

**DEFINING THE ROLE OF EFFLUX PUMP INHIBITORS ON
ANTI-TB DRUGS IN RIFAMPICIN RESISTANT CLINICAL
MYCOBACTERIUM TUBERCULOSIS ISOLATES**

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Cr tki' 2016

DECLARATION

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SUMMARY

Central dogma suggests that mutations in target genes is the primary cause of resistance to first and second-line anti-TB drugs in *Mycobacterium tuberculosis*. However, it was previously reported that approximately 5% of Rifampicin mono-resistant clinical *M. tuberculosis* did not harbor mutations in the *rpoB* gene. The present study hypothesized that active efflux plays a contributory role in the level of intrinsic resistance to different anti-TB drugs (Isoniazid, Ethionamide, Pyrazinamide, Ethambutol, Ofloxacin, Moxifloxacin, Ciprofloxacin, Streptomycin, Amikacin and Capreomycin in RIF mono-resistant clinical *M. tuberculosis* isolates with a *rpoB531* (Ser-Leu) mutation. This study aimed to define the role of Efflux pump inhibitors (verapamil, carbonylcyanide m-chlorophenylhydrazone and reserpine) in enhancing the susceptibility to different anti-TB drugs in the RIF mono-resistant clinical isolates.

The isolates were characterized by determining the level of intrinsic resistance to structurally related/unrelated anti-TB drugs; determining the effect of EPIs on the level of intrinsic resistance in the isolates and comparing the synergistic properties of the combination of EPIs and anti-TB drugs. To achieve this, genetic characterization was done by PCR and DNA sequencing. Phenotyping was done by the MGIT 960 system EpiCenter software to determine the MICs of the different anti-TB drugs and the effect of verapamil and carbonylcyanide m-chlorophenylhydrazone on determined MICs. Due to inability to test reserpine in a MGIT, a different technique (broth microdilution) was used for the reserpine experiment. Additionally; fractional inhibitory concentrations (FIC) indices were calculated for each of these drugs. The FIC assess the anti-TB drugs/inhibitor interactions. STATISTICA Software: version 11 was used for statistical analysis.

Results revealed that the RIF mono-resistant isolates were sensitive at the critical concentrations of all 10 drugs tested, with the exception of Pyrazinamide. This could be explained by the technical challenges of phenotypic Pyrazinamide testing. A significant growth inhibitory effect was observed between the combination of EPI and anti-TB drug exposure *in vitro*. This suggests that verapamil, carbonylcyanide m-chlorophenylhydrazone and reserpine play a significant role in restoring the susceptibility (decrease in intrinsic resistance level) of the RIF mono-resistant isolates to all anti-TB drugs under investigation. Additionally, a synergistic effect was observed by the combination treatment of the anti-TB drugs with the different EPIs.

Based on these findings, we proposed a model suggesting that efflux pumps are activated by the presence of anti-TB drugs. The activated pumps extrude multiple or specific anti-TB drugs out of the cell, this in

turn decrease the intracellular drug concentration, thereby causing resistance to various anti-TB drugs. In contrast, the addition of EPIs inhibits efflux pump activity, leading to an increase in the intracellular drug concentration and ultimate cell death. This is the first study to investigate the effect of different efflux pumps inhibitors on the level of intrinsic resistance to a broad spectrum of anti-TB drugs in drug resistant *M. tuberculosis* clinical isolates from different genetic backgrounds. The findings are of clinical significance as the combination of treatment with EPI and anti-TB drugs or use of EPIs as adjunctives could improve MDR-TB therapy outcome.

OPSOMMING

Sentrale dogma beweer dat mutasies in teiken gene die primêre oorsaak van die weerstandheid teen anti-TB-middels in *Mycobacterium tuberculosis* is. Vorige studies het getoon dat ongeveer 5% van Rifampisien enkelweerstandige kliniese *M. tuberculosis* isolate nie 'n mutasie in die *rpoB* geen het nie. Die hipotese van die huidige studie was dat aktiewe pompe 'n bydraende rol speel in die vlak van intrinsieke weerstandheid teen 10 verskillende anti-TB-middels (Isoniasied, Ethionamied, Pyrazinamied, Ethambutol, Ofloxacin, Moxifloxacin, Siprofloksasien, Streptomisien, Amikasien and Capreomycin) in RIF enkelweerstandige kliniese *M. tuberculosis* isolate met 'n *rpoB531* (Ser-Leu) mutasie. Die doel van hierdie studie was om die rol van uitpomp inhibeerders (verapamil, carbonylcyanide m-chlorophenylhydrazone en reserpien) te definieer in die verbetering van die werking vir verskillende anti-TB-middels in die RIF enkelweerstandige kliniese isolate.

Die doelstellings van die studie was om die vlak van intrinsieke weerstandigheid teen struktureel verwante/onverwante anti-tuberkulose middels asook die effek van die EPIs op die vlak van intrinsieke weerstand in die isolate is bepaal. Verder is sinergistiese eienskappe van die kombinasie van EPIs en anti-TB-middels ondersoek. Hierdie doelstellings is bereik deur genetiese karakterisering deur PKR en DNS volgorde bepaling. Fenotipering is gedoen deur gebruik te maak van MGIT 960 EpiCenter sagteware om die Minimum Inhibisie Konsentrasie (MIC) van die verskillende anti-TB-middels en die effek van verapamil en carbonylcyanide m-chlorophenylhydrazone op die MIC te bepaal. Reserpien kan nie in die MGIT sisteem getoets word nie, and daarom is 'n ander tegniek (mikro-verdunning) is gebruik om die effek van reserpien te toets. Fraksionele inhiberende konsentrasies (FIC) is bereken vir elk van hierdie middels die anti-TB-middels / inhibeerder interaksies te bepaal. STATISTICA v11 sagteware is gebruik vir alle statistiese analyses.

Resultate van hierdie studie toon dat die RIF enkelweerstandige isolate sensitief is teen kritieke konsentrasies van al die middels, met die uitsondering van Pyrazinamied. Weerstandigheid van Pyrazinamied kan wees as gevolg van welbekende tegniese probleme met die standaard fenotipiese pyrazinamied toets. 'n Beduidende groei inhiberende effek is waargeneem tussen die kombinasie van EPI en anti-TB middel blootstelling *in vitro*. Dit dui daarop dat verapamil, CCCP en reserpine 'n belangrike rol speel in die herstel van die sensitiwiteit (afname in intrinsieke weerstand vlak) van die RIF enkelweerstandige isolate aan alle anti-TB-middels wat ondersoek is. Daarbenewens is 'n sinergistiese effek waargeneem deur die kombinasie van die verskillende anti-TB-middels en die verskillende EPIs.

Op grond van hierdie bevindinge het ons 'n model voorgestel wat toon dat uitvloei pompe geaktiveer word deur die teenwoordigheid van anti-TB-middels en die geaktiveerde pompe dan verskeie of spesifieke anti-TB-middels uit die sel pomp. Dus verminder die intrasellulêre konsentrasie van die middel en veroorsaak daardeur weerstandigheid teen verskeie anti-TB-middels. Die byvoeging van EPIs inhibeer uitvloei pompe se werking en lei tot 'n toename in die intrasellulêre konsentrasie van die middels en uiteindelik die dood van die selle. Hierdie is die eerste studie wat die effek van verskillende uitvloei pompe inhibeerders op die vlak van intrinsieke weerstand teen 'n breë spektrum van anti-TB-middels in die middel-weerstandige kliniese isolate ondersoek. Die bevindinge kan van belangrike kliniese belang wees aangesien die kombinasie van behandeling met EPI en anti-TB-middels die uitkoms MDR-TB terapie kan verbeter.

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Jesus Christ my Lord, saviour and Holy Spirit my helper.

Psalm 119

LIST OF ABBREVIATIONS

°C	Degree Celsius
µl	microlitres
µg	micrograms
ABC	ATP binding cassette
ADC	Albumin dextrose catalase
AMI	Amikacin
AMINO	Aminoglycoside
bp	base pairs
BMM	Broth microdilution method
CAP	Capreomycin
CC	Critical concentration
CCCP	Carbonylcyanide <i>m</i> -chlorophenylhydrazone
CIP	Ciprofloxacin
DMSO	Distilled water
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
dNTP	Deoxyribonucleotide triphosphate
EMB	Ethambutol
EP	Efflux pump
EPI	Efflux Pump Inhibitor
ETH	Ethionamide
EtOH	Ethanol
FIC	Fractional Inhibitory Concentration
FQ	Fluoroquinolone
g	Grams
GC	Growth control
GU	Growth unit
INH	Isoniazid
LAM	Latin-American and Mediterranean
LCC	Low Copy Clade
LJ	Loewenstein Jensen

MTB	<i>Mycobacterium tuberculosis</i>
MATE	Multidrug And Toxic compounds Extrusion
MDR	Multi Drug Resistant
MFS	The Major Facilitator Super family
MIC	Minimum Inhibitory Concentration
MGIT	Middlebrook Growth Indicator Tube
ml	millilitres
mM	milliMolar
mRNA	Messenger RNA
MOXI	Moxifloxacin
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OADC	Oleic Acid Dextrose Catalase
OFL	Ofloxacin
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RND	Resistance-Nodulation-cell Division
RRDR	RIF Resistance Determining Region
rRNA	Ribosomal RNA
SA	South Africa
SDS	Sodium dodecyle sulphate
SMR	Small Multidrug Resistance
SNP	Single nucleotide polymorphism
STR	Streptomycin
TB	Tuberculosis
TBE	Tris/Borate/EDTA
TE	Tris/EDTA
Tm	Melting temperature
Tris	Trishydroxymethylaminomethane
U	Units

V

XDR

ZN

Volt

Extreme drug resistant

Ziehl-Neelsen

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STRUCTURE OF THE THESIS REFERENCES

- The references were formatted according to the guidelines of Journal of Clinical Microbiology.
- References of chapter 2 were listed after that chapter since chapter 2 will be submitted for review publication.
- References for other chapters are provided at the end of the Thesis.

CHAPTER 1
INTRODUCTION

1.1 BACKGROUND

Tuberculosis (TB), a deadly infectious disease caused by *Mycobacterium tuberculosis* remains a global health problem (1–3). The emergence of multidrug resistant tuberculosis (MDR-TB) and extensive drug resistant tuberculosis (XDR-TB) has led to an inadequate availability of more anti-tubercular (anti-TB) drugs and raises a worldwide threat to TB eradication (3–7, 9). MDR-TB is defined as an infection with *M. tuberculosis* bacilli resistant to first-line drugs isoniazid (INH) and rifampicin (RIF) (1) and XDR-TB with additional resistance to a fluoroquinolone (FQ) and one of the injectables i.e. amikacin (AMI), kanamycin (KANA) or capreomycin (CAP) (8). Central dogma suggests that the sole cause of resistance to first-line and second-line anti-TB drugs in *M. tuberculosis* is by evolution of spontaneous mutations in target genes, resulting in the selection of resistant mutants (3, 9–11) (Table 1.1). Previous studies report that the mutations in the specific target genes will change the structure of the target protein, thereby affecting the drug-target binding activity thus influencing the susceptibility to the specific drug (3, 6, 12).

However, it was previously observed that approximately 20-30% of INH resistant clinical *M. tuberculosis* isolates, harbored no mutations, in the known target genes (13, 14). Similarly, about 5% of RIF resistant clinical *M. tuberculosis* isolates did not harbour mutations in the RIF Resistance Determining Region (RRDR) of the *rpoB* gene (3, 15). Therefore, this suggests that alternative and or additional mechanisms could be conferring and/or defining the drug resistance level. These mechanisms include active efflux, the production of drug modifying enzymes and an increase in cell wall permeability (natural resistance). This shows that drug resistance in *M. tuberculosis* is more complex than previously assumed.

Recent studies revealed that mycobacteria might use active efflux systems such as multidrug resistant efflux pumps (MDR EPs) to extrude structurally/functionally related and unrelated drugs (16–19). These EPs are divided into different families, based on the energy source. These families include Major facilitator superfamily (MFS), Small multidrug resistant family (SMR), ATP binding cassette and Resistant nodulation cell division (RND) (17, 18, 20, 21). Independent studies showed that the exposure of clinical resistant *M. tuberculosis* cells to various drugs (INH, RIF and Ethambutol (EMB)) resulted in an up-regulation of different efflux pumps (8, 22). This in turn reduced the intracellular drug concentration which lead to clinical inefficiency (2, 3, 23, 24). Nevertheless, further addition of verapamil, cyanide *m*-chlorophenyl hydrazone (CCCP), and reserpine resulted in inhibition of these efflux pumps activity in *M. tuberculosis* cells and an increase in susceptibility (2, 5, 6, 25–27). The same phenomena were revealed in an *in vivo* macrophage-model where it was observed that the tap-like efflux pump, Rv1258c, was significantly up-regulated after RIF exposure leading to drug tolerance. The tolerance phenotype could be reversed after the addition of the efflux pump inhibitor, verapamil (5, 28).

Based on these findings, it is important to investigate the clinical relevance of the use of the different efflux pumps inhibitors (EPIs) in combination with various anti-TB drugs as treatment-shortening adjuncts (2, 5). These EPIs in combination with the anti-TB drugs might aid in improving the efficacy of the current TB treatment regimen and eradicate acquired and intrinsic resistance (20).

Table 1.1: Classes of structurally related/unrelated anti-TB drugs and the associated drug resistant conferring gene mutations*

Drugs studied	Mechanism of action	Gene target	Frequently mutated codons
RIF	Inhibits RNA synthesis	<i>rpoB</i>	531TTG, 526GAC
Fluoroquinolones OFL, MOXI, CIP	Introduces negative supercoils in DNA molecules	<i>gyrA, gyrB</i>	94GGC, 94TAC
Aminoglycosides STR, AMI, CAP (polypeptide)	Inhibits translation	<i>rrs, rpsL, tlyA</i>	1401G, 1402A
Structural analogs INH	Inhibits cell wall synthesis	<i>katG</i>	315ACA
ETH	Disrupts cell wall biosynthesis	<i>InhA</i>	inhA-15prom, inhA-17prom
EMB	Inhibits cell wall synthesis	<i>embCAB</i>	306GTG
PZA	Disrupts plasmamembrane and energy metabolism	<i>pncA</i>	14CGC, 103TAG, 13TTC

*The anti-TB drugs were classified according to their mechanism of action, gene target and most frequent mutations through literature searches (13, 29–33).

1.2 PROBLEM STATEMENT

The use of additional resistance mechanisms such as active efflux, by the *M. tuberculosis* bacillus, influences the efficacy of the current anti-TB treatment regimen. Recent reports indicate the involvement of such mechanisms in drug resistance, subsequently playing an intricate role in defining the level of RIF resistance. Patients infected with a RIF resistant *M. tuberculosis* strain requires treatment with second-line anti-TB drugs which is less effective, more toxic and more expensive. Furthermore, RIF, along with INH, PZA and EMB is administered to patients with undetected drug resistance, for up to 2 months, prior to drug susceptibility testing. These patients are essentially treated, during that time period, with drugs that

are ineffective. The prolonged treatment of these patients with drugs which is ineffective, due to resistance, may program the *M. tuberculosis* bacillus to become resistant to other frequently used second-line drugs. Subsequently, this results in the amplification of resistance and less effective MDR-TB treatment regimens. This scenario emphasizes the need for the development of new anti-TB drugs with novel modes of action, which is a long-term process. Alternatively, compounds that might boost the efficacy of the current TB treatment regimen might be of clinical importance.

The use of EPIs as potential adjuncts to improve the efficacy of existing anti-TB drugs regimens and restore their susceptibility has been the topic under investigation in many studies recently. However, the promiscuous nature of these efflux pumps complicates the simplicity of the concept, accentuating the knowledge gap. It is thus important to investigate the association between the efflux mechanism, the inhibition of this activity by EPI and the nature of the drugs (structurally related or unrelated) extruded, thereby studying cross-resistance (due to efflux).

1.3 HYPOTHESIS:

Active efflux plays a contributory and or causal role in the level of resistance to first and second-line anti-TB drugs in rifampicin mono-resistant clinical *M. tuberculosis* isolates with *rpoB531* (Ser-Leu) gene mutation.

1.4 OVERALL AIM:

The aim of this study is to define the role of verapamil, CCCP and reserpine in enhancing the susceptibility to first- and second line anti-TB drugs in RIF mono-resistant *M. tuberculosis* clinical isolates with the same *rpoB531* (Ser-Leu) mutation.

Specific aims:

1. To genotypically characterize the RIF mono-resistant *M. tuberculosis* clinical isolates with the same *rpoB531* (Ser-Leu) mutation
2. To determine the level of resistance (as reflected by the MIC's) of the first-line and second-line anti-TB drugs in these clinical isolates
3. To determine whether the addition of EPIs (verapamil, reserpine and CCCP):
 - a. Has an effect on the growth of the RIF mono-resistant clinical *M. tuberculosis* isolates
 - b. Changes the MICs of the structurally related/unrelated anti-TB drugs (INH, ETH, EMB, OFL, MOXI, OFL, STR, AMI and CAP)

4. To compare the overall synergistic properties of verapamil, reserpine and CCCP in combination with the first- and second-line anti-TB drugs at MIC.

1.5 EXPERIMENTAL APPROACH:

The RIF- resistant and sensitive clinical isolates were selected and genotypically characterized by targeted gene sequencing. Phenotypic characterization of these isolates included the Minimum inhibitory concentrations (MICs) determination of the various anti-TB drugs in the MGIT 960 system and EpiCenter software technology. Furthermore, using the same MGIT 960 system, the effect of the EPI's verapamil and CCCP on the MICs of the anti-TB drugs was determined. Additionally, the effect of reserpine on mycobacterial growth and the level of resistance were determined by Broth Microdilution Method (BMM). Lastly, to compare synergistic properties between EPIs and anti-TB drugs fractional inhibitory concentrations (FICs) were calculated. STATISTICA Software: version 11 was used to show statistical differences of EPIs experiment results data. All the work presented in this thesis was done according to standard operating procedures (SOP) in the Biosafety level III (P3) under safe conditions which are regulated by a safety officer (Appendix A). The project was approved by the ethics committee of the Faculty of Medicine and Health Science (N09/11/296)

CHAPTER 2
LITERATURE REVIEW

**THE *IN VIVO* AND *IN VITRO* EFFECT OF VARIOUS INHIBITORY COMPOUNDS ON
MYCOBACTERIAL GROWTH AND EFFLUX SYSTEMS: IMPLICATIONS FOR
TBTREATMENT**

2.1 INTRODUCTION

Mycobacterium tuberculosis, the causative agent of Tuberculosis (TB), was discovered by Robert Koch in 1882 (1, 2). The introduction of Streptomycin in 1943 to treat TB resulted in a decrease in death rates associated with TB disease worldwide (3). However, the concomitant emergence of drug resistance continues to plague TB treatment globally (4, 5). Moreover, multidrug resistant TB (MDR-TB) outbreaks have been described since the 1990s, emphasizing MDR-TB as a global health problem (5).

Moreover, different classes of TB drug resistance as per WHO definitions and guidelines (6) are summarized below. (Table 2.1). Additionally, MDR-TB is an infection with *M. tuberculosis* strains resistant to two main first-line anti-TB drugs rifampicin (RIF) and isoniazid (INH) (6, 7). XDR-TB is *M. tuberculosis* isolates resistant to INH and RIF (MDR-TB) in addition to one of the fluoroquinolones (FQs) and one of the injectables amikacin (AMI), kanamycin (KANA) and capreomycin (CAP) (6, 7). Recently, a somewhat controversial new term has been introduced in the literature, namely totally drug resistant (TDR) TB, defined as *M. tuberculosis* strains resistant to anti-TB drugs in addition to those which define XDR-TB, thereby encompassing nearly all current anti-TB drugs (8). TDR-TB, or as otherwise known, therapeutically destitute strains were first identified in Italy in 2007(9). This was followed by reports of its emergence in Iran in 2009 and India in 2011(10, 11).

Resistance to anti-microbial drugs covers a wide range of biological systems (12, 13), thus making it difficult to prevent resistance. It is suggested that the emergence of drug resistant bacilli is primarily attributed to genomic mutations in drug target genes (14, 15), however other mechanisms also confer the resistance phenotype (7, 14, 16–21). These mechanisms include: i) prevention of activating pro-drugs (e.g. INH) into active drugs (6, 23, 34, 35); ii) intrinsic resistance to a given drug by decreased permeability of the cell membrane (7, 23) and iii) activation of efflux pump systems, resulting in a decrease in the intracellular drug concentration (24). One major concern is that efflux pumps have the ability extrude a variety of toxic compounds out the bacterial cell. This may subsequently enable the bacilli to escape administered drug therapies (25, 26). During that past few years the molecular mechanisms underlying efflux pump activity and its phenotypic consequences has become a major focus in mycobacterial drug resistance studies (7, 17, 28, 29). Recently, the use of efflux pump inhibitors in anti-TB therapy has been demonstrated *in vitro* to aid in the restoration of drug susceptibility and improve MDR-TB treatment (19). This review aims to highlight the current understanding of efflux pump mediated drug resistance in mycobacteria (7, 30). In addition, literature will be reviewed on the growth inhibitory effect of efflux pump inhibitors either alone or in combination with other compounds.

Table 2.1: Classification of drug resistant treatment regimens as per WHO definitions and guidelines (9).

		MDR	Pre-XDR	XDR	TDR	
First-line drugs	Isoniazid	x	x	x	x	
	Rifampicin	x	x	x	x	
	Pyrazinamide				x	
	Streptomycin				x	
	Ethambutol				x	
	Injectables	Amikacin		(x)*	x	x
Second-line drugs		Kanamycin				
		Capreomycin				
	Fluoroquinolones	Ofloxacin		(x)*	x	x
		Moxifloxacin				
	Other anti-TB drugs	<i>p</i> -aminosalicylic acid				x
		Ethionamide				
	Cycloserine**					

* means either one of the 3 injectables or one of the fluoroquinolones; ** the drug is not fully standardized (there have not been clinical trials to confirm the actual role of this drug in drug resistance) (31).

