEC^R mutant *Mtb* strains were prepared in 7H9 ADC media from freezer stocks followed by sub-cultures (25ml, in triplicates) prepared with very low inoculums (OD_{600} 0.005). In two independent experiments, the growth kinetics of SPEC mutant strains were monitored in 7H9 ADC media at 37°C for 7 days by recording the OD_{600}.

7.4 Structural characterization of metabolites

An Agilent 1200 Rapid HPLC system coupled to an AB SCIEX 4000 QTRAP® mass spectrophotometer was used. A kinetex C_{18} HPLC column (150mm x 2.1mm, packed with 2.6µM fused core particles) was used for all analysis with a mobile phase flow rate of 0.4ml/min and a column temperature of 40°C. The mobile phase used in all experiments consisted of 5mM ammnonium formate buffer. Mobile phase A was mainly aqueous, consisting of 5% acetonitrile at pH 3 (pH adjusted using fomic acid), while mobile phase B consisted of 95% acetonitrile. Identification of the metabolites formed by all the systems was carried out using IDA-ESI experiments. A hybrid triple quadrupole-linear ion trap (QqQ_{LIT}) system (MS/MS) was used for comprehensive study of fragmentation mechanisms. All Nuclear Magnetic Resonance (NMR) experiments were performed on a 300MHz Bruker NMR spectrophotometer to confirm the structure of the metabolites.[12,13]

7.5 Scaled-up CPZ metabolites

7.5.1 7-HydroxyCPZ (M2)



$C_{17}H_{19}ClN_2OS$
Mol. Wt: 334.86

7-hydroxy CPZ **(M2)** was purchased from Sigma Aldrich. LC/MS analysis of **M2** generated MH^+ of the compound at m/z 335 and the retention time of this compound corresponded to **M2** in the HLM incubation sample of CPZ (Figure 7.1, Appendix 3A).

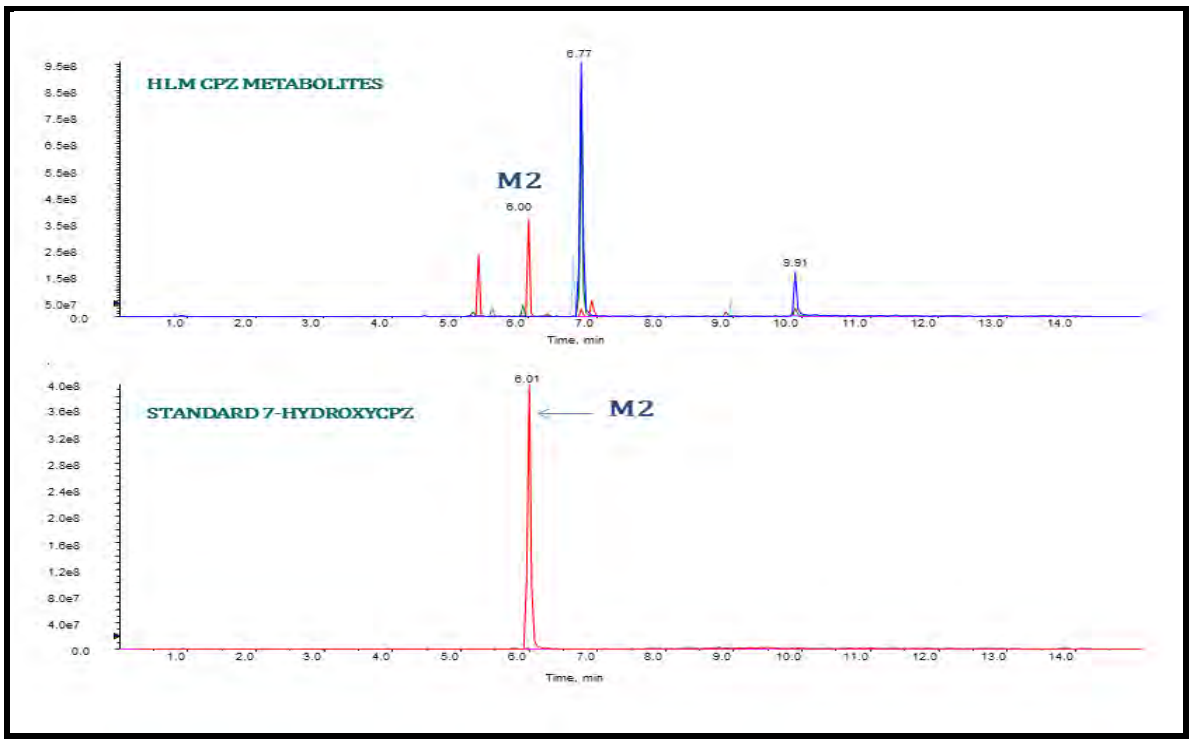
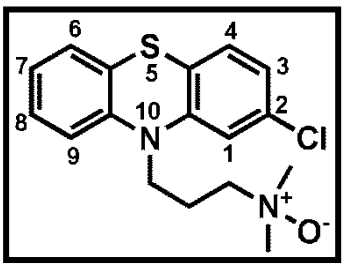


Figure 7.1 XIC chromatogram of the purchased standard, 7-hydroxyCPZ **(M2)** compared to the metabolite formed in HLM incubation sample of CPZ

7.5.2 CPZ-*N*-oxide (M3)



$C_{17}H_{19}ClN_2OS$
Mol. Wt: 334.86

m-CPBA (1.5g, 0.0084 mol) was added portion wise at 0°C to a solution of CPZ (2g, 0.0056 mol) in CH_2Cl_2 (50ml) and 4N NaOH solution (400µl) and the reaction mixture was stirred for 4h. The solvent was evaporated *in vacuo* before setting the pH of reaction at 10 using 4N NaOH solution and extracted with CH_2Cl_2 (3 x 15ml). The organic extracts were dried over $MgSO_4$ and concentrated under reduced pressure. Purification through column chromatography afforded target compound as oil (0.78g, 40%); R_f 0.35 (15% MeOH-CH_2Cl_2); 1H NMR (400MHz, $CDCl_3$) δ 7.17 (m, 2H, Ar*H*), 7.04 (d, *J* = 8.20 Hz, 1H, Ar*H*), 6.92 (m, 4H, Ar*H*), 4.02 (t, *J* = 6.30 Hz, 2H, CH_2N), 3.34 [t, *J* = 7.52 Hz, 2H, CH_2N$(CH_3)_2$], 3.05 [s, 6H, N$(CH_3)_2$], 2.36 (m, 2H, $CH_2CH_2CH_2$) (Appendix 4A); ^{13}C NMR (100MHz, $CDCl_3$) δ 128.2, 127.8, 127.7, 123.5, 122.9, 116.3, 68.4, 58.8, 44.9 and 21.3; LC/MS analysis generated MH^+ of the compound at m/z 335 and the retention time of this compound corresponded to **M3** in the HLM incubation sample of CPZ hence **M3** was confirmed to be CPZ-*N*-oxide (Figure 7.2, Appendix 4B).

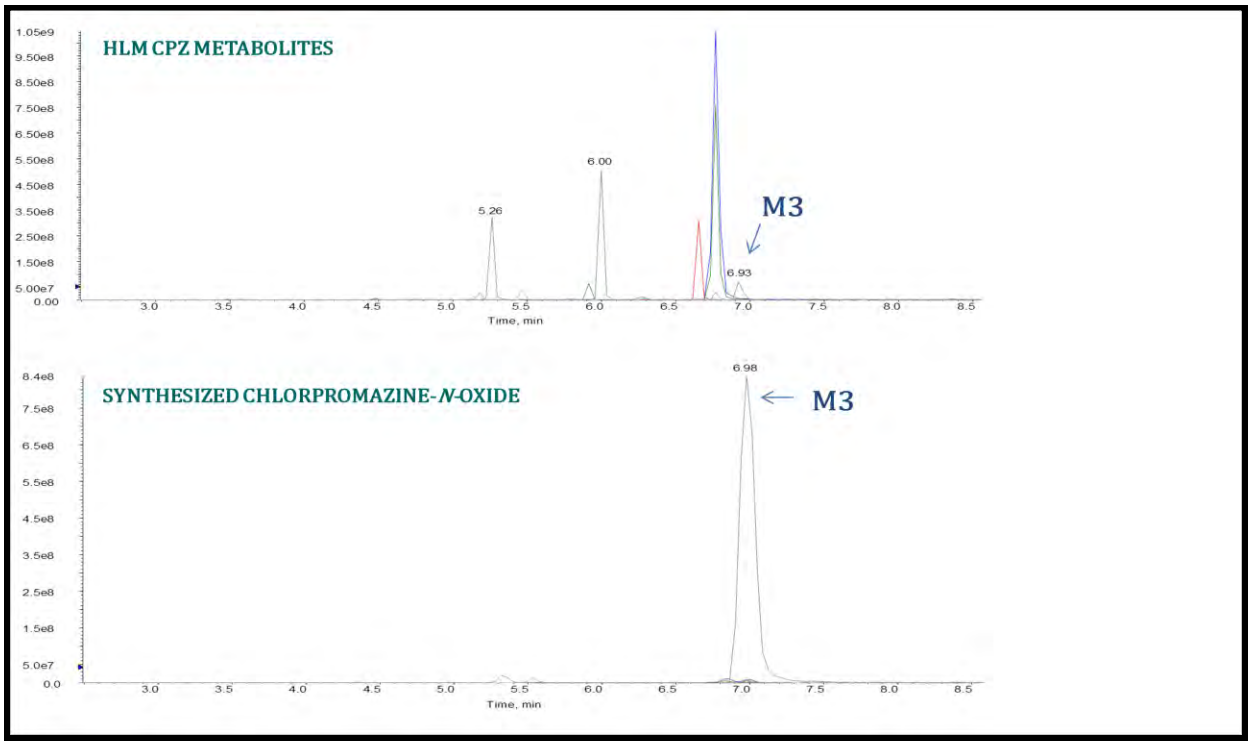
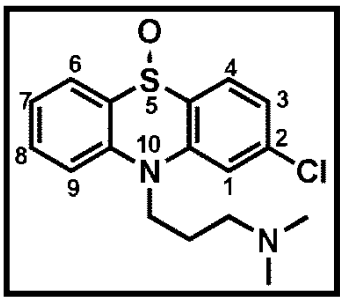


Figure 7.2 XIC chromatogram of CPZ-*N*-oxide **(M3)** synthesized compared to the metabolite formed in HLM incubation sample of CPZ

7.5.3 CPZ sulfoxide (M1)



$C_{17}H_{19}ClN_2OS$
Mol. Wt: 334.86

The target compound (0.2g, 10%) was obtained as side product in the reaction that led to the formation of CPZ-*N*-oxide **(M3)**. On thin layer chromatography (normal phase silica gel) and column chromatography, it eluted after the *N*-oxide **(M3)**. The structure was confirmed using NMR and LC/MS data (Appendices 2A-B). R_f 0.3 (15% MeOH-CH_2Cl_2); ^{1}H (400MHz, $CDCl_3$) δ 7.94 (m, 1H, ArH^6), 7.89 (d, J = 8.2 Hz, 1H, ArH^4), 7.63 (m, 1H, ArH^8), 7.54 (m, 2H, ArH^7), 7.30 (m, 1H, ArH^1), 7.24 (dd, J = 8.2 and 1.8 Hz, 1H, ArH^9), 4.42 (m, 2H, CH_2N), 2.75

[t, *J* = 7.2 Hz, 2H, C*H*$_2$N(CH$_3$)$_2$], 2.46 ([s, 6H, N(C*H*$_3$)$_2$], 2.25 (m, 2H, CH$_2$C*H*$_2$CH$_2$); ^{13}C (100MHz, CDCl$_3$) δ 133.1, 132.4, 131.2, 122.7, 122.3, 116.3, 55.5, 45.4, 44.2 and 23.9; LC/MS analysis generated MH$^+$ of the compound at m/z 335 but unlike the *N*-oxide **(M3)** the retention time of this compound (5.20) corresponded to **M1** in the HLM incubation sample of CPZ (Figure 7.3).

