Investigation of the activity of sulfonamide anti-bacterial drugs in Mycobacterium tuberculosis and the role of oxidative stress on the efficacy of these drugs

By

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Declaration

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Presentations and Publications

Publications

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Poster Presentations National and International

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Oral Presentations

Lubabalo Macingwana, Bienyameen Baker, Andile Ngwana , Mark Cotton, Anneke Hesseline, Paul Van Helden, and Ian Wiid (Investigation of the synergistic effect of SMX and Trimethoprim in combination with first-line TB drugs as potential first-line combination drug regimen against Mycobacterium tuberculosis). Stellenbosch University Medical Faculty Academic Year Day, 2011

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Abstract

Tuberculosis (TB) has become a global health epidemic affecting millions of people worldwide with a high incidence in third-world countries. The emergence of multi-drug and extremely-drug resistant *M. tuberculosis* strains together with the HIV/AIDS pandemic warrants the need for new drugs or new drug combinations.

The folic acid synthesis pathway is one of the key pathways that are essential for the survival of bacteria in general. Sulfonamides are a group of compounds that target folic acid synthesis, particularly dihydropteroate synthetase, the first enzyme in the folate pathway. Some of these sulfonamides were used during the introduction of chemotherapy for the treatment of TB in the 1930s, but had toxic side effects. Newer derivatives became safer, but were not employed again for TB treatment.

In a recent case study it was reported that the combination of trimethoprim-sulfamethoxazole (Bactrim), which is used to treat various bacterial infections, such as urinary tract infections, had activity against *M. tuberculosis*. In light of this and the fact that trimethoprim-sulfamethoxazole is well tolerated by humans, we have investigated their antimycobacterial activity with particular interest in the combinational effect of sulfamethoxazole and trimethoprim with the first-line anti-TB drugs, Isoniazid, Rifampicin and Ethambutol against *M. tuberculosis*. Since sulfonamides are known to produce oxidative stress, we also investigated the contribution of this factor to the efficacy of sulfamethoxazole using a mycothiol deficient strain of *M. tuberculosis*, *ΔmshA*. Though trimethoprim-sulfamethoxazole targets the folic acid pathway, we also investigated the

possibility that trimethoprim-sulfamethoxazole may have other cellular targets and applied proteomic analysis.

We have found that Trimethoprim-Sulfamethoxazole has activity against *M. tuberculosis* and that Sulfamethoxazole is the active compound. However, our observation was that not all sulfonamides are active against *M. tuberculosis*. In addition we observed that sulfamethoxazole enhances the activity of Rifampicin against *M. tuberculosis* in a synergistic way. We also observed that a mycothiol deletion mutant was more susceptible to Sulfamethoxazole compared to the wild type strain CDC 1551. Through global protein expression profiling (Proteomics) we were also able to show that sulfamethoxazole could also kill *M. tuberculosis* by oxidative stress production as we identified oxidative stress responsive proteins that were differentially regulated upon exposure to sulfamethoxazole. As trimethoprim-sulfamethoxazole is a registered drug combination, inexpensive and widely available, we propose that this regimen could be used in our fight against *M. tuberculosis* infection.

ABSTRAK

Tuberkulose (TB) is ‘n globale gesondheidsprobleem wat miljoene mense wêreldwyd affekteer met ‘n besoderse hoë voorkoms in die derdewêreld lande. Die voorkoms van multi-middel weerstandige en uitersweerstandige *M. tuberculosis* stamme, tesame met die HIV/VIGS pandemie, steun die erns vir die ontwikkeling van nuwe middels teen *M.tuberculosis*.

Die foliensuur sintesepad is essensieël tot die oorlewing van bakterieë in die algemeen. Vir daardie rede is daar vele middels ontwerp om hierdie metaboliese pad te teiken. Die sulfonamiedes is ‘n groep antibiotika wat foliensuursintese, spesifiek dihidropteroaatsintese, die eerste ensiem in die foliensuursintese pad, teiken. Van hierdie sulfonamiedes is voorheen in die 1930’s gebruik vir die behandeling van tuberkulose, maar het toksiese newe-effekte getoon. Nuwe, minder toksiese derivate, is later ontwikkel maar is nooit vir TB behandeling weer aangewend nie. In ‘n onlangse gevallestudie is daar gerapporteer dat die kombinasie trimethoprim-sulfamethoxazole (TMP/SMX. Handelsnaam: Bactrim), wat normaalweg gebruik word vir die behandeling van algemene bakteriële infeksies soos blaasinfeksies, aktiwiteit teen *M. tuberculosis* getoon het. Na aanleiding hiervan en dat Bactrim veilig in mense gebruik kan word, het ons die aktiwiteit van Bactrim komponente teen *M. tuberculosis* bepaal en in die besonder die aktiwiteite van SMX en TMP in kombinasie met die eerstelinie anti-tuberkulose middels Isoniasied, Rifampisien en Ethambutol. Aangesien sulfonamiedes ook oksidatiewe stres intrasellulêr genereer, het ons ook die bydrae van hiervan tot die doeltreffendheid van SMX bepaal deur gebruik te maak van ‘n mycothiol-gemuteerde *M.*

tuberculosis stam (ΔmshA). Omdat TMP/SMX die foliensuur-pad hoofsaaklik teiken het ons ook die moontlikheid ondersoek dat SMX ander sellulêre teikens het en het ons proteomiese (Proteomics) tegnieke hiervoor aangewend. Ons het gevind dat TMP/SMX aktiwiteit teen *M. tuberculosis* toon en dat SMX die aktiewe komponent van Bactrim is teen *M. tuberculosis*. Ons wys ook dat sulfonamiedes in die algemeen nie noodwendig ook aktiwiteit teen *M. tuberculosis* toon nie. Ons het ook waargeneem dat SMX die aktiwiteit van rifampisien bevorder en dat die twee middels saamwerk op ‘n sinergistiese wyse. Ons het ook getoon dat oksidatiewe stres ‘n rol speel deurdat‘n mycothiol delesie-mutant meer vatbaar was vir SMX in vergelyking met die wilde-tipe stam van *M. tuberculosis* (CDC1551). Met globale proteïen-kartering (Proteomics) het ons ook getoon dat SMX *M. tuberculosis* kan doodmaak deur oksidatiewe stres te genereer omdat ons oksidatiewe stres reaktiewe proteïne geïdentifiseer het wat differensieël gereguleer is gedurende blootstelling aan SMX. Aangesien Bactrim ‘n reeds geregistreerde middel is, goedkoop is en geredelik beskikbaar is, stel ons voor dat Bactrim moontlik geïnkorporeer kan word in die huidige behandeling van .Ttuberkulose.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

INH – Isoniazid

INH^R – Isoniazid resistant

RIF– Rifampicin

RIF^R–Rifampicin resistant

EMB– Ethambutol

SMX–Sulfamethoxazole

TMP–Trimethoprim

CCCP–Carbonyl cyanide m-chlorophenyl hydrazone

VEP–Verapamil

RES –Reserpine

DHF– Dihydropteroylglutamate/ Dihydrofolate

THF– Tetrahydropteroylmonoglutamate/Tetrahydrofolate

folP1– Dihydropteroate synthase

thyA– Thymidylate synthase

MIC–Minimum inhibitory concentration

bp – base pairs

BSA – bovine serum albumin

°C – degrees Celcius

cDNA – complementary DNA

cm – centimetre

Ct – threshold value

dH2O – double distilled water

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

dNTP – deoxynucleotide triphosphate

Tween -80– polyoxethlene sorbitan monooleate

kb - kilobase

M – molar

µg – microgram

MgCl2 – magnesium chloride

min - minute

µl – microliter

µM – micro Molar

mM – millimolar

NAD - nicotinamide adenine dinucleotide

NADP - nicotinamide adenine dinucleotide phosphate

ng – nanogram

OADC – oleic acid/albumin/dextrose/catalase

OD – optical density

PCR – polymerase chain reaction

RNA – ribonucleic acid

RNase – ribonuclease

rpm – revolutions per minute

RT-PCR – real time polymerase chain reaction/reverse transcriptase polymerase chain reaction

SB – di-Sodium tetraborate decahydrate buffer

SDS- sodium dodecyl sulphate

sec – second

TB – tuberculosis

Ta – annealing temperature

U – units

V – volts

CHAPTER 1

INTRODUCTION

1.1 General introduction

Tuberculosis remains one of the most deadly diseases in the world, second to Human Immunodeficiency Virus (WHO, 2002). It is caused by the bacillus *Mycobacterium tuberculosis.* There were about 8.7 million incident cases of new TB cases in 2011 of which 59% came from Asia and 26% came from Africa and 1.4 million TB deaths (WHO, 2012).

The spread of this disease is correlated with the socio-economic condition such as housing quality and overcrowding and is therefore extremely sensitive to changes in the standard of living and nutrition (Puranen Bi, 2003). Figure 1.1 shows a geographic estimation of new TB cases around the globe, even though there has been a significant decrease in new cases, South Africa is still placed among the top 5 countries with a high TB burden (WHO, 2012). The other factor that has greatly fuelled the deaths caused by TB is the HIV co-infection, with the highest HIV-TB cases in the African region (WHO, 2012) (figure 1.2).

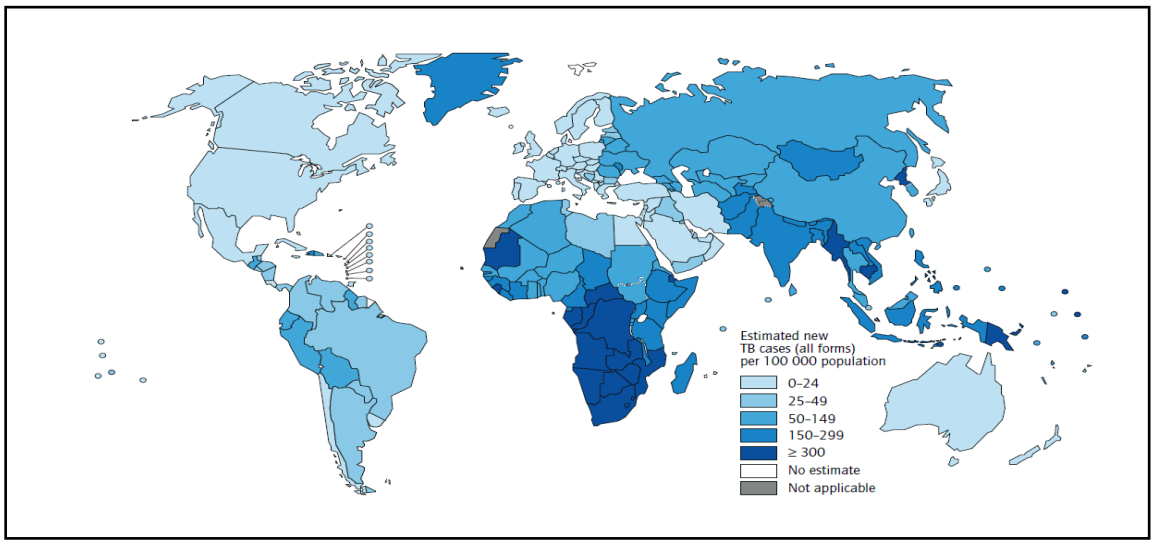


Figure 1.1: Estimated new TB cases per 100 000 population (WHO, 2012).

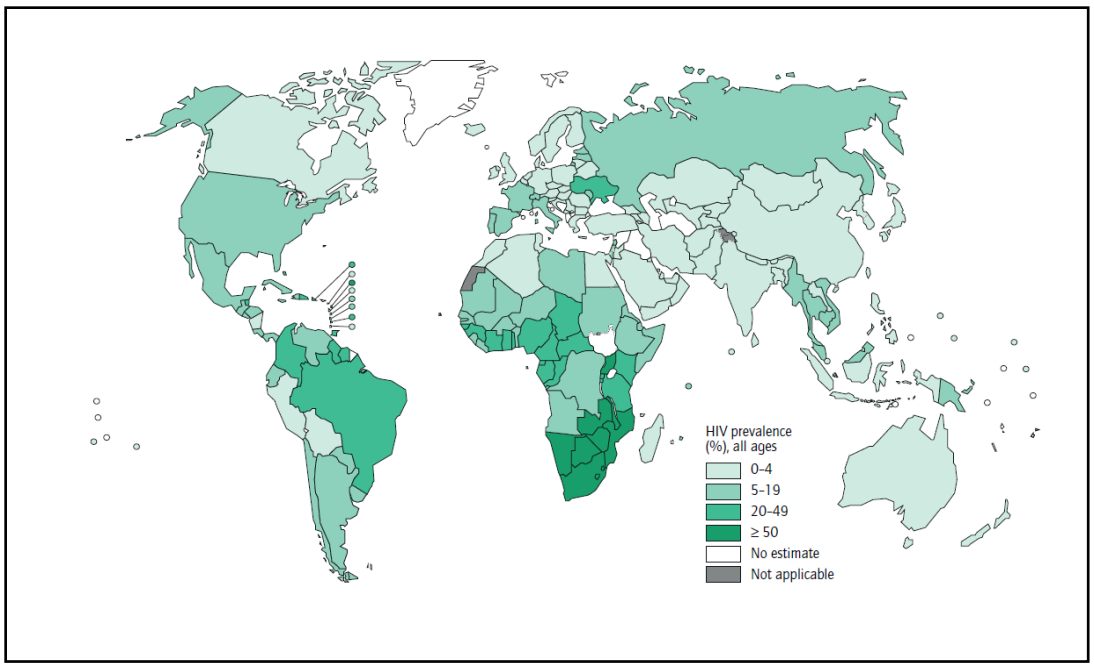


Figure 1.2: shows a global estimation of HIV/TB prevalence (WHO, 2012).

1.2 Historical point of view and epidemiology of Tuberculosis

Tuberculosis is one of the old human afflicts and is estimated that this disease has existed even before the dawn of humankind and may have infected early hominids (Gutierrez *et al.*,2005; Daniel, 2006). According to the evidence obtained using modern molecular genetics, sequencing of the genome of various strains of *Mycobacterium tuberculosis* and the archeological evidence, it could be that the ancestral home of tubercle bacilli and its human hosts was in East Africa (Daniel, 2006).

This disease attacks various parts of the body and is thus categorized into two forms, pulmonary (figure 1.3) and extra-pulmonary tuberculosis (Leung, 1999). Pulmonary tuberculosis is the most common form of TB, with extra-pulmonary TB constituting about 15 to 20 per cent in immune

competent individuals and about 50 per cent in HIV infected individuals (Sharma and Mohan 2004).

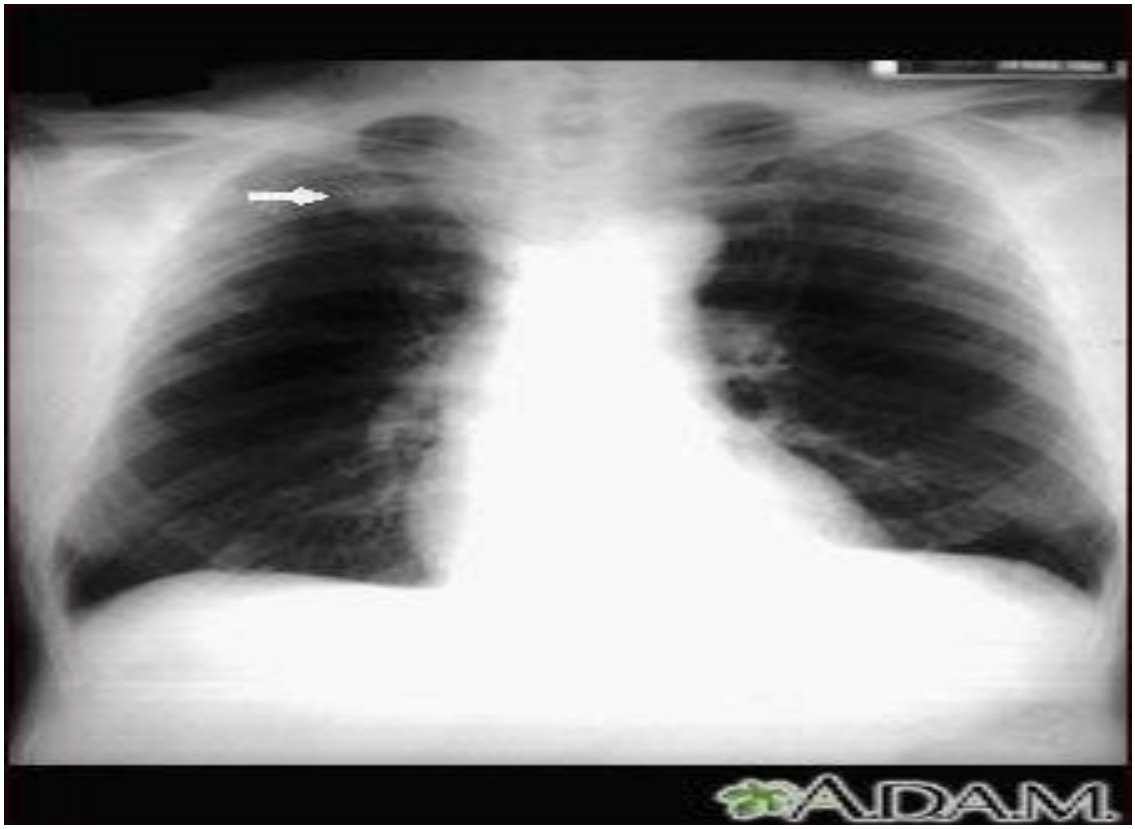


Figure 1.3: This x-ray shows a single lesion (pulmonary nodule) in the upper right (Board, 2012)

Extra-pulmonary Tuberculosis (EPT) develops in many organs and the risk of developing this type of TB increases with the decrease in immune competency (Golden and Vikram 2005).

Pleural Tuberculosis is another form of EPT which accounts for about 5% of all TB cases; its symptoms are usually pleuritic chest pain, fever, or dyspnea (Golden and Vikram 2005). Skeletal (Bone and joint), spinal and central nervous system (meningitis) tuberculosis are other forms of extra-pulmonary Tuberculosis and occur in different degrees figure 1.4 (Golden and Vikram 2005).

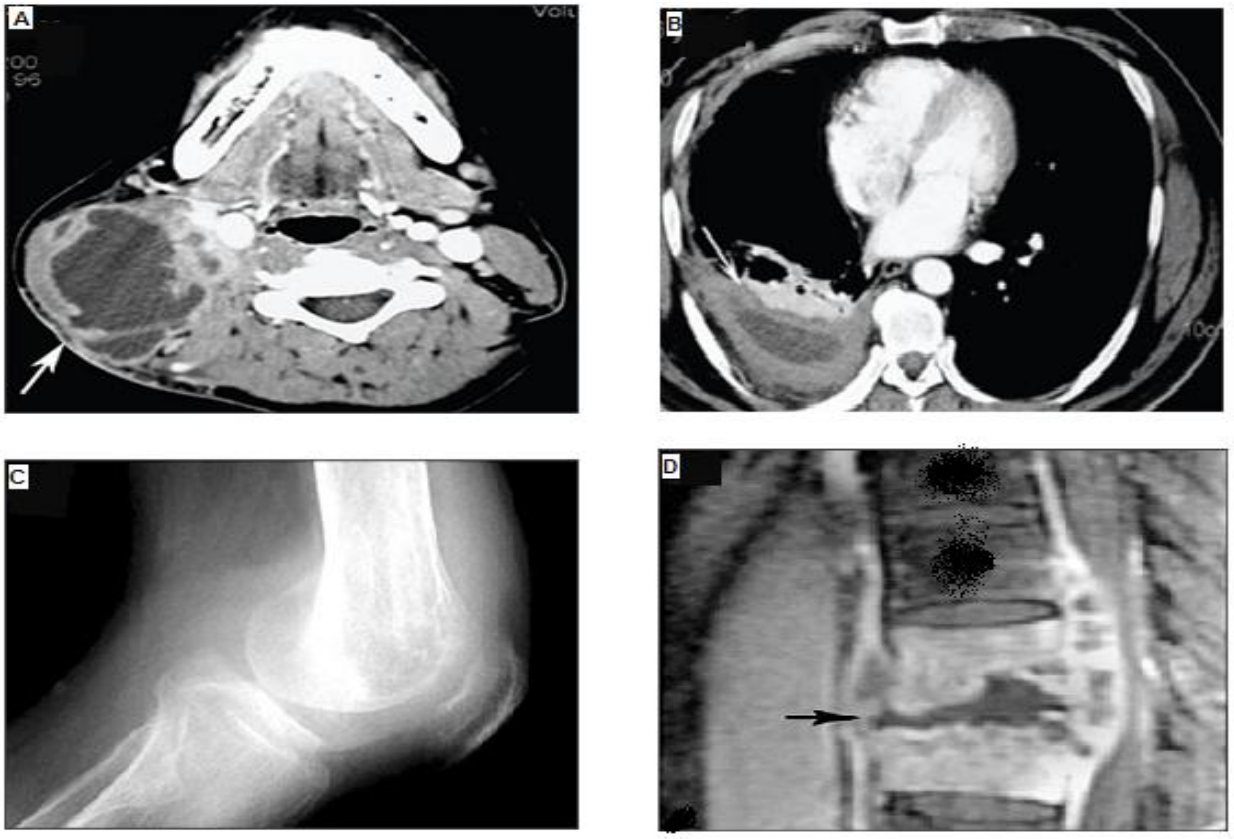


Figure 1.4: shows different forms of extra-pulmonary tuberculoses (A) Cervical tuberculoses, (B) Pleural tuberculosis, (C) Osteoarticular tuberculosis and (D) Spinal tuberculosis (Golden and Vikram, 2005).

The cases of these forms of extra-pulmonary tuberculosis vary from country to country and also depending on the origin of an individual within a country (Smith, 2003). Generally, extra-pulmonary tuberculosis arises from pulmonary tuberculosis through dissemination from an infected lung (Smith, 2003). A sequence of events illustrating how primary tuberculosis occurs has been generated (Grange and Zumla, 2008).

The first stage of Wallgren's time table begins from 3 to 8 weeks after inhalation of *M. tuberculosis* aerosols, which travel to alveoli and then disseminated by lymphatic circulation to lung lymph nodes forming the Ghon complex. The second stage begins when *M. tuberculosis*

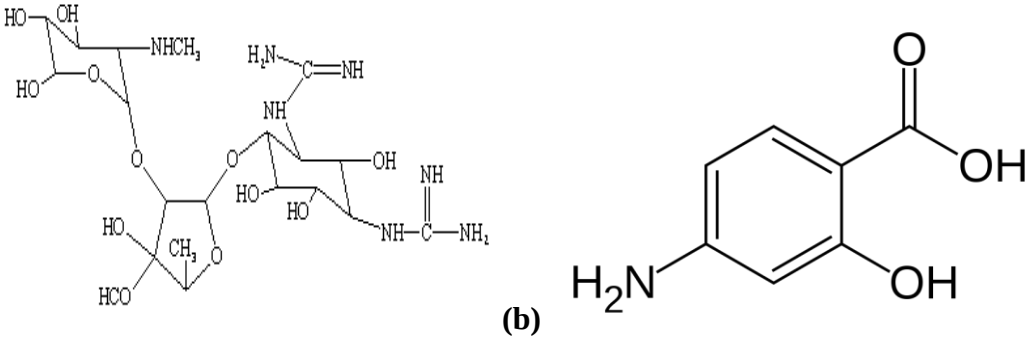
enters into the blood circulation to other parts of the body and organs that is termed Miliary tuberculosis that lasts for 3 months. The third stage is thought to be the result of release of *M. tuberculosis* from haematogenous dissemination or from the lungs to the pleural space giving rise to pleural tuberculosis. The last stage is the resolution of the Ghon complex or primary complex and might last for 3 years, this stage is marked by bone and joint pains (Smith, 2003).

1.3 Antibiotic treatment for Tuberculosis

Since the discovery of *Mycobacterium tuberculosis* by Robert Koch in 1882, the treatment of this disease was based mainly on resting, fresh air, good nutrition and improving social and hygienic conditions. These methods were later combined with artificial pneumothorax and other surgical methods to reduce the lung volume, which proved to be more effective at the time. Following these innovative methods of therapy, was an unexpected discovery of an attenuated form of *Mycobacterium bovis,* Bacillus Calmette–Guérin (BCG), which was used for preventive measures (Hsu *et al.*, 2003). Streptomycin discovered in 1943, was the first antibiotic active against *Mycobacterium tuberculosis* which was well tolerated by the body, with limited toxicity and was administered for the first time in patients in 1944 (Schatz *et al.*, 1944). A few years later it was realized that resistance to the single drug occurred rapidly thereby threatening the success gained from streptomycin (Graessle and Pietrowski, 1949).

Para-Aminosalicylic acid (PAS) was also discovered in 1943 and found to be active against *M. tuberculosis in vitro*, but its use in humans was delayed until 1948 due to the conflicting reports from clinical trials (Houghton, 1950).

Graessle and Pietrowski (1949) showed that addition of PAS to the TB treatment prevented *in vitro* development of resistance of *M. tuberculosis* to streptomycin (Graessle and Pietrowski, 1949). These results gave rise to the start of combined therapy against *Mycobacterium tuberculosis*.



(a) **(b)**

Figure 1.5: chemical structure of Streptomycin **(a)** and Para-Aminosalicylic acid **(b)** (structures obtained from PubChem).

Isoniazid was introduced in 1953 and improved the efficacy of the treatment. In 1960 Ethambutol replaced para-aminosalicylic acid and Rifampicin was introduced in 1970. The multidrug combination reduced the course of the treatment from 24 to 6 months (Almeida *et al.,* 2007).

1.4 Resistance of *M. tuberculosis* to current drug treatment

The current management system of the tuberculosis disease consists of two regimens; The front-line regimen (isoniazid, rifampicin, ethambutol and pyrazinamide) and the second-line regimen which is often toxic and expensive (amikacin or kanamycin, capreomycin and moxifloxacin) and their target sites are summarizes in table 1.1.

The search for new anti-tuberculosis drugs or measures have been renewed by the emergence of the multi-drug resistant *M. tuberculosis* strains (MDRs), defined as the strains of *M. tuberculosis* that are resistant to isoniazid and rifampicin, the most effective first-line drugs and extensively drug resistant TB strains (XDRs), defined as the MDRs that have gained resistance to fluoroquinolones and at least one of the injectable drugs, aminoglycosides or polypeptides (Basu and Galvani, 2008).

Apart from drug resistance, the current regimen is also marked by high levels of cytotoxicity and to some degree, antagonism of activity of drugs that are co-administered with the regimen (Lees *et al.,* 1971).

Drug	**Mode of action**	**(Target genes that contain alterations)**
Isoniazid	cell wall integrity	*KatG* and *InhA* genes
Rifampicin	Nucleic acid synthesis	*rpoB* gene
Ethambutol	Mycobacterial cell wall	*emb*B gene
Pyrazinamide	Disrupts membrane energetics and inhibit membrane transport functions	*pncA* gene
Amikacin	Inhibits translation	*rrs* gene
Kanamycin	Inhibits translation	*rrs* gene
Capreomycin	Translation in Mycobacteria	*tlyA* gene
Moxifloxacin	Release of DNA breaks	Quinolone resistance-determining region (QRDR) of *gyrA gene*

Table 1.1: A Summary of the mechanism of action of the first-line and the second-line anti-TB drugs, together with the mechanisms of resistance, Adapted from literature review (Alangaden *et al.*, 1998; Maus, Plikaytis, and Shinnick 2005; Mphahlele *et al.*, 2008; Sreevatsan *et al.*, 1997; Chen *et al.*, 1996; Ma *et al.*, 2010).

Further, for many years it was accepted that drug resistance develops through the process of spontaneous mutations in the target genes as shown in table 1.1 (Louw *et al.*, 2011). There are other mechanisms involved in the susceptibility of microorganisms to antibiotics. In recent years the focus has been in defining these mechanisms of drug resistance in order to effectively treat bacterial infections. This was prompted by the inability to detect the mechanism of resistance of isolates that did not harbor mutations in the target genes of the drugs.

For example, it is estimated that about 30% of isoniazid resistant isolates do not have mutations in the putative target genes and that about 5% of rifampicin resistant isolates do not harbor mutations in the RNA polymerase gene (Telenti *et al.*, 1993). It is now known that active efflux of drugs plays a major role in drug resistance (Li and Nikaido, 2004). These efflux pump systems can be drug specific and also transport various drugs from deferent classes (Higgins, 2007). They are categorized into five groups, namely ATP-binding cassette (ABC) superfamily, major facilitator superfamily (MFS), multidrug and toxic compound extrusion (MATE) family, small multidrug resistance (SMR) family and the resistance-nodulation-division (RND) superfamily (Li and Nikaido, 2009).

These mechanisms of drug resistance work together in helping microorganisms to successfully evade antibiotic stress. Therefore, there is a need for new antimycobacterial agents that are more effective, less toxic and that would shorten the treatment duration in order to prevent patient non compliance.

1.5 New anti-TB drugs in the pipe-line and new TB treatment regimen

The failure of the current TB treatment regimen are mainly due to the long periods of administration and adverse side effects leading to patient non compliance (Boogaard *et al.*, 2009), thus promoting mutations in the targets genes and the induction of other defence mechanisms in the organism that are exposed to low levels of antibiotics. Therefore, new drugs must have different mechanisms of action to the existing drugs to avoid cross resistance.

New effective anti-tuberculosis treatment regimens must be able to shorten the duration of treatment and allow co-administration with HIV and AIDS treatment and must have minimal cytotoxicity (Ma *et al.*, 2010). Progress has been made in the development of new compounds for TB treatment. In 2012, the Food and Drug Administration (FDA) announced that a Johnson & Johnson tuberculosis drug TMC207 has been approved, which is the first new effective TB drug in more than four decades (FDA, 2013).

There are other compounds with novel targets that are in the late stages of clinical trials and are anticipated to greatly improve the control of TB (Swindells, 2012). Figure 1.6 lists the new anti-TB drugs and the stages of clinical trials at which they are being evaluated. Figure 1.7 illustrates the different targets and mechanism of action of the new anti-tuberculosis drugs.