2.2 CONTRIBUTION OF EFFLUX PUMPS SYSTEMS IN THE MULTIDRUG RESISTANCE PHENOTYPE

The intrinsic resistome of mycobacteria is predominated by 2 properties: activated efflux pump and decreased compound/drug permeability due to the mycobacterial cell wall (7, 13, 32). Efflux pumps are defined as protein transporters in the plasma membrane involved in the export of toxic compounds (e.g. antibiotics, metabolites, antibiotic peptides and dyes etc.) through the bacterial cell envelope. This exportation results in a decrease in the accumulation of these compounds in the bacterial cell (27, 33). The *M. tuberculosis* genome possesses various efflux pump genes enabling the bacilli to evade the bactericidal or bacteriostatic effects of anti-TB drugs. Interestingly, Calgin *et al.* showed that expression levels of 15 putative multidrug efflux pump genes were the same in both MDR and drug-susceptible *M. tuberculosis* isolates (34). In contrast, resistant when compared to reference strains (H37Rv), the resistance isolates had high gene expression levels (34). Additionally, drugs at high concentrations can act as inducers of efflux pumps, resulting in increased drug efflux (33, 35). A limitation of clinical significance is that the over expression of efflux pump genes is regulated when the bacterium is under

selective pressure (36). This makes the reversal of drug efflux very difficult. Currently, efflux pumps were also revealed to play an important role in quorum sensing which signals between bacteria and in biofilm formation (13, 37, 38). Therefore, it is important to extrapolate how these efflux pumps function during extrusion of various types of structurally unrelated drugs.

2.3 CLASSIFICATION OF FIVE CLASSES OF BACTERIAL DRUG EFFLUX PUMPS

Bacterial efflux pumps are categorized into five distinct superfamilies with different structural morphologies, substrate specificities and energy sources (39–42). These include the ATP binding cassette (ABC), the Major facilitator superfamily (MFS), Small multidrug resistance (SMR), Resistance-nodulation-cell division (RND) and Multidrug and toxic compound extrusion (MATE) super families (130). All the families are encoded by chromosomal genes and require different energy sources (7, 36, 41). Efflux pumps of the ABC family are generally referred to as primary transporters and utilize the extra energy of ATP hydrolysis to extrude drugs from the cell (33). In contrast, the MFS, SMR, RND and MATE superfamilies are secondary multidrug transporters (43) which use transmembrane proton or sodium ion electrochemical gradient to energize the export drugs out of the cell (27, 39, 41, 42).

2.3.1 Primary transporters

The ABC transporters constitute a large superfamily of multi-subunit permeases that transport different molecules (44). These permeases are dependent on ATP as energy source (41, 45). ABC transporters are involved in the uptake of nutrients, the secretion of toxins and antibiotics through the cell membrane, also functionally equivalent to the human P-glycoprotein (MDR1) associated with multidrug resistance shown by tumor cells (41, 46–48). Thus, the same efflux mechanism is used to transfer a variety of substrates across the extra and intracellular membranes (48–50). The ABC transporters consists of two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs) that carry signature motifs engaged in ATP binding (41, 42, 51). These transporters can be classified as importers (when they serve to import molecules from the extracellular to the intracellular environment) and as exporters (when involved in drugs export from cytoplasm to the extracellular environment) (7, 51, 52). Surprisingly, only a few bacterial ABC transporters have been shown to be involved in multiple drug transport (41) (Table 2.2). In the sequenced *M. tuberculosis* H37Rv genome, the genes which encode ABC transporters constitute for about 2.5% of the entire genome content (7, 18, 41, 44, 48, 53).

A limited number of ABC transporters have been shown to be associated with drug resistance in *M. tuberculosis*. However, currently it has been reported that some ABC transporters are involved with reduced susceptibilities of MDR-TB clinical isolates to different antibiotics (2, 4, 18, 34). Such examples include *M. tuberculosis* Rv2686c-Rv2687c-Rv2688c operon encoding an ABC transporter accountable

for FQs efflux when overexpressed from a multicopy plasmid (41, 48). Susceptibility to FQs was subsequently restored with the addition of efflux pump inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), verapamil and reserpine (54). Furthermore, in Pang *et al.* study, the transcriptional level of Rv0933 was significantly upregulated in RIF mono-resistant strains (55). Additionally, it was shown that exposure of laboratory generated *M. smegmatis* mutants, resistant to ciprofloxacin (CIP), resulted in significant upregulation of the *pstB* gene encoding a putative nucleotide-binding subunit of the ABC transporter family. The authors concluded that this observation was primarily due to active efflux of CIP (6, 41). Moreover, currently it was demonstrated that the 3.1- and 5.4-fold over-expression of Rv1217c and Rv1218c (ABC transporters) at transcriptional level resulted in an increased MIC of RIF (OR = 1.01 of Rv1217c and 1.23 of Rv1218c; including INH (OR = 1.17)(56). It has also been shown that the exposure of a clinical isolate of *M. tuberculosis* to RIF lead to overexpression of ABC transporter *pstB* and upregulation of other putative efflux pumps (Rv2136c and Rv1819c) by quantitative real-time PCR analysis (7, 33, 57–60). This suggests that efflux plays a role in RIF resistance in *M. tuberculosis* (7) regardless that 95% of clinical RIF resistance strains harbour mutations in the RIF resistance-determining region (38). *M. tuberculosis* exposure to some drugs used in the treatment of TB has been shown to upregulate efflux pump genes e.g. STR and EMB upregulates the ABC transporter genes *drrAB* in *M. smegmatis* which are also responsible for aminoglycoside-related efflux (Table 2.2). Importantly, the addition of the efflux pump inhibitors reserpine and verapamil restored susceptibility to some of the above compounds (7, 18).

2.3.2 Secondary drug transporters

The secondary transporters can be sub-divided into distinct families of transport proteins that include the MFS, RND, SMR and MATE superfamilies encoded by chromosomal genes (35, 61–63). Each superfamily is characterized by the defined spectrum of antibiotic categories recognized (27, 39, 42). A current study by Dinesh *et al.* (64) demonstrated the involvement of Rv1258c and Rv0849 (MFS), Rv1218c (ABC) and Rv3065 (SMR) efflux pumps in intrinsic resistance to different peptidoglycan synthesis inhibitors (PSI) in *M. tuberculosis* (64) by gene knockout. In addition, they compared *in vitro* activities of the selected drugs (vancomycin, penicillin, meropenem and ceftriaxone) on wild-type (WT) *M. tuberculosis* and the efflux pumps knockout mutants. Interestingly, the PSI showed high potency for the knockout mutants with uniform 4- fold (0.5 µg/ml) drop in their MICs (64).

MFS are distributed in both gram-positive and negative bacteria (42, 65) and play a role in regulatory control by which their efflux mechanism is induced by the compound/drug that it exports (7, 63, 66, 67). Bioinformatics analysis indicate that the genome of H37Rv *M. tuberculosis* consists of 16 open reading

frames encoding putative MFS superfamily efflux pumps (41, 53, 68) (Table 2.2). Examples include the Rv1634 protein transporter which is associated with FQ transport in *M. tuberculosis* (41, 68). This was shown after cloning Rv1634 into different vectors and which resulted in a 2 to 4 fold increase (0.12 to 0.48 µg/ml) in the minimum inhibitory concentration (MIC) of CIP and norfloxacin in *M. smegmatis*. Contradictory studies exist regarding the effect of expression of the MFS transporter, LfrA, in FQ efflux and subsequent susceptibility in *M. smegmatis* (41, 69). The transcriptional regulator, *lfrR*, negatively regulates the expression of *lfrA* (70). Studies showed that the deletion of the *lfrR* gene resulted in an increase of *lfrA* expression. Consequently, the MIC's for CIP, norfloxacin and ethidium bromide increased by 4 to 16 fold in *M. smegmatis* (41, 70). No homolog of the *lfrA* gene exist in the *M. tuberculosis* genome (41, 68). Moreover, it was revealed in Pang *et al.* study that increased transcriptional level of Rv0783 and Rv2936 confers resistance to RIF in RIF mono-resistant *M. tuberculosis* strains (55).

RND superfamily transporters are known to be restricted to mycobacteria and are characterized by 12 transmembrane spans, thereby known as “mycobacterial membrane proteins” (mmpI). Bioinformatics analysis of the whole genome of *M. tuberculosis* H37Rv revealed 15 genes encoding for putative transmembrane proteins belonging to the RND superfamily (Table 2.2) (53). These transmembrane proteins elicit an identical structural sequence which play a role in regulatory control and extrude a variety of different compounds. Efflux depends on gene induction by the compound that they export (41), whereas their functionality depends on the presence and structural orientation of the outer membrane canal protein (OMP) and membrane fusion protein (MFP) to pump drugs out of the cell (36, 71, 72). These two proteins work in conjunction, allowing the bacterium to transport compounds through both cell membranes straight into the external medium (36, 41, 65). This mechanism of action is best described in *Escherichia coli* by the AcrAB/TolC drug efflux pump. The AcrAB (MFP) structural system elicits a broad spectrum of substrate specificity, thus allowing export of a variety of drugs out of the cell via TolC (OMP analogue/orthologue) (41, 73, 74). It has been shown that the MmpL7 protein extrudes INH in *M. smegmatis* (26) thus suggesting that overexpression of *mmpL7* in *M. tuberculosis* result in low-level INH resistance (26, 75). Additionally screening of genomic libraries and whole genome sequencing revealed that 1, 5-diarylpyrrole derivative (BM212) was active against MDR-TB clinical isolates. Furthermore when mapped BM212 to the MmpL3 protein; they found that all BM212 mutants which were characterized had mutations in the *mmpL3* gene (76) (Table 2.2).

SMR superfamily is part of the prokaryotic homo-oligomeric/hetero-oligomeric transport systems (41). These proteins are characteristically 100-120 amino acids in length with 4 membrane-spanning helices. In *M. tuberculosis*, only one protein (Mmr, Rv3065) of this superfamily has been identified by inserting the gene into a multicopy plasmid (Table 2.2). This resulted in a decrease in susceptibility of *M. smegmatis* to

ethidium bromide, erythromycin and acriflavine (39, 41, 77, 78). It was also reported that the deletion of the *mmr* homologue in *M. smegmatis*, increased the susceptibility to cationic dyes and the FQs yet it had no effect on the susceptibility to erythromycin (41, 70).

MATE superfamily transport proteins are the transporters with the least understood function (79, 80), due to their lack of sequence identity to the other 4 known families. This family is divided into 3 large subfamilies made up of 14 smaller groups of which only 3 consists of bacterial MATEs which are either NA^+ or H^+ antiporters (63, 79, 80). It is suggested that NorM of *Vibrio parahaemolyticus* putative protein belonging to MATE superfamily is a multidrug efflux system that extrude norfloxacin, CIP and structurally unrelated compounds kanamycin, streptomycin (25, 27, 79–82). This exporter was identified by phylogenic studies of more than 70 transport proteins families' from *Vibrio parahaemolyticus*, *Vibrio haemophilus* and *Bacillus* species (79, 83). However more research is needed to investigate the function on NorM in mycobacteria as no evidence exists of NorM's function in these bacteria. MATE transporters also export similar drugs transported by RND pumps. However, only few drugs exported by MATE transporters have been identified in mycobacterial species; these drugs include fluoroquinolones (42, 79).

Table 2.2: Putative mycobacterial efflux pumps and genes that might be associated with drug resistance in mycobacteria

Energy Source	Multi-drug pump super family	Gene encoded	Drug extruded	Function	references
Proton (H ⁺) or Na ⁺ exchange	MFS	<i>Rv2846/EfpA</i>	Multiple drugs	Drug export	(7, 41, 42, 53, 70, 84)
		<i>Rv0849</i>	Multiple drugs	Drug export	(43, 68)
		<i>Rv1410/p-55</i>	Aminoglycosides & tetracyclines	Drug export	(33, 41, 85)
		<i>Rv1634</i>	FQs	unknown	(7, 41, 53, 68, 70)
		<i>Rv1258/Tap</i>	Aminoglycosides & tetracyclines	Drug export	(16, 33, 41, 60)
		<i>LfrA</i>	FQs	unknown	(41, 69)
		<i>Rv0783</i>	RIF	Transcriptional regulation	(29–33, 55)
		<i>Rv2936</i>	RIF	Transcriptional regulation	(29–33, 55)
	SMR	<i>Rv3065/mmr</i>	Erythromycin & ethidium bromide	Export of multi drugs	(7, 33, 41, 55, 77)
		<i>emrB</i>	undetermined	Efflux of multiple-drugs	(7, 53, 77)
	Regulatory Protein	<i>whiB7</i>	RIF	Transcriptional regulation	(32, 58)
	RND	<i>mmpL-7, mmpL3</i>	INH, RIF	Export of antibiotic multicopy plasmid	(7, 26, 33, 41, 53, 75)
	ATP hydrolysis	ABC	<i>PstB (mtp1)</i>	FQs (specifically) CIP	Overexpression in CIP-resistant mutant & import of inorganic phosphate
<i>ddrAB</i>			Streptomycin, ethambutol, tetracycline, norfloxacin & erythromycin	unknown	(18, 33, 41)
<i>RV2686c-2687c-2688c</i>			FQs	Drug export	(7, 33, 41, 44, 48, 53)
<i>Rv1218c</i>			Multiple drugs	unknown	(43, 86)
<i>R1217c</i>			RIF, INH	Drug export	(44, 55)
<i>Rv0933</i>			RIF	Transcriptional regulation	(55, 87, 88)

2.4. DIFFERENT EFFLUX PUMP INHIBITORS' EFFECT ON MYCOBACTERIAL GROWTH

The emergence of MDR- and XDR-TB has increased the complexity of the TB treatment regimen. Furthermore, it has emphasized the urgency in drug development and accelerated progress in new drug regimen development pipelines to control TB disease and transmission (89). Recently, efflux pump inhibitors (EPIs) have been described as putative new drug compounds as they have the ability to restore susceptibility to antibiotics by blocking the activity of efflux pumps (25, 27). There are different types of efflux pumps inhibitors which include synthetic analogues and those derived from natural sources.

2.4.1 Proton-motive force and Ca^{2+} channel blockers

The proton motive force and Calcium channel blockers inhibit efflux pump activity by proton pump interaction and reduction in the trans-membrane potential (42, 90–92). These include carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (Figure 2.1A), dinitrophenol (DNP) (Figure 2.1B), valinomycin (Figure 2.1C), verapamil (Figure 2.1D) and phenothiazines (Figure 2.1E) (42, 48, 92–96). *In vitro* and molecular based research has shown that these compounds mainly inhibit the activity of efflux pumps belonging to the MFS superfamily (97, 98), with the exclusion of verapamil which inhibit the activity of the ABC superfamily.

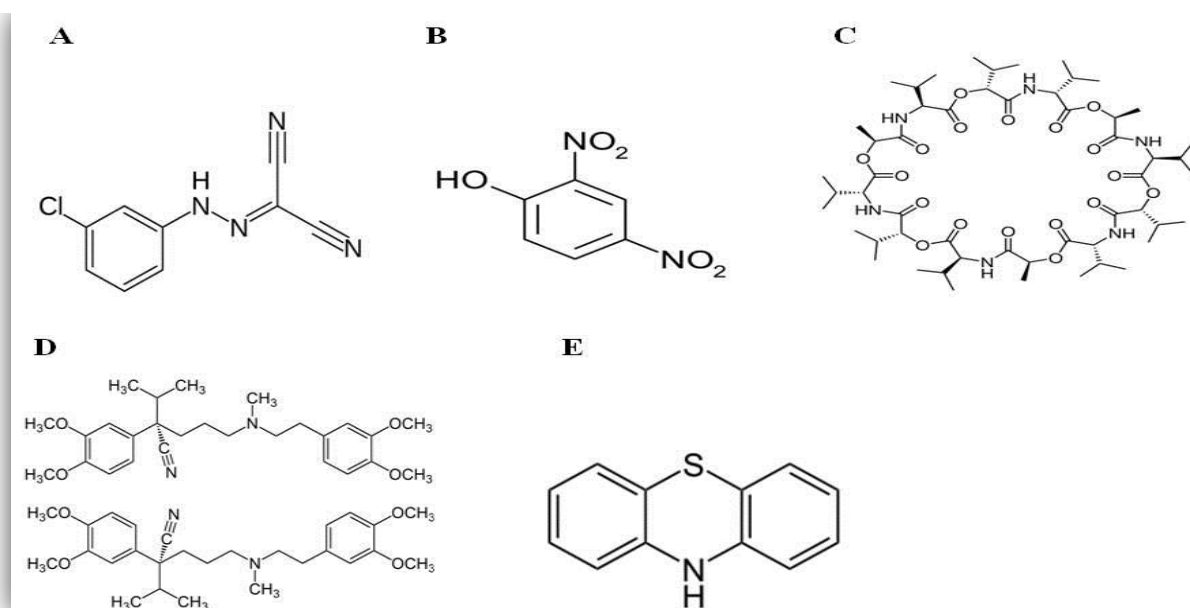


Figure 2.1: Chemical Structures of efflux pump inhibitors synthesised chemically. A) CCCP; B) DNP; C) Valinomycin; D) Verapamil; E) Phenothiazines (42, 48, 92–96).

2.4.1.1 CCCP and DNP

CCCP and DNP (Figure 2.1A and 2.1B respectively) disperse the membrane proton-motive force by modification of the trans-membrane electrochemical potential which results in cell death (27, 42, 99, 100). These compounds are rarely used commercially because of their toxic properties (101, 102). Additionally, these compounds are ionophores that act as chemical inhibitors of oxidative phosphorylation which in return inhibit the activity of ATP synthase (103).

In a recent *in vitro* study, it was observed that the addition of CCCP and DNP to ofloxacin resistant *M. tuberculosis* clinical strains with *gyrA* mutations resulted in a 2- to 8- fold decrease in the ofloxacin MIC. This therefore suggests that the level of ofloxacin resistance was increased by an efflux mechanism (98, 104). In addition, the same phenomena was reported in Gupta *et al.* study, the presence of CCCP and verapamil reversed resistance to RIF, INH, STR and OFL in *M. tuberculosis* isolates (33, 98). Further evidence to support CCCP as an effective efflux pump inhibitor includes recombinant *M. tuberculosis* H37Ra strains overexpressing Rv2459 (*jefA*), which resulted in an increase in the MIC of INH and ethambutol (EMB). The subsequent addition of CCCP to the cells resulted in a decrease in the resistance INH resistance (105, 106). Moreover, it was currently demonstrated that the overexpression of *mmr* (Rv3065) resulted in increased INH MIC (from 0.25 to 4 µg/ml) and decreased susceptibility to ethidium bromide, erythromycin and acriflavine (78). Furthermore, the addition of efflux pump inhibitors CCCP and verapamil was able to restore the decreased susceptibility (78).

P55 is a tap-like MFS multidrug efflux pump (Table 2.2) that confers low-level resistance to a broad range of compounds (33, 85, 107). Recent studies provided evidence of the ability of CCCP and DNP to inhibit P55-defined drug resistance (16, 93, 108, 109). Another study also illustrated that treating *M. tuberculosis* and *M. bovis* BCG wild type strains with ³H-enconazole + CCCP, inhibited MmpS5-MmpL5 efflux pumps. Additionally, that resulted in a rapid enconazole's accumulation increase in E3 and K7 mutant strains to the same levels as the wild-type strains (109, 110). Therefore it is clear that the inhibitory effect of CCCP and DNP might lead to a decrease in drug resistance and thus improving current TB treatment. However due to their toxicity, more novel research methods are needed in order to validate these findings *in vivo*.

2.4.1.2. Valinomycin

Valinomycin (Figure 2.1C) is an inhibitory compound which depletes the electrochemical gradient generated by potassium ions (K⁺) (27, 42, 99, 100). It is commercially known as dodecadeptide that is extracted the *Streptomyces* species (111). Valinomycin has a high selectivity for K⁺ over Na⁺ within the

cell membrane. It is suggested to be a potassium-specific transporter that facilitate the movement of K^+ through lipid membranes “down” an electrochemical potential gradient (111–113).

Limited reports exist to support valinomycin as an inhibitor of mycobacterial efflux pump activity. One of such reports provides evidence of valinomycin inhibiting P55-determined drug resistance in *M. tuberculosis* which prevents drug entry, thus proposing the active export of the compound as energy source using the transmembrane proton and electrochemical gradients (107, 109). Additionally, microarray analysis of *M. tuberculosis* strains treated with valinomycin showed a significant decrease of *p27* and *p55* expression levels (107, 109). The treatment of naturally PZA resistant *M. smegmatis* with valinomycin showed an increase in the accumulation of pyrazinoic acid at neutral pH in *M. tuberculosis* (114, 115), illustrating the effect of efflux activity on the natural resistant PZA phenotype in these cells.

2.4.1.3. Verapamil

The Ca^{2+} channel blocker Verapamil (Figure 2.1D) belongs to phenylalkylamines prototype class. Commercially verapamil is used to treat various disorders including angina pectoris, hypertension and cardiac arrhythmia, headaches and migraines (109, 116–118). Verapamil acts by inhibiting vesicular monoamine transporters and P-glycoprotein in mammalian cells (109, 119). In prokaryotes, it inhibits ATP-dependent multidrug transporters and MDR pumps of parasites (39, 42, 99, 120). Interestingly, reports also illustrate that this type of inhibitor interferes with the generation of the proton-motive force (121). To date, numerous studies showed that verapamil has a significant inhibitory effect on mycobacterial efflux pump activity (95, 96, 122).

Additionally, these studies demonstrate that verapamil inhibits active efflux of ethidium bromide in *M. avium* and *M. tuberculosis* strains (120, 121, and 150). Furthermore, macrolide resistance in clinical *M. avium* complex strains could also be reversed with the addition of verapamil (95, 96, 109). Further support for the restoration of susceptibility comes from a recent study which showed that induced induction of INH resistance in *M. tuberculosis* strains could be reversed with the addition of verapamil to these cultures (123). Similarly, a recent *in vitro* study demonstrate that RIF resistance in mono-resistant and MDR *M. tuberculosis* strains could be reversed with the addition of verapamil. Furthermore, RIF induced OFL resistance was reversed with the addition of verapamil (30).