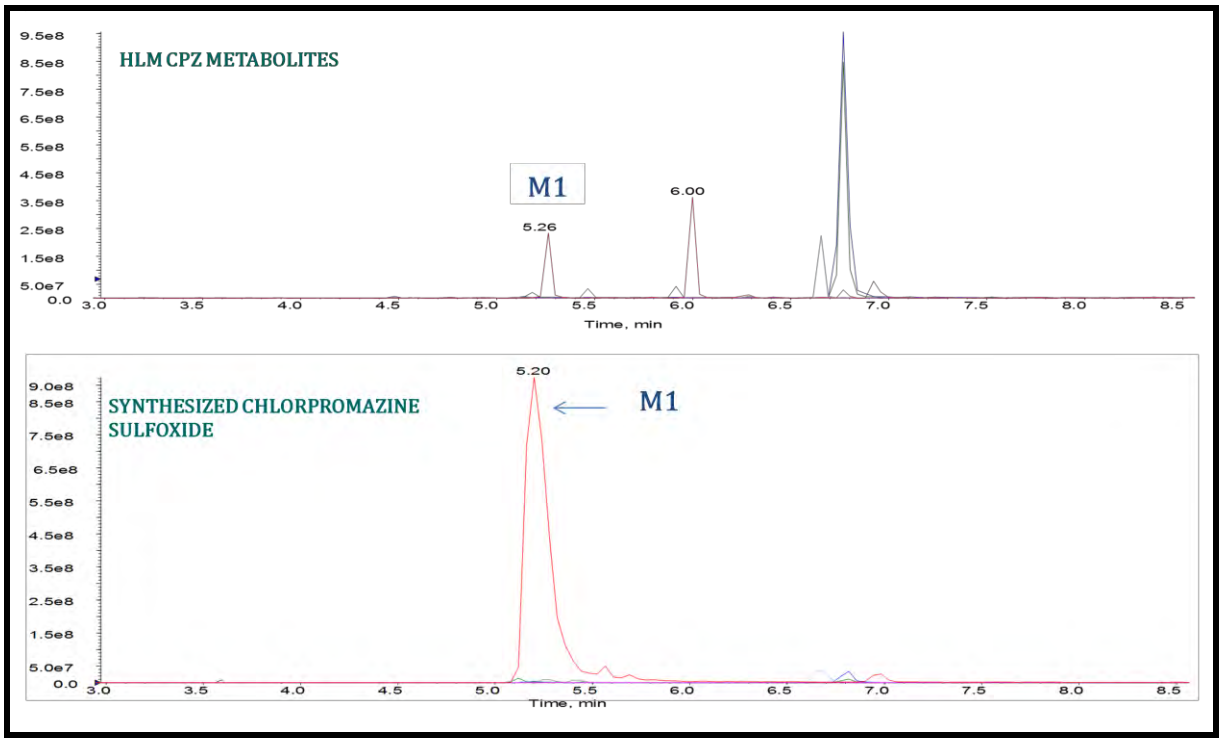
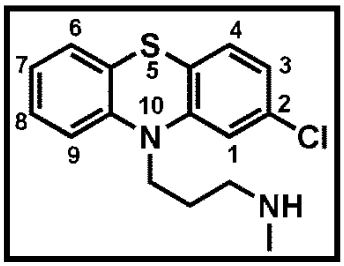


Figure 7.3 XIC chromatogram of sulfoxide **(M1)** synthesized compared to the metabolite formed in HLM incubation sample of CPZ

7.5.4 Nor-CPZ (M5)



$C_{16}H_{17}ClN_2S$
Mol. Wt: 304.84

Solution of CPZ-*N*-oxide **(M3)** (0.6g, 0.0018 mol) in MeOH (10ml) was cooled to 0°C followed by an addition of a solution of $FeSO_4$ (1g, 3.59 mol) in MeOH (1ml). The reaction was stirred at 0°C for 3h. The solvent was removed under reduced pressure to obtain reddish brown solid, which was dissolved in 0.1M EDTA solution (1.86g in 50ml H_2O) at pH 10 adjusted using NH_3 solution. Extraction was carried out using CH_2Cl_2 (3 x 15). Organic extracts were dried over anhydrous $MgSO_4$ and removed under reduced pressure. Purification through column chromatography afforded products as oil (65mg, 11%); R_f 0.3 (15% MeOH-CH_2Cl_2); ^{1}H (400MHz, $CDCl_3$) δ 7.2 (m, 2H, Ar*H*^{6,8}), 7.09 (m, 1H, Ar*H*^{4}), 6.96 (m, 4H, Ar*H*^{1,3,7,9}), 4.03 (t, *J* = 6.5 Hz, 2H, C*H*$_2$N), 2.95 (t, *J* = 7.1 Hz, 2H, C*H*$_2$NCH$_3$), 2.51 (s, 3H, NC*H*$_3$), 2.21 (m, 2H, $CH_2CH_2CH_2$) (Appendix 6A); ^{13}C (100MHz, $CDCl_3$) δ 128.2, 127.8, 127.7, 123.4, 122.8, 116.1, 47.9, 44.9, 34.1 and 24.6; LC/MS analysis of **M5** gave a m/z 305 and retention time 6.61 corresponding to the one in the HLM incubation of CPZ (Figure 7.4, Appendix 6B).

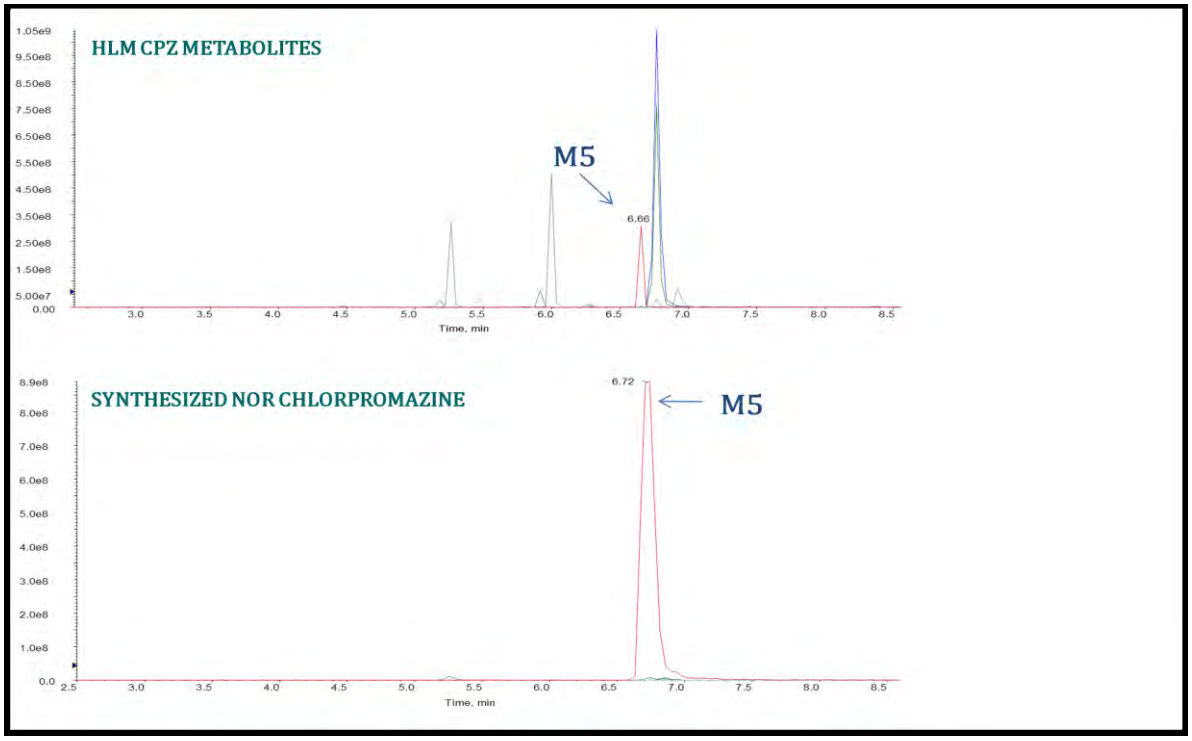
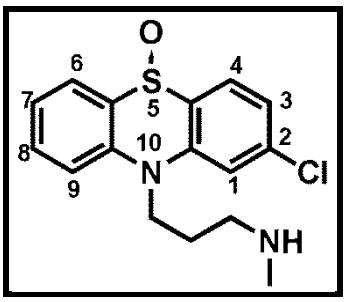


Figure 7.4 XIC chromatogram of nor-CPZ **(M5)** synthesized compared to the metabolite formed in HLM incubation sample of CPZ

7.5.5 Nor-CPZ sulfoxide (M6b)



$C_{16}H_{17}ClN_2OS$
Mol. Wt: 320.84

Nor-CPZ **(M5)** (0.2g, 0.0005 mol) was oxidized using *m*-CPBA (0.124g, 0.0007 mol) in a reaction that was carried out as described for CPZ-*N*-oxide **(M3)**. Purification on column chromatography yielded nor-CPZ sulfoxide **(M6b)** (46.7mg, 30%). 0.17 (80% MeOH-CH_2Cl_2); ^{1}H (400MHz, $CDCl_3$) δ 7.86 (m, 2H, Ar$H^{6,8}$), 7.57 (m, 1H, ArH^4), 7.25 (m, 4H, Ar$H^{1,3,7,9}$), 4.35 (m, 2H, CH_2N), 2.79 (t, *J* = 6.8 Hz, 2H, CH_2NCH$_3$), 2.48 (s, 3H, NCH_3), 2.11 (m, 2H, $CH_2CH_2CH_2$) (Appendix 7A); ^{13}C (100MHz, $CDCl_3$) 133.2, 132.2, 131.0, 122.8, 122.4, 116.6, 47.3, 45.2, 34.6 and 24.7; LC/MS analysis of **M6b** gave a m/z 320.9 at retention time 5.26 corresponding to the one in the HLM incubation of CPZ (Figure 7.5, Appendix 7A).

