Evaluation of the MTT and MABA assays for rapid screening of the *in vitro* activity of synthetic chalcones against *Mycobacterium tuberculosis*

By

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DECLARATION

I, Suventha Moodley, confirm that the work presented in this dissertation is my original work and has not been submitted to this or any other University. I confirm that work carried out by others or originated from other sources have been indicated and duly acknowledged in this dissertation.

Suventha Moodley

DEDICATION

When I started this journey I never in my wildest dreams imagined that you will not be here with me when I completed it. I know that you never left me alone and the reason I got through this was because of you, I just would like to thank you mummy for always being there for me till the end and you will always live on through me.

Dedicated to my beloved mother, Mrs Govindhamma (Saras) Moodley.

PRESENTATIONS

Conferences:

- **Moodley S,** Koorbanally NA, Ramjugernath D, Pillay M (13/09/2013) The activity of novel, synthetic chalcones against *Mycobacterium tuberculosis*. College of Health Sciences Research Symposium, Durban, South Africa. Oral presentation.

- **Moodley S**, Koorbanally NA, Ramjugernath D, Pillay M (24/11/2013) A Comparison of the Alamar Blue, MTT and Macroscopic broth assays to screen for anti-tuberculous activity of synthetic chalcones. 18th Biennial Conference of the South African Society of Microbiology (SASM), Bela-Bela, South Africa. Poster presentation.

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To my sister, Prinetha Moodley, thank you for motivating me and supporting me through everything. You are my role model and if one day I can be half the woman you are, I will consider myself lucky.

To my mum and dad (Saras and Vijiarajh Moodley), thank you for your sacrifice and support. I am the luckiest girl in the world to call both of you my parents and I hope to one day make you very proud of me.

ABSTRACT

Background: The chalcone scaffold (1,3-diaryl-2-propen-i-ones) has the advantage of easy chemical modification and has been shown to possess biological activity against a variety of organisms, including a wide range of anti-TB activity. The focus of this study was 2-fold: firstly, to compare the performance of the colorimetric MTT and MABA assays for screening synthetic chalcones, and secondly, to evaluate the activity of fluorinated and non-fluorinated chalcones against drug susceptible and resistant clinical strains of *M. tuberculosis*.

Materials and methods: Twenty seven chalcones and chromenochalcones were screened against the laboratory strain *M. tuberculosis* H37Rv, using a microtitre plate MTT assay at 7 days. The MIC for 20 active compounds was subsequently determined using the MABA, MTT and the macroscopic broth assays at 7, 14 and 21 days, extracellular activity against clinical isolates of varying drug susceptibility patterns and genotypes using the MTT assay, intracellular activity in a macrophage model and eukaryotic cytotoxicity using Vero cells.

Results and discussion: No significant difference in the MICs, or increase in the MICs was observed over time between the MABA ($p = 0.209$) and the MTT ($p = 0.207$) assays, in contrast to the gold standard, the macroscopic broth assay ($p = 0.000$). Fluorinated and non-fluorinated chalcones displayed moderate activity (32- 128 µg/mL) against MDR- and XDR-TB isolates, no significant activity against intracellular H37Rv and low selectivity for *M. tuberculosis*. The elevated MICs and lack of intracellular activity may be explained by the precipitation of the compounds indicating low solubility, with the exception of IV and XVI.

Conclusions: The MTT assay is a more cost effective drug susceptibility testing method than the MABA assay for the rapid *in vitro* screening of the activity of chalcones against *M. tuberculosis*.

Compound XIX and XI have the most potential for reformulation to improve their biological activity to yield a more potent drug candidate.

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LIST OF ABBREVIATIONS

AlrA	D-alanine racemase
AM	Arabinomannan
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
Bp	Base pair
CFU	Colony forming unit
CO_2	Carbon dioxide
COV	Coefficient of variation
Ddl	D-alanine: d-alanine ligase
DMEM	Dulbecco's modified eagle medium
DMF	N,N-dimethylformamide
DMSO	Dimethyl sulfoxide
DST	Drug susceptibility testing
EMB	Ethambutol
F	Fluorine
FADH	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug administration
FMNH	Flavin MonoNucleotide
HIV	Human immunodeficiency virus
IC_{50}	Concentration of drug that inhibits 50% of healthy vero cells

INH	Isonicotinic acid hydrazide (Isoniazid)
KZN	Kwazulu-Natal
LAM	Lipoarabinomannan
MABA	Microplate-based Alamar Blue Assay
MAP	Arabinogalactan and peptidoglycan complex
MDR-TB	Multi-drug-resistant tuberculosis
MGIT	Mycobacterial growth indicator tube method
MIC	Minimum inhibitory concentration
MIC_{50}	Median MIC
MOI	Multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
OADC	Oleic acid-albumin-dextrose-catalase
OD	Optical density
PAS	Para-aminosalicylic acid
PBS	Phosphate-buffered saline
PMA	Phorbol myristate acetate
PZA	Pyrazinamide
REMA	Resazurin microtitre assay
RIF	Rifampicin
RPMI	Roswell Park Memorial Institute medium
rRNA	Ribosomal ribonucleic acid

SDS	Sodium dodecyl sulphate
SDS-DMF	20% sodium dodecyl sulfate and 50% of N,N-dimethylformamide
SI	Selectivity index
TB	Tuberculosis
TDM	Trehalose dimycolate
TEMA	Tetrazolium microplate assays
UKZN	University of Kwazulu-Natal
v/v	Volume/volume
WHO	World health organization
XDR-TB	Extensively-drug-resistant tuberculosis

INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), latently infects over 30% of the global population (WHO, 2013a). HIV co-infection, the emergence of multiple and extensive drug resistance (MDR and XDR) and diagnostic delays have confounded the successful treatment of TB (Gillespie, 2002). Adverse effects of the first and second line TB drugs and prolonged treatment resulted in patient noncompliance with treatment regimen (O'Brien and Spigelman, 2005).

In the last approximately 40 years, only 1 new class of drug has been approved by the Food and Drug administration (FDA) for use against *M. tuberculosis*, especially MDR and XDR-TB. Although Bedaquiline offers new hope, like most of the new and existing TB drugs in the drug pipeline, it is disadvantaged by adverse effects (Stehr, *et al*., 2014). The challenge posed by rapidly evolving drug resistant *M. tuberculosis* strains (Gillespie, 2002) has to be overcome by consistently maintaining the TB drug pipeline with potential novel lead candidates.

In vitro screening is an important step in TB drug discovery because some synthetic compounds display difficulty in penetrating the lipid rich mycobacterial cell wall and display cytotoxicity against eukaryotic cells (Cole and Riccardi, 2011). In addition, compounds should also be screened for intracellular activity in macrophage models as *M. tuberculosis* is an intracellular pathogen that can infect and replicate in alveolar macrophages and epithelial cells (Smith, 2003).

Rapid, reliable *in vitro* screening of possible lead candidates is vital in the initial evaluation of compounds. The use of colorimetric assays such as Microplate-based Alamar Blue Assay (MABA) (Collins and Franzblau, 1997), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Martin, *et al*., 2005) and resazurin microtitre assay (REMA), also

referred to as the resazurin method (Martin, *et al*., 2005) in high throughput screening of possible lead compounds has negated the expense associated with the automated (BACTEC and MGIT) and agar and broth based conventional methods while maintaining the speed and sensitivity.

Synthetic compound libraries from pharmaceutical companies and medicinal and organic chemistry research groups represent a rich source of lead compounds and high throughput screening of similar libraries have led to the discovery of bedaquiline and SQ-109 (Kolyva and Karakousis, 2012). Chalcones, a class of flavonoids, provide an attractive scaffold for lead optimisation as it displays biological activity against various organisms (Li, *et al*., 1995; Lopez, *et al*., 2001; Wu, *et al*., 2003; Narender and Gupta, 2004; Nielsen, *et al*., 2004). Their characteristic amenability to structural alteration has resulted in derivatives with varying degrees of activity against *M. tuberculosis* (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013).

Complications associated with solubility and metabolic degradation of compounds has previously been resolved by fluorine substitution (Begue and Bonnet-Delpon, 2008). The inclusion of fluorine substituents was also shown to improve lipophilicity that facilitates permeation of lipid membranes (Smart, 2001) which is important in the case of *M. tuberculosis* with its complex, lipid rich cell wall and membrane (Brennan and Nikaido, 1995). The role of fluorine substitution on the *in vitro* activity of the chalcone scaffold against *M. tuberculosis* has not been previously evaluated.

Various drug susceptibility tests have been used to screen chalcones against *M. tuberculosis* including the MABA assay (Trivedi, *et al*., 2008; Hans, *et al*., 2010), luciferase reporter phage assay (Sivakumar, *et al*., 2007), agar proportion method (Sharma, *et al*., 2009a;

Marrapu, *et al*., 2011), BACTEC 460 radiometric assay (Lin, *et al*., 2002; Ahmad, *et al*., 2013), enzyme kinetic assays (Chiaradia, *et al*., 2008; Mascarello, *et al*., 2010) and the macroscopic broth assay using Sauton's media (Anand, *et al*., 2012). Due to the lack of a standard drug susceptibility test for screening, the level of improvement of substitutions is difficult to compare in different studies. The performance of the MTT colorimetric assay in comparison to the MABA has not yet been evaluated on chalcones.

This study compared the effectiveness of the MABA and MTT assays with the macroscopic broth assay as a gold standard in screening the activity of a series of synthetic chalcone compounds against *M. tuberculosis*. The MTT assay was then used to evaluate their *in vitro* extracellular activity against clinical strains of *M. tuberculosis* of varying drug susceptibility and genotype profiles. A few selected compounds were further evaluated for intracellular activity in macrophages and for cytotoxicity in the vero cell line.

DISSERTATION OUTLINE

This dissertation is written in a publication format. The first chapter presents a review of the relevant literature. Chapters 2 and 3 present 2 original research manuscripts that are ready for submission. Chapter 4 provides a general discussion which integrates the findings of both chapters 2 and 3 and highlights conclusions and recommendations.

CHAPTER 1: LITERATURE REVIEW

1.1. Epidemiology of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis, an airborne pathogen, is the causative agent of tuberculosis (TB). Human immunodeficiency virus (HIV) co-infection and the emergence of multiple and extensive drug resistance (MDR and XDR) have resulted in rapidly escalating burdens of TB, especially in developing countries. An estimated 8.6 million new cases of TB and 1.3 million deaths globally in 2012 were due to TB. South Africa has been identified as a country with high burdens of TB, MDR-TB and HIV (WHO, 2013a). This is evident from the TB prevalence, incidence (Fig. 1.1) and mortality rates (includes HIV+TB) per 100 000 population per year (857, 1 003, 227 cases respectively) in 2012 (WHO, 2013a). KwaZulu-Natal had the highest reported cases of TB per 100 000 population per year in 2012 (Table 1.1). The new smear positive cure rate in 2010 for South Africa, KwaZulu-Natal and the eThekwini District was 73.1%, 71.3% and 63.65% respectively (Massyn, *et al*., 2013).

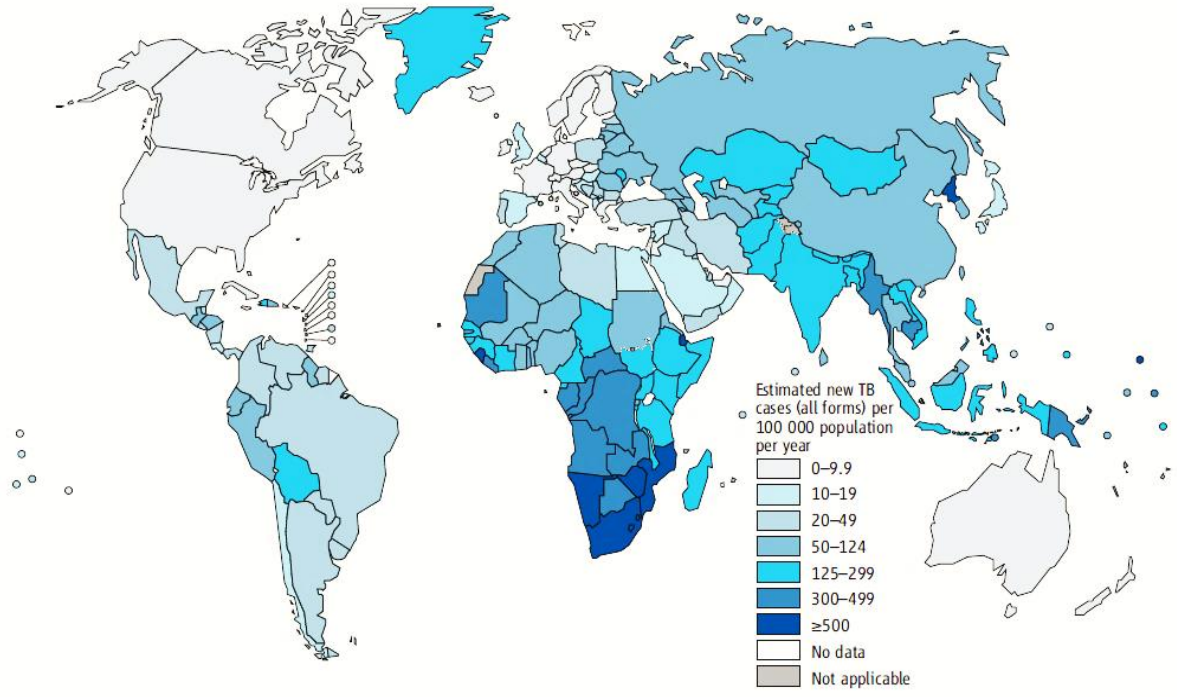


Fig. 1.1. Estimated TB incidence rates in 2012 (WHO, 2013a).

Table 1.1. Reported cases of TB per 100 000, 2012 (National Department of Health Notification System, 2012).

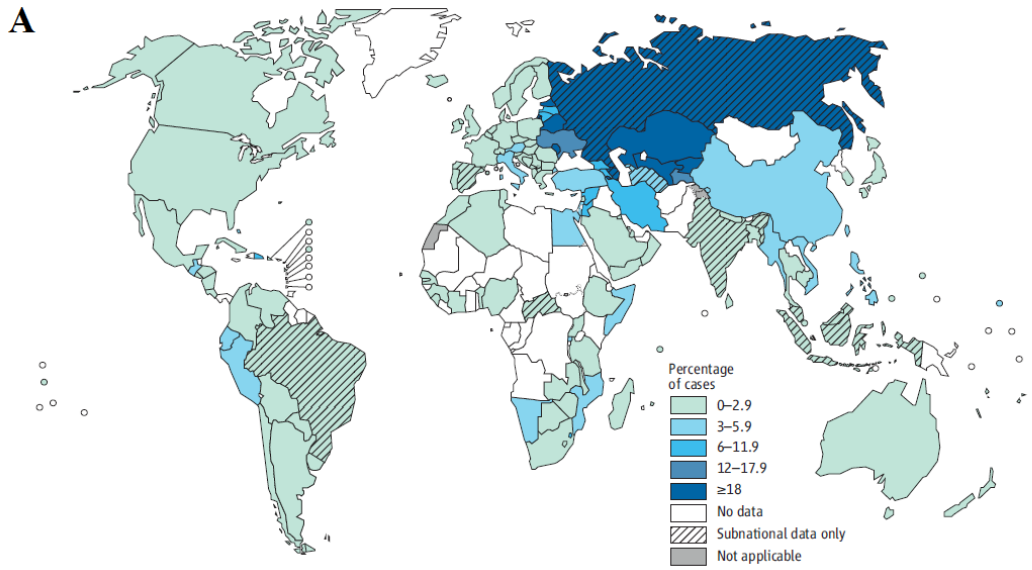
	EC	FS	GP	KZN	LP	MP	NC	NW	WC
Cases of TB per 100 000	831.7	708.5	448.3	971.0	382.3	551.8	712.1	720.2	800

EC: Eastern Cape, **FS**: Free State, GP: Gauteng, KZN: KwaZulu-Natal, LP: Limpopo, **MP**: Mpumalanga, **NC**: Northern Cape, **NW**: North West, **WC**: Western Cape.

1.1.1. MDR- and XDR-TB

Approximately 450 000 new cases of MDR-TB were reported globally in 2012 with 3.6% of these being new cases and 20% previously treated (WHO, 2013a). In 2012, 15 419 South Africans were diagnosed with MDR-TB, with 1.8% new (Fig. 1.2.A) and 6.7% previously treated cases (Fig. 1.2.B).

MDR is defined as *M. tuberculosis* strains that are "resistant to isoniazid (INH) and rifampicin (RIF)" and XDR refers to MDR strains that are also "resistant to any fluoroquinolone and to at least one of the three following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin" (WHO, 2013b).



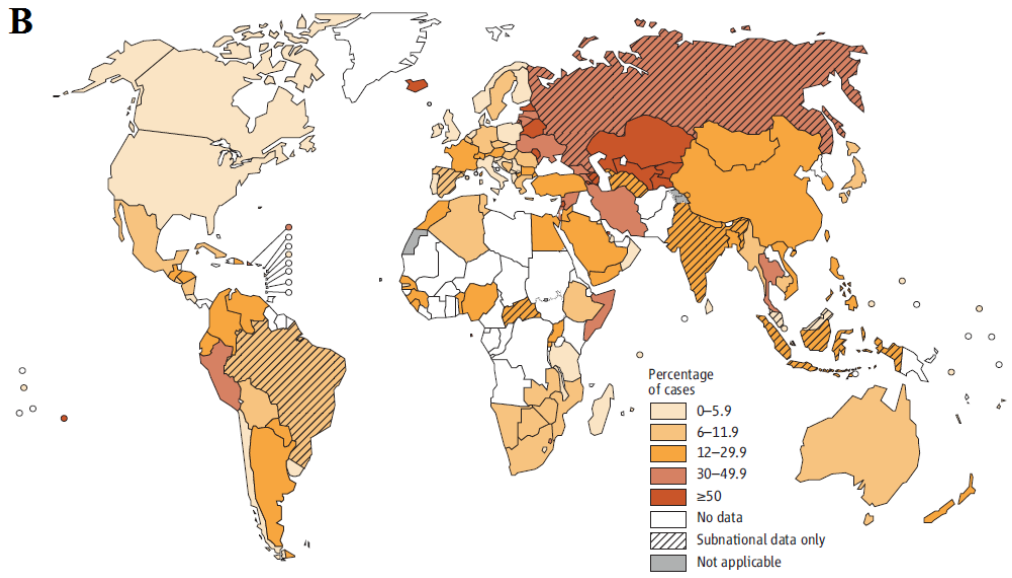


Fig. 1.2. Number of MDR-TB cases among new (A) and previously treated (B) cases of pulmonary TB, 2012 (WHO, 2013a).

1.1.2. Drug resistance mechanisms

Monotherapy and patient noncompliance with treatment regimens (Gillespie, 2002) often result in the selection of naturally occurring, drug resistant, spontaneous mutants from a population of *M. tuberculosis*. The mutation rate in the presence of anti-TB drugs is dependent on the specific drug, and the mutation type is dependent on the concentration of the drug (Gillespie, 2002). Drug resistance is a consequence of mutations in genes coding for drug targets or prodrug activating enzymes (Joloba and Bwanga, 2010).

Efflux pumps are associated with intrinsic resistance. Efflux pump encoding genes have been identified in *M. tuberculosis* for the following 5 families: the small multidrug resistance family, the resistance-nodulation-cell division family, the multidrug and toxic compounds extrusion ATP-binding cassette superfamily and the major facilitator superfamily (Li and Nikaido, 2004). Efflux pumps have been implicated in the resistance of anti-TB drugs; RIF and ofloxacin (Siddiqi, *et al*., 2004; Louw, *et al*., 2011), INH (Pasca, *et al*., 2005), fluoroquinolones (Pasca, *et al*., 2004), ethambutol (Colangeli, *et al*., 2005), streptomycin,

norfloxacin, chloramphenicol, tetracycline, erythromycin (Choudhuri, *et al*., 2002), aminoglycosides (Ainsa, *et al*., 1998) and β-lactams (Dinesh, *et al*., 2013); other drugs, anthracyclines (Choudhuri, *et al*., 2002) and other compounds; tetraphenyl phosphonium, ethidium bromide, acriflavine, safranin O, pyronin Y (De Rossi, *et al*., 1998) and pyrrole and pyrazolone (Balganesh, *et al*., 2012). Efflux pumps have been identified in clinical isolates (Siddiqi, *et al*., 2004; Gupta, *et al*., 2006; Jiang, *et al*., 2008; Gupta, *et al*., 2010; Hao, *et al*., 2011). Efflux inhibitors reduced *in vitro* Minimum Inhibitory Concentrations of isoniazid, ciprofloxacin, ofloxacin, streptomycin, and linezolid in resistant strains (Escribano, *et al*., 2007; Richter, *et al*., 2007; Spies, *et al*., 2008; Singh, *et al*., 2011; Machado, *et al*., 2012; Rodrigues, *et al*., 2012) and addition of verapamil restored the *in vivo* activity of isoniazid, rifampicin, and pyrazinamide against MDR isolates in the mice model (Louw, *et al*., 2011). Efflux pumps that are required for growth in macrophages facilitate a state of induced drug tolerance that is inhibited by the addition of efflux pump inhibitors (Adams, *et al*., 2011).

1.1.3. Lineages of *M. tuberculosis*

Specific lineages of *M. tuberculosis* have been implicated in MDR- and XDR-TB throughout the world (Glynn, *et al*., 2002; Pillay and Sturm, 2007; Chihota, 2011). Beijing strains are distributed worldwide but are most prevalent in Asia. These strains are associated with drug resistance in Cuba, Vietnam, New York and Estonia (Glynn, *et al*., 2002).

The presence of F15/LAM4/KZN strains in KwaZulu-Natal (KZN) and their strong association with MDR strains was detected as early as 1994 (Pillay and Sturm, 2007). This strain has been characterised with the spoligotype ST60 and has been found in 12 locations on the North American, South American and European continents (Filliol, *et al*., 2003).

In South Africa, the Beijing, S, and F15/LAM4/KZN strains have been commonly associated with MDR- and XDR-TB (Chihota, 2011). Of the MDR isolates (Table 1.2), the

Beijing strains were found predominantly in Eastern (42.5%) and Western Cape (47.9%), the S strains in KwaZulu-Natal (33.7%) and LAM in Gauteng (26.7%) (Chihota, 2011).

Table 1.2. Genotypes of MDR-TB strains isolated from the four provinces (Chihota, 2011).

M. tuberculosis genotype	Province Eastern Cape	Gauteng	KwaZulu Natal n (%)	WesternCape n (%)	Total
Beijing	57 (42.5)	128 (16.7)	15 (7.7)	1001 (47.9)	1201 (37.7)
LAM	33 (24.6)	204 (26.7)	49 (25.0)	212 (10.1)	498 (15.6)
T	18 (13.4)	135 (17.7)	33 (16.8)	250 (12.0)	436 (13.7)
X	2 (1.5)	46 (6.0)	16 (8.1)	386 (18.5)	450 (14.1)
S	8 (6.0)	54 (7.1)	66 (33.7)	49 (2.3)	177 (5.6)
Other	16 (11.9)	198 (25.9)	17 (8.7)	193 (9.2)	424 (13.3)
Total	134 (100.0)	765 (100.0)	196 (100.0)	2091 (100.0)	3186(100.0)

In KwaZulu-Natal, the LAM genotype was found in 24.9%, 41.7% and 80% of MDR, preXDR and XDR isolates respectively. Within this clade, the LAM4 specifically comprised 79.6%, 80% and 90% of MDR, preXDR and XDR isolates respectively (Chihota, 2011). This highlights the strong association of the F15/LAM4/KZN with drug resistance in KwaZulu-Natal (Fig. 1.3).

In Gauteng, LAM genotype was found in 26.7% of MDR isolates and of these, 63.2% was LAM4 (Chihota, 2011). The presence of F15/LAM4/KZN in Gauteng and KZN suggests cross border transmission of this strain due to migratory labour practices.

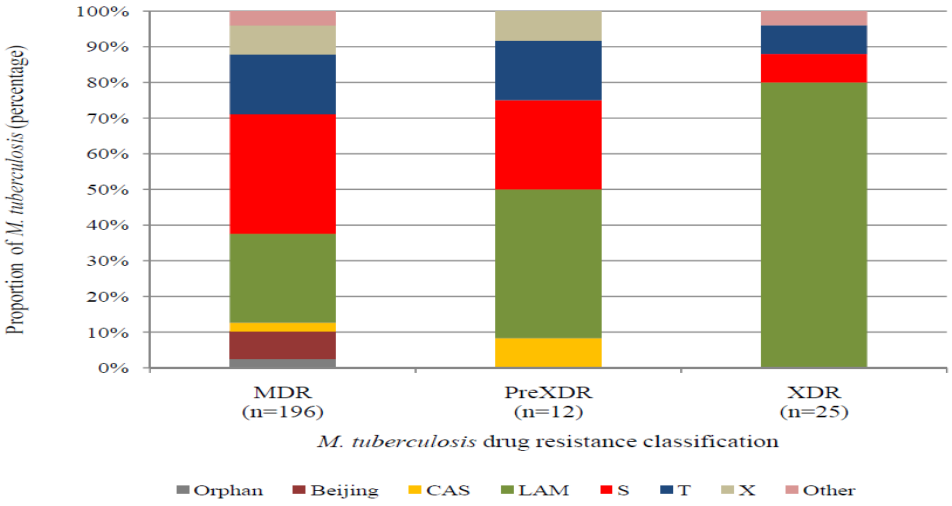


Fig. 1.3. Percentage of *M. tuberculosis* genotypes associated with MDR-TB, pre-XDR-TB and XDR-TB in KwaZulu-Natal, South Africa (Chihota, 2011).

1.2. Cell envelope of *M. tuberculosis* and its role in antibiotic resistance

The structure of the cell envelope of *M. tuberculosis* (Fig. 1.4) is composed of three major components: a plasma membrane; a mycolic acid, arabinogalactan and peptidoglycan complex (MAP) and a capsule-like material which is polysaccharide-rich (Brennan and Nikaido, 1995). A branched-chain arabinogalactan has been identified as a major cell wall polysaccharide in mycobacterial species. This polysaccharide is covalently attached to muramic residues through a phospho-diester linkage, arabinose residues forming the non-reducing termini to which mycolic acids are linked (Lederer, *et al*., 1975). The arabinose and galactose residues in the arabinogalactan are in the furanose configuration which is unusual due to the distinct structural motifs, lack of repeating units and elemental form which differs from other bacterial polysaccharides (Vilkas, *et al*., 1973; Daffe, *et al*., 1990).

The cell envelope polymers: MAP, lipoarabinomannan (LAM) and arabinomannan (AM) consist of unusual carbohydrate residues such as α-L-rhamnose, succinylated arabinose, and *N*-glycolylmuramic acid (Lederer, *et al*., 1975).

The plasma membrane consists of peptidoglycan which is linked to linear D-galactofuran via phophodiester linkage which is facilitated by N-acetylglucosamine and α-L-rhamnose. Highly branched D-Arabinose is attached to this peptidoglycan and linear D-galactofuran complex and mycolic acids are attached to the nonreducing terminus. The orientation of these mycolic acids is perpendicular to the plane of the plasma membrane (Nikaido, *et al*., 1993).

The capsule like layer is composed of free carbohydrates, lipids and proteins such as phthiocerol-containing mannan, lipids, glucan, and AM, lipomannan, sulfolipids, trehalose dimycolate (TDM), diacyl- and polyacyl-trehaloses, LAM, phosphatidylinositol mannosides and trehalose monomycolate (Nikaido, *et al*., 1993).