Recently, Adams *et al.* showed that inhibition of mycobacterial efflux pumps with verapamil reduced macrophage-induced tolerance by 2-fold in a *M. marinum*-infected zebrafish larval model (124). In addition, Gupta *et al.* revealed that addition of verapamil to standard TB chemotherapy accelerated the bacterial clearance close to sterilization and lower relapse rates (4 months treatment) in a mouse model infected with *M. tuberculosis* (124, 125). Therefore, the latter studies suggest the use of efflux pumps

inhibitors as adjunctive drugs which might significantly enhance potency of current anti-TB therapy. The potential clinical implication of using verapamil to restore susceptibility was demonstrated in a mouse infection model where treated of an MDR-TB infection with first-line TB drugs and verapamil, resulted in a significant decrease in the bacillary load (30, 98). However, the clinical use of verapamil in high concentrations is not advised due to adverse effects, such as headaches, swollen hands and legs, appetite loss, blurred vision, stomach pain, fever, flu-like symptoms, heartburn, constipation and nausea, despite these limitations, attention has been drawn to the potential use as efflux inhibitors to rejuvenate the efficacy of failing treatment regimens.

2.4.1.4 Phenothiazines

Phenothiazine (Figure 2.1E) is a yellow tricyclic compound that is a substituent in various antipsychotic and antihistaminic drugs. Phenothiazine has derivatives widely used as drugs commercially including chlorpromazine, piperidine and thioridazine. These compounds are well known for their *in vitro* and *in vivo* antimycobacterial activity (109, 126–128). Phenothiazines are potential inhibitors of K⁺ transport with the ability to reverse the MDR phenotype. They also inhibit the proton motive-force dependent pumps by interaction through reduction in the trans-membrane potential (42, 102, 129, 130). Due to the emergence of more MDR-TB cases, it is suggested that phenothiazines have potential for the treatment of tuberculosis (131, 132).

It is speculated that thioridazine has *in vitro*, *in vivo* and *ex vivo* activity against susceptible and resistant *M. tuberculosis* strains (127). Recently, it was demonstrated in an *ex vivo* experiment that thioridazine enhances the intracellular killing of phagocytised *M. tuberculosis* with a higher transport inhibition (126, 128, 132, 133). Furthermore, it was also shown that both thioridazine and chlorpromazine inhibit ethidium bromide efflux in *M. smegmatis* and *Mycobacterium avium complex* (MAC) (95, 96, 124, 128, 129). Moreover, thioridazine reduced clarithromycin resistance and elicited an effect on INH resistance in *M. tuberculosis* complex (121). More research is required to determine phenothiazine's ability to inhibit efflux in mycobacteria, as it is shown that these compounds have only a limited inhibitory effect *in vitro* (121, 123). It is suggested that improving phenothiazine basic structure might make this compound more effective *in vitro* (109).

2.4.2 Inhibitors from natural (plants) sources

Alkaloids inhibit multidrug transporters and act as potential targets to help improve TB therapy. These include: 1. the plant alkaloid reserpine (Figure 2.2A) 2. Piperine, trans-trans isomer of 1-piperoyl-piperine from the *Piperaceae* family (Figure 2.2B) and 3. Berberine from the *Berberis* family (Figure 2.2C) (134–

137). Basic molecular and clinical research is required on these compounds as no data exists that could elucidate their interaction *in vitro* and *in vivo*.

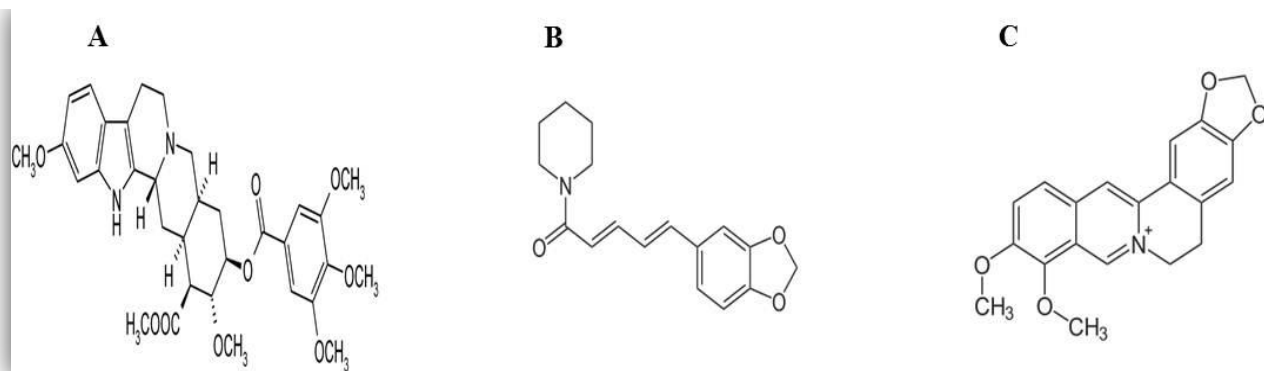


Figure 2.2: Chemical structures of efflux pump inhibitors derived from natural resources. A) Reserpine; B) Piperine; C) Berberine (134–137).

2.4.2.1. Reserpine

Reserpine (Figure 2.2A) is a naturally occurring compound isolated from the roots of *Rauwolfia vomitoria* Afz (42, 138). It is used commercially to treat wild hypertension, reduces blood pressure and has been shown in randomized controlled trials to reduce mortality of persons with diseases stated above (139). Furthermore, it irreversibly blocks the uptake (and storage) of dopamine into synaptic vesicles by inhibiting the vesicular monoamine transporters (140). Reserpine is also used to treat psychotic disease, but its use is restrained due to adverse effects it results in. These include nausea depression and nasal congestion. However, despite its property to act as carcinogen, it is still considered essential and as a promising efflux inhibitor (109, 141). Literature reports that it acts as drug potentiator by interacting directly with amino acids characteristic of some efflux proteins. One example is the Bmr protein responsible for tetracycline efflux. It is also reported that the addition of reserpine resulted in 4-fold MIC reduction to tetracycline in *B. subtilis* (137, 142, 143). Moreover, NorA-conferred resistance was fully reversed by reserpine, thus resulting in suppression of MDR transporters (144) in *Staphylococcus aureus* responsible for a decrease in FQs susceptibility (137).

Recent literature supports evidence of reserpine as an inhibitor in mycobacterial efflux activity (26, 145). It was observed that the addition of reserpine to *M. smegmatis* with high level of INH resistance stimulated by overexpression of the *M. tuberculosis mmpL7* gene (Table 2.2) resulting in a decrease of the level of INH resistance (19, 26, 145). Furthermore, it was also shown that reserpine inhibited the efflux pump responsible for pumping out active form of pyrazinamide (PZA); that is pyrazinoic acid (POA) in

M. tuberculosis, thereby increasing susceptibility to pyrazinamide (PZA) (19, 109, 115, 146). Numerous studies showed that reserpine increase sensitivity to both INH and ethidium bromide in *M. bovis* BCG and decrease isoniazid efflux in *M. tuberculosis* (109, 147, 148). Reserpine has also been shown to restore susceptibility to ofloxacin (OFL) from 53 to 81% in 15 MDR-TB isolates tested (30). Recent studies, identified the presence of antioxidant and antimycobacterial activities in reserpine which could be of value in inhibiting *M. tuberculosis* efflux (148, 149).

2.4.2.2. Piperine

Piperine (Figure 2.2B) is present in black pepper and isolated from *Piper nigrum* sp. (150). It was used in ancient times in some forms of traditional medicines. Piperine is a drug potentiator that inhibits the human P-glycoprotein (135, 150), particular cytochrome P450-mediated pathways and phase II reactions in animal models (135, 151, 152). Commercially, it inhibits enzymes important in drug metabolism, transport of metabolites and xenobiotics. At the same time, it subsequently increases the bioavailability of various compounds and alters the effectiveness of some medications (134, 135, 150). Studies done on piperine and piperidine, suggests its inhibitory action against bacterial efflux pumps (132, 135, 150, 153), including its role towards mycobacterial efflux pump activity (136). Furthermore studies have demonstrated the efficacy of piperine as potent inhibitor in NorA-overexpressing *S.aureus* strain 1199B, whereby the MIC of the ciprofloxacin-resistant was reduced 2-fold reduction with the addition of piperine (154).

A study demonstrated inhibitory effect of piperine on the putative multidrug efflux pump in *M. tuberculosis*, Rv1258c (136). In that study the expression level of *Rv1258c* was assessed after treating *M. tuberculosis* H37Rv, clinical and lab-generated RIF resistant mutants, with a combination of RIF and piperine. After treatment, the investigators observed synergy between the piperine and RIF resulting in a reduction in the RIF MIC by 4- to 8-fold (lower than 2 mg/L). Gene expression analysis of *Rv1258c* revealed a 3.6-fold increase on the transcript level of *R1258c* conferred by RIF in RIF-resistant *M. tuberculosis*. Additionally combination treatment of RIF and piperine could restore the RIF MIC phenotypically to its wild type (57, 136). Additionally it was observed from a modulation assay in *M. smegmatis* that piperine decreased the MIC of ethidium bromide by 4-fold (155). This suggests that piperine can inhibit mycobacterial efflux pumps.

2.4.2.3. Berberine

Berberine (Figure 2.2C) is isolated from *Berberis fremontii* and is a nucleic acid-binding isoquinolone alkaloid with broad spectrum therapeutic properties (156). Studies on Biberine were mainly focused on its

beneficial effects to the cardiovascular system (58), its anti-inflammatory properties (157) and its ability to suppress different tumour cells growth in cancer (158). It is used to treat neuro-inflammation-associated disorders (157). This compound has been shown to be an inhibitor of multidrug resistant pumps. It inhibits growth of *S. aureus in vitro* when used in combination with methoxyhydnocarpin. (159). Although it has been suggested previously that berberine has weak antibacterial activity alone, it was shown to have a synergic effect when used in combination with other compounds such as 5'-methoxyhydnocarpin-D, norfloxacin and other drugs which are NorA substrates in mycobacteria (137, 159). However, limited data exist of the significance of this compound in mycobacterial resistance studies.

2.5: NOVEL MYCOBACTERIAL GROWTH INHIBITORY COMPOUNDS

The focus of recent research has been geared towards the design of novel growth inhibitory compounds. These include drug candidates such as Farnesol, PA-824, OPC-67683, TMC 207, PNU-100480, SQ109 and new chemical entities which include IFN55, IFN271, IFN240 (92, 109, 160, 161). Studies report the growth inhibitory effect of these compounds either alone or in combination with already existing drugs to improve TB treatment (160, 162). Some of these novel compounds show significant growth inhibition properties against *M. tuberculosis* and therefore could potentially aid in the TB treatment regimen (163–170).

2.5.1 TMC207

TMC207 is a novel diarylquinolone anti- TB drug candidate with bactericidal and sterilizing activity against drug-susceptible and drug-resistant *M. tuberculosis in vitro* (164). This drug has advanced to phase II clinical trials, for instance an early bactericidal activity (EBA) study of different TMC207 doses performed on 75 smear-positive TB patients infected with drug-susceptible *M. tuberculosis* stains (164). This study showed potent bactericidal activity after 4 days after the start of treatment. Moreover TMC207 inhibits the activity of ATP synthase, an essential enzyme for *M. tuberculosis* ATP synthesis (164, 165, 169–172). It has an MIC ranging from 0.030 to 0.120 µg/ml in *M. tuberculosis* (Table 2.3) (165, 170, 171, 173). Spontaneous mutant selection and subsequent whole genome sequence analysis of the resistant *M. tuberculosis* and *M. smegmatis* mutants identified mutations (A63P and D32V) in the c-subunit of ATP synthase encoded by the *atpE* gene (Table 2.) (171). Mutations in *atpE* only partially account for the TMC207 resistance phenotype. No mutations in the *atpE* gene were observed in 38 out of 53 spontaneous mutants (168, 209). However, polymorphisms observed in c protein of ATP synthase did not influence the resistance phenotype (173). Some studies reported that TMC207 has a potent early and late bacterial activity, good pharmacokinetic and pharmacodynamic properties with a long half life, no effective

toxicity in mouse and preliminary human testing (165, 167, 169, 171). Studies demonstrate synergistic activity of TMC207 with SQ109, which showed a 4-fold to 8-fold TMC207 MIC decrease for *M. tuberculosis* H37Rv (179).

Interestingly in some studies (210), it was observed that TMC207 still exert significant bactericidal activity even with a 50% plasma concentrations reduction when exposed to RIF, thus suggesting low drug interaction relevance and potency of TMC207 (169). Moreover, an additive effect was observed with the treatment of the combination of RIF and TMC207 (179). In addition a significant synergistic effect is observed when PZA and TMC207 are combined (154, 155, 159, 199). This supports the findings of previous studies that demonstrated the strong bactericidal activity of TMC207 combined with first- or second-line anti-TB drugs (Table 2.3) (164, 165, 169, 174). Mice treated with the combination of TMC207 and PZA showed a significant decrease in lesions than those treated with RIF, INH or moxifloxacin (MOXI) alone (164, 174), moreover the colony forming units (CFU) decreased significantly in mice treated with the PZA-TMC207 combination than in mice treated with either TMC207 or PZA alone. Additionally more synergistic interactions included the following 3 drug combination, TMC207-INH-PZA, TMC207-RIF-PZA; TMC207-MOXI-PZA and PNU-100480 TMC207 (179, 211). This data indicates TMC207's clinical significance in TB treatment.

2.5.2 SQ109

SQ109 is a novel inhibitory compound discovered from a library based on the 1,2 ethylene diamide structure of EMB (212). SQ109 is less toxic and exhibit high potency against replicating *M. tuberculosis* (212). It has an MIC ranging from 0.16 µg/ml to 0.64 µg/ml in *M. tuberculosis* (Table 2.3) (164, 165, 177). Early clinical trial data mark it as a compound that could contribute significantly in susceptible and MDR-TB treatment and in addition was shown to have a significant activity against intracellular bacilli treated during first 2 months of intensive phase therapy (213). SQ109 disrupts cell wall synthesis by interfering with the incorporation of mycolic acids into the cell wall core of *M. tuberculosis* (169, 176). The target of SQ109 is mmpL3 (Table 2.3), a mycolic acid transporter required for the incorporation of mycolic acid into the *M. tuberculosis* cell wall (164, 180). Numerous studies provide evidence of synergistic properties of SQ109 in combination with anti-TB drugs used in the current TB treatment regimen and other inhibitory compounds (Table 2.3) (164, 165, 169, 170, 214). A significant interaction was observed between SQ109 and TMC207 *in vitro*, with a 4- to 8-fold TMC207 MIC decrease in *M. tuberculosis* H37Rv (Table 2.3) (179). Additionally antimycobacterial activity studies done in murine models reported similar SQ109 activity to that of INH but more potent to that of EMB (164, 178) and these suggests SQ109 might replace EMB in the future for susceptible *M. tuberculosis* strains.

Synergy was also observed between SQ109 and RIF in *M. tuberculosis*, whereby RIF caused the SQ109 MIC to be 16-fold lower than that obtained with SQ109 alone. The presence of SQ109 also resulted in a decrease in the MIC of RIF by 30-fold (176, 179). The synergistic activity between RIF and SQ109 brings hope for improving TB therapy as RIF is one of the essential front-line TB drugs used as a surrogate marker to detect of MDR-TB (Table 2.3). Most reported studies were done in *in vitro* assays; therefore studies are needed to assess the performance of this drug *in vivo*. Some *in vivo* studies using the chronic mouse model of TB showed improved efficacy of first-line drug therapy combination after 4 to 8 weeks of treatment when SQ109 (10 mg/kg) was substituted for EMB (100 mg/kg) (170, 214). Next step would be to test the efficacy and pharmacokinetics of this novel compound in combination with other antitubercular drugs in MDR-TB patients; to ensure less adverse events and toxicity which have a bad influence in immuno-compromised MDR-TB and TB patients.

2.5.3. PA-824 & OPC 67683

PA-824 and OPC-67683 are nitromidazoles. PA-824 exhibits bactericidal activity against actively replicating and non-replicating *M. tuberculosis*. PA-824 is a pro-drug that is activated by the mycobacterial nitroreductase Ddn, a deazaflavin F420-dependent enzyme (215). After activation, toxic forms of PA-824 are able to inhibit protein synthesis and cell wall lipid synthesis, resulting in cell death. Investigation on the modes of action of PA-824 has shown that intermediate metabolites of PA-824 act as intracellular nitrogen oxide donor, therefore encouraging intracellular killing of *M. tuberculosis* in anaerobic conditions (196, 216). It inhibits cell wall lipids and protein synthesis yet its target is not fully described up to date (164, 165, 169, 170). Its MIC range from 0.015 to 0.25 µg/ml in *M. tuberculosis* (Table 3) (165, 169, 193, 196). Early bactericidal activity (EBA) studies showed its synergistic properties with PZA and MOXI (217). In a (211) current study, discrepant results were obtained for PA-824, TMC207, PNU-100480 combinations; combinations of PNU-100480, TMC207, and SQ109 were fully additive, whereas those including PA-824 were less than additive or antagonistic (211, 218). It is speculated that the latter is due to fact that other drugs might be more “sensitive” to bacilli growth phase yet PA-824 action is concentration dependent (165, 169, 193, 196) including microbial culture conditions (219). Recent studies reports the promising efficacy of PA-824 (160, 214) without INH (169), that might be developed to treat latent TB in combination with MOXI (220) and no cross-resistance is observed with front-line anti-TB drugs (Table 2.3) (196).

OPC-67683 is a novel inhibitory prodrug candidate with strong activity against susceptible and MDR-TB (164, 165, 169, 170, 197, 198). It is structurally related to PA-824 and shares the same mechanism of action (165, 197), however with its MIC 10 times lower than MIC of PA-824. OPC-67683 is reported to be free of mutagenicity to cause permanent genetic alterations and exert highly potent activity against *M.*

tuberculosis and MDR-TB (197); Moreover its MIC ranges from as low as 0.006 to 0.024 µg/ml in *M. tuberculosis* (Table 2.3) (164, 165, 169, 170, 197, 198). It targets methoxy and keto-mycolic acids and inhibits the cell wall biosynthesis (164–167, 169, 170, 197, 198). Recent studies provide evidence of superior sterilizing activity of OPC-67683 in an *in vitro* model of drug-tolerant *M. tuberculosis* (169, 221). Combination treatment of OPC-67683 (2.5 mg/kg) with RIF (5mg/kg)/PZA (100 mg/kg) showed significant sterilizing activity with regards to time to negativity and lung bacillary load assessment over 2 months treatment in *M. tuberculosis* compared to low synergistic effect found for standard regimen RIF (5 mg/kg)/PZA (100mg/kg)/INH (10 mg/kg)/EMB (100 mg/kg) in mouse and murine model *in vivo* (164, 165, 169, 170, 197). Moreover preliminary human studies done with PA-824 and OPC-67683 (167, 222, 223), showed treatment with PA-824 to be more effective at following concentrations; 200 mg, 600 mg, 1000 mg or 1200 mg all per day for 14 days (224). Thus these findings emphasize the good synergistic properties of these nitroimidazoles with other anti-tubercular drugs. It could possibly have clinical implications in shortening TB treatment, however still more tests are required with the same reproducible results *in vitro* and *in vivo*.

2.5.4 SUTEZOLID & LINEZOLID

Sutezolid is an oxazolidinones with highly potent activity against drug-susceptible and resistant *M. tuberculosis* (225). It has an MIC as low as 0.03 to 0.50 µg/ml in *M. tuberculosis* (Table 2.3) (164–166, 169, 170, 185, 225). Sutezolid targets the 50S subunit of ribosomes and inhibits the protein synthesis in *M. tuberculosis* (164, 168, 170). Current studies report superior sterilizing activity of sutezolid in combination with the standard first-line drugs RIF, INH and PZA in murine models (164, 189, 226). This suggests that the duration of TB treatment could be shortened. In addition, a synergistic effect was observed for the sutezolid-MOXI/PZA/TMC207/SQ109 and PA-824 combination in *M. tuberculosis* (164, 170, 189). None of these novel drug compounds exhibits cross-resistance with existing TB drugs (Table 2.3) (164, 168). Some phase I studies have demonstrated that sutezolid is safe and well tolerated at all doses and shows synergy with PZA in an *ex vivo* whole-blood culture assay (164, 165, 170, 188, 211). This suggests that new regimens comprised of this drug compound might aid in treating both drug sensitive and resistant *M. tuberculosis* strains because of its synergistic effect with PZA.

Linezolid was the first class of oxazolidinone approved for clinical use, yet its use in TB has some limitations due to adverse effects (227). It is a structural analogue of sutezolid with broad activity against mycobacteria and gram-positive bacteria (164). Linezolid has an MIC that ranges from 0.125 to 1.0 µg/ml in *M. tuberculosis* and it has a unique mechanism of action which is supported by the absence of cross-resistance to other antibiotics (164, 165, 184, 186, 186). It inhibits the early steps of protein synthesis by

binding to 23S RNA in the 50S ribosomal subunit (164, 168, 170, 190). Even though clinical resistance to linezolid has been found to be rare, a study by Richter *et al* reported 1.9% resistance among 210 MDR strains (168, 228). *In vitro*-selected mutants with high level resistance to linezolid (MIC = 16 – 32 mg/l) have been reported to have mutations a G2061T and G2576T in the 23S rRNA gene (Table 2.3) (186, 228). Linezolid efficacy to treat MDR-TB in combination regimens was evaluated in two studies with a total of 11 patients. Some patients were cured after treatment (sputum conversion and culture negativity) when administered with doses ranging from 600 to 1200 mg/day (160, 214). However toxic side effects, such as peripheral and optic neuropathy were common in these patients. In addition, linezolid is used off-label as third-line drug in combination regimens to treat MDR-TB and XDR-TB (170, 182).