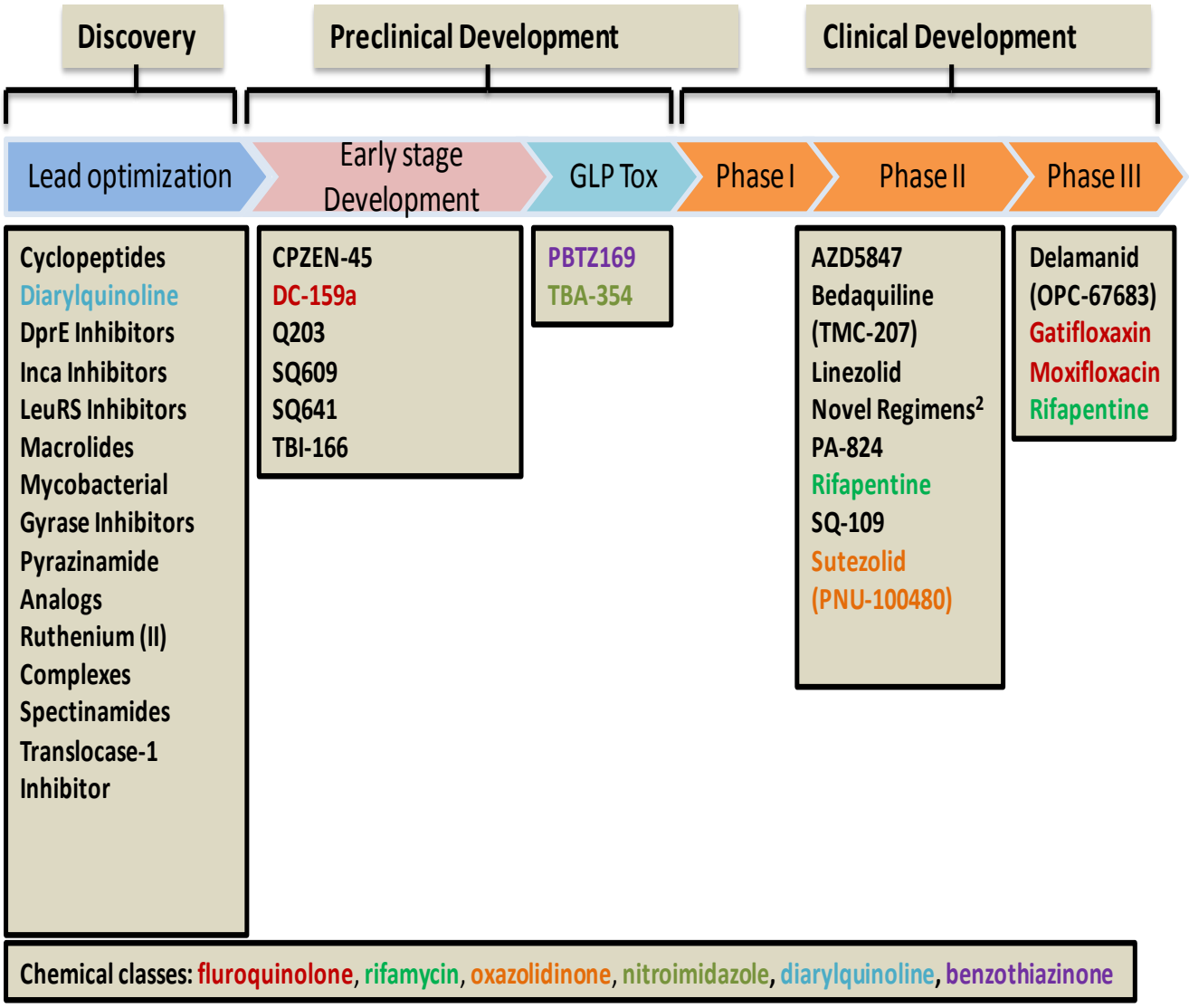


Figure 1.6: Summary of the drug clinical trials in oder to determine the safety and eficacy of the new anti-TB drugs (Figure adpted from http://www.newtbdrugs.org/pipeline)

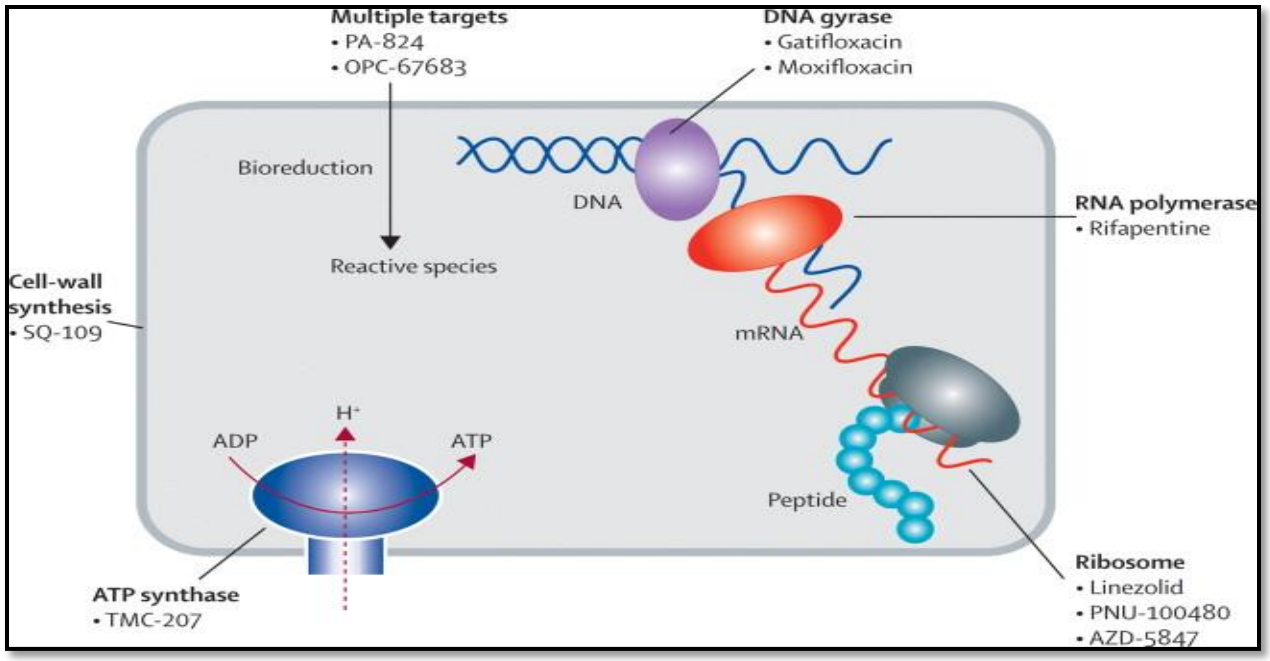


Figure 1.7: Summary of the targets of new anti-tuberculosis agents (Ma *et al.*, *2010*). One of the key requirements of the new anti-TB drug is that it must have completely different target to the existing drugs. As illustrated on the figure, these promising anti-TB drugs have that valuable feature.

A new TB regimen that does not contain rifampicin, PaMZ, that is composed of PA-824 which is a nitroimidazo-oxazine, moxifloxacin, (a fluoroquinolone) and pyrazinamide cured mice faster than the first-line regimen (which contains INH and RIF) and an experimental regimen RIF-MXF-PZA (Stover *et al.*, 2000).

This is the first time a regimen that does not contain rifampicin and isoniazid was able to prevent relapse more effectively than the first-line regimen and also reduce the treatment duration to 4 months (Nuermberger *et al.*, 2008). Diacon and his colleagues also confirmed the efficacy of this regimen, and showed that this combination could kill MDR-TB within 2 weeks (Diacon *et al.*, 2012).

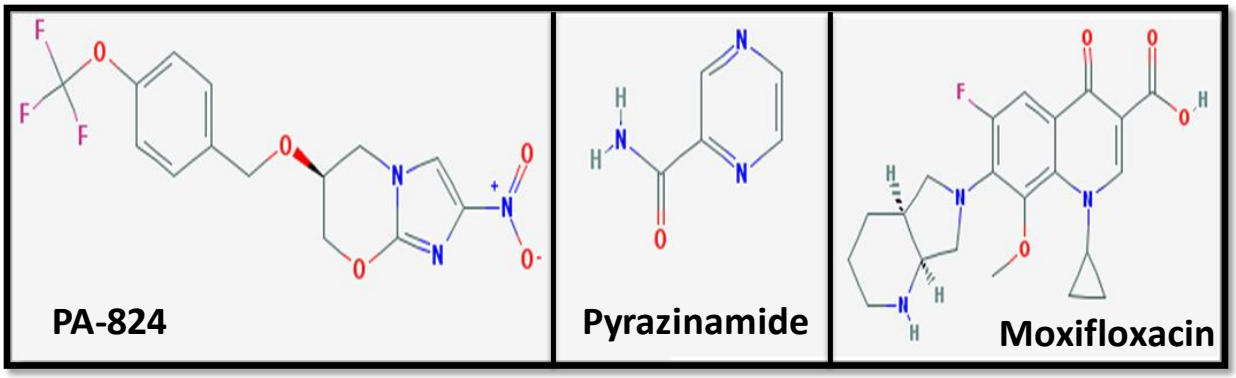


Figure 1.8: chemical structures of the compounds in the novel TB regimen (structures obtained from PubChem).

Thus far, progress has been made in the search for new drugs. Figure 1.9 summaries this progress from the discovery of streptomycin to the present day and the development of new TB regimens.

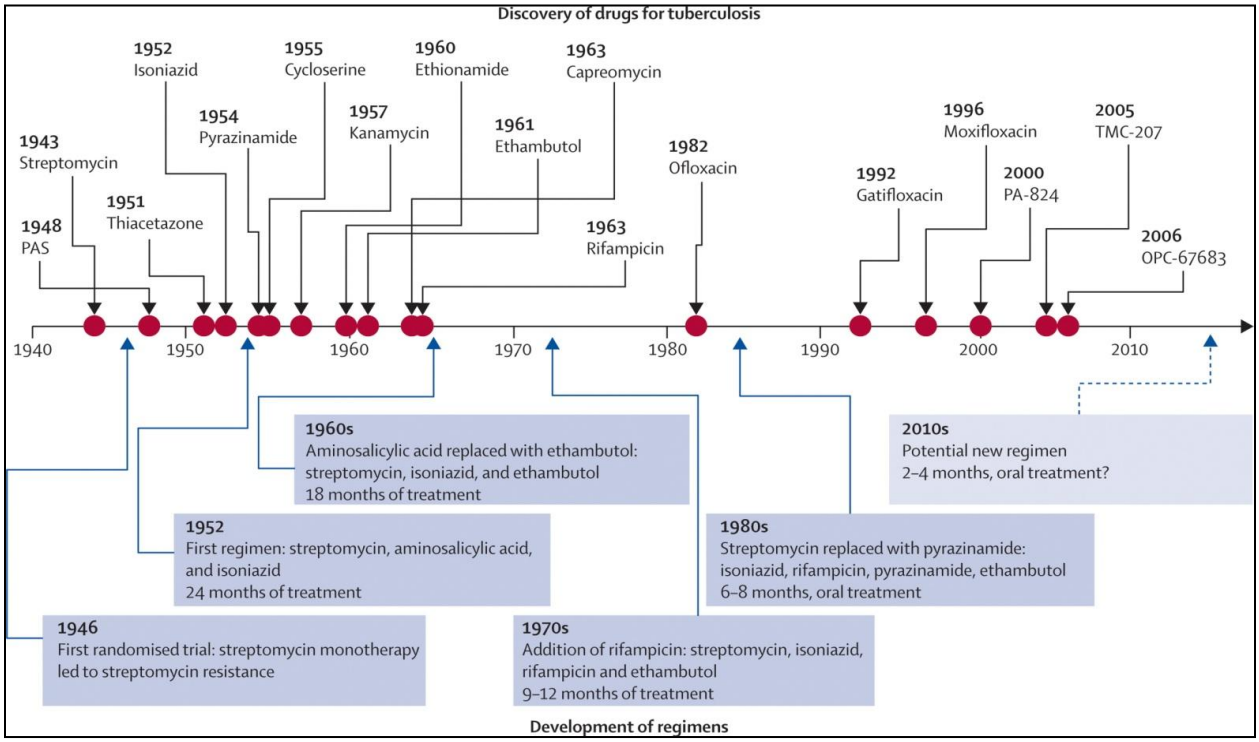


Figure1.9: Time line of TB drug discovery and development of TB regimens for tuberculosis. Arrow with dashed line represents future regimen. Red dots represent when the drugs were first reported (Ma *et al.,* 2010).

While new drugs are being searched for, the recall of old and forgotten drugs has found a place in many pharmaceutical companies, because it takes about 10 to 15 years to find a new compound that is effective, safe and this process is very expensive. While on the other hand, the repurposing of old and forgotten drugs is cheaper and faster. Figure 1.10 illustrates the process of drug development.

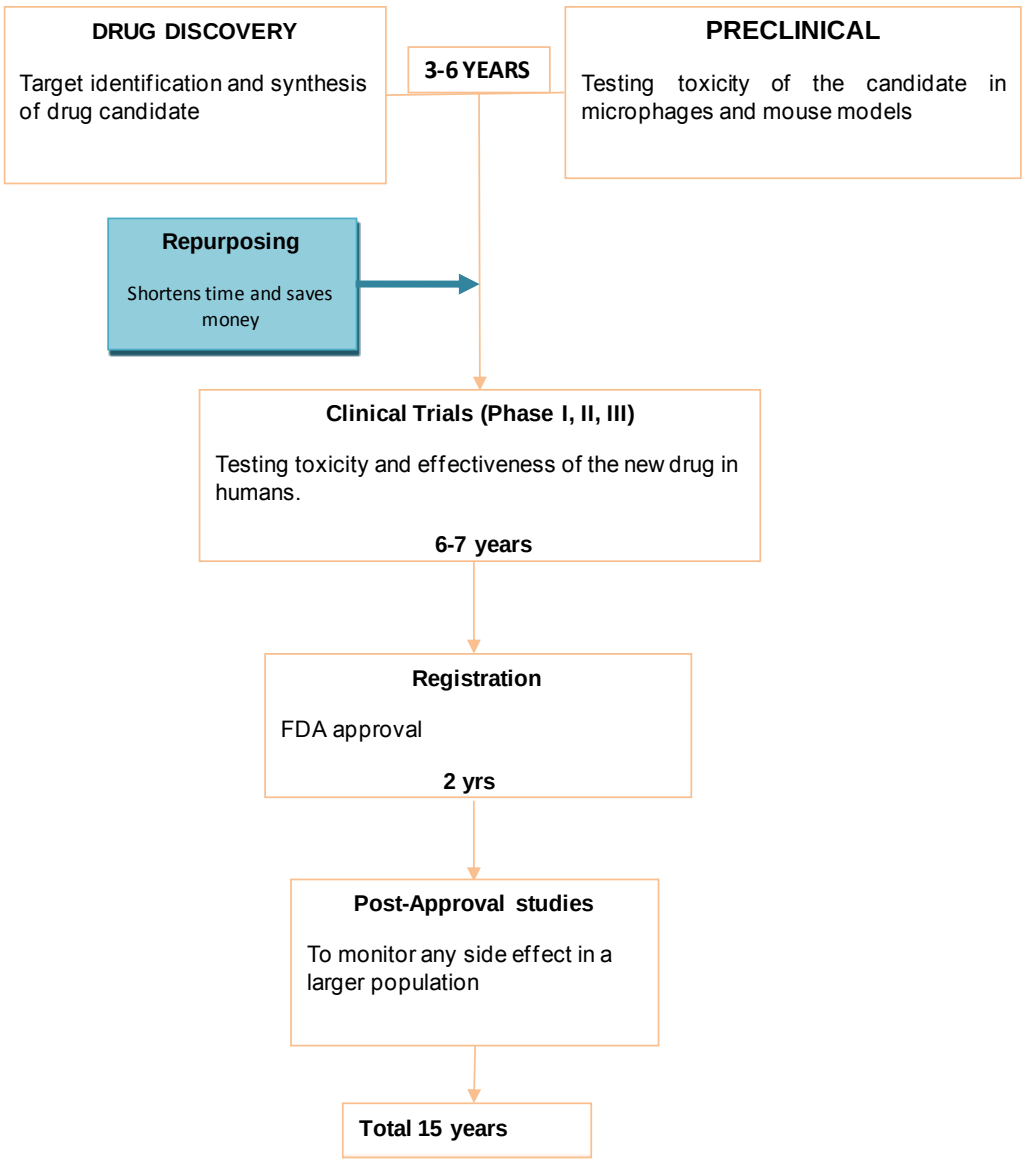


Figure 1.10: Time-line for discovery of new TB-drugs

1.6 History of sulfonamides and the treatment of TB

The repurposing of one class of FDA approved drugs for treating tuberculosis, the sulfonamides, have recently drawn attention. These compounds were the first chemical substances to have a real antibacterial activity discovered in the 1940s (Woods, 1940).

Recently, Forgacs and co-workers observed that a patient that was thought to have had nocardiosis and was placed on TMP-SMX improved after the start of the treatment. The patient was later found to have tuberculosis and not nocardiosis. They then decided to evaluate more samples from this patient and their results concluded that *Mycobacterium tuberculosis* is susceptible to TMP-SMX whose putative targets are dihydrofolate reductase and dihydropteroate synthase, respectively (see figure 1.12) (Forgacs *et al.*, 2009).

The first sulfonamides that showed an inhibitory effect against *M. tuberculosis* was sulfanilamide and sulphapyridine, these compounds however required high concentrations, which were very toxic to the host to achieve sterilization (Follis 1940; Smith *at al.*, 1942).

Subsequently, Smith and co-workers also discovered other sulfonamides (sulfathiazole, and sulfadiazine) that inhibited the growth of *Mycobacterium tuberculosis* (Smith *at al.*, 1942). Figure 1.11 shows the chemical structures of some sulfonamides that are active against *Mycobacterium tuberculosis*.

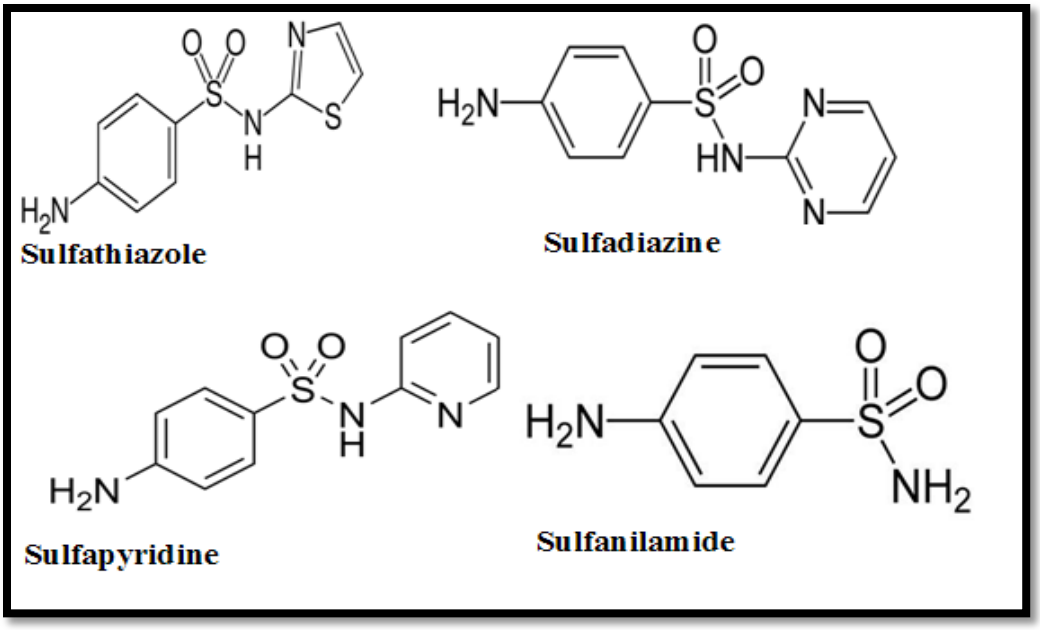


Figure 1.11: Chemical structures of the early sulfonamides that have inhibitory action against *Mycobacterium tuberculosis* (structures obtained from PubChem).

1.7 Mechanism of action of Sulfonamides

Sulfonamides target the folic acid pathway and inhibit the first enzyme in the pathway, dihydropteroate synthase and they are structural analogs of the substrate, para-aminobenzoic acid (figure 1.12) (Follis, 1940). The inhibition of this pathway results in the depletion of purines, thymine and serine whose synthesis depends on tetrahydrofolate (Hitchings, 1973).

Dihydropteroate synthase does not exist in higher organisms and therefore depend on dietary sources for dihydrofolate. Woods-Fildes showed that the addition of para-aminobenzoic acid to the medium suppressed the inhibitory effect of the sulfonamides. However, some researchers

argued that the inhibitory effect of sulfonamides can not only be centered on the competitive action with para-aminobenzoic acid.

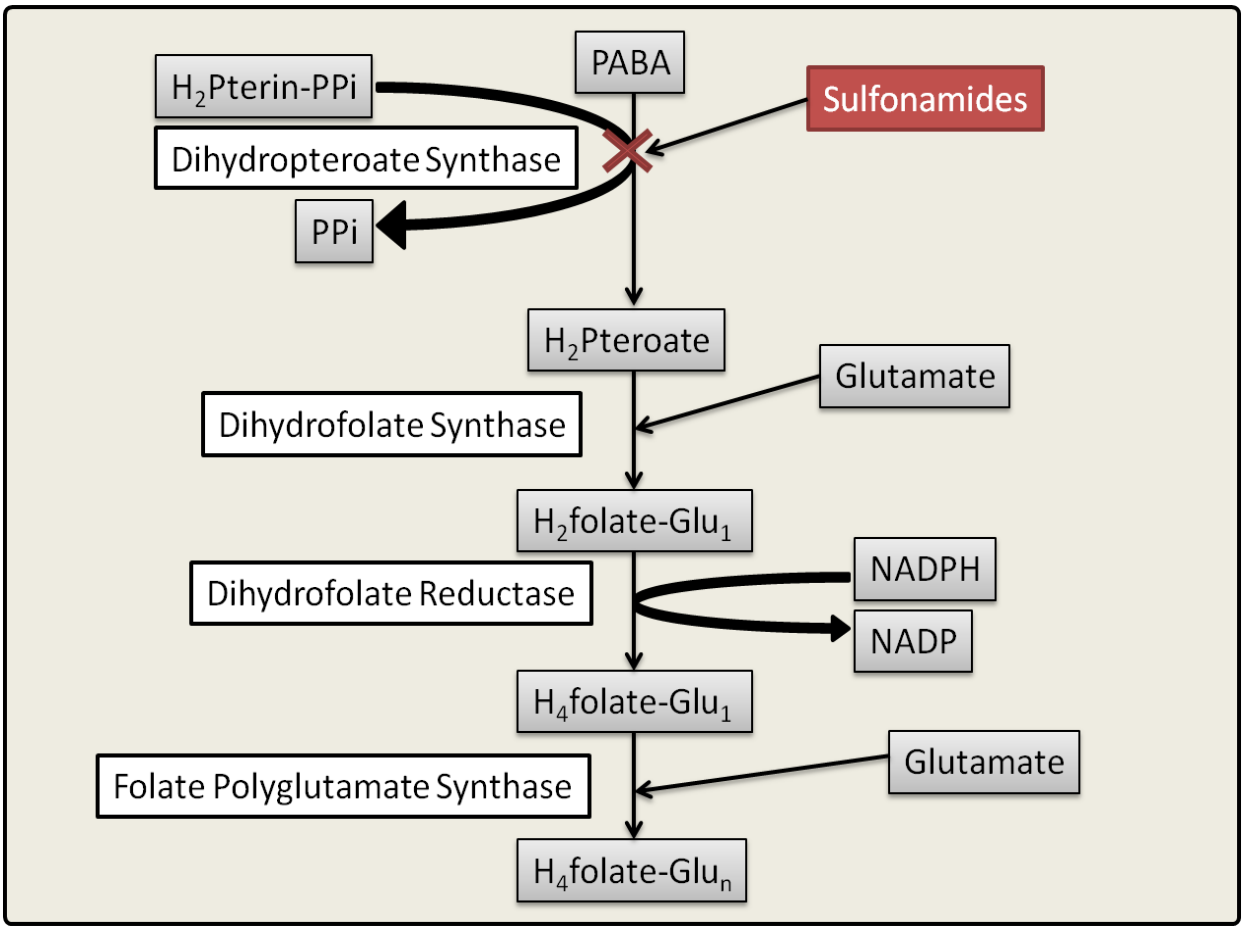


Figure 1.12: Schematic representation of the mode of action of sulfonamides.

Sulfonamides also block the functioning of various pathways including pyruvate dismutation, oxidation, and the synthesis of amino acids, succinate and lactate. They also inhibit enzymes such as bacterial dehydrogenase, cytochrome reductase, cytochrome oxidase, flavoproteins, bacterial luciferase, staphylococcal coagulase, yeast sucrase and amylase (Yegian and Long, 1951). Para-aminobenzoic acid antagonises the inhibitory effect of sulfonamides and other antagonisers include cocarboxylase, flavine-adenine dinucleotide, riboflavin and methylene blue.

In 1980, it was reported that homocysteine sulfonamide (figure 1.13), is a competitive inhibitor of *Escherichia coli* and *Saccharomyces cerevisiae* glutamine synthetase, an enzyme that catalyses the synthesis of glutamine from glutamate, a physiological important reaction in central nitrogen metabolism of living organisms (Meek and Villafranca, 1980; Masters and Meister, 1982).

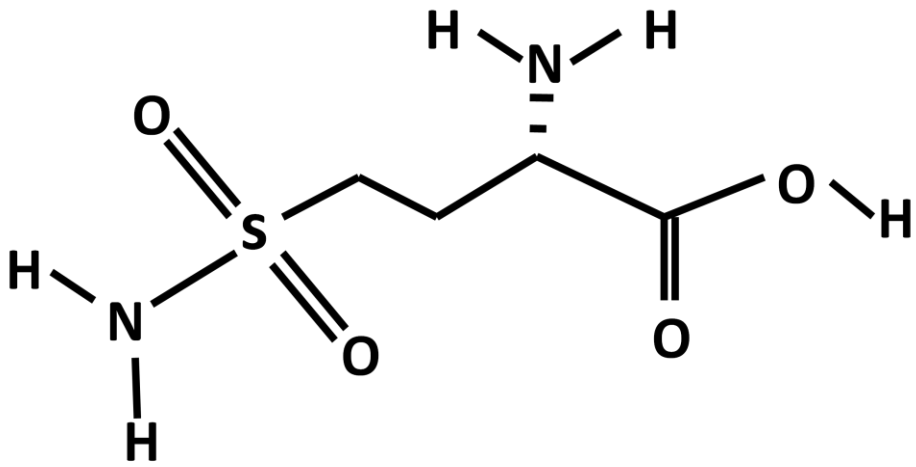


Figure 1.13: chemical structure of a sulfonamide that inhibits glutamine synthetase, homocysteine sulfonamide.

Recently, carbonic anhydrases have also been identified as targets of sulfonamides; these enzymes catalyze the hydration of carbon dioxide to form bicarbonate (Meldrum and Roughton, 1933). Bicarbonate is very important in the synthesis of long chain fatty acids, pH homeostasis and other small molecules (Covarrubias *et al.*, 2005). Various sulfonamides that effectively inhibit carbonic anhydrases have been identified (figure 1.14) (Vullo *et al.*, 2003; Winum *et al.*, 2003; Weber *et al.*, 2004).

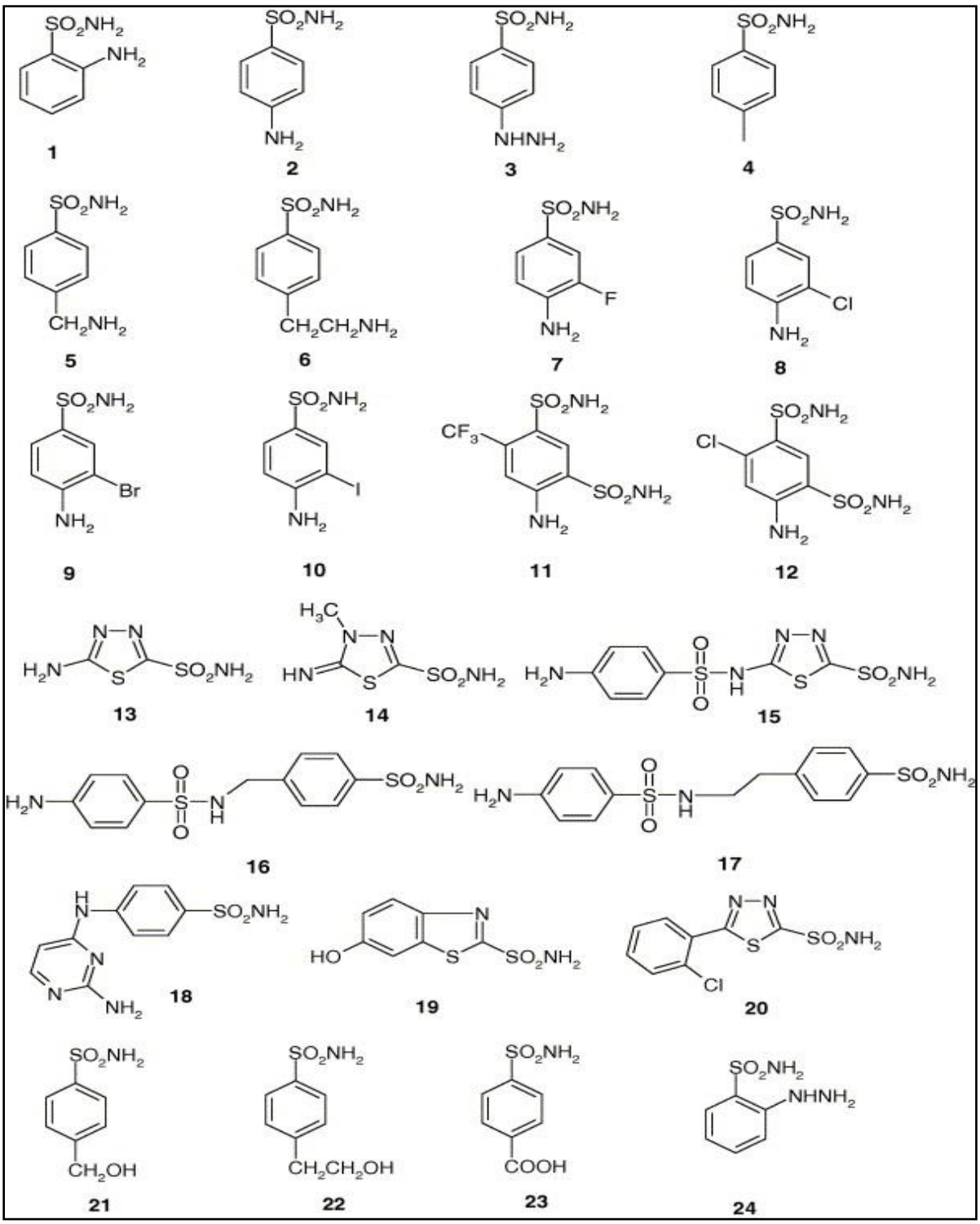


Figure 1.14: Chemical structures of sulfonamides that inhibit carbonic anhydrases (Vullo *et al.*, 2005).

Carbonic anhydrases are widely distributed throughout living organisms and there are at least five classes (α-, β-, γ-, δ-, and ζ carbonic anhydrases) with α- found in humans and β found mainly in bacteria (Supuran, 2011). It has been reported that one of the three carbonic anhydrases in *Mycobacterium tuberculosis,* Rv3588c, is essential for survival in vivo and all *Mycobacterium tuberculosis* carbonic anhydrases are inhibited by sulfonamides (Sassetti and Rubin, 2003). Further, it has been reported that oxidative stress also plays a role in the mechanism of action of sulfonamides, possibly due to their bio-activation (Coleman *et al.*, 1989; Cribb *et al.*, 1990).

Microarray, proteomics and other techniques have facilitated the identification of drug targets. Global proteomic profiling has been carried out in many studies in order to identify possible proteins and pathways contributing to a specific phenotype such as cross resistance (Sleno and Emili, 2008).

1.8 Study design

This study was undertaken in the understanding that the work reported by Forgacs and co-workers 2009 warranted further investigation and that the new generation of sulfonamides could offer a great benefit in tuberculosis treatment. We systematically designed a study in order to investigate the inhibitory effect of TMP-SMX and other sulfonamides on *Mycobacterium tuberculosis*. Secondly, we investigated the combinational effect of SMX with the existing anti-tuberculosis drugs and also set out to identify the target(s) of SMX in *M. tuberculosis*. These studies were conducted in the Biosafety level 3 facility and an ethical clearance for this was

obtained from the Health Research Ethics Committee of Stellenbosch University (Ethics reference no. N11/07/230).

1.8.1 Hypothesis

SMX, a sulfonamide drug, has antimycobacterial activity through numerous targets and could interact synergistically with first-line anti-tuberculosis drugs.

1.8.2 **Objective 1: Susceptibility testing of *M. tuberculosis* to TMP-SMX, TMP, SMX including other sulfonamides and SMX in combination with the first-line anti-TB drugs. The following will be determined:**

1.8.3 Objective 2*:* Sequence analysis and expression of genes essential in the folate pathway in drug sensitive and drug resistant strains *of M.tuberculosis*.