The mycolic acids were thought to be covalently bound and the inner leaflet of an asymmetrical bilayer and other lipids form the outer leaflet, either intercalating with the mycolates or forming a more clearly defined interlayer plane (Minnikin, 1982). An alternate model suggests that the noncovalently-linked lipids form a monolayer that does not intercalate with the mycolic acids (Rastogi, 1991). The use of cryo-electron tomography to study the *Mycobacterium smegmatis* cell envelope has challenged these models of mycobacterial outer membrane and suggested the following possible revisions with regards to the positions of mycolic acids: the meromycolates may spread across the entire hydrophobic region and only the α-chain is covered by fatty acids from the other leaflet or the mycolic acid layer contributes to the inner leaflet by the extended branches of the meromycolates, whereas the major part is located below the outer membrane (Hoffmann, *et al*., 2008). Antigen 85 complex which is secreted protein is involved in the transfer of mycolates onto the cell wall of the *M. tuberculosis* (Jackson, *et al*., 1999). The cell wall lipid structure is disrupted by Mce1 proteins that are involved in the re-importering of mycolic acid in limited conditions of growth (Forrellad, *et al*., 2014). The structure of the central domain of the

protein Rv0899 (OmpATb) suggests that it may employ an alternate mode of membrane association than the outer membrane porin that was previously described (Teriete, *et al*., 2010). UDP-galactopyranose mutase and galactofuranosyl transferase are essential enzymes for growth and are involved in the attachment of the outer lipid layer by arabinogalactan to peptidoglycan (Pan, *et al*., 2001). Lipoglycans that are exposed at the cell surface of mycobacteria are most likely inserted in the outer leaflet of the outer membrane (Pitarque, *et al*., 2008). Phosphatidylinositol and phosphatidylinositol mannosides have essential functions in the mycobacterial cell wall (Jackson, *et al*., 2000). Phosphatidyl-*myo*-inositol mannosides, lipomannan and LAM are suggested to be involved in more than one critical function in mycobacterial cells (Guerin, *et al*., 2009).

The presence and organisation of the mycolic acids in the cell envelope is essential for the cell wall function as a permeability barrier which confers intrinsic drug resistance to *M. tuberculosis* (Wang, *et al*., 2000). Therefore, INH and ethambutol that target the biosynthesis of mycolic acid and arabinogalactan respectively are important first line TB drugs. β-lactam and aminoglycoside resistance of *Mycobacterium chelonae* has been attributed to this permeability barrier (Jarlier and Nikaido, 1990; Jarlier, *et al*., 1991).

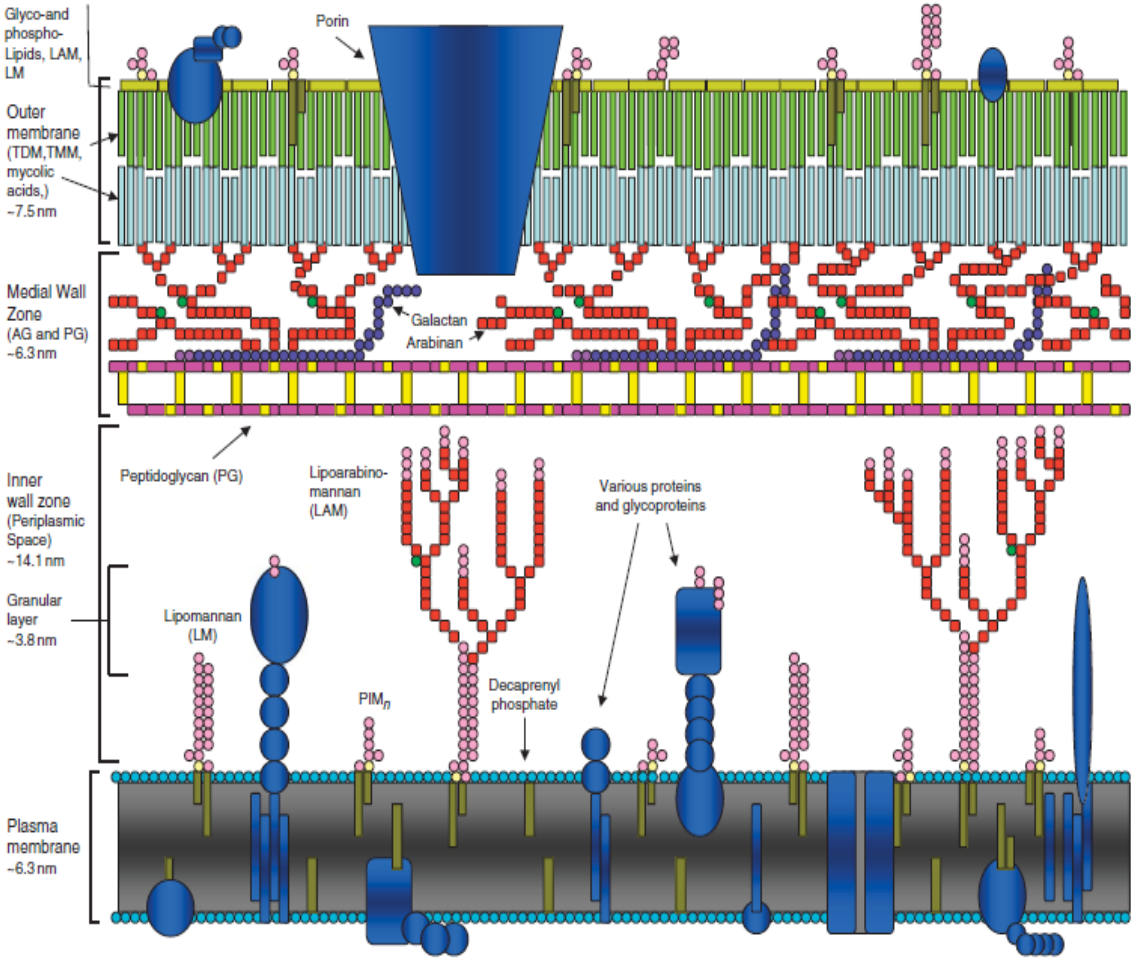


Fig. 1.4. A schematic diagram of the cell envelope of mycobacteria (Crick, *et al*., 2010).

1.3. Interaction between macrophage and *Mycobacterium tuberculosis*: antimicrobial activity

Contact of the bacteria with the mannose or complement receptors on the macrophage results in phagocytosis (Schlesinger, 1993). In addition, the Human toll-like receptor 2 also participates in the phagocytosis of the bacteria (Noss, *et al*., 2001).

Upon entry into the macrophage, the bacteria reside in a phagosome, which is an endocytic vacuole. The maturation of the phagsome results in the formation of a phagosome-lysosome. The accumulation of V-ATPase complexes in the phagosome membrane results in acidification, that is important for the functioning of the phagosome (Hackam, *et al*., 1997).

The phagosome maturation is modulated by mycobacterial cell wall lipids such as LAM (Hayakawa, *et al*., 2007), the phenolic glycolipid phenolphthiocerol diester (Robinson, *et al*., 2007), the isoprenoid edaxadiene (Mann, *et al*., 2009), and TDM (Axelrod, *et al*., 2008). The phagosome-lysosome contains an acidic pH, reactive oxygen intermediates, lysosomal enzymes, toxic peptides and reactive nitrogen intermediates resulting in a hostile environment (Nathan and Hibbs, 1991).

Upon infection of the macrophage, *M. tuberculosis* upregulates expression of genes for adaptation to oxidative and nitrosative stresses, growth limitation and alternative nutrient sources (Fig. 1.5) (Schnappinger, *et al*., 2003). The initial antimicrobial activity of the macrophage is the superoxide burst, mediated by the Nicotinamide adenine dinucleotide phosphate (NADPH) complex I as part of phagocytosis (El-Benna, *et al*., 2009). However, *M. tuberculosis* is able to overcome the effect of superoxide, by various mechanisms, including the secretion of superoxide dismutase (Spagnolo, *et al*., 2004) and the scavenging properties of lipidoglycans present in the cell wall (Chan, *et al*., 1989).

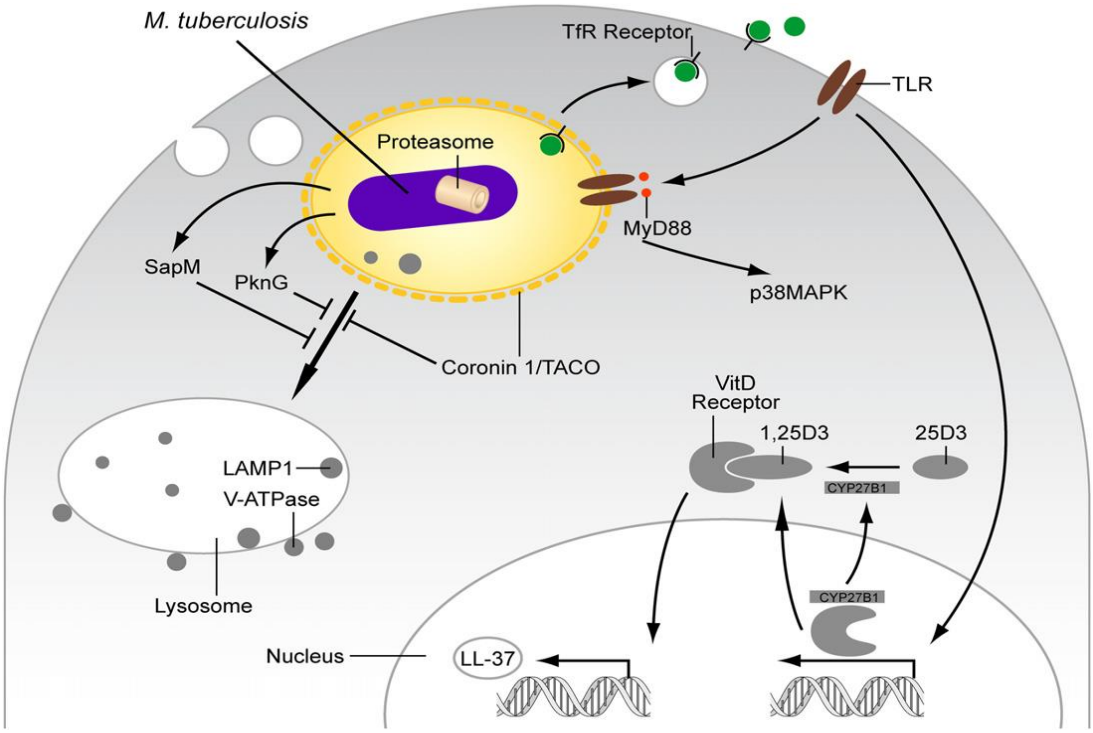


Fig. 1.5. The interaction between macrophage and mycobacterial cell. The active recruiting of the host protein coronin 1/TACO (yellow), secretion of phosphatase SapM and serine/threonine kinase PknG to prevent the mycobacterial phagosome (light-yellow circle) fusion with lysosomes. The effect of nitric oxide is neutralised by the *M. tuberculosis* proteasome. Phagosome-lysosomal fusion can be mediated via the Toll-like receptor signalling activation of p38MAPK however; it is unclear if mycobacteria counteract this pathway. The activation of the vitamin D receptor by 1, 25-dihydroxyvitamin D3 results in TLR signalling which induces cathelicidin (antimicrobial peptide) (Pieters, 2008).

1.4. Current recommended treatment regimen

The current WHO recommended regimen for drug susceptible TB involves treatment with first line drugs namely: rifampicin, isoniazid, pyrazinamide and ethambutol for the initial two months followed by 4 months with isoniazid and rifampicin (WHO, 2010). The treatment of MDR- and XDR-TB involves a combination of first and second line drugs namely; aminoglycosides, cycloserine, terizidone, ethionamide, protionamide, capreomycin, para-

aminosalicylic acid and fluoroquinolones. The treatment duration for MDR- and XDR-TB is substantially longer at 18 months or more, which contributes to patient non-compliance (WHO, 2010) leading to the development of drug resistance incurring mutations. The severe adverse side effects of both first and, especially, second line drugs also exacerbate non-compliance.

1.5. First line Anti-TB drugs

1.5.1. Isoniazid

The clinical activity of isonicotinic acid hydrazide (isoniazid or INH) was first reported in 1952 (Seilkoff and Robitzek, 1952). The structure of INH contains a pyridine ring and hydrazide group (Fig. 1.6). INH, ethionamide and pyrazinamide are structural analogs. INH is one of the important first line drugs for TB treatment. INH, which is initially in a pro-drug form, is oxidised by the *M. tuberculosis* catalase-peroxidase enzyme KatG resulting in its active form (Zhang, *et al*., 1992). The main mechanism of action of INH is inactivation of the enoyl-acyl carrier protein reductase (inhA) during mycolic acid synthesis (Winder and Collins, 1970). Resistance to INH has been most frequently observed compared to any other anti-TB drug (Karakousis, 2009). Mutations in the *katG* (Marttila, *et al*., 1998), *inhA* (Basso, *et al*., 1998), *ndh* (Miesel, *et al*., 1998), *nat* (Ramaswamy, *et al*., 2003), *furA* (Pym, *et al*., 2001), *ahpC* (Sherman, *et al*., 1996), *fasE24* (Wilson, *et al*., 1999) and *efpA* (Wilson, *et al*., 1999) genes have been reported to be responsible for the development of resistance to INH.

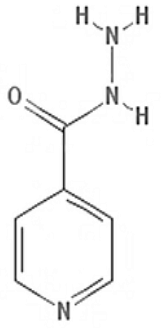


Fig. 1.6. The structure of INH (http://pubchem.ncbi.nlm.nih.gov).

1.5.2. Rifampicin

Rifamycin was isolated from *Amycolatopsis mediterranei* in 1957. The initial extraction of rifamycin contained rifamycin A, B, C and D (Sensi, *et al*., 1959). Rifamycin B was isolated from the mixture and *A. mediterranei* was able to produce rifamycin B together with a small amount of inactive rifamycin Y when exposed to 0.2% sodium diethylbarbiturate (Margalith and Pagani, 1961). Rifamcyin B demonstrated a low level of toxicity and therapeutic activity against infections in animals. Rifamycin B is converted to rifamycin O which is hydrolyzed to rifamycin S, which in turn is reduced to rifamycin SV (Sensi, *et al*., 1961). Rifamycin SV displayed activity against *M. tuberculosis* but was not well absorbed in the gastrointestinal tract and rapidly excreted by the liver (Bergamini and Fowst, 1965). This led to the chemical modification of rifamycin SV to form rifampicin (RIF). RIF (Fig. 1.7) is the hydrazone of 3-formylrifamycin SV with N-amino-N'-methylpiperazine (Maggi, *et al*., 1966). Rifampicin retains activity against *M. tuberculosis* with increased oral absorption compared to rifamycin SV (Binda, *et al.*, 1971). The mechanism of action of rifampicin is by the inhibition of bacterial DNA-dependent RNA polymerase encoded by the *rpoB* gene (Wehrli and Staehelin, 1971). Resistance to RIF is rare, and is normally accompanied by resistance to INH, and thus, is often used as a surrogate marker for MDR-TB. Resistance is commonly due to single point mutations in the *rpoB* gene, with more than 90% occurring in the 81 bp region (Telenti, *et al*., 1993).

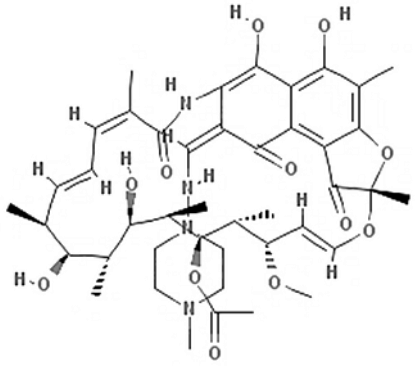


Fig. 1.7. The structure of RIF (http://pubchem.ncbi.nlm.nih.gov).

1.5.3. Pyrazinamide

Pyrazinamide (PZA) was first discovered in 1952, alongside isoniazid and ethionamide (Yeager, *et al*., 1952). PZA (Fig. 1.8) is an amide derivative of pyrazine-2-carboxylic acid and nicotinamide analog (Zhang and Mitchison, 2003). PZA possessed activity in *in vivo* studies in murine models but not *in vitro* due to the requirement of an acidic pH environment (Yeager, *et al*., 1952). PZA is converted to pyrazinoic acid by the action of bacterial pyrazinamidase (Konno, *et al*., 1967). The target of pyrazinoic acid is ribosomal protein S1 (RpsA), which is participates in protein translation and the ribosome-sparing process of trans-translation (Shi, *et al*., 2011). Resistance to PZA is often due to mutations in the *pncA* gene that encodes pyrazinamidase (Scorpio and Zhang, 1996).

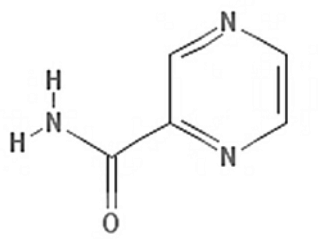


Fig. 1.8. The structure of PZA (http://pubchem.ncbi.nlm.nih.gov).

1.5.4. Ethambutol

The anti-TB activity of ethambutol (EMB) was first discovered in 1961 (Thomas, *et al*., 1961). EMB (Fig. 1.9) is a D-2, 2'- (ethylenediimino) -di- 1-butanol (Kolyva and Karakousis, 2012). EMB inhibits the cell wall arabinan polymerization thus affecting the arabinogalactan biosynthesis (Mikusova, *et al*., 1995). Resistance to EMB has most often been attributed to mutations in the *embCAB* operon (Belanger, *et al*., 1996). Varying degrees of resistance have been conferred by mutations in the different loci: *embB306*- variable (Safi, *et al*., 2008), *embCAB*- low to moderate (Safi, *et al*., 2010), *Rv3806c*- high-level, *embC* highest level and *Rv3792*- increases expression of *embC* (Safi, *et al*., 2013).

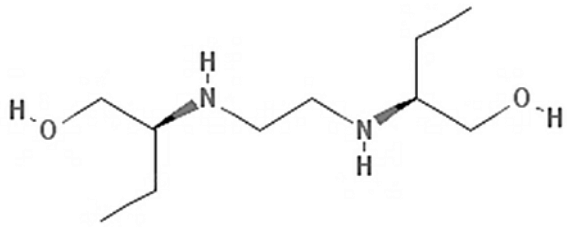


Fig. 1.9. The structure of EMB (http://pubchem.ncbi.nlm.nih.gov).

1.6. Second line Anti-TB drugs

1.6.1. Aminoglycosides

Aminoglycosides (streptomycin, kanamycin and amikacin) form part of the second line treatment regimen. The aminoglycoside (Fig. 1.10) basic structure comprises of different aminated sugars joined in glycosidic linkage to a dibasic cyclitol (Mingeot-Leclercq, *et al*., 1999). Streptomycin was the first drug with anti-TB activity that was discovered in the 1940s (Kolyva and Karakousis, 2012). The dibasic cyclitol in streptomycin (Fig. 1.10.A) is 2-deoxystreptidine (Mingeot-Leclercq, *et al*., 1999). Aminoglycosides inhibit protein translation by attaching to the 30S ribosomal subunit (Kolyva and Karakousis, 2012). Resistance to streptomycin has resulted mainly through mutations in the following genes:

rpsL gene (Nair, *et al*., 1993), the *rrs* (Douglass and Steyn, 1993) and the *gidB* gene (Wong, *et al*., 2011). However, a study in Cape Town, South Africa showed the presence of mutations in the *rrs* gene in clinical isolates that do not display resistance to streptomycin (Victor, *et al*., 2001).

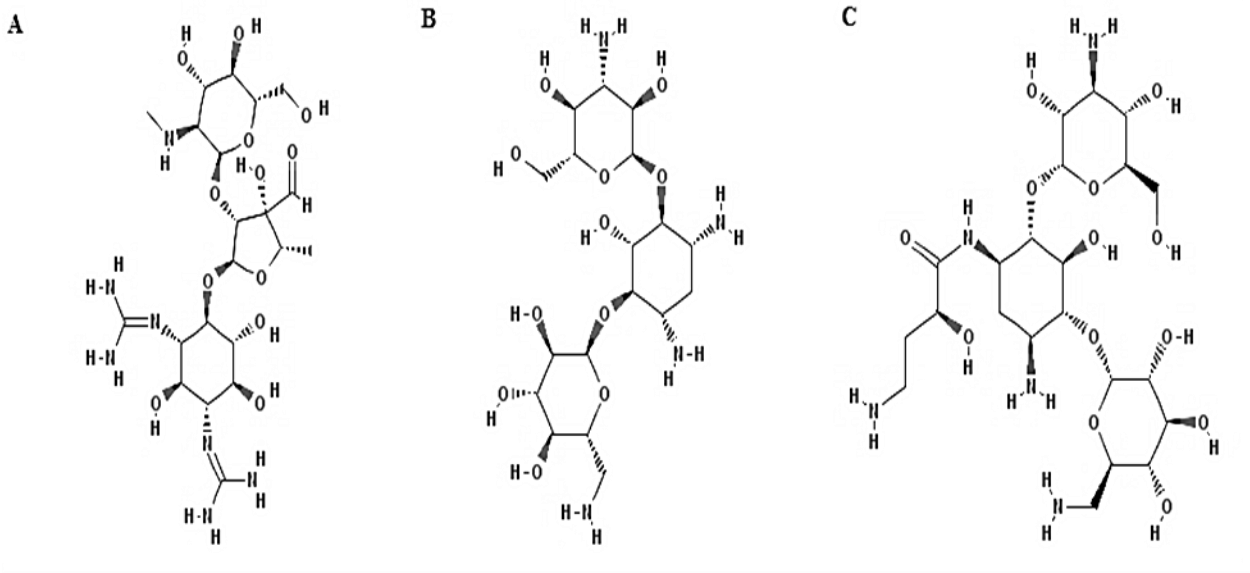


Fig. 1.10. Structure of aminoglycosides; A: Streptomycin, B: Kanamycin and C: Amikacin (http://pubchem.ncbi.nlm.nih.gov).

1.6.2. Fluoroquinolones

Fluoroquinolones are nalidixic acid derivatives that contain fluorine (Neu, 1987). The structures of quinolones contain a 4-oxo-1,4-dihydroquinolone nucleus (Fig. 1.11). The new generation fluoroquinolones (gatifloxacin and moxifloxacin) possess a methoxy addition to the carbon 8 and display lower MICs than the older generation (levofloxacin, ciprofloxacin, and ofloxacin) (Ji, *et al*., 1998). The new generation fluoroquinolones are currently in clinical trials with the aim of reducing the 6 month treatment to 4 months (Stehr, *et al*., 2014). Fluoroquinolones act on the bacterial topoisomerases II and IV, thus inhibiting DNA synthesis (Cole, *et al*., 1998). Resistance is commonly due to mutations in the quinolone

resistance-determining region where the interaction between the DNA gyrase and drug takes place (*gyrA and gyrB)* (Ginsburg, *et al*., 2003).

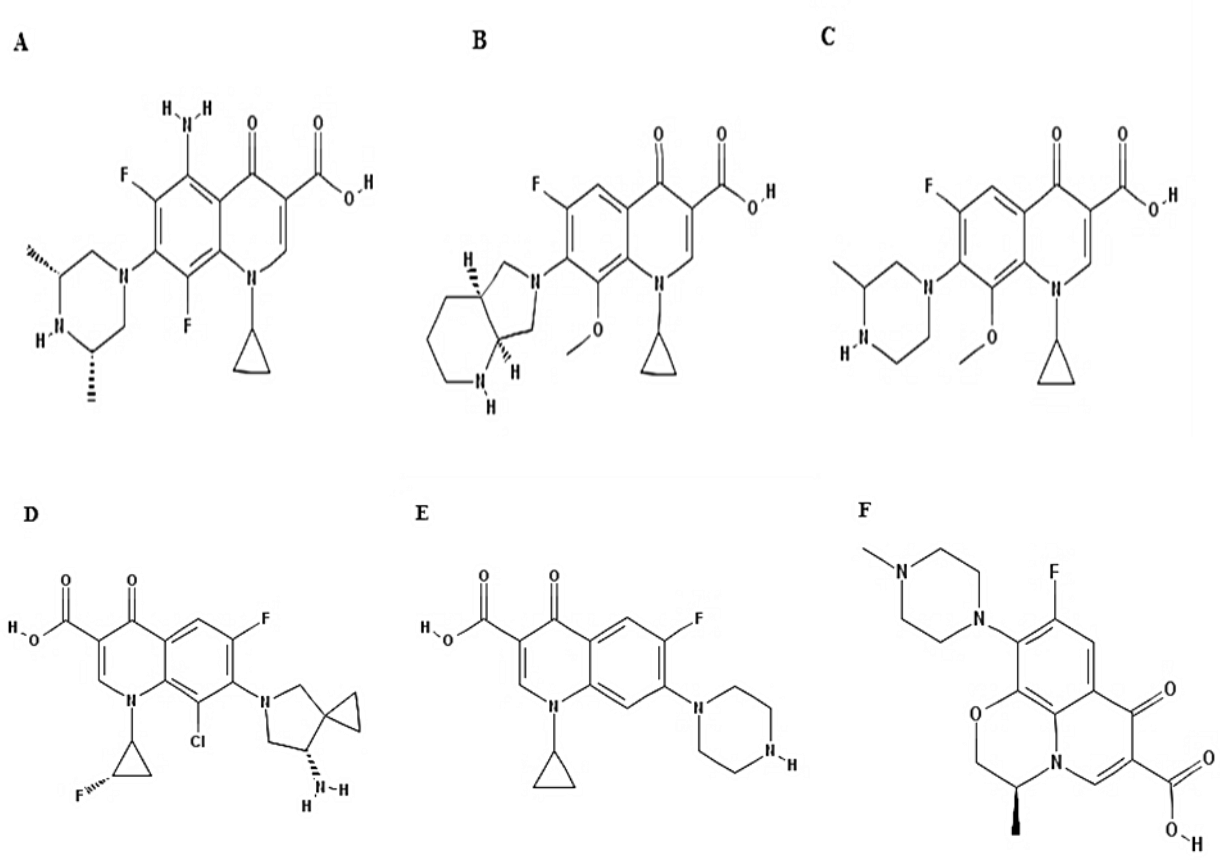


Fig. 1.11. Fluoroquinolone structures; A: Sparfloxacin, B: Moxifloxacin, C: Gatifloxacin, D: Sitafloxacin, E: Ciprofloxacin, F: Ofloxacin (http://pubchem.ncbi.nlm.nih.gov).

1.6.3. Ethionamide

Ethionamide is a structural analog of INH. INH contains a carbonyl functional group and ethionamide contains sulphur double bonded to the carbon at same position (Fig. 1.12). Monooxygenase EthA (*ethA*) is responsible for the activation of ethionamide (Baulard, *et al*., 2000). The mechanism of action of ethionamide is similar to INH. Common mutations in the

ethA or *inhA* genes lead to the development of resistance in this antibiotic (Morlock, *et al*., 2003).

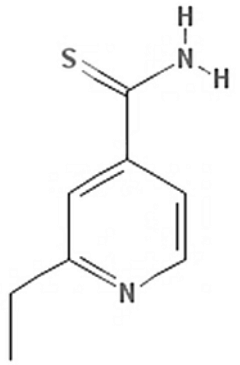


Fig. 1.12. The structure of ethionamide (http://pubchem.ncbi.nlm.nih.gov).

1.6.4. Capreomycin

Capreomycin (Fig. 1.13) was isolated from *Streptomyces capreolus* and is a macrocyclic polypeptide antibiotic (Karakousis, 2009). The mechanism of action of capreomycin involves the inhibition of protein synthesis via alteration of ribosomal structures at the 16S rRNA (Wade and Zhang, 2004). Resistance to capreomycin has been attributed largely to mutations in the *tlyA* gene which encodes 2'-O-methyltransferase of 16S rRNA (Johansen, *et al*., 2006) and the *rrs* gene (Taniguchi, *et al*., 1997).

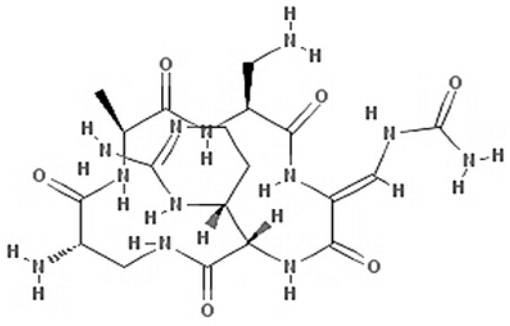


Fig. 1.13. The structure of capreomycin (http://pubchem.ncbi.nlm.nih.gov).