2.5.5 MOXIFLOXACIN AND GATIFLOXACIN

Moxifloxacin (MOXI) and Gatifloxacin are fluoroquinolones (FQs), which are promising novel growth inhibitory compounds of *M. tuberculosis* for shortening TB treatment. (229, 230). MOXI is a broad-spectrum 8-methoxy FQ with potent bactericidal activity against *M. tuberculosis* and has a low MIC ranging from 0.18 to 0.5 µg/ml (169, 170, 200, 201, 203, 230). It targets the mycobacterial topoisomerase II DNA gyrase and blocks the movement of replication forks and transcription complexes (Table 2.3) (165, 168–170, 200, 231–233). Cross-resistance was reported within the FQs group, including mutations in QRDR of *gyrA* and *gyrB* genes encoding the A and B subunits of DNA gyrase (53, 168, 202, 234). Current *in vitro* and mice studies provide evidence about enhanced bactericidal activity when MOXI and INH is administered together (169, 235–237). Furthermore it is reported that the synergistic activity of RIF/MOXI with PZA could result in a novel short TB treatment regimen of 2 months, followed by 4 months of RIF/MOXI alone, then another regimen of 2 month RIF/INH and PZA followed by 4 months of RIF/INH (169, 238). Moreover MOXI is also synergistic when combined with PZA and TMC207 (164). Even more important MOXI efficacy is also evident in human studies (169) hence there are a number of EBA studies still on-going (51).

Gatifloxacin, has a low MIC which ranges from 0.12 to 0.25 µg/ml (169, 170, 200, 201, 203, 230). It also targets and blocks mycobacterial DNA gyrase, thus preventing chromosomal replication of *M. tuberculosis* (Table 2.3) (169, 233). Cross-resistance was observed and the mechanism of resistance is similar to that of MOXI (169, 199, 202, 204). Gatifloxacin shows synergistic activity in RIF/INH with combination PZA (164, 169, 205, 206, 208) *in vitro* and mice studies in *M. tuberculosis*. Moreover, greater bactericidal activity was observed when gatifloxacin (100 mg/kg) was combined with ethionamide (ETH) (75 mg/kg) and EMB (100 mg/kg) in a mice model (169, 170, 207, 239).

Table 2.3: Functional properties and promising combinations of novel mycobacterial inhibitory compounds (*in vitro* studies).

Drug Class	Compound name	Mechanism of action	Target, mechanism of resistance and drug interaction	MIC range in <i>M.tuberculosis</i> ($\mu\text{g/ml}$)	Drug combination property	References
Diarylgui-nolones	TMC207	Inhibits its proton pumps	Target AtpE (ATP synthase); no cross-resistance with Anti-TB drugs; mutations in the <i>atpE</i> gene	0.030 to 0.120	Synergistic with SQ109; PNU1000480; PZA; PZA+INH; PZA+RIF; PZA+MXF;	(165, 169–175)
	SQ109	Disrupts cell wall assembly and block efflux pump activity	Target MmpL3; no cross-resistance; no mutations except up regulation of <i>ahpC</i> expression	0.16 to 0.64	Synergistic with RIF; INH; TMC207	(164, 165, 168–170, 176–180)
Oxazolidinones	Linezolid	Inhibits protein synthesis	Target 50S subunit of ribosomes; unknown cross-resistance; mutations at G2061T and G2576T in the 23S rRNA gene	0.125 to 1.0	Results in serious adverse events (181–183)	(165, 166, 170, 184–191)
	PNU-100480	Inhibits protein synthesis	Target 50S subunit of ribosomes; unknown cross-resistance; mutations at G2061T, G2576T in the 23S rRNA gene	0.03 to 0.50	Synergistic with PZA; TMC207; RIF + INH + PZA	(165, 166, 170, 188–192)
Nitrimid-azoles	PA-824	Prodrug activated by a nitroreductase (Ddn): inhibits cell wall lipids and protein synthesis	No cross-resistance; mutations in the <i>Rv3547</i> gene	0.015 to 0.25	Synergistic with RIF+ PZA	(164–166, 168–170, 193–196)
	OPC-67683	Inhibits cell wall biosynthesis	Target methoxy- and keto-mycolic acids; no cross-resistance; mutations in the <i>Rv3547</i> gene	0.006 to 0.024	Synergistic with RIF+PZA	(164–166, 169, 170, 196–198)
FQs	Moxifloxacin	Inhibit bacterial DNA replication	Target DNA gyrase and topoisomerase IV; cross-resistance within FQs group; mutations in QRDR of <i>gyrA</i> and <i>gyrB</i>	0.18 to 0.5	Synergistic with TMC207 + PZA; RIF + PZA; Rifapentine	(53, 165, 166, 169, 170, 199–206)
	Gatifloxacin	Inhibit bacterial DNA replication	Cross-resistance within FQs group: mutations similar to Moxifloxacin	Range from 0.12 to 0.25 $\mu\text{g/ml}$	Synergistic with RIF + INH + PZA; Ethionamide + EMB	(53, 166, 169, 201, 202, 205, 207, 208)

2.6. Concluding Remarks

Novel resistance mechanisms such as efflux are making it difficult to pin-point weaknesses in the genome of *M. tuberculosis* for drug design, because *M. tuberculosis* seems to be able to evolve in such a way that it has become resistant to most anti-TB drugs and in certain instances it also developed a TDR phenotype. These challenges therefore highlight the need for new treatment regimens. These include the use EPI's in combination with already existing drugs to help increase the potency of current anti-TB drugs. Since, to discover and develop a new drug is a lengthy process which takes years. Potentially, it could take more than 10 years from discovery, efficacy studies and to clinical trials (phase I, II and III). There have been some promising *in vitro* and *in vivo* reports suggesting the importance of using EPI's as treatment-shortening adjunctives to improve TB treatment control. Additionally, recent advances have provided new hope for the development of new treatment regimens which also targets efflux activity. These include SQ109 and TMC207 examples of drugs currently in clinical trials. Additionally, there have been promising reports about Q203 as a new clinical candidate for the treatment of TB.

Moreover, the observation that the new compounds seem to be enhancing the activity of existing anti-TB drugs, either through synergism or additive effects as was seen in animal models and EBA studies, will aid in the design of efflux pump inhibitor/antibiotic combination regimens to improve TB treatment. Additionally, natural sources e.g. plants, can be exploited for medicinal purposes as exemplified by biberine, piperine and reserpine etc. However, a key aspect is the collaboration between scientists from different disciplines (molecular biologists, clinicians etc), to establish a good foundation for the progression of translational research. It is important to validate *in vitro* findings in an *in vivo* model. In brief, due to increasing MDR-TB transmission cases (man-made MDR-TB), eradication requires more than just novel drug regimens but also most significant, managed drug delivery, patient care and the support from research consortia especially in resource-poor countries.

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CHAPTER 3
MATERIALS AND METHODS

3.1 EXPERIMENTAL STRATEGY

Complementary strategies were used to determine the effect of the EPIs on anti-TB drugs in RIF resistant *M. tuberculosis* clinical isolates (Figure 3).

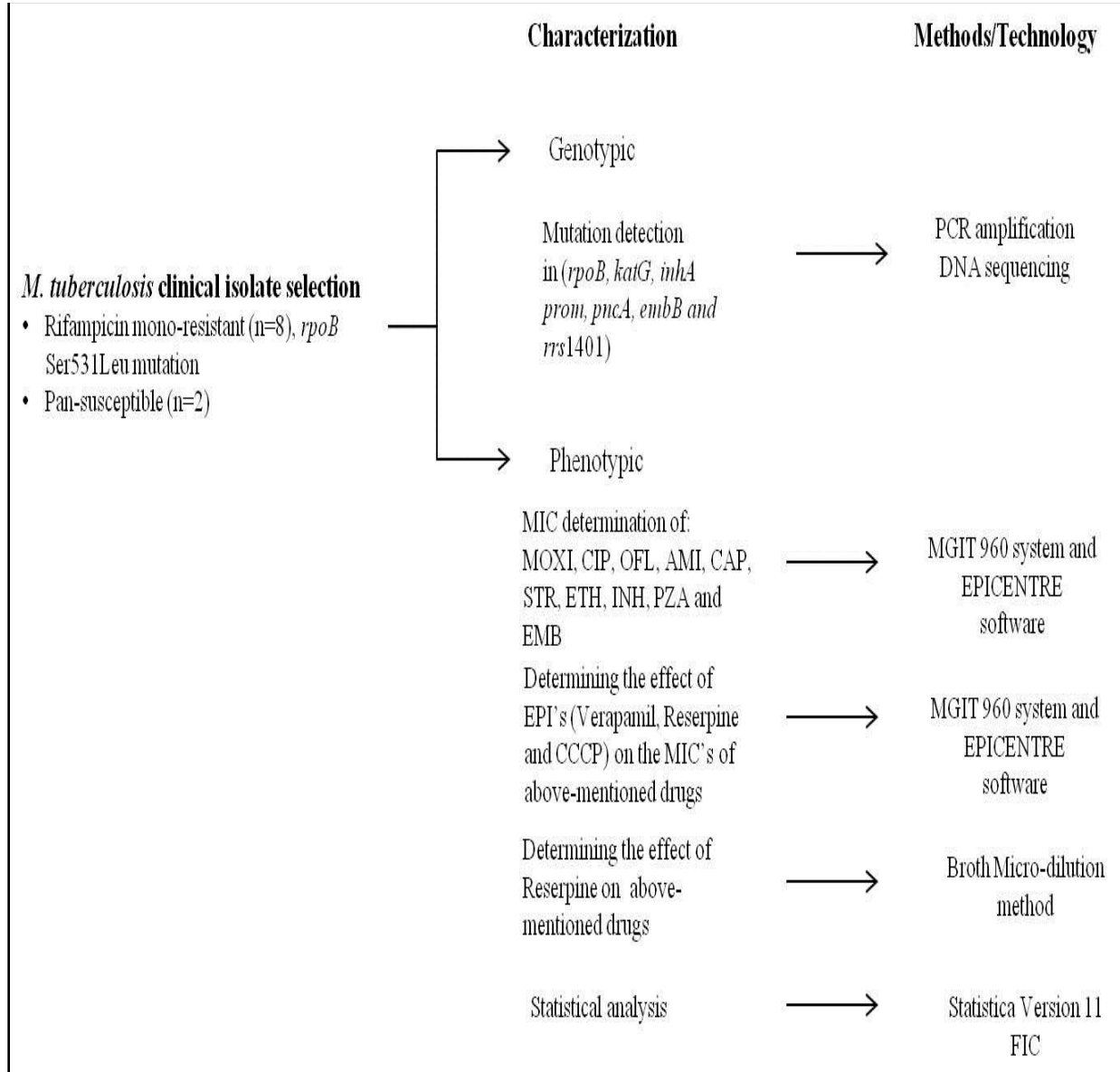
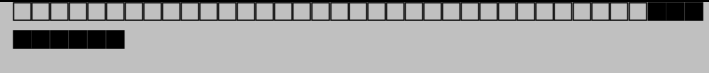

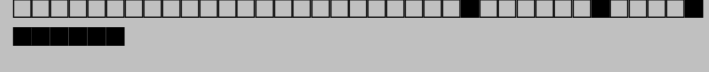




Figure 3: Summary of the complementary experimental strategies used in this study.

3.2 STRAIN SELECTION

Eight RIF mono-resistant *M. tuberculosis* clinical isolates were selected from an existing sample bank maintained at the Stellenbosch University, Western Cape, South Africa. Isolates from this sample bank were previously phenotypically and genotypically characterized. The selected isolates had different genetic backgrounds based on IS6110 RFLP fingerprinting and spoligotyping and were from the following families: Beijing (n= 4), LCC (n=1), F13 (n=1) and Haarlem (n=1). These clinical isolates had identical *rpoB531* (Ser-Leu) mutation with varying levels of RIF resistance, as determined in by MGIT (25) (PhD thesis, Gail E. Louw, 2009) (Table 3.1). In addition, the sensitive laboratory H37Rv ATCC27294 strain and a clinical isolate K636 were used as susceptible controls.

Table 3.1: Genotypic and phenotypic characteristic of the selected RIF mono-resistant *M. tuberculosis* clinical isolates with *rpoB531* (Ser-Leu) mutation.

Family	Spoligotype pattern	Spoligotype number	Range of RIF MIC ($\mu\text{g/ml}$)
Beijing (n=4)		2	70-160
LCC (n=1)		118	80
Haarlem (n=1)		3	130
F13 (n=1)		18	130
Unclassified (n=1)		214	120

n= 8; LCC = Low Copy Clade; MIC = minimum inhibitory concentration

3.3 CULTURE OF *M.TUBERCULOSIS* STRAINS

M. tuberculosis isolates were cultured on Lowenstein-Jensen (LJ) solid media and incubated at 37°C for 3-4 weeks with continuous aeration. Subsequently, colonies were scraped from the LJ slants and starter cultures were prepared by inoculating the colonies into 10 ml 7H9 Middlebrook medium (Becton, Dickinson Microbiology system, Sparks, USA), supplemented with 10 % albumin-dextrose-catalase (ADC), 0.2 % (v/v) glycerol (Merck Laboratories, Saarchem, Gauteng, SA) and 0.1 % Tween80 (Becton,

Microbiology systems, Sparks, USA). Cultures were grown in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany) without shaking and incubated at 37°C until an optical density (OD₆₀₀) of 0.6 - 0.8 was reached. Contamination of these cultures was assessed by Ziehl-Neelsen (ZN) staining and culture on blood agar plates (APPENDIX). Subsequently, 80% glycerol stock-cultures were prepared from uncontaminated starter cultures and stored at -80°C.

3.4 GENOTYPIC CHARACTERISTICS OF CLINICAL ISOLATES

The phenotypically confirmed RIF mono-resistant *M. tuberculosis* clinical isolates were characterized by PCR amplification and targeted gene sequencing to confirm the presence of the *rpoB531* (Ser-Leu) mutation and the absence of other drug resistance conferring mutations.

3.4.1 DNA extraction

A volume of 500 µl of each strain, sub-cultured in MGIT vial supplemented with OADC (Becton, Dickinson and Company, Sparks, USA), was incubated at 100°C for 20-30 min. The extracted crude DNA was then stored at 4°C for subsequent analysis.

3.4.2 Primers of TB drug resistance conferring genes for PCR amplification

The primers (Table 3.2) used for PCR amplification and targeted gene sequencing were obtained from an oligonucleotide primer set bank maintained at Stellenbosch University, Western Cape, South Africa.

Table 3.2: Primers used for the amplification of TB drug resistance conferring genes

Anti-TB drugs	Gene	Primer	Sequence (5-3')	T _m (°C)	Fragment length
RIF	<i>rpoB</i>	rpoB For	TGGTCCGCTTGCACGAGGGTCAGA	78°C	437 bp
		rpoB Rev	CTCAGGGGTTTCGATCGGGGCACAT		
INH	<i>katG</i>	RTB 59	TGGCCGCGGCGGTCGACATT	62°C	419 bp
		RTB 38	GGTCAGTGGCCAGCATCGTC		
ETH	<i>inhA prom</i>	inhA P5	CGCAGCCAGGGCCTCGCTG	55°C	246 bp
		inhA P3	CTCCGGTAACCAGGACTGA		
PZA	<i>pncA</i>	pncAFor	AGTCGCCCGAACGTATGGTG	62°C	615 bp
		pncARev	CAACAGTTCATCCCGTTTCG		
EMB	<i>embB</i>	emb 151	CGGCATGCGCCGGCTGATTC	65°C	260 bp
		emb 131	TCCACAGACTGGCGTCGCTG		
AMI, CAP, STR	<i>rrs1401</i>	rrs_F	GTAATCGCAGATCAGCAACG	62°C	124 bp
		rrs_R	GTGATCCAGCCGCACCTT		
OFL, CIP, MOXI	<i>gyrA</i>	GyrAFor	TGACATCGAGCAGGAGATGC	62°C	344 bp
		GyrARev	GGGCTTCGGTGTACCTCATC		

3.4.3 PCR amplification conditions and fragment visualisation

The PCR master-mix comprised of 5 µl 10X buffer (Qiagen), 1 µl of MgCl₂ (2.5 mM), 10 µl of Q-solution (Qiagen), 4 µl deoxyribonucleotide triphosphates (dNTP's) (Promega) (0.2 mM of each dNTP), (0.25 µl of Forward primer (50pmol/µl) , 0.25 µl Reverse primer (50 pmol/µl) (of specific gene amplified), 0.15 µl Hotstar Taq polymerase (5 units/µl) (Qiagen). Subsequently, 2.5 µl of the crude DNA template was added to the PCR master mix. No template controls were included in each reaction to assess possible contamination. Additionally, DNA from the laboratory strain, H37Rv ATCC27294 was included as a positive control. The PCR reactions were carried out in the GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, USA) and thermal cycling conditions were as follows: an initial denaturing

step at 95°C for 15 minutes, followed by 45 cycles of denaturation at 94°C for 1 minute, annealing at the T_m of the specific gene primer (Table 3.2) for 1 minute, extension at 72°C for 1 minutes and final extension step at 72°C 15 minutes. Successful amplification of the amplified fragments were visualised on a 1.5% agarose gel, stained with ethidium bromide.

3.4.5 DNA Sequencing and mutation detection

The PCR products were submitted for PCR clean-up and sequencing at the Central Analytic Facility (CAF) of Stellenbosch University. Gene sequences were then aligned using DNA MAN Version 4.1 and the respective gene sequence of *M. tuberculosis* H37Rv reference strain (<http://genolist.pasteur.fr/Tuberculist>).

3.5: COMPOUND SELECTION

3.5.1 ANTI-TB DRUG:

Ten anti-TB drugs were selected for this study based on their use in TB treatment (as defined by the WHO) and from different structural and functional classes. These anti-TB drugs included: the fluoroquinolones (FQs) [OFL, MOXI, and CIP]; aminoglycosides [STR, AMI, and CAP (polypeptide)]; INH, ETH, EMB and PZA (Sigma-Aldrich Chemie, St Louis, USA). Drugs were dissolved in specific diluents according to the instructions by the manufacturers. The aminoglycosides (CAP, AMI and STR), INH, PZA and EMB were dissolved in sterile distilled water (dH₂O), while the FQs (OFL, MOXI and CIP) were dissolved in 0.1% sodium hydroxide (NaOH) (Sigma-Aldrich Chemie, St Louis, USA). RIF and ETH were dissolved in 100% dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany).

Table 3.3: Classification of different groups of anti-TB drugs

Drug group	Drug name
Group 1 First-line oral anti-TB agents	Isoniazid Rifampicin Ethambutol Pyrazinamide
Group 2 Injectable anti-TB agents	Streptomycin Amikacin Capreomycin
Group 3 Fluoroquinolones	Ciprofloxacin Ofloxacin Moxifloxacin
Group 4 Oral bacteriostatic second-line anti-TB agents	Ethionamide

3.5.2 EFFLUX PUMP INHIBITORS:

Three efflux pump inhibitors were selected based on their reported structural and functional properties. These efflux pump inhibitors included: Verapamil (calcium channel blocker) antagonist), Reserpine (an alkaloid) and carbonyl cyanide 3-chlorophenyldrazone (CCCP) (protonophore) (Sigma-Aldrich, St Louis, USA). Verapamil was dissolved in sterile dH₂O, whereas reserpine and CCCP were dissolved in 100% DMSO (Merck KGaA, Darmstadt, Germany). The optimal concentration of verapamil was previously determined as 40 µg/ml for anti-TB drugs resistant isolates and 10 µg/ml for pan-susceptible isolates (25). The optimal concentrations of CCCP and reserpine were determined in this study at concentrations ranging from 10 µg/ml to 80 µg/ml.

3.6 DRUG MIC DETERMINATION

3.6.1 Anti-TB drugs

The MICs for the respective anti-TB drugs for the clinical isolates were determined in the BACTEC MGIT 960 instrument (BD Bioscience, MD, USA) according to the manufacturer instructions. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of a antimicrobial compound needed to eliminate 99% of the bacterial cells in a culture (34). In order to determine the MICs for ten drugs (CAP, AMI, STR, MOXI, CIP, OFL, ETH, INH, PZA and EMB), dilutions of these drugs

were made in small increments. The dilutions started at 2 x lower followed by 10 x lower than the critical concentration for the specific anti-TB drugs (Table 3.4). Sub-cultures were prepared by inoculating 200 µl of previously prepared *M. tuberculosis* stock-cultures in MGIT 960 media supplemented with 800 µl of OADC. These sub-cultures were then incubated at 37°C in the MGIT 960 instrument until a positive growth reading was obtained, which is typically a GU > 400. Two days after obtaining positive growth, the growth control tubes were prepared by inoculating 500 µl of the 1:100 diluted sub-cultures into OADC enriched MGIT tubes.

Subsequently, 500 µl of the undiluted positive sub-cultures were inoculated into MGIT tubes enriched with 800µl OADC and 100 µl of the anti-TB drug specific drug concentrations (Table 3.4).The MGIT tubes were then registered on the EpiCenter (version 5.75A) TBeXist software (BD Bioscience, Erembodegem, Belgium) and placed in BACTEC MGIT 960 instrument to continuously monitor the growth for 14 consecutive days. PZA MIC determination was done under different conditions, due to PZA being active at pH 5.5 *in vitro*, thus influencing the growth of the mycobacteria. For this reason, PZA MIC testing was done in a PZA MGIT 960 kit was used as per manufacture's instruction (35, 36) in modified MGIT 960 PZA medium (37).

3.6.2 Efflux pump inhibitor

3.6.2.1 EPI optimal concentration determination

a) MGIT 960

The optimal concentration of the EPI was defined as the highest concentration of drug that resulted in <10% growth inhibition. This was done in the MGIT 960 system and EpiCenter software as described above. The concentrations for the different efflux pump inhibitor used ranged from 10 µg/ml to 80 µg/ml (Table 3.4).

b) Broth Micro dilution

The preliminary findings from our group, demonstrated that reserpine influences the turbidity of the MGIT media, thus having a direct effect on determining the growth based on fluorescence (38). For this reason, the non-inhibitory concentration of reserpine and its effect on the MICs of the anti-TB drugs were determined using the Broth Microdilution Method in a 96-well v-bottom plate (Greiner Bio-one, Maybachstreet, Germany). Briefly, *M. tuberculosis* isolates were grown to an OD_{600nm} of 0.2- 0.3 (~10⁶ CFU/ml) and subsequently diluted 1:100 in 7H9 Middlebrook media. Subsequently, 100 µl 7H9 medium

(drug free control) were pipetted in row 1 well 1, 1.6% DMSO (diluent) were pipetted in row 1 well 2, and reserpine (inhibitor) were pipetted in row 1 well 3-4 at 4 x the initial required concentration (Table 3.4). Afterwards, 50 µl of 7H9 medium was added to all wells from row 2- 12 and reserpine was serially diluted (2 fold) in the 96 well plates. Finally, 50 µl of the 1:100 diluted culture ($\sim 10^4$ cells/well) was added to rows 2-12 and all was done in duplicates. The inoculated 96-well plates were placed in zip lock bags and incubated at 37°C for 7 to 14 days.