1.8.4 Objective 3: Protein expression profiling in SMX treated and untreated drug resistant strains of *M.tuberculosis*.

CHAPTER 2

Susceptibility testing of *Mycobacterium tuberculosis* to folate inhibitors and to combinations of folate inhibitors with anti-TB drugs

2.1 Background

SMX-TMP is a combination drug that is commercialised under the trade names such as Bactrim® or Purbac®. This combination is active against most of the gram positive and gram negative bacteria and it is used to treat various infections including opportunistic infections in HIV patients (Klein *et al.,* 1992). These compounds target enzymes in the folic acid synthesis pathway. SMX is a structural analogue of para-aminobenzoic acid (PABA), and it inhibits dihydropteroate synthase preventing the production of dihydropteroate, while TMP inhibits dihydrofolate reductase (the last enzyme in the pathway) (Hitchings, 1973).

In 2009, it was reported that TMP/SMX combination has activity against *Mycobacterium tuberculosis* clinical isolates (Forgacs *et al.*, 2009). These findings triggered further investigation of this compound as a potential anti-TB drug. TMP-SMX has been shown to inhibit the clearance of compounds such as tolbutamide and phenytoin, by inhibiting cytochrome P450 enzymes that are involved in oxidative metabolism of compounds in humans (Wing and Miners, 1985). A detailed study in vitro investigated the effects of TMP and SMX on the major P450 isoform activities in human liver microsomes and recombinant P450s (Wen *et al.,* 2002). This study found that these compounds selectively inhibited the cytochrome P450 enzymes in a concentration dependent manner, with TMP concentrations ranging from 5 to 100 µM and SMX concentrations ranging from 50 to 500 µM. This indicates that TMP is more toxic than SMX and that ideal MICs of these drugs must be less than these concentrations that affect cytochrome P450 enzymes.

In this study, we sought to investigate the antimycobacterial activity of the TMP-SMX combination, the individual activity of the drugs and also evaluate any possible interactions between SMX and the first-line anti-tuberculosis drugs against drug susceptible and drug resistant strains of *Mycobacterium tuberculosis*. Since sulfonamides have been reported to produce oxidative stress, we also evaluated the activity of SMX against the mycothiol mutant strain (*mshA*) that is susceptible to oxidative stress.

Efflux pumps are the major role players in drug resistance in many organisms (Romanova *et al.*, 2006; Balganesh *et al.*, 2012). Extensive research has been done on the effect of efflux pumps on the activity of many anti-mycobacterial drugs. These studies have identified several types of efflux pumps, which include proton dependent ATP dependent efflux pumps. These types of systems constitute a broad mechanism of drug resistance, which is capable of conferring resistance to a variety of drugs (Silva *et al.*, 2001). We also evaluated the possible involvement of efflux pumps in the cross-resistance of isoniazid mono-resistant clinical isolates to SMX through the use of various inhibitors that inhibit different types of efflux pumps in combination with SMX.

In this study, we used the BACTEC 460 TB system to evaluate all drug activities. This system measures radio-labelled carbon dioxide produced by mycobacteria that is obtained from metabolism of radio labelled palmitic acid in the BACTEC vial. This labelled carbon dioxide is equivalent to the amount of bacteria in the vial and each carbon dioxide detected is assigned a growth index value of 1 (Siddiqi, 1989). We employed BACTEC 460 rather than BACTEC 960

(MGIT), because this system is faster and results are obtained within 5 days and it also has a lower rate of contamination than the BACTEC 960 (MGIT) system (Whyte *et al.* 2000).

2.1.1 Objective of this part of the study: Susceptibility testing of *M. tuberculosis* to TMP-SMX, TMP, SMX including other sulfonamides and SMX in combination with the first-line anti-TB drugs. The following will be determined:

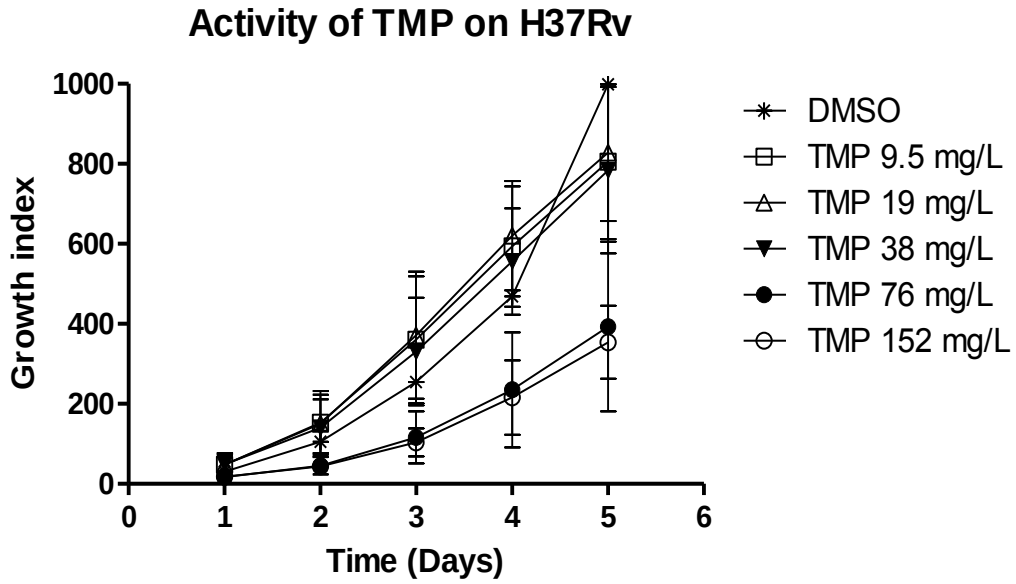
a) Test the activity of TMP-SMX, TMP and SMX on the drug susceptible reference strain of *Mycobacterium tuberculosis* H37Rv.
b) To determine the combinational effect of SMX with first-line anti-TB drugs; Isoniazid, Rifampicin and Ethambutol on the drug susceptible reference strain of *Mycobacterium tuberculosis*.
c) Test the activity of SMX on *M. tuberculosis* drug resistant clinical isolates
d) Evaluation of the role of oxidative stress in SMX efficacy by testing the susceptibility of a deletion mutant strain of *M. tuberculosis* (*mshA*) to SMX compared to the wild type strain
e) Evaluation of the activity of other sulfonamides and antifolates on *M. tuberculosis*
f) Test the activity of SMX in isoniazid resistant strains in the presence of efflux pump inhibitors

2.2 Results and Discussion

2.2.1 Test the activity of TMP-SMX, TMP and SMX on a drug susceptible strain of *Mycobacterium tuberculosis*

To evaluate the possible interactions between several compounds, it is important to first determine their individual MICs (see materials and methods section 5.4). The activities of TMP and SMX individually on H37Rv are shown in figure 2.1. TMP had negligible activity against *M. tuberculosis*, showing only 22% growth inhibition at 76 µg/ml, doubling to 44% at 152 µg/ml (Figure 2.1a). In contrast, SMX showed 93% growth inhibition at 76 µg/ml and 95% growth inhibition at 152 µg/ml (Figure 2.1b). At 9.5 µg/ml SMX still showed 90% growth inhibition, which was determined as the MIC of SMX (see section 2.1) for *M. tuberculosis* H37Rv (Figure 2.1). MIC was defined as the lowest concentration that inhibited 90% of bacterial growth.

(a)



(b)

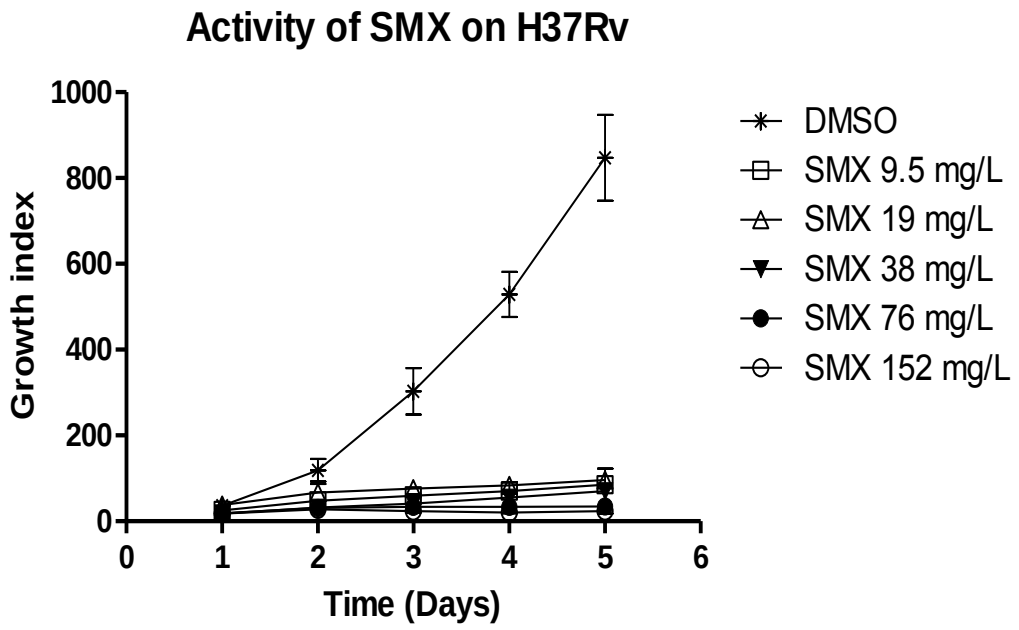


Figure 2.1: (a) Shows the activity of TMP on H37Rv. (b) shows the activity of SMX on H37Rv. Results was obtained from three separate experiments and standard deviations were calculated using Excel.

Our findings are in agreement with other previous studies in that it is only the sulfonamide component of the BACTRIM combination that is active against *M. tuberculosis*. It is not surprising that TMP exhibits minimal inhibition of the growth of *M. tuberculosis* as it has been reported in several studies that it does not inhibit *M. tuberculosis* growth (Wallace *et al.*, 1986; Ong *et al.*, 2010). TMP is a weaker inhibitor of mycobacterial enzymes and as result it is used in the cultivation method of mycobacterial strains in MGIT medium as a supplement together with other antibiotics (BBL™, MGIT™ and PANTA™ Antibiotic Mixture) to eliminate non-mycobacterium contaminating organisms (Rengarajan *et al.*, 2004; Suling *et al.*, 1998).

One of the reasons TMP is not active against *Mycobacterium tuberculosis* is that this organism contains a distinct class of dihydrofolate reductase that is inherently less susceptible to this compound (Burchall, 1975). *Mycobacterium tuberculosis* is not the only bacterium that contains this naturally insensitive dihydrofolate reductase to TMP. There are other species that are also naturally resistant to TMP, including *Bacillus anthracis* and *Cryptosporidium hominis* (Zhou *et al.,* 2013).

Studies have shown that the reason for this low potency is that the trimethoxyphenyl ring of TMP does not form maximal van der waal contacts with the hydrophobic pocket that normally houses the *para*-aminobenzoic acid moiety of dihydrofolate, leaving a gap between the trimethoxyphenyl ring and specific residues of the enzyme (Liu *et al.*, 2009).

The ideal distance between interacting residues and an inhibitor for van der Waals force to occur must be less than 4.2 Å, (Tan *et al.*, 2013) and only one residue that has a distance less than 4.2 Å, (3.40 Å in figure 2.3), which may explain the low potency of TMP in *Mycobacterium tuberculosis*. Thus, it seems inappropriate to suggest the introduction of TMP to the TB drug regimen as its contribution to the killing effect of the combination is minimal.

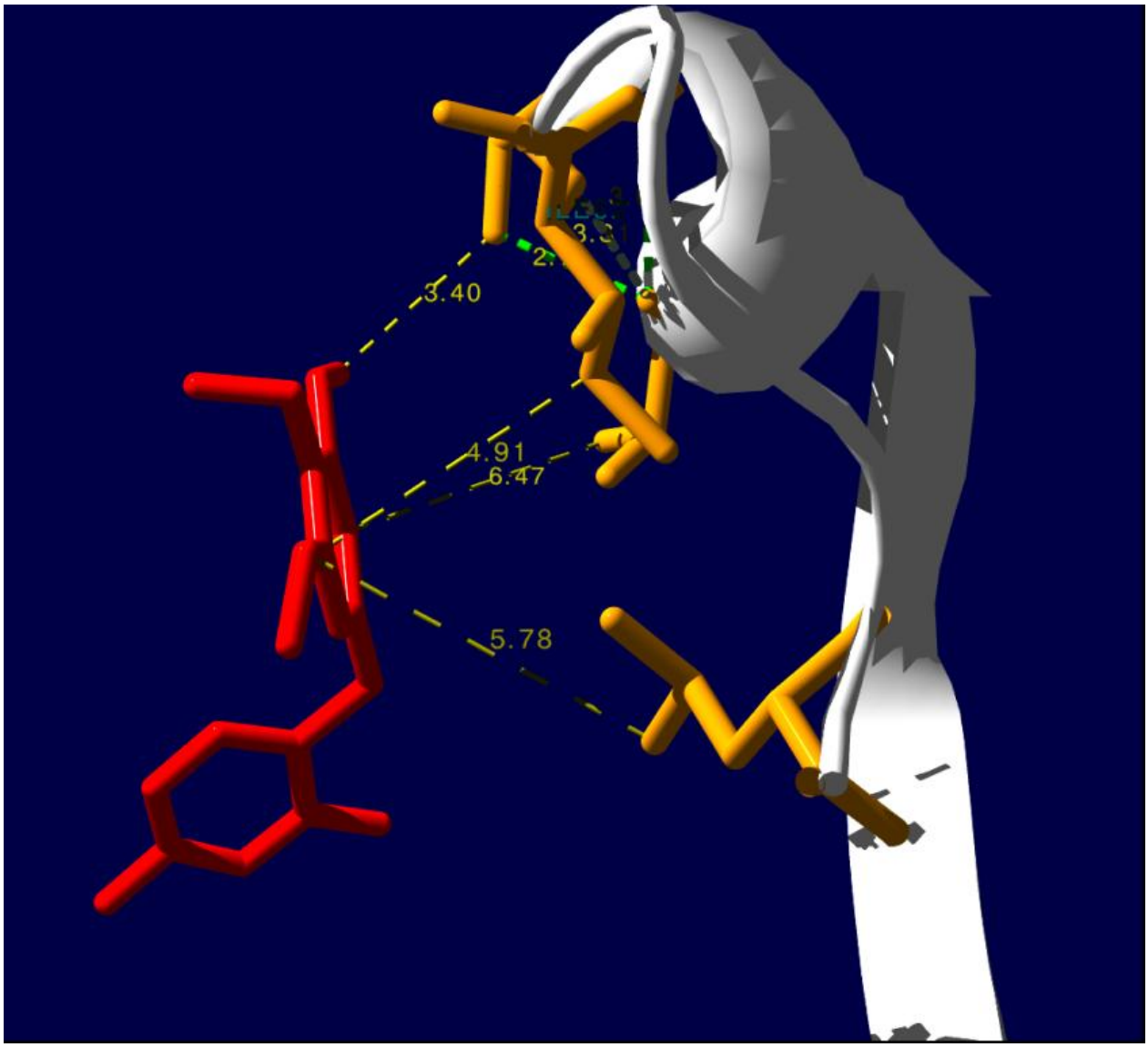


Figure2.3: Shows TMP on the active site *M. tuberculosis* DHFR and the distances between the residues and the inhibitor. Images were created using DeepView and POV-ray (SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis* 18, 2714-2723.) (Persistence of Vision Pty. Ltd. (2004), Persistence of Vision Raytracer Version 3.6, Computer software) (Guex and Peitsch 1997).

Using X/Y<1/Z (see material and methods section 5.4.1) for interpretation of drug interactions, we showed that there is no synergistic interaction between SMX and TMP but an additive effect (quotient 0.62) against *M. tuberculosis* (Table 2.2).

2.2.2 Determine the combinational effect of SMX with first-line anti-TB drugs; Isoniazid, Rifampicin and Ethambutol on the drug susceptible strain of *Mycobacterium tuberculosis,* H37Rv

We evaluated how SMX would interact with the current anti-tuberculosis agents. We first determined the MICs of the individual drugs using the BACTEC 460 TB system (see section 5.4) against the reference stain H37Rv (Table 1). The MICs of the first-line ant-TB drugs were in agreement with other published reports of the MICs of these drugs for H37Rv (Chen *et al.,* 2006).

Table2.1. MICs of the first-line drugs and SMX against Mycobacterium tuberculosis strain H37Rv*

Drugs	MICs of H37Rv (µg/ml)
INH	0.05
EMB	1.6
RIF	0.8
SMX	9.5

INH-isoniazid; EMB-ethambutol; RIF-rifampicin; SMX-sulfamethoxazole. The MICs were determined using the BACTEC 460TB system following the manufacturer's recommendations.

Table 2.2 lists the drug interactions evaluated in this study. We showed that SMX has a synergistic effect with RIF (quotients less than 0.5), an additive effect with ethambutol and no interaction with isoniazid. It is evident from these results for in vitro testing, no antagonism was observed between SMX and the tested compounds. This is clinically important as antagonistic activity would interfere with co-administration of SMX with the first-line anti-TB drugs should it be included in the TB regimen.

The MIC of SMX was reduced to 2 µg/ml in the combination and RIF reduced to two and three times less than it's MIC (table 2.2). The synergy between SMX and RIF is not unexpected, since SMX indirectly inhibits RNA synthesis through inhibiting tetrahydrofolate production, a co-factor

in the synthesis of thymidine and RIF directly inhibits RNA synthesis by inhibiting the DNA dependent RNA polymerase (Libecco and Powell, 2004; McIlleron *et al.*, 2007). These results could have a valuable implication in the anti-TB regimen due to the benefits that this combination offers, which include the reduction of toxic side effects of both compounds, while retaining their efficacy.

The combination SMX-RIF could also have valuable clinical relevance, especially to the co-administration of a tuberculosis regimen with HIV treatment. It has been reported that high concentrations of Rif induced P450 up-regulation and reduces Protease inhibitor exposure (Decloedt *et al.*, 2011). The reduced concentrations potentially result in the reduction of antiviral efficacy leading to the development of drug resistance (McIlleron *et al.*, 2007).

Table 2.2: Interaction between SMX and TMP, rifampicin, ethambutol and isoniazid

SMX (µg/ml)	TMP (µg/ml)	Quotients (mean x/y +/- SD)
9.5	0.5	0.62 +/- 0.03
4.75	0.25	1.06 +/- 0.02
2.4	0.1	1.18 +/- 0.26
1.2	0.1	1.02 +/- 0.12
	RIF (µg/ml)	
2	0.3	0.16 +/- 0.19
	0.4	0.19 +/- 0.16
	EMB (µg/ml)	
2	0.4	0.49 +/- 0.02
	INH (µg/ml)	
2	0.025	1.06

Interactions between SMX; TMP, RIF, EMB and INH. All results were obtained from three separate experiments and standard deviations were calculated using Excel.

2.2.3 Activity of SMX on drug resistant clinical isolates

We further evaluated the effect of SMX in the growth of drug-resistant clinical strains of *Mycobacterium tuberculosis*. Table 2.3 shows the activity of SMX at various concentrations in three drug resistant clinical isolates, two INH^R (R1129 and R1845) strains and a RIF^R (R5182) strain (see materials and methods section 5.1). SMX inhibited the growth of the RIF resistant isolate at concentrations between 9.5 µg/ml and 19 µg/ml. INH^R clinical isolates were also resistant to SMX, with an MIC higher than 19 µg/ml.

Table2.3. ***Activity of SMX in clinical isolates***

SMX (µg/ml)	**Rif mono-resistant**	**% inhibition**
4.75	R5182 (rpoB)	34.9
9.5	R5182 (rpoB)	76.5
19	R5182 (rpoB)	96.6
SMX (µg/ml)	**INH mono-resistant**	
4.75	R1129 (KatG)	19.9
9.5		43.6
19		86.1
4.75	R1845 (InhA)	1.6
9.5		18.9
19		44.4

The activity of SMX on RIF mono-resistant clinical isolate R5182 and INH mono-resistant clinical isolates R1129 and R1845 were obtained from three separate experiments.

The higher MIC of this drug on the INH mono-resistant clinical isolates may indicate a multiple drug overlapping mechanism of resistance. Drug resistance may be attributed to a number of factors, which may include mutations in the target gene, see chapter 3. Efflux pumps may also contribute to drug resistance, both in intrinsic and acquired drug resistance (Rossi *et al.*, 2006).

Intrinsic drug resistance involves efflux pumps that are naturally active in the cell, synergistically working with membrane permeability, which restrict drug passage (Nikaido, 2001).

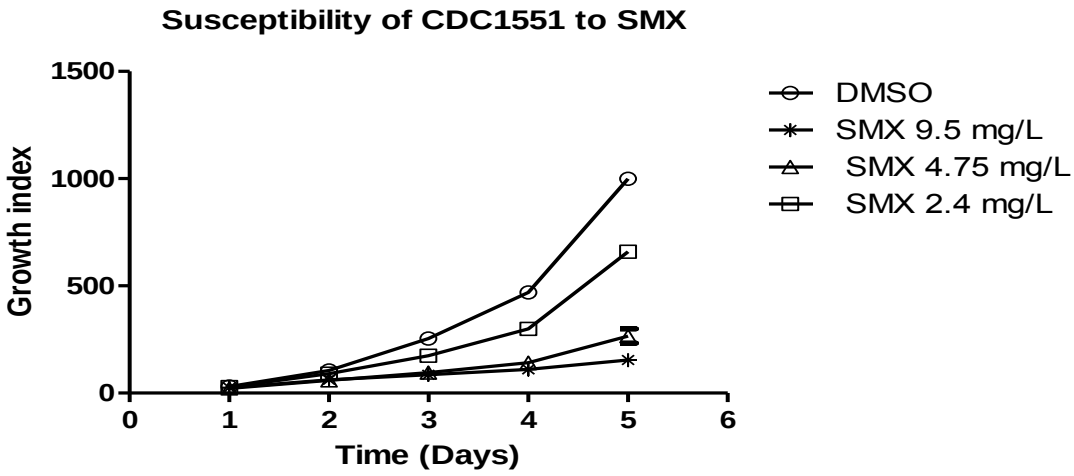
Examples of these efflux pumps are AcrB of *E. coli,* MexB of *P. aeruginosa* and MtrD of *N. gonorrhoeae*, that confer natural resistance to various antibiotics, including tetracyclines, chloramphenicol and macrolides (Nikaido, 1996). On the other hand, antibiotics can serve as inducers, regulating the expression of efflux pumps at the level of gene transcription resulting in the acquired drug resistance conferred by efflux pumps (Rossi *et al.*, 2006).

2.2.4 Evaluating the role of oxidative stress in SMX efficacy

Some antimycobacterial agents have been reported to produce oxidative stress as part of their mechanism of action. These compounds include INH, via the production of various adducts and RIF via unknown mechanisms (Sodhi *et al.,* 1997). Sulfonamides have also been reported to produce oxidative stress as their secondary mechanism of action (Rieder *et al.*, 1988).

To evaluate the role of oxidative stress in the efficacy of SMX, we employed the *mshA* CDC 1551 mutant (see section 5.1) and compared the growth of this strain to the wild type parent strain CDC1551 in the presence of varying concentrations of SMX (9.5, 4.75, 2.4 mg/L). The MIC of CDC1551 was not different from the MIC in H37Rv, 9.5 mg/L and a decrease in the MIC of SMX was observed in the *M. tuberculosis* Δ*mshA* mutant, where the MIC was decreased four-fold (from 9.5 mg/L to 2.4 mg/L), see figure 2.4

(a)



(b)

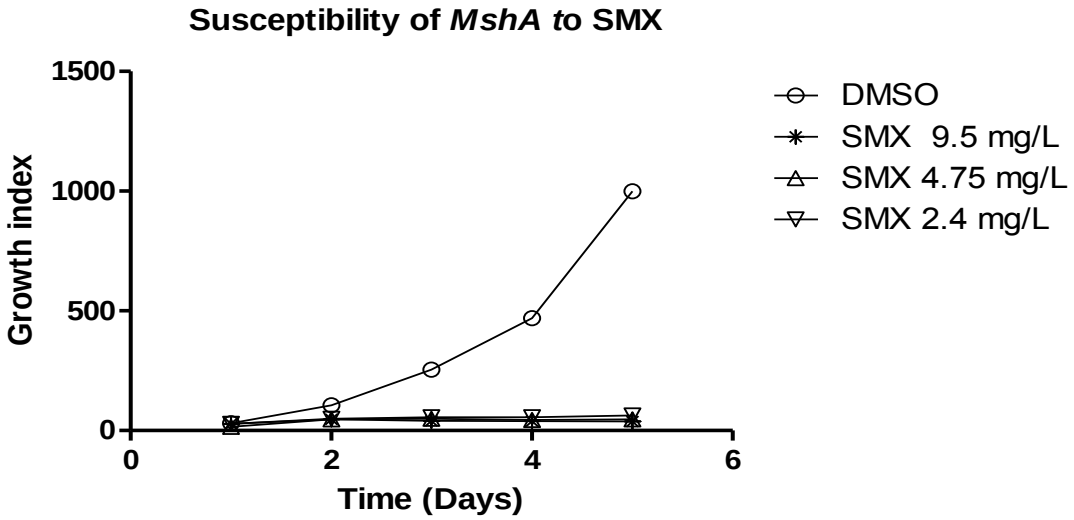


Figure 2.4: (a) The growth profile of Δ*mshA* mycothiol mutant and **(b)** CDC1551 reference strain, exposed to SMX. Growth was monitored by BACTEC 460 TB system and GI values were obtained after the first day of inoculation until the GI of the 1:100 culture was more than 30. Vials were incubated at 37^0 C and each point represents a mean value of duplicates.

A possible explanation for the increased susceptibility of the mycothiol mutant could be that SMX is converted to intermediates that eventually produce oxidative stress (figure 2.5). In

human keratinocytes, SMX is converted to arylhydroxylamine (SMXNOH) by flavin-containing monooxygenases (Vyas *et al.*, 2005). This less stable metabolite is auto-oxidised to a nitroso metabolite (SMXNO) which generates oxidative stress (Figure 2.6) (Reilly *et al.*, 2000; Roychowdhury and Svensson, 2005; Vyas *et al.*, 2005).

A similar enzyme encoded by the *etaA* gene exists in *M. tuberculosis*. This putative flavin-containing monooxygenase is responsible for the activation of the second-line anti-TB pro-drug ethionamide, which is a structural analog of INH, and inhibits mycolic acid synthesis (Baulard *et al.*, 2000; DeBarber *et al.*, 2000). We postulate that the observed Δ*mshA* mutant phenotype is potentially as a result of the lack of mycothiol which would normally neutralize these free radicals (Buchmeier *et al.,* 2003) and that the accumulation of these intermediates results in the increased sensitivity observed.

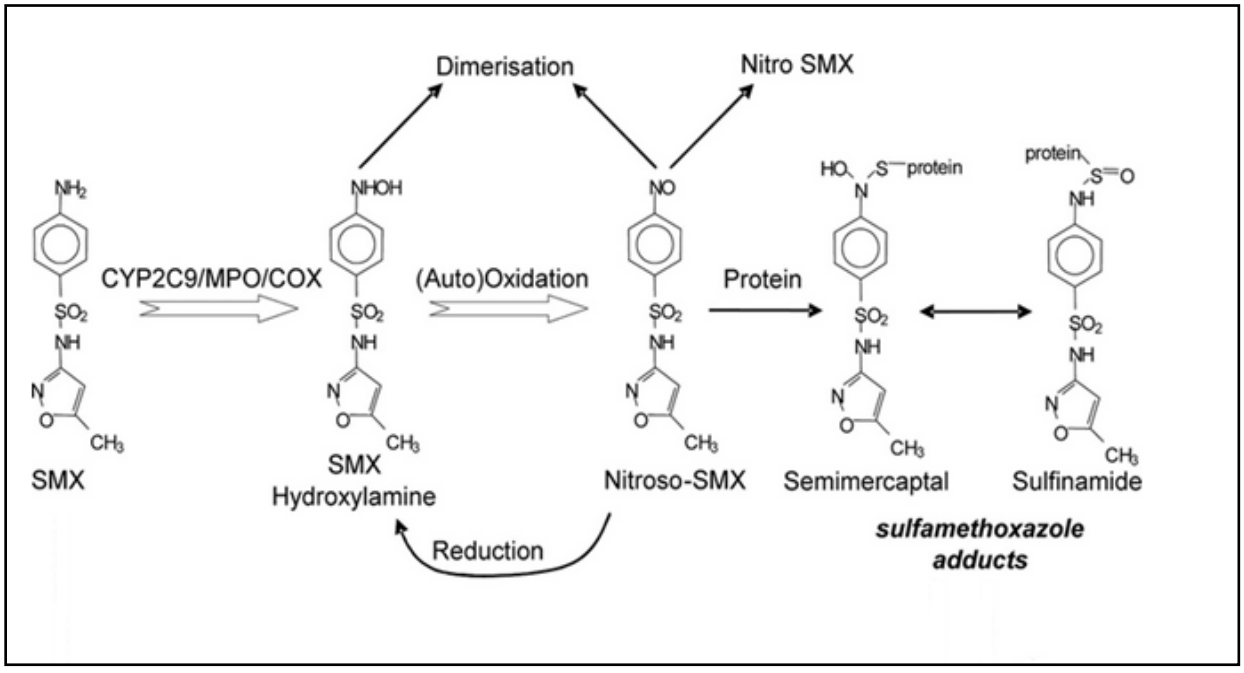


Figure 2.5: Shows a schematic representation of the activation of SMX by various enzymes in human cells. This schematic representation was modified from Sanderson *et al.*, *2006* (Sanderson *et al.*, 2006).