1.6.5. Cycloserine

Cycloserine (Fig. 1.14) is a structural analogue of d-alanine and it acts by inhibiting d-alanine racemase (AlrA) and d-alanine: d-alanine ligase (Ddl) enzymes that play a role in peptidoglycan synthesis (Caceres, *et al*., 1997). In *M. smegmatis* and *Mycobacterium bovis BCG*, resistance was due to a multicopy vector overexpressing AlrA and Ddl. However, it is still unclear if this mechanism is also present in *M. tuberculosis* **(**Feng and Barletta, 2003).

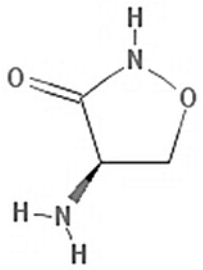


Fig. 1.14. The structure of cycloserine (http://pubchem.ncbi.nlm.nih.gov).

1.6.6. Para-aminosalicylic acid

The antituberculous activity of para-aminosalicylic acid (PAS) was discovered in the 1940s. PAS (Fig. 1.15) contains an aromatic amide with carboxyl and hydroxyl substitution (Eastlake and Barach, 1949). However, due to gastrointestinal toxicity, its use has been limited. The mechanism of action of PAS involves the inhibition of the folate biosynthesis by replacing the substrate of dihydropteroate synthase (Chakraborty, *et al*., 2013; Zheng, *et al*., 2013). In addition, folate biosynthesis is inhibited by dihydrofolate synthase, which generates a hydroxyl dihydrofolate antimetabolite which inhibits dihydrofolate reductase enzymatic activity (Zheng, *et al*., 2013). Resistance to PAS is due to mutations in the *thyA*, *folC* and *ribD* genes. The *thyA* gene encodes the enzyme thimidylate synthetase which plays a role in the folate biosynthesis pathway (Rengarajan, *et al*., 2004), whilst the *folC* and *ribD* genes

encode dihydrofolate synthase and riboflavin biosynthesis protein respectively (Zheng, *et al*., 2013).

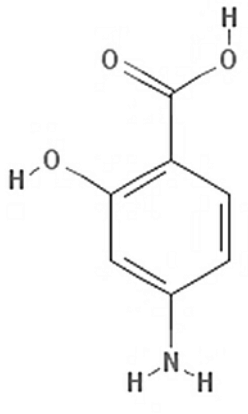


Fig. 1.15. The structure of PAS (http://pubchem.ncbi.nlm.nih.gov).

1.7. Drug pipeline

Bedaquiline is the first new class of drug to be approved by the FDA in the last 40 years. This is mainly due to the decline of TB incidence in industrialized countries like the United States of America and in Europe. The result of this decline was the reduction of active research and development of new drugs despite the increasing TB burden in the developing nations in sub-Saharan Africa, India and China. When XDR-TB emerged in 2005, it was virtually untreatable because of the lack of effective drugs. However, in recent years there have been 10 compounds that are being processed in the clinical development pipeline (Fig. 1.16) (Working group on new TB drugs, 2013).

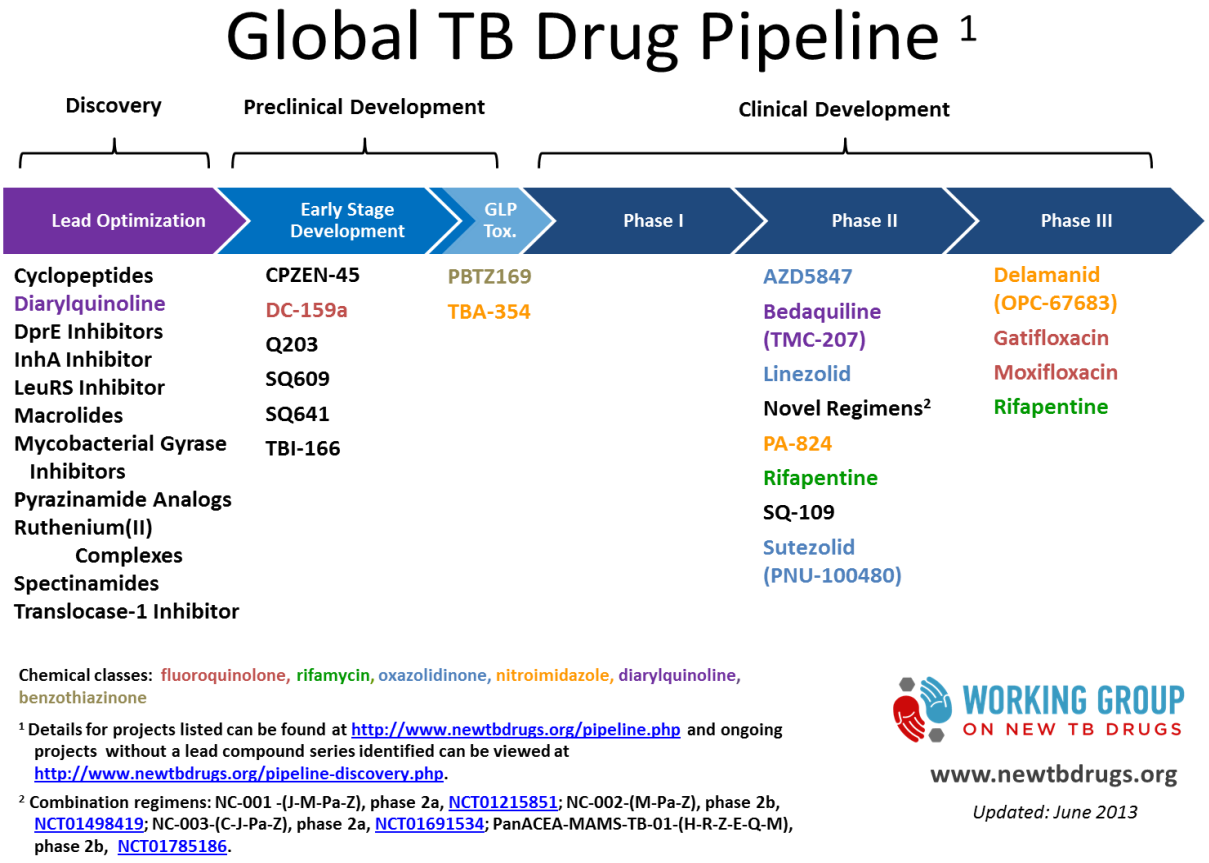


Fig. 1.16. Current TB drug pipeline (Working group on new TB drugs, 2013).

1.7.1. Rifamycin

Rifapentine (Fig. 1.17) belongs to the rifamycin class of antibiotics and has demonstrated long-lasting ability for intracellular accumulation. When rifamycin is compared to rifapentine the latter shows better bacteriostatic and bactericidal activities than the former in both broth and agar culture. This action was also observed in human monocyte-derived macrophages that were infected by *M. tuberculosis* (Rosenthal, *et al*., 2007).

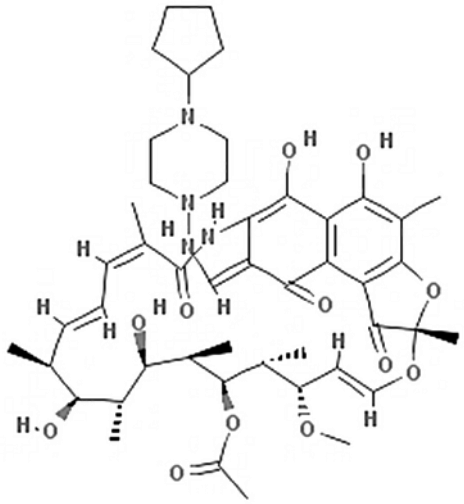


Fig. 1.17. The structure of rifapentine (http://pubchem.ncbi.nlm.nih.gov).

1.7.2. Nitroimidazopyrans

PA-824 (Fig. 1.18) which belongs to the nitroimidazopyrans class, displays activity against clinical isolates of MDR as well as drug susceptible *M. tuberculosis.* Activity was also observed against *M. tuberculosis* grown in an oxygen depleted model; thus, it could potentially be active against latent TB (Lenaerts, *et al*., 2005). The mechanism of action of PA-824 involves the inhibiting of the terminal step in mycolic acid synthesis (Stover, *et al*., 2000) and in addition, the generation of reactive nitrogen species by des-nitroimidazole metabolites (Singh, *et al*., 2008). Resistance is attributed to mutations in the *Rv0407* and *Rv3547* genes that encode the activating enzymes, F420-dependent glucose-6-phosphate dehydrogenase and deazaflavin-dependent nitroreductase respectively (Kolyva and Karakousis, 2012).

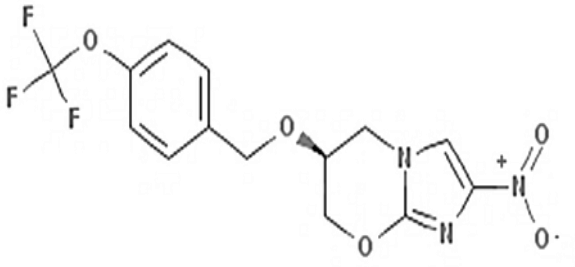


Fig. 1.18. The structure of PA-824 (http://pubchem.ncbi.nlm.nih.gov).

1.7.3. Ethylenediamine

SQ-109 (Fig. 1.19) belongs to the ethylenediamine class and displays activity against drug susceptible, ethambutol-resistant and MDR- *M. tuberculosis* (Protopopova, *et al*., 2005). The mechanism of action of SQ-109 involves the inhibition of the membrane transporter (MmpL3) of trehalose monomycolate which is the precursor of cell wall mycolates (Tahlan, *et al*., 2012).

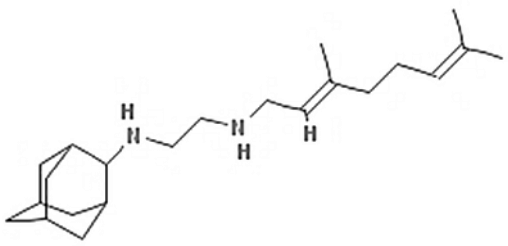


Fig. 1.19. The structure of SQ-109 (http://pubchem.ncbi.nlm.nih.gov).

1.7.4. Diarylquinoline

Bedaquiline (Fig. 1.20), a diarylquinoline, previously called R207910 and TMC-207, demonstrated activity against drug susceptible and MDR- *M. tuberculosis* (Timmerman, *et al*., 2005). Bedaquiline inhibits adenosine tri-phosphate (ATP) synthase (Andries, *et al*., 2005). Bedaquiline is the first new class of drug to be approved by the FDA in the last 40 years. Side effects of bedaquiline include tissue accumulation, induction of arrhythmia and elevated transaminase levels (Cohen, 2013).

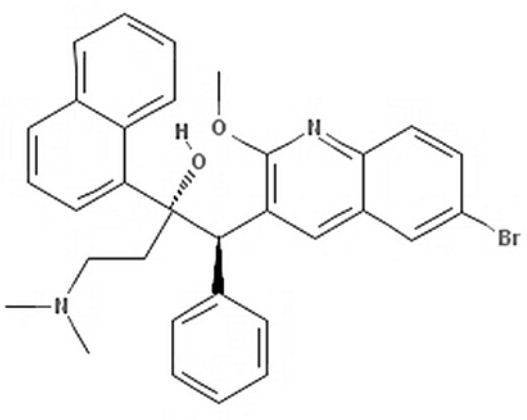


Fig. 1.20. The structure of bedaquiline (http://pubchem.ncbi.nlm.nih.gov).

1.7.5. Nitroimidazoles

OPC-67683 (Delamanid) (Fig. 1.21), belonging to the nitroimidazoles class, demonstrated activity against both drug susceptible and MDR-*M. tuberculosis* (Matsumoto, *et al*., 2006). In addition to inhibiting mycolic acid biosynthesis, its use in combination drug treatment regimens was reported to potentially result in shorter treatment duration (Matsumoto, *et al*., 2006). Delamanid displays mild to moderate QT interval (measure of the time between start of the Q wave and the end of the T wave in the heart) prolongation (Diacon, *et al.*, 2011).

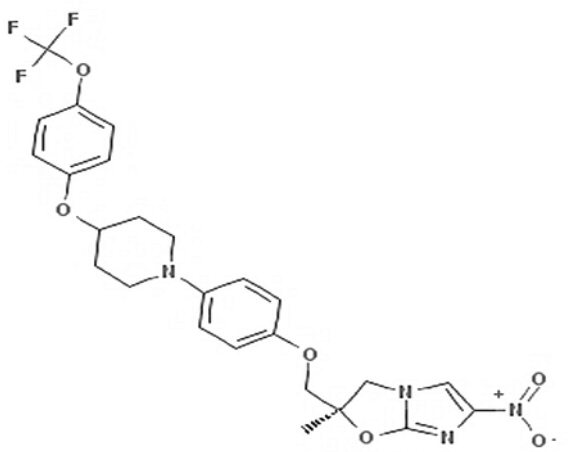


Fig. 1.21. The structure of OPC-67683 (http://pubchem.ncbi.nlm.nih.gov).

1.7.6. Oxazolidinone

Linezolid (Fig. 1.22.A), AZD-5847 and PNU-100480 (sutezolid) (Fig. 1.22.B) belong to the oxazolidinone class. Linezolid demonstrated activity against drug susceptible as well as MDR- *M. tuberculosis* (Erturan and Uzun, 2005). The mechanism of action of linezolid is the inhibition of protein synthesis (Zhang, 2005). AZD-5847 was expected to display better activity against *M. tuberculosis* than linezolid. Sutezolid use in combination drug treatment regimens with second line drugs was also expected to result in shorter treatment duration, including against MDR- and XDR-TB strains (Williams, *et al*., 2009). Linezolid has displayed numerous adverse effects such as anemia, neutropenia, optic neuropathy, peripheral neuropathy and rhabdomyolysis (Lee, *et al*., 2012).

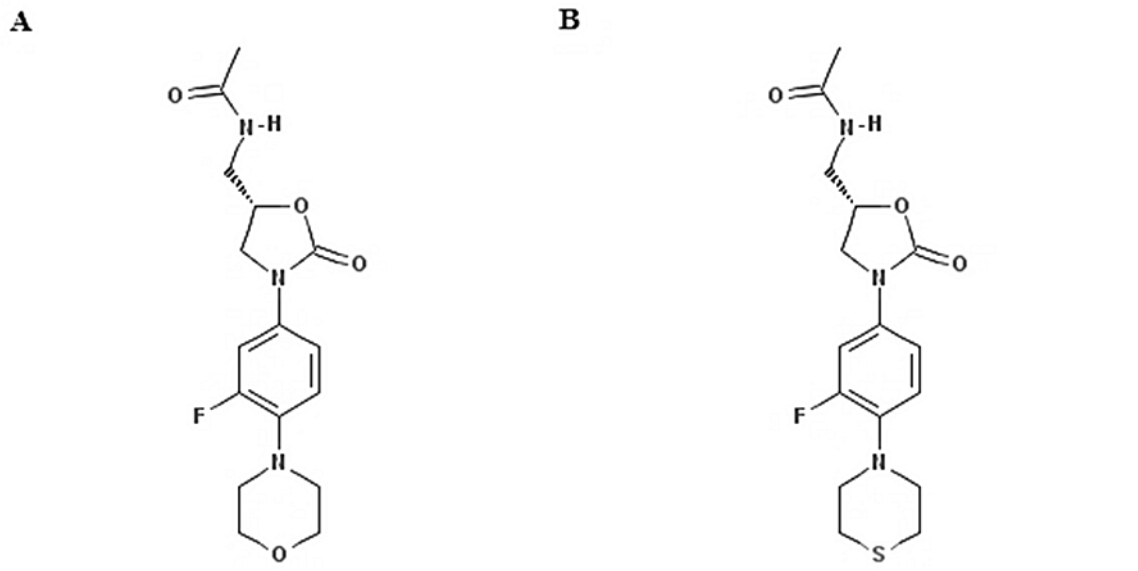


Fig. 1.22. The structure of A: linezolid and B: Sutezolid (http://pubchem.ncbi.nlm.nih.gov).

1.8. Synthetic Compounds

Synthetic compound libraries are an important reservoir of possible lead compounds. Synthetic compounds offer the advantage of chemical modification leading to improvement of lead compound properties (for example: fluoroquinolones (Sharma, *et al*., 2009b)), aiding

in the fight against resistance (Bevan, *et al*., 1995). In an effort to reduce the current treatment regimen duration, the ideal drug or combination of drugs should be able to permeate the bacterial cell wall, lesions and immune cells that are characteristic niches of bacterial subpopulations (Dartois and Barry 3rd, 2013; Dartois, 2014). However, some synthetic compounds also display inability to permeate the cell wall of bacteria and might display cytotoxicity against eukaryotic cells (Cole and Riccardi, 2011) and therefore *in vitro* screening is a vital step in drug discovery. Screening of synthetic compound libraries have yielded some promising anti-TB drug candidates such as bedaquiline and SQ-109 (Kolyva and Karakousis, 2012).

1.8.1. Chalcones

Chalcones belong to the class of flavonoids (Dhar, 1981). The basic structure of a chalcone is "C-15, α, β, unsaturated carbonyl compounds that contain two aromatic rings" (Fig. 1.23) (Nowakowska, 2007). Chalcones with various substitution patterns have displayed a range of biological activity against bacteria (Nielsen, *et al*., 2004), malaria (Li, *et al*., 1995), fungi (Lopez, *et al*., 2001), cancer (Narender and Gupta, 2004), *leishmania* (Wu, *et al*., 2003) and viruses (Mishra, *et al*., 2001). The crystal structure of chalcones has a dihedral angle between the two phenyl rings of 13.0 (1)°, and a dihedral angle from the plane of C7/C8/C9 to the C1 to C6 of A ring of 2.6 (1)° and C1 to C6 of B ring of 13.8 (1)°, respectively. This indicates that the central C7-C8-C9 fragment lies nearly in the phenyl ring plane of B ring carbons, but rather further away from the other benzene ring of A ring. The arene hydrogen bonds along the *c* axis make the molecule form a zigzag chain and a three-dimensional network is formed by the intermolecular hydrogen bonding interactions (Wu, *et al*., 2006).

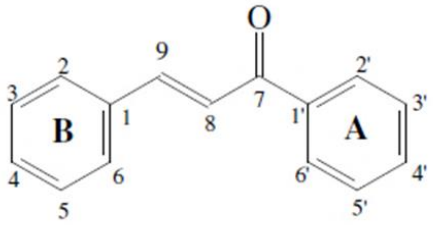


Fig. 1.23. Basic chalcone structure (Moodley, 2011).

1.8.1.1. Anti-Cancer activity of chalcones

Hydroxy substituted chalcones displayed increased activity against tumours and methyl substituted chalcones decreased activity against tumours (Anto, *et al*., 1995). Methoxy substitution at positions 3' and 5' contributed to inhibition of breast cancer resistance protein, but had a negative effect at position 4'. However, methoxy groups at positions 3', 4', and 5' of the B-ring markedly increased cytotoxicity (Valdameri, *et al*., 2012). Thiophene chalcones exhibited better inhibition of MDA-MB231 breast cancer cells than chalcones derived from bioisoteric replacement of furan chalcones (Solomon and Lee, 2012). Indolyl chalcones; 3,4,5-trimethoxyphenyl, 4-pyridyl and N,N-dimethylphenyl displayed superior selectivity and activity against cancer (Kumar, *et al*., 2010). A chalcone with an A ring amino substitute displayed potent and selective inhibition of breast, cervical, ovarian, lung, liver, colorectal, nasopharyngeal, erythromyeloblastoid and T-lymphoblastoid cancer cells (Mai, *et al*., 2014). Halogenated chalcones; 1-(4-Bromo-phenyl)-3-(2,4-dimethoxy-phenyl)-propenone, 3-(2-Fluoro-6-methoxy-phenyl)-1-(4-phenoxyphenyl)-propenone and 3-(2-Bromo-5-methoxy-phenyl)-1-(4-methoxyphenyl)-propenone displayed inhibition of colon cancer cell line (Jain, *et al*., 2014).

1.8.1.2. Antioxidant activity of chalcones

Chalcones with 2,4,5-Trimethoxy substitutions (Shenvi, *et al*., 2013), olefin chalcones (Bandgar, *et al*., 2012), chalcones that contained a hydroxy and methoxy group on the phenyl moiety (Naik, *et al*., 2013) and chalcone fatty acid esters (Lahsasni, *et al*., 2014) possess antioxidant activity. Zinc chalcone complexes are better antioxidants than copper chalcone complexes (El Sayed Aly, *et al*., 2014). Studying the antioxidant activity of a series of polyphenolic 2'-hydroxychalcones revealed that the hydroxyl group in position 5' induced the highest antioxidant activity and 2,2',5'-trihydroxychalcone is a potent and selective antioxidant (Rossi, *et al*., 2013).

1.8.1.3. Antibacterial activity of chalcones

The following substitutions in the chalcones: C-4 hydroxyl, C-40 oxygenated and C-30 isoprenoid have demonstrated antibacterial activity and the substitution of C-20 hydroxyl group could be involved in the stability of the chalcone structure (Ávila, *et al*., 2008). The following substitutions have demonstrated increased activity: hydroxyl in B ring, any substitution at R4, and benzyloxy in the A-ring. The substitution of chlorine in A-ring leads to decreased activity (Sivakumar, *et al*., 2010). Chalcones with bromo and chloro substitutions displayed potent antibacterial activity (Abdullah, *et al*., 2014). Hydrazone derivatives displayed improved antibacterial activity compared to their chalcone precursors (Fadare, *et al*., 2014). Thiazole-based chalcones displayed activity against both gram negative and positive bacteria (Liaras, *et al*., 2011). Substitutions of electron donating groups like methoxy and hydroxy, exhibited potent antibacterial activity (Parikh and Joshi, 2013). Activity of heterocyclic chalcone analogues on *Staphylococcus aureus* seems to require a free hydroxyl group in position 2'/3' in the B ring (Tran, *et al*., 2012). Fluorine substitution on the

phenyl ring in the 5-chlorothiophene moiety displayed increased antibacterial activity (Kumar, *et al*., 2013).

1.8.1.4. Antifungal activity of chalcones

Electron withdrawing groups that were substituted in their para substitution in B-ring resulted in antifungal activity. Disruption of the cell wall could possibly be the possible mechanism of action (Sivakumar, *et al*., 2009). Bromo substituents in chalcones containing 5-chlorothiophene moiety displayed decreased antifungal activity (Kumar, *et al*., 2013). Chalcones containing a benzimidazole motif and chloro, bromo, and nitro substitutions displayed weak antifungal activity (Parikh and Joshi, 2013). Chalcone based sulfone and bisulfone derivatives with chloro, bromo and nitro susbstitutions displayed potent activity against *Candida albicans* (Konduru, *et al*., 2013). Chalcones containing a phenothiazine ring and electron donating group substitutions (Saranya and Ravi, 2013) wand 6-quinolinyl N-oxide chalcones (De Carvalho Tavares, *et al*., 2011) displayed antifungal activity.

1.8.1.5. Anti-HIV activity of chalcones

The chalcone analogs displayed decreased activity compared to their unsubstituted thiophenyl derivatives, whilst the derivatives that contain a methyl and halo group substition at position 5’ of thiophenyl ring displayed improved activity against HIV (Rizvi, *et al*., 2012). The 3-keto salicylic acid chalcone derivatives with aryl ring B with a 2,3,6-trichloro substitution display activity against HIV (Sharma, *et al*., 2011). Quinolinyl chalcones with 2,5-dichlorothiophenyl moieties or benzofuran ring only displayed potent anti-HIV-1 activity (Rizvi, *et al*., 2014).

1.8.1.6. Anti-malarial activity of chalcones

Acetylenic chalcones with a methoxy group ortho to the acetylenic group displayed activity against the W2 strain of *Plasmodium falciparum* (Hans, *et al*., 2010). Sulfonamide 4-metychalcone derivatives require a morpholine ring substitution for optimal binding site interaction (De Oliveira, *et al*., 2013). The 4-aminoquinolinyl-chalcone amides displayed activity against *P. falciparum* (Smit and N'da, 2014). Chalcone derivatives with methoxy groups at position 2' and 4' displayed better antimalarial activity than other methoxy-substituted chalcones (Yadav, *et al*., 2012).

1.8.1.7. Antileishmanial activity of chalcones

Chalcones with methoxy di-ortho substitution at phenyl ring A and chlorine atom at phenyl ring B displayed antileishmanial activity (Bello, *et al*., 2011). Sulfonamide 4-methoxychalcone derivatives with benzylamino derivative displayed better antileishmanial activity than pentamidine derivatives (Andrighetti-Frohner, *et al*., 2009). Oxygen analogs of dihydrochalcones displayed antileishmanial activity (Ansari, *et al*., 2012).

1.8.1.8. Anti-TB activity of chalcones

Naphthyl groups (Chiaradia, *et al*., 2008), quinolinyl (Sharma, *et al*., 2009a), acetylenic (Hans, *et al*., 2010), benzyloxyl and imidazolyl (Marrapu, *et al*., 2011), triazolyl (Anand, *et al*., 2012), halogens (Lin, *et al*., 2002), dimethylamino, methoxy, thiomethyl, nitro and hydroxyl groups (Sivakumar, *et al*., 2007), nitro and methoxy (Trivedi, *et al*., 2008) substitutions have displayed the ability to increase the activity of chalcones against *M. tuberculosis*. Substitutions of halogens at a specific position on the A ring (R4) resulted in the inactivation of the activity of the derivatives (Sivakumar, *et al*., 2007). Recently, chalcones that were conformationally restricted displayed enhanced activity when compared to the

analogues containing open chains. This could be a result of the molecular flexibility of these compounds (Ahmad, *et al*., 2013). Chalcones with a benzyloxy moiety and 2,4-difluoro and 2,4-dichloro substituent displayed high selectivity for *M. tuberculosis* (Marrapu, *et al*., 2011). The effect of fluorine substitution on the basic structure on the activity of chalcones against *M. tuberculosis* has not yet been fully explored. The variation of drug susceptibility testing methods in different studies makes it challenging to ascertain the exact level of activity.

1.8.2. Fluorine substitution

Fluorine substitution in compounds has been previously shown to resolve solubility and metabolic degradation problems (Begue and Bonnet-Delpon, 2008). In addition, it confers lipophilicity to the compound, thereby allowing for improved permeation of lipid membranes (Smart, 2001). The MIC of a fluorinated chalcone against *Staphylococcus aureus* was 2 μM compared to 75 μM of its non-fluorinated derivative (Nielsen, *et al*., 2004).

1.9. Drug susceptibility testing (DST)

Screening of high volumes of compounds entails rapid detection of the growth of *M. tuberculosis* when different compounds at varying concentrations are used, thus enabling rapid Minimum Inhibitory Concentration (MIC) determination. Methods for detection of the growth of *M. tuberculosis* include conventional agar and broth methods, automated methods and colorimetric methods. Colorimetric methods such as the MTT, Alamar Blue and resazurin dyes have bridged the gap between rapid, automated methods such as BACTEC and mycobacterial growth indicator tube method (MGIT) and low cost conventional methods such as the proportion agar and broth based methods. Recently, a survey amongst investigators at laboratories involved with *in vitro* evaluation of potential anti-TB drugs showed that one third of them used visual readouts in liquid culture, and alamar blue or resazurin was used for assays in microtitre plate format (Franzblau, *et al*., 2012). Although

bedaquiline was discovered using *M. smegmatis*, studies have shown that only 20- 50% of compounds that displayed activity against *M. smegmatis* are active against *M. tuberculosis* and therefore it is recommended that *M. tuberculosis* be used for screening new anti-TB drugs (Goldman, 2013).

1.9.1. Conventional Methods

1.9.1.1. Agar methods

The absolute, resistant ratio and proportion agar methods have been used as conventional drug susceptibility testing methods for *M. tuberculosis* (Canetti, *et al*., 1963). The absolute method can be carried out using broth, agar media or Lowenstein-Jensen medium. MICs are obtained via comparison of growth in drug free media and media with varying concentrations of drug.