3.6.2.2 EPI in combination with anti-TB drugs

a) MGIT 960 system

The effect of the EPI on the MIC of the anti-TB drugs were determined in the MGIT 960 system, based on the instructions of the manufacturer. Briefly, sub-cultures were prepared by inoculating 200 µl of previously prepared *M. tuberculosis* stock-cultures in MGIT 960 media supplemented with 800 µl of OADC. These sub-cultures were then incubated at 37°C in the MGIT 960 instrument until a positive growth reading was obtained. After two days of obtaining positive growth, the growth control tubes were prepared by inoculating 500 µl of the 1:100 diluted sub-cultures into OADC enriched MGIT tubes. Subsequently, 500 µl of the undiluted positive sub-cultures were inoculated into MGIT tubes enriched with 800µl OADC and 100 µl of each of the ten drugs (CAP, AMI, STR, MOXI, CIP, OFL, ETH, INH, PZA and EMB) separately in the presence of verapamil (40 µg/ml (resistant strains) and 10 µg/ml (sensitive strains). Additionally, in the presence of CCCP (7.5 µg/ml (resistant) and 4.0 µg/ml (sensitive) at determined MICs and 10-fold lower respectively. Moreover, verapamil and CCCP only controls were included to show that the EPI had minimal effect on the growth of the bacterial population. In addition drugs-only controls were included for all the anti-TB drugs tested to show and validate that the change in growth was due to the presence of verapamil and CCCP. Finally, the MGIT tubes were then registered on the Epicenter and placed in BACTEC MGIT 960 instrument to continuously monitor the growth for 14 consecutive days.

b) Broth Micro dilution

The Broth Micro dilution (BMD) was used to determine the effect of reserpine on the MICs of the anti-TB drugs as described in section 3.5 (EPI optimal concentration determination). This was done by incubating reserpine (80 µg/ml) in combination with the ten drugs (CAP, AMI, STR, MOXI, CIP, OFL, ETH, INH, PZA and EMB) at determined MICs. The reserpine-only control was included to show that the reserpine had minimal effect on the growth of the bacterial population, also a drug-only control for all the anti-TB drugs tested to show and validate that the change in MIC was due to the presence of

reserpine. Further other controls included 7H9 medium and the drug diluents (1.6% DMSO and dH₂O) as positive controls to show that the bacterial growth was independent of them. All experiments were done in triplicate for verification purposes.

Table 3.4: Concentrations of anti-TB drugs used for MIC determination

Anti-TB drug		Critical concentration (µg/ml) ^a	MIC (µg/ml) ^b	Range of concentrations tested	Reference
	INH	0.1/0.4	0.02 - 0.2	0.01 - 0.4	(39-42)
	ETH	5.0/12.5	2.5 - 10	1.25 - 12.5	(39-42)
	EMB	5.0	1 - 5	0.5 - 5.0	(39-42)
	PZA	100	16 - 50	10 - 100	(39-42)
	OFL	2.0	0.5 - 2.0	0.2 - 2.0	(39-42)
	MOXI	0.25	0.18 - 0.2	0.025 - 0.25	(43-45)
	CIP	1.0	0.5	0.1 - 1.0	(41, 42)
	STR	1.0	< 1.0	0.1 - 1.0	(39-42)
	AMI	1.0	< 1.0	0.1 - 1.0	(41, 42)
	CAP	2.5	1.25	0.25 - 2.5	(41, 42)
Efflux pump inhibitor *		Typical active concentration (µg/ml) ^c	Sub inhibitory concentration (µg/ml) ^d	Range of concentrations tested	Reference
	Verapamil	25 - 50	5 - 50	10 - 40	(2, 25, 46-48)
	CCCP	n/a	1 - 7.5	1.25 - 15	(2, 46, 49)
	Reserpine	n/a	80	1.25 - 100	(25, 38, 46, 47)

a) Critical concentration as defined by the WHO (41) The critical concentration (CC) was defined as the drug concentration that discriminates between drug sensitivity and resistance, as defined by the WHO; **b)** MIC that was reported in the literature ; **c)** EPIs typical active concentrations of the (concentration at which do not inhibit growth); **d)** EPIs sub-inhibitory concentrations reported was reported in literature;*The EPIs active concentration ranges were obtained from literature searches and used as starting point to determine the final non-inhibitory concentrations used in the present study

3.6.2.3 Interpretation of results

a) MGIT 960/EpiCenter

Results were read when the growth control reached a growth unit (GU) > 400. When a GU of the tested drug tube was ≥ 100 , the isolate was interpreted as being resistant (R). In contrast the isolate was sensitive (S) if the GU of the tested drug tube was <100 and remained the same after being incubated for more 7 days. The isolates was interpreted as intermediate (I) if the GU of the tested drug tube was > 100 during 7 days after the growth control GU reached > 400 (38, 42). The same procedure was used to read the growth units of the EPI's and drugs combination.

b) Broth Micro dilution

The plates were read by a visual inspection after 7 days of incubation to determine whether bacterial pellets have formed formation at the bottom of the plates. If no bacterial pellets were visible, the plates we left for another 12-14 days. On day 13 a blue resazurin reagent was added to the 96-well plate and left overnight at 37°C in the incubator (24 hrs). The blue non-fluorescent resazurin reagent is reduced to highly fluorescent resorufin by dehydrogenase enzymes in metabolically active cells. This conversion only occurs in viable cells and thus, the amount of resorufin produced is proportional to the number of viable cells in the sample. The conversion from resazurin to resorufin would result in a colour change from blue to pink, which represents bacterial growth (which was scored as either growth (+++), no growth (-) or partial growth (+/-) if less than 50% growth.

3.7 STATISTICAL ANALYSIS

3.7.1 ANOVA: F-test (STATISTICA VERSION 11)

Descriptive statistics (ANOVA: F-test) was used to determine the significance of Verapamil or CCCP on the growth of *M. tuberculosis*, either alone or in combination with anti-TB drugs.

3.7.2 Fractional inhibitory concentration formula index (FIC)

The fractional inhibitory concentration (FIC) formula index was used, to assess the anti-TB drugs/inhibitor interactions. FIC formula index is an isobologram mathematical expression used to analyze the synergistic properties in drug/inhibitor combination experiments. The FIC is defined as a ratio of the MIC observed in an inhibitor/drug combination to the MIC of the same drug tested alone [Equation 3.6] (50, 51). The FIC indices determination was done for all the EPIs (reserpine, verapamil and CCCP). In the case of verapamil and CCCP experiments which was done in the MGIT system, the FIC was defined as a ratio of the GU assessed in an inhibitor and drug combination to the GU of the same drug tested alone (Equation 3.7a). In addition, for reserpine experiments based on a BMD method, the FIC was defined as a ratio of the MIC assessed in an inhibitor and drug combination to the MIC of the same drug tested alone (Equation 3.7b). In addition, the interaction FIC indices were classified as follows: synergistic when FIC index ≤ 0.5 -0.9, indifferent/additive when FIC index = 1-1.9 and antagonistic when FIC index ≥ 2 FIC (50–53).

Equation 3.7: FIC mathematical calculation used to assess drug/inhibitor interactions

$$\text{a) FIC (Verapamil and CCCP)} = \text{GU in } \frac{\text{combination (anti-TB drug and Verapamil OR CCCP)}}{\text{anti-TB drug alone}}$$

and

$$\text{b) FIC(reserpine)} = \text{MIC in } \frac{\text{combination (anti-TB drug and reserpine)}}{\text{anti-TB drug alone}}$$

CHAPTER 4
RESULTS

4.1 THE GENOTYPIC CHARACTERISTICS OF CLINICAL ISOLATES

Genotypic characterization identified the *rpoB531* (Ser-Leu) mutation in the 8 RIF mono-resistant clinical *M. tuberculosis* isolates analysed. In contrast, no mutation was identified in the rifampicin resistant determining region (RRDR) *rpoB* gene for the clinical susceptible *M. tuberculosis* isolate (K636) or the H37Rv laboratory strain. Moreover, isolate R810 was found to harbour an *inhA* promoter mutation, therefore this isolate does not represent a RIF mono-resistant phenotype but an MDR phenotype. For this reason, this isolate was excluded from subsequent experiments (Appendix C).

4.2 ANTI-TB DRUG MIC DETERMINATION

4.2.1 Anti-TB drugs

The MICs of the anti-TB drugs in RIF mono-resistant (n=7) and pan-susceptible isolates (n=2) were determined. Five out of seven RIF mono-resistant isolates with the same *rpo531* mutation showed sensitivity at the critical concentration of the various drugs assessed (Table 4.1), confirming the RIF mono-resistant phenotype. However, two isolates (Haarlem and unclassified families) showed resistance at the critical concentration of PZA (100 µg/ml), thus suggesting a multiple resistance phenotype. Genotypic analysis of the *pncA* gene showed no mutations suggesting alternative resistance mechanisms conferring the PZA resistance phenotype. Both pan-susceptible strains (K636 and H37Rv) showed sensitivity at the critical concentration of the various drugs assessed.

Table 4.1: The range of MICs for the different anti-TB drugs in the RIF mono-resistant clinical isolates (n=9)

Anti-TB drugs	Critical conc. (µg/ml) ^a	Range of MIC (µg/ml) in the different strain families					
		Beijing (n=3) ^b	LCC (n=1)	Haarlem (n=1)	F13 (n=1)	Unclassified (n=1)	Pan-susceptible isolates (n=2)
RIF ^c	2.0	70-160	80	130	130	130	0.2- 0.5
INH	0.1/0.4	0.03- 0.05	0.03	0.03	0.05	0.05	0.05- 0.2
ETH	5.0/12.5	1.0 – 10.0	1.50	0.60	10.0	1.25	3.5 – 10.0
EMB	5.0	1.5- 2.5	1.0	0.50	0.50	2.00	1.0 – 3.0
PZA	100	50	50	100 ^d	50	100 ^d	22.5 -25
OFL	2.0	0.20	0.20	0.20	0.20	0.20	0.25 – 0.50
MOXI	0.25	0.04	0.04	0.06	0.04	0.04	0.06 – 0.13
CIP	1.0	0.1- 0.25	0.10	0.10	0.10	0.25	0.13 – 0.25
STR	1.0	0.07- 0.1	0.10	0.10	0.25	0.10	0.13 – 0.80
AMI	1.0	0.1-0.25	0.25	0.25	0.25	0.25	0.25 – 0.50
CAP	2.5	0.4 -0.6	0.60	0.60	0.60	0.60	1.00 – 1.25

a) critical conc. of the anti-TB drugs in a MGIT (µg/ml) (41), **b)** number of isolates in specific family, **c)** previously determined by G. Louw *et al* (25) **d)** The MICs showing resistant at critical concentrations.

4.2.2 Efflux pump inhibitor

4.2.2.1 Determined EPI optimal concentrations

i) The optimal sub-Inhibitory concentration of CCCP.

The optimal sub-inhibitory concentration of CCCP was determined to assess the highest concentration of efflux pump inhibitor where $\geq 90\%$ of *M. tuberculosis* growth was observed.

To determine the sub-inhibitory concentration of CCCP, clinical resistant isolates from the different strain families (LCC, Beijing and F13) were selected. The CCCP concentrations tested ranged from 5 to 15 µg/ml for the resistant isolates and 2.5 to 5 µg/ml for the susceptible isolates (Table 4.2). From this assay it was determined that the sub-inhibitory optimal concentration for CCCP for the resistant and susceptible isolates were, 7.5 µg/ml and 4.0 µg/ml, respectively (Table 4.2).

ii) *The optimal sub-Inhibitory concentration of Reserpine*

Our group previously determined the optimal concentrations 40 and 80 µg/ml for the efflux pump inhibitors verapamil and reserpine respectively for the resistant isolates (25). For the susceptible isolates, it was previously determined to be 10 and 30 µg/ml for verapamil and reserpine, respectively (25). The above assays were done by the BACTEC 460TB method. To assess the sub-inhibitory optimal concentration for reserpine in resistant isolates, by the Broth Microdilution Method, the concentrations tested ranged from 1280 µg/ml to 1.25 µg/ml. It was observed that 80 µg/ml reserpine was the optimal concentration, thus confirming previous findings (25).

Table 4.2: The optimal sub- inhibitory concentrations of CCCP in clinical isolates

Strain Family		CCCP conc.'s (µg/ml)						
		15	10	7.5	5	4.0	3.0	2.5
Resistant isolates	LLC	0.0%	72.0	98.7%^b	100%	ND ^a	ND	ND
	BEIJING	0.0%	70.3%	92.1%^b	95.0%	ND	ND	ND
	F13	0.0%	69.3%	94.4%^b	98.3%	ND	ND	ND
Pan-susceptible isolates	K636	ND	ND	ND	86.6%	90.9%^b	92.1%	94.6%
	H37Rv	ND	ND	ND	90.6%	95.0%^b	96.4%	99.8%

a) Concentration not done for the specific phenotypic group, b) the chosen sub-inhibitory concentrations for CCCP were at concentrations where $\geq 90\%$ of the *M. tuberculosis* still grew.

4.2.2.2 EPI in combination with anti-TB drugs

a) MGIT 960

i) *The effect of verapamil on M. tuberculosis growth at the MICs of different anti-TB drugs*

Significant overall growth inhibition was observed ($p = 0.0026$, 95% confidence interval) when *M. tuberculosis* RIF resistant isolates were grown in the presence of a combination of verapamil (40 µg/ml) and the different anti-TB drugs relative to anti-TB drugs alone (Figure 4 A). This growth inhibition was

specifically observed for RIF resistant isolates grown in the presence of the combination of verapamil (40 $\mu\text{g/ml}$) and INH (0.03- 0.05 $\mu\text{g/ml}$), ETH (0.6 – 10.0 $\mu\text{g/ml}$), EMB (0.5 – 2.5 $\mu\text{g/ml}$), PZA (50 $\mu\text{g/ml}$), CIP (0.1 – 0.25 $\mu\text{g/ml}$), STR (0.07 – 0.25 $\mu\text{g/ml}$), AMI (0.1- 0.25 $\mu\text{g/ml}$) or CAP (0.4 – 0.6 $\mu\text{g/ml}$). This effect was independent of the genetic background of the studied isolates. In contrast, the addition of verapamil (40 $\mu\text{g/ml}$) significantly stimulated growth in the presence of OFL (0.2 $\mu\text{g/ml}$) MOXI (0.04 – 0.06 $\mu\text{g/ml}$) (Figure 4 A).

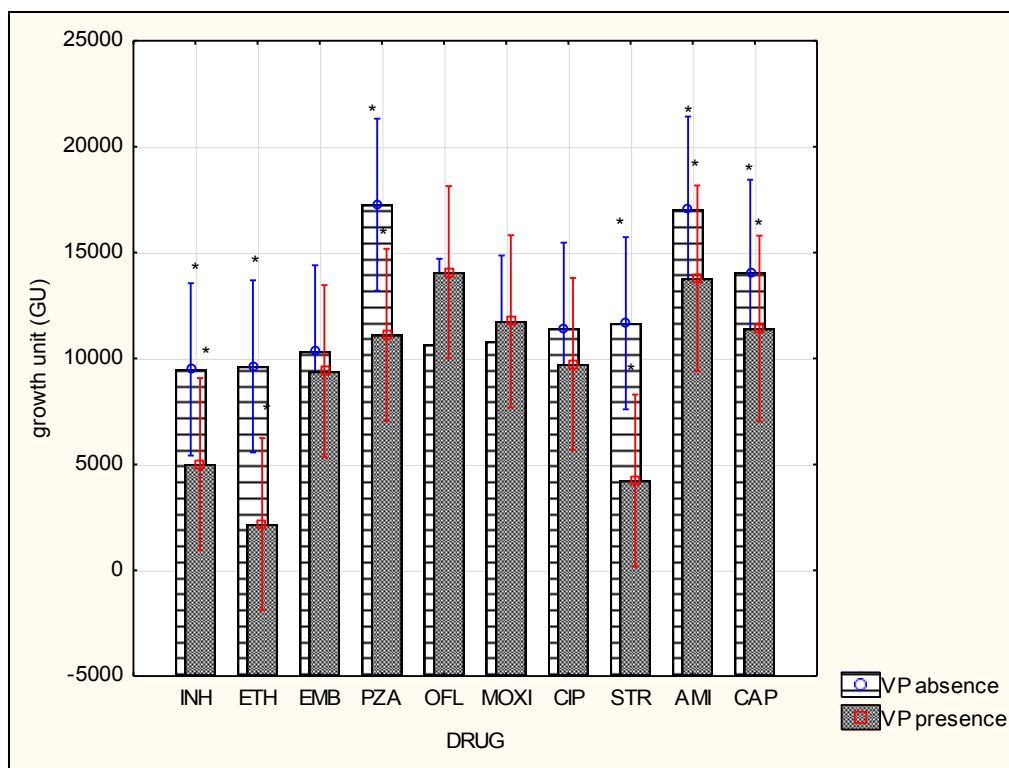


Figure 4 A: Growth of RIF resistant isolates in different anti-TB drugs in the presence or absence of verapamil (40 $\mu\text{g/ml}$). * denotes statistical significant difference; Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(9, 52) = 4.4080$, $p = 0.0026$, Type III decomposition; vertical bars denotes 95% confidence intervals (the mean growth units obtained for the different RIF resistant isolates tested). RIF resistant isolates were cultured in the presence of ETH (0.6 – 10.0 $\mu\text{g/ml}$), PZA (50 $\mu\text{g/ml}$), STR (0.07 – 0.25 $\mu\text{g/ml}$), AMI (0.1- 0.25 $\mu\text{g/ml}$), CAP (0.4 – 0.6 $\mu\text{g/ml}$), INH (0.03- 0.05 $\mu\text{g/ml}$), EMB (0.5 – 2.5 $\mu\text{g/ml}$), CIP (0.1 – 0.25 $\mu\text{g/ml}$), OFL (0.2 $\mu\text{g/ml}$) or MOXI (0.04 – 0.06 $\mu\text{g/ml}$).

Verapamil (10 $\mu\text{g/ml}$) did not significantly alter the growth of the drug susceptible *M. tuberculosis* isolates ($p = 0.5464$; 95% confidence interval) when cultured in the presence of INH (0.05- 0.2 $\mu\text{g/ml}$), ETH (3.5 – 10.0 $\mu\text{g/ml}$), PZA (22.5- 25 $\mu\text{g/ml}$), CIP (0.13 – 0.25 $\mu\text{g/ml}$), STR (0.13 – 0.8 $\mu\text{g/ml}$), AMI or

(0.25- 0.5 $\mu\text{g/ml}$) (Figure 4 B). However, a significant decrease in growth was observed for these isolates grown in the presence of verapamil (10 $\mu\text{g/ml}$) and EMB (1.0-3.0 $\mu\text{g/ml}$), MOXI (0.06 – 0.13 $\mu\text{g/ml}$) or OFL (0.25-0.5 $\mu\text{g/ml}$) while an increased in growth was observed in the presence of verapamil and CAP (1.0- 1.25 $\mu\text{g/ml}$).

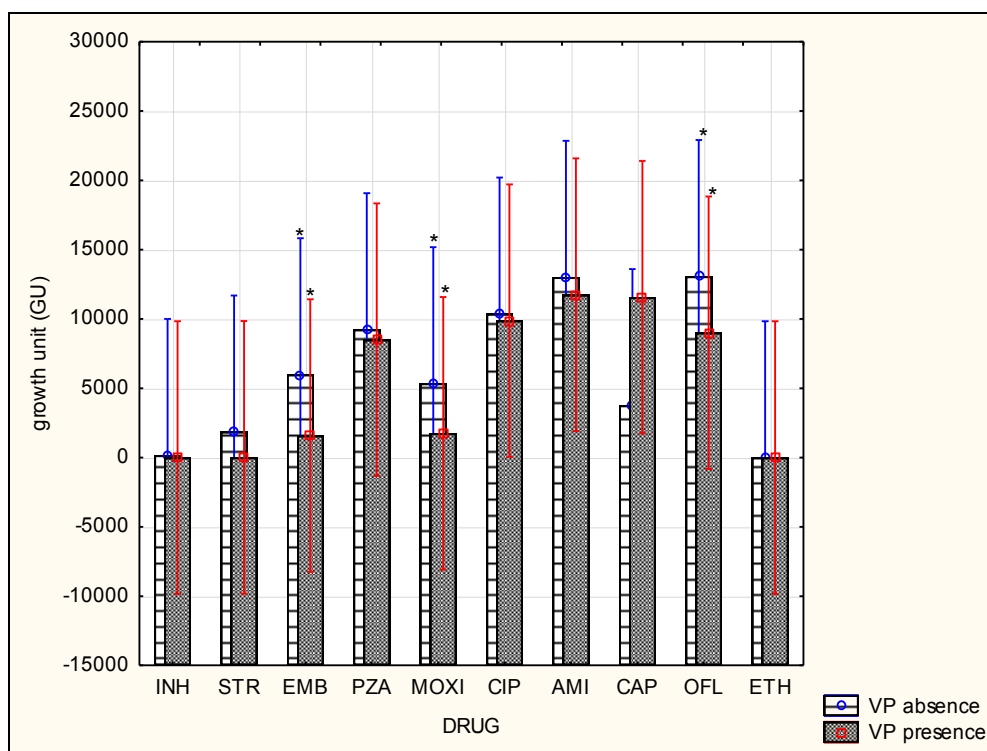


Figure 4 B: Growth of RIF susceptible isolates cultured in the presence of different anti-TB drugs and in the presence or absence of verapamil (10 $\mu\text{g/ml}$). * denotes statistical significant difference; Descriptive statistics (ANOVA: F-test) plot: LS means, $F(9, 9) = 0.92318$, $p = 0.5464$, Type III decomposition; vertical bars denotes 95% confidence intervals (the mean growth units obtained for the different RIF resistant isolates tested). RIF susceptible isolates were cultured in ETH (3.5 – 10.0 $\mu\text{g/ml}$), PZA (22.5 - 25 $\mu\text{g/ml}$), STREP (0.1 – 0.8 $\mu\text{g/ml}$), AMI (0.25- 0.5 $\mu\text{g/ml}$), CAP (1.0 – 1.25 $\mu\text{g/ml}$), INH (0.05 – 0.2 $\mu\text{g/ml}$), EMB (1.0 – 3.0 $\mu\text{g/ml}$), OFL (0.25 – 0.5 $\mu\text{g/ml}$), MOXI (0.06 - 0.13 $\mu\text{g/ml}$) or CIP (0.13 – 0.25 $\mu\text{g/ml}$).