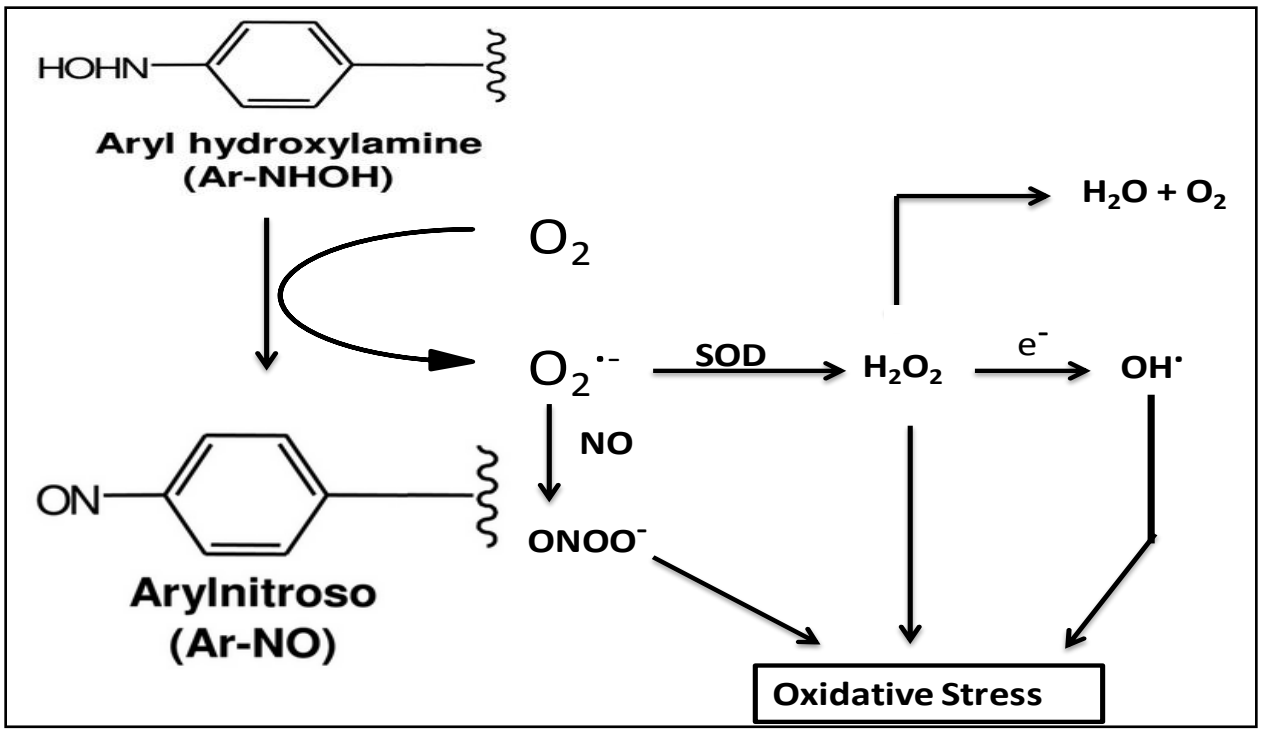
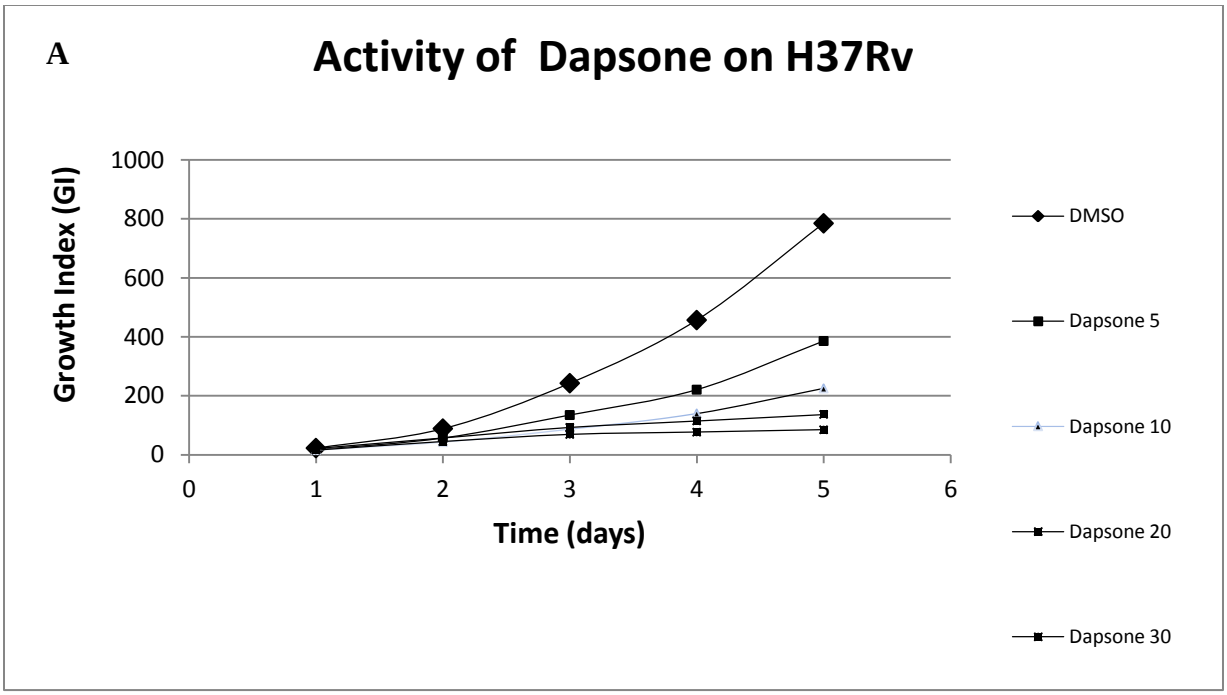


Figure 2.6: A schematic representation of oxidative stress generated by SMX/Sulfonamides. (Adapted from Vyas *et al.*, 2006).

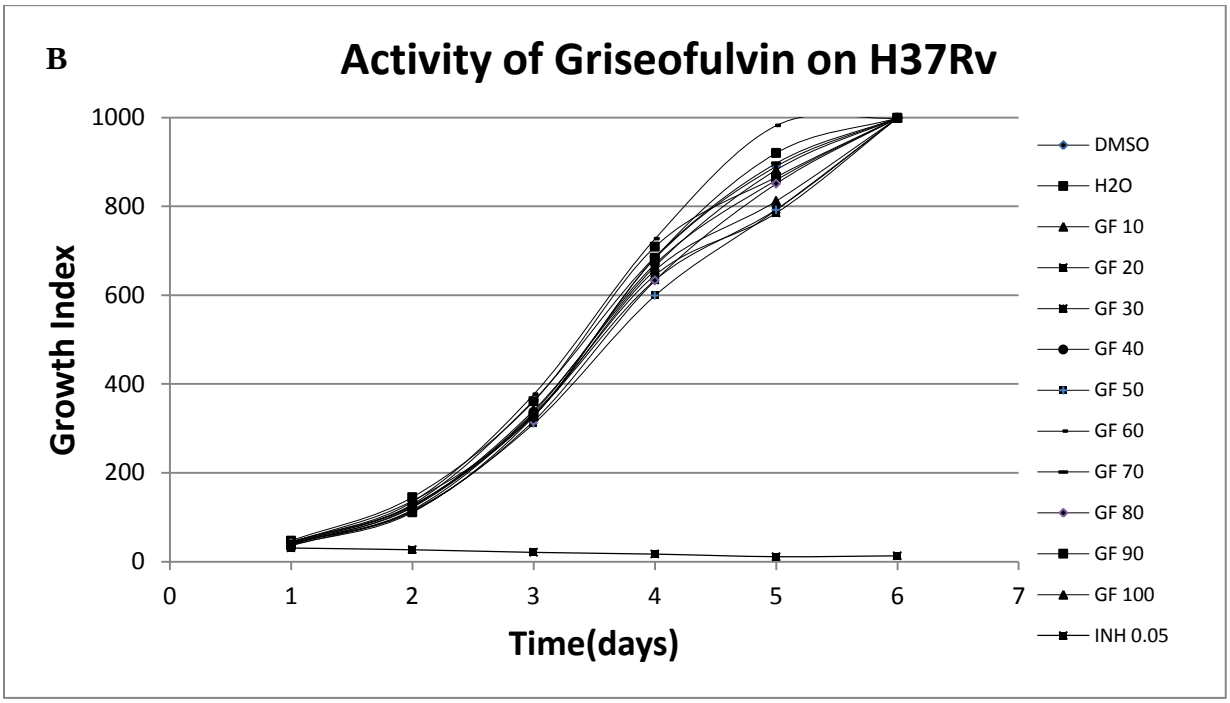
2.2.5: Evaluation of the activity of other sulfonamides and antifolates on *M. tuberculosis*

Since SMX displayed bacteriostatic activity against *M. tuberculosis* (Macingwana *et al.*, 2012; Vilchèze and Jacobs, 2012), we investigated the activity of other sulfonamides against *Mycobacterium tuberculosis.* We evaluated the antimycobacterial activity of some of the clinically approved sulfonamides dapsone, griseofulvin (Grifulvin V) and sulfasalazine (Azulfidine) against the *M. tuberculosis* reference strain H37Rv. Figure 2.7 (a, b & c), shows activities of these sulfonamides. We observed that Griseofulvin and Sulfasalazine exhibited no activity up to 100 µg/ml and 30 µg/ml respectively, whereas INH used as a control maintained its MIC of 0.05 µg/ml.

A



B



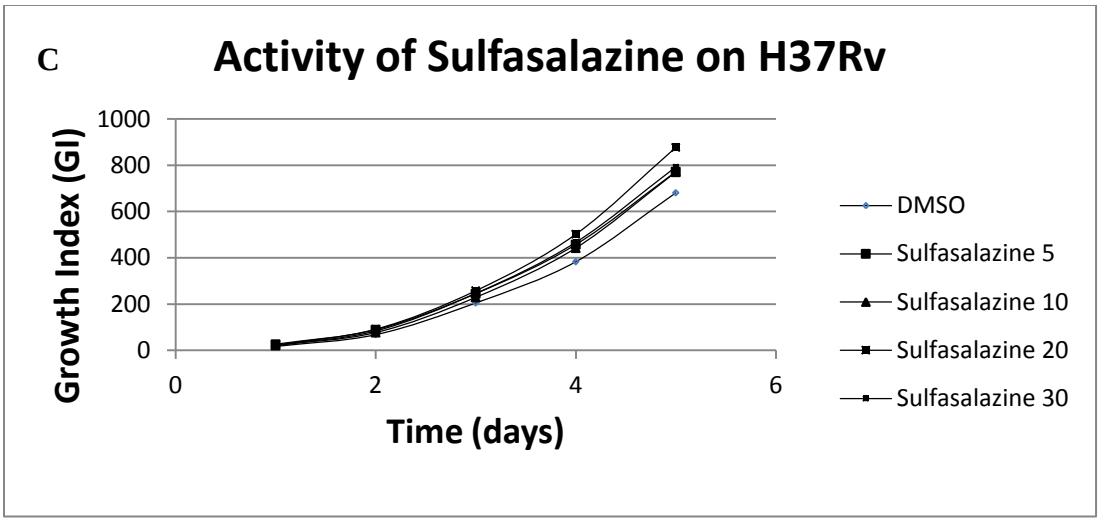


Figure 2.7: A Growth curves showing the effect of Dapsone on H37Rv as tested with BATEC 460 TB system (see M&M 5.4). The numbers on the legend are the concentrations (µg/ml) that were tested for each drug. **B:** Growth curves showing the effect of Griseofulvin on H37Rv. The numbers on the legend are the concentrations (µg/ml) that were tested for each drug. **C:** Growth curves showing the effect of sulfasalazine on H37Rv. The numbers on the legend are the concentrations (µg/ml) that were tested for each drug.

Dapsone (DDS) is the most effective sulfonamide against *Mycobacterium leprae,* malaria and against *Pneumocystis pneumonia* in patients with HIV disease (Shepard, 1967). The reports of susceptibility of *M. tuberculosis* to dapsone have been reported (Rastogi *et al.*, 1993; Opravil *et al.*, 1995; Nopponpunth *et al.*, 1999; Gonzalez *et al.*, 1989), but there are few studies that have evaluated the activities of dapsone against *Mycobacterium tuberculosis.* We therefore evaluated the activity of dapsone against *M. tuberculosis,* using the reference strain H37Rv. Using BACTEC TB 460 TB system, we determined the MIC of dapsone, which was defined as the lowest concentration that inhibited more than 90% of *M. tuberculosis* growth (Reddy *et al.*, 2010). The MIC of dapsone ranged between 20 to 30 µg/ml (see figure 2.7 A), which is in agreement with previous results (≥ 32 mg/l) that were obtained by the agar disk elution method (Gonzalez *et al.*,

1989).When DDS was combined with various drugs at concentrations that inhibited less than 50% of *M. tuberculosis* growth, the combination of DDS-SMX and DDS-EMB showed an additive effect (see table 4) and the addition of DDS to RIF and INH did not result in any positive interaction, but no antagonistic effect was observed. However, DDS has been associated with various dose-dependent side effects such as hemolysis, methemoglobinemia, peripheral neuropathy, agranulocytosis and aplastic anemia (Coleman, 1995). It has been reported that the plasma concentration of DDS that exceed 5 µg/ml increase the risk of developing these adverse side effects (Reilly *et al.,* 1999; Vieira *et al.,* 2010). Therefore, DDS is not a suitable candidate drug for tuberculosis treatment since the MIC for H37Rv is very high (≥ 32 mg/l), that is more than six fold above the critical concentration.

Table 2.4: Interactions between dapsone (DDS) and various antituberculosis drugs

Drugs	Concentration (µg/ml)	Quotients
DDS +SMX	5+2	0.6
	5+4.75	0.5
	10+2	0.6
	10+4.75	0.7
DDS +EMB	5+0.8	0.5
	5+0.4	0.5
	5+0.2	0.5
	10+0.8	0.5
	10+0.4	0.5
	10+0.2	0.6
DDS +RIF	5+0.3	-
	5+0.01	-
DDS +INH	5+0.025	-
	10+0.025	-

*DDS- Dapsone, EMB-Ethambutol, RIF-Rifampicin, INH-Isoniazid

Griseofulvin is an antifungal agent that is used to treat many dermatophyte infections and exhibit insignificant toxicity to humans (De Carli and Larizza, 1988; Chan and Friedlander, 2004). It inhibits mitosis by disrupting mitotic spindles in susceptible strains and recently it was reported that it can also inhibit cancer cells and does not affect healthy cells (Jordan and Wilson, 2004; Rebacz *et al.*, 2007). We hypothesize that interference with the mechanisms involved in cell division will result in killing of *Mycobacterium tuberculosis*. We evaluated the activity of griseofulvin and we could not find any inhibition of *M. tuberculosisM. tuberculosis* growth (see figure 2.7 B)

Sulfasalazine is prescribed for the treatment of inflammatory bowel disease (Wahl *et al.*, 1998; Das and Dubin, 1976; Svartz, 1942). The drug is metabolised by intestinal bacteria, releasing two components (Peppercorn, 1984). We postulated that the sulfonamide component sulfapyridene, which is also structurally related to dapsone, will inhibit the growth of *Mycobacterium tuberculosis* (Paniker and Levine, 2001). Sulfasalazine also did not have any in vitro activity against *M. tuberculosis* even at concentrations that were higher than the mean peak concentration (14 µg/ml) in the treatment of inflammatory bowel disease (see figure 2.7 C).

2.2.6 Testing the effect of efflux pump inhibitors on the activity of SMX in *M. tuberculosis* INH^R (R1129) strain

We investigated the possible involvement of efflux pumps in the cross resistance of the isoniazid mono-resistant clinical *M. tuberculosis* isolate (R1129) to SMX. The MIC of SMX for the R1129 isoniazid resistant strain was determined to be more than 19 µg/ml (see table 3). Three efflux pump inhibitors (verapamil, reserpine and CCCP) were investigated. The concentrations of

verapamil and reserpine chosen were 50 µg/ml and 80 µg/ml respectively, which did not directly affect the growth of the bacterial strain tested (Louw *et al.*, 2011). These concentrations were then added individually to the INH^R cultures to assess their inhibitory effect and were also combined with various concentrations of SMX (see figure 2.8). The MIC of CCCP was 20 µg/ml for the R1129 strain. We used various concentrations of CCCP that were lower than 20 µg/ml; 2, 4, 8, 12 and 14 µg/ml in combination with SMX and interpreted the results based on the effect they had on the growth of R1129 individually and in combination with SMX.

The synergy between efflux pump inhibitor and SMX was interpreted using X/Y<1/Z (see material and methods section 5.4.1). It was observed that the MIC of SMX on the isoniazid mono-resistant clinical *M. tuberculosis* isolate was decreased to between 9.5 µg/ml and 19 µg/ml by the addition of 80 µg/ml reserpine. The addition of 50 µg/ml verapamil did not have an effect on the MIC of SMX for this strain. Using concentrations of CCCP (12 µg/ml and 14 µg/ml), lowered the MIC of SMX for the resistant strain R1129 to that of the drug susceptible H37Rv lab strain, that is 9.5 µg/ml (see figure 2.9 B).

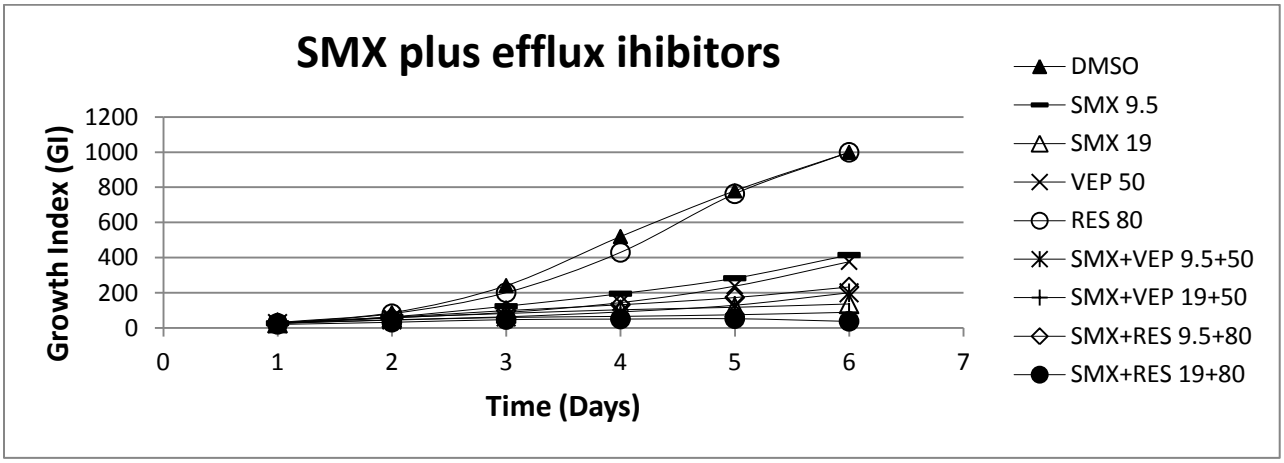


Figure 2.8: Growth curves showing the effect of combination of SMX with Reserpine (RES) and Verapamil (VEP) against H37Rv. The numbers on the legend are the concentrations (µg/ml) that were tested for each drug.

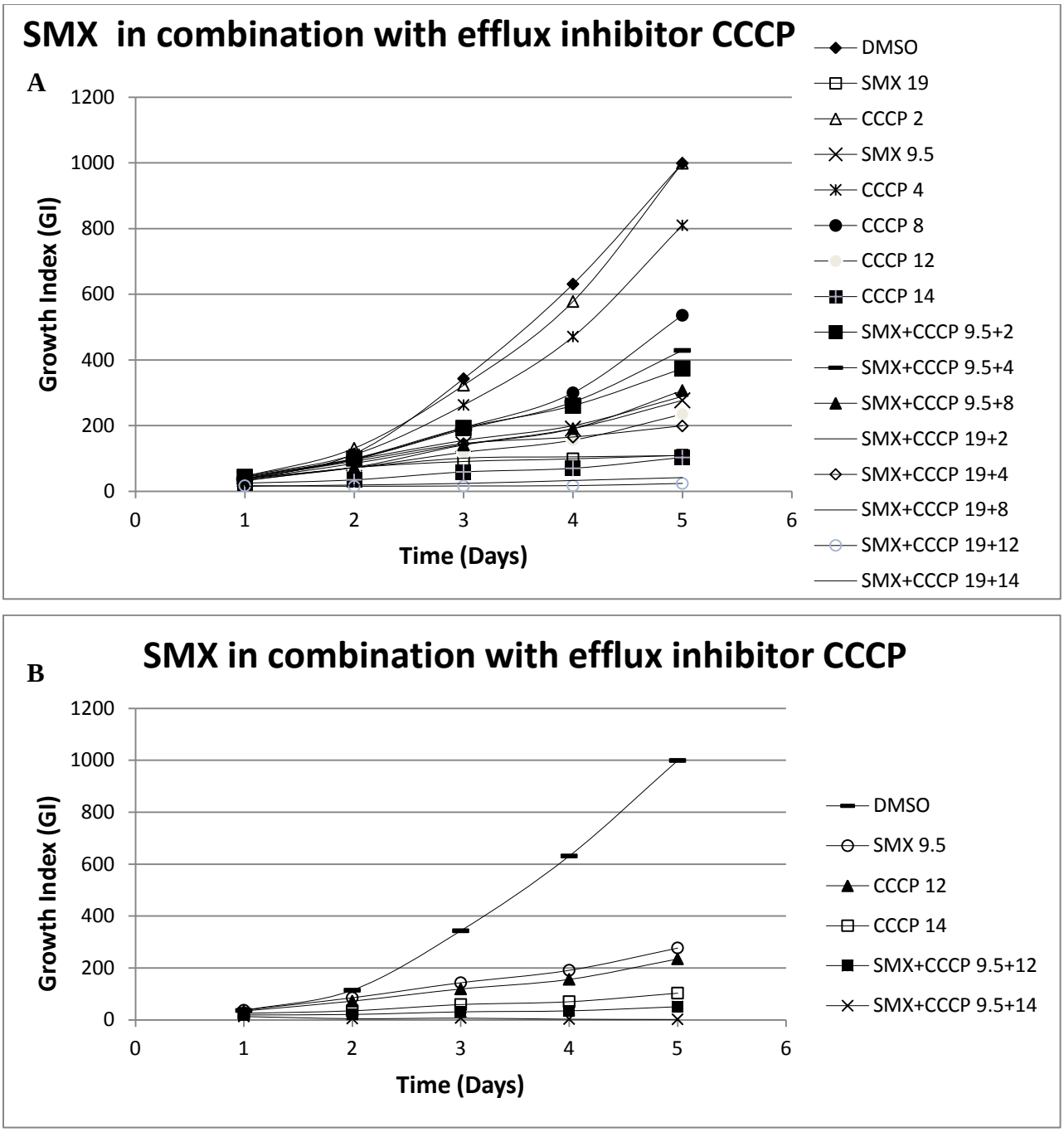


Figure 2.9: (a) Growth curves showing the effect of combination of SMX with CCCP against H37Rv. The numbers on the legend are the concentrations (µg/ml) that were tested for each drug. **(b)** Growth curves showing the effect of combination of SMX with CCCP against H37Rv. The numbers on the legend are the concentrations (µg/ml) that were tested for each drug.

These results obtained suggest that there are efflux pumps that are involved in the cross resistance and particularly those belonging to the ATP Binding Cassette transporters (ABC transporters) inhibited by reserpine and CCCP (Klyachko *et al.*, 1997; Pasca *et al.*, 2004). Thus, high concentrations of SMX would be required in order to overcome the effect of the efflux pumps.

Cross-resistance may result from exposure to one agent that belongs to the substrate profile of a particular efflux pump, inducing its over-expression and subsequently leading to the cross-resistance to all other substrates of that particular efflux pump (Webber and Piddock, 2003). For example, over-expression of the MexAB-OprM efflux system in *P. aeruginosa* due to the exposure to triclosan resulted in cross-resistance to TMP, ciprofloxacin and other antibiotics (Chuanchuen *et al.*, 2001).

Our results together with previous reports suggest that efflux pump inhibitors may play a critical role in the treatment of tuberculosis, particularly MDR and XDR tuberculosis (Gupta *et al.*, 2006; Amaral *et al.*, 2008; Louw *et al.*, 2011). We are aware that CCCP used in this study had a direct effect on the growth of *M. tuberculosis* and that these experiments were done with one clinical isolate. More clinical isolates (INH resistant) must be tested to substantiate these results. Developments are under way to generate efflux pump inhibitors that do not inhibit growth on their own and that are selective for bacterial efflux pumps. Currently, most of the available efflux pump inhibitors are not suitable for treatment application, for example verapamil, which also inhibits human P-glycoprotein and cytochrome P450 (Prakash *et al.*, 2003).

In conclusion, our findings support reports that SMX is the active compound in the TMP-SMX combination. SMX has synergistic activity with RIF and an additive effect with EMB. TMP-SMX is a registered drug combination for other indications, is inexpensive and widely available. Clinical trials should be initiated to clarify the potential of SMX and SMX-RIF in drug susceptible TB and of SMX as an additional option for patients with highly resistant strains.

Furthermore, a potential new mechanism of action of SMX has been identified, which shows that SMX produces oxidative stress and thus plays a role in its efficacy. The fact that *M. tuberculosis ΔmshA* mutants are more susceptible to SMX, suggest that it may be useful in combination with mycothiol synthesis inhibitors against *M. tuberculosis*. We have also determined that efflux pumps may potentially play a role to the cross resistance of an isoniazid mono-resistant clinical isolate to SMX.

CHAPTER3

Effect of SMX on the folic acid pathway and global expression protein profile in an SMX resistant clinical isolate

3.1 Background

The folic acid pathway is one of the essential pathways that help bacteria survive inside their host (figure 1.12). Sulfonamides are presumed to inhibit this pathway as discussed chapter 1 section 1.7, either as their main target or one of several pathways (Nopponpunth *et al.*, 1999). This part of the study was designed to verify the target of SMX and possibly also discover new targets of SMX in *M. tuberculosis* and also to try to uncover the underlying mechanisms of cross resistance of the isoniazid mono-resistant *M. tuberculosis* isolates to SMX.

We monitored the levels of metabolites formed or utilised by the folic acid pathway as a measure of the activity of SMX towards its target dihydropteroate synthase (DHFS). It was assumed that the binding of SMX to DHFS would result in high levels of PABA and reduced levels of dihydrofolate and tetrahydrofolate. We also monitored the expression of the gene that codes for DFHS, *folP1* in the SMX resistant *M. tuberculosis*.

Furthermore, we monitored the global protein expression in the SMX resistant clinical isolate after exposure to sub-lethal concentrations of SMX. This approach has been used before in *Mycobacterium tuberculosis* studies to identify targets for specific compounds and elucidate new genes that confer resistance to known anti-TB drugs owing its ability to provide a broad overview of proteomic changes (Singhal *et al.*, 2012).

Objectives of this part of the study:

(1) Sequence analysis and expression of genes essential in the folate pathway in drug sensitive and drug resistant strains *of M.tuberculosis*. (2) Protein profiling in SMX treated and untreated drug resistant strains of M.tuberculosis. These will include the following;

a) To evaluate folate levels in *Mycobacterium tuberculosis* treated with SMX
b) To sequence and search for possible mutations in the putative target gene of SMX and genes that may impede the effect of SMX in the isoniazid mono-resistant clinical isolates
c) To evaluate gene expression of the *folP1* gene in response to SMX treatment in isoniazid mono-resistant clinical isolates
d) To evaluate the global protein expression profile in isoniazid mono-resistant clinical *M. tuberculosis* isolate treated with and untreated SMX employing High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS)

3.2 Results and Discussion

3.2.1 Measurement of folate in Mycobacterium tuberculosis *treated with SMX*

To determine whether SMX inhibits dihydropteroate synthase in *Mycobacterium tuberculosis*, folate species were extracted as described in materials and methods section 5.7.2 and quantified using high performance liquid chromatography- mass spectrometry (LC-MS) under the conditions described in section 5.7.4 of materials and methods.

We used *Mycobacterium bovis* BCG as a model of *M. tuberculosis* in order to optimise our methods of folate extraction. We extracted folate using two methods and in the first method we

used EDTA, perchloric acid and acetonitrile, routinely used in our laboratory to extract metabolites (Emani *et al.*, 2013). This method yielded no detectable folate species, THF and DHF and we thought this was due to the fact that folates are unstable and sensitive to oxidation (Lu *et al.*, 2007). Figure 3.1 (a & b), shows the results from the first method of extraction. We therefore used the second method as described in materials and methods section 5.7.3, which includes an antioxidant ascorbic acid, to protect folate from oxidation (See figure 3.2 & 3.3).

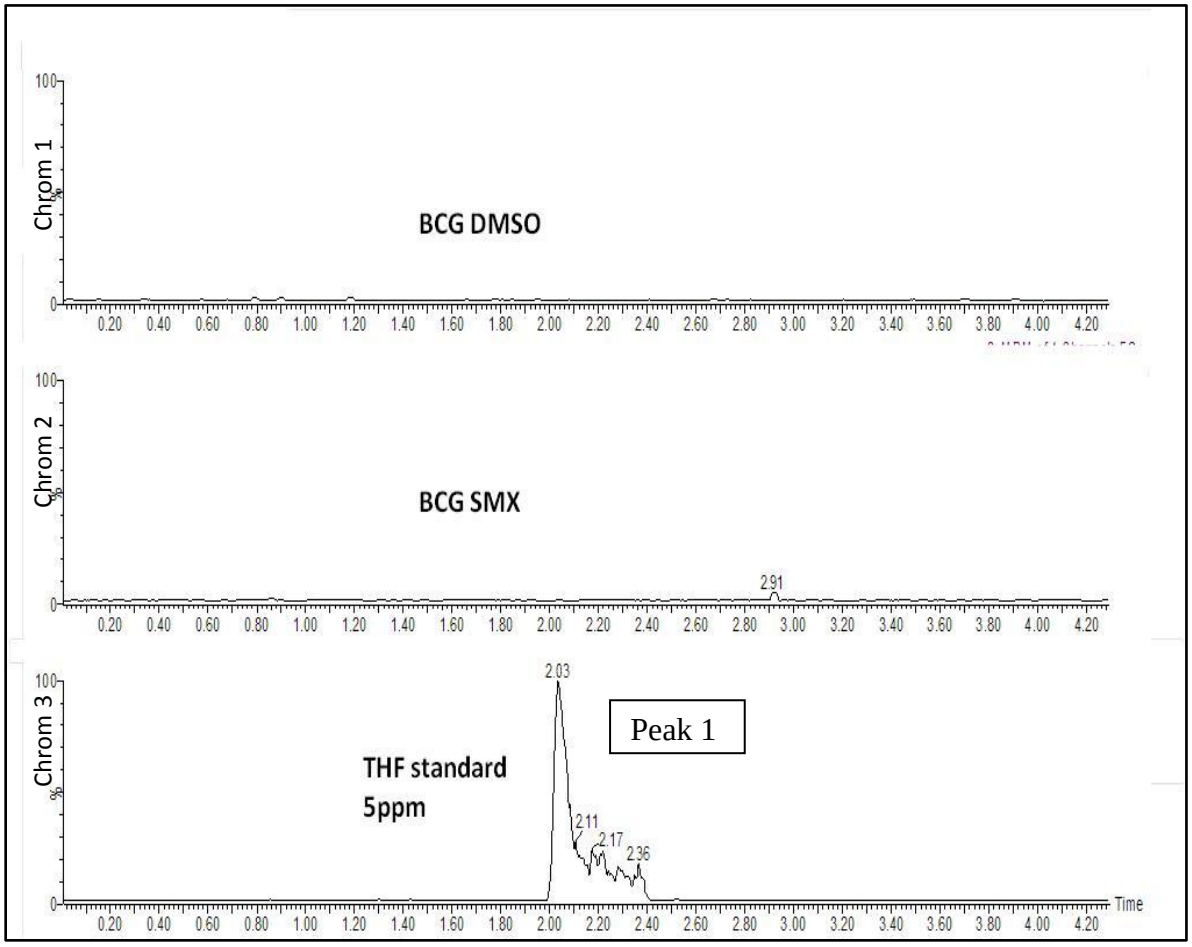


Figure 3.1 A: Shows the measurement of the total THF in samples treated with 9.5 µg/ml SMX or DMSO. The chromatograms are as follows; (Chrom 1) DMSO treated sample, (Chrom 2) SMX treated sample, (Chrom 3) THF standard and its peak.

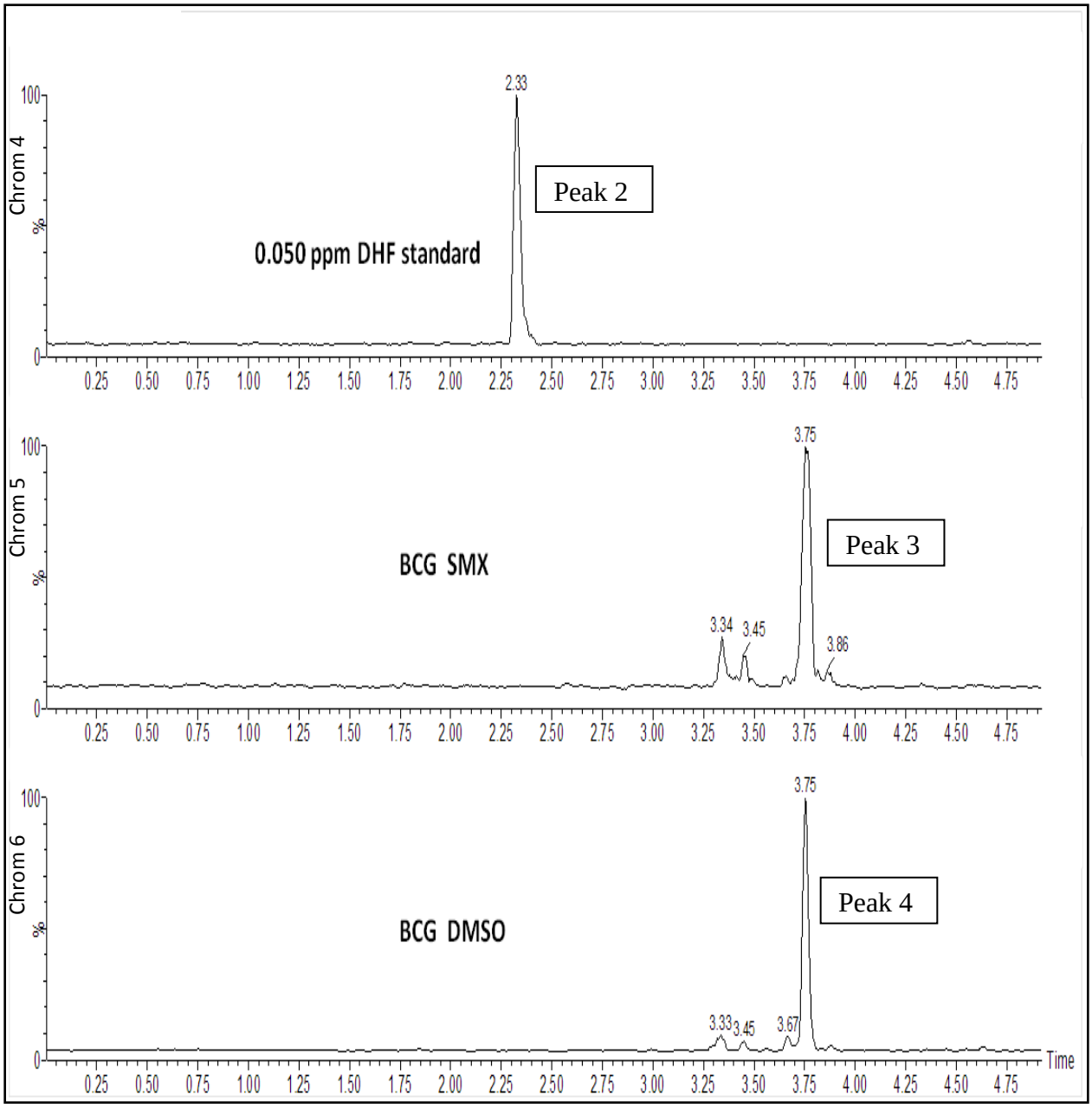


Figure 3.1 B: Shows the measurements of the total DHF in samples treated with 9.5 µg/ml SMX, (Chrom 4) DHF standard and its peak, (Chrom 5) SMX treated sample, (Chrom 6) DMSO treated sample. Peak 3 and 4 are unknown.