The resistant ratio method involves reporting a ratio of MICs of the test isolate and a susceptible laboratory strain (H37Rv) (Canetti, *et al*., 1963). The proportion method calculates the drug resistant mutants in a population from comparison of growth on media with and without drugs. The agar methods are cumbersome and labour intensive, taking up to 21 days or longer for results to be obtained (Canetti, *et al*., 1963).

1.9.1.2. The macroscopic broth method

The macroscopic broth method entails the observation of turbidity of the broth that contains the inoculum in the presence of the test compound. However, a result is available after 21 days and, thus, is not rapid enough as a screening method (Heifets, 1996).

1.9.2. Automated methods

1.9.2.1. Mycobacterial grow indicator tube method (MGIT)

The mycobacterial growth indicator tube method utilizes tubes that contain silicone embedded with a fluorescent oxygen-quenched sensor at the bottom. Growth of mycobacteria results in use of oxygen. This expenditure of oxygen causes fluorescence which is subsequently measured (Palomino, *et al*., 1999).

1.9.2.2. BACTEC 460

The BACTEC 460 is a radiometric method of measuring the growth of bacteria. C14 labelled fatty acid is utilized by the bacteria producing radiolabeled CO_2 which is subsequently measured by the machine. Results are obtained in 5 to 7 days but the use of radiometric material makes it expensive (Roberts, *et al*., 1983).

1.9.3. Colorimetric methods

1.9.3.1. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)

In 1983, Mossmann developed a colorimetric method using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for the measurement of cell survival. This method has since been modified by Mshana, *et al*., 1998 to an assay using a 96-well microtitre plate for the detection of drug susceptibility in *M. tuberculosis*. MTT is an oxidation-reduction indicator that undergoes a colour change from yellow to violet which denotes the presence of bacterial viability (Mosmann, 1983). MTT is reduced to form formazan by NADPH, flavin adenine dinucleotide (FADH), flavin MonoNucleotide (FMNH), and nicotinamide adenine dinucleotide (NADH), but not by the cytochromes (Rampersad, 2012).

1.9.3.2. Resazurin Microtiter Assay (REMA)

Resazurin is an oxidation- reduction indicator, which is similar in mechanism to MTT but a colour change from blue to pink is indicative of bacterial viability. This method has been previously used for detection of contamination and since been adapted into a microtitre plate for detection of drug susceptibility in *M. tuberculosis* (Palomino, *et al*., 2002). MTT and REMA displayed similar efficiencies for the drug susceptibility testing against the first line anti-TB drugs. On the other hand, the colour change displayed by MTT was noted to be more pronounced, which assisted in data capture (Martin, *et al*., 2005). Resazurin was identified to be a component of Alamar blue (O'Brien, *et al*., 2000). Resazurin is reduced to resorufin by NADPH, FADH, FMNH, NADH, cytochromes, dihydrolipoamine dehydrogenase, NAD(P)H:quinone oxidoreductase and flavin reductase (Rampersad, 2012).

1.9.3.3. Alamar blue (MABA)

Alamar blue is also an oxidation- reduction indicator which works on a similar principle to the MTT assay but a colour change from blue to pink is indicative of bacterial viability (Yajko, *et al*., 1995). Resazurin, the main component of alamar blue, is reduced to resorufin by NADPH, FADH, FMNH, NADH, cytochromes, dihydrolipoamine dehydrogenase, NAD(P)H:quinone oxidoreductase and flavin reductase (Rampersad, 2012). A comparative study demonstrated that the efficiencies of both the BACTEC system and MABA assay were similar (Collins and Franzblau, 1997). Compared to the MGIT, the MABA was found to be cheaper and required less equipment. The MTT assay was reported to be a potentially more cost effective assay than Alamar blue (Collins and Franzblau, 1997).

1.10. Significance of this work

Koul, *et al.*, 2011 stated that the "ideal TB drug" should possess the ability to treat susceptible, MDR- and XDR-TB with a shorter duration, lower dose, reduced pill burden and

limited interaction with other drugs taken. In the current regimen, however, there is no drug that satisfies all of these criteria. This also applies to the promising candidates in the drug pipeline, many of which do not offer a reduction of the pill burden as they require combination treatment regimens and thus, will still not satisfy these criteria. Therefore, there is a global need for efforts to be channelled into the development of novel, more powerful and effective drugs that could possibly shorten the current drug regimen and be effective against MDR and XDR-TB, and this has been supported by the WHO initiated STOP TB STRATEGY. This has resulted in the synthesis of a large number of synthetic compounds and drug candidates that require rapid screening.

In this study the following hypotheses were formulated:

1. Fluorine substitution would improve the anti-tuberculosis activity of chalcones.
2. MTT would perform better than, and be more cost effective than the MABA for the drug susceptibility testing of chalcones.
3. The series of fluorinated and non-fluorinated chalcone compounds (Chapter 2, Page 45) that exhibited activity against H37Rv, would also be active against clinical strains of *M. tuberculosis* of varying genotype and drug susceptibility.
4. The compound activity in *M. tuberculosis* infected macrophages would be increased due to the antimicrobial activity of these cells.

Aims of study:

1. To evaluate the *in vitro* activity of novel, synthetic fluorinated and non-fluorinated chalcones against *Mycobacterium tuberculosis*.

2. To compare colorimetric methods, Alamar Blue and MTT and the macroscopic broth method for the evaluation of the *in vitro* anti-tuberculous activity of chalcones.

Specific objectives:

1. To determine the Minimum Inhibitory Concentration (MIC) of the compounds against laboratory and clinical strains, including multi- and extensively drug resistant *M. tuberculosis*.
2. To evaluate the utility of the MTT and alamar blue assays as rapid screening tests for synthetic compounds using the broth macroscopic test as the gold standard.
3. To determine the cytotoxicity of active compounds.
4. To determine the *in vitro* activity of active compounds in infected macrophages.

CHAPTER 2:

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is a rapid, cheap, screening test for the *in vitro* anti-tuberculous activity of chalcones

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Abstract:

Rapid and reliable drug susceptibility testing facilitates replenishment of the TB drug pipeline in the fight against drug resistant *M. tuberculosis*. This study compared the performance of the MTT and MABA assays on the anti-tuberculous activity of a set of chalcones. Twenty seven chalcones and chromenochalcones were screened against the laboratory strain *M. tuberculosis* H37Rv, using a microtitre plate MTT assay at 7 days. The MIC for 20 active compounds was subsequently determined using the MABA, MTT and the macroscopic broth assays at 7, 14 and 21 days. No significant difference in the MICs, or increase in the MICs was observed over time between the MABA (p = 0.209) and the MTT (p = 0.207) assays, in contrast to the gold standard, the macroscopic broth assay (p = 0.000). The MICs (16 to >128

µg/ml) were much higher than the currently used TB drugs. In conclusion, the MTT assay is a cost effective method (R0.06/well) for the rapid *in vitro* screening of chalcones against *M. tuberculosis*, producing reliable results in 8 days. The chalcone with a MIC of 16 µg/mL shows promise as a potential lead compound and should be investigated further.

Introduction:

Mycobacterium tuberculosis, the etiological agent of tuberculosis (TB) has resulted in an estimated 8.6 million new cases and 1.3 million deaths in 2012 (WHO, 2013a). Diagnostic delays, due to the slow growth rates of the pathogen and a lack of rapid point of care tests leading to delays in treatment, have been largely responsible for this high disease burden. High rates of HIV co-infection and the emergence of multiple and extensive drug resistance (MDR and XDR) have compounded this problem and pose a serious threat to TB control (Gillespie, 2002). MDR- and XDR-TB are associated with prolonged treatment periods that favour patient noncompliance (Duncan, 2003), second line drug therapy which is toxic and costly (O'Brien and Spigelman, 2005), and low cure and high mortality rates (WHO, 2013a).

Limited progress had been achieved in identifying and developing new drugs and compounds to combat TB in the past 40 years. The emergence of virtually untreatable MDR and XDR strains of *M. tuberculosis* (Zager and McNerney, 2008) has stimulated a renewed interest in drug discovery and development, resulting in a number of promising candidates. The current drug pipeline consists of the repurposed drugs gatifloxacin, moxifloxacin, linezolid and metronidazole, as well as new compounds OPC-67683, PA-824, SQ-109, PNU-100480, AZD-5847 and TMC-207 (Stehr, *et al*., 2014). TMC-207 (Bedaquiline) is a diarylquinoline and the first new class of drug to be approved by the FDA in the last 40 years (Cohen, 2013). The fluoroquinolones, gatifloxacin and moxifloxacin are currently in phase III TB clinical trials, whilst the nitroimidazoles, metronidazole, PA-824 and OPC-67683 are in

phase II and III clinical TB trials respectively. SQ-109, an analogue of ethambutol is currently in phase II clinical trials. Linezolid, PNU-100480 (phase II clinical trials) and AZD-5847 (phase I clinical trials) belong to the class of oxazolidinones (Stehr, *et al.*, 2014).

Major pharmaceutical companies possess compound libraries of thousands of compounds (Goldman, 2013). Similarly, medicinal and organic chemistry research groups synthesise compounds in efforts to target various diseases and these compounds form a library of possible lead compounds. High throughput screening of 70 000 compounds using a whole cell assay against *Mycobacterium smegmatis* led to the discovery of bedaquiline (Andries, *et al*., 2005). This highlights the role of high throughput screening as an essential step in the rapid production of lead compounds. The expense associated with high throughput screening has been overcome by the use of colorimetric methods such as Microplate-based Alamar Blue Assay (MABA) (Collins and Franzblau, 1997), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Martin, *et al*., 2005) and resazurin microtitre assay (REMA), also referred to as resazurin method (Martin, *et al*., 2005). Antimicrobial testing of compounds using these methods have the advantage of speed, low cost and ease of performance compared to the BACTEC, and agar and broth based methods (Yajko, *et al*., 1995; Collins and Franzblau, 1997; Abate, *et al*., 1998).

Chalcones belong to the class of flavonoids (Dhar, 1981). Chalcones with various substitution patterns have displayed a range of biological activity against bacteria (Nielsen, *et al*., 2004), malaria (Li, *et al*., 1995), fungi (Lopez, *et al*., 2001), cancer (Narender and Gupta, 2004) and *leishmania* (Wu, *et al*., 2003), as well as *M. tuberculosis* (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013). Fluorine substitution in compounds has been previously shown to resolve solubility and metabolic degradation problems (Begue and Bonnet-Delpon, 2008). In

addition, it confers lipophilicity to the compound, thereby allowing for improved permeation of lipid membranes (Smart, 2001). The MIC of a fluorinated chalcone against *Staphylococcus aureus* was reduced to 2 μM compared to that of 75 μM displayed by its non-fluorinated derivative (Nielsen, *et al*., 2004).

Drug susceptibility methods used to screen chalcones include the MABA assay (Trivedi, *et al*., 2008; Hans, *et al*., 2010), luciferase reporter phage assay (Sivakumar, *et al*., 2007), agar proportion method (Sharma, *et al*., 2009a; Marrapu, *et al*., 2011), BACTEC 460 radiometric assay (Lin, *et al*., 2002; Ahmad, *et al*., 2013), enzyme kinetic assays (Chiaradia, *et al*., 2008; Mascarello, *et al*., 2010) and macroscopic broth assay using Sauton's media (Anand, *et al*., 2012). The performance of the MTT colorimetric assay in comparison to the MABA has not yet been evaluated on chalcones. Comparison of the MTT and REMA assay noted that the distinct colour change of the MTT assay facilitated visual reading and REMA assay did not require the solubilisation of precipitate step (Martin, *et al*., 2005). A comparison of MABA and MTT performance in cytotoxicity assays on eukaryotic cells displayed that the former was slightly more sensitive than the latter assay for most compounds tested (Hamid, *et al*., 2004). However, unpublished data by Collins and Franzblau suggest that the MTT assay is more cost effective than the Alamar Blue assay (Franzblau, *et al*., 1998), an important consideration in high throughput testing in poorly resourced countries. In the present study, we hypothesized 1: that fluorine substitution would improve the anti-tuberculosis activity of chalcones and 2: that both MTT would perform better than, and be more cost effective than the MABA for the drug susceptibility testing of chalcones. We screened 27 synthetic chalcone compounds, including those with fluorine substituents for anti-TB activity by the MTT assay initially. We then compared the performance of the MABA and MTT assays by measuring the MIC's of the 20 active compounds for anti-TB activity using the macroscopic broth assay as the gold standard.

Materials and Methods:

Compounds

The chalcones (Table 2.1) were synthesized by Moodley, 2011, using the base catalysed Claisen –Schmidt condensation method with the exception of compounds IV and XVI (acid catalysed).

Preparation of compounds

Stock solutions (51.2 mg/mL) of the compounds dissolved in dimethyl sulfoxide (DMSO) (Merck) were stored at -80 ^{o}C.

Preparation of standardized inoculum

Experimental work was carried out in Biosafety 2+ Laboratory at Medical Microbiology and Infection Control at the University of KwaZulu-Natal, South Africa. Infectious material was handled only in Class II biosafety cabinets and personal protective equipment (N95 masks or Powered air-purifying respirators) were worn during this time.

The laboratory strain *M. tuberculosis* H37Rv (ATCC 27294) was grown aerobically at 37 ^{o}C with shaking in Middlebrook 7H9 (Difco) broth containing 0.2% (v/v) glycerol (Sigma), 0.05% (v/v) Tyloxapol (Sigma), and 10% (v/v) oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson) to an optical density $(OD)_{600nm}$ of 1 (approximately 3 x 10^8 organisms per mL) (Larsen, *et al*., 2007). This was serially diluted in broth to a concentration of 1 x 10^6 organisms per mL for use in the assays. Serial dilutions of an aliquot was plated onto Middlebrook 7H11 (Difco) agar plates to obtain the number of colony forming units (CFU) per mL (Larsen, *et al*., 2007).

Assay controls

Positive (isoniazid (Sigma) and rifampicin (Sigma) at 1 µg/mL with inoculum, negative (inoculum only and no compound/drug) and solvent only (inoculum only and DMSO (Merck)) controls were included in all assays. A drug only control was included for macroscopic broth test.

MTT screening assay

Prior to performing the comparative assays, the activity of all 27 compounds (Table 2.1), including the intermediate, was evaluated against the laboratory strain *M. tuberculosis* H37Rv, using the MTT assay (Martin, *et al*., 2005). The assay was performed in triplicate on the same day. Briefly, aliquots of 100 µL of Middlebrook 7H9 (Difco) broth were added to each well of a 96-well microtitre plate (Porvair). The stock compounds were diluted two-fold in the broth, resulting in a range of 128 µg/mL to 0.125 µg/mL. The inoculum (100 µL) was added to the appropriate wells (final concentration of 1 x 10^6 organisms/mL). Plates were incubated at 37 °C for 7 days. Thereafter, 10 µL of the MTT (Sigma) solution (5 mg/mL) was added to the wells followed by overnight incubation at 37 °C. If a violet precipitate was observed, 50 µL SDS-DMF [20% sodium dodecyl sulfate (SDS) (Sigma) and 50% of N,N-dimethylformamide (DMF) (Sigma)] was added and the plate was incubated for another 3 h. The MIC was defined as the lowest concentration devoid of a colour change from yellow to purple (Fig. 2.1).

Comparison of the MABA, MTT and macroscopic broth assays

In order to compare performance of the 3 techniques, MICs of the 20 active compounds were determined in triplicate in 3 separate biological assays.

MABA and MTT assays

The MABA (Collins and Franzblau, 1997) and MTT assays (Martin, *et al*., 2005) were carried out with minor modifications. The 96 well microtitre plates were prepared as described for the screening assay. Plates were incubated at 37 °C for 7, 14 and 21 days respectively. For the MABA assay, after the addition of 10 µL of Alamar blue (Invitrogen) to the wells, plates were incubated at 37 °C for 24 h. The MIC was defined as the lowest concentration without a colour change from blue to pink (Fig. 2.1). The MTT conditions were described in the screening assay.

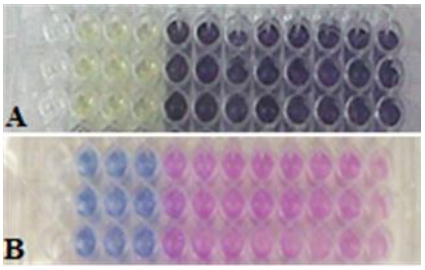


Fig. 2.1. Example of colour changes of MTT (A) and MABA (B). A positive test is denoted by a colour change to purple for the MTT and pink in the MABA assays.

Macroscopic broth based tube method

The macroscopic broth based tube method was carried out as previously described with minor modifications (Onajole, *et al*., 2009). Two-fold dilutions of the compounds were prepared in sterile 30 mL universal tubes (Sterilin) containing Middlebrook 7H9 (Difco) broth supplemented with 0.2% (v/v) glycerol (Sigma), 1.0 g of Casitone (Difco) per litre, and 10% (v/v) OADC (Becton Dickinson). Aliquots of 100 µL of standardized inoculum were added to each tube, followed by incubation for 21 days at 37 °C. Results were documented macroscopically every 7 days. The MIC was defined as the tube equal in turbidity to the drug only control.

Statistical analysis

The MIC values were converted to log (base 2). All statistics were calculated using SPSS software 21.0. (IBM). One-way analysis of variance (ANOVA) was used for comparisons of mean log2 (MIC) for each method at different time points and to each other.

Ethics approval

Approval was granted by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (BE107/12).

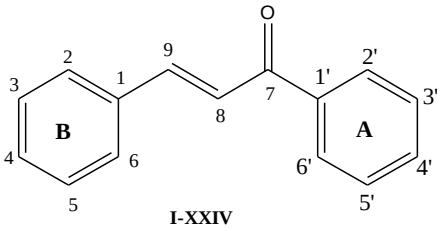
Results:

MTT screening assay

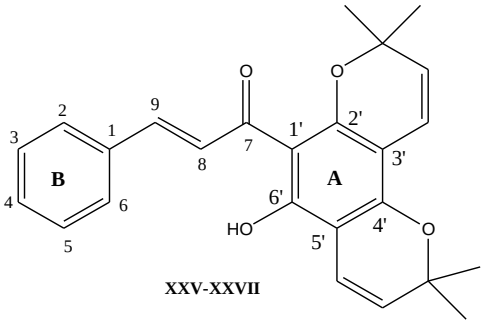
Of the 27 compounds screened, 20 possessed activity against H37Rv with MICs ranging from 32 to 64 µg/mL (Table 2.1). The remaining 7 compounds, including the intermediate (I), were inactive against the susceptible laboratory strain at the highest concentration tested.

Table 2.1. The *in vitro* anti-tuberculous activity of chalcones and the effect of substituents on activity against *M. tuberculosis* H37Rv using the MTT assay.

Compound numeral	Name of compound	A ring Substituents	B ring Substituents	MIC (µg/mL)
I	Chromene Intermediate			No activity
II	1,3-di(phenyl) prop-2-ene-1-one	-	-	32
III	1-(4-methoxyphenyl)-3-phenyl prop-2-ene-1-one	4'-OMe	-	32
IV	1-(4-hydroxyphenyl)-3-phenyl prop-2-ene-1-one	4'-OH	-	32
V	1-(4-fluorophenyl)-3-(phenyl) prop-2-ene-1-one	4'-F	-	32
VI	1-(2-fluoro-4-methoxyphenyl)-3-phenyl prop-2-ene-1-one	4'-OMe, 2'-F	-	32
VII	1-(3-fluoro-4-methoxyphenyl)-3-phenyl prop-2-ene-1-one	4'-OMe, 3'-F	-	No activity
VIII	1-phenyl-3-(2-chlorophenyl) prop-2-ene-1-one	-	2-Cl	32
IX	1-phenyl-3-(4-fluorophenyl) prop-2-ene-1-one	-	4-F	32
X	1-phenyl-3-(4-fluoro-2-chlorophenyl) prop-2-ene-1-one	-	2-Cl, 4-F	32
XI	1-phenyl-3-(6-fluoro-2-chlorophenyl) prop-2-ene-1-one	-	2-Cl, 6-F	32
XII	1-(4-fluorophenyl)-3-(4-fluorophenyl) prop-2-ene-1-one	4'-F	4-F	32
XIII	1-(4-methoxyphenyl)-3-(4-fluorophenyl) prop-2-ene-1-one	4'-OMe	4-F	No activity
XIV	1-(2-fluoro-4-methoxyphenyl)-3-(4-fluorophenyl) prop-2-ene-1-one	4'-OMe,2'-F	4-F	32
XV	1-(3-fluoro-4-methoxyphenyl)-3-(4-fluorophenyl) prop-2-ene-1-one	4'-MeO,3'-F	4-F	64
XVI	1-(4-hydroxyphenyl)-3-(4-fluorophenyl) prop-2-ene-1-one	4'-OH	4-F	32
XVII	1-(4-fluorophenyl)-3-(2-fluoro-3-methoxyphenyl) prop-2-ene-1-one	4'-F	2-F, 3-OMe	32
XVIII	1-(4-fluorophenyl)-3-(2-chlorophenyl) prop-2-ene-1-one	4'-F	2-Cl	32
XIX	1-(4-fluorophenyl)-3-(4-fluoro-2-chlorophenyl) prop-2-ene-1-one	4'-F	2-Cl, 4-F	32
XX	1-(4-fluorophenyl)-3-(2-chloro-6-fluorophenyl) prop-2-ene-1-one	4'-F	2-Cl, 6-F	64
XXI	1-(4-methoxyphenyl)-3-(2-chlorophenyl) prop-2-ene-1-one	4'-OMe	2-Cl	32
XXII	1-(2-fluoro-4-methoxyphenyl)-3-(2-chlorophenyl) prop-2-ene-1-one	4'-OMe, 2'-F	2-Cl	32
XXIII	1-(4-methoxyphenyl),3-(4-fluoro-2-chlorophenyl) prop-2-ene-1-one	4'-OMe	2-Cl,4-F	32
XXIV	1-(2-fluoro-4-methoxyphenyl)-3-(4-fluoro-2-chlorophenyl)prop-2-ene-1-one	4'-OMe, 2'-F	2-Cl,4-F	No activity
XXV	1-(dichromenophenyl)-3-(phenyl) prop-2-ene-one	-	-	No activity
XXVI	1-(dichromenophenyl)-3-(4-fluorophenyl) prop-2-ene-one	-	4-F	No activity
XXVII	1-(dichromenophenyl)-3-(2-fluorophenyl) prop-2-ene-one	-	2-F	No activity



I-XXIV



XXV-XXVII

Comparison of the MICs of the MABA, MTT and Macroscopic broth assays at 7, 14 and 21 days

Day 7

The median MICs of thirteen compounds were identical in all 3 assays at 7 days (Table 2.2). A 2-fold dilution change was observed in the MICs of compounds II and XVII in the MABA and VI, XI and XXIII in the macroscopic broth assay compared to the other assays. The MIC of compound XV in the macroscopic broth assay differed from the others by a 4-fold dilution. The MICs of compound XIX were discordant in all 3 assays with a difference of a 2-fold and 4-fold dilution in the MTT and Macroscopic Broth Assays respectively compared to MABA.

Day 14

The median MICs of nine compounds were identical in all 3 assays at 14 days (Table 2.3). The MICs of compounds (XII, XVIII, XX, XXI and XXII) and (II, VIII, XI, XIV and XIX) and compound III in the macroscopic broth assay differed from the MTT and MABA by a 2-; 4; and >4-fold dilution respectively.

Day 21

The median MICs of only 2 compounds were concordant in all 3 assays at this time-point (Table 2.4). The MICs in the macroscopic broth assay differed from the other 2 by a 4-; 8-; >2-; >4-fold dilution for the rest of the compounds respectively: (II, VIII, IX, X, XIV, XVII and XXII); (XI); (XX and XXIII); and (III, V, VI, XVIII and XIX). There was a 4-fold and 2-fold difference in the MIC of XII in the macroscopic broth assay compared to the MABA and the MTT assay respectively, whilst that of XV was greater than the MICs of both colorimetric assays. Compound XXI MIC for the macroscopic broth assay was >4 times that of MABA and >2 of MTT.

Table 2.2. Comparison of the MIC of 20 compounds using the MABA, MTT and Macroscopic Broth assays on Day 7.

	MIC (µg/mL)					
	MABA Day 7		MTT Day 7		Macroscopic Broth Day 7	
Compounds	**Median**	**% COV**	**Median**	**% COV**	**Median**	**% COV**
II	16	12	32	12	32	0
III	32	9	32	9	32	7
IV	32	0	32	0	32	0
V	32	7	32	0	32	12
VI	16	12	16	12	32	0
VIII	32	11	32	11	32	10
IX	32	9	32	11	32	9
X	32	0	32	0	32	7
XI	16	12	16	12	32	7
XII	32	0	32	0	32	7
XIV	32	11	32	7	32	7
XV	128	8	128	8	32	0
XVI	32	7	32	14	32	0
XVII	16	12	32	11	32	7
XVIII	32	0	32	11	32	0
XIX	16	8	32	19	64	7
XX	64	9	64	9	64	0
XXI	32	11	32	11	32	0
XXII	32	12	32	11	32	7
XXIII	32	9	32	9	64	0
Average		7.95		8.85		4.35

Table 2.3. Comparison of the MIC of 20 compounds using the MABA, MTT and Macroscopic Broth assays on Day 14.

	MIC (µg/mL)					
	MABA Day 14		MTT Day 14		Macroscopic Broth Day 14	
Compounds	**Median**	**% COV**	**Median**	**% COV**	**Median**	**% COV**
II	32	0	32	8	128	5
III	32	13	32	9	>128	14
IV	32	7	32	7	32	0
V	32	0	32	9	32	9
VI	32	7	32	7	32	8
VIII	32	9	32	11	128	8
IX	32	11	32	0	32	0
X	32	9	32	0	32	0
XI	16	12	16	12	64	8
XII	32	13	32	9	64	8
XIV	32	9	32	9	128	0
XV	128	13	128	7	128	7
XVI	32	0	32	7	32	0
XVII	32	12	32	12	32	9
XVIII	32	7	32	0	64	17
XIX	32	12	32	9	128	8
XX	64	10	64	8	128	7
XXI	32	11	32	7	64	12
XXII	32	11	32	16	64	0
XXIII	64	9	64	9	64	8
Average		8.75		7.8		6.4

Table 2.4. Comparison of the MIC of 20 compounds using the MABA, MTT and Macroscopic Broth assays on Day 21.

	MIC (µg/mL)					
	MABA Day 21		MTT Day 21		Macroscopic Broth Day 21	
Compounds	**Median**	**% COV**	**Median**	**% COV**	**Median**	**% COV**
II	32	11	32	11	128	0
III	32	9	32	13	>128	7
IV	32	9	32	10	32	0
V	32	8	32	9	>128	12
VI	32	9	32	0	>128	0
VIII	32	11	32	11	128	10
IX	32	7	32	0	128	9
X	32	0	32	0	128	7
XI	16	12	16	12	128	7
XII	32	10	64	9	128	7
XIV	32	9	32	9	128	7
XV	128	7	128	7	>128	0
XVI	32	0	32	7	32	0
XVII	32	9	32	11	128	7
XVIII	32	0	32	0	>128	0
XIX	32	11	32	13	>128	7
XX	64	8	64	8	>128	0
XXI	32	9	64	16	>128	0
XXII	32	9	32	9	128	7
XXIII	64	9	64	9	>128	0
Average		7.85		8.2		4.35

Comparison of the MABA assay at 7, 14 and 21 days

The MICs of 15 compounds were identical at all three time points (Table 2.5). A 2-fold increase in the MIC of compounds II, VI, XVII, XIX and XXIII was observed on day 14 compared to day 7. However, the MIC of these 5 compounds remained the same on day 21 compared to day 14.

Comparison of the MTT assay at 7, 14 and 21 days

The MICs of 16 compounds were identical at the three time points (Table 2.5). The MICs of compounds VI and XXIII increased by a 2-fold dilution on day 14 when compared to day 7 and remained the same on day 21. A 2-fold dilution increase in the MIC was observed for compounds XII and XXI on day 21 compared to days 7 and 14.