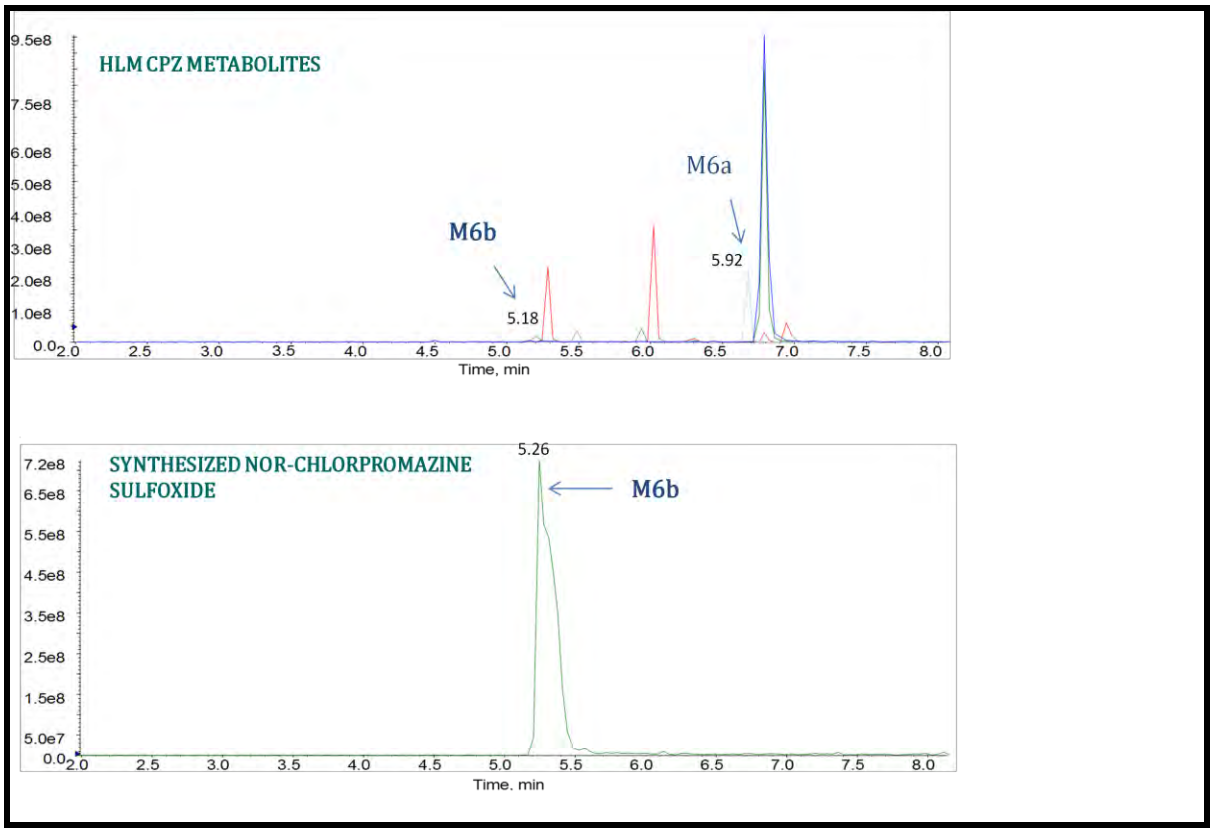
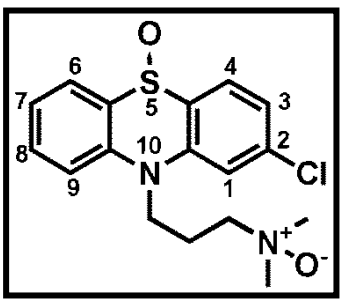


Figure 7.5 XIC chromatogram of nor-CPZ sulfoxide **(M6b)** synthesized compared to the metabolite formed in HLM incubation sample of CPZ

7.5.6 CPZ-*N*-*S*-dioxide (M4b)



$C_{17}H_{19}ClN_2O_2S$
Mol. Wt: 350.86

CPZ sulfoxide **(M1)** (0.4g, 1.1 mol) was oxidized using *m*-CPBA (0.3g, 1.7 mol). The reaction was carried out at 0°C in CH_2Cl_2 (50ml) and 4N NaOH solution (400 µl) for 4h with stirring. Extraction and purification was carried out as described for CPZ-*N*-oxide **(M3)** yielding CPZ-*N*-*S*-dioxide **(M4b)** as a white crystalline solid (0.241g, 65%); R_f 0.17 (20% MeOH-CH_2Cl_2); M. pt. 105-107°C; 1H NMR (400MHz, $CDCl_3$) δ 7.88 (m, 2H, Ar$H^{6,8}$), 7.60 (d, *J* = 8.20 Hz,

1H, ArH^{4}), 7.28 (m, 4H, Ar$H^{1,3,7,8}$), 4.56 (m, 2H, CH_2N), 3.30 [t, J = 7.6 Hz, 2H, CH_2N(CH_3)$_2$], 3.01 [s, 6H, N(CH_3)$_2$], 2.25 (m, 2H, $CH_2CH_2CH_2$) (Appendix 5A); ^{13}C(100MHz, $CDCl_3$) δ 133.1, 131.9, 130.7, 122.9, 122.6, 117.2, 67.0, 59.2, 44.3 and 21.8; LC/MS analysis of **M4b** gave a m/z 351.1 and retention time 5.98 corresponding to the one in the HLM incubation of CPZ (Figure 7.6, Appendix 5B).

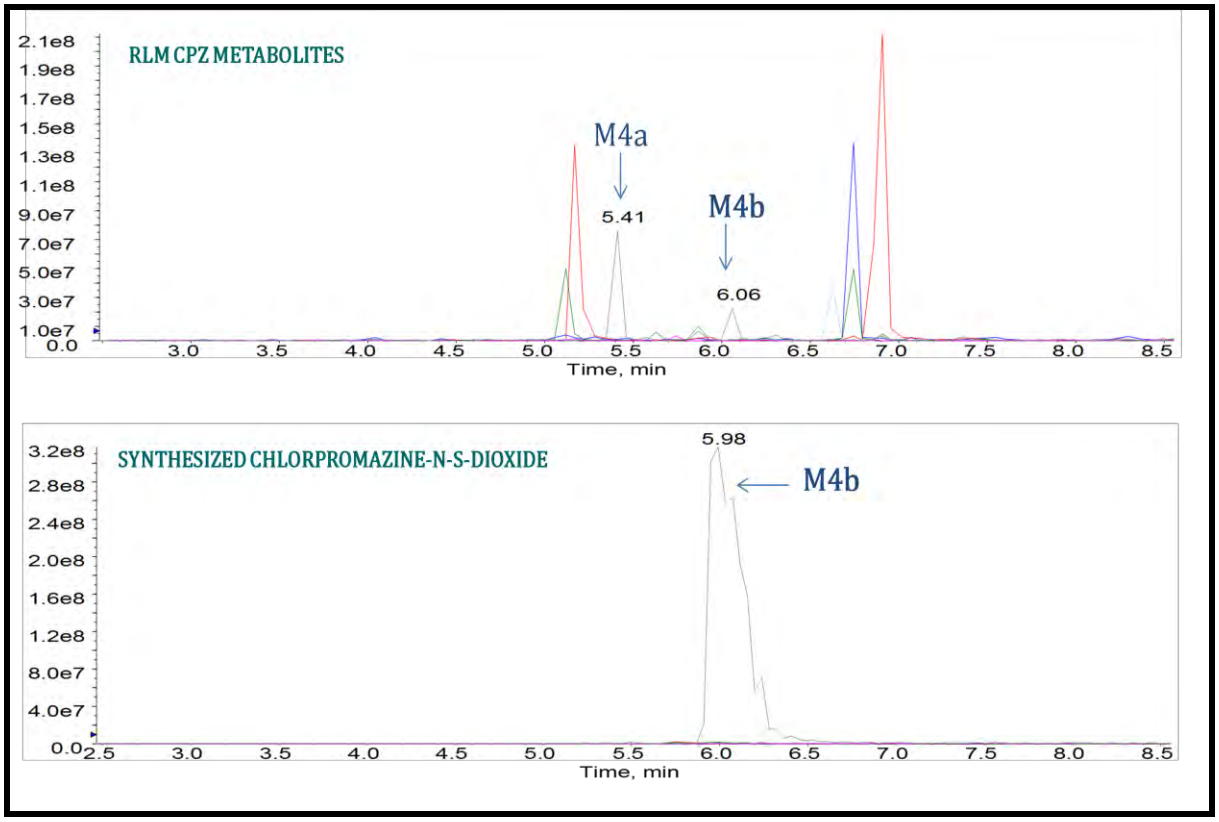


Figure 7.6 XIC chromatogram of CPZ-*N*-*S*-dioxide **(M4b)** synthesized compared to the metabolite formed in RLM incubation sample of CPZ