In theory, *M. tuberculosis* samples treated with SMX at specific concentrations should have decreased THF and DHF levels compared to the controls, because SMX inhibits their synthesis, but in our experiments we were not able to detect any of these metabolites with both methods. There is a peak that was detected in our samples in the measurement of DHF that appeared at the retention time of 3.75 minutes (figure 3.1 b); however this peak was not identified successfully. We then added 1 μg/ml of each of the metabolites (DHF and THF) before or after extraction for evaluation and measured THF and DHF as before, but we could not detect these metabolites as well (see figure 3.2 & 3.3). This indicates that the folate species are very unstable. Previously, it has been shown that folates are not good biomarkers because of their instability which result from the cleavage of the bond between pterin and *p*ABA moieties induced by light (Scott, *et al.*, 2000; Brain *et al.*, 2008).

Many studies that have analysed folates have used ascorbic acid as an antioxidant to prevent their loss due to oxidation. In our study, we used ascorbic acid as the sole antioxidant in the buffer solution in the second method, but we were not able to detect any folate species in our samples, even in the spiked samples. It has been reported that folates under elevated temperature undergo interconversion even in the presence of ascorbic acid due to the formation of formaldehyde from the oxidation of ascorbic acid (Wilson and Horne, 1983). The presence of formaldehyde in the solution promotes the conversion of THF to other folate species (5,10-CH2THF) and DHF is very unstable at pH 8 and below and at elevated temperatures even at 37^0C when kept for an extended period (De Brouwer *et al.*, 2007).

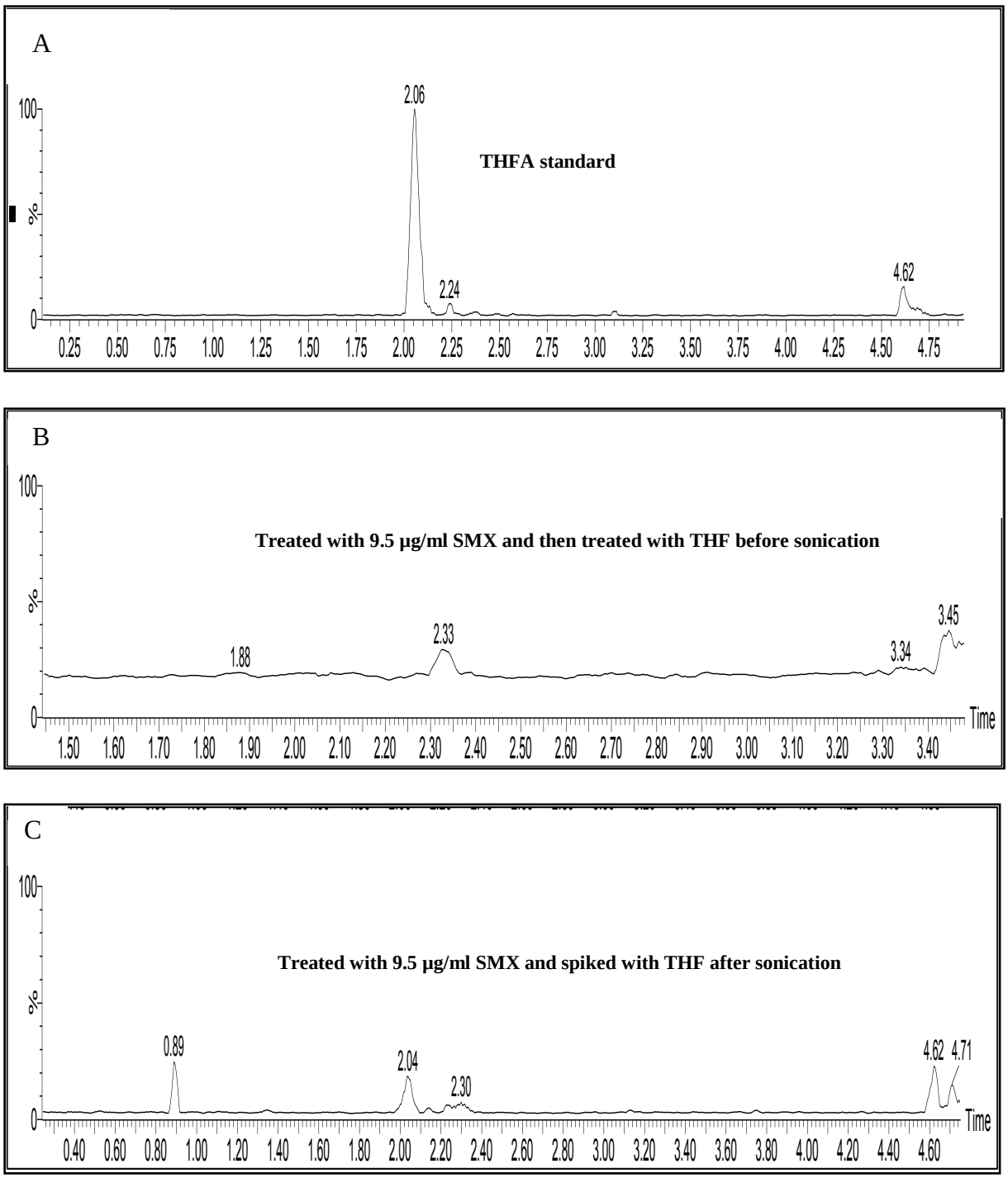


Figure 3.2 (a, b & c): Shows chromatograms of total THF extracts from BCG samples that were spiked with 1µg/ml of each of the folate standards before or after sonication.

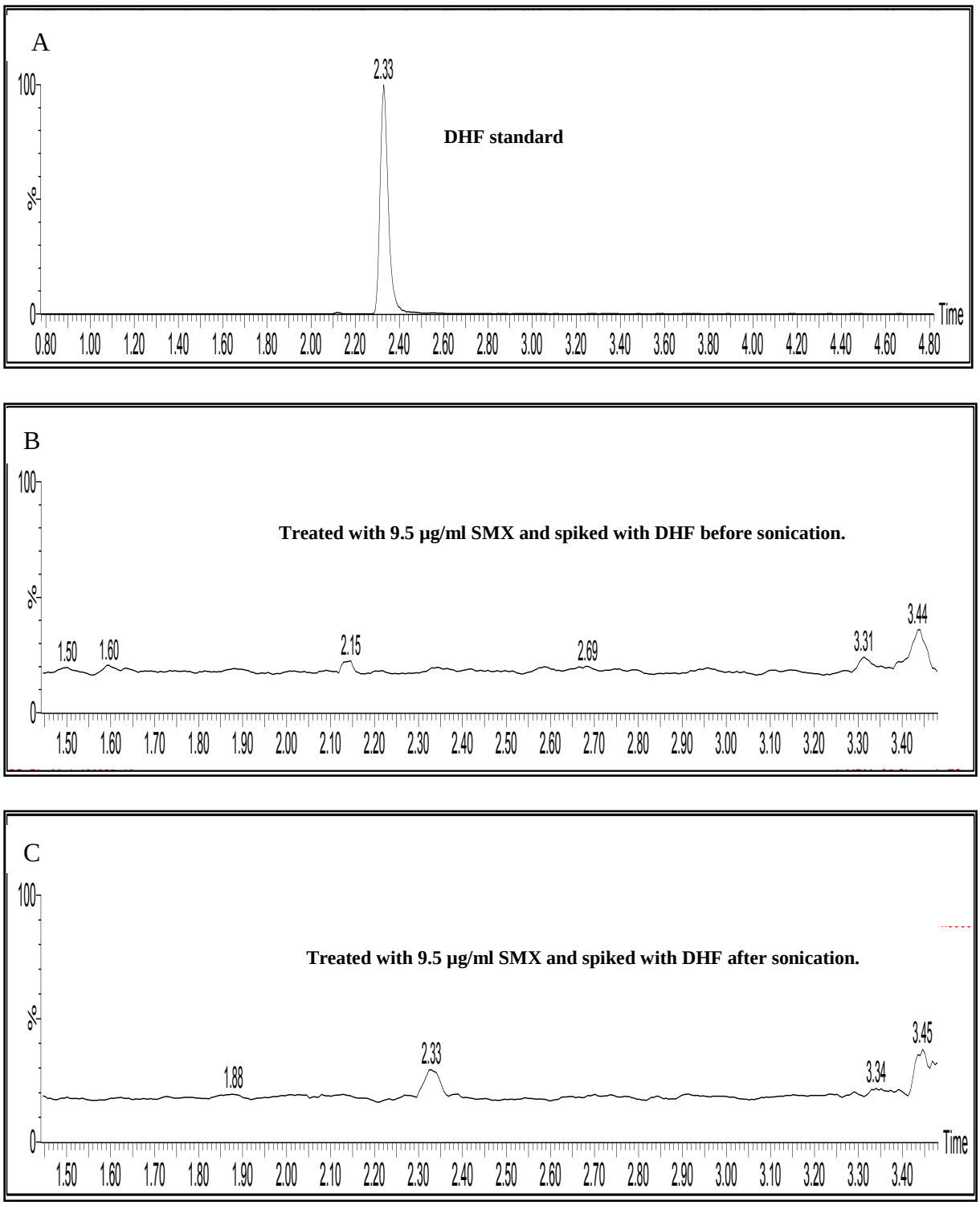


Figure 3.3 (a, b & c): Shows chromatograms of total DHF extracts from BCG samples that were spiked with 1µg/ml of each of the folate standards before or after sonication.

The other factor that could have affected our analysis of DHF and THF is that these folates are the least abundant forms and the most abundant forms are polyglutamated folates with 4 to 5 glutamates attached (Quinlivan *et al.*, 2006; Arcot and Shrestha, 2005). The polyglutamation of folate is necessary for the retention of folates inside the cell and folate exporters, such as reduced folate carriers, have low affinity for polyglutamate folate and it has also been reported that many folate dependent enzymes, such as thymidine synthase prefer polyglutamated forms (Shane and Stokstad, 1975; Lu *et al.*, 1984; Schirch and Strong, 1989).

We then measured the levels of para-aminobenzoic acid (PABA), a substrate of dihydropteroate synthase and an analog of SMX (Brain *et al.*, 2008). In this experiment we expected to detect PABA in SMX treated samples, because SMX will block its utilisation resulting in the accumulation of PABA (see figure 1.12).

We were able to detect PABA in SMX treated samples although peaks had a low signal-to-noise ratio (4.5:1) (see figure 3.4) whereas a ratio of 10:1 is required for quantitation purposes (Shan *et al.,* 2012). In figure 3.4, the first chromatogram shows PABA detected in the sample with a retention time of 2.11 as for the standard.

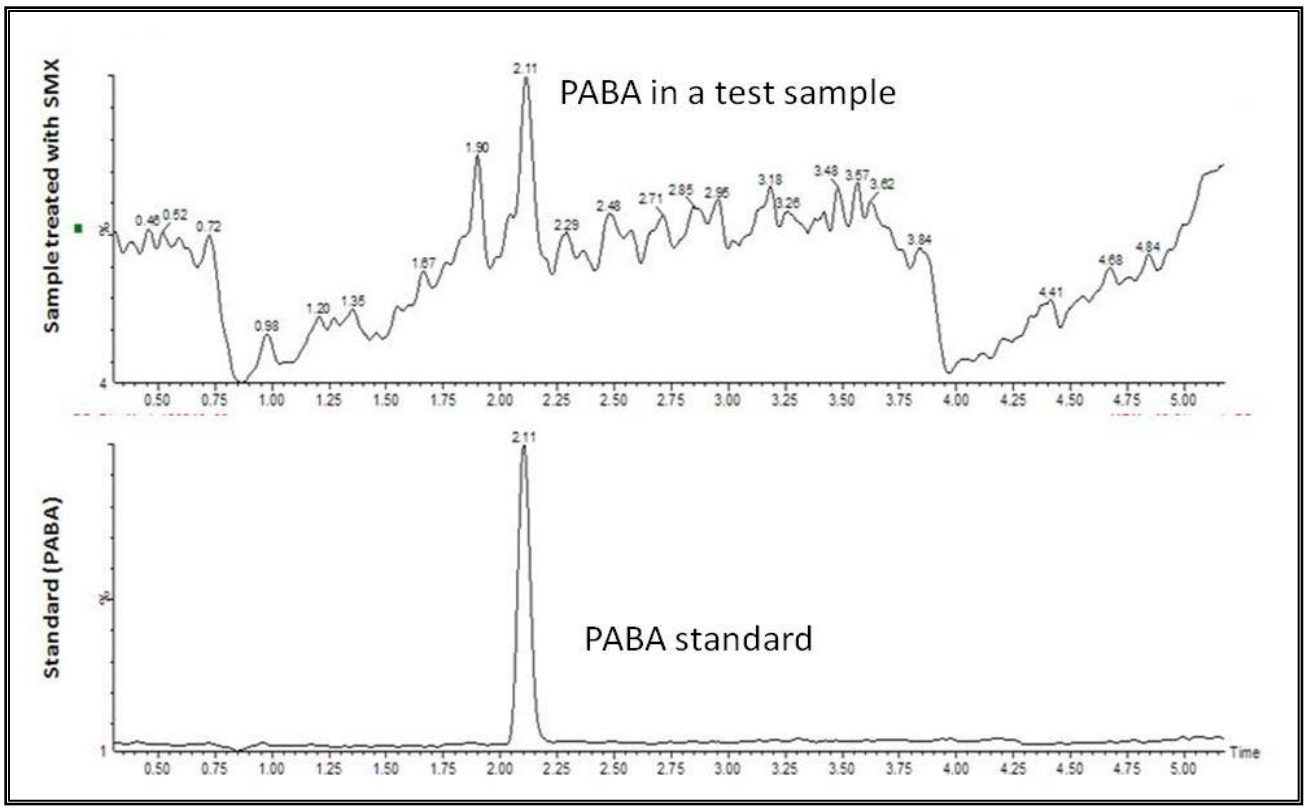


Figure 3.4: Shows chromatograms of PABA from BCG extracts. BCG cultures were treated with 9.5 µg/ml of SMX and extracted after 2hrs of incubation at 37^{o}C.

Table 3.1 shows the amount of PABA that was present in samples treated with various concentrations of SMX (4.75, 2.4 and 0.6 µg/ml). Experiments were repeated at least three times and yielded the same results, each sample had two technical replicates (see table 3.1). From these experiments we could detect PABA in samples treated with SMX, but not in samples treated with DMSO (control samples). However, we could not detect PABA from intracellular extracts (see table 3.1 samples 24 to 31). We then added SMX to 7H9 medium in order to be certain that it was not SMX that was detected in SMX treated samples (Yun *et al.*, 2012). In table 3.1, samples 32 and 33; show that there was no detection of SMX as PABA in the medium.

Name	Sample	Peak area	Conc. (ppm).	µg/vial
1	PABA 0.0004 ppm	567.8		
2	PABA 0.004 ppm	4842.8		
3	PABA 0.020 ppm	23747.4		
4	PABA 0.040 ppm	48092.2		
8	4.75 R1 M	3.7	0.0003	0.0002
9	4.75 R2 M	399.4	0.0003	0.0002
10	2.4 R1 M	Not detected		
11	2.4 R2 M	Not detected		
12	0.6 R1 M	477.2	0.0004	0.0002
13	0.6 R2 M	349.3	0.0003	0.0002
14	DMSO R1 M	Not detected		
15	DMSO R2 M	Not detected		
16	4.75 R1 E	688.0	0.0006	0.0003
17	4.75 R2 E	819.2	0.0007	0.0004
18	2.4 R1 E	544.5	0.0005	0.0003
19	2.4 R2 E	605.3	0.0005	0.0003
20	DMSO R1 E	Not detected		
21	DMSO R2 E	Not detected		
22	0.6 R1 E	394.9	0.0003	0.0002
23	0.6 R2 E	9.8	0.0003	0.0002
24	4.75 R1 I	Not detected		
25	4.75 R2 I	Not detected		
26	2.4 R1 I	Not detected		
27	2.4 R2 I	Not detected		
28	DMSO R1 I	Not detected		
29	DMSO R2 I	Not detected		
30	0.6 R1 I	Not detected		
31	0.6 R2 I	Not detected		
32	9.5 R1 SMX	Not detected		
33	9.5 R2 SMX	Not detected		

Table 3.1: Concentrations of PABA detected in samples treated with SMX; M- PABA was measured from the medium after cells were pelleted; E- cells were re-suspended in the extraction buffer and incubated on ice for 15 mins, cells were then centrifuged and PABA was measured in the supernatant; I- intracellular PABA was measured from sonicated cells.

A study using a filter culture system to support metabolomic profiling of *M. tuberculosis* in response to PAS treatment, found that treatment of *M. tuberculosis* with SMX and DDS resulted in low accumulation of PABA compared to PAS and they suggested that *M. tuberculosis* may inactivate sulfonamides through unknown mechanisms and they concluded that these results indicated that dihydropteroate synthase inhibition alone cannot explain the growth inhibitory activity of SMX (Chakraborty *et al.*, 2013).

In this study, the difficulty of quantifying DHF and THF was also acknowledged, because they exhibit a half-life of less than an hour and are prone to oxidation/reduction, methylation, and polyglutamylation (Chakraborty *et al.*, 2013). Since we could not detect folate levels successfully, the next step was to detect mutations and expression regulation of the genes involved in the folate pathway in *M. tuberculosis*.

3.2.2 Sequencing of the putative target gene of SMX and the *thyA* gene that may impede the effect of SMX in an isoniazid mono-resistant clinical isolate

DNA sequencing was performed to determine whether mutations occurred in the *folP1* and *thyA* (a gene that codes for an enzyme that catalyses the biosynthesis of thymidylate and also known to cause resistance to folate inhibitors when mutated) (Zhang *et al.*, 2007; Carreras and Santi 1995) genes in the Isoniazid mono-resistant clinical isolate R1129, (see section 5.6.3). Sequences of the fragments of *folP1* and *thyA* were aligned to *Mycobacterium tuberculosis* sequences obtained from Tuberculist (http://tuberculist.epfl.ch/) using Sequencher™ software version 4.10.1-Build 5828 Ref number 1010033.

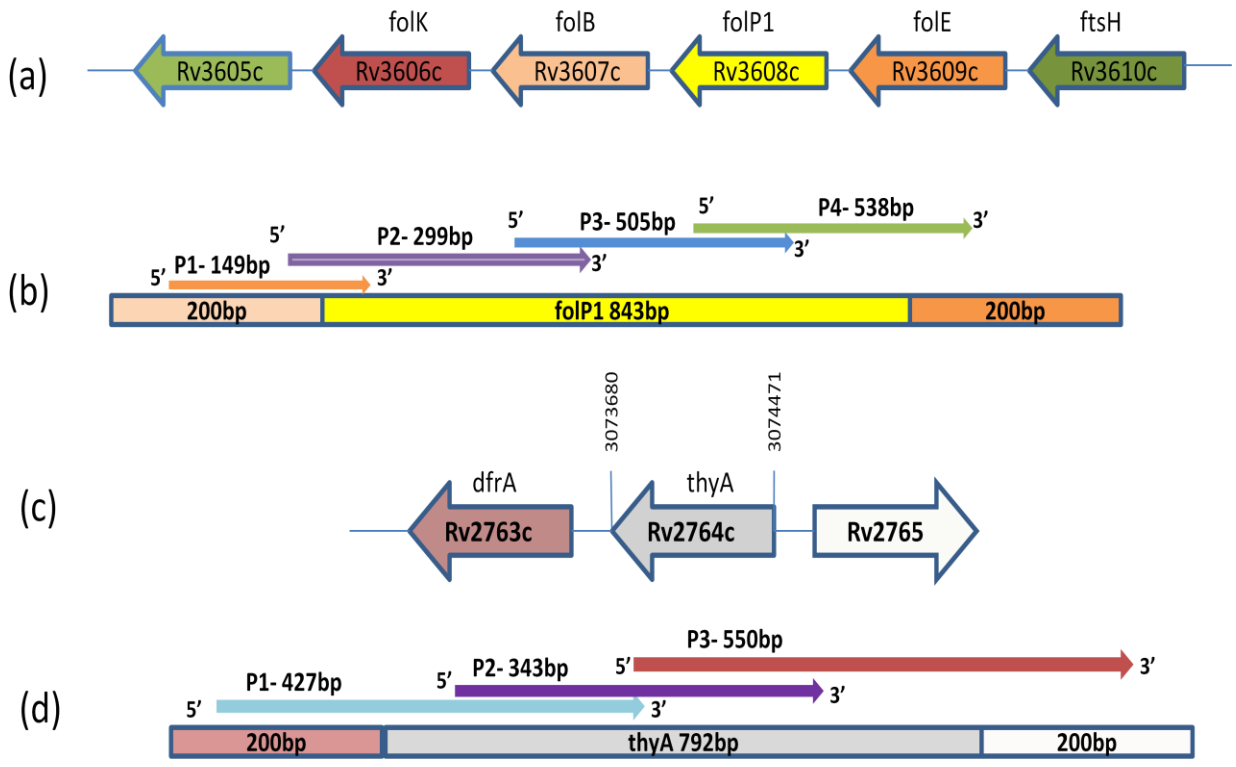


Figure 3.5: Schematic representation of the locations of the two genes sequenced (*folP1* & *thyA*) in the *M. tuberculosis* genome; **(a)** Shows the six genes of the folate pathway operon, **(b)** shows a method used to PCR amplify the entire *folP1* gene using multiple primer pairs (P1-4) and the resulting fragments were sequenced. **(c)** Shows the location of the *thyA* gene in the *M. tuberculosis* genome, **(d)** shows the method used for sequencing.

When sequencing *folP1*, which is part of the folate operon, we included 200bp upstream and downstream to cover the entire gene, however the sequenced region is not likely to contain a promoter, as the promoter of an operon is located close to the first gene and *folP1* is located in the middle of the operon (figure 3.3). In the sequencing of the *thyA* gene, we also included 200bp in the upstream and downstream regions in order to cover the promoter sequence which lies a few base pairs (+/- 30bp) upstream (Newton-Foot and Gey van Pittius, 2013). No

mutations were detected in the folP1 and *thyA* genes. DNA sequences were identical to those obtained from Tuberculist.

3.2.3 Relative gene expression of folP1 gene in response to SMX treatment in the INH^R clinical isolate

In order to survive the effect of a drug, *Mycobacterium tuberculosis* induces the over-expression of a drug's target as an adaptive strategy (Karakousis *et al.*, 2008). In order to gain insight into the phenotypic tolerance of the isoniazid mono-resistant isolate to SMX, we monitored the expression the *folP1* gene in response to SMX treatment. We hypothesised that the expression of this gene would differ significantly upon exposure to SMX compared to the unexposed bacilli and might explain the phenotypic tolerance to SMX.

We treated the mid log phase liquid cultures of the INH^R isolate with SMX at 2 µg/ml (approximately 4x below the MIC which inhibited approximately 50% of H37Rv growth) and harvested the cells at defined time points (see section 5.6.2). Total RNA collected from the cells treated with DMSO at time zero was used as the reference sample (Wilson *et al.*, 1999).

The quality of the extracted RNA was found be at an acceptable standard (RNA Quality Indicator (RQI) values raging from 7-10) according to the manufacturer's recommendation (Experion™, Bio-RAD). The gene expression of the *folP1* gene was monitored by semi-quantitative real time RT-PCR using SYBR Green I and 16s rRNA as the housekeeping gene (Pfaffl *et al.,* 2002; Harper *et al.*, 2010). The difference between the control at time zero and the experiment treated with SMX was analyzed using the REST® program (Pfaffl *et al.,* 2002).

No change in the *folP1* gene expression upon exposure to SMX after 30 minutes, 1hour and four hours was observed.

Table 3.2: RT–PCR of INH^R *folP1*

	Gene- ***folP1***	
SMX treatment (µg/ml)	**Time**	**Fold Change**
2	30 minutes	1.1228
2	1hour	1.185
2	4hours	1.076

The table shows gene regulation in INH-mono-resistant clinical isolate exposed to 2µg/ml of SMX at different time points versus untreated cultures, where 16s was used as the internal control. The data was obtained from two separate experiments.

The finding that the expression of the target gene of SMX is not differentially expressed upon SMX treatment, may suggest that SMX activity in *M. tuberculosis* may be via other mechanisms that does not involve the folate pathway inhibition. To address this possibility, we applied a global protein profiling approach in order to identify other potential target genes involved in the mode of action of SMX.

3.2.4 To evaluate the proteomic profile of the isoniazid resistant isolate treated with SMX using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS, LTQ Orbitrap)

In order to gain understanding on the potential mechanisms involved in the cross-resistance of the isoniazid mono-resistant *M. tuberculosis* strain to SMX and also identify other possible targets of SMX, we evaluated the variation of the global protein expression in response to the treatment with SMX (9.5 µg/ml) that inhibited about 50% of the growth of the strain (see chapter 2, table 2.3). Cultures were exposed to 9.5 µg/ml of SMX at the mid log phase and intracellular proteins were extracted at two time points (see section 8.1). We identified 162

proteins that were differentially regulated upon exposure of isoniazid mono-resistant *M. tuberculosis* strain to SMX, of which, 96 were induced within the first 30 minutes of exposure to SMX and the remaining 66 were induced after 24 hours of exposure to this sub-inhibitory concentration of SMX (Table 3.3-3.6). A total of 65 proteins were up-regulated and 97 proteins were down-regulated and belong to the following groups; proteins of cell wall synthesis and cell processes, lipid metabolism, information pathways, regulatory, virulence, detoxification and adaptation, conserved hypothetical and intermediary metabolism and respiration category (Figures 3.4 and 3.5).

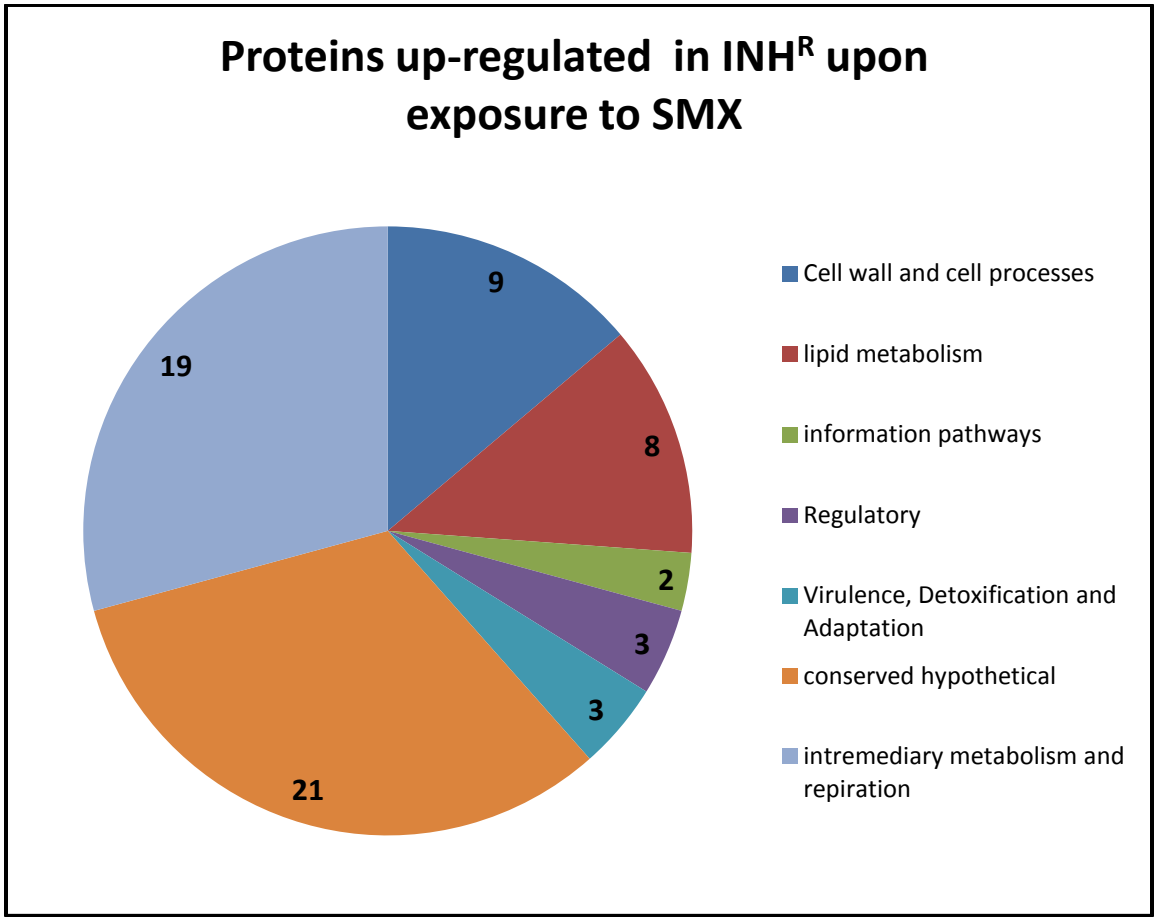


Figure 3.6: Functional groups of proteins up-regulated upon exposure to 9.5 µg/ml of SMX, 22 proteins within 30 minutes and 43 proteins after 24 hours.