Comparison of the macroscopic broth assay at 7, 14 and 21 days

Only 2 of the 20 compounds displayed identical MICs at the three time points (Table 2.5). MICs increased with time for 18 compounds: (II, VIII and XIV) 4-fold dilution increase on days 14 and 21; (III) >4-fold dilution increase on days 14 and 21; (V and VI) >4-fold dilution increase on day 21 compared to days 7 and 14; (IX, X and XVII) 4-fold dilution on day 21 compared to days 7 and 14; (XI, XII and XXII) 2-fold dilution increase on each of days 14 and 21; (XV) 4-fold and >4-fold dilution increases on days 14 and 21; (XVIII and XXI) 2-fold and >4-fold dilution increases on days 14 and 21; (XIX and XX) 2-fold and >2-fold dilution increases on days 14 and 21; (XXIII) >2-fold dilution increase on day 21 compared to days 7 and 14.

MABA, MTT and Macroscopic Broth assay comparison

The mean log2 (MIC) for the MABA and MTT method were similar (p = 1.000), whereas that of the macroscopic broth assay compared to MABA (p = 0.000), and MTT (p = 0.000) were significantly different. The mean log2 (MIC) for the macroscopic broth assay (p = 0.000) increased significantly over time compared to MABA (p = 0.209) and MTT (p = 0.207).

Inter and Intra assay variability

The greatest variation observed among the 3 technical repeats within each assay and among assays repeated on 3 different days, was a 2x fold increase or decrease. The average percentage coefficient of variation between replicates for the MABA, MTT and Macroscopic broth assay at the three time points were under 10% (Tables 2.2, 2.3 and 2.4).

Table 2.5. Comparison of the MIC of 20 compounds using the MABA, MTT and Macroscopic Broth assay on Days 7, 14 and 21.

	MIC (Median, µg/mL)								
	MABA			MTT			Macroscopic Broth		
Compound	**Day 7**	**Day 14**	**Day 21**	**Day 7**	**Day 14**	**Day 21**	**Day 7**	**Day 14**	**Day 21**
II	16	32	32	32	32	32	32	128	128
III	32	32	32	32	32	32	32	>128	>128
IV	32	32	32	32	32	32	32	32	32
V	32	32	32	32	32	32	32	32	>128
VI	16	32	32	16	32	32	32	32	>128
VIII	32	32	32	32	32	32	32	128	128
IX	32	32	32	32	32	32	32	32	128
X	32	32	32	32	32	32	32	32	128
XI	16	16	16	16	16	16	32	64	128
XII	32	32	32	32	32	64	32	64	128
XIV	32	32	32	32	32	32	32	128	128
XV	128	128	128	128	128	128	32	128	>128
XVI	32	32	32	32	32	32	32	32	32
XVII	16	32	32	32	32	32	32	32	128
XVIII	32	32	32	32	32	32	32	64	>128
XIX	16	32	32	32	32	32	64	128	>128
XX	64	64	64	64	64	64	64	128	>128
XXI	32	32	32	32	32	64	32	64	>128
XXII	32	32	32	32	32	32	32	64	128
XXIII	32	64	64	32	64	64	64	64	>128

Discussion:

The TB drug pipeline requires a constant supply of new drug candidates due to the efficiency of *M. tuberculosis* in acquiring resistance to anti-TB drugs. Sensitive and rapid drug susceptibility testing methods are essential to test the vast number of compounds produced in response to this need and to obtain valuable structure-activity relationship information timeously.

Numerous drug susceptibility testing (DST) methods have been used to evaluate the activity of chalcones against *M. tuberculosis* (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Sharma, *et al*., 2009a; Hans, *et al*., 2010; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013). Both the MABA (Collins and Franzblau, 1997) and the MTT (Martin, *et al*., 2005) assays that are based on colorimetric dyes have been reported to significantly reduce the time to determine *in vitro* activity of novel drug candidates compared to conventional DSTs. However, to our knowledge, they have not been compared for use with chalcones.

In this study, no significant difference in the MICs, or increase in the MICs over time, was observed between the MABA and the MTT assays in contrast to the gold standard, the macroscopic broth assay. Concordance between the MABA and tetrazolium microplate (TEMA) assays was also reported for the MICs of rifampin and isoniazid against *M. tuberculosis*. The difference between TEMA and other MTT assays is that the MTT salt in the former is dissolved in ethanol and solubilisation with SDS-DMF is not required (Caviedes, *et al*., 2002).

The MABA and MTT assay displayed 75% and 80% concordance respectively at the 7 day time intervals. For both assays, a 7 day initial incubation and 1 day incubation with dye seemed to suffice for MIC determination, similar to other studies on standard anti-TB drugs (Collins and Franzblau, 1997; Martin, *et al*., 2005). In our study, the MICs of 15 (75%) and

18 (90%) of 20 compounds in the MABA and MTT assays respectively, remained identical on days 7 and 14, suggestive of the superiority of the latter. On day 14, concordance of both assays was 100%. Therefore, the potential for inaccurate lower MIC reporting has to be taken in cognisance of the context of the need for rapid turnaround times. These findings indicate the need for evaluation of rapid tests on novel anti-tuberculous candidates in specific settings prior to their implementation as high throughput screening assays.

MICs obtained by the gold standard macroscopic broth assay at day 7 were identical to those on day 14 for 18 compounds for the MABA/MTT assays. The MICs of 15 compounds on day 7 were identical to the MABA/MTT assays at the same interval. Macroscopic broth results for days 14 and 21 remained the same as day 7 for only 8 (40%) and 2 (10%) of the compounds respectively. This increase in the MICs with time may have resulted from the precipitation of all compounds, with the exception of IV and XVI in Middlebrook 7H9 broth, despite completely dissolving in DMSO during stock preparation. The precipitation may have been due to binding to protein components in the 7H9 broth or solubility problems. The ensuing challenges posed by the precipitation in macroscopic visualisation of the results were overcome by the inclusion of a drug and broth only control. It is highly likely that the precipitation could have led to time dependent sub-optimal availability of the active ingredient and/or evolution and selection of drug resistant mutants over the incubation period leading to the high MICs. This effect could have been significantly amplified in the larger volume of broth used in the macroscopic broth assay compared to the microtitre plate methods. Reliance solely on the macroscopic broth assay to evaluate the activity of this subset of compounds would have led to the false results, thus highlighting the significant role of compound properties in the choice of a drug susceptibility test. Modification of compounds with even slight activity can be potentially yield a potent drug candidate.

The MTT reagent is significantly cheaper (R0.06/well) compared to the MABA reagent (R1.31/well). Moreover, the colour change is more outstanding even in absence of the SDS-DMF, such that the insoluble precipitate allows for an unequivocal interpretation of MICs. Unpublished, preliminary data by Collins and Franzblau cited by Franzblau *et al*. 1998 also alluded to the cost effectiveness of the MTT assay.

The *in vitro* anti-tuberculous activity of the subset of chalcones evaluated in this study displayed MICs much higher than the TB drugs in current treatment regimen. However, one compound, XI, with constant activity at 16 μg/mL, at all 3 time intervals for both MABA and MTT, holds great promise as a potential lead compound. This compound had chloro and fluoro groups respectively at both ortho positions on the phenyl ring adjacent to the double bond. It is possible that reformulation of all the compound structures may lead to lower MICs, particularly, in light of the incomplete solubility of the compounds in the assay medium. The reformulation could include retaining the substitution on the B ring and varying the substitutions on the A ring. It must be noted however; that inclusion of a fluorine atom in the para position of the A ring (compound XX) resulted in a 4-fold decrease in activity to XI. It would be interesting to see the effects of combining this substitution pattern (as in XI) with that of the A ring in compounds III, IV and VI to see the effects of these combinations in the same assays.

Similar to the findings of Hans, *et al*., 2010, but contradictory to that of Lin, *et al*., 2002, the activity against *M. tuberculosis* in this study was not restricted to halogen substituted compounds. Addition of fluorine (F) at the 4’ position of A (V) and B ring (IX) had no effect on the activity of the parent compound (II). F substituent addition, removal and movement in compounds resulted in a loss of activity completely or increased MIC in compounds: VII, XIII, XV, XX and XXIV. The addition of F at the 4’ position of the B ring resulted in activity (XV) when compared to VII (no activity) but decreased when compared to

XIV. Both compounds VII and XV have an A ring 3'-F substituent. The addition of F at the 2' position of the A ring resulted in activity (XIV) when compared to XIII (no activity) and the activity was equivalent to XII. The addition of F at the 6' position of the B ring resulted in decreased activity (XX), but addition of F at the 4' position of the B ring instead of 6' position had no effect on activity (XIX) when compared to XVIII. The addition of F at the 2' position of the A ring resulted in a loss of activity (XXIV) when compared to XXIII, but XXII with a methoxy at 4', F at 2'of the A ring and chlorine at 2' position of the B ring and XXI with a methoxy at the 4' position of the A ring and chlorine at 2' position of the B ring displayed equivalent activity to XXIII. None of the substitutions (methoxy, hydroxyl, chlorine or F) improved activity by more than a 2-fold dilution.

Substitutions, other than fluorine, have been shown to improve activity of chalcones against *M. tuberculosis*: naphthyl groups (Chiaradia, *et al*., 2008), nitro (Trivedi, *et al*., 2008), quinolinyl (Sharma, *et al*., 2009a), acetylenic (Hans, *et al*., 2010), benzyloxyl (Marrapu, *et al*., 2011) and triazolyl (Anand, *et al*., 2012). Due to the variation of DST methods, it is difficult to compare the level of improvement of these substitutions. It is worth investigating whether these substitutions together with the substitutions made in this study, would improve chalcone activity.

In conclusion, the MTT assay is a cost effective method for the rapid *in vitro* screening of chalcones against *M. tuberculosis*, producing reliable results in 8 days. Further *in vitro*, including intracellular studies of the activity of this subset of chalcones against drug susceptible and resistant clinical isolates would be worth performing despite the high MICs. If the permeability properties of the compounds in macrophages are optimal, the combined effect of the antimicrobial properties of the cells and compound activity may serve to significantly reduce MICs. Early cytotoxicity tests would also determine the worthiness of continued investigations into these compounds.

References:

Abate G, Mshana RN, Miörner H (1998) Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 2:1011–1016

Ahmad I, Thakur JP, Chanda D, Saikia D, Khan F, Dixit S, Kumar A, Konwar R, Negi AS, Gupta A (2013) Syntheses of lipophilic chalcones and their conformationally restricted analogues as antitubercular agents. Bioorg Med Chem Lett 23:1322–1325

Anand N, Singh P, Sharma A, Tiwari S, Singh V, Singh DK, Srivastava KK , Singh BN, Tripathi RP (2012) Synthesis and evaluation of small libraries of triazolylmethoxy chalcones, flavanones and 2-aminopyrimidines as inhibitors of mycobacterial FAS-II and PknG. Bioorg Med Chem Lett 20:5150–5163

Andries K, Verhasselt P, Guillemont J, Göhlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V (2005) A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science 307:223-227

Begue JP, Bonnet-Delpon D (2008) Bioorganic and medicinal chemistry of fluorine, John Wiley and Sons, New York, pp 2-22

Caviedes L, Delgado J, Gilman RH (2002) Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. J Clin Microbiol 40:1873–1874

Chiaradia LD, Mascarello A, Purificação M, Vernal J, Cordeiro MNS, Zenteno ME, Villarino A, Nunes RJ, Yunes RA, Terenzi H (2008) Synthetic chalcones as efficient inhibitors of *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpA. Bioorg Med Chem Lett 18:6227-6230

Cohen J (2013) Approval of novel TB drug celebrated—with restraint. Science 339:130

Collins L, Franzblau SG (1997) Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 41:1004–1009

Dhar DN (1981) The chemistry of chalcones and related compounds, John Wiley & Sons, New York, pp 169

Duncan K (2003) Progress in TB drug development and what is still needed. Tuberculosis 83:201-207

Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH (1998) Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36:362-366

Gillespie SH (2002) Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob Agents Chemother 46:267-274

Goldman RC (2013) Why are membrane targets discovered by phenotypic screens and genome sequencing in *Mycobacterium tuberculosis*? Tuberculosis (Edinb) 93:569-588

Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P (2004) Comparison of alamar blue and MTT assays for high through-put screening. Toxicol In Vitro 18:703-710

Hans RH, Guantai EM, Lategan C, Smith PJ, Wan B, Franzblau SG, Gut J, Rosenthal PJ, Chibale K (2010) Synthesis, antimalarial and antitubercular activity of acetylenic chalcones. Bioorg Med Chem Lett 20:942-944

Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs WR Jr (2007) Laboratory Maintenance of *Mycobacterium tuberculosis*. Curr Protoc Microbiol 6:10A.1

Li R, Kenyon GL, Cohen FE, Chen X, Gong B, Dominguez JN, Davidson E, Kurzban G, Miller RE, Nuzum EO, Rosenthal PJ, Mckerrow JH (1995) *In vitro* antimalarial activity of chalcones and their derivatives. J Med Chem 38:5031-5037

Lin YM, Zhou Y, Flavin MT, Zhou LM, Nie W, Chen FC (2002) Chalcones and flavonoids as antituberculosis agents. Bioorg Med Chem Lett 10:2795-2802

Lopez SN, Castelli MV, Zacchino SA, Dominguez JN, Lobo G, Charris-Charris J, Cortes JCG, Ribas JC, Devia C, Rodriguez AM, Enriz RD (2001) *In vitro* antifungal evaluation and structure–activity relationships of a new series of chalcone derivatives and synthetic analogues with inhibitory properties against polymers of the fungal cell wall. J Bioorgan Med Chem 9:1999-2013

Marrapu VK, Chaturvedi V, Singh S, Singh S, Sinha S, Bhandari K (2011) Novel aryloxy azolyl chalcones with potent activity against *Mycobacterium tuberculosis* H37Rv. Eur J Med Chem 46:4302-4310

Martin A, Morcillo N, Lemus D, Montoro E, da Silva Telles MA, Simboli N, Pontino M, Porras T, León C, Velasco M, Chacon L, Barrera L, Ritacco V, Portaels F, Palomino JC (2005) Multicenter study of MTT and resazurin assays for testing susceptibility to firstline anti-tuberculosis drugs. Int J Tuberc Lung Dis 9:901–906

Mascarello A, Chiaradia LD, Vernal J, Villarino A, Guido RV, Perizzolo P, Poirier V, Wonge D, Martins PGA, Nunes RJ, Yunes RA, Andricopulo AD, Av-Gay Y, Terenzi H (2010) Inhibition of *Mycobacterium tuberculosis* tyrosine phosphatase PtpA by synthetic chalcones: Kinetics, molecular modeling, toxicity and effect on growth. Bioorgan Med Chem 18:3783-3789

Moodley T (2011) The synthesis and characterisation of fluorinated chalcones. Masters Dissertation, University of KwaZulu-Natal

Narender T, Gupta S (2004) A convenient and biogenetic type synthesis of few naturally occurring chromeno dihydrochalcones and their *in vitro* antileishmanial activity. Bioorg Med Chem Lett 14:3913-3916

Nielsen TF, Boesen T, Larsen M, Schønning K, Kromann H (2004) Antibacterial chalcones-biosteric replacement of the 4'-hydroxy group. J Bioorgan Med Chem 12:3047-3054

O'Brien RJ, Spigelman M (2005) New drugs for tuberculosis: current status and future prospects. Clin Chest Med 26:327-340

Onajole OK, Govender K, Govender P, van Helden PD, Kruger HG, Maguire GEM, Muthusamy K, Pillay M, Wiid I, Govender T (2009) Pentacyclo-undecane derived cyclic tetra-amines: Synthesis and evaluation as potent anti-tuberculosis agents. Eur J Med Chem 44:4297–4305

Sharma M, Chaturvedi V, Manju YK, Bhatnagar S, Srivastava K, Puri SK, Chauhan P (2009a) Substituted quinolinyl chalcones and quinolinyl pyrimidines as a new class of anti-infective agents. Eur J Med Chem 44:2081-2091

Sivakumar PM, Seenivasan SP, Kumar V, Doble M (2007) Synthesis, antimycobacterial activity evaluation, and QSAR studies of chalcone derivatives. Bioorg Med Chem Lett 17:1695-1700

Smart BE (2001) Fluorine substituent effects (on bioactivity). J Fluorine Chem 109:3-11

Stehr M, Elamin AA, Singh M (2014) Filling the Pipeline-New Drugs for an Old Disease. Curr Top Med Chem 14:110-129

Trivedi AR, Dodiya DK, Ravat NR, Shah VH (2008) Synthesis and biological evaluation of some new pyrimidines via a novel chalcone series. Arkivoc 11:131-141

World Health Organization (2013a) Global Tuberculosis Report 2013. http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf. Accessed 14 January 2014

Wu JH, Wang XH, Yi YH, Lee KH (2003) Anti-AIDS agents 54. A potent anti- HIV chalcone and flavonoids from genus desmos. J Bioorgan Med Chem 13:1813-1815

Yajko DM, Madej JJ, Lancaster MV, Sanders CA, Cawthon VL, Gee B, Babst A, Hadley WK (1995) Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J Clin Microbiol 33:2324–2327

Zager EM, McNerney R (2008) Multidrug-resistant tuberculosis. BMC Infect Dis 8:10

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CHAPTER 3:

Fluorinated and non-fluorinated chalcones display moderate *in vitro* extracellular activity, but poor selectivity against multiple and extensive drug resistant *Mycobacterium tuberculosis*

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Abstract:

The chalcone scaffold (1,3-diaryl-2-propen-i-ones) has the advantage of easy chemical modification and has been shown to possess biological activity against a variety of organisms, including a wide range of anti-TB activity. A series of synthetic fluorinated and non-fluorinated chalcones that previously displayed activity against the laboratory strain *M. tuberculosis* H37Rv was evaluated for extracellular activity against clinical isolates of varying drug susceptibility patterns and genotypes using the MTT assay, intracellular activity in a macrophage model and eukaryotic cytotoxicity using Vero cells. Fluorinated and non-fluorinated chalcones displayed moderate activity (32- 128 µg/mL) against MDR- and XDR-TB isolates, no significant activity against intracellular H37Rv and low selectivity for *M.*

tuberculosis. Reformulation of these compounds is essential to improve their biological activity before further testing can be conducted. Compound XIX displays the most promise in this study for reformulation to yield a potential lead compound.

Introduction:

Global Tuberculosis (TB) control is threatened by the escalating disease burden (8.6 million new cases) and corresponding high mortality (1.3 million deaths) in 2012 (WHO, 2013a). This is largely due to the prolonged, multi-drug treatment regimen, diagnostic delays, HIV co-infection (Gillespie, 2002) and the emergence of multiple and extensive drug resistance (MDR and XDR) (Zager and McNerney, 2008). Globally, approximately 450 000 new cases of MDR-TB were reported in 2012. New and previously treated cases comprised 3.6% and 20% of these respectively (WHO, 2013a). In South Africa, 15 419 patients were diagnosed in 2012 with MDR-TB, with 1.8% new and 6.7% previously treated cases. XDR-TB was detected in 9.6% of MDR-TB cases worldwide and 10% of MDR-TB cases in South Africa (WHO, 2013a). The Beijing, S and F15/LAM4/KZN strains have been commonly associated with MDR- and XDR-TB in South Africa (Chihota, *et al*., 2012). Strains with differing drug resistance profiles, phenotypic and genotypic characteristics co-exist in patients (Warren, *et al*., 2004; van Rie, *et al*., 2005) and this is associated with poor outcomes due to the fact that incorrect treatment regimens are being used (van Rie, *et al*., 2005).

This high burden of virtually untreatable drug resistant TB has escalated global efforts in developing new drugs to meet this challenge. Despite the availability of many promising repurposed drugs as well as new compounds (Stehr, *et al*., 2014), the ability of *M. tuberculosis* to rapidly acquire drug resistance (Kolyva and Karakousis, 2012) highlights the need for a steady stream of potential candidates in the drug pipeline.

Synthetic compound libraries serve as important reservoirs of possible lead compounds, with the advantage of potential chemical modification leading to improvement of antimicrobial properties such as in the evolution of fluoroquinolones (Sharma, *et al*., 2009b). Screening of synthetic compound libraries have yielded promising anti-TB drug candidates such as bedaquiline and SQ-109 (Kolyva and Karakousis, 2012). However, some synthetic compounds also display an inability to permeate the bacterial cell wall and might display cytotoxicity against eukaryotic cells (Cole and Riccardi, 2011) and therefore *in vitro* screening is a vital step in drug discovery.

Chalcones, belonging to the class of flavonoids (Dhar, 1981), have been reported to have a wide repertoire of biological activity (Li, *et al*., 1995; Lopez, *et al*., 2001; Wu, *et al*., 2003; Narender and Gupta, 2004; Nielsen, *et al*., 2004). The amenability of the chalcone scaffold (1,3-diaryl-2-propenones) that allows for easy chemical modification has been exploited in several studies to develop series of derivatives with a wide range of anti-TB activity (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013). Chalcones with promising activity include those with iodo- or chloro groups (Lin, *et al*., 2002), methoxyl and naphthyl groups (Chiaradia, *et al*., 2008), amino linked quinolinyl groups (Sharma, *et al*., 2009a), hydroxyl, methyl and methoxyl groups (Pavan, *et al*., 2009), acetylenic groups (Hans, *et al*., 2010), chloro and thiomethyl substituents (Sivakumar, *et al*., 2007) and aryloxy azolyl moieties (Marappu, *et al*., 2011).

The addition of fluorine substitutents in synthetic compounds increases solubility (Begue and Bonnet-Delpon, 2008) and lipophilicity in compounds, which improves permeability of lipid membranes (Smart, 2001). The latter property is important given the complex, highly lipid nature of the *M. tuberculosis* cell wall and membrane.

M. tuberculosis, an intracellular pathogen, encounters numerous host cells upon infection, including alveolar macrophages and epithelial cells (Smith, 2003). The organism not only survives, but also replicates in these immune cells (Armstrong and Hart, 1971). Within macrophages, *M. tuberculosis* up-regulates the expression of genes for adaptation to oxidative and nitrosative stresses, growth limitation and alternative nutrient sources and is able to resist the antimicrobial action of this cell (Schnappinger, *et al*., 2003). It is imperative therefore, that drugs targeting this pathogen possess both extracellular as well as intracellular activity. Thus, *in vitro* anti-TB screening of new compounds should also include macrophage infected models. In addition, since synthetic compounds may be cytotoxic against eukaryotic cells (Cole and Riccardi, 2011), early *in vitro* cytotoxicity screening is a vital step in drug discovery.

Evaluation of chalcones against methicillin-resistant and susceptible *Staphylococus aureus* showed that some compounds can display resistance to resistant strains even though they active against susceptible strains and interestingly even though most compounds display elevated MICs against resistant strains, two compounds displayed reduced MIC against the resistant strains compared to the susceptible strain (Osório, *et al*., 2012).

In order for a compound to be used as a possible drug candidate, it would be necessary that the compounds be active against panel of globally representative strains from different genetic clades (Franzblau, *et al*., 2012). In addition, activity against MDR and XDR-TB strains and intracellular bacteria in macrophages are desirable properties of drug candidates.

In this study, we hypothesized that the series of fluorinated and non-fluorinated chalcone compounds (Chapter 2, Page 45) that exhibited activity against H37Rv, would also be active against clinical strains of *M. tuberculosis* of varying genotype and drug susceptibility. Therefore, we evaluated the extracellular activity against clinical isolates of

varying drug susceptibility patterns and genotypes using the MTT assay, as well as intracellular activity in a macrophage model and host cytotoxicity using vero cells. We hypothesized that the compound activity in *M. tuberculosis* infected macrophages would be increased due to the antimicrobial activity of these cells.

Materials and methods:

Compounds

The chalcones were synthesised according to the procedures outlined by Moodley, 2011, and the preparation and storage of the stock solutions were described previously (Chapter 2, Page 41).

Preparation of standardized inoculum

The genotypes and the respective drug resistance profiles of 9 clinical isolates included in this study are listed in Table 3.1. Standardization of the inocula of the laboratory strain *M. tuberculosis* H37Rv and the clinical isolates was performed as previously described (Chapter 2, Page 41). The number of colony forming units (CFU) per mL of the initial inoculum was verified by plating onto Middlebrook 7H11 (Difco) agar plates.

Table 3.1. Genotype and drug susceptibility patterns of clinical strains tested in the MTT assays.

Genotype family	Strain	Resistance	Drug Resistance Profile
F15/LAM/KZN	V9124	Susceptible	
	V2475	MDR	IR
	605	XDR	IRSEOKCN
	X162	XDR	IRSEOKCN
F11	R271	MDR	IR
F28	R104	Susceptible	
	R262	XDR	IRSEOKN
Beijing	B910	Susceptible	
	R283	MDR	IR

I: isoniazid; R: rifampicin; S: Streptomycin; E: Ethambutol; O: Ofloxacin; K: Kanamycin; C: Capreomycin; N: Niacinamide

***In vitro* activity of compounds against clinical isolates (MTT assay)**

The MTT assay (Martin, *et al*., 2005) was carried out in triplicate on 3 separate occasions with minor modifications as described previously (Chapter 2, Page 42). The compounds were tested in a range of 128 µg/mL to 8 µg/mL, based on the activity against H37Rv in the screening assay. Assay controls for susceptible isolates were isoniazid (INH) (Sigma) and rifampicin (RIF) (Sigma) at 1 µg/mL. MICs were determined for INH (Sigma) and RIF (Sigma) against MDR and XDR isolates using a range of 32- 0.125 µg/mL. A range of 128-0.5 µg/mL was tested to ascertain the MICs of kanamycin (KANA) (Sigma) and ofloxacin (OFLOX) (Sigma). The inoculum (100 µL) was added to the appropriate wells (final concentration of 1 x 10^6 organisms/mL). Plates were incubated at 37 °C for 7 days. Thereafter, 10 µL of the MTT (Sigma) solution (5 mg/mL) was added to the wells followed by overnight incubation at 37 °C. Growth only control (negative) and solvent controls were also included for all the compounds tested. The MIC was defined as the lowest concentration in which a purple precipitate was absent.

Intracellular activity compounds IV, VI, XI, XVI and XIX in a macrophage model

The intracellular activities of 5 compounds (IV, VI, XI, XVI and XIX) that displayed activity against all strains tested in the MTT assay were evaluated in the human THP-1 macrophage model with modifications (Matsumoto, *et al*., 2006). The THP-1 monocytic cell line (ATCC TIB-202) was cultured in RPMI-1640 medium (Lonza) containing 10% fetal bovine serum (FBS) (Biowest). Ninety six- well plates (Porvair) were seeded with 1 x 10^6 cells/mL and were differentiated into macrophages with 20 ng/mL phorbol myristate acetate (PMA) (Sigma) for 1 day at 37 °C and 5% CO_2.

Log phase cultures of the laboratory strain *M. tuberculosis* H37Rv were centrifuged at 2,000*g* for 20 min (Heraeus Multifuge 3S-R Centrifuge, Thermo Scientific) and reconstituted in fresh tissue culture media. The differentiated macrophages were infected at a multiplicity of infection (MOI) of 10 for 4 h at 37 °C. Post-infection, cells were washed twice with 200 µL phosphate-buffered saline (PBS) (Oxoid) in order to remove non-adherent bacteria and treated with each of the above mentioned compounds at concentrations of 128 µg/mL to 8 µg/mL. After incubation for 3 days, cells were lysed using 0.1% Triton X-100 (Sigma) and serial dilutions were plated on 7H11 (Difco) agar to obtain a count of viable bacteria. Colony forming units were counted after 21 days incubation at 37 °C and 5% CO_2. Assay controls included a negative (untreated), solvent only control and RIF and INH at 1 µg/mL activity against intracellular H37Rv.

The cytotoxicity of compounds IV, VI, XI, XVI and XIX on vero cells

The cytotoxicity of compounds on vero cells was assessed according to Falzari, *et al*., 2005 with modification. Five compounds (IV, VI, XI, XVI and XIX) used in the intracellular assay were evaluated for cytotoxicity against vero cells. The vero cell line (ATCC number: CRL-

1586) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Lonza) and 10% FBS (Biowest) at 37 °C until confluent.