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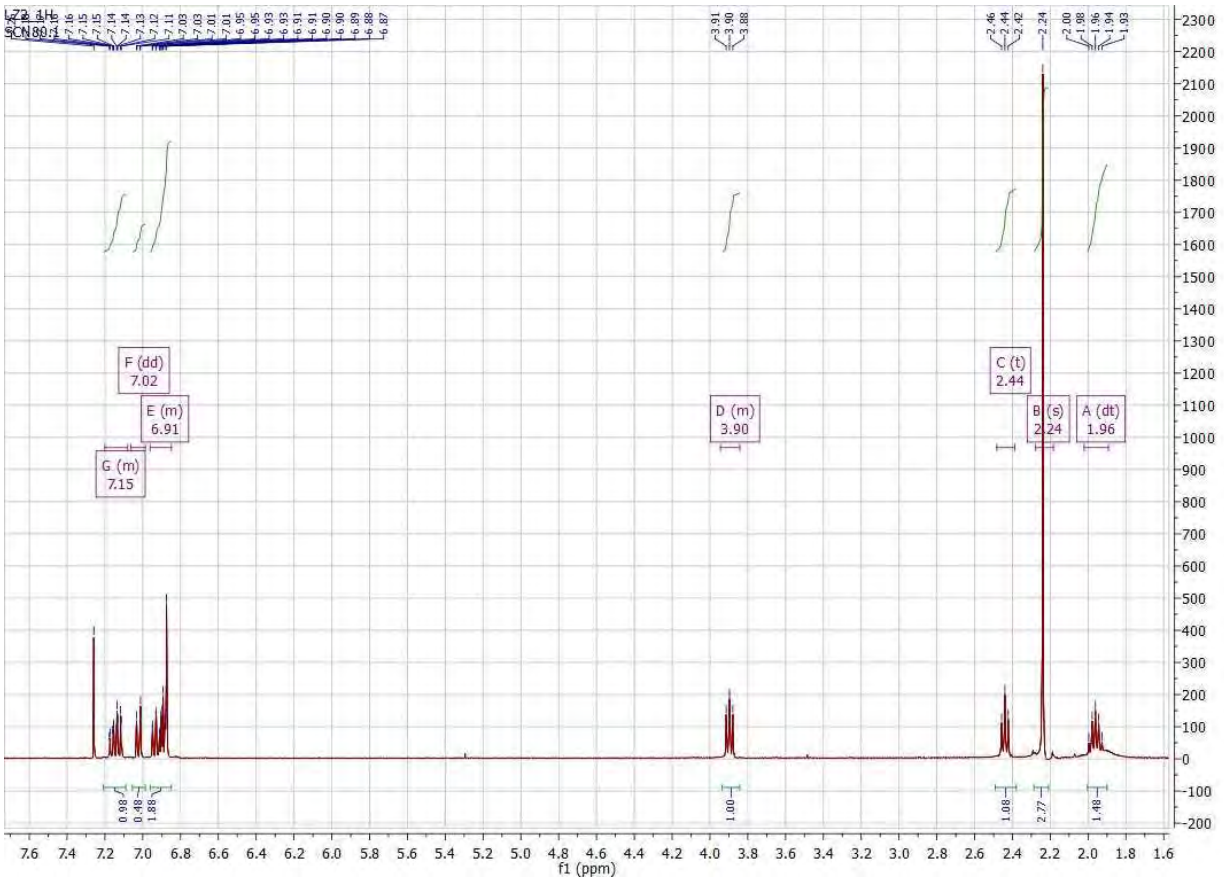
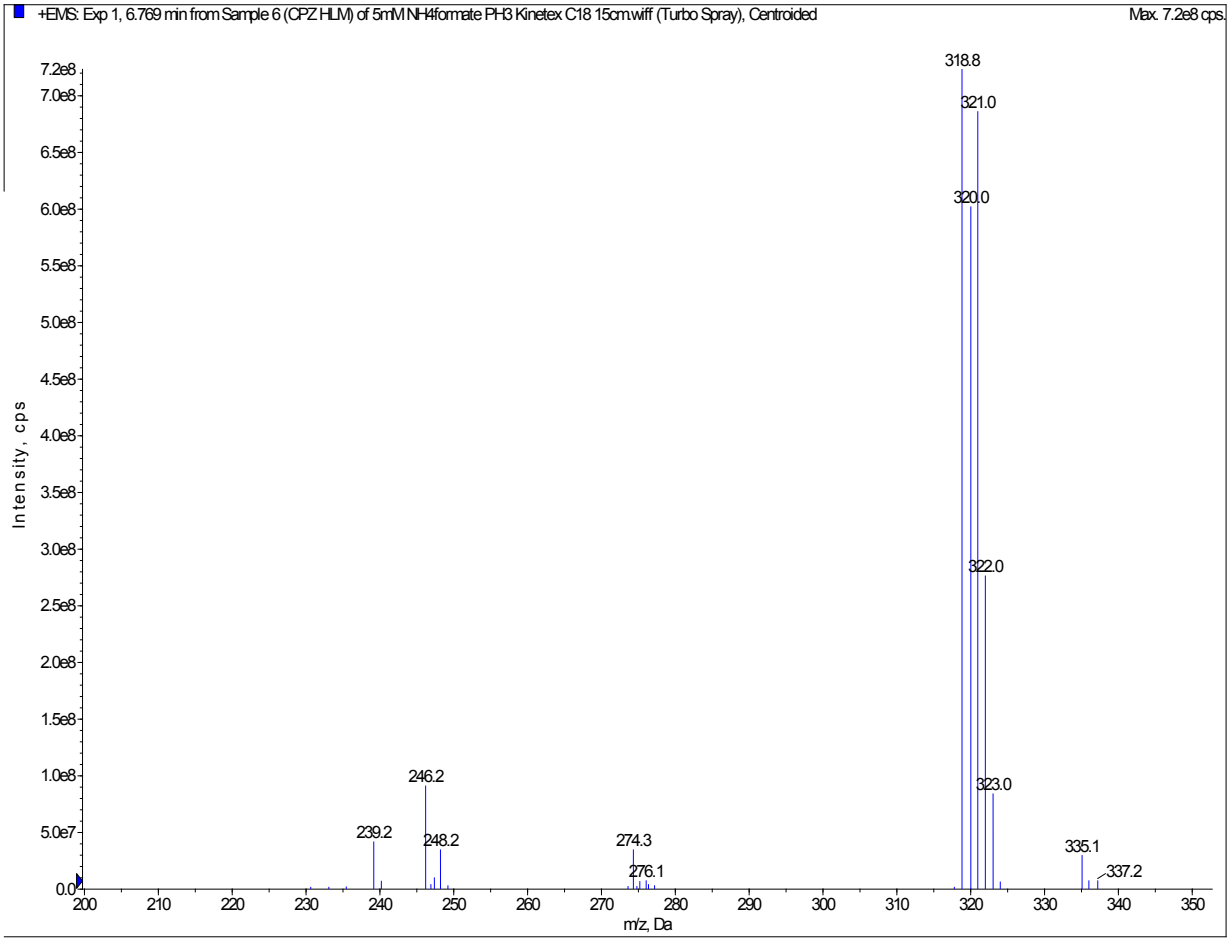
APPENDICES

Appendix 1A: ^{1}H NMR of chlorpromazine (CPZ)

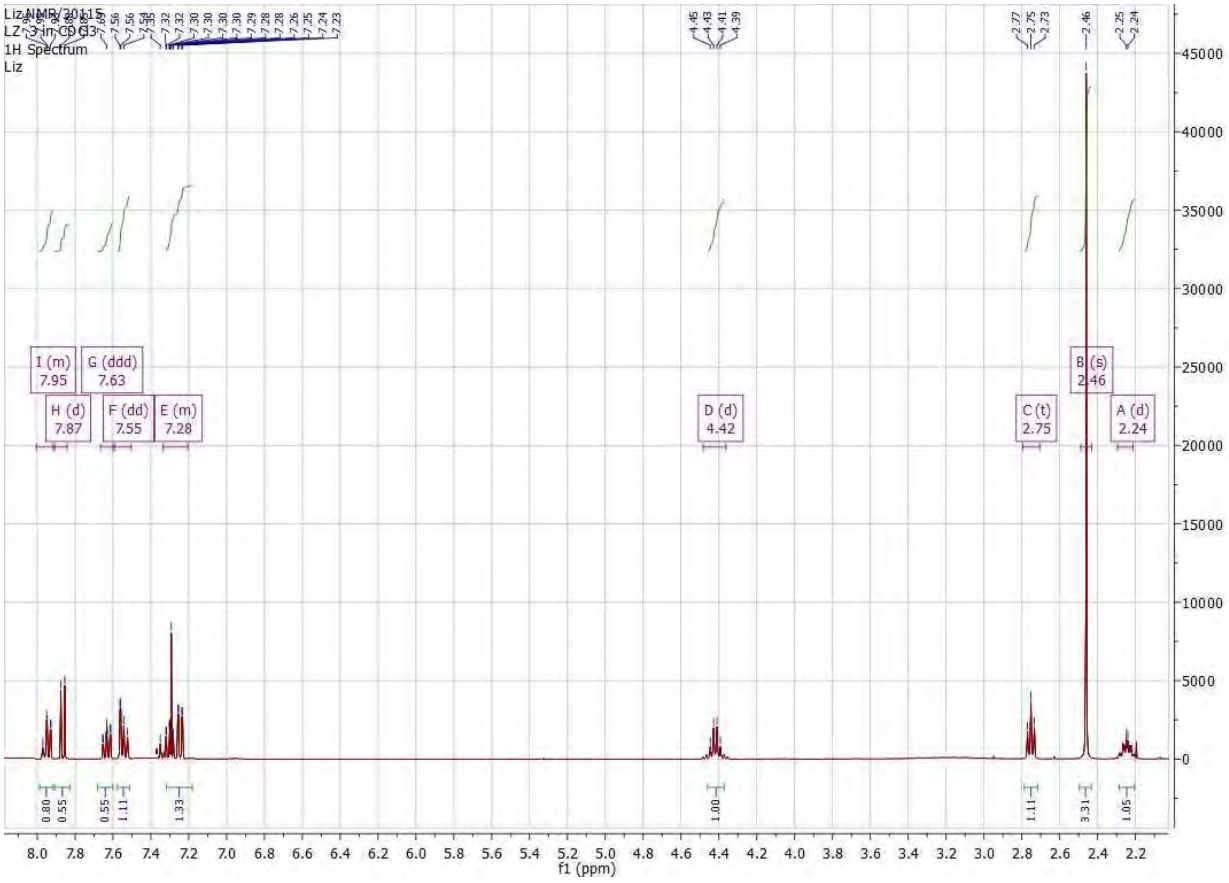


Appendix 1B: MS/MS spectrum of CPZ

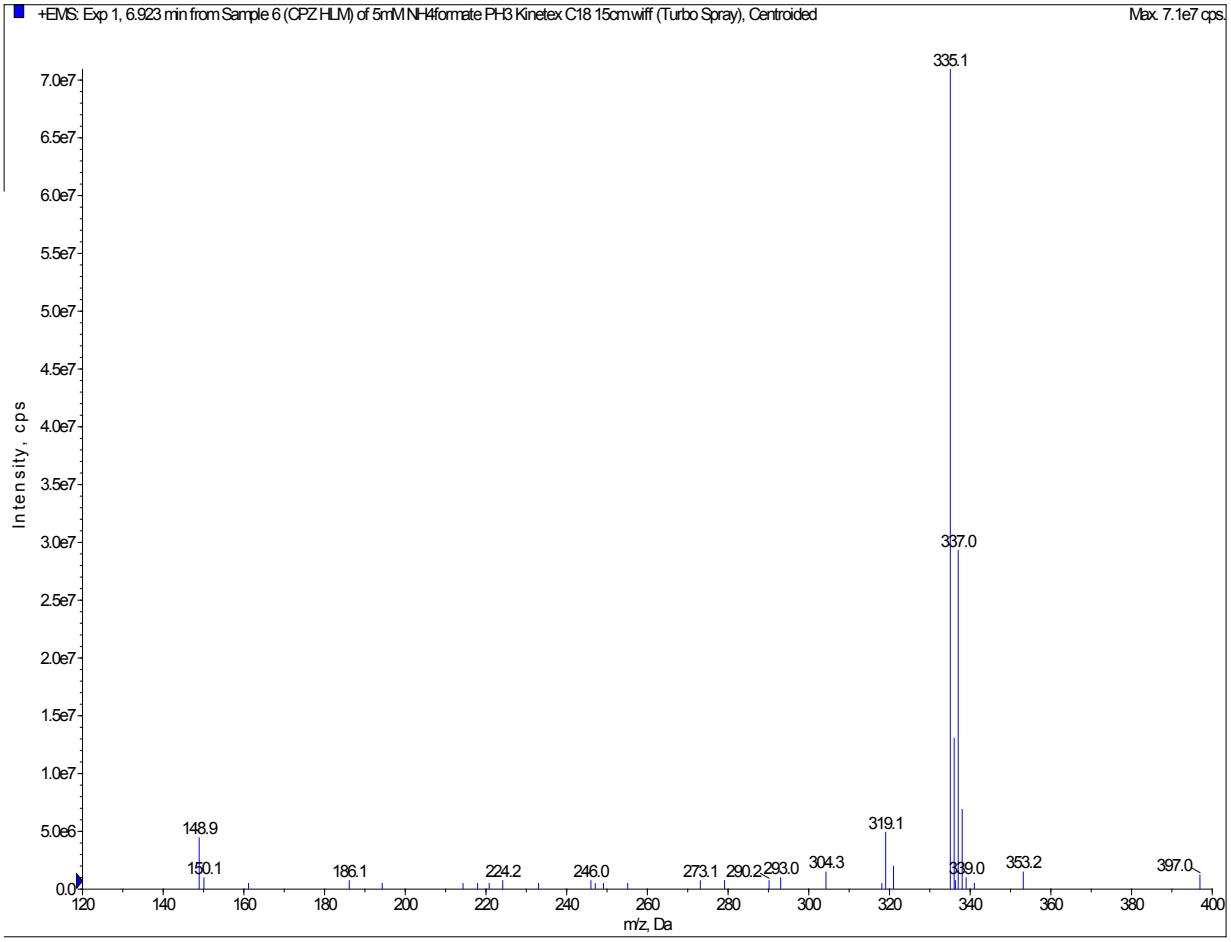


Parent ion: *m/z* - 318.8

Appendix 2A: ^{1}H NMR of CPZ sulfoxide (M1)