Further analysis of the effect of structurally related and unrelated compounds in combination with verapamil showed that the structurally related anti-TB drugs (OFL, MOXI and CIP) behave similarly to one another but differently to those that are not structurally related (STR, AMI and CAP), (INH, ETH) or EMB and PZA (Figure 4 C).

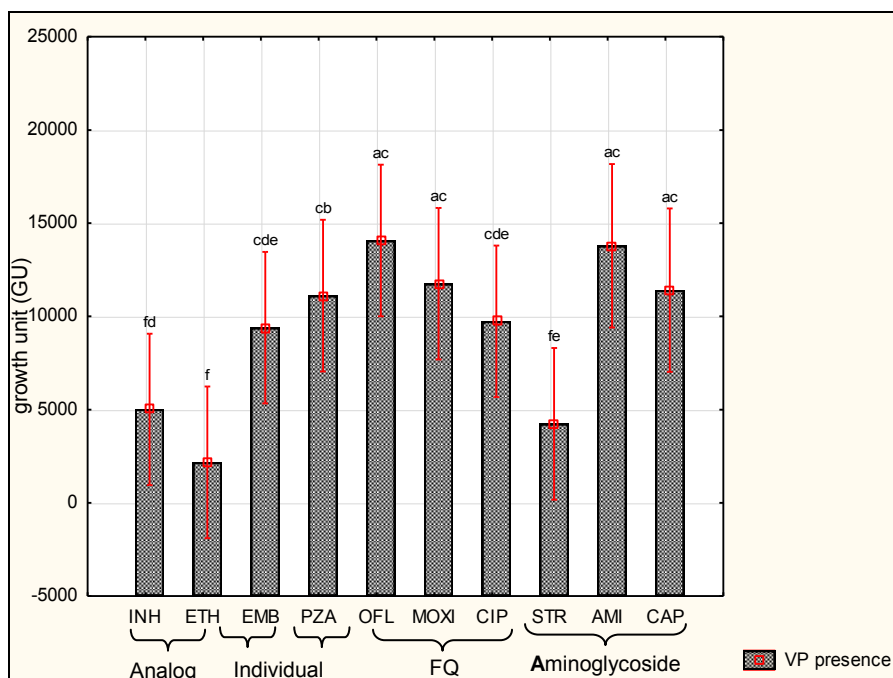


Figure 4 C: Graphic representation of the similarities and differences in growth of RIF resistant isolates when cultured in the presence of structural analogs and structurally unrelated anti-TB drugs together with verapamil. Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(9, 52) = 4.4080$, $p = 0.0026$, Type III decomposition; vertical bars denotes 95% confidence intervals. Overlapping of the letters abcdef shown in the graph represents the similar and different behavior of anti-TB drugs in the presence of verapamil. If the anti-TB drugs in the graph show similar letters, they behave similarly. However, if they show different letters, they behave differently.

ii) *The effect of CCCP on M. tuberculosis growth at the MICs of different anti-TB drugs*

The addition of CCCP (7.5 $\mu\text{g/ml}$) had a significantly overall growth inhibition ($p = 0.01196$, 95% confidence interval) on the growth of RIF resistant isolates cultured in the presence of different anti-TB drugs (Figure 4 D). This synergy was specifically observed for resistant isolates grown in the presence of CCCP (7.5 $\mu\text{g/ml}$) and INH (0.03 – 0.05 $\mu\text{g/ml}$), PZA (50 $\mu\text{g/ml}$ or CAP (0.4 – 0.6 $\mu\text{g/ml}$) TB drugs. Moreover, indifference/additive was observed for resistant isolates grown in the presence of CCCP (7.5 $\mu\text{g/ml}$) and ETH (0.6 – 10.0 $\mu\text{g/ml}$), MOXI (0.04 – 0.06 $\mu\text{g/ml}$), EMB (0.5 – 2.5 $\mu\text{g/ml}$) or STR (0.07 – 0.25 $\mu\text{g/ml}$). However, the presence of CCCP (7.5 $\mu\text{g/ml}$) had an antagonistic effect when OFL (0.2 $\mu\text{g/ml}$), CIP (0.1 – 0.25 $\mu\text{g/ml}$) or AMI (0.1- 0.25 $\mu\text{g/ml}$) were present in the medium. The influence of CCCP on growth was independent of the genetic background of the studied isolates.

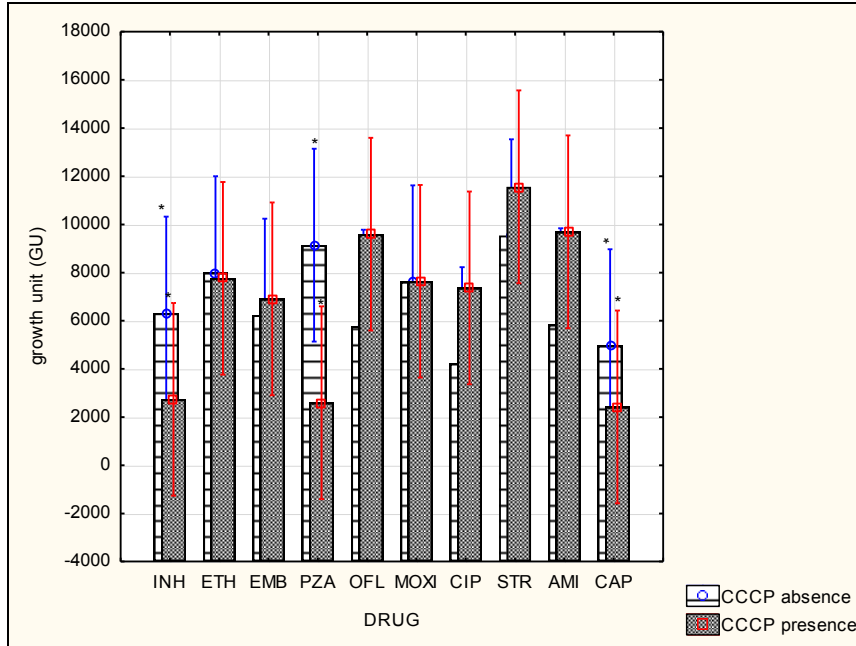


Figure 4 D: Growth RIF resistant isolates cultured in the presence of different anti-TB drugs and in the presence or absence of CCCP (7.5 µg/ml). * denotes statistical significant difference; Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(9, 52) = 1,7771$, $p = 0.01196$; vertical bars denotes 95% confidence intervals (the mean growth units obtained for the different RIF resistant isolates tested). RIF resistant isolates were cultured in the presence of (ETH (0.6 – 10.0 µg/ml), PZA (50 µg/ml), STR (0.07 – 0.25 µg/ml), CAP (0.4 – 0.6 µg/ml), INH (0.03- 0.05 µg/ml), EMB (0.5 – 2.5 µg/ml), MOXI (0.04 – 0.06 µg/ml), OFL (0.2 µg/ml), CIP (0.1 – 0.25 µg/ml) or AMI (0.1- 0.25 µg/ml).

In contrast, CCCP (4.0 µg/ml) did not significantly alter the growth of the sensitive *M. tuberculosis* isolates ($p = 0.5085$, 95% confidence interval) when cultured in the presence of certain anti-TB drugs (Figure 4 E). However, a significant decrease in growth was observed for these isolates when grown in the presence of CCCP (4.0 µg/ml) and EMB (1.0 – 3.0 µg/ml) or PZA (22.5 - 25 µg/ml).

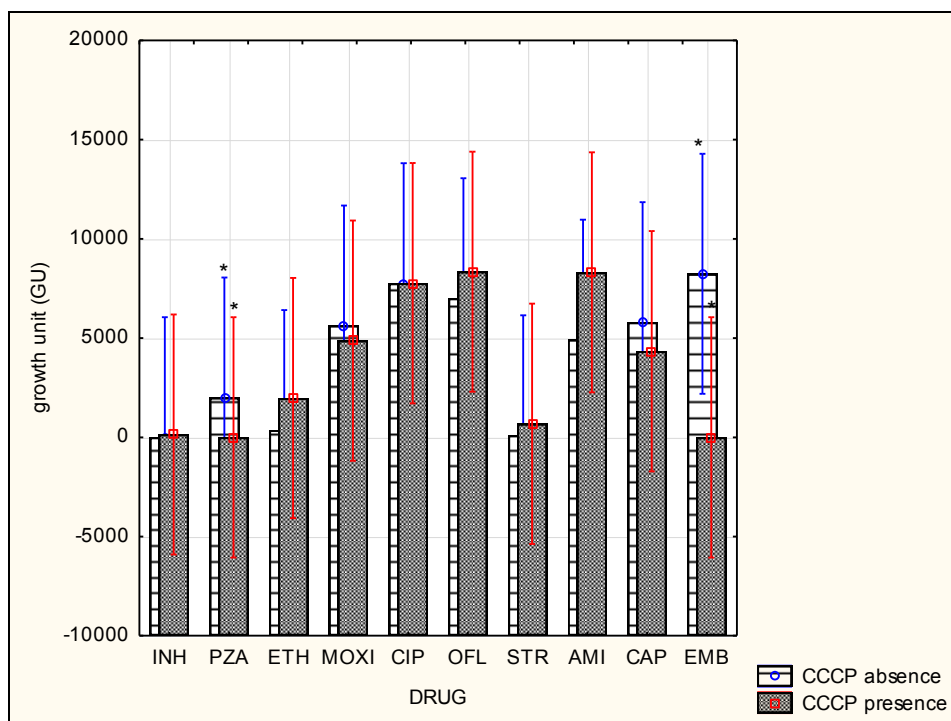


Figure 4 E: Growth of RIF susceptible isolates cultured in the presence of different anti-TB drugs and in the absence and presence of CCCP (4.0 µg/ml). * denotes statistical significant difference; Descriptive statistics (ANOVA: F-test) plot: LS means, $F(9, 9) = 0.98548$, $p = 0.50851$, Type III decomposition; vertical bars denotes 95% confidence intervals (the mean growth units obtained for the different RIF resistant isolates tested). RIF susceptible isolates were cultured in the presence of ETH (3.5 – 10.0 µg/ml), PZA (22.5 - 25 µg/ml), STR (0.1 – 0.8 µg/ml), CAP (1.0 – 1.25 µg/ml) INH (0.05 – 0.2 µg/ml), EMB (1.0 – 3.0 µg/ml), OFL (0.25 – 0.5 µg/ml), MOXI (0.06 - 0.13 µg/ml) or AMI (0.25- 0.5 µg/ml).

Further analysis of the effect of structurally related and unrelated compounds in combination with CCCP showed that; the structurally related anti-TB drugs either (OFL, MOXI and CIP), (STR, AMI and CAP), (INH, ETH) or EMB and PZA behave similarly to one another but differently to those that are not structurally related (Figure 4 F). The same observation was seen as per verapamil.

It was observed that the analogs INH, ETH have a similar effect on the growth of *M. tuberculosis* strains in combination with CCCP (Figure 4F). This is expected as they are structural analogs and share common gene targets. Furthermore, a similar observation was seen for MOXI and CIP with slight difference for OFL. Interestingly, the latter anti-TB drugs are all FQs and with the same mechanism of action (inhibit DNA replication). Nevertheless, different behavior was observed to other structurally unrelated (STR, AMI and CAP), (INH, ETH) or EMB and PZA.

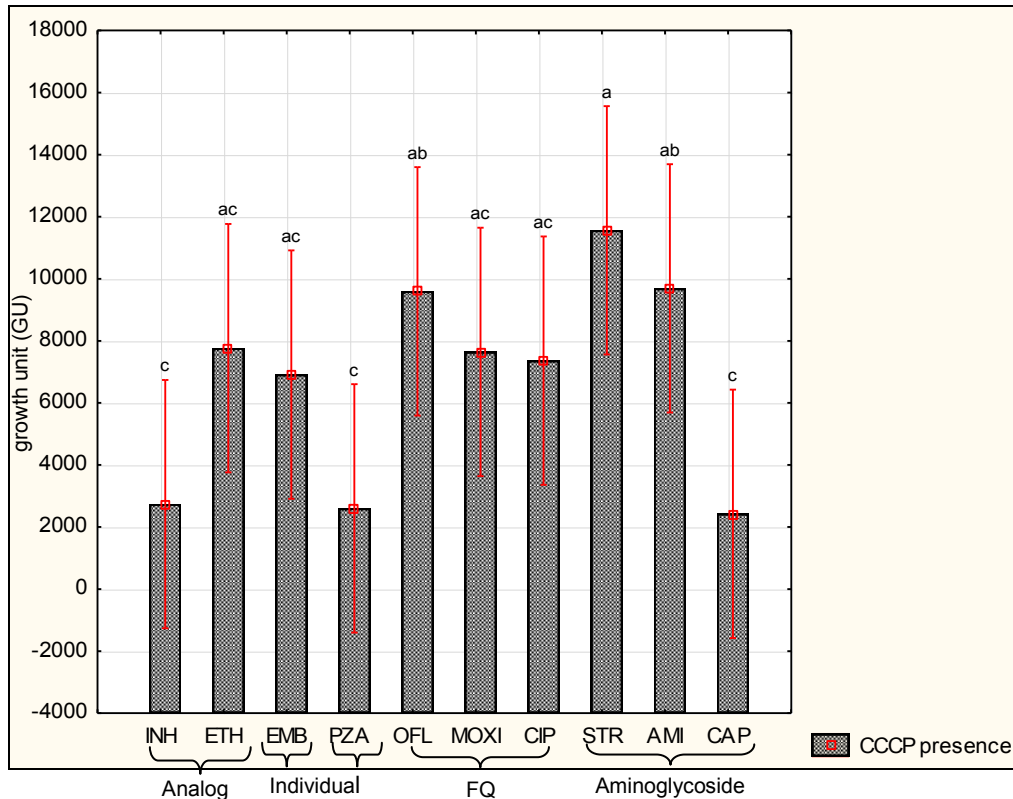


Figure 4 F: Graphic representation of the similarities and differences in growth of RIF resistant isolates when cultured in the presence of structural analogs and structurally unrelated anti-TB drugs together with CCCP. Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(9, 52) = 1,7771$, $p = 0.01196$; vertical bars denotes 95% confidence intervals. Overlapping of the letters abc shown in the graph represents the similar and different behavior of anti-TB drugs in the presence of verapamil. If the anti-TB drugs in the graph show similar letters, they behave similarly. However, if they show different letters, they behave differently.

iii) *The effect of verapamil on M. tuberculosis growth at the critical conc. of RIF (2.0 µg/ml)*

The effect of verapamil (40 µg/ml) on the growth of RIF resistant isolates cultured in the presence of RIF (2.0 µg/ml) was determined. From figure 4G it is evident that verapamil (40 µg/ml) significantly enhanced the growth of the RIF resistant isolates in the presence or absence of RIF (2.0 µg/ml) ($p = 0.0001$, 0.95 CI). This effect was independent of the genetic background of the studied isolates. Conversely, verapamil (10 µg/ml) did not significantly ($p = 0.0048$) alter the growth of the pan-susceptible isolates in the presence of RIF (2.0 µg/ml) and this was done in duplicates.

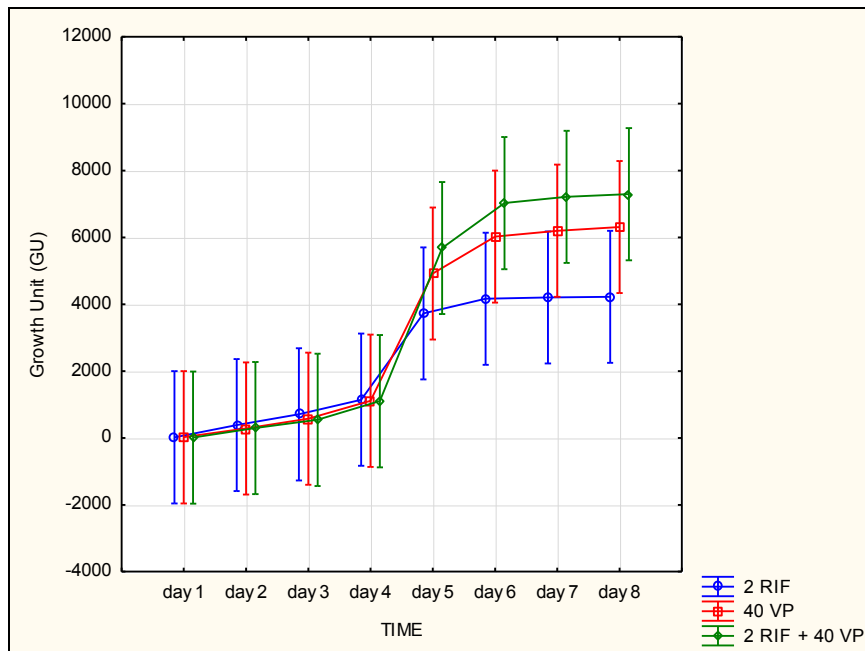


Figure 4 G: Growth of RIF resistant isolates. Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(10, 98) = 10.231$, $p = 0.0001$, Type III decomposition; vertical bars denotes 95% confidence intervals (the model is based on the data from all of the isolates tested). RIF resistant isolates were cultured in the presence of RIF (2.0 $\mu\text{g/ml}$), verapamil (40 $\mu\text{g/ml}$) or RIF (2.0 $\mu\text{g/ml}$) and verapamil (40 $\mu\text{g/ml}$) and this was done in duplicates.

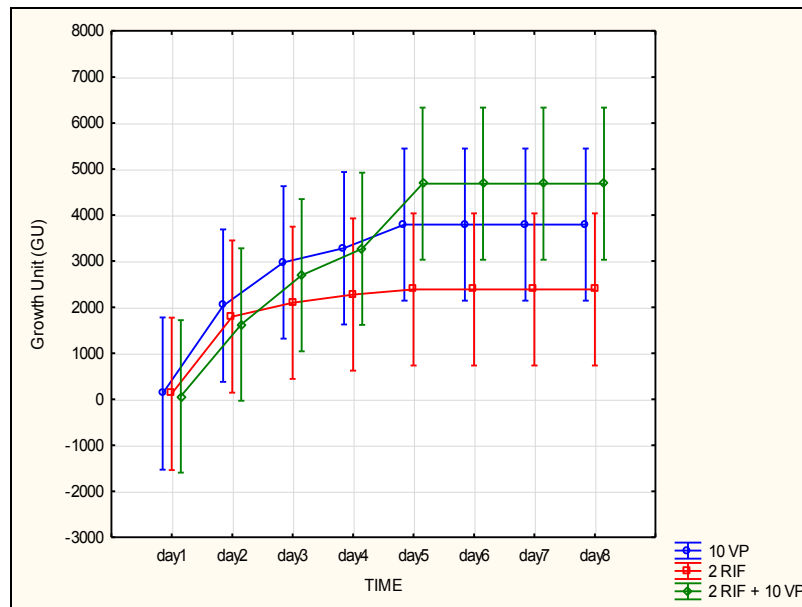


Figure 4 H: Growth of pan-susceptible isolates. Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(14, 14) = 4.3296$, $p = 0.0048$, Type III decomposition; vertical bars denotes 95% confidence intervals (the model is based on the data from all of the isolates tested). Pan-susceptible isolates were cultured in the presence of RIF (2.0 $\mu\text{g/ml}$), verapamil (10 $\mu\text{g/ml}$) or RIF (2.0 $\mu\text{g/ml}$) and verapamil (10 $\mu\text{g/ml}$) and this was done in duplicates.

iv) *The effect of CCCP on M. tuberculosis growth at the critical concentration of RIF (2.0 $\mu\text{g/ml}$).*

The effect of CCCP (7.5 $\mu\text{g/ml}$) on the growth of RIF resistant isolates cultured in the presence of RIF (2.0 $\mu\text{g/ml}$) was determined. From, Figure 4I it is evident that CCCP (7.5 $\mu\text{g/ml}$) significantly enhanced the growth of the RIF resistant isolates in the presence or absence of RIF (2.0 $\mu\text{g/ml}$) ($p = 0.0001$, 0.95 CI). Moreover, it was shown to increase the lag phase and this effect was independent of the genetic background of the studied isolates. Conversely, CCCP did not significantly ($p > 0.05$) alter the growth of the pan-susceptible isolates in the presence of RIF (2.0 $\mu\text{g/ml}$) as expected (data not shown).and this was done in duplicates.