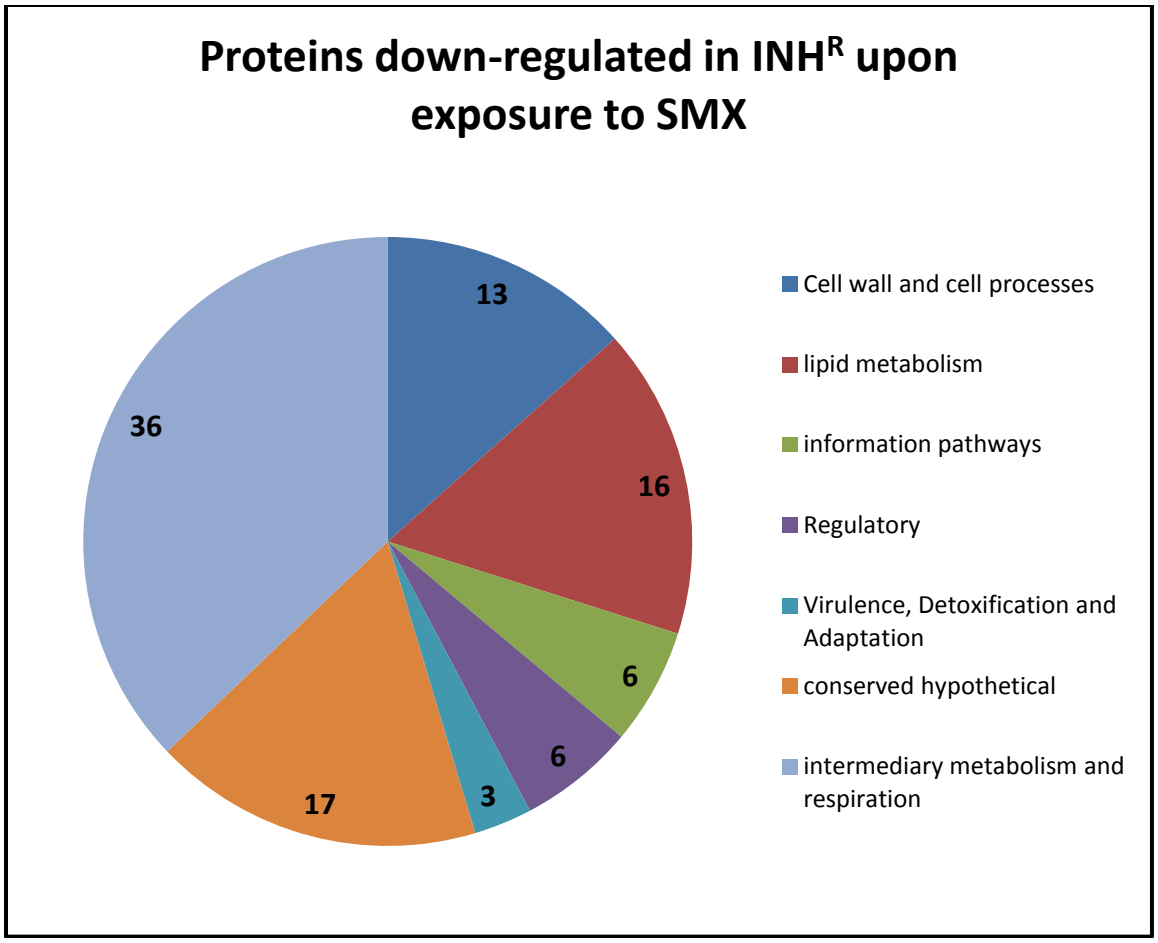


Figure 3.7: Functional groups of proteins down-regulated upon exposure to 9.5 µg/ml of SMX, 74 proteins within 30 minutes and 23 proteins after 24 hours. No members of information pathways and Virulence, Detoxification and Adaptation were down-regulated after 24 hours.

Among the proteins that were up-regulated, were proteins involved in oxidative stress response, toxin and antitoxin proteins (Rv0117, OxyS- oxidative stress protein, Rv1242, vapC33-toxin and Rv0300, vapB2- antitoxin). *OxyS* is a regulatory gene, belonging to the LysR family in Mycobacteria and it is associated with the mediation of oxidative stress responses (Domenech *et al.*, 2001; Philipp *et al.*, 1996; Cole *et al.*, 1998).

OxyS directly regulates *katG* by binding directly to the *katG* promoter region and the conserved binding site for OxyS in the promoter region of *katG* has been mappad (Li and He, 2012). It was

shown that oxidative stress up-regulates the expression of OxyS which directly down-regulates the expression of *katG* and concluded that OxyS is a negative regulator of *katG* in response to oxidative stress in mycobacteria (Li and He, 2012). Together with the over-expression of *OxyS* and down-regulation of *KatG*, is the up-regulation of *mca* that encodes for Mycothiol S-conjugate amidase. It catalyzes the hydrolysis of an amide bond in MSH-toxin conjugates, producing a mercapturic acid which is excreted from mycobacteria and glucosaminyl inositol is recycled back to mycothiol (Newton *et al.*, 2000). It has been reported that oxidative stress conditions that deplete the levels of thiols (e.g. mycothiol) induce over-expression of *sigR*, *rsrA*, *mshA* and *mca* (Newton and Fahey, 2008).

These observations together with our findings that a strain of *M. tuberculosis* lacking mycothiol (*mshA*) was more susceptible to SMX (see chapter 2, section 2.2.4), suggest that SMX kills *M. tuberculosis* via oxidative stress and that the up-regulation of *mca* is the means of mopping up the reactive intermediate species produced by SMX which would damage cell wall, DNA and cellular proteins (Zhou and Moore, 1997). Furthermore, the up-regulation of these proteins suggests their possible involvement in drug resistance in *M. tuberculosis,* making them potential drug targets (Provvedi *et al.*, 2009). It has been reported that a *Mycobacterium smegmatis* mutant strain lacking the *mca* gene was more susceptible to a number of antibiotics including rifampicin and streptomycin (Rawat *et al.*, 2004).

We also observed up-regulation of toxin (Rv1242, VapC33-toxin) and antitoxin (Rv0300, VapB2-antitoxin) in the isoniazid mono-resistant *M. tuberculosis* strain treated with SMX. The toxin-antitoxin systems (TA) are present in almost all prokaryotes and they function as a stress

response system that induces a nonreplicative persistent state or cell cycle arrest that result in bacterial tolerance (Gerdes, 2000; Hayes, 2003; Gerdes *et al.*, 2005). The genes coding for these systems are grouped in an operon of two genes, one coding for the antitoxin and the other coding for the toxin (Provvedi *et al.*, 2009). Unlike the plasmid encoded TA, which target plasmid-free cells and cause cell death or growth restriction, the chromosomal encoded TA, which exist in mycobacteria and other advanced pathogens, adjust the state of the cell in response to an external stimulus (Hayes, 2003). Antitoxin regulates toxin, but it gets degraded rapidly giving an increase to toxin levels, which mediates toxicity by RNA cleavage, inhibiting translation thereby resulting in a metabolic change of the bacteria (Provvedi *et al.*, 2009). Toxins which contain a PIN domain, like VapC, have also been shown to have RNase activity and are involved in growth regulation (Moyed and Bertrand, 1983; Ramage *et al.*, 2009; Provvedi *et al.*, 2009; Ahidjo *et al.*, 2011). Figure 3.6 shows the mode of oxidative stress production by SMX.

The induction of VapC33 and VapB2 (TA), upon treatment with SMX suggests their involvement in the growth rate of isoniazid mono-resistant *M. tuberculosis* strain in response to oxidative stress. In this scenario, the inhibition of tetrahydrofolate involved in the thymine synthesis required for DNA and RNA synthesis would not be effective as the organism is slowing growth shown by the down-regulation of *gyr*B, a DNA gyrase (see table 3.4).

We also observed down-regulation of many proteins involved in fatty acid metabolism (see table 3.4 and 3.6). Many studies have indicated that *M. tuberculosis* in vivo uses cholesterol or host fatty acids as a preferred carbon source rather than glycerol and glucose (Schnappinger *et*

al., 2003). This process requires different classes of enzymes, such as fadD genes, which code for enzymes that are responsible for fatty acid activation (Black *et al.,* 1992), producing acyl-CoA thioesters that are degraded to acetyl-CoA by fadE, fadB and fadA enzymes (Campbell and Jr, 2002; Binstock *et al.,* 1977). FadD enzymes have been reported to also activate endogenous fatty acids (Pech-Canul *et al.,* 2011), therefore these enzymes are capable of triggering degradation of membrane lipids of the bacterium. The down-regulation of proteins involved in beta oxidation (fadE20, fadD7, fadD16, fadD13, fadA2, fadD36, fadB, fadE32, fadD15) (see table 3.4) indicates that *M. tuberculosis* prevents further destruction of its cell wall.

Furthermore, down regulation of proteins involved in mycolic acid synthesis (fabG4, accD2, accD4, pks16) (Gande *et al.,* 2004; Gurvitz, 2009) and repression of the FtsK protein, an ATP-dependent DNA translocase, which is involved in the translocation of DNA from the mother cell compartment into the smaller compartment during cell division (Massey *et al.,* 2006), suggests that the bacterium is slowing down all energy consuming processes.

The up-regulation of recC (see table 3.3 and 3.5), which is a protein involved in DNA repair (Wigley, 2013) and an energy requiring process, suggests that the energy reserved from the fatty acid or mycolic acid synthesis pathways is shifted to cell protection processes. Furthermore, the rec gene cluster has been reported to be up-regulated in response to DNA damaging external stress, such as H_2O_2 (Voskuil and Bartek, 2011).

The suppression of *nuoI* (table 3.4), which is part of the three subunits (NuoL, NuoM, and NuoN) of NADH dehydrogenase I that is involved in proton translocation, expressed during rapid growth. A deletion or loss of function of either NuoL or NuoM, results in the total loss of

activity of the enzyme, further suggests the scaling down of energy expensive metabolic pathways and suggests slowing of growth, but not cell death (Provvedi *et al.*, 2009; Torres-Bacete *et al.*, 2011).

In conclusion, these results suggest that the isoniazid mono-resistant clinical isolate, upon exposure to SMX experiences stress, resulting in the induction of stress response systems in order to protect itself. Some of the proteins that were induced upon exposure to SMX are also induced by some antibiotics that inhibit cell wall synthesis, such a isoniazid and vancomycin (Provvedi *et al.,* 2009), suggesting that SMX might also be targeting similar pathways as these drugs. This could explain the cross resistance in the isoniazid resistant *M. tuberculosis* clinical isolates to SMX.

Furthermore, in this study we have identified proteins that could be potential drug targets, such as the *mca* gene. This proteomic approach has allowed us to be able to identify proteins that may be involved in the protection of *Mycobacterium tuberculosis* from the oxidative stress imposed by SMX and could potentially be involved in the cross-resistance of the isoniazid mono-resistant clinical isolate to SMX.

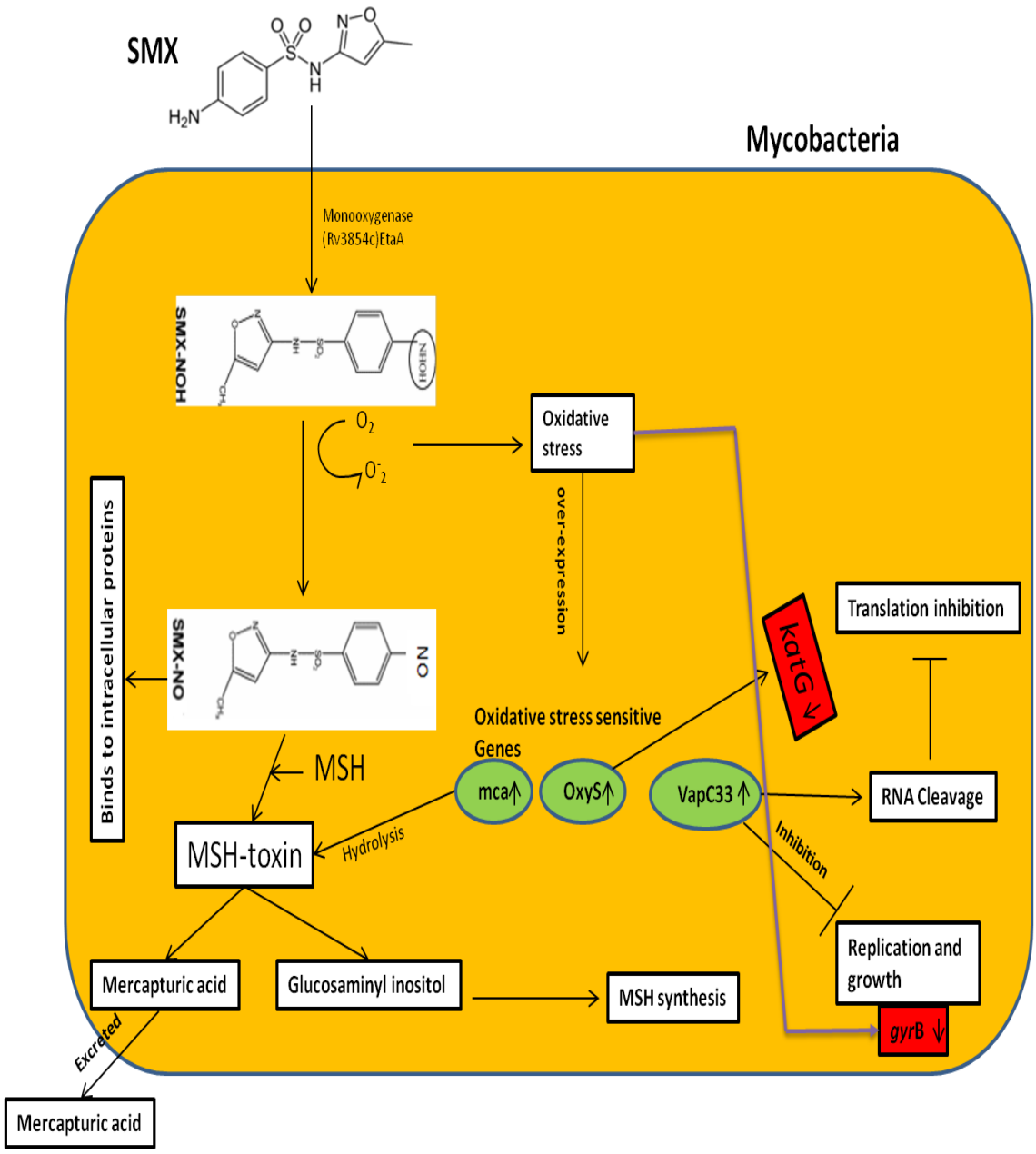


Figure 3.8: Proposed mode of oxidative stress induction by SMX and the response thereof in *Mycobacterium tuberculosis*. The ↑ green colour indicates up-regulated and ↓ red colour indicates down-regulated proteins.

Table 3.3: Proteins that are deferentially regulated in SMX resistant clinical isolate upon exposure to SMX, classified according to biological function. Up +/-30mins*

Rv number	Protein Name	Function	Functional Group	p-value	Fold change
Rv0190	Rv0190	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.002	3.383
Rv3504	fadE26	PROBABLE ACYL-CoA DEHYDROGENASE FADE26	lipid metabolism	0.003	3.351
Rv3229c	desA3	POSSIBLE LINOLEOYL-CoA DESATURASE (DELTA(6)-DESATURASE)	lipid metabolism	0.004	3.335
Rv3090	Rv3090	HYPOTHETICAL ALANINE AND VALINE RICH PROTEIN	Conserved hypothetical	0.004	3.320
Rv0291	mycP3	PROBABLE MEMBRANE-ANCHORED MYCOSIN MYCP3 (SERINE PROTEASE) (SUBTILISIN-LIKE PROTEASE) (SUBTILASE-LIKE) (MYCOSIN-3)	Intermediary metabolism and respiration	0.010	3.185
Rv0451c	mmpS4	PROBABLE CONSERVED MEMBRANE PROTEIN MMPS4	Cell wall and cell processes	0.017	3.096
Rv0117	oxyS	OXIDATIVE STRESS RESPONSE REGULATORY PROTEIN OXYS	Regulatory	0.019	3.068
Rv1914c	Rv1914c	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.022	3.038
Rv1242	vapC33	POSSIBLE TOXIN VAPC33. CONTAINS PIN DOMAIN.	Virulence, detoxification and adaptation	0.023	3.017
Rv2345	Rv2345	POSSIBLE CONSERVED TRANSMEMBRANE PROTEIN	Cell wall and cell processes	0.024	3.014
Rv0501	galE2	POSSIBLE UDP-GLUCOSE 4-EPIMERASE GALE2 (GALACTOWALDENASE) (UDP-GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHATE GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHO-GALACTOSE 4-EPIMERASE)	Intermediary metabolism and respiration	0.026	2.993
Rv2536	Rv2536	PROBABLE CONSERVED TRANSMEMBRANE PROTEIN	Cell wall and cell processes	0.031	2.942
Rv0631c	recC	PROBABLE EXONUCLEASE V (GAMMA CHAIN) RECC (EXODEOXYRIBONUCLEASE V GAMMA CHAIN)(EXODEOXYRIBONUCLEASE V POLYPEPTIDE)	Information pathways	0.036	2.900
Rv1729c	Rv1729c	POSSIBLE S-ADENOSYLMETHIONINE-DEPENDENT METHYLTRANSFERASE	lipid metabolism	0.038	2.884
Rv2257c	Rv2257c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.040	2.869
Rv1211	Rv1211	CONSERVED HYPOTHETICAL PROTEIN	Conserved	0.036	2.855

			hypothetical		
Rv1990c	Rv1990c	PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN	Regulatory	0.047	2.821
Rv3565	aspB	POSSIBLE ASPARTATE AMINOTRANSFERASE ASPB (TRANSAMINASE A) (ASPAT) (GLUTAMIC--OXALOACETIC TRANSAMINASE) (GLUTAMIC--ASPARTIC TRANSAMINASE)	Intermediary metabolism and respiration	0.048	2.812
Rv1530	adh	PROBABLE ALCOHOL DEHYDROGENASE ADH	Intermediary metabolism and respiration	0.048	2.805
Rv1362c	Rv1362c	POSSIBLE MEMBRANE PROTEIN	Cell wall and cell processes	0.048	2.804
Rv0690c	Rv0690c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.052	2.786

***Proteins that were up-regulated within 30 minutes of exposure to 9.5 µg/ml of SMX**

Table 3.4: Proteins with that are deferentially regulated in SMX resistant clinical isolate upon exposure to SMX, classified according to biological function. Down +/-30mins*

Rv number	Protein Name	Function	Functional Group	p-value	Fold change
Rv3240c	secA1	PROBABLE PREPROTEIN TRANSLOCASE SECA1 1 SUBUNIT	Cell wall and cell processes	0.001	3.417
Rv1830	Rv1830	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.002	3.407223
Rv2982c	gpdA2	PROBABLE GLYCEROL-3-PHOSPHATE DEHYDROGENASE [NAD(P)+] GPDA2 (NAD(P)H- DEPENDENT GLYCEROL-3-PHOSPHATE DEHYDROGENASE)	lipid metabolism	0.002	3.404
Rv1480	Rv1480	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.002	3.369
Rv0974c	accD2	PROBABLE ACETYL-/PROPIONYL-CoA CARBOXYLASE (BETA SUBUNIT) ACCD2	lipid metabolism	0.003	3.343
Rv3799c	accD4	PROBABLE PROPIONYL-CoA CARBOXYLASE BETA CHAIN 4 ACCD4 (PCCASE) (PROPANOYL-CoA:CARBON DIOXIDE LIGASE)	lipid metabolism	0.003	3.341
Rv3153	nuoI	PROBABLE NADH DEHYDROGENASE I (CHAIN I) NUOI (NADH-UBIQUINONE OXIDOREDUCTASE CHAIN I)	Intermediary metabolism and respiration	0.004	3.324
Rv3866	espG1	ESX-1 SECRETION-ASSOCIATED PROTEIN ESPG1	Cell wall and cell processes	0.004	3.321
Rv1908c	katG	CATALASE-PEROXIDASE-PEROXYNITRITASE T KATG	Virulence, detoxification and adaptation	0.004	3.319

Rv0583c	lpqN	PROBABLE CONSERVED LIPOPROTEIN LPQN	Cell wall and cell processes	0.004	3.306
Rv3808c	glfT2	BIFUNCTIONAL UDP-GALACTOFURANOSYL TRANSFERASE GLFT2	Cell wall and cell processes	0.006	3.278
Rv1771	Rv1771	L-GULONO-1,4-LACTONE DEHYDROGENASE	Intermediary metabolism and respiration	0.008	3.238
Rv2192c	trpD	PROBABLE ANTHRANILATE PHOSPHORIBOSYLTRANSFERASE TRPD	Intermediary metabolism and respiration	0.008	3.225
Rv1340	rphA	PROBABLE RIBONUCLEASE RPHA (RNase PH) (tRNA NUCLEOTIDYLTRANSFERASE)	Information pathways	0.011	3.177
Rv1001	arcA	PROBABLE ARGININE DEIMINASE ARCA (ADI) (AD) (ARGININE DIHYDROLASE)	Intermediary metabolism and respiration	0.012	3.157
Rv1559	ilvA	PROBABLE THREONINE DEHYDRATASE IlvA	Intermediary metabolism and respiration	0.013	3.146
Rv2187	fadD15	PROBABLE LONG-CHAIN-FATTY-ACID-CoA LIGASE FADD15 (FATTY-ACID-CoA SYNTHETASE) (FATTY-ACID-CoA SYNTHASE)	lipid metabolism	0.014	3.129
Rv1894c	Rv1894c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.015	3.116
Rv1638	uvrA	PROBABLE EXCINUCLEASE ABC (SUBUNIT A - DNA-BINDING ATPase) UVRA	Information pathways	0.016	3.108
Rv0005	gyrB	DNA GYRASE (SUBUNIT B) GYRB (DNA TOPOISOMERASE (ATP-HYDROLYSING)) (DNA TOPOISOMERASE II) (TYPE II DNA TOPOISOMERASE)	Information pathways	0.016	3.100
Rv2298	Rv2298	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.017	3.090
Rv0896	gltA2	PROBABLE CITRATE SYNTHASE I GLTA2	Intermediary metabolism and respiration	0.019	3.069
Rv1865c	Rv1865c	PROBABLE SHORT-CHAIN TYPE DEHYDROGENASE	Intermediary metabolism and respiration	0.020	3.056
Rv3671c	Rv3671c	POSSIBLE MEMBRANE-ASSOCIATED SERINE PROTEASE	Intermediary metabolism and respiration	0.020	3.051
Rv0408	pta	PROBABLE PHOSPHATE ACETYLTRANSFERASE PTA (PHOSPHOTRANSACETYLASE)	Intermediary metabolism and respiration	0.021	3.041
Rv3563	fadE32	PROBABLE ACYL-CoA DEHYDROGENASE FADE32	lipid metabolism	0.022	3.031
Rv3048c	nrdF2	RIBONUCLEOSIDE-DIPHOSPHATE REDUCTASE (BETA CHAIN) NRDF2 (RIBONUCLEOTIDE REDUCTASE SMALL SUBUNIT) (R2F PROTEIN)	Information pathways	0.022	3.030
Rv0860	fadB	PROBABLE FATTY OXIDATION PROTEIN FADB	lipid metabolism	0.025	3.005
Rv3212	Rv3212	CONSERVED HYPOTHETICAL ALANINE VALINE RICH PROTEIN	Conserved hypothetical	0.025	2.998

Rv1692	Rv1692	PROBABLE PHOSPHATASE	Intermediary metabolism and respiration	0.026	2.993
Rv1229c	mrp	PROBABLE MRP-RELATED PROTEIN MRP	Intermediary metabolism and respiration	0.026	2.986
Rv2030c	Rv2030c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.027	2.982
Rv1193	fadD36	PROBABLE FATTY-ACID-CoA LIGASE FADD36 (FATTY-ACID-CoA SYNTHETASE) (FATTY-ACID-CoA SYNTHASE)	lipid metabolism	0.028	2.969
Rv3220c	Rv3220c	PROBABLE TWO COMPONENT SENSOR KINASE	Regulatory	0.028	2.968316
Rv0307c	Rv0307c	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.029	2.956
Rv1106c	Rv1106c	3-BETA-HYDROXYSTEROID DEHYDROGENASE	Intermediary metabolism and respiration	0.030	2.952
Rv3464	rmlB	dTDP-GLUCOSE 4,6-DEHYDRATASE RMLB	Intermediary metabolism and respiration	0.030	2.950
Rv1018c	glmU	PROBABLE UDP-N-ACETYLGLUCOSAMINE PYROPHOSPHORYLASE GLMU	Cell wall and cell processes	0.030	2.946
Rv3305c	amiA1	POSSIBLE N-ACYL-L-AMINO ACID AMIDOHYDROLASE AMIA1 (N-ACYL-L-AMINO ACID AMINOHYDROLASE)	Intermediary metabolism and respiration	0.031	2.938
Rv0242c	fabG4	PROBABLE 3-OXOACYL-[ACYL-CARRIER PROTEIN] REDUCTASE FABG4 (3-KETOACYL-ACYL CARRIER PROTEIN REDUCTASE)	lipid metabolism	0.032	2.931
Rv1475c	acn	PROBABLE IRON-REGULATED ACONITATE HYDRATASE ACN (CITRATE HYDRO-LYASE) (ACONITASE)	Intermediary metabolism and respiration	0.032	2.930
Rv0466	Rv0466	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.033	2.920
Rv0423c	thiC	PROBABLE THIAMINE BIOSYNTHESIS PROTEIN THIC	Intermediary metabolism and respiration	0.034	2.919
Rv1257c	Rv1257c	PROBABLE OXIDOREDUCTASE	Intermediary metabolism and respiration	0.034	2.914
Rv2178c	aroG	PROBABLE 3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHASE AROG (DAHP SYNTHETASE, PHENYLALANINE-REPRESSIBLE)	Intermediary metabolism and respiration	0.034	2.913
Rv1297	rho	PROBABLE TRANSCRIPTION TERMINATION FACTOR RHO HOMOLOG	Information pathways	0.035	2.904
Rv2540c	aroF	PROBABLE CHORISMATE SYNTHASE AROF (5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE PHOSPHOLYASE)	Intermediary metabolism and respiration	0.036	2.897
Rv2005c	Rv2005c	UNIVERSAL STRESS PROTEIN FAMILY PROTEIN	Virulence, detoxification and adaptation	0.037	2.891
Rv3120	Rv3120	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.038	2.881

Rv0479c	Rv0479c	PROBABLE CONSERVED MEMBRANE PROTEIN	Cell wall and cell processes	0.038	2.880
Rv1422	Rv1422	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.039	2.874
Rv2916c	ffh	PROBABLE SIGNAL RECOGNITION PARTICLE PROTEIN FFH (FIFTY-FOUR HOMOLOG) (SRP PROTEIN)	Cell wall and cell processes	0.039	2.873
Rv0243	fadA2	PROBABLE ACETYL-CoA ACYLTRANSFERASE FADA2 (3-KETOACYL-CoA THIOLASE) (BETA-KETOTHIOLASE)	lipid metabolism	0.040	2.867
Rv2786c	ribF	PROBABLE BIFUNCTIONAL FAD SYNTHETASE/RIBOFLAVIN BIOSYNTHESIS PROTEIN RIBF: RIBOFLAVIN KINASE (FLAVOKINASE) + FMN ADENYLYLTRANSFERASE (FAD PYROPHOSPHORYLASE) (FAD SYNTHETASE)(FAD DIPHOSPHORYLASE) (FLAVIN ADENINE DINUCLEOTIDE SYNTHETASE)	Intermediary metabolism and respiration	0.038	2.855
Rv2748c	ftsK	POSSIBLE CELL DIVISION TRANSMEMBRANE PROTEIN FTSK	Cell wall and cell processes	0.043	2.847
Rv2989	Rv2989	PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN	Regulatory	0.043	2.846
Rv1013	pks16	PUTATIVE POLYKETIDE SYNTHASE PKS16	lipid metabolism	0.044	2.836
Rv3918c	parA	PROBABLE CHROMOSOME PARTITIONING PROTEIN PARA	Cell wall and cell processes	0.044	2.836
Rv2457c	clpX	PROBABLE ATP-DEPENDENT CLP PROTEASE ATP-BINDING SUBUNIT CLPX	Intermediary metabolism and respiration	0.045	2.827
Rv2299c	htpG	PROBABLE CHAPERONE PROTEIN HTPG (HEAT SHOCK PROTEIN) (HSP90 FAMILY PROTEIN) (HIGH TEMPERATURE PROTEIN G)	Virulence, detoxification and adaptation	0.045	2.827
Rv20c	Rv20c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.046	2.824
Rv0952	sucD	PROBABLE SUCCINYL-CoA SYNTHETASE (ALPHA CHAIN) SUCD (SCS-ALPHA)	Intermediary metabolism and respiration	0.046	2.820
Rv0750	Rv0750	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.047	2.811
Rv0265c	Rv0265c	PROBABLE PERIPLASMIC IRON-TRANSPORT LIPOPROTEIN	Cell wall and cell processes	0.048	2.809
Rv1293	lysA	PROBABLE DIAMINOPIMELATE DECARBOXYLASE LYSA (DAP DECARBOXYLASE)	Intermediary metabolism and respiration	0.047673	2.809
Rv0932c	pstS2	PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS2 (PBP-2) (PSTS2)	Cell wall and cell processes	0.048	2.805
Rv2605c	tesB2	PROBABLE ACYL-CoA THIOESTERASE II TESB2 (TEII)	lipid metabolism	0.049	2.803
Rv2922A	acyP	PROBABLE ACYLPHOSPHATASE ACYP (ACYLPHOSPHATE PHOSPHOHYDROLASE)	Intermediary metabolism and respiration	0.049	2.801
Rv3602c	panC	PROBABLE PANTOATE--BETA-ALANINE LIGASE PANC (PANTOTHENATE SYNTHETASE) (PANTOATE ACTIVATING ENZYME)	Intermediary metabolism and respiration	0.050	2.795

Rv0818	Rv0818	TRANSCRIPTIONAL REGULATORY PROTEIN	Regulatory	0.051	2.784
Rv1383	carA	PROBABLE CARBAMOYL-PHOSPHATE SYNTHASE SMALL CHAIN CARA (CARBAMOYL-PHOSPHATE SYNTHETASE GLUTAMINE CHAIN)	Intermediary metabolism and respiration	0.051	2.782
Rv3089	fadD13	PROBABLE CHAIN -FATTY-ACID-CoA LIGASE FADD13 (FATTY-ACYL-CoA SYNTHETASE)	lipid metabolism	0.052	2.776
Rv3634c	galE1	UDP-GLUCOSE 4-EPIMERASE GALE1 (GALACTOWALDENASE) (UDP-GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHATE GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHO-GALACTOSE 4-EPIMERASE)	Intermediary metabolism and respiration	0.054	2.767
Rv0078A	Rv0078A	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.054	2.767

***Proteins that were down-regulated within 30 minutes of exposure to 9.5 µg/ml of SMX**

3.5: Proteins with that are deferentially regulated in SMX resistant clinical isolate upon exposure to SMX, classified according to biological function. Up 24hrs*

Rv number	Protein Name	Function	Functional Group	p-score	Fold change
Rv0070c	glyA2	PROBABLE SERINE HYDROXYMETHYLTRANSFERASE GLYA2 (SERINE METHYLASE 2) (SHMT 2)	Intermediary metabolism and respiration	0.001	3.419
Rv1511	gmdA	GDP-D-MANNOSE DEHYDRATASE GMDA (GDP-MANNOSE 4,6 DEHYDRATASE) (GMD)	Intermediary metabolism and respiration	0.006	3.272
Rv3671c	Rv3671c	POSSIBLE MEMBRANE-ASSOCIATED SERINE PROTEASE	Intermediary metabolism and respiration	0.006	3.265
Rv2171	lppM	PROBABLE CONSERVED LIPOPROTEIN LPPM	Cell wall and cell processes	0.006	3.265
Rv1311	atpC	PROBABLE ATP SYNTHASE EPSILON CHAIN ATPC	Intermediary metabolism and respiration	0.010	3.190
Rv1423	whiA	PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN WHIA	Regulatory	0.011	3.181
Rv3535c	hsaG	PROBABLE ACETALDEHYDE DEHYDROGENASE (ACETALDEHYDE DEHYDROGENASE [ACETYLATING])	Intermediary metabolism and respiration	0.013	3.149