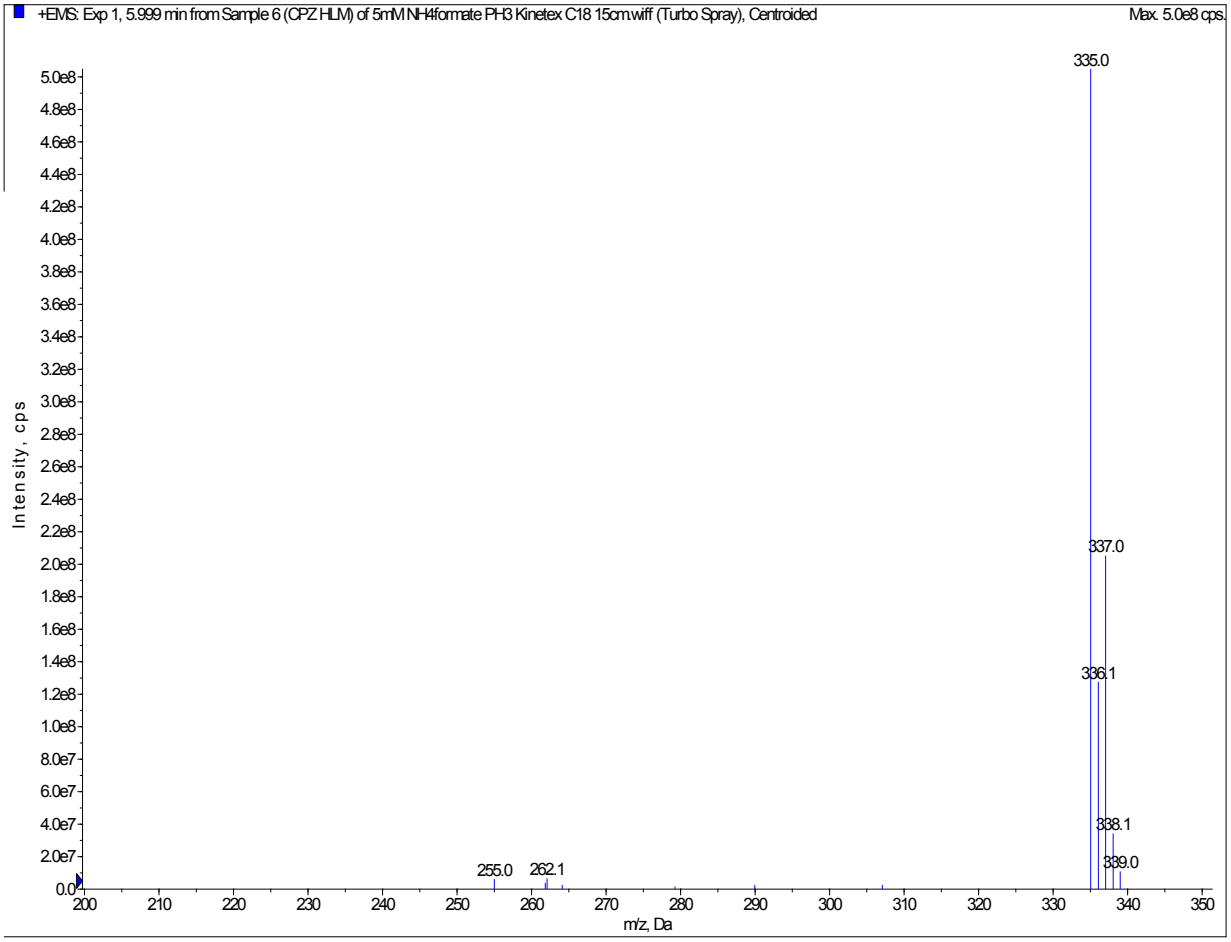


Appendix 2B: MS/MS spectrum of CPZ sulfoxide (M1)



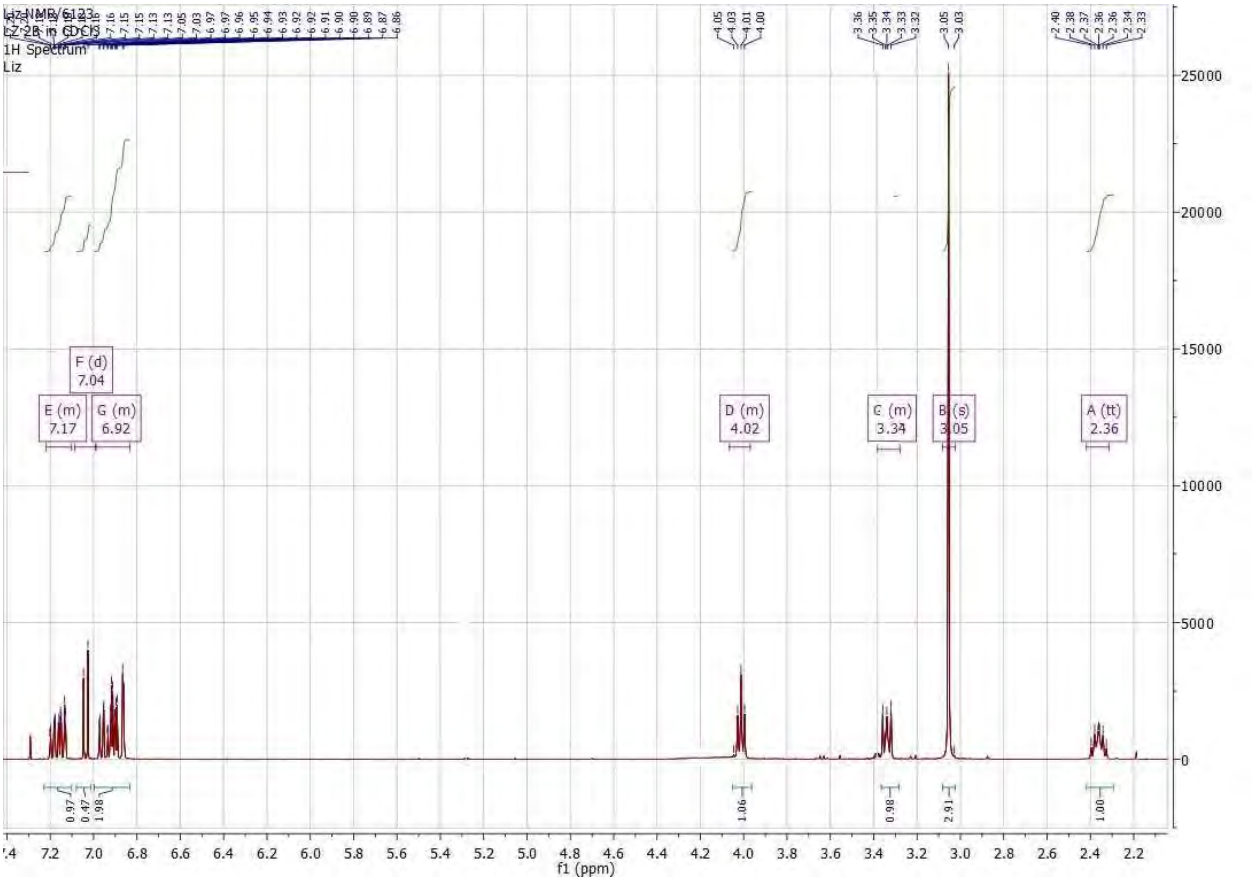
Parent ion: *m/z* – 335.1

Appendix 3A: MS/MS spectrum of 7-hydroxyCPZ (M2)

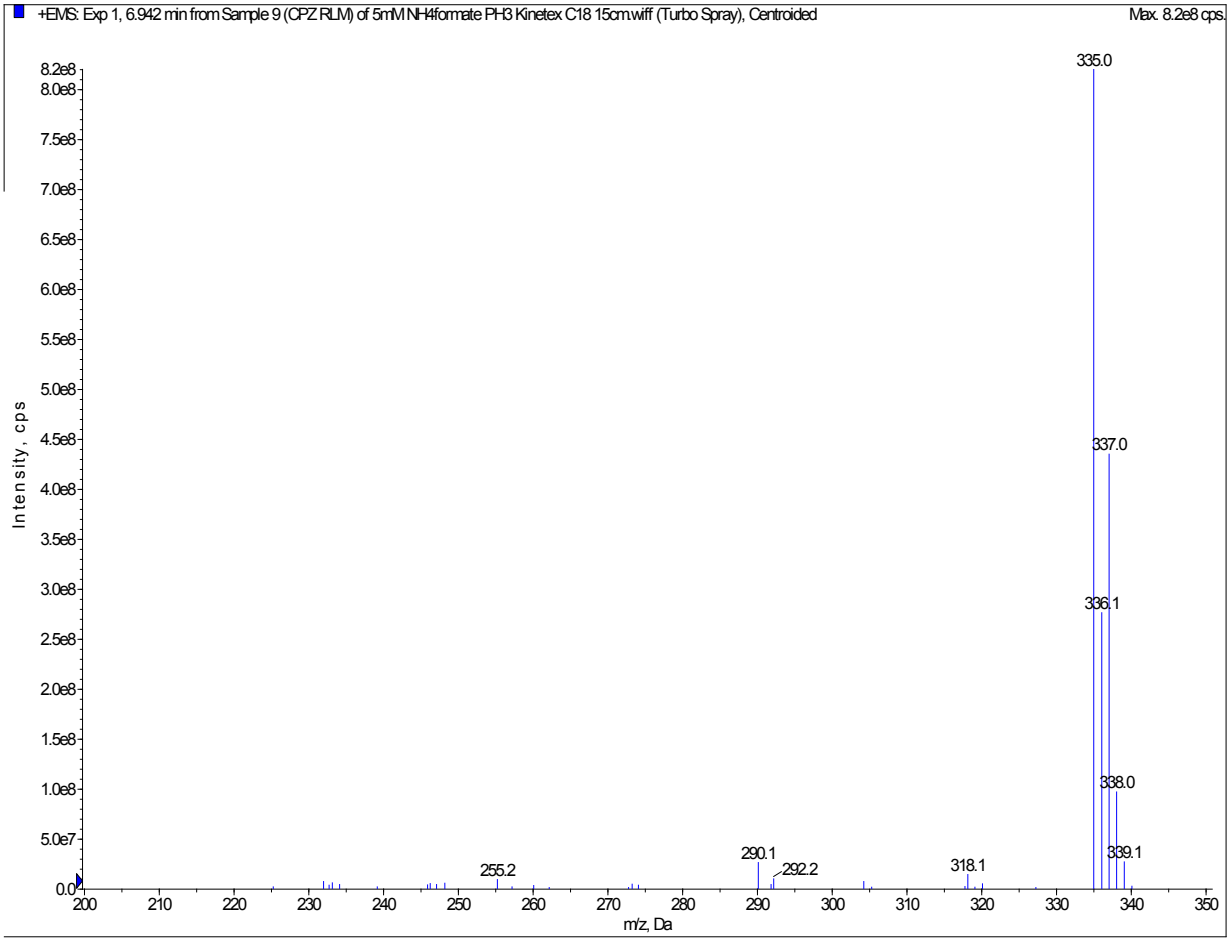


Parent ion: *m/z* – 335.0

Appendix 4A: ^{1}H NMR of CPZ-*N*-oxide (M3)