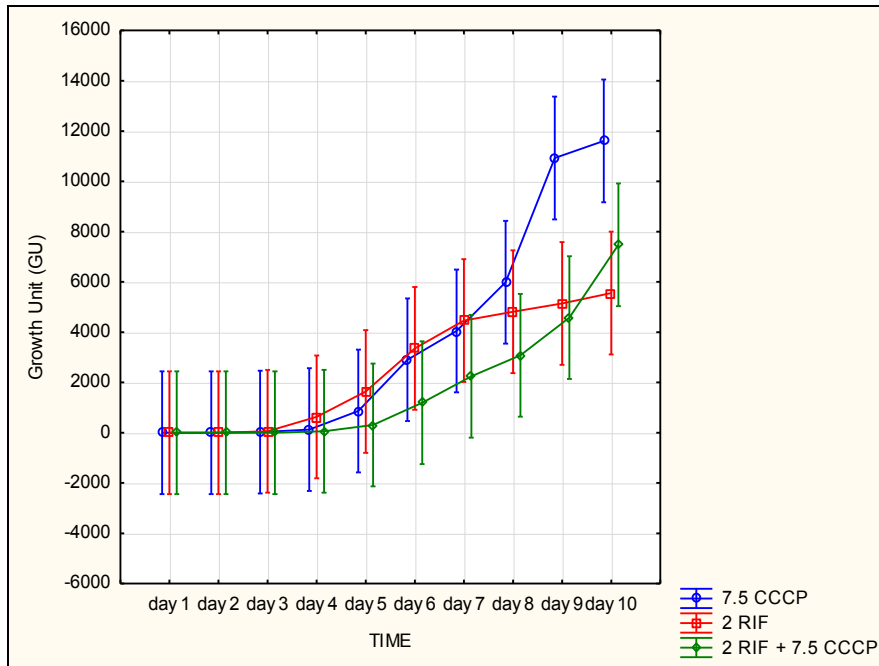


Figure 4 I: Growth of RIF resistant isolates. Descriptive statistics (ANOVA: F-test) plot: LS means, current effect: $F(18, 54) = 3.7098$, $p = 0.0001$, Type III decomposition; vertical bars denotes 95% confidence intervals (the model is based on the data from all of the isolates tested). RIF resistant isolates were cultured in the presence of RIF (2.0 $\mu\text{g/ml}$), CCCP (7.5 $\mu\text{g/ml}$) or RIF (2.0 $\mu\text{g/ml}$) and CCCP (7.5 $\mu\text{g/ml}$) and this was done in duplicates.

b) Broth Microdilution Method

i) The effect of reserpine on the growth *M. tuberculosis* cultured in the presence of different anti-TB drug.

The inclusion of reserpine (80 $\mu\text{g/ml}$) in the culture media had a significant influence on the MICs of some tested anti-TB drugs in the RIF resistant isolates. This was reflected by observed different MICs fold changes (2-6 folds) in the presence of reserpine and INH, ETH, MOXI, CIP, STR, AMI, CAP, EMB and PZA respectively (Table 4.3). In contrast, reserpine did not influence the OFL MIC significantly (Table 4.3). The inclusion of reserpine (30 $\mu\text{g/ml}$) in the culture media did not alter the MICs of the sensitive *M. tuberculosis* (H37Rv) laboratory strain for some of the anti-TB drugs (Table 4.3). However, the presence of reserpine (30 $\mu\text{g/ml}$) significantly decreased the MIC for INH (6-fold), EMB (4-fold), and OFL, MOXI, CIP, STR and CAP by 2-fold (Table 4.3).

Table 4.3: Effect of reserpine on the MICs of different anti-TB drugs (MIC fold changes) as measured using different RIF resistant and susceptible isolates.

Clinical isolates	R 160	R 637	R 376	H37Rv
Drug	MIC ($\mu\text{g/ml}$) up-fold changes in the presence of reserpine			
INH	4	2	6	6
ETH	0	0	4	0
EMB	2	4	0	4
PZA	0	2	0	0
OFL	0	0	0	2
MOXI	2	4	2	2
CIP	4	4	0	2
STR	2	2	4	2
AMI	2	2	2	0
CAP	2	0	0	2

ii) The effect of reserpine at the critical concentration of RIF (2.0 $\mu\text{g/ml}$).

The influence of reserpine (80 $\mu\text{g/ml}$) on the MIC for RIF was determined in the panel of RIF resistant *M. tuberculosis* isolates. The findings from the present showed that reserpine caused a drastic increase in the RIF MIC (2.0 – 32 $\mu\text{g/ml}$) (10-fold increase). However, the inclusion of reserpine (30 $\mu\text{g/ml}$) did not influence the RIF MIC of the drug sensitive *M. tuberculosis* (H37Rv) laboratory strain (Table 4.4).

Table 4.4 The influence of reserpine on the RIF critical conc. for RIF resistant and susceptible isolates.

Clinical isolates	R 160 ^a	R 637 ^a	R 376 ^a	H37Rv ^b
Drug treatment	RIF critical conc. (µg/ml)			
RIF	2.0	2.0	2.0	0.5
RIF+reserpine	32	32	32	0.5
MIC fold change	10	10	10	0

a) Denotes tested RIF resistant isolates b) Denotes tested RIF susceptible isolate

4.3 The synergistic properties of EPIs and anti-TB drugs MICs combination

The fractional inhibitory concentrations (FIC) of RIF resistant isolates were calculated to assess the interaction of verapamil (40 µg/ml), CCCP (7.5 µg/ml) or reserpine (80 µg/ml) with the different anti-TB drugs MICs. The FIC indices were classified as follows: synergistic when FIC index ≤ 0.5 -0.9, indifference/additive when FIC index = 1-1.9 and antagonistic when FIC index ≥ 2 FIC (25,26). Different equations were used to calculate the FIC indices because two different technologies used (MGIT 960 and Broth Microdilution). For verapamil (40 µg/ml) and CCCP (7.5 µg/ml) Equation 3.6A with growth units was used, while for reserpine (80 µg/ml) Equation 3.6B with MIC valued was used (chapter 3). The FIC indices for verapamil (40 µg/ml) and CCCP (7.5 µg/ml) are tabulated in Table 4.5 and for reserpine (80 µg/ml) interaction tabulated in Table 4.6.

Based on the calculated FIC indices, synergistic interaction was observed in the presence of verapamil (40 µg/ml) or CCCP (7.5 µg/ml) in combination with INH, ETH, PZA and CAP (Table 4.5). Conversely, indifference interaction was observed in combination with EMB, MOXI and OFL. Additionally, synergistic interaction was observed in the presence of verapamil (40 µg/ml) but indifference interaction in the presence of CCCP (7.5 µg/ml) in combination with STR, AMI and CIP. Moreover, synergistic interaction was observed in the presence of reserpine (80 µg/ml) in combination with INH, STR and AMI in all clinical isolates (Table 4.6). However, for other anti-TB drugs (CAP, PZA, EMB, CIP, MOXI, ETH and OFL) various interactions were observed for different clinical isolates and this illustrated in Table 4.6.

Table 4.5: FIC indices for verapamil and CCCP in combination with different anti-TB drug as determined in different RIF resistant isolates.

Anti-TB drug	Verapamil (FIC)	Inhibitor/drug interaction	CCCP (FIC)	Inhibitor/drug interaction
INH	0.5	synergistic	0.4	synergistic
ETH	0.2	synergistic	0.9	synergistic
EMB	1.3	indifference	1.5	indifference
PZA	0.6	synergistic	0.3	synergistic
OFL	1.1	indifference	1.6	indifference
MOXI	1.0	indifference	1.1	indifference
CIP	0.8	synergistic	1.7	indifference
STR	0.4	synergistic	1.2	indifference
AMI	0.7	synergistic	1.7	indifference
CAP	0.8	synergistic	0.5	synergistic

Table 4.6: FIC indices for reserpine in combination with different anti-TB drug as determined in different RIF resistant isolates.

Clinical isolates		R160		R637		R376	
Anti-TB drug	reserpine (FIC)	Interaction	reserpine (FIC)	Interaction	reserpine (FIC)	Interaction	Interaction
INH	0.2	synergistic	0.5	synergistic	0.1	synergistic	
ETH	2	antagonistic	2	antagonistic	0.3	synergistic	
EMB	0.5	synergistic	0.3	synergistic	2	antagonistic	
PZA	1	indifference	0.5	synergistic	1	indifference	
OFL	1	indifference	1	indifference	1	indifference	
MOXI	0.5	synergistic	0.3	synergistic	2	antagonistic	
CIP	0.3	synergistic	0.2	synergistic	1	indifference	
STR	0.5	synergistic	0.5	synergistic	0.3	synergistic	
AMI	0.5	synergistic	0.5	synergistic	0.5	synergistic	
CAP	0.5	synergistic	1	indifference	1	indifference	

CHAPTER 5

DISCUSSION

DISCUSSION

Central dogma suggests that mutations in target genes are the main cause of drug resistance in *M. tuberculosis* (3, 9–11, 54). In addition, previous studies report that efflux related mechanisms may contribute to intrinsic resistance and to the process whereby drug resistance is acquired (2, 5, 25, 28, 32, 38, 55, 56). Furthermore, recent studies have shown that efflux mechanisms may impact on the level of resistance following the mutation in defined target genes (*rpoB* and *gyrA*) (25, 57). This phenomenon has been shown by an increase in susceptibility to anti-TB drugs in the presence of efflux pump inhibitors (2, 5, 21, 28, 58–60). In this study, we aimed to test the hypothesis that the addition of efflux pump inhibitors could synergize with first- and second-line anti-TB drugs to enhance their bacteriocidal/bacteriostatic effect. Enhancing the activity of current anti-TB drugs could significantly shorten treatment thereby easing the management of TB patients and decreasing the risk of default.

In this study, all of the isolates tested exhibited a susceptible phenotype when cultured at the critical concentration for INH, ETH, EMB, PZA, OFL, MOXI, OFL, STR, AMI and CAP. However, two isolates showed resistance towards PZA implying multiple resistance and were subsequently excluded from further analysis. The remaining clinical isolates showed minor variations in their level of intrinsic resistance to the respective anti-TB drugs. This variation in the level of intrinsic resistance is commonly observed and forms the basis for defining the critical concentration used to differentiate between susceptible and resistant phenotypes (41). Previous studies have suggested that genetic background may define the level of intrinsic resistance (25, 61); however we were unable to demonstrate an association between the level of intrinsic resistance and the genetic background of the strains tested given the small number of strains tested.

Our study showed that the addition of the efflux pump inhibitor Verapamil, decreased bacterial growth in the presence of INH, ETH, PZA, CIP, STR, AMI or CAP suggesting an increased susceptibility towards these drugs. This implies that the addition of the efflux pump inhibitor blocked the active transport of these molecules out of the cell leading to the intracellular accumulation of these drugs and thereby enhancing their inhibitory effect. Conversely, verapamil increased resistance to OFX and MOX, as measured by the observed increased growth, suggesting that verapamil inhibited the uptake of these drugs. Together these results demonstrate the involvement of MFS and ABC superfamilies efflux pumps in defining the level of intrinsic resistance to the various anti-TB drugs. Similarly, by depriving energy that drives efflux through inhibition of the proton transport chain with CCCP we showed that susceptibility toward INH, ETH, PZA and CAP could be restored. However, reserpine resulted in restored susceptibility towards INH, STR and AMI in all three RIF mono-resistant strains tested. Together this

implies that different classes of efflux pumps may be involved in defining the level of intrinsic resistance to specific drugs. These observations were not restricted to RIF mono-resistant isolates suggesting that efflux is also involved in defining the level of intrinsic resistance. However, these results are not directly comparable given that the concentrations of efflux pump inhibitors used were significantly lower for the susceptible strains.

Our results were in concordance with previous findings that suggested that high-level INH resistance was mainly driven by efflux when RIF mono-resistant clinical isolates were exposed to the combination of INH and verapamil or chlorpromazine (60). The authors also noted that exposure of RIF mono-resistant strains to INH significantly increased expression of *mmpL7*, *P55*, *efpA*, *Rv1258c*, *Rv2459* and *mmr* genes, as well as inhibited in the presence of verapamil or chlorpromazine. Based on our findings, we suggest that INH is extruded by members of the MFS, ABC and RND efflux pump families which are inhibited by CCCP, verapamil and reserpine. This is supported by a previous study which showed that *iniA* (membrane protein) extruded INH and that reserpine blocked this efflux mechanism (6, 63, 64). Additionally, the overexpression of *inhA* induce efflux of INH (65) and this was reversed by CCCP, verapamil and reserpine. In the current study, we observed that susceptibility towards ETH was also significantly increased in the presence of CCCP, and verapamil (synergy observed). This is in agreement with previous reports which have shown that CCCP inhibits P55-dependent drug resistance to ETH and other anti-TB drugs in *M. tuberculosis* (27, 66, 67). The P55-efflux pump (MFS superfamily) utilizes the electrochemical and transmembrane proton gradients as source of energy (6, 20, 67) which is targeted by CCCP.

Interestingly, our data also showed that verapamil and reserpine increased susceptibility towards the aminoglycosides STR and AMI in RIF mono-resistant clinical isolates. In addition, CCCP increased susceptibility towards CAP. This suggests that the aminoglycosides and cyclic peptides are extruded multiple efflux pumps (ABC, RND and MFS superfamilies). These findings may aid in the development of novel inhibitors to target these efflux pump targets and thereby improve/strengthen MDR-TB treatment regimens.

The present study revealed that reserpine reduced the MIC for EMB by 2-4 fold, suggesting synergy. Conversely, verapamil and CCCP showed indifference in their effect on EMB, suggesting that efflux of EMB was restricted to a define group of efflux pump inhibitors. This was supported by a study by Gupta *et al.* who reported that an increase in the expression of *jefA* increased EMB and INH resistance in *M. tuberculosis* by MFS superfamily efflux pumps and the this resistance could be reversed with the addition of reserpine (58).

Our findings contrast with previous studies (2, 25, 68) concerning the synergy of efflux pump inhibitor and fluoroquinolone susceptibility. This in part could be explained by differences in the genetic backgrounds of the strains tested, extent of resistance and the concentration of the inhibitors used. Our results also conflicted with a previous finding which showed that the addition of verapamil and reserpine enhanced susceptibility to RIF in RIF resistant clinical isolates of *M. tuberculosis* (25, 28). Interestingly, we observed an increase in mycobacterial growth in the presence of verapamil suggesting that this inhibitor prevents RIF from entering the cell thereby increasing resistance to RIF. In concordance with previous studies we showed that CCCP increased susceptibility to RIF (2.0 µg/ml), suggesting the involvement of MFS, ABC and RND class/group of efflux pumps. A similar effect was not observed for RIF susceptible isolates confirming the absence of efflux defining the level of intrinsic resistance (2, 69). This suggests that the RIF mono-resistant clinical isolates have adapted mechanisms to survive environmental stresses of RIF.

Based on the overall findings of the present study, we propose a model: “Inhibition of efflux = restored anti-TB drug susceptibility”. The model demonstrates that the inhibition of efflux pumps contributes to the decrease in intrinsic resistance. Moreover, intrinsic resistance results when efflux pumps are activated and decrease intracellular drug concentration (Figure 5: Senerio A). This mechanism is driven by the efflux pump inhibitors that inhibit the activity of efflux pump (Figure 5: Senerio B), thereby resulting restored susceptibility to the efflux pump substrates (anti-TB drugs).

The clinical explanation of the presented model suggest that treating a RIF resistant strain with first and second line anti-TB drugs, might stimulate overexpression of the bacterial ABC, MFS and RND superfamilies. This would result in the up-regulation of encoded efflux pump genes to extrude the various anti-TB drugs. In return, the intracellular anti-TB drug concentration decreases thereby the concentration is not sufficient to cause a killing response. The addition of verapamil, CCCP and reserpine blocks the efflux pumps, leading to an increase in the intracellular drug concentration. This results in the anti-TB drug being able to bind to its target, and thus restore susceptibility.

PROPOSED A MODEL: INHIBITION OF EFFLUX = RESTORED ANTI-TB DRUG SUSCEPTIBILITY

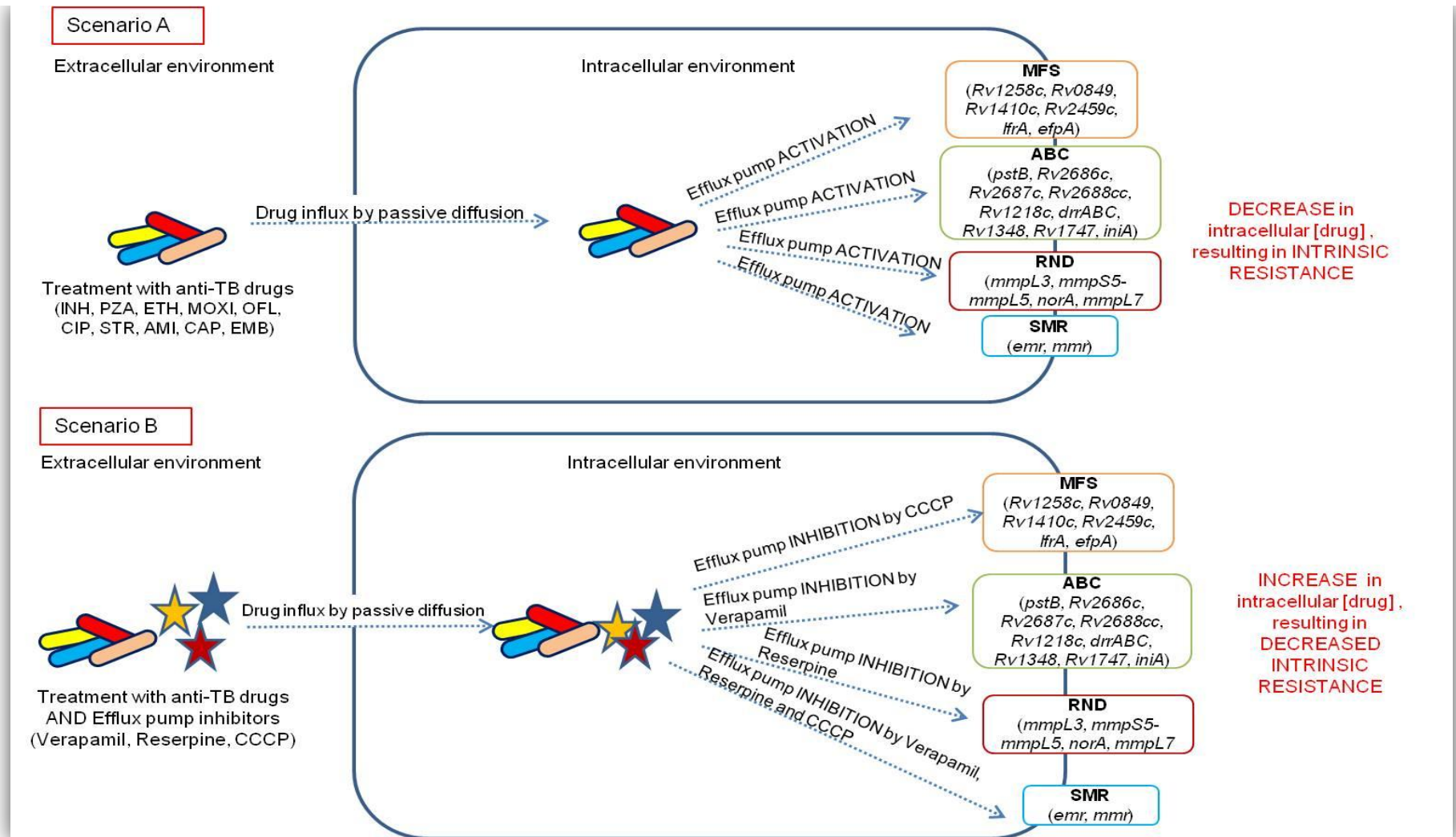


Figure 5: Diagrammatic illustration of “Inhibition of efflux = restored anti-TB drug susceptibility”; the inhibition of EPs responsible for intrinsic resistance by use of EPIs, results in restored susceptibility to extruded anti-TB drugs (substrate to the EP).

CHAPTER 6
CONCLUSION

6. CONCLUSION

The current study provided evidence that an efflux related mechanism contributes to the level of intrinsic drug resistance in RIF mono-resistant *M. tuberculosis* clinical isolates with an *rpoB531* (Ser-Leu) mutation. The MICs to the various drugs were confirmed to be sensitive at the respective critical concentrations, with the exception of PZA. The variable intrinsic resistance of the RIF mono-resistant isolates was independent of the genetic background. This could be explained by the technical challenges of phenotypic PZA testing and alternative resistance mechanisms. This study showed that PZA resistance could be reversed with the addition of efflux pump inhibitors, thus supporting previous findings.

We observed that combination therapy with EPIs, first and second line anti-TB drugs had a significant inhibitory effect on the growth of *M. tuberculosis* isolates. This suggests that the addition of verapamil, CCCP and reserpine plays a significant role in restoring the susceptibility (change in MIC and decrease in intrinsic resistance level) of the RIF mono-resistant isolates to these anti-TB drugs. A synergistic effect was also observed by the combination treatment of the anti-TB drugs with the different efflux pump inhibitors. These findings have clinical implications as combination treatment with EPI and anti-TB drugs could improve MDR-TB therapy outcome. The proposed model is based on the data obtained from this study and by incorporating evidence from the literature. We proposed that suggests that the overexpression of EPs in the clinical isolates studied, results in the up-regulation of encoded EP genes followed by the extrusion of multiple anti-TB drugs out of the cell. This decrease in intracellular drug accumulation results in decreased drug-target affinity, thus causing multidrug resistance. Additionally, the use of EPIs can inhibit active efflux and increase anti-TB drug concentration.

Reserpine and verapamil were observed to be the most potent efflux pump inhibitor, with CCCP being the least effective. These findings (i) provided a proof of principle for the synergistic effect of drugs and EPI combination in MDR-TB treatment (ii) emphasized the need to design novel compounds which will also target EPs and reduce spread of drug resistance and tolerance. An example is SQ109 which targets MmpL3 involved in mycolic acid, the disruption of cell wall assembly and TMC207 which showed significant synergistic effect when combined with PZA and TMC207 (chapter 2) and both are currently in clinical trials.

In summary, based our the findings and published literature, the present study confirms what was suggested by other studies, namely that efflux pumps play a crucial role in the level of intrinsic resistance. However, this is the first to investigate the effect of different efflux pumps on the level of intrinsic resistance to a broad spectrum of anti-TB drugs (first- and second-line) in drug resistant *M. tuberculosis* clinical isolates from different genetic backgrounds. Even more important, the present study also showed

that the variation in the level of intrinsic resistance is independent of the genetic background. Thus, this also emphasizes that drug resistance is more complex than previously thought.