Ninety six-well plates (Porvair) were seeded with 1 x 10^6 cells/mL and incubated till 80% confluent. Monolayers were washed with PBS (Oxoid) and DMEM (Lonza) containing compounds at concentrations (32- 8 µg/mL) was added to the respective wells, followed by incubation for 3 days at 37 °C and 5% CO_2. Post-incubation, compound containing-media was removed and monolayers washed with PBS (Oxoid). This was followed by the addition of 100 µl of DMEM (Lonza) and 20 µl of the dye MTS-PMS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and phenylmethasulfazone] (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit, Promega). After incubation for 3 h at 37 °C and 5% CO_2, absorbance was determined at 450 nm using the GloMax system (Promega). Percent cytotoxicity was calculated using the following equation: percent cytotoxicity =100 - [(OD of sample/OD of control) x 100%].

Calculation of the selectivity index of compounds for *M. tuberculosis*

The selectivity index (SI) of compounds for *M. tuberculosis* is the ratio of IC_{50} divided by MIC_{50}. The MIC_{50} is defined as the median MIC of 50% of the isolates tested, whilst the IC_{50} is the concentration of drug that inhibits 50% of healthy vero cells based on dose response cytotoxicity data.

Statistical analysis

All experiments were performed in triplicate at three independent times. MTT assay results were expressed as the median of replicates. Intracellular activities in the macrophage model were expressed as mean CFU/mL and analysed by one-way analysis of variance (ANOVA) to ascertain significant differences between treated and untreated macrophages. Cytotoxicity

in vero cells was expressed as a percentage and analysed using ANOVA to ascertain significant differences between compounds at specific concentrations. All statistical analysis was performed using SPSS software 21.0. (IBM) and a *p*-value of less than 0.05 was considered statistically significant.

Ethics approval

Approval was granted by the Biomedical Research Ethics Committee, University of KwaZulu-Natal (BE107/12).

Results:

***In vitro* activity of compounds against clinical isolates**

Susceptible clinical isolates: R104, V9124 and B910

All compounds with the exception of 2 inhibited all 3 strains at MICs of either 32 or 64 ug/mL (Table 3.2). Identical MICs were observed for all compounds against R104 and V9124, with 2 of the compounds at 64 and >128 µg/mL, and the rest at 32 µg/mL respectively. The MICs of 9 compounds against B910 were identical to the other two strains at 32 µg/mL. A 2-fold increase in the MIC at 64 µg/mL was obtained for 9 compounds against B910 compared to the two susceptible strains. Compound XV was inactive at the highest concentration tested against all three strains whilst compound XXIII was inactive against B910 only.

MDR isolates: V2475, R271 and R283

Twelve compounds were able to inhibit all three MDR strains at MICs of 32 or 64 µg/mL (Table 3.2). Identical MICs were obtained by 17 of the 19 active compounds against V2475 and R283. A 2-fold dilution difference in the MIC was observed for the other 2 compounds.

No activity was observed for 8 compounds against R271. The MICs of 4 and 1 compounds were identical for all three strains at 32 and 64 µg/mL respectively. The MIC of 1 compound was identical at 64 µg/mL against both R271 and R283. Eight compounds were inactive against V2475. A 2-fold increase in the MIC was observed for 6 compounds against R271 compared to the other two strains. All three MDR strains were resistant to RIF and INH with MICs at >32 µg/mL for V2475 and R271 and 16 µg/mL for R283.

XDR isolates: 605, X162 and R262

Only 2 compounds, II and VIII, inhibited all three XDR strains at 32 µg/mL, whilst 1, XV was inactive at the highest concentration against all three strains (Table 3.2). Six compounds were active against both 605 and R262 at a MIC of 32 µg/mL. MICs of 64 µg/mL were observed for 4 compounds against 605 and X162, and for 1 compound against X162 and R262. Two compounds, XX and XXIII, were active at a MIC of 128 µg/mL against 605 and X162, and 605 and R262 respectively. MICs of 128 and >128 µg/mL were also observed for compounds XXI and XXII respectively against X162. The MIC of III varied for all 3 strains. RIF and KANA were inactive at the highest concentration tested and OFLOX showed inhibition at a MIC of 8 µg/mL for all three XDR-strains. INH was inactive at the highest concentration tested for 605 and X162 and displayed a MIC of 32 µg/mL against R262.

Table 3.2. The *in vitro* activity (MIC) of 20 compounds[1] against the laboratory strain H37Rv and clinical isolates determined by the MTT assay.

	Median MIC (µg/mL)									
		Susceptible			MDR			XDR		
Compound	**H37Rv**	**R104$^{\alpha}$**	**V9124***	**B910$^{\#}$**	**V2475***	**R271$^{\beta}$**	**R283$^{\#}$**	**605***	**X162***	**R262$^{\alpha}$**
II	32	32	32	64	32	64	32	32	32	32
III	32	32	32	64	32	>128	32	64	>128	32
IV	32	32	32	32	32	64	32	32	64	32
V	32	32	32	64	32	64	64	32	64	64
VI	16	32	32	64	32	>128	32	64	64	32
VIII	32	32	32	32	32	64	32	32	32	32
IX	32	32	32	32	32	32	32	64	64	32
X	32	32	32	64	32	32	32	64	64	64
XI	16	32	32	32	32	64	32	32	64	32
XII	32	32	32	32	32	>128	32	32	64	32
XIV	32	32	32	64	32	>128	32	32	64	32
XV	128	>128	>128	>128	>128	>128	>128	>128	>128	>128
XVI	32	32	32	32	32	32	32	32	64	32
XVII	32	32	32	32	32	32	32	32	64	32
XVIII	32	32	32	64	32	64	32	64	64	32
XIX	32	32	32	32	32	64	32	64	64	32
XX	64	64	64	64	64	64	64	128	128	64
XXI	32	32	32	64	32	>128	32	32	128	64
XXII	32	32	32	64	64	>128	32	64	128	64
XXIII	32	32	32	>128	64	>128	64	128	>128	128
RIF	ND	ND	ND	ND	>32	>32	16	>32	>32	>32
INH	ND	ND	ND	ND	>32	>32	16	>32	>32	32
OFLOX	ND	ND	ND	ND	ND	ND	ND	8	8	8
KANA	ND	ND	ND	ND	ND	ND	ND	>128	>128	>128

[1] Compounds I, VII, XII, XIV, XXV, XXVI and XXVII were not tested against clinical isolates as they displayed no activity against H37Rv (Chapter 2, Page 45).

α : F28; * :F15/LAM/KZN; # : Beijing; β: F11; ND: Not Determined

Intracellular activity compounds IV, VI, XIX and XVI in macrophage model

Five compounds were selected for determining intracellular activity based on their performance against all isolates tested. None of the compounds at all concentrations tested displayed significant reduction of mean CFU/mL when compared to untreated macrophages (Fig. 3.1). Intracellular growth in the 5 lower concentrations of compound IV was higher than the untreated control. Overall, the average activity of the other 4 compounds was lower than or similar to the untreated controls, with the exception of XVI with higher intracellular growth at 8 ug/mL. However, none of these intracellular growth increases was statistically different from the untreated control. RIF (p = 0.002) and INH (p = 0.000) displayed significant reduction of the mean CFU/mL compared to untreated macrophages.

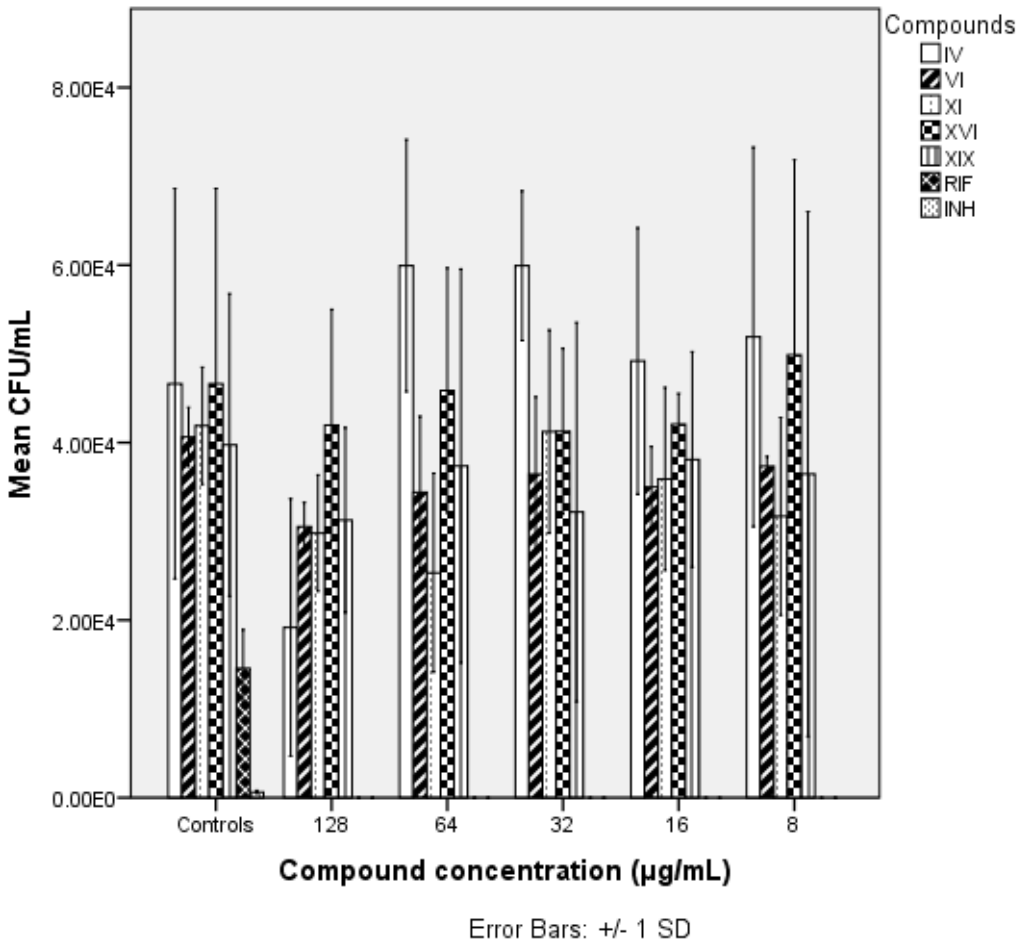

Fig. 3.1. The intracellular activity of compounds IV, VI, XI, XVI, XIX, RIF and INH in the THP-1 macrophage model. THP-1 macrophages were exposed to compounds at concentrations ranging from 128- 8 µg/mL and RIF and INH at 1 µg/mL for 3 days at 37 °C. Results are expressed as mean CFU/mL derived from triplicate assays on 3 separate days. The error bars represent ± standard deviation. Treated and untreated mean CFU/ mL were evaluated with one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant.

The cytotoxicity of compounds IV, VI, XI, XVI and XIX on vero cells

The cytotoxicity of the 5 compounds ranged between 64-74% at 32 µg/mL, 13-66% at 16 µg/mL, and 8-14% at 8 µg/mL respectively (Fig. 3.2). At 32 µg/mL, compound XIX was significantly less toxic than IV (p = 0.012), XI (p = 0.009) and XVI (p = 0.023), but was similar to VI (p = 1.000). At 16 µg/mL, compound XIX displayed significantly lower cytotoxicity than all 4 others (p = 0.000). At 8 µg/mL, cytotoxicity of compound XIX was significantly lower than IV (p = 0.000) and XVI (p = 0.000), but similar to compounds VI (p = 1.000) and XI (p = 1.000). The MIC_{50} of the 5 compounds range between 32 to 64 µg/mL, whilst the cytotoxicity levels are also correspondingly high at 64 to 74%. The MIC_{50} of the 5 compounds was 32 µg/mL and the IC_{50} ranged between 10.06- 28.25 µg/mL (Table 3.2). The selectivity index of the compounds for *M. tuberculosis* over the vero cells ranged from 0.31-0.88.

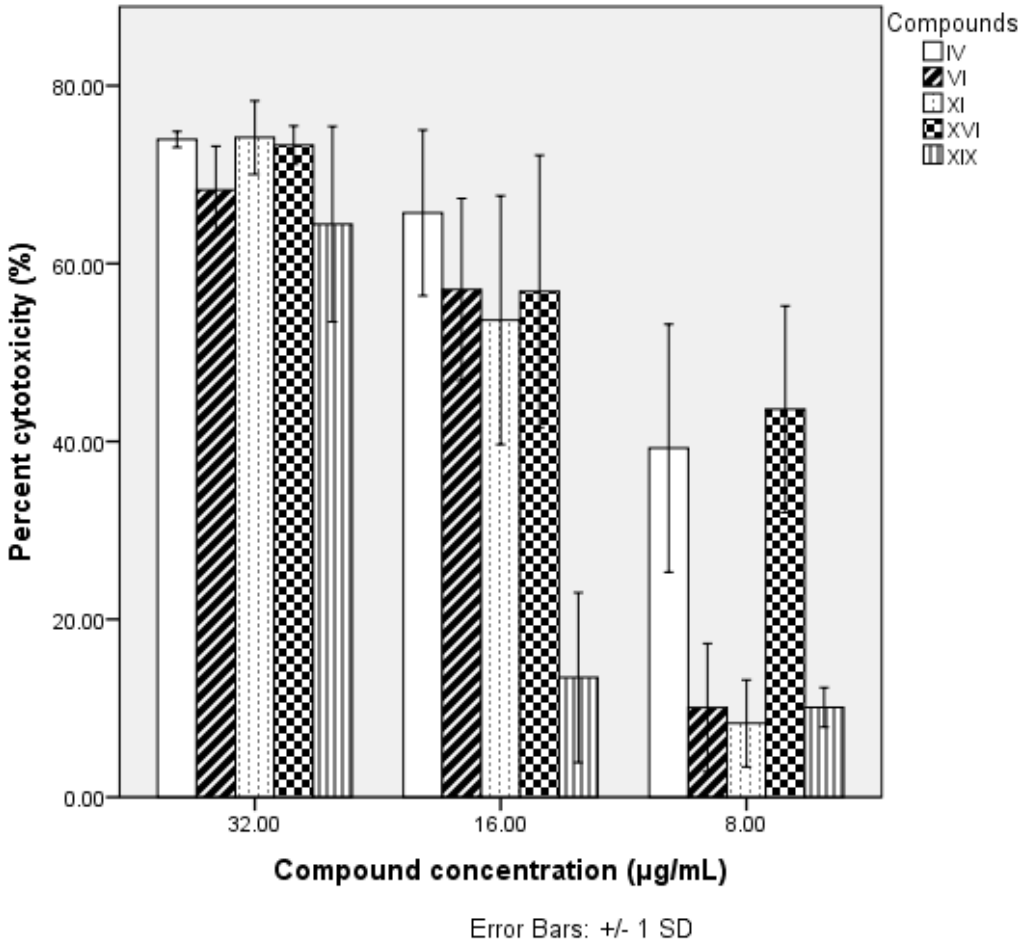

Fig. 3.2. Cytotoxicity of compounds IV, VI, XI, XVI and XIX on the vero cell model. Vero cells were exposed to compounds at concentrations ranging from 128- 8 µg/mL for 3 days at 37 °C and 5% CO_2. After removal of compound containing-media post-incubation, monolayers washed, and DMEM and MTS-PMS were added. Following incubation for 3 h at 37 °C and 5% CO_2, absorbance was determined at 450 nm using the GloMax system. Results are expressed as mean percent cytotoxicity of treated relative to untreated vero cells and are derived from triplicate measurements on 3 separate days. Error bars represent ± standard deviation. The mean percent cytotoxicity of each compound was evaluated for significant differences with one-way analysis of variance (ANOVA). A *p*-value of less than 0.05 was considered statistically significant.

Table 3.3. The MIC_{50}, IC_{50}, and selectivity index of compounds IV, VI, XI, XVI and XIX against clinical strains of *M. tuberculosis*.

Compound	MIC_{50}	IC_{50}	SI
IV	32	10.06	0.31
VI	32	18.00	0.56
XI	32	17.15	0.54
XVI	32	10.93	0.34
XIX	32	28.25	0.88

MIC_{50}: Median MIC of all isolates IC_{50}: Concentration that inhibits 50% of vero cells.

Discussion:

Effective treatment regimens that are active against MDR- and XDR-TB require drugs with fewer adverse effects and shorter duration of treatment. Chalcones have not been previously tested for activity against susceptible, MDR and XDR clinical isolates of *M. tuberculosis* (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013).

In this study, strains with different drug resistance as well as genotype profiles were tested. Amongst the susceptible strains, the compounds were less active against Beijing than the F15/LAM4/KZN and F28. The F11 was more resistant than the other 2 among the MDR strains, whilst the XDR-F15/LAM4/KZN strains were more resistant than the F28. Although no trend was observed, the results serve to show that differences do exist in the activity of some chalcones against different genotypes. This was further confirmed by the promising low MIC of 16 ug/mL of compound XI against H37Rv, but which increased by 2 to 4-fold for the clinical strains. This suggests that testing of chalcones should take strain identity into consideration. Of the 20 compounds that previously displayed activity against H37Rv

(Chapter 2, Page 45), 95% displayed activity against R104, V9124, V2475, R283, 605 and R262 and 90%, 85% and 60% displayed activity against B910, X162 and R271 respectively. MICs ranged between 32-128 µg/mL, which is comparable to the compounds' activity against H37Rv. Compound XV displayed activity against H37Rv at 128 µg/mL but did not display activity against any of the clinical isolates tested. This compound contains a fluorine substitution at the 3' position on the A ring and a methoxy group at the 4'-position, which is common with compound VII that previously displayed no activity against H37Rv. Compound III and XXIII, displayed no activity at the highest concentration tested for R271 and X162 and B910, R271 and X162 respectively. Both these compounds contain a methoxy substituent at the 4' position of the A ring. The differences in MIC of some compounds when susceptible, MDR and XDR isolates are compared could be due to the latter 2 possessing a thicker cell wall than susceptible isolates (Velayati, *et al*., 2009).

The MICs displayed by this subset of chalcones (32-128 µg/mL) are high compared to first line anti-TB drugs; RIF (0.02-1.02 µg/mL), INH (0.02-0.48 µg/mL), ethambutol (1.3-1.8 µg/mL) (Stehr, *et al*., 2014) and drugs in the drug pipeline; bedaquiline (0.002-0.06 µg/mL), PA-824 (0.013-0.81 µg/mL), OPC-67683 (0.006-0.024 µg/mL), SQ-109 (0.06-0.2 µg/mL), linezolid (0.99 µg/mL), PNU-100480 (0.49 µg/mL), AZD5847 (1.00 µg/mL), gatifloxacin (0.007-0.25 µg/mL) and moxifloxacin (0.031-0.5 µg/mL) (Stehr, *et al*., 2014). However, some of the compounds display MICs (32 µg/mL) which are within the range displayed by pyrazinamide (6.25–50 µg/mL) (Zhang and Mitchison, 2003). Pyrazinamide displays no *in vitro* activity at neutral pH but displays *in vitro* activity at pH 5.5 and activity in the murine model and has played an important role in reducing TB treatment from 9 to 6 months (Zhang and Mitchison, 2003).

The high MIC values could be due to several factors, including poor solubility of the compounds due to binding of media components, thus reducing permeability of the

compound through the cell wall of *M. tuberculosis*, intrinsic mechanism of resistance displayed by *M. tuberculosis* such as efflux pumps and pH sensitive action of compounds such as pyrazinamide. Alternate drug susceptibility tests employing different media such as Sauton's media and alteration of pH could be explored to counteract these factors.

The similar activity of some of the chalcones against susceptible as well as MDR and XDR isolates may result from a different mechanism of action compared to the first line and second line drugs they are resistant to. All of these strains that display resistance to some of the chalcones tested belong to different genotypes but it is possible that they have common resistance mechanisms to these compounds.

The mechanism of action of different chalcones varies widely against different organisms. Naphthyl substituted chalcones inhibit the *M. tuberculosis* protein tyrosine phosphatase PtpA (Chiaradia, *et al*., 2008) by competition with substrate for the active site of the enzyme (Mascarello, *et al*., 2010). Licochalcone A, 2,4-dimethoxy-49-allyloxychalcone and 2,4-dimethoxy-49-butoxychalcone inhibit *Leishmania major* and *Leishmania donovani* fumarate reductase, and affect the respiratory pathway, which results in alteration of ultrastructure and function of mitochondria, leading to death of the parasite (Chen, *et al*., 2001). Cationic chalcones that contain aliphatic amino substituents disrupt cell membranes of gram-positive and -negative pathogens (Nielsen, *et al*., 2005).

The mechanism of action of the subset of chalcones in this study could be similar to that of naphthyl substituted chalcones or Licochalcone A, 2,4-dimethoxy-49-allyloxychalcone and 2,4-dimethoxy-49-butoxychalcone described above as acetylenic chalcones display activity against non-replicating *M. tuberculosis* that is equal to, or greater than replicating *M. tuberculosis* (Hans, *et al*., 2010) and fumurate reductase activity has been shown to be elevated under conditions of anaerobic persistence of *M. tuberculosis* (Watanabe, *et al*., 2011).

In this study, 5 compounds; IV, VI, XI, XVI and XIX, which displayed activity against all the clinical isolates were evaluated in a macrophage model. No significant difference was found between the macrophages treated with all concentrations of compounds and untreated infected macrophages. The high standard deviations displayed in the assays could be due to clumping of *M. tuberculosis* as a result of omission of tween or tyloxapol in PBS used in serial dilutions. Addition of detergent at this stage could have influenced permeability of *M. tuberculosis* cell wall (Masaki, *et al*., 1990) and may have resulted in loss of CFU through direct exposure to intracellular compounds present in lysate. Other factors that may have affected the intracellular activity of the compounds include the duration of assay and permeability of compounds into the THP-1 cell due to solubility problems. Since slight reductions were observed, it is possible that a longer assay duration may have resulted in significant reduction of the bacterial load. However, preliminary experiments indicated that longer incubation (5 or 7 days) of infected macrophages led to an increase in the number of extracellular bacteria exposed to and inhibited by the drug in media, thus possibly leading to false results (Data not shown). Chalcones with naphthyl (Mascarello, *et al*., 2010) and benzyloxy with 2,4 dichloro or 2,4 difluoro (Marappu, *et al*., 2011) substitutions have displayed significant activity against intracellular *M. tuberculosis*.

The 5 compounds evaluated in the macrophage model were evaluated for cytotoxicity against vero cells which originated from kidney cells of the African green monkey (Ammerman, *et al*., 2008). Slight differences were observed in the low SI of the different compounds which reflect the poor selectivity of compounds for *M. tuberculosis*. The lowest selectivity of compounds IV and XVI (SI = 0.31 and 0.34 respectively) may be due to the hydroxyl substitutions at the 4’ position on the A ring. Compounds VI (SI = 0.56) (with a methoxy at C- 4’ and fluorine at C-2' on ring A) and XI (SI = 0.54) (with a chlorine at C-2' and fluorine at C-6' on ring B) had increased SI values compared to IV and VI, probably due

to different functional groups and atoms on the chalcones skeleton. Fluorine *para* substituted on each of the rings in addition to a chlorine at C-2' of ring B (compound XIX) was seen to be the best substitution pattern with regard to selectivity (SI =0.88).

The very low selectivity of the compounds (0.31- 0.88) for *M. tuberculosis* is a strong indicator of high toxicity in the human host in their current form. Thus, further testing is not recommended without reformulation of these compounds and until they achieve a SI of greater than 10 (Tuberculosis Drug Screening Program, 2001). Chalcones with quinolinyl (Sharma, *et al*., 2009a), benzyloxy, imidazolyl and triazolyl (Marappu, *et al*., 2011) substituents have previously displayed lower cytotoxicity for vero cells thus displaying better selectivity for *M. tuberculosis* than chalcones tested in this study. Therefore, it is worth derivatising these compounds as this may increase the SI.

A limitation in this study was that the bacteriostatic or bactericidal action of compounds was not determined by measuring the Minimal Bactericidal Concentration (MBC). Since the MIC's of this subset of compounds were higher than most anti-TB drugs in initial screening, and flavonoids have previously caused aggregation of bacterial cells which confounded MBC determination (Cushnie, *et al*., 2007), intracellular activity and cytotoxicity were determined instead.

In conclusion, fluorinated and non-fluorinated chalcones display moderate activity against clinical, including MDR- and XDR- *M. tuberculosis* isolates, no significant activity against intracellular H37Rv and low selectivity indices. These results clearly show the need for reformulation of this compound series to improve activity against *M. tuberculosis*. Compound XIX with the highest selectivity index, shows the most potential for reformulation to improve its biological activity to yield a more potent drug candidate.