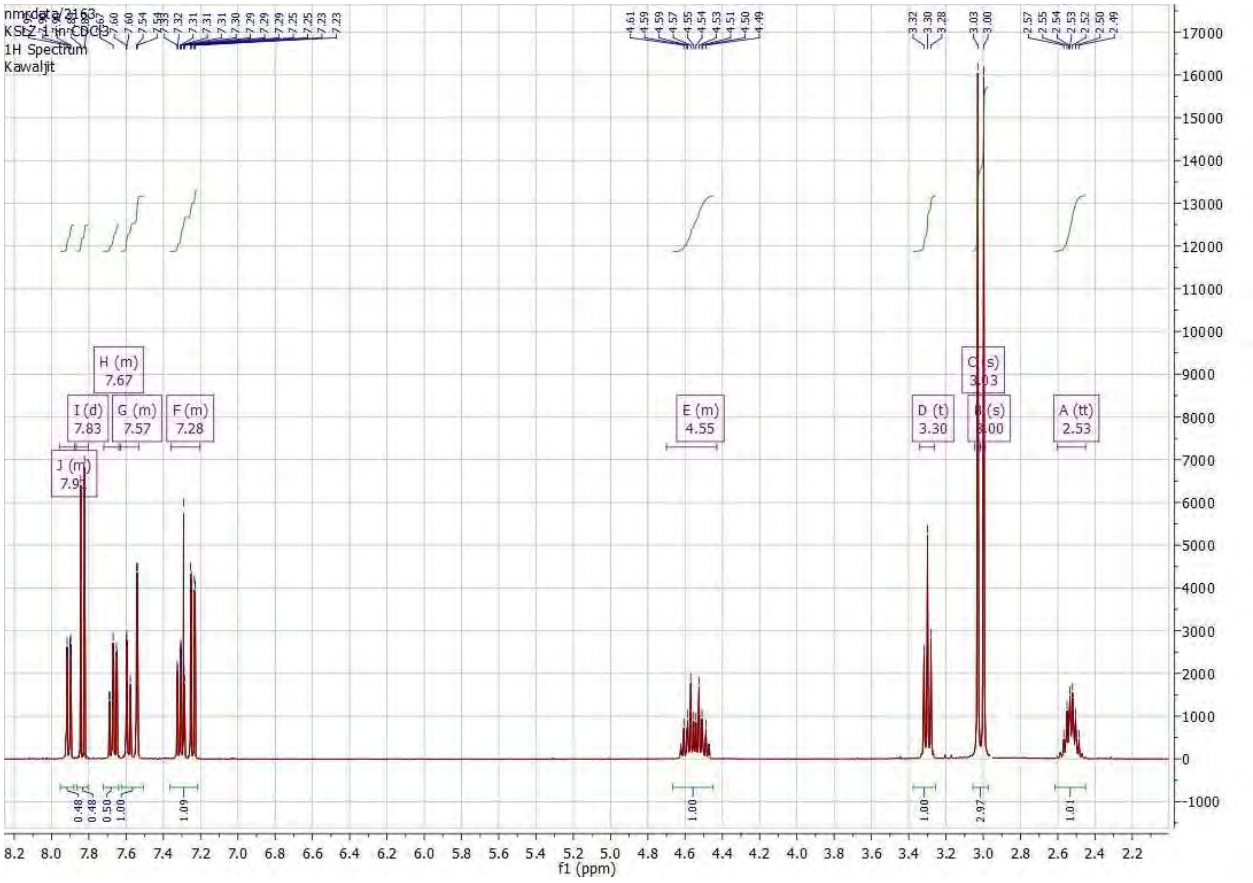


Appendix 4B: MS/MS spectrum of CPZ-*N*-oxide (M3)

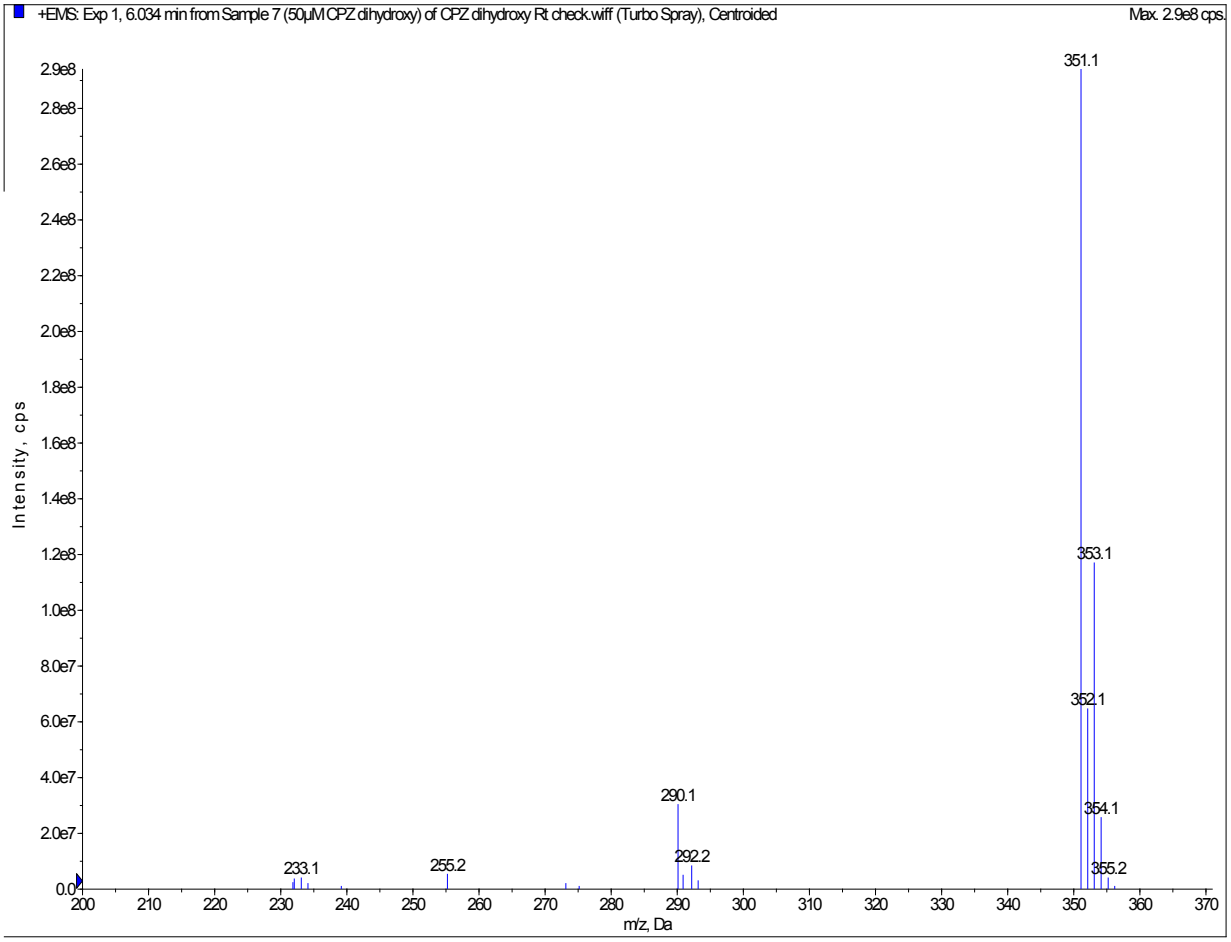


Parent ion: *m/z* – 335.0

Appendix 5A: ^{1}H NMR of CPZ-*S*-*N*-dioxide (M4b)

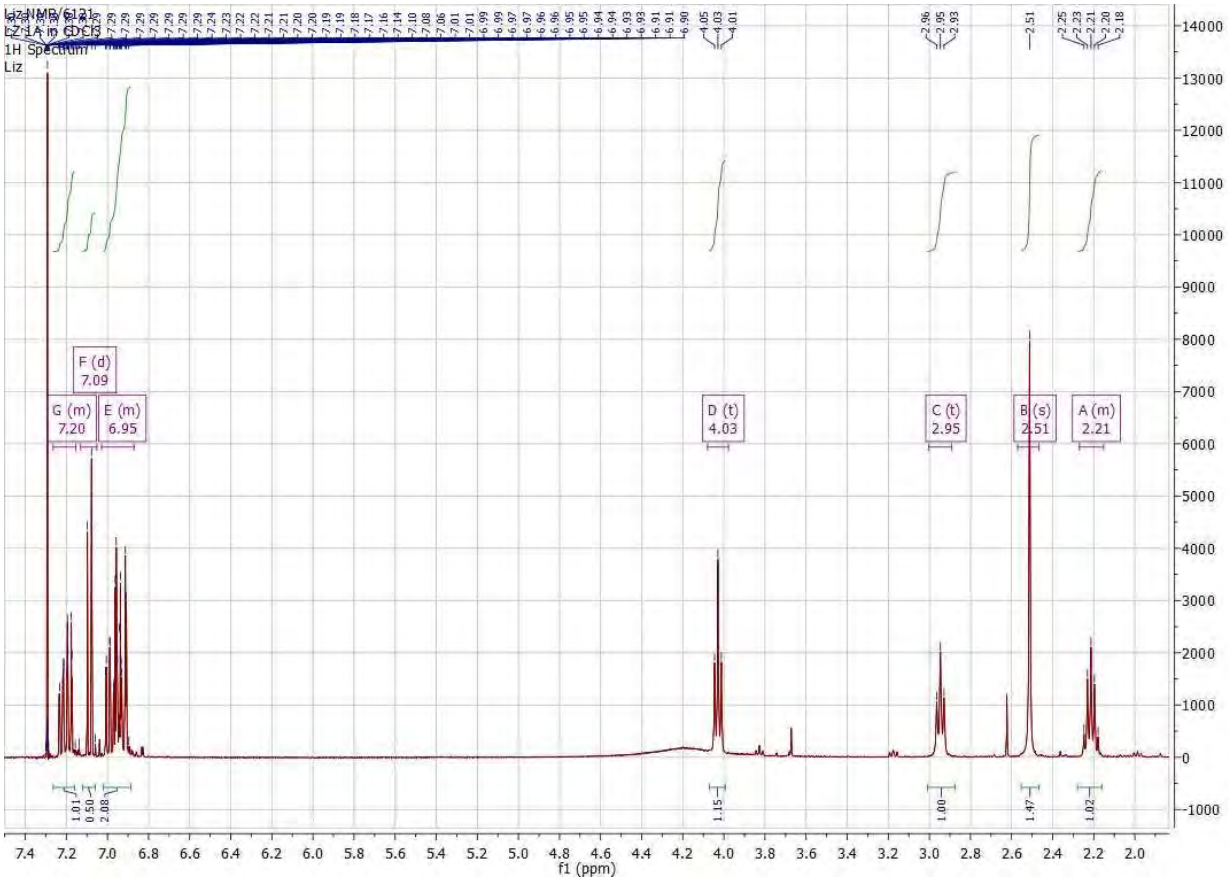


Appendix 5B: MS/MS spectrum of CPZ-*N*-*S*-dioxide (M4b)