A limitation in the present study is the lack of efflux pump gene expression analysis. However, future work would entail investigating the gene expression of the different efflux pumps in RIF mono-resistant *M. tuberculosis* clinical isolates by quantitative real-time polymerase chain reaction (qRT-PCR). This assessment would identify the specific efflux pump genes (individual or combination) that might aid in the extrusion of the various structurally related/unrelated anti-TB drugs. Functional studies on the different efflux pump genes would also provide evidence of the extent to which a specific gene contribute to intrinsic drug resistance. As, only few *in vivo* studies have been reported in this topic; this include Mooy J *et al* study that showed reduction in the bioavailability of the calcium antagonist in patients treated for 6 month with verapamil in combination with RIF, EMB and INH. Additionally, G. Louw *et al* study in BALB/c mice showed that treatment with verapamil in combination with first-line drugs significantly reduces pulmonary CFUs after 1 and 2 months. Hence, the correlation of the *in vitro* combination therapy data to *in vivo* studies would be informative. This would provide data that might be of clinical significance. In short, in-depth knowledge of efflux /inhibitor mechanisms in correlation with anti-TB drug resistance will help in the design and development of novel drugs/ inhibitors therapy and eradication of the MDR-TB Spread.

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APPENDICES

APPENDIX A: BIO-SAFETY LEVEL III (P3) LABORATORY

Good microbiological laboratory practices

The risk of being infected with *M. tuberculosis* pathogen is high for personnel who work in laboratory that handle live *M. tuberculosis* processing. Hence the P3 laboratory was designed with specific engineering controls, administrative procedures and appropriate personal work practices that will ensure the organism containment and protection of the workers. The P3 laboratory is a facility used to facilitate safety when working with the Class III pathogens. Furthermore the facility is made up of main laboratory area which can be accessed through an access controlled airlock system. To ensure that the air from inside the P3 laboratory doesn't escape to the entire building, the negative pressure is controlled throughout the laboratory.

To comply with section 43 of the Occupational Health and Safety Act for dealing with hazardous biological agents (esp. *M. tuberculosis*) regulations: before personnel could be allowed to work in P3, had to undergo a complete medical examination to ensure personnel vaccinated and fit to work in P3. Then the safety training was done by safety officer and after the personnel was provided with the access code together with a safety manual on how to handle *M. tuberculosis* cultures when working in P3.

P3 laboratory: Good laboratory Practice administered when working with of live *M. tuberculosis*

Protective clothing was worn prior working in P3 laboratory, these included impervious quality long sleeves wrap-around gown, nitrile gloves, overshoes and masks for safety purposes when working with *M. tuberculosis*.

All *M. tuberculosis* culturing was prepared aseptically in biological safety cabinets (BSC) and the BSC was disinfected as follows:

- Firstly 10% incidin was prepared and used to decontaminate the BSC hood work surface, then sprayed and wiped with 70 % ethanol also to completely disinfect the BSC hood working surface. In addition this was done daily when processing cultures in order to avoid the use of old incidin.
- Secondly all equipments used, reagents and cultures were also sprayed and wiped with 70% ethanol before entered in BSC hood; then wiped with 10% incidin before taken out of the BSC hood.
- All waste materials were double bagged into autoclave plastic bags and autoclaved at 121°C for 30 minutes before being disposed.

- Moreover after handling infectious *M. tuberculosis* cultures, gloves were removed and hands were washed with disinfectant soap in P3 working area, then gown was removed in P3 changing room and washed the hands again before leaving P3 laboratory.
- The BSCs were serviced and certified by a trained external contractor for every six months. Also the pressure gauge and airflow in these cabinets were maintained daily prior starting work in order to ensure that the BSC is operating at optimum level.
- Lastly all the procedures were carried out discreetly, without overcrowding the cabinet, keeping the air intake and exhaust grilles free to minimize any formation of splashes or aerosols.

APPENDIX B: REAGENTS, MEDIA AND SOLUTIONS

CULTIVATION OF *M.TUBERCULOSIS* STRAINS

7H9 Liquid medium:

4.7 7H9 Middlebrook medium

0.5 Tween 80

2 ml glycerol

900 ml dH₂O

Autoclave at 121°C for 15 minutes

Enrich with 100 ml OADC/ADC

7H10 Middlebrook Agar Medium:

19 g of 7H10 agar powder

900 ml dH₂O

5 ml glycerol

Boil for 1 min and autoclave at 121°C for 10 min

Enrich with 100 ml OADC (at cooled temperature of 50 - 55°C)

GEL ELECTROPHORESIS

10 X TBE Buffer:

108 g of 0.45M Tris

55 g of 0.44M boric acid

7.4 g of 10mM EDTA

1 L dH₂O

1X TBE Buffer:

100 ml of 10X TBE

900 ml dH₂O

SOLUTIONS

Saline:

8.5 g NaCl

1L dH₂O

Tween saline:

8.5 g NaCl

0.10 g Tween 80

1 L dH₂O

REAGENTS

0.1M NaOH:

2 g of NaOH

500 ml dH₂O

0.02 % Resazurin NaCL:

10 ml of the resazurin powder

50 ml of dH₂O

DRUGS CONCENTRATIONS

Ofloxacin, Moxifloxacin and Ciprofloxacin:

80 mg of drug powder each in 0.1M NaOH

Streptomycin, Capreomycin, Amikacin, Ethambutol and Isoniazid:

80 mg of drug powder each in 10 ml dH₂O

Ethionamide and Rifampicin:

80 mg of drug powder each in 10% (RIF) and 100% (ETH) DMSO

Pyrazinamide:

20 000 µg in 2.5 ml dH₂O

EFFLUX PUMP INHIBITORS CONCENTRATIONS

Verapamil:

80 mg of inhibitor powder in 10 ml dH₂O

Reserpine and CCCP:

80 mg of inhibitor powder each in 10 ml DMSO

BLOOD AGAR PLATES AND ZN STAINING

The blood agar and ZN staining were done to monitor possible contamination. These included plating *M. tuberculosis* cultures onto blood agar for period of two days as *M. tuberculosis* does not grow on blood agar for that period. Secondly, ZN staining was done as follows. The smear cultures were prepared and heat fixed at 100°C for 2 hours. then they were stained with carbol-fuchsin (Becton, Dickinson and Company, Maryland, USA), followed by decolorizing them with acid alcohol and finally counterstaining them with methylene blue (Becton, Dickinson and Company, Maryland, USA). The counterstained smears were visualized at 100x (oil immersion) magnification under the microscope for acid-fast bacilli. As *M. tuberculosis* is an acid-fast bacilli which retain dyes when handled and heated with acid containing compounds, the bacilli were expected to appear pink.

GEL ELECTROPHORESIS

An agarose gel (1.5 %) was prepared by dissolving 1.5 g agarose (Sigma-Aldrich Chemie, St Louis, USA) in 100 ml 1X Tris/Borate/EDTA (TBE) buffer. After the solution was boiled in a microwave oven for 2-3 minutes, 5 µl of ethidium bromide was added to it. The agarose was poured into the gel rack and left for 30 minutes to solidify. Subsequently the PCR products were mixed with an equal volume (5 µl) of loading dye (0.25 % Xylene Cyanol, 30% glycerol) and loaded onto the gel. The gel was run at 160 V for 1hours in 1X TBE Buffer. The gel was visualized under ultra violet light using the Kodak, Digital Science Electrophoresis Documentation and Analysis System (Vilber Lourmat, France).

BACTEC MGIT 960 SYSTEM AND EPICENTER, (VERSION 5.75A) TBEXIST SOFTWARE OPERATION

The BACTEC MGIT 960 is a complete automated system. It utilizes a fluorescence-quenching-based oxygen sensor to detect growth in combination with EpiCenter software fitted with the TB eXiST module (42, 70). This system was used to determine the MICs of the selected drugs and the effect of verapamil and CCCP on MICs of these drugs in RIF mono-resistant *M. tuberculosis* clinical isolates as recommended by the manufacturer. The MGIT 960 system comprised of the supplement kit which included the Mycobacteria Growth Indicator fluorescence Tubes (MGIT) containing 7 ml of modified Middlebrook 7H9 Broth base; Oleic acid, Albumin, Dextrose and Catalase (OADC) enrichment (71) and the MGIT instrument (Becton, Dickinson and Company, Sparks, MD, USA); which monitors the growth of cultivated *M. tuberculosis* strains. Subsequently, the data generated by the MGIT 960 instrument was analysed on the EpiCenter, (version 5.75A) TBExist software (BD Bioscience, Erembodegem, Belgium).

The MGIT tubes were entered into the MGIT instrument and incubated at 37°C and the fluorescence reading were captured every 60 min. In addition, the analysis of the fluorescence readings were used to determine whether the tube showed positive growth and thereby determine whether the sample tested contained viable organisms (72). According to the manufacturer, an instrument positive tube had to contain about 10^5 to 10^6 colony forming units per milliliter (CFU/ml). In addition, the culture vials which remained negative for minimum of 42 days (up to 56 days) showing no clear signs of positivity were stabilized and removed from the instrument as negatives.

BROTH MICRODILUTION METHOD

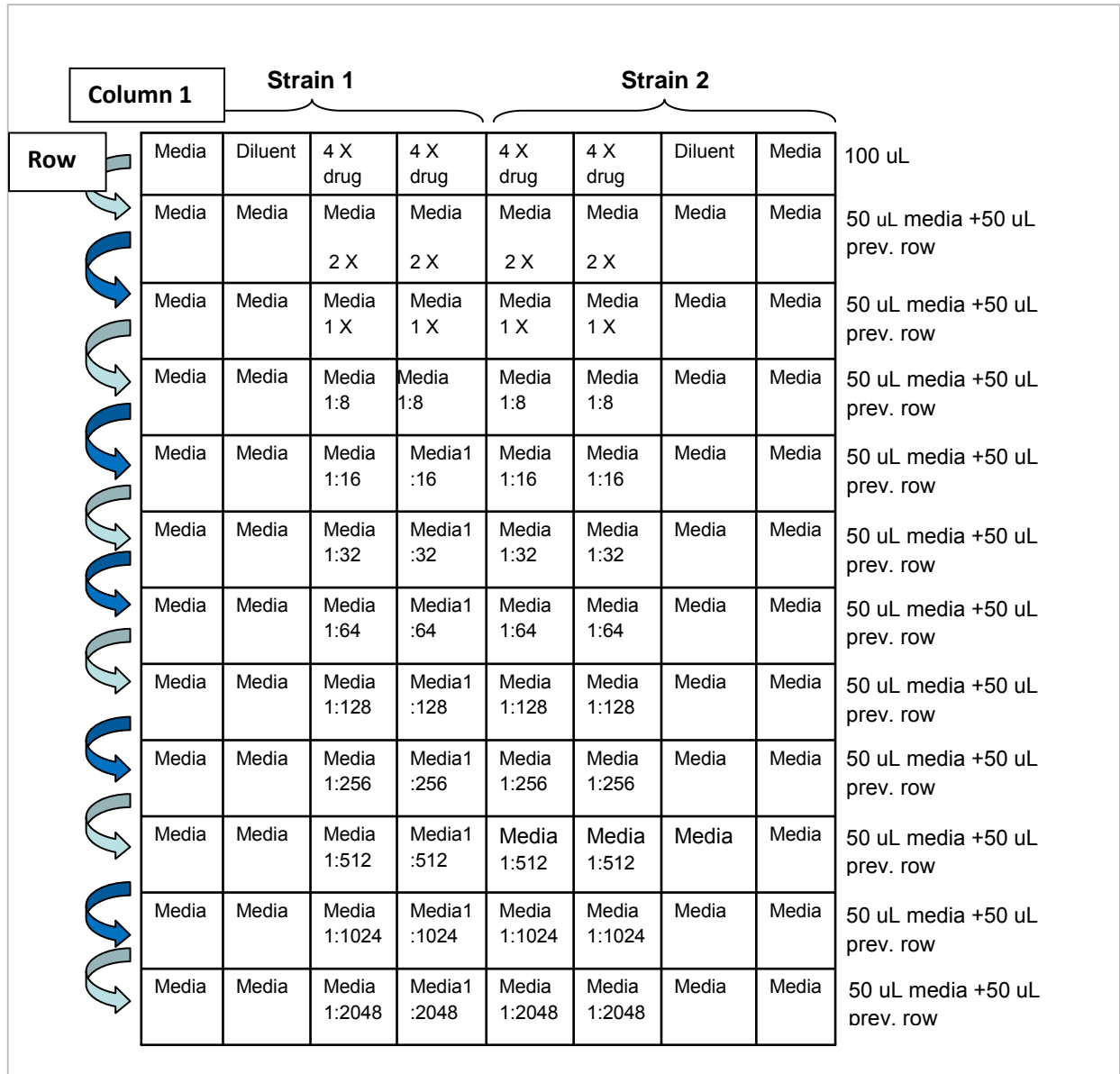


Figure B: demonstration how the 96- well drug containing plate is prepared for MIC and non inhibitory concentration determination by broth microdilution method

APPENDIX C: SUPPLEMENTARY RESULTS DATA

THE GENOTYPIC CHARACTERISTICS OF CLINICAL ISOLATE

Table C: The summary of characteristics of the clinical isolates

Clinical isolate s n	RIF MICs ($\mu\text{g/ml}$)	RIF	INH	ETH	OFL	MO XI	CIP	AMI	CAP	STR	EM B	PZA
Resistance causing genes		<i>rpoB</i>	<i>katG</i>	<i>INHpr om</i>	<i>gyrA</i>	<i>gyrA</i>	<i>gyrA</i>	<i>rrs</i>	<i>rrs</i>	<i>rrs</i>	<i>emb B</i>	<i>pncA</i>
R160	80		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R376	130		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R637	160		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R721	70	<i>rpoB53 1</i>	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R810	150	(Ser to Leu)	wt	<i>inhA- 15prom</i>	wt	wt	wt	wt	0wt	wt	wt	wt
R966	120		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R1035	130		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
R458	130		wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
K636	0.2	No <i>rpoB53 1</i>	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt
H37Rv	0.5	mutatio n	wt	wt	wt	wt	wt	wt	wt	wt	wt	wt

MGIT RESULT EXAMPLE FROM EPICENTER TB eXIST FOR AN ISOLATE

Below it's a typical result from the raw data obtained from EpiCenter TB eXIST for isolate R1035, as an example describing how MIC results are interpreted. These firstly includes the TB eXIST worklist (Figure 3.5.1) which shows the MICs of three anti-TB drugs; ethionamide at 2.5 µg/ml (resistant [R]); 0.25 µg/ml (R represented by undefined 3.1) and 0.625 µg/ml (R represented by undefined 3.2). Then ofloxacin at 2.0 µg/ml (Sensitive [S]); 1.0 µg/ml (S); 0.5 µg/ml (S) and last Ciprofloxacin at 1.0 µg/ml (S represented by undefined 2.1); 0.5 µg/ml (S represented by undefined 2.2) and 0.25 µg/ml (S represented by undefined 2.3). Secondly includes MGIT 960 TB eXIST graph plot (Figure 3.5.2). This shows the MIC plots for Ethionamide (where the GUs ≥ 100 for all tested concentrations); for both ofloxacin and ciprofloxacin (where the GUs = 0 at all concentrations).

TB eXIST Worklist					
Filter Name: TB eXIST Worklist.ftt			28/03/2012 15:08:43		
Enter Accession Number = Caroli1035			Page 1 Of 1		
Sorted By: None					
Antimicrobial	Concentration	GU when GC reached 400	Actual or Last GU (GC on board)	Extended GU (GC removed)	Interpretation — Test Status
Patient Info:					
Accession #: caroli1035					
Organism: Unspecified					
Growth Control (days;hours):		403 (7;17)	14075 (13;23)		
Start DT: 14/03/2012 16:02		*			
Ethionamide	...2.5 µg/mL	6744 (7;17)	9172 (13;23)		R — Complete-R
Ofloxacin	...0.5 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Ofloxacin	...1.0 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Ofloxacin	...2.0 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Undefined 2	...1.0 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Undefined 2	...2.0 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Undefined 2	...3.0 µg/mL	0 (7;17)	0 (13;23)		S — Complete-S
Undefined 3	...1.0 µg/mL	6173 (7;17)	7278 (13;23)		R — Complete-R
Undefined 3	...2.0 µg/mL	5910 (7;17)	6816 (13;23)		R — Complete-R

Figure C1: Illustration of TB eXIST Worklist MICs raw data result for isolate R1035 as an example.* denotes that GC: reached 400 and confirms the interpretation of the tested anti-TB drugs MIC wordlist results

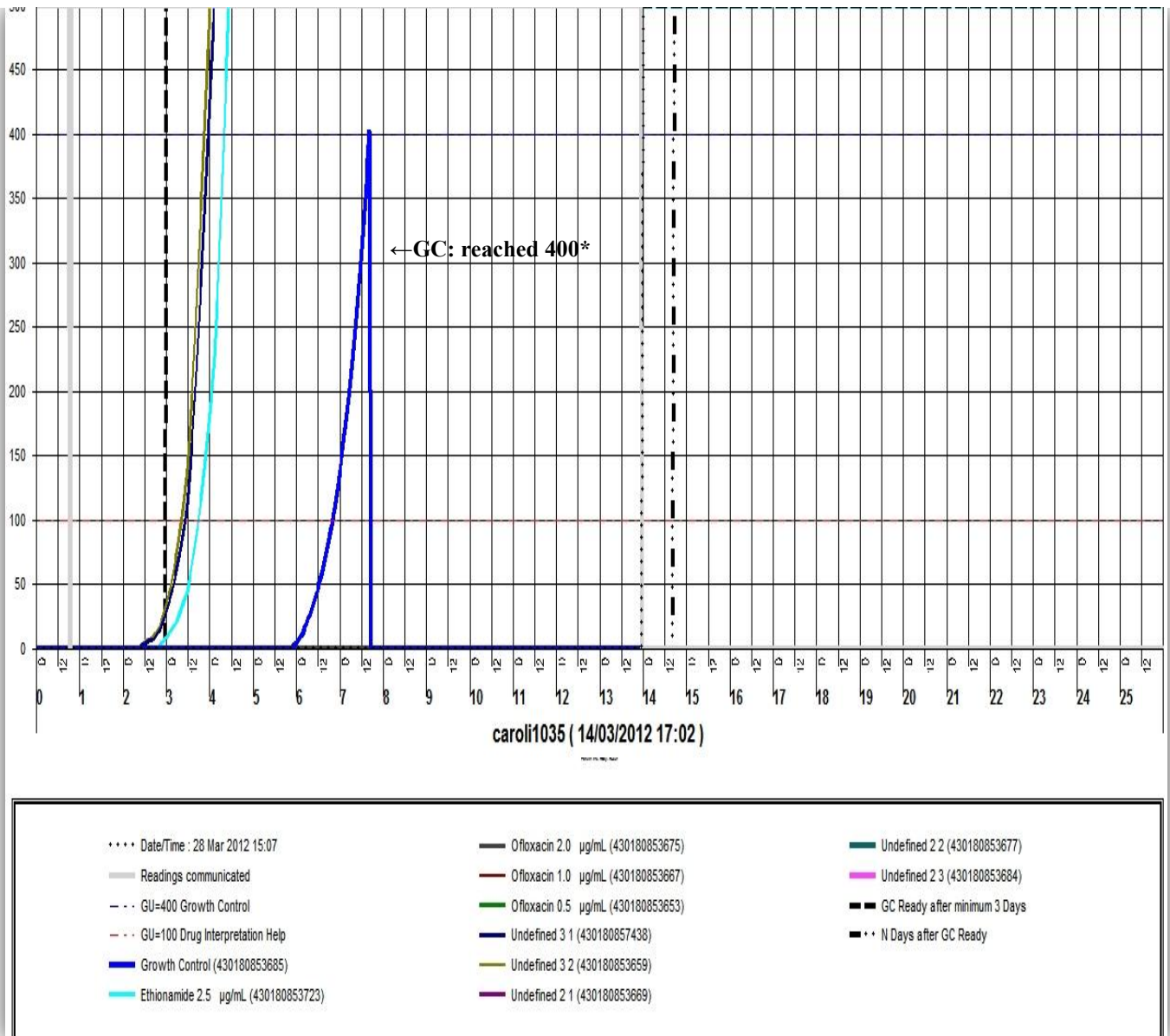


Figure C2: MGIT 960 TB eXIST plot MICs raw data result for isolate R1035.* the blue line plot denotes that GC: reached 400 and confirms the interpretation of the tested anti-TB drugs MIC plots.

BROTH MICRODILUTION RESULTS

To to determine whether reserpine change the MICs of various anti-TB drugs in studied clinical isolates , the following conditions were assessed: the conversion of resazurin to resorufin by resulting in a colour change from blue to pink, this representrd bacterial growth and was scored as either growth (+++), no growth (-) or partial growth (+/-) if less than 50% growth.

Table B: Representation of reserpine inhibitory effect (MIC changes) at the MICs of different anti-TB drugs in *M. tuberculosis* isolates*

Clinical isolates	R 160	R 637	R 376	H37Rv
Drug treatment	Anti-TB drugs MIC ($\mu\text{g/ml}$) in absence/presence reserpine			
INH	0.05	0.05	0.05	0.4
INH+Reserpine	0.01	0.03	0.006	0.05
ETH	1.5	1	0.6	3.5
ETH +Reserpine	3	2	0.15	3.5
EMB	2.5	2.5	0.5	1
EMB +Reserpine	1.25	0.63	1	0.25
PZA	150	50	200	22.5
PZA + Reserpine	150	25	200	22.5
OFL	0.2	0.2	0.2	0.5
OFL +Reserpine	0.2	0.2	0.2	0.25
MOXI	0.04	0.04	0.06	0.13
MOXI +Reserpine	0.02	0.01	0.12	0.07
CIP	0.1	0.25	0.1	0.25
CIP+ Reserpine	0.03	0.06	0.1	0.13
STR	0.1	0.1	0.1	0.8
STR +Reserpine	0.05	0.05	0.03	0.1
AMI	0.25	0.25	0.5	0.5
AMI +Reserpine	0.13	0.13	0.25	0.5
CAP	0.6	0.6	0.6	1.25
CAP +Reserpine	0.3	0.6	0.6	0.625

*the table demonstrates the change in MIC levels of various anti-TB drugs in the presence of reserpine in RIF mono-resistant clinical *M. tuberculosis* isolates and susceptible isolates.