References:

Ahmad I, Thakur JP, Chanda D, Saikia D, Khan F, Dixit S, Kumar A, Konwar R, Negi AS, Gupta A (2013) Syntheses of lipophilic chalcones and their conformationally restricted analogues as antitubercular agents. Bioorg Med Chem Lett 23:1322–1325

Anand N, Singh P, Sharma A, Tiwari S, Singh V, Singh DK, Srivastava KK, Singh BN, Tripathi RP (2012) Synthesis and evaluation of small libraries of triazolylmethoxy chalcones, flavanones and 2-aminopyrimidines as inhibitors of mycobacterial FAS-II and PknG. Bioorg Med Chem Lett 20:5150–5163

Ammerman NC, Beier-Sexton M, Azad AF (2008) Growth and maintenance of Vero cell lines. Curr Protoc Microbiol A-4E

Armstrong JA, Hart PA (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J Exp Med 134:713-740

Begue JP, Bonnet-Delpon D (2008) Bioorganic and medicinal chemistry of fluorine. John Wiley and Sons, New York, pp 2-22

Chen M, Zhai L, Christensen SB, Theander TG, Kharazmi A (2001) Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. Antimicrob Agents Chemother 45:2023-2029

Chiaradia LD, Mascarello A, Purificação M, Vernal J, Cordeiro MNS, Zenteno ME, Villarino A, Nunes RJ, Yunes RA, Terenzi H (2008) Synthetic chalcones as efficient inhibitors of *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpA. Bioorg Med Chem Lett 18:6227-6230

Chihota VN, Müller B, Mlambo CK, Pillay M, Tait M, Streicher EM, Marais E, van der Spuy GD, Hanekom M, Coetzee G, Trollip A, Hayes C, Bosman ME, Gey van Pittius NC, Victor TC, van Helden PD, Warren RM (2012) Population structure of multi-and extensively drug-resistant *Mycobacterium tuberculosis* strains in South Africa. J Clin Microbiol 50:995-1002

Cole ST, Riccardi G (2011) New tuberculosis drugs on the horizon. Curr Opin Microbiol 14:570-576

Cushnie TPT, Hamilton VES, Chapman DG, Taylor PW, Lamb AJ (2007) Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonol galangin. J Appl Microbiol 103:1562-1567

Dhar DN (1981) The chemistry of chalcones and related compounds. John Wiley & Sons, New York, pp 169

Falzari K, Zhu Z, Pan D, Liu H, Hongmanee P, Franzblau SG (2005) *In vitro* and *in vivo* activities of macrolide derivatives against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49:1447–1454

Franzblau SG, DeGroote MA, Cho SH, Andries K, Nuermberger E, Orme IM, Mdluli K, Angulo-Barturen I, Dick T, Dartois V, Lenaerts AJ (2012) Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 92:453-488

Gillespie SH (2002) Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob Agents Chemother 46:267-274

Hans RH, Guantai EM, Lategan C, Smith PJ, Wan B, Franzblau SG, Gut J, Rosenthal PJ, Chibale K (2010) Synthesis, antimalarial and antitubercular activity of acetylenic chalcones. Bioorg Med Chem Lett 20:942-944

Kolyva AS, Karakousis PC (2012) Old and New TB Drugs: Mechanisms of Action and Resistance. In: Cardona P (ed) Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance. Intech Open Access Publisher, Reijek

Li R, Kenyon GL, Cohen FE, Chen X, Gong B, Dominguez JN, Davidson E, Kurzban G, Miller RE, Nuzum EO, Rosenthal PJ, Mckerrow JH (1995) *In vitro* antimalarial activity of chalcones and their derivatives. J Med Chem 38:5031-5037

Lin YM, Zhou Y, Flavin MT, Zhou LM, Nie W, Chen FC (2002) Chalcones and flavonoids as anti-tuberculosis agents. Bioorg Med Chem Lett 10:2795-2802

Lopez SN, Castelli MV, Zacchino SA, Dominguez JN, Lobo G, Charris-Charris J, Cortes JCG, Ribas JC, Devia C, Rodriguez AM, Enriz RD (2001) *In vitro* antifungal evaluation and structure–activity relationships of a new series of chalcone derivatives and synthetic analogues with inhibitory properties against polymers of the fungal cell wall. J Bioorgan Med Chem 9:1999-2013

Marrapu VK, Chaturvedi V, Singh S, Singh S, Sinha S, Bhandari K (2011) Novel aryloxy azolyl chalcones with potent activity against *Mycobacterium tuberculosis* H37Rv. Eur J Med Chem 46:4302-4310

Martin A, Morcillo N, Lemus D, Montoro E, da Silva Telles MA, Simboli N, Pontino M, Porras T, León C, Velasco M, Chacon L, Barrera L, Ritacco V, Portaels F, Palomino JC (2005) Multicenter study of MTT and resazurin assays for testing susceptibility to firstline anti-tuberculosis drugs. Int J Tuberc Lung Dis 9:901–906

Masaki S, Sugimori G, Okamoto A, Imose J, Hayashi Y (1990) Effect of Tween 80 on the growth of *Mycobacterium avium* complex. Microbiol Immunol 34:653–663

Mascarello A, Chiaradia LD, Vernal J, Villarino A, Guido RV, Perizzolo P, Poirier V, Wonge D, Martins PGA, Nunes RJ, Yunes RA, Andricopulo AD, Av-Gay Y, Terenzi H (2010) Inhibition of *Mycobacterium tuberculosis* tyrosine phosphatase PtpA by synthetic chalcones: Kinetics, molecular modeling, toxicity and effect on growth. Bioorgan Med Chem 18:3783-3789

Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M (2006) OPC-67683 a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. PLoS Med 3:e466

Moodley T (2011) The synthesis and characterisation of fluorinated chalcones. Masters Dissertation, University of KwaZulu-Natal

Narender T, Gupta S (2004) A convenient and biogenetic type synthesis of few naturally occurring chromeno dihydrochalcones and their *in vitro* antileishmanial activity. Bioorg Med Chem Lett 14:3913-3916

Nielsen SF, Larsen M, Boesen T, Schønning K, Kromann H (2005) Cationic chalcone antibiotics. Design, synthesis, and mechanism of action. J Med Chem 48:2667-2677

Nielsen TF, Boesen T, Larsen M, Schønning K, Kromann H (2004) Antibacterial chalcones-biosteric replacement of the 4'-hydroxy group. J Bioorgan Med Chem 12:3047-3054

Osório TM, Monache FD, Chiaradia LD, Mascarello A, Stumpf TR, Zanetti CR, Silveira DB, Barardi CRM, de Fatima Albino Smânia E, Viancelli A, Garcia LAT, Yunes RA, Nunes RJ, Smânia A Jr (2012) Antibacterial activity of chalcones, hydrazones and oxadiazoles against methicillin-resistant *Staphylococcus aureus*. Bioorg Med Chem Lett 22:225-230

Pavan FR, Leite CQR, Coelho RG, Coutinho ID, Honda NK, Cardoso CAL, Vilegas W, Leite SRDA, Sato DN (2009) Evaluation of anti-*Mycobacterium tuberculosis* activity of *Campomanesia adamantium* (MYRTACEAE). Quim Nova 32:1222-1226

Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK (2003) Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages insights into the phagosomal environment. J Exp Med 198:693-704

Sharma M, Chaturvedi V, Manju YK, Bhatnagar S, Srivastava K, Puri SK, Chauhan P (2009a) Substituted quinolinyl chalcones and quinolinyl pyrimidines as a new class of anti-infective agents. Eur J Med Chem 44:2081-2091

Sharma PC, Jain A, Jain S (2009b) Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. Acta Pol Pharm 66:587-604

Sivakumar PM, Seenivasan SP, Kumar V, Doble M (2007) Synthesis, antimycobacterial activity evaluation, and QSAR studies of chalcone derivatives. Bioorg Med Chem Lett 17:1695-1700

Smart BE (2001) Fluorine substituent effects (on bioactivity). J Fluorine Chem 109:3-11

Smith I (2003) *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 16:463-496

Stehr M, Elamin AA, Singh M (2014) Filling the Pipeline-New Drugs for an Old Disease. Curr Top Med Chem 14:110-129

Trivedi AR, Dodiya DK, Ravat NR, Shah VH (2008) Synthesis and biological evaluation of some new pyrimidines via a novel chalcone series. Arkivoc 11:131-141

Tuberculosis Drug Screening Program (2001) Search for new drugs for treatment of tuberculosis. Antimicrob Agents Chemother 45:1943-1946

van Rie A, Victor TC, Richardson M, Johnson R, van der Spuy GD, Murray EJ, Beyers N, Pittius NC, van Helden PD, Warren RM (2005) Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Resp Crit Care 172:636

Velayati AA, Farnia P, Ibrahim TA, Haroun RZ, Kuan HO, Ghanavi J, Farnia P, Kabarei AN, Tabarsi P, Omar AR, Varahram M, Masjedi MR (2009) Differences in cell wall thickness between resistant and nonresistant strains of *Mycobacterium tuberculosis*: using transmission electron microscopy. Chemotherapy 55:303-307

Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, van Pittius NCG, van Helden PD (2004) Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Resp Crit Care 169:610-614

Watanabe S, Zimmermann M, Goodwin MB, Sauer U, Barry 3rd CE, Boshoff HI (2011) Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. PLoS Pathog 7:e1002287

World Health Organization (2013a) Global Tuberculosis Report 2013. http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf. Accessed 14 January 2014

Wu JH, Wang XH, Yi YH, Lee KH (2003) Anti-AIDS agents 54. A potent anti- HIV chalcone and flavonoids from genus desmos. J Bioorgan Med Chem 13:1813-1815

Zager EM, McNerney R (2008) Multidrug-resistant tuberculosis. BMC Infect Dis 8:10

Zhang Y, Mitchison D (2003) The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis 7:6-21

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CHAPTER 4: GENERAL DISCUSSION

Bedaquiline is the first drug in 40 years to be approved by the Food and Drug administration (FDA) for use against *M. tuberculosis* (Cohen, 2013). Adverse effects of bedaquiline and the evolution of resistance of *M. tuberculosis* to potent first- and second line TB drugs highlights the need for continual replenishment of the TB drug pipeline with novel potential lead candidates.

Synthetic compound libraries have been an attractive source of lead candidates and led to discovery of both bedaquiline and SQ-109 (Kolyva and Karakousis, 2012). Chalcone derivatives have demonstrated promising activity as anti-bacterial (Nielsen, *et al*., 2004), anti-viral (Li, *et al*., 1995), anti-fungal (Lopez, *et al*., 2001), anti-parasitic (Wu, *et al*., 2003), anti-cancer (Narender and Gupta, 2004) and anti-TB agents (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013). Substitutions such as halogens (Lin, *et al*., 2002), dimethylamino, methoxy, thiomethyl, nitro and hydroxyl groups (Sivakumar, *et al*., 2007), naphthyl groups (Chiaradia, *et al*., 2008), methoxy, nitro (Trivedi, *et al*., 2008), quinolinyl (Sharma, *et al*., 2009a), acetylenic (Hans, *et al*., 2010), benzyloxyl and imidazolyl (Marrapu, *et al*., 2011) and triazolyl (Anand, *et al*., 2012) have demonstrated improved activity against *M. tuberculosis*. However, the effect of fluorine substitution on the anti-TB activity of chalcones has not yet been evaluated.

The lack of a standardised drug susceptibility testing technique for screening chalcones has posed an obstacle in evaluating the impact of specific substitution patterns on compound activity for comparative purposes. Athough a variety of techniques (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013)

have been used for screening chalcone activity against *M. tuberculosis*, the efficacy and cost effectiveness of the MTT assay has until now, not been evaluated against the MABA assay.

The focus of this study was 2-fold: firstly, to compare the performance of the colorimetric MTT and MABA assays for screening synthetic chalcones, and secondly, to evaluate the activity of fluorinated and non-fluorinated chalcones against *M. tuberculosis*.

Initially, the MICs of 27 compounds against laboratory strain, H37Rv was determined using the MTT assay. The rationale for using this assay was based on its widespread use in anti-microbial testing (Yajko, *et al*., 1995; Caviedes, *et al*., 2002; Martin, *et al*., 2005). Twenty of the 27 compounds which were active against H37Rv were subsequently retested using the MTT, MABA and the gold standard Macroscopic broth assays at time intervals of 7, 14 and 21 days. The *in vitro* extracellular activities of these 20 compounds against clinical isolates of various drug susceptibility and genotype profiles were evaluated using the MTT assay. Intracellular activity in macrophages and cytotoxicity in the vero cell line was further investigated for selected compounds.

No significant difference was observed in the MIC obtained in the MABA and MTT assays, whereas this was significantly increased in the macroscopic broth assay compared to the other 2. This is possibly a result of compound precipitation in Middlebrook 7H9 broth of all compounds except compounds IV and XVI. The ensuing difficulty in the interpretation of the results was overcome by comparison with a drug and broth only control. Solubility problems and/or binding of compounds to protein components of the Middlebrook 7H9 broth are possible reasons for the observed precipitation. Furthermore, this precipitation could have resulted in both sub-optimal availability of the active ingredient and/or evolution and selection of drug resistant mutants resulting in elevated MICs over the incubation period. This implies that the sole reliance

on the macroscopic broth assay for the evaluation of the *in vitro* activity of this subset of compounds would have led to false negative results thus excluding potentially valuable candidates for lead optimisation. Therefore, compound properties play a significant role in the determination of an appropriate drug susceptibility test method.

The ease, duration, plastic consumable requirements and performance of both the colorimetric assays in this study were comparable. However, MTT reagent (Sigma) was significantly cheaper (R0.06/well) than the MABA reagent (Invitrogen) (R1.31/well) and the MICs were definitive even without the SDS-DMF solubilisation, due to the distinctive colour of the insoluble precipitate. Thus, the MTT assay is a more cost effective assay compared to the MABA for rapid screening of chalcone and possibly other synthetic compounds.

In previous studies, the activity of chalcones had been tested against the laboratory strain H37Rv (Lin, *et al*., 2002; Sivakumar, *et al*., 2007; Chiaradia, *et al*., 2008; Trivedi, *et al*., 2008; Hans, *et al*., 2010; Sharma, *et al*., 2009a; Mascarello, *et al*., 2010; Marrapu, *et al*., 2011; Anand, *et al*., 2012; Ahmad, *et al*., 2013). In this study, the compound series were tested against dominant KwaZulu-Natal susceptible, MDR and XDR strains belonging to different genotype families. Among the susceptible group, the chalcone compounds were less active against the Beijing than the F15/LAM4/KZN and F28 strains. The F11 was less susceptible to the compounds than the F15/LAM4/KZN and Beijing within the MDR strains, whilst both the XDR F15/LAM4/KZN strains were more susceptible than F28 XDR strain. This suggests that despite no trend being observed, the activity of chalcones differ between genotypes. Ninety five percent of the compounds were active against H37Rv and clinical strains R104, V9124, V2475, R283, 605 and R262, whilst 90%, 85% and 60% were active against B910, X162 and R271 respectively. The variation in MICs of some compounds when the different strains are compared

could be due to the denser cell wall of MDR and XDR isolates (Velayati, *et al*., 2009). The MICs displayed by this subset of chalcones are high compared to that of most of the standard anti-TB drugs except pyrazinamide. The high MICs values could be due to numerous factors including low solubility, binding to media components (Chung, *et al*., 1995), *M. tuberculosis* intrinsic mechanism of resistance (Kolyva and Karakousis, 2012) or pH dependent action similar to pyrazinamide (Zhang and Mitchison, 2003).

The analysis of the structure-activity relationships of this subset of chalcones revealed that none of the substitutents (methoxy, hydroxyl, chlorine or fluorine) improved activity by more than a 2-fold dilution. Fluorine substitution at the 3' position of the A ring seems to attenuate activity, as is seen in compounds VII (Chapter 2, Page 45) and XV (Chapter 3, Page 71) in comparison to VI and XIV. Our findings are in agreement with previous studies showing activity of methoxyl and naphthyl (Chiaradia, *et al*., 2008), amino linked quinolinyl (Sharma, *et al*., 2009a), hydroxyl, methyl and methoxyl (Pavan, *et al*., 2009), acetylenic (Hans *et al*., 2010), thiomethyl (Sivakumar, *et al*., 2007) and aryloxy azolyl (Marappu, *et al*., 2011) substituted chalcones against *M. tuberculosis*. This is in contrast to the findings of Lin, *et al*., 2002, which showed that only halogenated chalcones displayed activity against *M. tuberculosis*.

The mechanism of action of the subset of chalcones in this study can only be speculated to be the inhibition *M. tuberculosis* protein tyrosine phosphatase PtpA (Chiaradia, *et al*., 2008) or fumarate reductase (Chen, *et al*., 2001) as described in previous studies, since chalcones were reported to display activity against anaerobic *M. tuberculosis* (Hans, *et al*., 2010) and fumarate reductase has been shown to be active in these conditions (Watanabe, *et al*., 2011).

Compounds IV, VI, XI, XVI and XIX, which displayed activity against all the clinical isolates were selected for evaluation in a macrophage model and for cytotoxicity against vero

cells. Comparison between treated and untreated macrophages displayed no significant difference for all compounds tested. Factors that may have influenced the intracellular activity include the duration of the assay and solubility problems that decreased permeation of compounds into the THP-1 cell. Extension of the assay duration may have resulted in false results due to the extracellular bacterial exposure to compounds. Other studies have reported significant intracellular activity by naphthyl (Mascarello, *et al*., 2010) and benzyloxy with 2,4 dichloro or 2,4 difluoro (Marappu, *et al*., 2011) substituted chalcones.

The 5 compounds tested for cytotoxicity displayed very low selectivity for *M. tuberculosis* (0.31- 0.88). Of these, XIX displayed the highest selectivity for *M. tuberculosis* (SI = 0.88). This may be due to a particular substitution pattern, having the presence of two fluorine substituents at the 4' position of the A and B rings and a chlorine atom at the 2' position of the B ring. Quinolinyl (Sharma, *et al*., 2009a), benzyloxy, imidazolyl and triazolyl (Marappu, *et al*., 2011) substituted chalcones are known to possess higher selectivity for *M. tuberculosis* than vero cells when compared to chalcones tested in this study. Based on the requirement of SI >10 (TB drug screening program, 2001), the extremely low SI values indicate that despite the moderate activity of this chalcone series against clinical strains of MDR- and XDR- *M. tuberculosis*, they are extremely toxic to the human host and therefore, unsuitable as lead compounds in their current formulation.

A limitation in this study was that the bactericidal activity of the compounds was not determined. However, the rationale behind this was that the MIC values were much higher than those of the standard anti-TB drugs used as controls, as well as the bacterial aggregative property of flavonoids that would negatively affect the MBC values (Cushnie, *et al*., 2007).

CONCLUSION AND FUTURE WORK

The MTT assay is a more cost effective drug susceptibility testing method than the MABA assay for the rapid *in vitro* screening of the activity of chalcones against *M. tuberculosis*. Fluorinated and non-fluorinated chalcones display moderate extracellular activity against clinical isolates, including MDR- and XDR- *M. tuberculosis* isolates, but no significant activity against intracellular H37Rv.

Since the selectivity index is an indicator of toxicity, these compounds with low selectivity indices would be highly toxic to the human host in their current formulation. Because these chalcones do have moderate anti-TB activity, reformulation is recommended till they reach a SI of greater than 10 (Tuberculosis Drug Screening Program, 2001) before further testing is continued. Compound XIX with the highest selectivity index, shows the most potential for reformulation to improve its biological activity to yield a more potent drug candidate.

Since compound XI displayed an MIC of 16 μg/mL against H37Rv, reformulation could include variation of the substitutions (methoxy, hydroxyl or chlorine) on the A ring. Fluorination as in XX did not enhance activity. Combinations of substitutions for compound XIX including naphthyl (Mascarello, *et al*., 2010), benzyloxy with 2,4 dichloro or 2,4 difluoro imidazolyl and triazolyl (Marappu, *et al*., 2011) or quinolinyl (Sharma, *et al*., 2009a) substitutions that have previously displayed significant activity against intracellular *M. tuberculosis* or less cytotoxicity against vero cells may result in a subset of chalcones displaying more potent and selective activity against *M. tuberculosis*.

REFERENCES:

Abate G, Mshana RN, Miörner H (1998) Evaluation of a colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) for rapid detection of rifampicin resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 2:1011–1016

Abdullah MI, Mahmood A, Madni M, Masood S, Kashif M (2014) Synthesis, characterization, theoretical, anti-bacterial and molecular docking studies of quinoline based chalcones as a DNA gyrase inhibitor. Bioorg Chem 54:31-37

Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, Edelstein PH, Cosma CL, Ramakrishnan L (2011) Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell 145:39-53

Ahmad I, Thakur JP, Chanda D, Saikia D, Khan F, Dixit S, Kumar A, Konwar R, Negi AS, Gupta A (2013) Syntheses of lipophilic chalcones and their conformationally restricted analogues as antitubercular agents. Bioorg Med Chem Lett 23:1322–1325

Aínsa JA, Blokpoel MC, Otal I, Young DB, De Smet KA, Martín C (1998) Molecular cloning and characterization of Tap, a putative multidrug efflux pump present in *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*. J Bacteriol 180:5836-5843

Ammerman NC, Beier-Sexton M, Azad AF (2008) Growth and maintenance of Vero cell lines. Curr Protoc Microbiol A-4E

Anand N, Singh P, Sharma A, Tiwari S, Singh V, Singh DK, Srivastava KK, Singh BN, Tripathi RP (2012) Synthesis and evaluation of small libraries of triazolylmethoxy chalcones, flavanones and 2-aminopyrimidines as inhibitors of mycobacterial FAS-II and PknG. Bioorg Med Chem Lett 20:5150–5163

Andries K, Verhasselt P, Guillemont J, Göhlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V (2005) A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science 307:223-227

Andrighetti-Fröhner CR, de Oliveira KN, Gaspar-Silva D, Pacheco LK, Joussef AC, Steindel M, Simões CM, de Souza AM, Magalhaes UO, Afonso IF, Rodrigues CR, Nunes RJ, Castro HC (2009) Synthesis, biological evaluation and SAR of sulfonamide 4-methoxychalcone derivatives with potential antileishmanial activity. Eur J Med Chem 44:755-763

Ansari M, Emami S, Khalilzadeh MA, Maghsoodlou MT, Foroumadi A, Faramarzi MA, Samadi N, Ardestani SK (2012) Replacement of the methylene of dihydrochalcones with oxygen: synthesis and biological evaluation of 2-phenoxyacetophenones. Chem Biol Drug Des 80:591-597

Anto RJ, Sukumaran K, Kuttan G, Rao MN, Subbaraju V, Kuttan R (1995) Anticancer and antioxidant activity of synthetic chalcones and related compounds. Cancer Lett 97:33-37

Armstrong JA, Hart PA (1971) Response of cultured macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes with phagosomes. J Exp Med 134:713-740

Avila HP, Smânia Ede F, Monache FD, Smânia A Jr (2008) Structure-activity relationship of antibacterial chalcones. Bioorg Med Chem 16:9790-9794

Axelrod S, Oschkinat H, Enders J, Schlegel B, Brinkmann V, Kaufmann SH, Haas A, Schaible UE (2008) Delay of phagosome maturation by a mycobacterial lipid is reversed by nitric oxide. Cell Microbiol 10:1530-1545

Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U (2012) Efflux Pumps of *Mycobacterium tuberculosis* Play a Significant Role in Antituberculosis Activity of Potential Drug Candidates. Antimicrob Agents Chemother 56:2643–2651

Bandgar BP, Jalde SS, Adsul LK, Shringare SN, Lonikar SV, Gacche RN, Dhole NA, Nile SH, Shirfule AL (2012) Synthesis of new olefin chalcone derivatives as antitumor, antioxidant and antimicrobial agents. Med Chem Res 21:4512–4522

Basso LA, Zheng R, Musser JM, Jacobs WR Jr, Blanchard JS (1998) Mechanisms of isoniazid resistance in *Mycobacterium tuberculosis*: enzymatic characterization of enoyl reductase mutants identified in isoniazid-resistant clinical isolates. J Infect Dis 178:769-775

Baulard AR, Betts JC, Engohang-Ndong J, Quan S, McAdam RA, Brennan PJ, Locht C, Besra GS (2000) Activation of the pro-drug ethionamide is regulated in mycobacteria. J Biol Chem 275:28326-28331

Begue JP, Bonnet-Delpon D (2008) Bioorganic and medicinal chemistry of fluorine. John Wiley and Sons, New York, pp 2-22

Belanger AE, Besra GS, Ford ME, Mikusová K, Belisle JT, Brennan PJ, Inamine JM (1996) The *embAB* genes of *Mycobacterium avium* encode an arabinosyl transferase involved in cell wall arabinan biosynthesis that is the target for the antimycobacterial drug ethambutol. Proc Natl Acad Sci 93:11919-11924

Bello ML, Chiaradia LD, Dias LR, Pacheco LK, Stumpf TR, Mascarello A, Steindel M, Yunes RA, Castro HC, Nunes RJ, Rodrigues CR (2011) Trimethoxy-chalcone derivatives inhibit growth of *Leishmania braziliensis*: synthesis, biological evaluation, molecular modeling and structure-activity relationship (SAR). Bioorg Med Chem 19:5046-5052

Bergamini N, Fowst G (1965) Rifamycin SV. A review. Arzneimittelforschung 15:Suppl:951-1002

Bevan P, Ryder H, Shaw I (1995) Identifying small-molecule lead compounds: the screening approach to drug discovery. Trends Biotechnol 13:115-121

Binda G, Domenichini E, Gottardi A, Orlandi B, Ortelli E, Pacini B, Fowst G (1971) Rifampicin, a general review. Arzneimittelforschung 21:1907-1977

Brennan PJ, Nikaido H (1995) The envelope of mycobacteria. Annu Rev Biochem 64:29-63

Caceres NE, Harris NB, Wellehan JF, Feng Z, Kapur V, Barletta RG (1997) Overexpression of the D-alanine racemase gene confers resistance to D-cycloserine in *Mycobacterium smegmatis*. J Bacteriol 179:5046-5055

Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler HT, Meissner G, Mitchison DA, Gula L (1963) Mycobacteria: laboratory methods for testing drug sensitivity and resistance. B World Health Organ 29:565–578

Caviedes L, Delgado J, Gilman RH (2002) Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of *Mycobacterium tuberculosis*. J Clin Microbiol 40:1873–1874

Chakraborty S, Gruber T, Barry CE 3rd, Boshoff HI, Rhee KY (2013) Para-aminosalicylic acid acts as an alternative substrate of folate metabolism in *Mycobacterium tuberculosis*. Science 339:88-91

Chan J, Fujiwara T, Brennan P, McNeil M, Turco SJ, Sibille JC, Bloom BR (1989) Microbial glycolipids: possible virulence factors that scavenge oxygen radicals. Proc Natl Acad Sci 86:2453-2457

Chen M, Zhai L, Christensen SB, Theander TG, Kharazmi A (2001) Inhibition of fumarate reductase in *Leishmania major* and *L. donovani* by chalcones. Antimicrob Agents Chemother 45:2023-2029

Chiaradia LD, Mascarello A, Purificação M, Vernal J, Cordeiro MNS, Zenteno ME, Villarino A, Nunes RJ, Yunes RA, Terenzi H (2008) Synthetic chalcones as efficient inhibitors of *Mycobacterium tuberculosis* protein tyrosine phosphatase PtpA. Bioorg Med Chem Lett 18:6227-6230

Chihota V (2011) The Molecular Epidemiology of *Mycobacterium tuberculosis*: Role in understanding disease dynamics in high prevalence settings in Southern Africa Region. Ph.D. Thesis, The University of Stellenbosch.