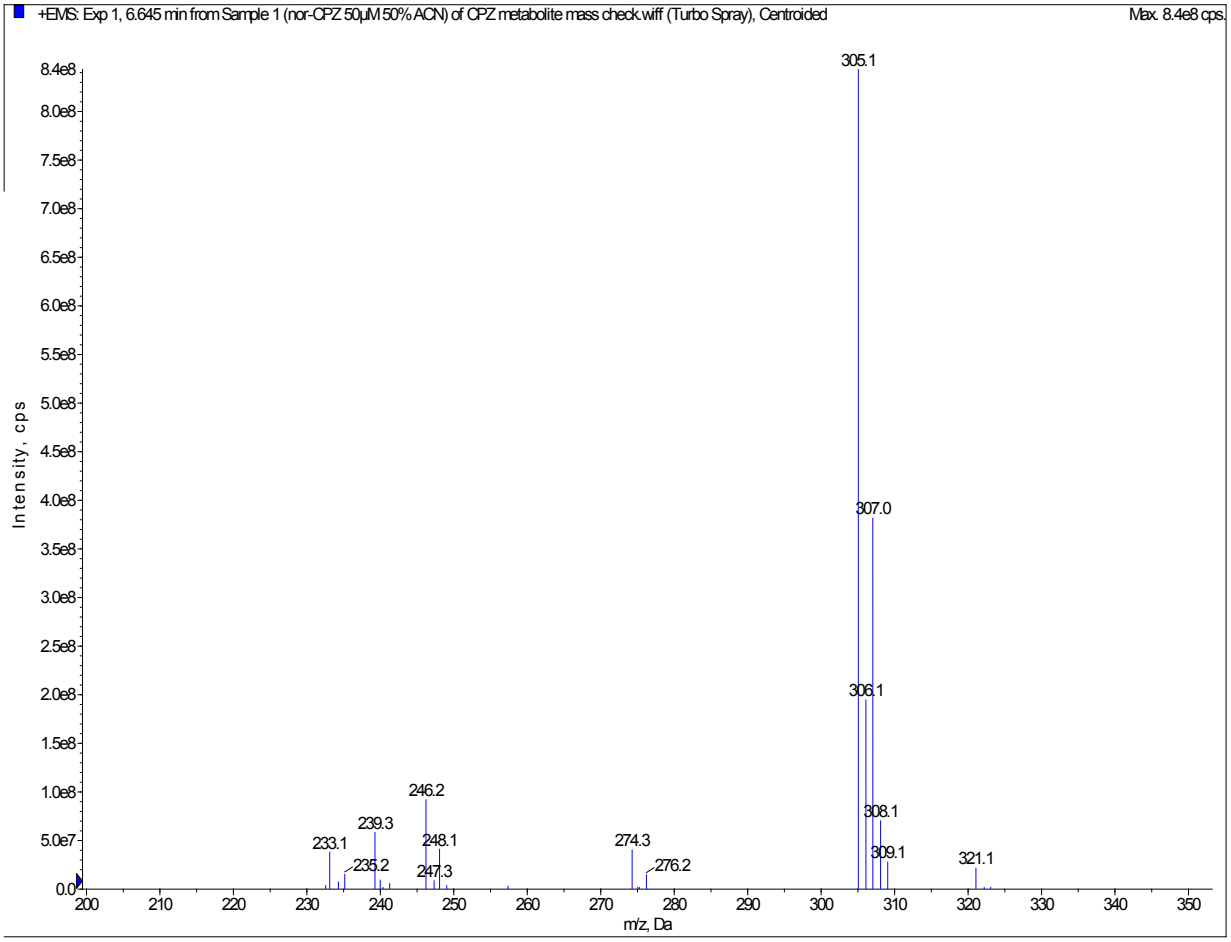


Parent ion: *m/z* – 351.1

Appendix 6A: ^{1}H NMR of nor-CPZ (M5)

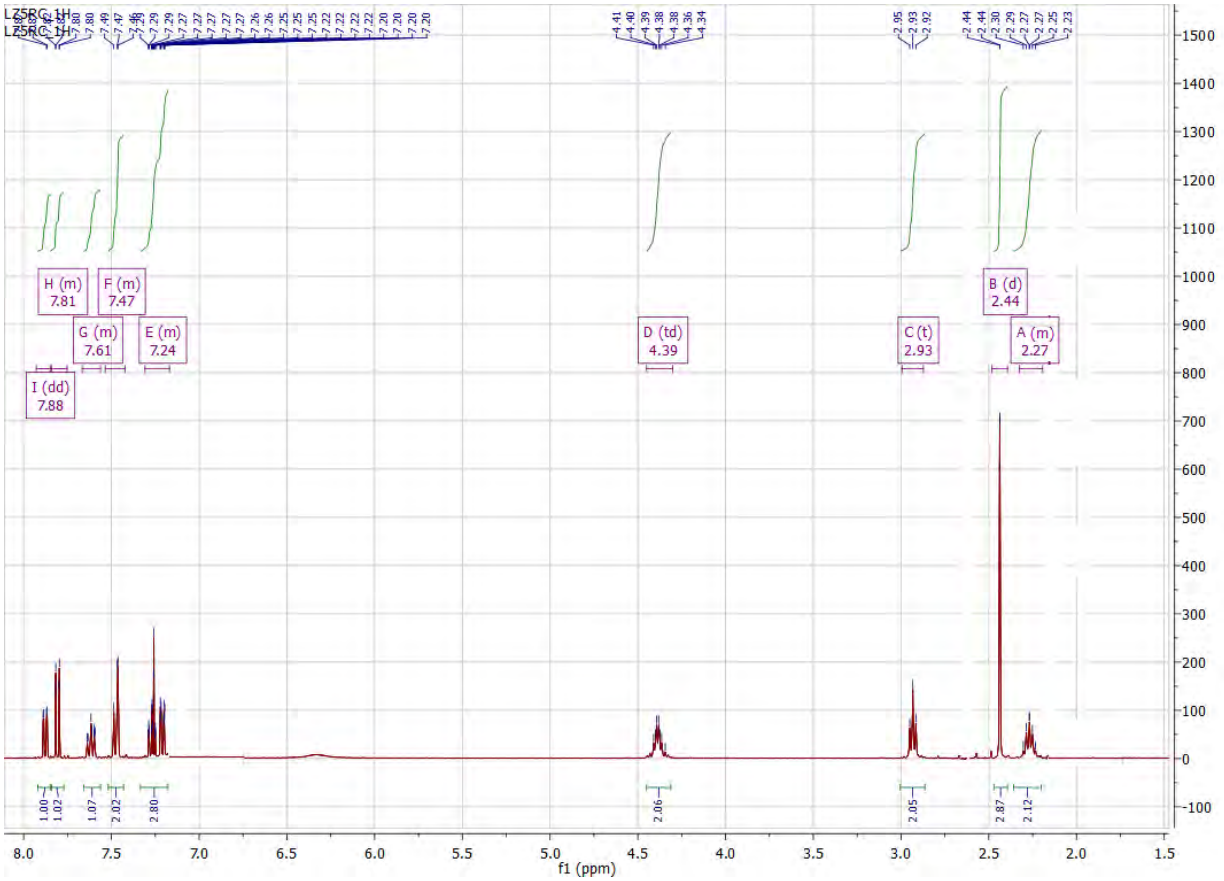


Appendix 6B: MS/MS spectrum of nor-CPZ (M5)

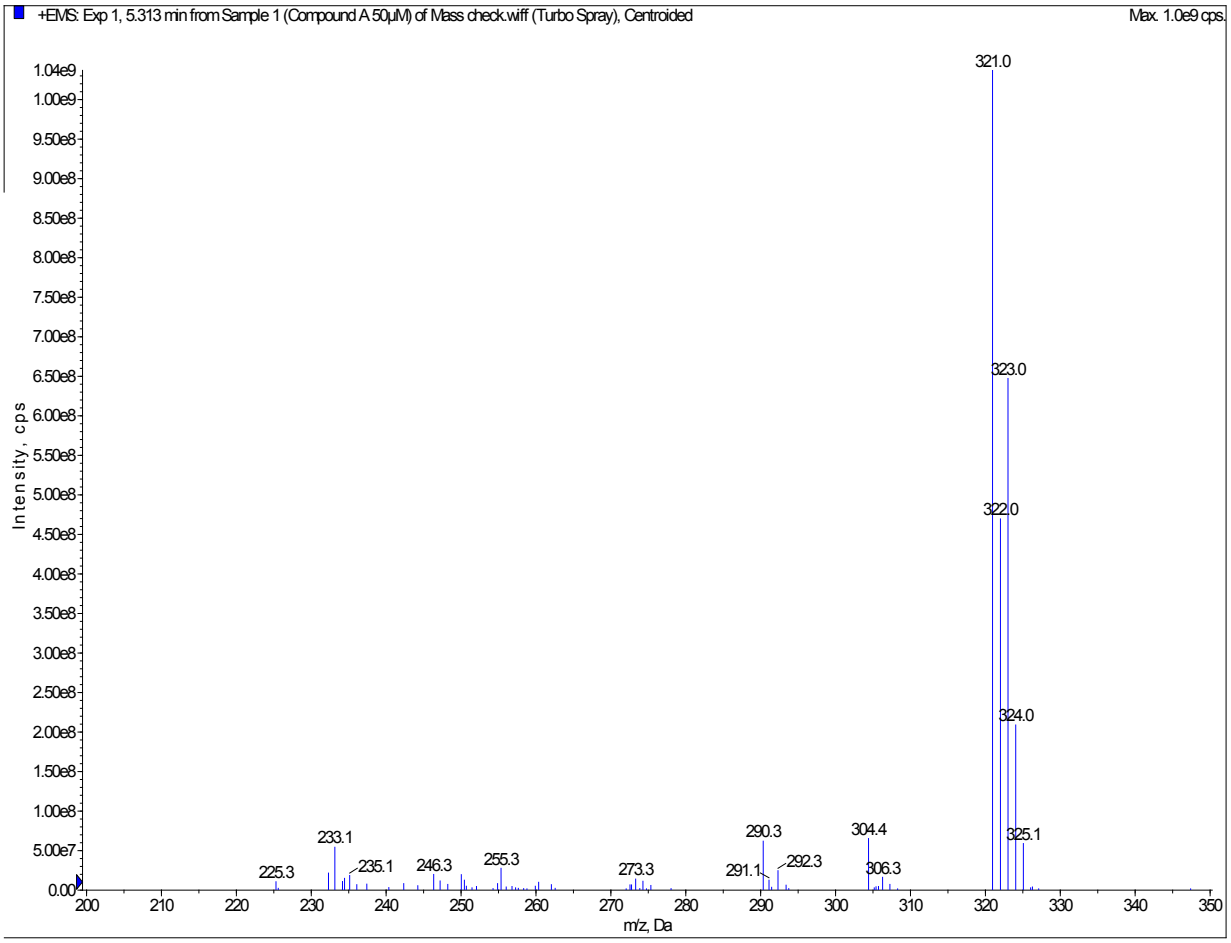


Parent ion: *m/z* – 305.1

Appendix 7A: ^{1}H NMR of nor-CPZ sulfoxide (M6b)