Rv3281	accE5	PROBABLE BIFUNCTIONAL PROTEIN ACETYL-/PROPIONYL-COENZYME A CARBOXYLASE (EPSILON CHAIN) ACCE5	lipid metabolism	0.013	3.139
Rv23c	Rv23c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.013	3.138
Rv0645c	mmaA1	METHOXY MYCOLIC ACID SYNTHASE 1 MMAA1 (METHYL MYCOLIC ACID SYNTHASE 1) (MMA1) (HYDROXY MYCOLIC ACID SYNTHASE)	lipid metabolism	0.015	3.115
Rv2954c	Rv2954c	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.015	3.110
Rv2237	Rv2237	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.015	3.110
Rv1754c	Rv1754c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.018	3.084
Rv3226c	Rv3226c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.018	3.077
Rv0300	vapB2	POSSIBLE ANTITOXIN VAPB2	Virulence, detoxification and adaptation	0.020	3.050
Rv0564c	gpdA1	PROBABLE GLYCEROL-3-PHOSPHATE DEHYDROGENASE [NAD(P)+] GPDA1 (NAD(P)H-DEPENDENT GLYCEROL-3-PHOSPHATE DEHYDROGENASE) (NAD(P)H-DEPENDENT DIHYDROXYACETONE-PHOSPHATE REDUCTASE)	lipid metabolism	0.023	3.019
Rv3231c	Rv3231c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.023	3.019
Rv3030	Rv3030	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.024	3.010
Rv0500	proC	PROBABLE PYRROLINE-5-CARBOXYLATE REDUCTASE PROC (P5CR) (P5C REDUCTASE)	Intermediary metabolism and respiration	0.025	2.994
Rv1296	thrB	PROBABLE HOMOSERINE KINASE THRB	Intermediary metabolism and respiration	0.026	2.986
Rv0928	pstS3	PERIPLASMIC PHOSPHATE-BINDING LIPOPROTEIN PSTS3 (PBP-3) (PSTS3) (PHOS1)	Cell wall and cell processes	0.027	2.979
Rv1594	nadA	PROBABLE QUINOLINATE SYNTHETASE NADA	Intermediary metabolism and respiration	0.027	2.977
Rv3634c	galE1	UDP-GLUCOSE 4-EPIMERASE GALE1 (GALACTOWALDENASE) (UDP-GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHATE GALACTOSE 4-EPIMERASE) (URIDINE DIPHOSPHO-GALACTOSE 4-EPIMERASE)	Intermediary metabolism and respiration	0.028	2.972

Rv2008c	Rv2008c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.030	2.951
Rv0161	Rv0161	POSSIBLE OXIDOREDUCTASE	Intermediary metabolism and respiration	0.032	2.936
Rv1082	mca	MYCOTHIOL CONJUGATE AMIDASE MCA (MYCOTHIOL S-CONJUGATE AMIDASE)	Virulence, detoxification and adaptation	0.034	2.914
Rv2826c	Rv2826c	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.037	2.914
Rv0394c	Rv0394c	POSSIBLE SECRETED PROTEIN	Cell wall and cell processes	0.034	2.913
Rv0562	grcC1	PROBABLE POLYPRENYL-DIPHOSPHATE SYNTHASE GRCC1 (POLYPRENYL PYROPHOSPHATE SYNTHETASE)	Intermediary metabolism and respiration	0.035	2.909
Rv2714	Rv2714	CONSERVED HYPOTHETICAL ALANINE AND LEUCINE RICH PROTEIN	Conserved hypothetical	0.038	2.883
Rv1295	thrC	PROBABLE THREONINE SYNTHASE THRC (TS)	Intermediary metabolism and respiration	0.038	2.883
Rv1421	Rv1421	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.038	2.880
Rv3566c	nat	ARYLAMINE N-ACETYLTRANSFERASE NAT (ARYLAMINE ACETYLASE)	Intermediary metabolism and respiration	0.039	2.876
Rv2403c	lppR	PROBABLE CONSERVED LIPOPROTEIN LPPR	Cell wall and cell processes	0.043	2.843
Rv2188c	pimB	MANNOSYLTRANSFERASE PIMB	lipid metabolism	0.044	2.836
Rv1493	mutB	PROBABLE METHYLMALONYL-CoA MUTASE LARGE SUBUNIT MUTB (MCM)	lipid metabolism	0.046	2.821
Rv0911	Rv0911	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.047	2.812
Rv0449c	Rv0449c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.047	2.812
Rv1596	nadC	PROBABLE NICOTINATE-NUCLEOTIDE PYROPHOSPHATASE NADC	Intermediary metabolism and respiration	0.048	2.810

Rv1006	Rv1006	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.049	2.802
Rv0631c	recC	PROBABLE EXONUCLEASE V (GAMMA CHAIN) RECC (EXODEOXYRIBONUCLEASE V GAMMA CHAIN)(EXODEOXYRIBONUCLEASE V POLYPEPTIDE)	Information pathways	0.049	2.799
Rv2564	glnQ	PROBABLE GLUTAMINE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER GLNQ	Cell wall and cell processes	0.050	2.794
Rv3780	Rv3780	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.050	2.794

***Proteins that were up-regulated after 24 hours of exposure to 9.5 µg/ml of SMX**

Table 3.6: Proteins with that are deferentially regulated in SMX resistant clinical isolate upon exposure to SMX, classified according to biological function. Down 24hrs*

Rv number	Protein Name	Function	Functional Group	p-score	Fold change
Rv1408	rpe	PROBABLE RIBULOSE-PHOSPHATE 3-EPIMERASE RPE (PPE) (R5P3E) (PENTOSE-5-PHOSPHATE 3-EPIMERASE)	Intermediary metabolism and respiration	0.001	3.442
Rv3607c	folB	PROBABLE DIHYDRONEOPTERIN ALDOLASE FOLB (DHNA)	Intermediary metabolism and respiration	0.002	3.389
Rv0130	htdZ	PROBABLE 3-HYDROXYL-THIOESTER DEHYDRATASE	Intermediary metabolism and respiration	0.009	3.314
Rv2404c	lepA	PROBABLE GTP-BINDING PROTEIN LEPA (GTP-BINDING ELONGATION FACTOR)	Intermediary metabolism and respiration	0.005	3.305
Rv0604	lpqO	PROBABLE CONSERVED LIPOPROTEIN LPQO	Cell wall and cell processes	0.005019	3.292
Rv1676	Rv1676	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.006	3.282
Rv1480	Rv1480	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.007	3.249
Rv2495c	bkdC	PROBABLE BRANCHED-CHAIN KETO ACID DEHYDROGENASE E2 COMPONENT BKDC	Intermediary metabolism and	0.007	3.244

			respiration		
Rv2724c	fadE20	PROBABLE ACYL-CoA DEHYDROGENASE FADE20	lipid metabolism	0.008	3.220
Rv0119	fadD7	PROBABLE FATTY-ACID-CoA LIGASE FADD7 (FATTY-ACID-CoA SYNTHETASE) (FATTY-ACID-CoA SYNTHASE)	lipid metabolism	0.011	3.183
Rv3614c	espD	ESX-1 SECRETION-ASSOCIATED PROTEIN ESPD	Cell wall and cell processes	0.018	3.083
Rv0023	Rv0023	POSSIBLE TRANSCRIPTIONAL REGULATORY PROTEIN	Regulatory	0.020036	3.054
Rv0852	fadD16	POSSIBLE FATTY-ACID-CoA LIGASE FADD16 (FATTY-ACID-CoA SYNTHETASE) (FATTY-ACID-CoA SYNTHASE)	lipid metabolism	0.021	3.045
Rv1332	Rv1332	PROBABLE TRANSCRIPTIONAL REGULATORY PROTEIN	Regulatory	0.023	3.021
Rv3528c	Rv3528c	HYPOTHETICAL PROTEIN	Conserved hypothetical	0.0234	3.017
Rv3455c	truA	PROBABLE TRNA PSEUDOURIDINE SYNTHASE A TRUA (PSEUDOURIDYLATE SYNTHASE I) (PSEUDOURIDINE SYNTHASE I) (URACIL HYDROLYASE)	Information pathways	0.029	2.958
Rv2409c	Rv2409c	CONSERVED HYPOTHETICAL PROTEIN	Conserved hypothetical	0.031	2.938
Rv3058c	Rv3058c	POSSIBLE TRANSCRIPTIONAL REGULATORY PROTEIN (PROBABLY TETR-FAMILY)	Regulatory	0.035	2.907
Rv1187	rocA	PROBABLE PYRROLINE-5-CARBOXYLATE DEHYDROGENASE ROCA	Intermediary metabolism and respiration	0.035	2.904
Rv3302c	glpD2	PROBABLE GLYCEROL-3-PHOSPHATE DEHYDROGENASE GLPD2	Intermediary metabolism and respiration	0.037	2.891
Rv1011	ispE	PROBABLE 4-DIPHOSPHOCYTIDYL-2-C-METHYL-D-ERYTHRITOL KINASE ISPE (CMK) (4-(CYTIDINE-5'-DIPHOSPHO)-2-C-METHYL-D-ERYTHRITOL KINASE)	Intermediary metabolism and respiration	0.040	2.869
Rv0939	Rv0939	POSSIBLE BIFUNCTIONAL ENZYME: 2-HYDROXYHEPTA-2,4-DIENE-1,7-DIOATE ISOMERASE (HHDD ISOMERASE) + CYCLASE/DEHYDRASE	Intermediary metabolism and respiration	0.046	2.819
Rv3667	acs	ACETYL-COENZYME A SYNTHETASE ACS (ACETATE--CoA LIGASE) (ACETYL-CoA SYNTHETASE) (ACETYL-CoA SYNTHASE) (ACYL-ACTIVATING ENZYME) (ACETATE THIOKINASE) (ACETYL-ACTIVATING ENZYME) (ACETATE--COENZYME A LIGASE) (ACETYL-COENZYME A SYNTHASE)	lipid metabolism	0.049	2.798

***Proteins that were down-regulated after 24 hours of exposure to 9.5 µg/ml of SMX**

CHAPTER 4

Conclusion

The rise in drug resistance and the economic strain that many countries experience affects the control of tuberculosis. A new and less expensive drug would help in the eradication of the disease. The search for less expensive compounds has focussed attention on existing drugs that are used for other diseases other than tuberculosis or drugs that were once used for tuberculosis, but were abandoned due to the discovery of newer drugs. Sulfonamides are a class of antibiotics that are used effectively to treat many bacterial infections and were once used to treat tuberculosis, but were abandoned due to toxicity of the early derivatives.

In this study, we evaluated the effect of some of the newer, less toxic sulfonamides on the growth of *Mycobacterium tuberculosis* and also attempted to identify their mechanism of action. The *Mycobacterium tuberculosis* reference strains, H37Rv, CDC1551, *mshA* and an INH^R clinical isolate were used in this study for the evaluation of drug activity, drug interaction and identification of possible modes of action. *Mycobacterium bovis* (BCG) was also used in some parts where the use of *Mycobacterium tuberculosis* was deemed inconvenient, such as in the optimisation of a protocol, due to the non-pathogenic nature of the organism.

In this study, it was found that SMX, which is available in combination with TMP, is active against *Mycobacterium tuberculosis*. We also showed that it has a synergistic effect in combination with rifampicin, one of the key drugs in the current tuberculosis regimen. We then investigated the effect of SMX on drug resistant *Mycobacterium tuberculosis* clinical isolates. We found that a rifampicin resistant *Mycobacterium tuberculosis* strain was also susceptible to SMX, although the minimum inhibitory concentration was between 9.5 µg/ml and 19 µg/ml. We also found that two isoniazid resistant Mycobacterium tuberculosis strains were also

resistant to SMX with a minimum inhibitory concentration that was more than 19 µg/ml. We then attempted to uncover the underlying mechanisms that are involved in the cross-resistance. We sequenced the putative target of SMX (*folP1*) in these resistant strains and genes that may have a negative effect on the activity of this drug when they are mutated, such as *thyA*. We did not find any mutations in the genes that we sequenced (*folP1* and *thyA*) from an isoniazid mono-resistant strain of *Mycobacterium tuberculosis* that was also resistant to SMX. We also evaluated the expression levels of *folP1* in this strain and the expression of this gene was not increased upon exposure to the drug.

Efflux pump systems are one of the factors that have been reported to play a major role in drug resistance, conferring resistance to a number of structurally unrelated compounds (Webber and Piddock, 2003). We used efflux pump inhibitors in combination with SMX against the isoniazid mono-resistant *M. tuberculosis* strain that was also resistant to SMX. We were able to restore the susceptibility of this strain to SMX to the level that was similar to the reference strain. This data indicates the involvement of efflux pump systems in the cross resistance of the tested isoniazid resistant *M. tuberculosis* strain to SMX.

We also tried to measure folate levels in *Mycobacterium bovis* BCG treated with SMX in an attempt to verify that this drug also targets the folate pathway in mycobacteria, however this strategy proved unsuccessful, due to the instability of these metabolites and their very short half-life.

To further investigate the underlying mechanisms that are involved in the cross resistance of this strain and also try to identify other possible modes of action of SMX on *Mycobacterium tuberculosis,* we employed a global protein profiling approach. We treated an isoniazid mono resistant *M. tuberculosis* clinical isolate with a sub-lethal concentration of SMX and evaluated changes in protein expression using LC-MS/MS. Our data showed that *Mycobacterium tuberculosis* upon treatment with SMX induced over-expression of genes that are involved oxidative stress response and also down-regulation of genes that are involved in energy consuming pathways, such as fatty acid synthesis, proton translocation and replication in order to counter the effect of SMX.

These results are in agreement with the data obtained from using a strain of *Mycobacterium tuberculosis* that lacked mycothiol (*mshA*). We treated this strain with various concentration of SMX and compared it to the parental strain CDC1551 strain of *Mycobacterium tuberculosis*. We found that the *mshA* mutant strain was more susceptible to SMX than the wild-type strain, indicating that SMX produces oxidative stress (see figure 2.4).

This study has elucidated more aspects on the action of SMX and the possible application in tuberculosis treatment. TMP/SMX is a registered drug combination for other bacterial infections, is inexpensive and is widely available. Further studies, such as drug efficacy mouse studies will be undertaken before proceeding to clinical trials to clarify the potential of SMX and SMX/rifampicin in drug-susceptible tuberculosis.

CHAPTER 5

Materials and Methods

5.1 Mycobacterium tuberculosis strains

H37Rv (ATCC 27294) and CDC 1551 laboratory strains were used as references in this study.

Two strains of isoniazid mono-resistant strain (INH^R) were used in the study, one harboured mutation in the *KatG* gene +S/315ACC (R1129) and the other one in *inhA* promoter (R1845) and rifampicin mono-resistant (RIF^R) harboured a mutation in *rpoB* gene +S/531TTG (R5182) (see table 5.1). These clinical isolates were randomly selected from the *Mycobacterium tuberculosis* strain bank of our division.

The Beijing phenotype was chosen because of its association with drug resistance and its association with outbreaks (Glynn *et al.*, 2002; Buu *et al.*, 2012). Classification of these clinical isolates was based on IS6110 restriction fragment length polymorphism patterns (Mathema *et al.*, 2006). The drug resistance of these strains was tested by a routine laboratory (NHLS, green point, Cape Town) using the standard method on MGIT 960 TB system (Scarparo *et al.*, 2004).

The Mycothiol mutant stain Δ*mshA* and its parent strain CDC1551 were generously provided by Prof William R Jacobs, Jr (Howard Hughes Medical Institute, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA). Δ*mshA* was generated by specialized transduction with phAE222 (Vilcheze *et al.*, 2008).

Table 5.1: Genetic characteristics and drug susceptibility profile of the clinical isolates

Spoligotype	Spoligotype Family	Family	IS6110	Resistance	Mutation region
R1129	2Beijing	11	1038	Inh mono	katG +S/315ACC
R1845	2Beijing	29	7037	Inh mono	InhAProm +S/15
R5182	2Beijing			Rif mono	rpoB +S/531TTG

5.2 Bacterial culturing condition and stock preparation

(Refer to appendix for buffer and solution preparation).

All *M. tuberculosis* strains, (drug susceptible and drug resistant) were prepared by inoculating 1ml of 15 % glycerol frozen stock obtained from the division to 5ml of 7H9 Mycobacterial medium (Difco, Detroit, USA) supplemented with 0.2% (v/v) glycerol, 10%(v/v) OADC, and 0.025% (v/v) Tween 80 and incubated at 37^0C until the culture reached an optical density (OD_{600}) of 0.5. These cultures were then transferred to various 50ml cell culture flaks (CELL STAR®, fricknhause, Germany) containing 50 ml 7H9, such that the initial OD_{600} was 0.005 and incubated at 37^0C until the culture reached an OD_{600} 0.8. The cultures were then tested for contamination by ZN stain and blood agar.

Briefly, aliquots of cultures were heat-fixed to microscope slides at 100^0C for 2hrs. Slides were flooded with ZN Carbol Fuchsin (BD), heated with a flame until steaming, and allowed to stand for 5 minutes. Slides were rinsed with water, decolorized with 5% acid-alcohol (see appendix) solution for 2 minutes and rinsed with water. Slides were then counterstained with Methylene Blue (BD) for 1-2 minutes and rinsed with water and allowed to air dry. Slides were then observed using a light microscope under the 100X (oil immersion) magnification.

5.2.1 Blood agar

Following ZN staining, cultures were screened for contamination using blood agar (5% blood content, Diagnostic media products, NHLS). Briefly, aliquots of Mycobacterial cultures were spread on the blood agar plates. Plates were then incubated at 37^0C and were observed after two days. When the cultures were found to be clean with no contamination, they were frozen

in 1ml of 15% glycerol aliquots at -80^{0}C and these aliquots were used in future experimental procedures.

5.3 Compounds used in this study

Sulfamethoxazole, trimethoprim, Ethambutol (EMB), Para-aminosalicylic acid (PAS), 4-Aminophenyl sulfone, Thymidine, (±) - Verapamil hydrochloride, Reserpine and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) were purchased from Sigma-Aldrich, St. Louis, MO. Isoniazid (INH) and Pyrazinamide were purchased from BD (Becton, Dickinson and Company); Rifampicin (RIF) was obtained from Novartis. Griseofulvin tablets were kindly donated by Prof Mark Cotton.

TMP, SMX, RIF, PAS, *Griseofulvin,* CCCP, Verapamil and Reserpine were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to make stock concentrations and these compounds were serially diluted in sterile water to the desired concentrations. INH, EMB and Thymidine were dissolved in sterile water to make stock concentrations.

5.4 Drug susceptibility testing in BACTEC 460 TB system

The susceptibility of *M. tuberculosis* strains to a various drugs was evaluated using BACTEC 460 TB system as described by (Tortoli and Simonetti 1995). Briefly, an inoculum was prepared by inoculating 0.1ml from a frozen stock of a *M. tuberculosis* strain into a BACTEC vial and the vial was incubated at 37^{0}C. This vial was read in BACTEC 460 until it reached a Growth Index (GI) of 500. 0.1ml from this vial was inoculated into a new BACTEC vial containing 0.1ml of a drug tested for activity.

The control vials contained only drug solvent (DMSO or H_2O) and an inoculum of bacteria (the final concentration of DMSO in the growth medium was less than 2%) and a 1:100 dilution of the inoculum was added to a separate drug free vial (Lenaerts *et al.*, 2005). The GI of bacteria was monitored every 24hrs, until the ΔGI of the 1:100 culture was more than 30 (Siddiqi, 1989). The susceptibility of *M. tuberculosis* strains to a specific drug was interpreted using a standard method (Springer *et al.*, 2009). A strain was regarded as susceptible, when the GI of the drug-exposed culture at a specific concentration on the final day of the experiment (GI 1:100>30) was below or equal to the GI of the preceding day, indicating a reduction of metabolic activity in the test vial. The GI of the drug-exposed culture on the last day was also compared to the GI of the drug free vial containing undiluted culture on the same day and MIC was defined as minimum concentration of a drug that inhibited 90% of bacteria (Ollinger *et al.*, 2012).

5.4.1 Drug Interactions

The interactions between the compounds were evaluated at concentrations that were below their MICs or concentrations that inhibited less than 50% of the bacterial growth. Two drugs were combined in one tube to a total volume of 0.1ml to give a final concentration of each drug that is 1X, 2X or 3X less that the MIC of the respective drug. The 0.1ml of the combined drugs was then injected into the BACTEC vial containing 0.1ml of the inoculum to give a total of 4.2 ml in the vial. Vials were incubated at 37^0C and GI of bacteria was monitored every 24hrs, until the ΔGI of the 1:100 culture was more than 30.

The effect of the two drugs in combination was interpreted using a formula described by Hoffner and Källenius, (1987), in which synergism is defined as X/Y<1/Z, where X is the GI of the vial with two drugs on the final day; Y is the lowest GI of the single drug in the combination and

Z the number of the drugs in the combination. We used two drugs in a combination, therefore, Z=2. X/Y<0.5=synergy; X/Y>0.5 but <0.75=additive; XY/=1 no interaction and when X/Y>2=antagonism (Hoffner *et al.*, 1987).

5.4.2 Effect of Efflux inhibitors on the activity of SMX in INH^R

Role of efflux pumps on the cross resistance of isoniazid mono-resistant isolates to SMX was evaluated using efflux inhibitors. We used the commonly used inhibitors, verapamil, reserpine and CCCP at non lethal concentrations. The procedure in section 5.4.1 was followed substituting the first-line drugs with efflux inhibitors.

5.4.3 Statistical analysis

The data of *M. tuberculosis* susceptibility to SMX or other drugs and drug interaction in this study is presented as the mean of two or three separate experiments and the data was analyzed using prism GraphPad Prism®, prism 5, version 5.01, August 7, 2007

5.5 Extraction of Genomic DNA (gDNA)

Genomic DNA was extracted and purified from isoniazid mono-resistant clinical isolates (Inh^R) obtained from the Division of Molecular Biology and Human Genetics using our lab protocol. Briefly, 300 µl Inh^R *M. tuberculosis* culture was spread on two 7H11 plates (9 cm diameter) and incubated at 37^0C for a month. The cultures were then heat inactivated by incubating the plates at 80^0C for 1hr and samples were allowed to cool down for 20mins. A 50ml polypropylene tube was filled with approximately 20ml of glass beads (5mm Dia) and 7ml of extraction buffer (50mM TRIS-HCL pH7.4, 25mM EDTA and 5% Monosodium glutamate) was added to it.

Colonies were carefully scraped from the plates and re-suspended in the 50ml tube containing the buffer and the beads. The tube was then vortexed vigorously for 2 mins and 500µl of lysozyme (Roche) with a final concentration of 100mg/ml was added. The vortexed tube was incubated for 2hrs at 37^0C and gently mixed every 30mins and then 650 µl of 10x Proteinase K buffer and 300 µl of Proteinase K (10mg/ml) were added to the mixture, mixed and the incubated overnight at 45^0C. To the overnight mixture, 5ml of phenol/chloroform/isoamylalcohol (25:24:1) was added and the mixture was incubated for 2hrs at room temperature with gentle mixing every 30mins. The mixture was then centrifuged at 2500 xg for 20 mins at room temperature.

The top liquid phase was removed and transferred to a new tube containing 5ml of chloroform/isoamylacohol (24:1) and mixed by inverting and centrifuged as the above step. The top liquid phase was again transferred into a new tube containing 600 µl of 3M sodium-acetate at pH 5.2 and mixed well. Ice-cold isopropanol (7ml) was then added to the mixture and slowly inverted back and forth until DNA became visible.

The precipitated DNA was fished out immediately with a thin glass rod and the rod was placed into a 1.5ml eppendoeff® tube containing 1ml of 70% ethanol for 10mins. The rod was then placed in an empty 1.5ml tube at room temperature until DNA was air dried. DNA was re-hydrated by adding 300 µl of TE buffer at pH 8.0 and the DNA was stored at -20^0C for future use.

5.5.1 PCR amplification of target genes from gDNA

Target genes were PCR-amplified from the purified DNA with the primers listed in table 2. These primers were designed using sequences of *Mycobacterium tuberculosis* obtained from Tuberculist (TubercuList Web Server, 2013) using a web based program, Primer3 (version. 0.4.0). Briefly, a master mix was made from the PCR reagents (Qiagen) as follows; 5 µl (n) 2x Q buffer, 2.5 µl (n) 10x Buffer, 2 µl (n) $MgCl_2$, 2 µl (n) dNTPs, forward and reverse primer (table 5.2) to a final concentration of 0.5 µM and 0.25 µl (n) Hot Star Taq polymerase, where n is total number of reactions. PCR conditions were as follows; 15 min activation at 95^0C followed by 35 amplification cycles and each cycle conditions as follows; 94^0C for 1 min denaturation, 60^0C for 1 min annealing, 1 min extension at 72^0 C and the final elongation step of 10 min at 72^0C in a thermal cycler (Gene Amp® PCR system 9700, Applied Bio-systems). PCR amplification was confirmed by 1% agarose gel electrophoresis stained with ethidium bromide separated at 100 Volts for 1 hour. The PCR amplicons obtained were sequenced at the Central Analytic Facility of Stellenbosch University using primers in table 5.2.

Table 5.2. Primers used for sequencing.

Gene	Forward (5´-3´)	Reverse(5´-3´)	Product Size(bp)
thyA	gcctccgttgtactcctgtg	gcctgtatcacttgcccatt	427
	aatcggtagcctacgagctg	ctgcgttggtagagctgaca	343
	tccatccggtgagcacat	acacgcgtcactccttgatt	550
folP1	gcggactgttcaaaaccaat	cgtcgagatcgagataacacc	149
	gcggactgttcaaaaccaat	cttgtgctgcaagctctttg	299
	gcgggtgttatctcgatctc	caattatgttgcgccgtctt	505
	gcccagatggtcaacgac	catcgcgttctatcctttcc	538

5.6 Gene expression of inh^R *M. tuberculosis* exposed to SMX

5.6.1 Growth conditions and drug treatment

The bacterial growth conditions and drug treatment procedures were designed to match those of Betts *et al.,* (2003) with the following modifications; 1ml of frozen stock of *M. tuberculosis* strain was grown in 50ml of 7H9 Mycobacterial medium supplemented with 0.2% (v/v) glycerol, 10%(v/v) OADC, and 0.025% (v/v) Tween 80 at 37°C to an optical density (OD_{600}) of 0.8 and then a 1:100 dilution of the culture was inoculated in 120ml of the same medium in roller bottles (Betts *et al.*, 2003).

The cultures were then placed in a roller culture apparatus (Wheaton) and rolled at 2rpm at 37°C until they reached an OD_{600} of 0.3. Cultures were then incubated overnight at 37°C standing. 30ml of the culture was transferred to 125ml sterile storage bottles (corning) and treated with 10µl Dimethyl Sulfoxide (DMSO) and incubated at 37°C while slowly stirring with a sterile stirrer bar. The remaining 90ml in the roller bottle was then treated with 30µl of SMX to give a final concentration of 2µg/ml and was also incubated at 37°C while agitated on a roller machine.

5.6.2 RNA extraction and cDNA synthesis

RNA was extracted with the Trizol™ (Invitrogen) extraction method as described by Harper and colleagues briefly; Cells from the 30ml control cultures were harvested at time zero by centrifugation at 18626×g for 10 minutes (Harper *et al.*, 2010). In drug treated cultures, cells

were harvested as above after 30 minutes, 1hr and 4hrs of treatment. Cells were suspended in 1ml Trizol in 2ml screw-cap cell ribolysation tubes containing 0.5mm glass beads and ribolysed (Fastprep FP120, Bio101 Savant) at the speed of 6m/s for 20 seconds and ribolysation was repeated 3 times with cooling in-between on ice for 1 minute.

The cell lysate was then allowed to incubate at room temperature for 5 minutes. Ribolysed cells were centrifuged at 18626×g for 10 min at 4°C. The supernatant containing nucleic acids was transferred to a 2ml tube. 200µl of absolute chloroform was added to the ribonucleic acid containing fluid and was mixed rapidly for the first 20 seconds and then inverted periodically for an additional 2-3 minutes at room temperature. The mixture was then centrifuged 18626×g for 10 min at 4°C. The clear aqueous phase was transferred to a new 1.5ml tube and 750µl of absolute ethanol was added to the 1.5ml tube. Nucleic acids were allowed to precipitate overnight at -20°C. Precipitated nucleic acids were collected by centrifugation 18626×g for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 1ml of 75% ethanol. Samples were then centrifuged at 18626×g for 5 min at 4°C. The supernatant was discarded and the pellet allowed to air dry for 10 minutes.

Ribonucleic acids were dissolved in 50µl RNase–free water (Ambion™). Agarose gel was used to check for DNA contamination, 1% gel was prepared and 4µl ethidium-bromide (see appendix) was added to it. 5 µl of MassRuler DNA Ladder (Fermentas) was run along with the samples. 3 µl of loading buffer (fermentas) plus 2 µl of a sample were loaded in the wells for separation in SB running buffer. Furthermore, PCR using 16S primers (table 3), was also performed to identify contaminating DNA, (see section 6.3 for PCR procedures).

Any DNA contamination was removed by TURBO DNA-free™ kit (Ambion) with the following modifications; 15µl of total RNA was added to a new RNase-free 1.5ml tube, 2µl of TURBO DNase and 2µl of 10X TURBO DNase Buffer was added to the tube and incubated for 1hr at 37°C. RNase free water was added to the DNase treated RNA to a final volume of 200µl and an equal volume of cold Phenol: chloroform (4:1) was added, mixed and stored in ice for 10min.

The mixture was centrifuged at 13400×g for 10min at room temperature and 150µl of the top aqueous layer was transferred to a new tube. 0.1 volumes of cold RNase-free sodium acetate pH 5.2 and 2.5 volumes of cold RNase-free 100% ethanol was added to the tube and incubated at 4^0C for 1hr. RNA was collected by centrifugation at 13400×g for 30min at 4^0C.

The total RNA was washed with 1ml of cold RNase-free 70% ethanol and centrifuged at 13400×g for 10min at 4°C. Ethanol was aspirated and the pellet was air-dried for 20min and re-dissolved in 20µl RNase free water. Following phenol precipitation, PCR was again performed to detect DNA contamination using 16S primers (table 3).

The quality and the quantity of the total RNA was determined by Experion™ (Bio-RAD), according to the manufacturer's instructions. cDNA was synthesized from 0.5µg of the extracted total RNA using Quantitect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

5.6.3 PCR

Qiagen PCR kit with HotStar *Taq* polymerase was used in all PCR reactions according to manufacturer's instructions with the following amplification parameters; 15min activation step

at 95°C for 15 minutes followed by 35 to 40 cycles of (i) a 30sec denaturation step at 72°C (ii) a 30 sec annealing step at the required annealing temperature (Ta) of the specific set of primers and (iii) an elongation or synthesis step at 72°C for one minute. A final elongation step at 72°C for 10min. In all PCR reactions, a negative control (No templet) and positive controls (genomic DNA) were included. Products of the PCR reaction were analyzed with 1% agarose gel electrophoresis.

5.6.4 Real-Time RT-PCR

Real time PCR was performed in a LightCycler version 1.5 (Roche) using 20µl capillaries (Roche). A 10µl reaction mixture was prepared, which included 1:10 diluted cDNA, 0.5µM of each primer (listed in table 3) and LightCycler Master^{Plus} SYBR Green PCR master mix (Roche). PCR parameters; (1) Activation at 95°C for 15 min (2) Amplification 40 cycles, Quantification analysis mode (95°CI15s, 60°CI30s, 72°CI06s) with a single fluorescence measurement (3) Melting curve analysis, 60-99°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement (4) Cooling at 40°C.

RNA template and non template controls were included to determine DNA contamination in the samples. PCR efficiency for each transcript was calculated from serially diluted H37Rv genomic DNA. In order to determine the relative transcript levels in the samples, the statistical programme REST-384 Version 1 was used to evaluate relative quantification of gene expression (Pfaffl *et al.*, 2002). The expression levels of the target genes were normalized to 16s which is the internal standard (Eleaume and Jabbouri, 2004).