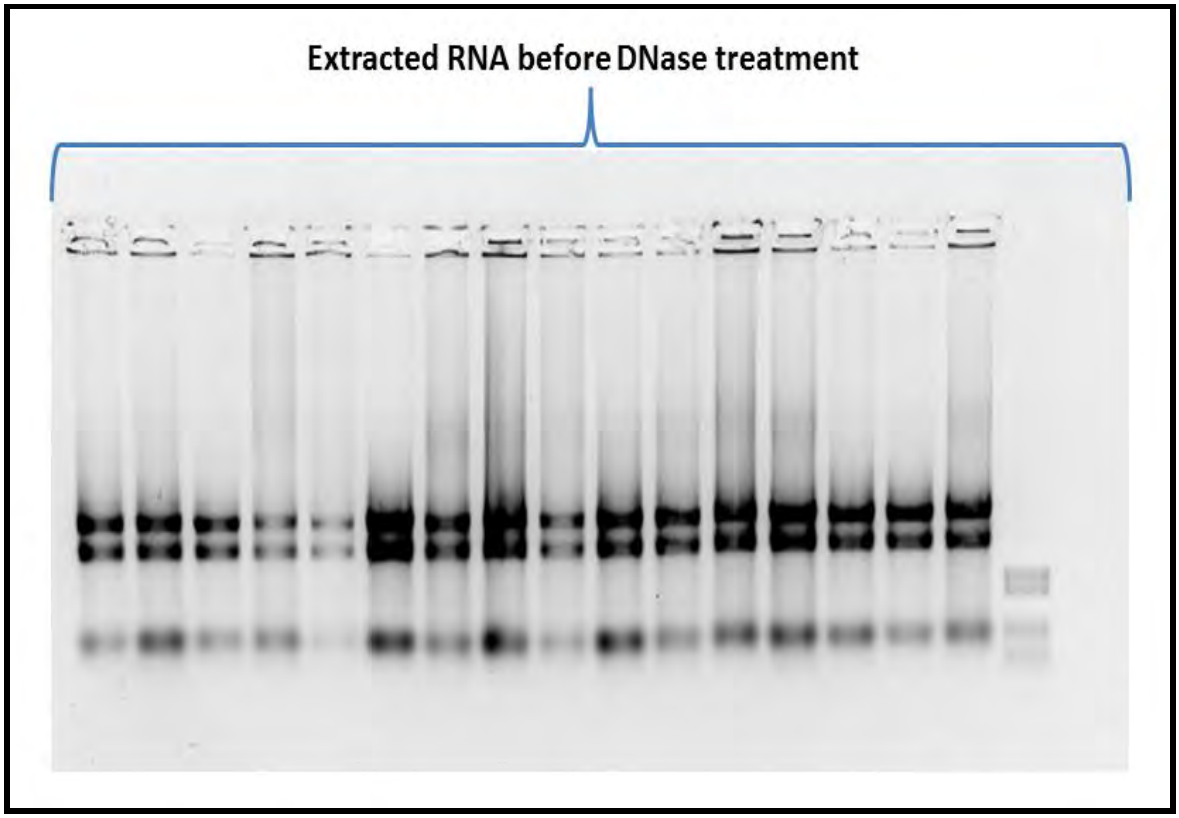


Appendix 7B: MS/MS spectrum of nor-CPZ sulfoxide (M6b)

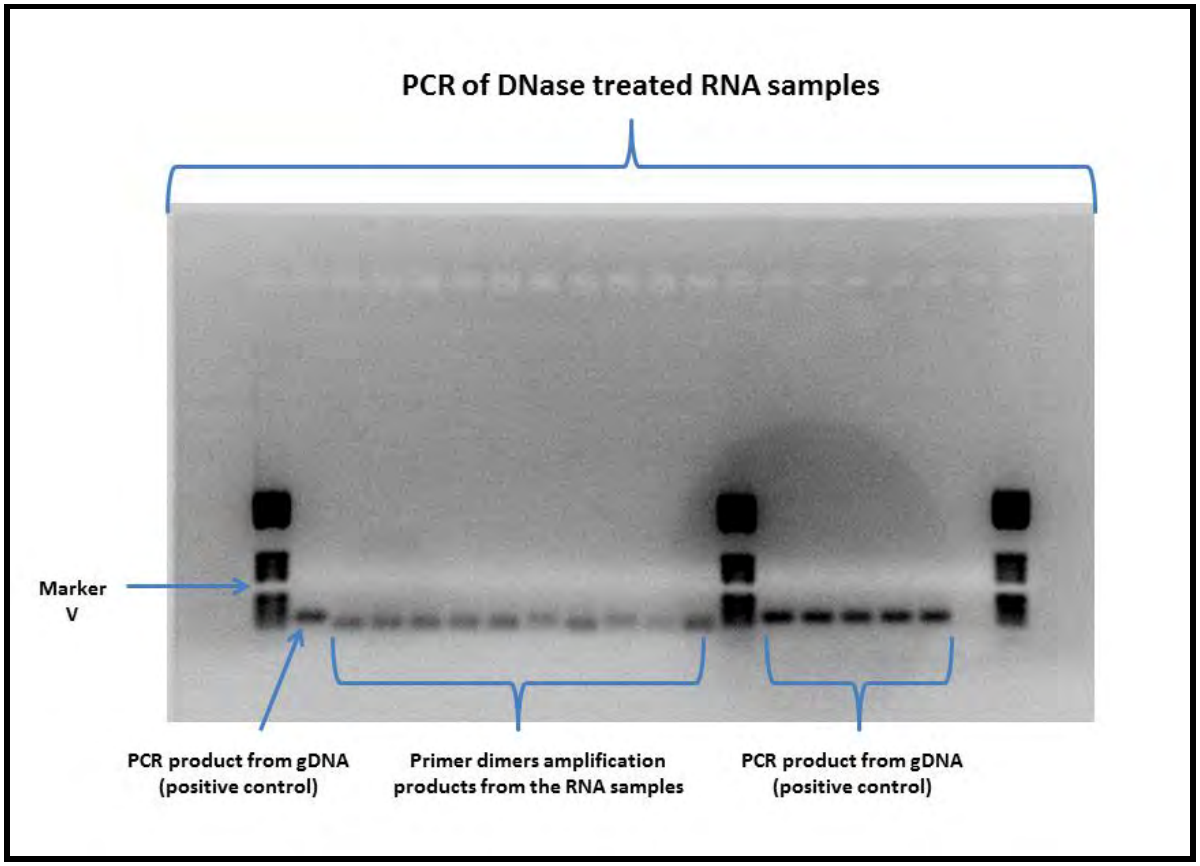


Parent ion: *m/z* – 321.0

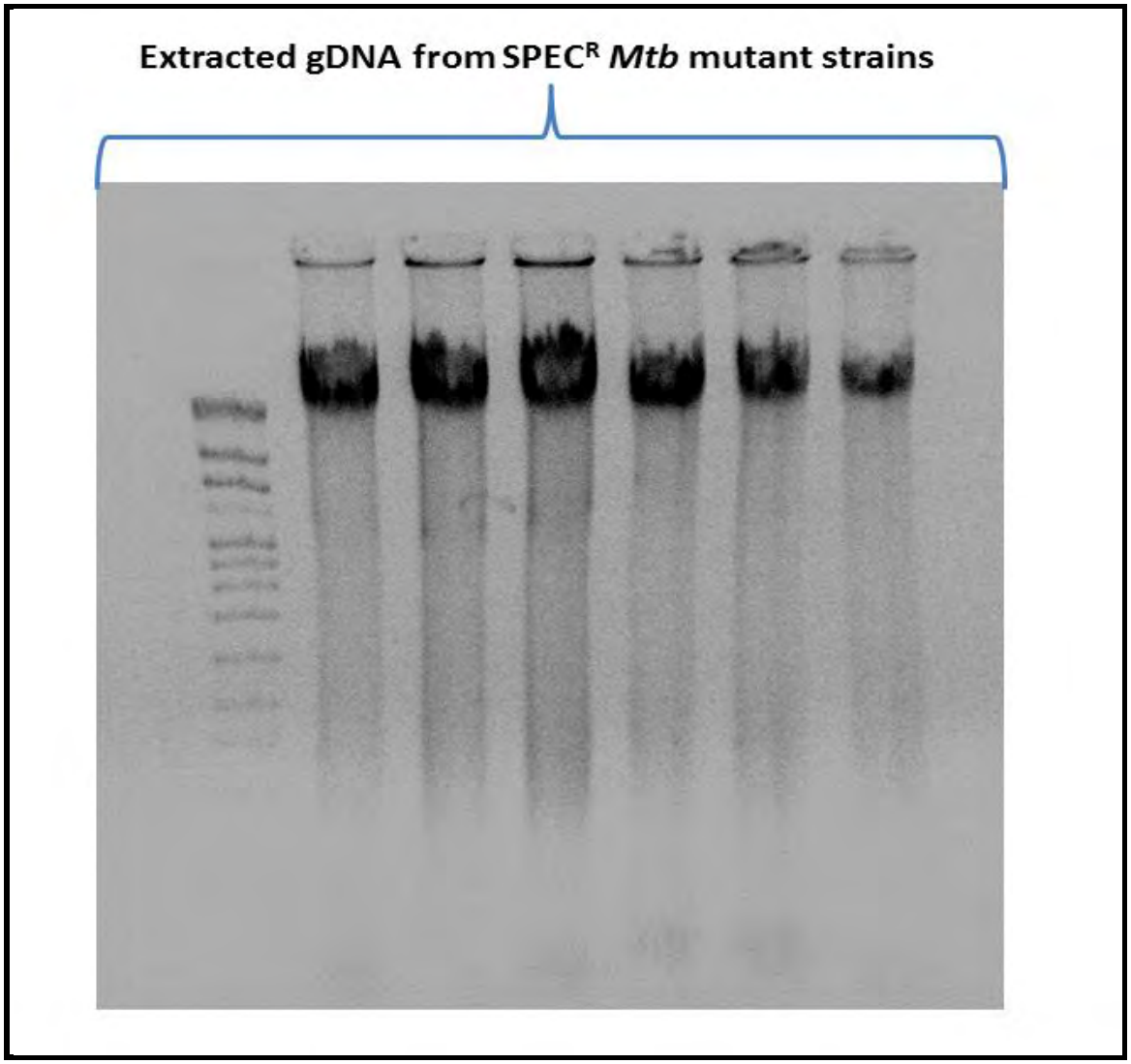
Appendix 8A: Extracted RNA samples before DNase treatment



Appendix 8B: PCR of the RNA samples after DNase treatment



Appendix 9A: gDNA isolated from SPEC^{R} *Mtb* mutant strains



Appendix 10A: Morphology of SPEC^{R} *Mtb* mutant strains