Table3. Primers for gene expression

Gene	Forward (5'-3')	Reverse(5'-3')	Product size(bp)	Annealing Tem(°C)
FolP1	cgtcacggacgactctttct	cttgtgctgcaagctctttg	200	56.7
16s	tcatgttgccagcacgtaat	ctggacataaggggcatgat	102	55

5.7 Measurement of folate in *Mycobacterium tuberculosis* treated with SMX

5.7.1 Bacterial culturing conditions

M.bovis BCG was used as model for *M. tuberculosis* to optimize our protocols for the assessment folate levels in response to the treatment of SMX. Folate was extracted from *in vitro* growing BCG under the stress of SMX using the following two methods. The first one was adapted from Steenkamp & Vogt and the second method was adapted from Lu and colleagues (Lu *et al.,* 2007; Steenkamp and Vogt, 2004). All the standards and the reagents used in this part of the study were purchased from Sigma-Aldrich (St. Louis, MO).

5.7.2 First method

BCG was grown in 200ml of 7H9 to an OD_{600} of 0.6 and the culture was split into four 50ml tubes and the tubes were centrifuged at 2755×g for 15mins at 4^0C. Supernatant was discarded and the pellets from the four tubes were combined in one 50ml tube (total mass of wet cells ≈ 1g). The pellet was re-suspended in 875 µl of 5.7mM EDTA, 625 µl of 1M perchloric acid and

1000 µl of acetonitrile. The solution was sonicated (Amplitude 30 for 20 seconds, 6 times with 1min cooling intervals) and the lysate was centrifuged at 2755×g for 15mins at 4^0C. pH was adjusted to 8-9 using potassium carbonate and the solution was centrifuged for 15mins at 4^0C at 2755×g. The supernatant was freeze dried and sent for analysis by the Central Analytical Facility, Mass Spectrometry Unit.

5.7.3 Second method

BCG was grown in 300ml of 7H9 to an OD_{600} of 0.6 and the culture was also split into four 50ml tubes and the tubes were centrifuged at 2755×g for 15mins at 4^0C. Supernatant was discarded and the pellet in each 50ml was re-suspended into 300 µl of 80:20 Methanol: Water + 0.1% ascorbic acid + 20mM ammonium acetate Solution (-75^0C cold and prepared fresh daily). The solution was kept in ice for 15min.

The solution was then centrifuged at 2755×g for 5min at 4^0C and the supernatant was transferred to a 1.5ml tube and was taken as the first extraction. The pellet was then re-suspended in 200 µl of the solution and sonicated (QSONICA) (Amplitude 30 for 20 seconds, 6 times with 1min cooling intervals). The lysate was centrifuged at 18626×g for 5min at 4^0c and supernatant was transferred to a 1.5ml tube and was taken as the second extraction. The extracts were then lyophilized and sent for analysis by the Central Analytical Facility, Mass Spectrometry Unit.

5.7.4 Analysis

For the quantification of folate levels in BCG cultures treated with SMX and untreated cultures, dihydrofolate, tetrahydrofolate and Para-Aminobenzoic Acid (PABA) were used as standards

and LC-MS analysis was performed by the Central Analytical Facility with the following settings; The lyophilized material was re-dissolved in a solution containing 25% acetonitrile and 0.05% formic acid and analyzed by ultraperformance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). UPLC-ESI-MS/MS analysis was performed with a Waters Acquity UPLC system coupled to a Waters Xevo TQ MS system (Waters Corporation, Milford, MA, USA). Compounds (THF, DHF and PABA) were separated on a Waters Acquity BEH phenyl column (100 by 2.1 mm; 1.7 µm) at 50°C using a 1% formic acid (in water) (solvent A)–acetonitrile (solvent B) gradient, starting with 100% solvent A for 0.1 min at a flow rate of 0.3 ml/min. The acetonitrile concentration was increased linearly to 40% over 3.5 min at a flow rate of 0.3ml/min and then increased to 95% over 5.1 min at a flow rate of 0.5 ml/min and maintained for 0.2 min. The column was re-equilibrated for 2 min (the total run time was 8 min). Compounds were analyzed in the ESI-positive mode, and the multiple-reaction-monitoring (MRM) transition *m/z* 446.1 > 120.2 (cone voltage = 15 V; collision energy = 30 V) for THF, *m/z* 444.1 > 178.1(cone voltage = 15 V; collision energy = 15 V) for DHF and *m/z* 138.0 > 65.1 for PABA (cone voltage = 30 V; collision energy = 25 V). The source capillary was at 3.5 kV. The source and desolvation temperatures were 140°C and 400°C, respectively and standard curves were generated for quantification of these compounds.

5.8 Proteomics

5.8.1 Culturing conditions and drug treatment of Isoniazid mono-resistant clinical isolate (R1129)

Isoniazid mono-resistant clinical isolate (R1129) was grown in eight 50ml flasks with 7H9 liquid medium enriched with DC (Glucose and catalase) to an OD_{600}≈ 0.6. The cultures were mixed together to make a homogenous culture and then split into four 50ml cultures. Four 50ml cultures were treated with 20µl of SMX each in order to give final concentration of 9.5µg/ml SMX in the flask and the other four was treated with DMSO. Cultures were harvested at time zero and 24 hours by centrifugation as follows: Briefly, each 50ml liquid culture was aliquoted into two 25ml aliquots in 50ml conical tubes (SPL Lifesciences) and centrifuged at 2500g for 10min at 4°C.

Supernatant was frozen at -80^{0}C for future use which contained culture secretion proteins. Cells were washed with 1ml extraction buffer (100µl of a Tris-HCL 1M, pH 7.4), 200µl Protease inhibitor (Roche), 3µl Tween 80 and H20 to a final volume 10ml and transferred to 2ml screw cap tubes (Quality Scientific Plastics). The suspension was centrifuged at 14000g for 4min and kept on Ice for 1min and centrifuged again for 2min and kept on ice for 1min. The supernatant was removed and cells were kept. An Equal amount of glass beads (0.1mm), 300µl extraction buffer and 10µl DNase were added to the cells and the mixture was rybolised (Fastprep FP120, Bio101 Savant) at 4 m/s for 20sec for 6 intervals and tubes were kept on ice after every run. The lysate was centrifuged at 14000g for 2 min and the tubes were kept on ice for 0.5min for two intervals.

Only the supernatant was kept (contains cellular proteins). 20µl of extraction buffer was filtered through the filter (0.22 µm, 33mm Low Protein Binding Durapore® Membrane, Millex®-GV) using a syringe in order to prepare the filter before using (because if it is dry, you will lose your proteins). The whole-cell lysate (supernatant) was filter sterilized through the prepared filter.

5.8.2 Protein quantification

Protein concentrations were determined spectrophotometrically using RCDC Protein assay (Bio-Rad Laboratories). Briefly; 2mg/ml of Bovine Serum Albumin (BSA) working stock was prepared by adding 80 µl of extraction buffer to 20 µl of 10mg/ml BSA stock. The concentrations of BSA used to generate standard curve are listed in table 4.

Table 4: BSA standards for the standard curve

Standard concentration	µl for 2mg/ml BSA stock	µl for Protein extraction buffer
0	0	25
0.2	2.5	22.5
0.5	6.25	18.75
1.0	12.5	12.5
1.5	18.75	6.25
2.0	25	0

Samples were diluted by 1:5 by taking 5 µl of a sample together with 20 µl of protein extraction buffer. From the kit, the following buffers were prepared according to manufacturer's instructions. Buffer A+S was prepared by adding 2000 µl buffer A and 5 µl of buffer S and this buffer was enough for 16 samples including BSA standards. 125 µl RC Reagent I was added into each sample tube, vortexed and incubated for 1 minute at room temperature. 125 µl RC

Reagent II was then added into each tube, vortexed and tubes were centrifuged at room temperature at 18626×g for 5 minutes.

The supernatant was discarded by inverting the tubes on clean absorbent tissue paper and liquid was allowed to drain completely from tubes. 127 µl of Reagent A was added (prepared from Reagents S and A) to each tube, vortexed and the tubes were incubated at room temperature for 5 minutes, (or until precipitate was completely dissolved). Tubes were vortexed before proceeding to next step. One ml of DC reagent B was added to each tube, vortexed immediately and the tubes were then incubated at room temperature for 15 minutes. After 15 minutes incubation, absorbance was read at OD=595 nm. A standard curve was drawn on Excel as linear regression to determine protein sample concentrations.

5.8.3. SDS Polyacrylamide Gel Electrophoresis

5.8.3.1 Preparation of the Samples

Loading buffer (4X) from Bio-Rad was added to the samples such that the final concentration of the buffer in the sample is 1X. Loading buffer and protein samples were vortexed and the tubes were placed in a 95^0C heating block for 5min. Samples where then centrifuged at 18626×g for 5 seconds and loaded on the gel along with PageRuler™ Prestained Protein Ladder (Thermo Scientific).

5.8.3.2 Protein separation

Criterion™ XT precast gels (4-12% Bis-Tris, 12+2 well comb, 45 µl, 1.0 mm, Bio-Rab were used for the separation of the intracellular proteins. Proteins were separated at 150V (Voltage) until the dye front reached the end of the gel. XT MOPS (Bio-Rad) running buffer was used.

5.8.3.3 Staining of the gels

Gels were stained with Coomassie blue for two hours with gentle shaking at room temperature. The stain was prepared as follows; 2.5 g Coomassie Brilliant blue, 450ml absolute methanol, 100ml of glacial acetic acid and 250ml of water.

8.3.4 De-staining of the gels

Gels were de-stained with the de-staining solution overnight, which was prepared as follows; 100ml 100% Glycerol, 375ml glacial acetic acid, 250ml absolute Methanol and volume was made up to 5L with sterile water.

5.8.4 Protein Identification

LC-MS/MS analysis was carried out by Dr S. Smit on the LTQ Orbitrap Velos (Thermo Scientific) at the proteomic reach group at the Central Analytical Facility, Stellenbosch University.

5.8.4.1 In-gel trypsin digestion

The gel pieces were cut into smaller cubes and washed twice with water followed by 50% (v/v) acetonitrile for 10 min. The acetonitrile was replaced with 50 mM ammonium bicarbonate and incubated for 10 min, and repeated two more times. All the gel pieces were then incubated in 100% acetonitrile until they turned white, after which the gel pieces were dried *in vacuo*. Proteins were reduced with 10 mM DTT for 1 h at 57 °C. This was followed by brief washing steps of ammonium bicarbonate followed by 50% acetonitrile before proteins were alkylated with 55 mM iodoacetamide for 1 h in the dark.

Following alkylation the gel pieces were washed with ammonium bicarbonate for 10 min followed by 50% acetonitrile for 20 min, before being dried *in vacuo.* The gel pieces were digested with 100 µl of a 10 ng/µl trypsin solution at 37^{o}C overnight. The resulting peptides

were extracted twice with 70% acetonitrile in 0.1% trifluoroacetic acid for 30 min and then 100% acetonitrile for 30 min, and then dried. The dried peptides were then dissolved in 5% formic acid and cleaned using Stage Tips (Thermo Scientific) according to the instructions. The peptides were again dried and stored at -20^{o}C. Dried peptides were dissolved in 5% acetonitrile in 0.1% formic acid and 10 µl injections were made for nano-LC chromatography.

5.8.4.2 Mass spectrometry

All experiments were performed on a Thermo Scientific EASY-nLC II connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nano-electropsray source. For liquid chromatography, separation was performed on a EASY-Column (2 cm, ID 100µm, 5 µm, C18) pre-column followed by XBridge BEH130 NanoEase column (15 cm, ID 75 µm, 3.5 µm, C18) column with a flow rate of 300 nl/min. The gradient =7used was from 5-17 % B in 5 min, 17-25% B in 90 min, 25-60% B in 10 min, 60-80% B in 5 min and kept at 80% B for 10 min. Solvent A was 100% water in 0.1 % formic acid, and solvent B was 100 % acetonitrile in 0.1% formic acid.

The mass spectrometer was operated in data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Data were acquired using the Xcaliber software package. The precursor ion scan MS spectra (*m/z* 400 – 2000) were acquired in the Orbitrap with resolution R = 60000 with the number of accumulated ions being 1 x 106. The 20 most intense ions were isolated and fragmented in linear ion trap (number of accumulated ions 1.5 x 104 using collision induced dissociation.

The lock mass option (polydimethylcyclosiloxane; *m/z* 445.120025) enabled accurate mass measurement in both the MS and MS/MS modes. In data-dependent LC-MS/MS experiments,

dynamic exclusion was used with 60 s exclusion duration. Mass spectrometry conditions were 1.8 kV, capillary temperature of 250 °C, with no sheath and auxiliary gas flow. The ion selection threshold was 500 counts for MS/MS and an activation Q-value of 0.25 and activation time of 10 ms were also applied for MS/MS.

5.8.4.3 Data analysis

MaxQuant 1.2.2.5 was used to identify proteins via automated database of all tandem mass spectra against the TBDB H37 July 2013 database. Carbamidomethyl cysteine was set as fixed modification, and oxidized methionine, N-acetylation and deamidation (NQ), Pyro-Gln, Pyro-Glu was used as variable modifications. The precursor mass tolerance was set to 20 ppm, and fragment mass tolerance set to 0.8 Da.

Two missed tryptic cleavages were allowed. Proteins were considered positively identified when they were identified with at least 1 tryptic peptide per protein, and protein and peptide FDR of 0.01. Statistical analysis was done using Perseus. Data were transformed (log 2) and imputated (width 0.3, Down shift 1.8) to replace missing values. ANOVA testing was performed using a p-value with threshold of 0.05. Z-scoring normalisations were also done.

APPENDIX

6.1) Culture media

6.1.1) DC mycobacterial liquid culturing media supplement:

10g Glucose, 0.75ml Catalase and the mixture was dissolved in 500ml double distilled water. The solution was sterilised by it passing through a 0.22 micron filter. The solution was aliquoted into 50ml tubes and stored at 4^0C for 1 month.

6.2) Buffers and solutions

6.21) 1M Tris-HCl buffer:

12.114g Tris (Sigma) was dissolved in 80mL water. Adjust pH to the desired value by adding concentrated HCl. The final volume was adjusted to 100ml with water and sterilized by autoclaving.

6.2.2) 0.5M EDTA:

9.306g was dissolved in 50ml water

6.2.3) DNA extraction buffer:

(5% sodium glutamate, 50 mM Tris-HCl [pH 7.4], and 25 mM EDTA)

6.2.4) 10X proteinase K buffer:

(5% sodium dodecyl sulfate, 100 mM Tris-HCl [pH 7.8], 50mM EDTA)

6.2.5) Phenol-chloroform-isoamyl alcohol (25/24/1):

400ml phenol, 384ml chloroform, 16ml isoamyl alcohol

6.2.6) Chloroform-isoamyl alcohol (24/1):

384ml chloroform, 16ml isoamyl alcohol

6.2.7) 3 M sodium acetate (pH 5.2) (500ml):

204.1g NaOAc was dissolved in 400mL water. Adjust pH to the desired value by adding concentrated glacial acetic acid. The final volume was adjusted to 500ml with water and stored at room temperature.**TE:** (10 mM Tris-HCl [pH 8.0], 1 mM EDTA)

6.2.8) Agarose gel: (1X Tris-borate-EDTA, pH 8.3)

All agarose gels were prepared using 1X TE buffer and was also used as a running buffer

6.2.9) 20× SB Buffer:

19.1g di-Sodium tetraborate decahydrate dissolved in 500ml water.

6.2.10) 10% SDS: 10g in 100ml distilled water:

A 10% SDS stock solution (Sigma) was diluted to the desired working solution.

6.2.11) 15% glycerol (for bacterial stock):

7.5ml of 100% glycerol into 42.5ml of bacterial culture.

6.2.12) 5% Acid alcohol (ZN-staining):

5% HCl in 100% methanol

6.2.13) Folat extraction buffer (100ml):

80:20 Methanol: Water + 0.1% ascorbic acid + 20mM ammonium acetate Solution

0.2g Ammonium acetate

0.1g Ascorbic acid

The solution was made fresh every day and stored at -80^{0}C

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Sulfamethoxazole enhances the antimycobacterial activity of rifampicin

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Objectives: To investigate the effect of trimethoprim/sulfamethoxazole on the survival of *Mycobacterium tuberculosis* and trimethoprim and sulfamethoxazole individually and combined with the first-line tuberculosis drugs (isoniazid, rifampicin and ethambutol).

Methods: *M. tuberculosis* strains were exposed to either trimethoprim/sulfamethoxazole combination or sulfamethoxazole and trimethoprim alone at various concentrations. The strains were also exposed to sulfamethoxazole in combination with existing antibiotics to assess the combined effect on the growth of *M. tuberculosis* in the BACTEC 460TB system. The effect of the drugs was compared with vehicle-treated controls. Drug interactions were interpreted using quotient values obtained from the growth index of cultures treated with a single drug or the combination.

Results: Trimethoprim showed a negligible effect on the growth of *M. tuberculosis* while sulfamethoxazole inhibited 80% of the growth of *M. tuberculosis* at 4.75 mg/L. There was no synergistic activity between sulfamethoxazole and trimethoprim, although an additive effect was observed. A statistically significant synergistic effect was observed between sulfamethoxazole and rifampicin. Sulfamethoxazole also had an additive effect with ethambutol, but there was no interaction with isoniazid.

Conclusions: Sulfamethoxazole is the main active compound against *M. tuberculosis* in the combination trimethoprim/sulfamethoxazole and has a synergistic effect with rifampicin. These findings suggest that sulfamethoxazole has potential in the multidrug regimen against *M. tuberculosis*.

Keywords: trimethoprim, isoniazid, ethambutol, synergy

Introduction

The emergence of extensively drug-resistant *Mycobacterium tuberculosis* strains (although quite rare in some parts of the world) is a medical and public health concern as the inexpensive and easily administered first-line drugs lose efficacy. Therefore, there is a need for new drugs and drug combinations. While new drugs are being sought, it is important to re-examine available, registered and inexpensive compounds for their potential role as part of antituberculosis regimens.

Trimethoprim/sulfamethoxazole is an old drug combination used in the last few decades for treating various bacterial infections, such as urinary tract infection.[1] More recently, however, trimethoprim/sulfamethoxazole has been used extensively in HIV-infected patients for the prevention and treatment of *Pneumocystis jirovecii* and *Toxoplasma gondii* infections.[2] Trimethoprim inhibits dihydrofolate reductase, thereby blocking the reduction of dihydrofolate to tetrahydrofolate. Sulfamethoxazole is the structural analogue of *para*-aminobenzoic acid (PABA) and inhibits dihydropteroate synthetase, a key enzyme in folate biosynthesis, encoded by *folP1*.[3] A recent study reported that a patient, initially thought to have nocardiosis, improved on trimethoprim/sulfamethoxazole, but later was found to have had tuberculosis without nocardiosis or any other infection. This study suggested that *M. tuberculosis* was susceptible to the combination of sulfamethoxazole and trimethoprim.[4]

Sulfonamides were used for the treatment of tuberculosis in the 1940s, but toxicity from early sulfonamides and the fact

that the newer antimycobacterial compounds were much safer and more effective resulted in sulfonamides being abandoned for the treatment of tuberculosis.[4] With the new knowledge about trimethoprim/sulfamethoxazole and the fact that this combination is well tolerated, this study presents the investigation of the antimycobacterial activity of trimethoprim/sulfamethoxazole. The combined effect of sulfamethoxazole with trimethoprim or with the first-line antituberculosis drugs isoniazid, rifampicin and ethambutol against *M. tuberculosis* was also studied.

Materials and methods

Ethics approval for this study was obtained from the Health Research Ethics Committee of Stellenbosch University (reference no. N11/07/230).

Reagents and antibiotics

Drugs were purchased from Sigma-Aldrich (St Louis, MO, USA). Stock solutions of 76 000 mg/L sulfamethoxazole, 40 mg/L trimethoprim and 332 mg/L rifampicin were prepared in 100% sterile DMSO. Stock solutions of 332 mg/L isoniazid and 200 mg/L ethambutol were prepared in deionized water and filter sterilized with a 0.2 μm Supor® membrane Acrodisc® syringe filter (Pall Corporation, USA). All stock solutions were prepared under sterile conditions and stored at −20°C.

M. tuberculosis strains

The reference strain of *M. tuberculosis* H37Rv (ATCC 27294) was used for drug evaluation. The H37Rv strain was cultured at 37°C in 7H9 Middlebrook medium supplemented with 10% (v/v) oleic acid/albumin/dextrose/catalase (OADC) (Becton Dickinson, Sparks, MD, USA) and 0.025% (v/v) Tween 80 to an optical density (OD_{600}) of 0.7. Ziehl–Neelsen staining and blood agar cultures were performed to control for contamination of the bacterial stocks. Bacterial stocks were stored at −80°C until further use.

Inoculum preparation

A volume of 0.1 mL of each *M. tuberculosis* frozen stock was inoculated in BACTEC 12B medium (Becton Dickinson). The cultures were incubated at 37°C and the growth monitored daily in a BACTEC 460TB system (Becton Dickinson) to establish a working culture. The growth was monitored until the culture reached a maximum growth index (GI) of 999. A volume of 0.1 mL of the working culture was subsequently inoculated into a new vial containing BACTEC 12B medium and grown to a GI of 500 (GI_{500}). This culture was used for susceptibility testing and synergistic effect determinations.

Drug susceptibility testing

The MICs of trimethoprim/sulfamethoxazole, sulfamethoxazole, trimethoprim, isoniazid, rifampicin and ethambutol for *M. tuberculosis* H37Rv were determined using the BACTEC 460TB system as described by Tortoli *et al.*[5] Briefly, 0.1 mL from a culture at GI_{500} was inoculated into BACTEC vials containing a drug at the required concentration. The control vials contained a drug solvent with undiluted bacterial inoculum and a 1:100 diluted bacterial inoculum. The cultures were monitored daily until the GI of the 1:100 control culture was ≥30.[6]

M. tuberculosis strains were categorized as susceptible to a specific drug when the GI of the drug-exposed culture at a specific concentration on the final day of the experiment (GI 1:100 > 30) was below or equal to the GI of the preceding day, indicating a reduction of metabolic activity in the test vial. The GI of the drug-exposed culture on the final day was also compared with the GI of the drug-free vial containing undiluted inoculum on the same day and MIC was defined as the minimum concentration of a drug that inhibited 99% of bacteria.

Sulfamethoxazole in combination with trimethoprim and the first-line antituberculosis drugs isoniazid, rifampicin and ethambutol

The drug–drug interaction of sulfamethoxazole in combination with isoniazid, rifampicin and ethambutol was assessed by evaluating drug combinations that were two to four times less than the MICs of the individual drugs and for sulfamethoxazole and trimethoprim in a 19:1 ratio. Growth was monitored daily in the BACTEC 460TB system, as described earlier.

Synergism

The effect of the drug combinations was interpreted using a formula in which synergism is defined as $x/y < 1/z$, where x is the GI of the vial with two drugs on the final day (day 5), y is the lowest GI of the single drug in the combination and z is the number of drugs in the combination. In this instance, two drugs were used in a combination, hence $z=2$. Therefore, $x/y < 0.5$ = synergy, $x/y \geq 0.5$ and < 0.75 = additive, $x/y \geq 0.75$ and < 2 = no interaction, and $x/y \geq 2$ = antagonism.[7]

Results

Sulfamethoxazole and trimethoprim using a fixed ratio of 19 sulfamethoxazole to 1 trimethoprim[8] displayed antimycobacterial activity. In order to determine which of the two drugs was responsible for the observed antimycobacterial effect, the individual drugs were tested for growth inhibition of *M. tuberculosis*. The antimycobacterial activities of these compounds on *M. tuberculosis* strain H37Rv are shown in Figure 1. Trimethoprim (Figure 1a) had negligible activity against *M. tuberculosis*, showing only 22% growth inhibition at 76 mg/L, doubling to 44% at 152 mg/L. In contrast, sulfamethoxazole (Figure 1b) showed 93% growth inhibition at 76 mg/L and 95% growth inhibition at 152 mg/L. At 9.5 mg/L, sulfamethoxazole still showed 90% growth inhibition, which was determined as the MIC of sulfamethoxazole for *M. tuberculosis* (Figure 1).

Combined effect of sulfamethoxazole and trimethoprim on H37Rv

Table 1 lists the quotient values for the combination of sulfamethoxazole and trimethoprim. The drug–drug interaction was assessed at concentrations that were below the cytotoxic level in a 19:1 ratio.[9] There was no synergistic killing between the two compounds. However, an additive effect was observed between 9.5 mg/L sulfamethoxazole (MIC) and 0.5 mg/L trimethoprim.

Combined effect of sulfamethoxazole with first-line drugs on H37Rv

The MICs of the compounds for the laboratory strain H37Rv were determined using the BACTEC 460TB system and Table 2 lists the MIC of each drug. A concentration below the MIC of sulfamethoxazole (2 mg/L, approximately five times less than the MIC) was tested in combination with the first-line

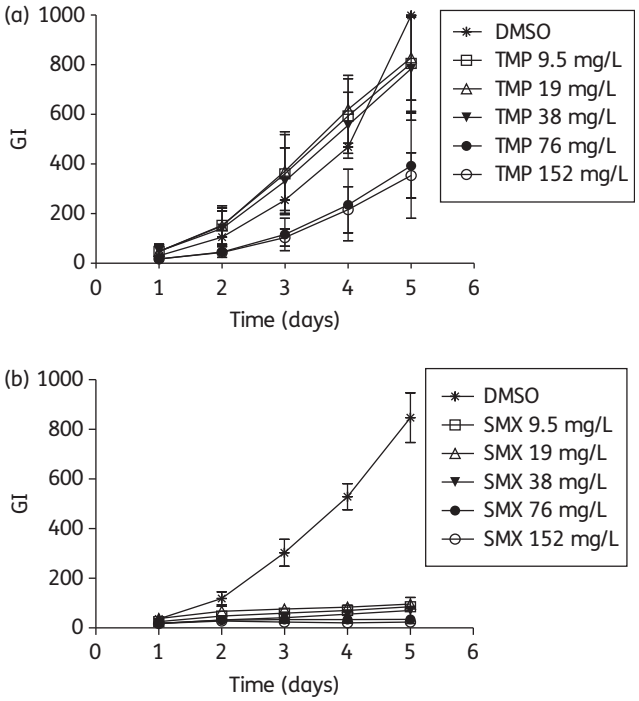

Figure 1. Growth profile of *M. tuberculosis* H37Rv in the presence of various concentrations of sulfamethoxazole and trimethoprim plus DMSO (control). The GIs of H37Rv in BACTEC vials with various drug concentrations were measured using the BACTEC 460TB system. The BACTEC vials were incubated at 37°C and GI readings were obtained after the first day until the ΔGI of the 1:100 culture was >30. (a) Activity of trimethoprim. (b) Activity of sulfamethoxazole. Results were obtained from three separate experiments and standard deviations were calculated using Excel. TMP, trimethoprim; SMX, sulfamethoxazole.

tuberculosis drugs isoniazid, rifampicin and ethambutol. Table 1 includes only concentrations where synergy or an additive effect was observed. For combinations where no interaction was noted, only the quotient obtained from half the MIC is listed. A strong and concentration-dependent synergistic interaction between rifampicin and sulfamethoxazole was observed, indicated by the quotients. There was no synergistic killing effect or antagonistic effect observed between sulfamethoxazole and isoniazid. The combination of sulfamethoxazole (2 mg/L) with 0.4 mg/L ethambutol showed a borderline synergistic effect. It therefore appears that there was no antagonism in any of the combinations of sulfamethoxazole with the first-line drugs.

Discussion

We have shown that sulfamethoxazole possesses antimycobacterial activity that could be explored further for clinical use. Trimethoprim/sulfamethoxazole is readily available and is an inexpensive combination. A single dose of trimethoprim/sulfamethoxazole (160/800 mg) yields a sulfamethoxazole C_{max} of 45 mg/L at ~2 h and a sulfamethoxazole C_{min} of

Table 1. Interaction between sulfamethoxazole and trimethoprim, rifampicin, ethambutol and isoniazid

SMX (mg/L)	TMP (mg/L)	Quotients (mean $x/y \pm$SD)
9.5	0.5	0.62±0.03
4.75	0.25	1.06±0.02
2.4	0.125	1.18±0.26
SMX (mg/L)	RIF (mg/L)	
2	0.3	0.16±0.19
	0.4	0.19±0.16
SMX (mg/L)	EMB (mg/L)	
2	0.4	0.49±0.02
SMX (mg/L)	INH (mg/L)	
2	0.025	1.03±0.05

The table shows the interaction between sulfamethoxazole (SMX) and trimethoprim (TMP), rifampicin (RIF), ethambutol (EMB) and isoniazid (INH). The data were obtained at day 5 when the GI of the 1:100 culture was ≥30 or when the GI of the control treated with DMSO (solvent) reached 999. Synergy was defined as $x/y < 1/z$, where x is the GI value of the combination, y is the lowest GI of the single drug in the combination and z is the number of drugs combined (which was two in our case). Synergy, $x/y < 0.5$; additive, $x/y \geq 0.5$ and < 0.75; no interaction, $x/y \geq 0.75$ and < 2; antagonism, $x/y \geq 2$. All results were obtained from three separate determinations and standard deviations were calculated using Excel.

Table 2. MICs of the first-line drugs and sulfamethoxazole for *M. tuberculosis* strain H37Rv

Drug	MIC (mg/L)
INH	0.05
EMB	1.6
RIF	0.8
SMX	9.5

INH, isoniazid; EMB, ethambutol; RIF, rifampicin; SMX, sulfamethoxazole. MICs were determined using the BACTEC 460TB system following the manufacturer's recommendations.

13 mg/L 24 h after ingestion.[10] Both are in excess of the MIC for 90% inhibition for *M. tuberculosis* strain H37Rv in this study. An early randomized study in HIV-infected adults with active tuberculosis in Coté d'Ivoire comparing trimethoprim/sulfamethoxazole with placebo showed efficacy in reducing mortality and morbidity, largely due to reduced septicaemia and enteritis.[11] There was a modest, non-significant reduction in mycobacterial disease in subjects receiving trimethoprim/sulfamethoxazole [hazard ratio 0.6 (0.3–1.2)]. An antimycobacterial effect may have contributed to the benefit. Forgacs *et al.*[4] recently reported that this combination showed activity against *M. tuberculosis*. After documenting a clinical response in a patient treated only with trimethoprim/sulfamethoxazole for suspected nocardiosis, who was subsequently shown to have drug-susceptible

tuberculosis, they then showed an antituberculosis effect in clinical isolates, including multidrug-resistant tuberculosis.[4]

Our data support the findings of Ong and colleagues,[12] who reported that in the combination of trimethoprim and sulfamethoxazole, it is only sulfamethoxazole that has an activity against *M. tuberculosis*. We evaluated the interaction of sulfamethoxazole with trimethoprim and the first-line drugs isoniazid, rifampicin and ethambutol and there was no synergistic killing between sulfamethoxazole and trimethoprim. Sulfamethoxazole showed a strong synergistic effect with rifampicin, one of the two key drugs in the tuberculosis regimen.[13] This synergism was achieved at very low concentrations of both drugs, where sulfamethoxazole at four times less than its MIC of 9.5 mg/L in combination with rifampicin between two and three times its MIC of 0.8 mg/L gave the same killing effect as rifampicin at its MIC. The combination of sulfamethoxazole and ethambutol showed an additive effect, with no positive or negative interaction between sulfamethoxazole and isoniazid. There was no antagonism in all combinations involving sulfamethoxazole. The synergy between sulfamethoxazole and rifampicin may be the result of their respective targets; sulfamethoxazole indirectly inhibits RNA synthesis through inhibiting tetrahydrofolate production, a co-factor in the synthesis of thymidine, and rifampicin directly inhibits the RNA synthesis by inhibiting DNA-dependent RNA polymerase.[3,14]

Our findings support reports that sulfamethoxazole is the active compound in the trimethoprim/sulfamethoxazole combination. Sulfamethoxazole has synergistic activity with rifampicin and an additive effect with ethambutol. Trimethoprim/sulfamethoxazole is a registered drug combination for other indications, is inexpensive and is widely available. Mouse studies will be undertaken before proceeding to clinical trials to clarify the potential of sulfamethoxazole and sulfamethoxazole/rifampicin in drug-susceptible tuberculosis. Also, we plan to evaluate clinical isolates.

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Transparency declarations

None to declare.

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