Chihota VN, Müller B, Mlambo CK, Pillay M, Tait M, Streicher EM, Marais E, van der Spuy GD, Hanekom M, Coetzee G, Trollip A, Hayes C, Bosman ME, Gey van Pittius NC, Victor TC, van Helden PD, Warren RM (2012) Population structure of multi-and extensively drug-resistant *Mycobacterium tuberculosis* strains in South Africa. J Clin Microbiol 50:995-1002

Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P (2002) Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. Biochem J 367:279–285

Chung GA, Aktar Z, Jackson S, Duncan K (1995) High-throughput screen for detecting antimycobacterial agents. Antimicrob Agents Chemother 39:2235-2238

Cohen J (2013) Approval of novel TB drug celebrated—with restraint. Science 339:130

Colangeli R, Helb D, Sridharan S, Sun J, Varma-Basil M, Hazbón MH, Harbacheuski R, Megjugorac NJ, Jacobs Jr WR, Holzenburg A, Sacchettini JC, Alland D (2005) The *Mycobacterium tuberculosis iniA* gene is essential for activity of an efflux pump that confers drug tolerance to both isoniazid and ethambutol. Mol Microbiol 55:1829-1840

Cole S, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Barrell BG (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature 393:537-544

Cole ST, Riccardi G (2011) New tuberculosis drugs on the horizon. Curr Opin Microbiol 14:570-576

Collins L, Franzblau SG (1997) Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. Antimicrob Agents Chemother 41:1004–1009

Crick DC, Chatterjee D, Scherman MS, McNeil MR (2010) 6.13 - Structure and Biosynthesis of the Mycobacterial Cell Wall. In: Liu HW and Mander L (ed) Comprehensive Natural Products II. Elsevier, Oxford, pp 400

Cushnie TPT, Hamilton VES, Chapman DG, Taylor PW, Lamb AJ (2007) Aggregation of *Staphylococcus aureus* following treatment with the antibacterial flavonol galangin. J Appl Microbiol 103:1562-1567

Daffe M, Brennan PJ, McNeil M (1990) Predominant structural features of the cell wall arabinogalactan of *Mycobacterium tuberculosis* as revealed through characterization of oligoglycosyl alditol fragments by gas chromatography/mass spectrometry and by 1H and 13C NMR analyses. J Biol Chem 265:6734-6743

Dartois V, Barry CE 3rd (2013) A medicinal chemists' guide to the unique difficulties of lead optimization for tuberculosis. Bioorg Med Chem Lett 23:4741-4750

Dartois V (2014) The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat Rev Microbiol 12:159-167

De Carvalho Tavares L, Johann S, Maria de Almeida Alves T, Guerra JC, Maria de Souza-Fagundes E, Cisalpino PS, Bortoluzzi AJ, Caramori GF, de Mattos Piccoli R, Braibante HT, Braibante ME, Pizzolatti MG (2011) Quinolinyl and quinolinyl N-oxide chalcones: synthesis, antifungal and cytotoxic activities. Eur J Med Chem 46:4448-4456

De Oliveira ME, Cenzi G, Nunes RR, Andrighetti CR, de Sousa Valadão DM, Dos Reis C, Simões CM, Nunes RJ, Júnior MC, Taranto AG, Sanchez BA, Viana GH, de Pilla Varotti F (2013) Antimalarial activity of 4-metoxychalcones: docking studies as falcipain/plasmepsin inhibitors, ADMET and lipophilic efficiency analysis to identify a putative oral lead candidate. Molecules 18:15276-15287

De Rossi E, Branzoni M, Cantoni R, Milano A, Riccardi G, Ciferri O (1998) *mmr*, a *Mycobacterium tuberculosis* gene conferring resistance to small cationic dyes and inhibitors. J Bacteriol 180:6068–6071

Dhar DN (1981) The chemistry of chalcones and related compounds. John Wiley & Sons, New York, pp 169

Diacon AH, Dawson R, Hanekom M, Narunsky K, Venter A, Hittel N, Geiter LJ, Wells CD, Paccaly AJ, Donald PR (2011) Early bactericidal activity of delamanid (OPC-67683) in smear-positive pulmonary tuberculosis patients. Int J Tuberc Lung Dis 5:949-954

Dinesh N, Sharma S, Balganesh M (2013) Involvement of efflux pumps in the resistance to peptidoglycan synthesis inhibitors in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 57:1941-1943

Douglass J, Steyn LM (1993) A ribosomal gene mutation in streptomycin-resistant Mycobacterium. J Infect Dis 167:1505-1506

Duncan K (2003) Progress in TB drug development and what is still needed. Tuberculosis 83:201-207

Eastlake C, Barach AL (1949) Use of para-aminosalicylic acid in chronic pulmonary tuberculosis. CHEST J 16:1-14

El-Benna J, Dang PMC, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F (2009) p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. Exp Mol Med 41:217-225

El Sayed Aly MR, Abd El Razek Fodah HH, Saleh SY (2014) Antiobesity, antioxidant and cytotoxicity activities of newly synthesized chalcone derivatives and their metal complexes. Eur J Med Chem 76:517-530

Erturan Z, Uzun M (2005) *In vitro* activity of linezolid against multidrug-resistant *Mycobacterium tuberculosis* isolates. Int J Antimicrob Agents 26:78–80

Escribano I, Rodriguez JC, Llorca B, Garcia-Pachon E, Ruiz M, Royo G (2007) Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid. Chemotherapy 53:397–401

Fadare OA, Akinpelu DA, Obafemi CA (2014) 1-Indanone chalcones and their 2, 4-Dinitrophenylhydrazone derivatives: Synthesis, physicochemical properties and *in vitro* antibacterial activity. Afr J Pure Appl Chem 8:68-77

Falzari K, Zhu Z, Pan D, Liu H, Hongmanee P, Franzblau SG (2005) *In vitro* and *in vivo* activities of macrolide derivatives against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 49:1447–1454

Feng Z, Barletta RG (2003) Roles of *Mycobacterium smegmatis* D-alanine: D-alanine ligase and D-alanine racemase in the mechanisms of action of and resistance to the peptidoglycan inhibitor D-cycloserine. Antimicrob Agents Chemother 47:283-291

Filliol I, Driscoll JR, van Soolingen D, Kreiswirth BN, Kremer K, Valétudie G, Dang DA, Barlow R, Banerjee D, Bifani PJ, Brudey K, Cataldi A, Cooksey RC, Cousins DV, Dale JW, Dellagostin OA, Drobniewski F, Engelmann G, Ferdinand S, Gascoyne-Binzi D, Gordon M, Gutierrez MC, Haas WH, Heersma H, Kassa-Kelembho E, Ho ML, Makristathis A, Mammina C, Martin G, Moström P, Mokrousov I, Narbonne V, Narvskaya O, Nastasi A, Niobe-Eyangoh SN, Pape JW, Rasolofo-Razanamparany V, Ridell M, Rossetti ML, Stauffer F, Suffys PN, Takiff H, Texier-Maugein J, Vincent V, de Waard JH, Sola C, Rastogi N (2003) Snapshot of moving and expanding clones of *Mycobacterium tuberculosis* and their global distribution. J Clin Microbio l41:1963-1970

Forrellad MA, McNeil M, Santangelo Mde L, Blanco FC, García E, Klepp L, Huff J, Niederweis M, Jackson M, Bigi F (2014) Role of the Mce1 transporter in the lipid homeostasis of *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 94:170-177

Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH (1998) Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 36:362-366

Franzblau SG, DeGroote MA, Cho SH, Andries K, Nuermberger E, Orme IM, Mdluli K, Angulo-Barturen I, Dick T, Dartois V, Lenaerts AJ (2012) Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*. Tuberculosis (Edinb) 92:453-488

Gillespie SH (2002) Evolution of drug resistance in *Mycobacterium tuberculosis*: clinical and molecular perspective. Antimicrob Agents Chemother 46:267-274

Ginsburg AS, Grosset JH, Bishai WR (2003) Fluoroquinolones, tuberculosis, and resistance. Lancet Infect Dis 3:432-442

Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D (2002) Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: a systematic review. Emerg Infect Dis 8:843-849

Goldman RC (2013) Why are membrane targets discovered by phenotypic screens and genome sequencing in *Mycobacterium tuberculosis*? Tuberculosis (Edinb) 93:569-588

Guerin ME, Kaur D, Somashekar BS, Gibbs S, Gest P, Chatterjee D, Brennan PJ, Jackson M (2009) New insights into the early steps of phosphatidylinositol mannoside biosynthesis in mycobacteria: PimB' is an essential enzyme of *Mycobacterium smegmatis*. J Biol Chem 284:25687-25696

Gupta AK, Chauhan DS, Srivastava K, Das R, Batra S, Mittal M, Goswami P, Singhal N, Sharma VD, Venkatesan K, Hasnain SE, Katoch VM (2006) Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. J Commun Dis 38:246–254

Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, Sharma VD (2010) Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. Microb Drug Resist 16:21–28

Hackam DJ, Rotstein OD, Zhang WJ, Demaurex N, Woodside M, Tsai O, Grinstein S (1997) Regulation of Phagosomal Acidification Differential Targeting Of Na+/H+ Exchangers, Na+/K+-ATPases, And Vacuolar-Type H+-ATPases. J Biol Chem 272:29810-29820

Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P (2004) Comparison of alamar blue and MTT assays for high through-put screening. Toxicol In Vitro 18:703-710

Hans RH, Guantai EM, Lategan C, Smith PJ, Wan B, Franzblau SG, Gut J, Rosenthal PJ, Chibale K (2010) Synthesis, antimalarial and antitubercular activity of acetylenic chalcones. Bioorg Med Chem Lett 20:942-944

Hao P, Shi-Liang Z, Ju L, Ya-Xin D, Biao H, Xu W, Min-Tao H, Shou-Gang K, Ke W (2011) The role of ABC efflux pump, Rv1456c-Rv1457c-Rv1458c, from *Mycobacterium tuberculosis* clinical isolates in China. Folia Microbiol (Praha) 56:549–553

Hayakawa E, Tokumasu F, Nardone GA, Jin AJ, Hackley VA, Dvorak JA (2007) A *Mycobacterium tuberculosis* derived lipid inhibits membrane fusion by modulating lipid membrane domains. Biophys J 93:4018-4030

Heifets L (1996) Susceptibility testing of *Mycobacterium avium* complex isolates. Antimicrob Agents Chemother 40:1759

Hoffmann C, Leis A, Niederweis M, Plitzko JM, Engelhardt H (2008) Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc Natl Acad Sci USA 105:3963-3967

Jackson M, Raynaud C, Lanéelle MA, Guilhot C, Laurent-Winter C, Ensergueix D, Gicquel B, Daffé M (1999) Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the Mycobacterium tuberculosis cell envelope. Mol Microbiol 31:1573-1587

Jackson M, Crick DC, Brennan PJ (2000) Phosphatidylinositol is an essential phospholipid of mycobacteria. J Biol Chem 275:30092-30099

Jain UK, Bhatia RK, Rao AR, Singh R, Saxena AK, Sehar I (2014) Design and Development of Halogenated Chalcone Derivatives as Potential Anticancer Agents. Trop J Pharm Res 13:73-80

Jarlier V, Nikaido H (1990) Permeability barrier to hydrophilic solutes in *Mycobacterium chelonei*. J Bacteriol 172:1418-1423

Jarlier V, Gutmann L, Nikaido H (1991) Interplay of cell wall barrier and beta-lactamase activity determines high resistance to beta-lactam antibiotics in *Mycobacterium chelonae*. Antimicrob Agents Chemother 35:1937-1939

Ji B, Lounis N, Maslo C, Truffot-Pernot C, Bonnafous P, Grosset J (1998) *In vitro* and *in vivo* activities of moxifloxacin and clinafloxacin against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 42:2066–2069

Jiang X, Zhang W, Zhang Y, Gao F, Lu C, Zhang X, Wang H (2008) Assessment of efflux pump gene expression in a clinical isolate *Mycobacterium tuberculosis* by real-time reverse transcription PCR. Microb Drug Resist 14:7–11

Johansen SK, Maus CE, Plikaytis BB, Douthwaite S (2006) Capreomycin binds across the ribosomal subunit interface using *tlyA* encoded 2′-O-methylations in 16S and 23S rRNAs. Mol Cell 23:173-182

Joloba M, Bwanga F (2010) Drug resistance in *Mycobacterium tuberculosis*. Antimicrobial Resistance in Developing Countries. Springer, New York, pp 117-135

Karakousis PC (2009) Mechanisms of Action and Resistance of Antimycobacterial Agents. In: Mayers DL (ed) Antimicrobial Drug Resistance. Springer, pp 271-291

Kolyva AS, Karakousis PC (2012) Old and New TB Drugs: Mechanisms of Action and Resistance. In: Cardona P (ed) Understanding Tuberculosis - New Approaches to Fighting Against Drug Resistance. Intech Open Access Publisher, Reijek

Konduru NK, Dey S, Sajid M, Owais M, Ahmed N (2013) Synthesis and antibacterial and antifungal evaluation of some chalcone based sulfones and bisulfones. Eur J Med Chem 59:23-30

Konno K, Feldmann FM, Mcdermott W (1967) Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am Rev Respir Dis 95:461-469

Koul A, Arnoult E, Lounis N, Guillemont J, Andries K (2011) The challenge of new drug discovery for tuberculosis. Nature 469:483-490

Kumar D, Kumar NM, Akamatsu K, Kusaka E, Harada H, Ito T (2010) Synthesis and biological evaluation of indolyl chalcones as antitumor agents. Bioorg Med Chem Lett 20:3916-3919

Kumar CS, Loh WS, Ooi CW, Quah CK, Fun HK (2013) Heteroaryl chalcones: design, synthesis, X-ray crystal structures and biological evaluation. Molecules 18:12707-12724

Lahsasni SA, Al Korbi FH, Aljaber NAA (2014) Synthesis, characterization and evaluation of antioxidant activities of some novel chalcones analogues. Chem Cent J 8:32

Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs WR Jr (2007) Laboratory maintenance of *Mycobacterium tuberculosis*. Curr Protoc Microbiol 6:10A.1

Lederer E, Adam A, Ciorbaru R, Petit JF, Wietzerbin J (1975) Cell walls of mycobacteria and related organisms; chemistry and immunostimulant properties. Mol Cell Biochem 7:87-104

Lee M, Lee J, Carroll MW, Choi H, Min S, Song T, Via LE, Goldfeder LC, Kang E, Jin B, Park H, Kwak H, Kim H, Jeon HS, Jeong I, Joh JS, Chen RY, Olivier KN, Shaw PA, Follmann D, Song SD, Lee JK, Lee D, Kim CT, Dartois V, Park SK, Cho SN, Barry CE 3rd (2012) Linezolid for treatment of chronic extensively drug-resistant tuberculosis. N Engl J Med 367:1508-1518

Lenaerts AJ, Gruppo V, Marietta KS, Johnson CM, Driscoll DK, Tompkins NM, Rose JD, Reynolds RC, Orme IM (2005) Preclinical testing of the Nitroimidazopyran PA-824 for activity against *Mycobacterium tuberculosis* in a series of *in vitro* and *in vivo* models. Antimicrob Agents Chemother 49:2294–2301

Li R, Kenyon GL, Cohen FE, Chen X, Gong B, Dominguez JN, Davidson E, Kurzban G, Miller RE, Nuzum EO, Rosenthal PJ, Mckerrow JH (1995) *In vitro* antimalarial activity of chalcones and their derivatives. J Med Chem 38:5031-5037

Li XZ, Nikaido H (2004) Efflux-mediated drug resistance in bacteria. Drugs 64:159-204

Liaras K, Geronikaki A, Glamočlija J, Cirić A, Soković M (2011) Thiazole-based chalcones as potent antimicrobial agents. Synthesis and biological evaluation. Bioorg Med Chem 19:3135-3140

Lin YM, Zhou Y, Flavin MT, Zhou LM, Nie W, Chen FC (2002) Chalcones and flavonoids as anti-tuberculosis agents. Bioorg Med Chem Lett 10:2795-2802

Lopez SN, Castelli MV, Zacchino SA, Dominguez JN, Lobo G, Charris-Charris J, Cortes JCG, Ribas JC, Devia C, Rodriguez AM, Enriz RD (2001) *In vitro* antifungal evaluation and structure–activity relationships of a new series of chalcone derivatives and synthetic analogues with inhibitory properties against polymers of the fungal cell wall. J Bioorgan Med Chem 9:1999-2013

Louw GE, Warren RM, Gey van Pittius NC, Leon R, Jimenez A, Hernandez-Pando R, McEvoy CR, Grobbelaar M, Murray M, van Helden PD, Victor TC (2011) Rifampicin Reduces Susceptibility to Ofloxacin in Rifampicin-resistant *Mycobacterium tuberculosis* through Efflux. Am J Respir Crit Care Med 184:269–276

Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M (2012) Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PloS one 7:e34538

Maggi N, Pasqualucci CR, Ballotta R, Sensi P (1966) Rifampicin: a new orally active rifamycin. Chemotherapy 11:285-292

Mai CW, Yaeghoobi M, Abd-Rahman N, Kang YB, Pichika MR (2014) Chalcones with electron-withdrawing and electron-donating substituents: anticancer activity against TRAIL resistant cancer cells, structure-activity relationship analysis and regulation of apoptotic proteins. Eur J Med Chem 77:378-387

Mann FM, Prisic S, Hu H, Xu M, Coates RM, Peters RJ (2009) Characterization and inhibition of a class II diterpene cyclase from *Mycobacterium tuberculosis*: implications for tuberculosis. J Biol Chem 284:23574-23579

Margalith P, Pagani H (1961) Rifomycin XIV. Production of Rifomycin B. Appl Microbiol 9:325-334

Marrapu VK, Chaturvedi V, Singh S, Singh S, Sinha S, Bhandari K (2011) Novel aryloxy azolyl chalcones with potent activity against *Mycobacterium tuberculosis* H37Rv. Eur J Med Chem 46:4302-4310

Martin A, Morcillo N, Lemus D, Montoro E, da Silva Telles MA, Simboli N, Pontino M, Porras T, León C, Velasco M, Chacon L, Barrera L, Ritacco V, Portaels F, Palomino JC (2005) Multicenter study of MTT and resazurin assays for testing susceptibility to firstline anti-tuberculosis drugs. Int J Tuberc Lung Dis 9:901–906

Marttila HJ, Soini H, Eerola E, Vyshnevskaya E, Vyshnevskiy BI, Otten TF, Vasilyef AV, Viljanen, MK (1998) A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia. Antimicrob Agents Chemother 42:2443-2445

Masaki S, Sugimori G, Okamoto A, Imose J, Hayashi Y (1990) Effect of Tween 80 on the growth of *Mycobacterium avium* complex. Microbiol Immunol 34:653–663

Mascarello A, Chiaradia LD, Vernal J, Villarino A, Guido RV, Perizzolo P, Poirier V, Wonge D, Martins PGA, Nunes RJ, Yunes RA, Andricopulo AD, Av-Gay Y, Terenzi H (2010) Inhibition of *Mycobacterium tuberculosis* tyrosine phosphatase PtpA by synthetic chalcones: Kinetics, molecular modeling, toxicity and effect on growth. Bioorgan Med Chem 18:3783-3789

Massyn N, Day C, Barron P, Haynes R, English R, Padarath A (2013) District Health Barometer 2011/12. http://www.health-e.org.za/wp-content/uploads/2013/04/DHB2011_12lowres.pdf. Accessed 24 January 2014

Matsumoto M, Hashizume H, Tomishige T, Kawasaki M, Tsubouchi H, Sasaki H, Shimokawa Y, Komatsu M (2006) OPC-67683 a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis *in vitro* and in mice. PLoS Med 3:e466

Miesel L, Weisbrod TR, Marcinkeviciene JA, Bittman R, Jacobs WR Jr (1998) NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. J Bacteriol 180:2459-2467

Mikusová K, Slayden RA, Besra GS, Brennan PJ (1995) Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob Agents Chemother 39:2484-2489

Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM (1999) Aminoglycosides: activity and resistance. Antimicrob Agents Chemother 43:727-737

Minnikin D (1982) The biology of the mycobacteria. Vol. 1. Physiology, identification and classification. Tatledge C, Stanford J (eds), Academic, London, pp 94–184

Mishra L, Sinha R, Itokawa H, Bastow KF, Tachibana Y, Nakaniski Y, Kilgore N, Lee KH (2001) Anti-HIV and cytotoxic activities of Ru(II)/Ru(III) polypyridyl complexes containing 2,6-(2'-Benzimidazolyl)-pyridine/chalcone as co-ligand. J Bioorgan Med Chem 9:1667-1671

Moodley T (2011) The synthesis and characterisation of fluorinated chalcones. Masters Dissertation, University of KwaZulu-Natal

Morlock GP, Metchock B, Sikes D, Crawford JT, Cooksey RC (2003) *ethA, inhA, and katG* loci of ethionamide-resistant clinical *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 47:3799-3805

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

Mshana RN, Tadesse G, Abate G, Miörner H (1998) Use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide for rapid detection of rifampin-resistant *Mycobacterium tuberculosis*. J Clin Microbiol 36:1214–1219

Nair J, Rouse DA, Bai GH, Morris SL (1993) The *rpsL* gene and streptomycin resistance in single and multiple drug-resistant strains of *Mycobacterium tuberculosis*. Mol Microbiol 10:521-527

Naik N, Kumar HV, Dias S, Swamy JR (2013) Novel 4-methoxy-2-acetyl benzofuran based chalcones: a new perceptivity into their antioxidant potentials. Int J Pharm Pharm Sci 5:242-247

Narender T, Gupta S (2004) A convenient and biogenetic type synthesis of few naturally occurring chromeno dihydrochalcones and their *in vitro* antileishmanial activity. Bioorg Med Chem Lett 14:3913-3916

Nathan CF, Hibbs JJB (1991) Role of nitric oxide synthesis in macrophage antimicrobial activity. Curr Opin Immunol 3:65-70

National Department of Health Notification System (2012) Reported cases of TB (all types) (per 100 000). http://indicators.hst.org.za/healthstats/16/data. Accessed 24 January 2014

Neu H (1987) Clinical use of the quinolones. Lancet 330:1319-1322

Nielsen SF, Larsen M, Boesen T, Schønning K, Kromann H (2005) Cationic chalcone antibiotics. Design, synthesis, and mechanism of action. J Med Chem 48:2667-2677

Nielsen TF, Boesen T, Larsen M, Schønning K, Kromann H (2004) Antibacterial chalcones-biosteric replacement of the 4'-hydroxy group. J Bioorgan Med Chem 12:3047-3054

Nikaido H, Kim SH, Rosenberg EY (1993) Physical organization of lipids in the cell wall of *Mycobacterium chelonae*. Mol Microbiol 8:1025-1030

Noss EH, Pai RK, Sellati TJ, Radolf JD, Belisle J, Golenbock DT, Boom WH, Harding CV (2001) Toll-like receptor 2-dependent inhibition of macrophage class II MHC expression and antigen processing by 19-kDa lipoprotein of *Mycobacterium tuberculosis*. J Immunol 167:910–918

Nowakowska Z (2007) A review of anti-infective and anti-inflammatory chalcones. Eur J Med Chem 42:125-137

O'Brien J, Wilson I, Orton T, Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur J Biochem 267:5421–5426

O'Brien RJ, Spigelman M (2005) New drugs for tuberculosis: current status and future prospects. Clin Chest Med 26:327-340

Onajole OK, Govender K, Govender P, van Helden PD, Kruger HG, Maguire GEM, Muthusamy K, Pillay M, Wiid I, Govender T (2009) Pentacyclo-undecane derived cyclic tetra-amines: Synthesis and evaluation as potent anti-tuberculosis agents. Eur J Med Chem 44:4297–4305

Osório TM, Monache FD, Chiaradia LD, Mascarello A, Stumpf TR, Zanetti CR, Silveira DB, Barardi CRM, de Fatima Albino Smânia E, Viancelli A, Garcia LAT, Yunes RA, Nunes RJ, Smânia A Jr (2012) Antibacterial activity of chalcones, hydrazones and oxadiazoles against methicillin-resistant *Staphylococcus aureus*. Bioorg Med Chem Lett 22:225-230

Palomino JC, Traore H, Fissette K, Portaels F (1999) Evaluation of Mycobacteria Growth Indicator Tube (MGIT) for drug susceptibility testing of *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis 3:344–348

Palomino JC, Martin A, Camacho M, Guerra H, Swings J, Portaels F (2002) Resazurin microtiter assays plate: simple and inexpensive method for detection of drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 46:2720–2722

Pan F, Jackson M, Ma Y, McNeil M (2001) Cell wall core galactofuran synthesis is essential for growth of mycobacteria. J Bacteriol 183:3991-3998

Parikh K, Joshi D (2013) Antibacterial and antifungal screening of newly synthesized benzimidazole-clubbed chalcone derivatives. Med Chem Res 22:3688-3697

Pasca MR, Guglierame P, Arcesi F, Bellinzoni M, De Rossi E, Riccardi G (2004) Rv2686c–2687c–2688c, an ABC fluoroquinolone efflux pump in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 48:3175–3178

Pasca MR, Guglierame P, De Rossi E, Zara F, Riccardi G (2005) *mmpL7* gene of *Mycobacterium tuberculosis* is responsible for isoniazid efflux in *Mycobacterium smegmatis*. Antimicrob Agents Chemother 49:4775-4777

Pavan FR, Leite CQR, Coelho RG, Coutinho ID, Honda NK, Cardoso CAL, Vilegas W, Leite SRDA, Sato DN (2009) Evaluation of anti-*Mycobacterium tuberculosis* activity of *Campomanesia adamantium* (MYRTACEAE). Quim Nova 32:1222-1226

Pieters J (2008) *Mycobacterium tuberculosis* and the macrophage: maintaining a balance. Cell Host Microbe 3:399-407

Pillay M, Sturm AW (2007) Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. Clin Infect Dis 45:1409-1414

Pitarque S, Larrouy-Maumus G, Payré B, Jackson M, Puzo G, Nigou J (2008) The immunomodulatory lipoglycans, lipoarabinomannan and lipomannan, are exposed at the mycobacterial cell surface. Tuberculosis (Edinb) 88:560-565

Protopopova M, Hanrahan C, Nikonenko B, Samala R, Chen P, Gearhart J, Einck L, Nacy CA (2005) Identification of a new antitubercular drug candidate, SQ109, from a combinatorial library of 1, 2-ethylenediamines. J Antimicrob Chemother 56:968–974

Pym AS, Domenech P, Honore N, Song J, Deretic V, Cole ST (2001) Regulation of catalase-peroxidase (KatG) expression, isoniazid sensitivity and virulence by *furA* of *Mycobacterium tuberculosis*. Mol Microbiol 40:879–889

Ramaswamy SV, Reich R, Dou SJ, Jasperse L, Pan X, Wanger A, Quitugua T, Graviss EA (2003) Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 47:1241-1250

Rampersad SN (2012) Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors (Basel) 12:12347-12360

Rastogi N (1991) Recent observations concerning the structure and function relationships in the mycobacterial cell envelope: elaboration of a model in terms of mycobacterial pathogenicity, virulence and drug-resistance. Res Microbiol 142:464–476

Rengarajan J, Sassetti CM, Naroditskaya V, Sloutsky A, Bloom BR, Rubin EJ (2004) The folate pathway is a target for resistance to the drug para-aminosalicylic acid (PAS) in mycobacteria. Mol Microbiol 53:275-282

Richter E, Rusch-Gerdes S, Hillemann D (2007) First linezolid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 51:1534–1536

Rizvi SUF, Siddiqui HL, Johns M, Detorio M, Schinazi RF (2012) Anti-HIV-1 and cytotoxicity studies of piperidyl-thienyl chalcones and their 2-pyrazoline derivatives. Med Chem Res 21:3741-3749

Rizvi SUF, Ahmad M, Bukhari MH, Montero C, Chatterjee P, Detorio M, Schinazi RF (2014) Anti-HIV-1 screening of (2E)-3-(2-chloro-6-methyl/methoxyquinolin-3-yl)-1-(aryl) prop-2-en-1-ones. Med Chem Res 23:402-407

Roberts GD, Goodman NL, Heifets L, Larsh HW, Lindner TH, McClatchy JK, McGinnis MR, Siddiqi SH, Wright P (1983) Evaluation of the BACTEC radiometric method for recovery of mycobacteria and drug susceptibility testing of *Mycobacterium tuberculosis* from acid-fast smear positive specimens. J Clin Microbiol 18:689–696

Robinson N, Wolke M, Ernestus K, Plum G (2007) A mycobacterial gene involved in synthesis of an outer cell envelope lipid is a key factor in prevention of phagosome maturation. Infect Immun 75:581-591

Rodrigues L, Machado D, Couto I, Amaral L, Viveiros M (2012) Contribution of efflux activity to isoniazid resistance in the *Mycobacterium tuberculosis* complex. Infect Genet Evol 12:695–700

Rosenthal IM, Zhang M, Williams KN, Peloquin CA, Tyagi S, Vernon AA, Bishai WR, Chaisson RE, Grosset JH, Nuermberger EL (2007) Daily dosing of rifapentine cures tuberculosis in three months or less in the murine model. PLoS Med 4:e344

Rossi M, Caruso F, Crespi EJ, Pedersen JZ, Nakano G, Duong M, McKee C, Lee S, Jiwrajka M, Caldwell C, Baffour F, Karlin DA, Lidoff G, Leone S, Balducci V, Miler J, Incerpi S (2013) Probing antioxidant activity of 2'-hydroxychalcones: crystal and molecular structures, *in vitro* antiproliferative studies and in vivo effects on glucose regulation. Biochimie 95:1954-1963

Safi H, Sayers B, Hazbón MH, Alland D (2008) Transfer of *embB* codon 306 mutations into clinical *Mycobacterium tuberculosis* strains alters susceptibility to ethambutol, isoniazid, and rifampin. Antimicrob Agents Chemother 52:2027-2034

Safi H, Fleischmann RD, Peterson SN, Jones MB, Jarrahi B, Alland D (2010) Allelic exchange and mutant selection demonstrate that common clinical *embCAB* gene mutations only modestly increase resistance to ethambutol in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 54:103-108

Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, McNeil M, Peterson SN, Chatterjee D, Fleischmann R, Alland D (2013) Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat Genet 45:1190-1197

Saranya AV, Ravi S (2013) *In vitro* antifungal activity of chalcones with phenothiazine ring. Int J Org Bioorg Chem 3:9-12

Schlesinger LS (1993) Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J Immunol 150:2920–2930

Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK (2003) Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages insights into the phagosomal environment. J Exp Med 198:693-704

Scorpio A, Zhang Y (1996) Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat Med 2:662-667

Selikoff IJ, Robitzek EH (1952) Tubercu1osis chemotherapy with hydrazine derivatives of isonicotinic Acid. CHEST J 21:385-438

Sensi P, Greco AM, Ballotta R (1959) Rifomycin. I. Isolation and properties of rifomycin B and rifomycin complex. Antibiotics annual 7:262-270

Sensi P, Ballotta R, Greco AM, Gallo GG (1961) Rifomycin. XV. Activation of rifomycin B and rifomycin 0. Production and properties of rifomycin S and rifomycin SV. Farmaco Sci 16:165-80

Sharma M, Chaturvedi V, Manju YK, Bhatnagar S, Srivastava K, Puri SK, Chauhan P (2009a) Substituted quinolinyl chalcones and quinolinyl pyrimidines as a new class of anti-infective agents. Eur J Med Chem 44:2081-2091

Sharma PC, Jain A, Jain S (2009b) Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. Acta Pol Pharm 66:587-604

Sharma H, Patil S, Sanchez TW, Neamati N, Schinazi RF, Buolamwini JK (2011) Synthesis, biological evaluation and 3D-QSAR studies of 3-keto salicylic acid chalcones and related amides as novel HIV-1 integrase inhibitors. Bioorg Med Chem 19:2030-2045

Shenvi S, Kumar K, Hatti KS, Rijesh K, Diwakar L, Reddy GC (2013) Synthesis, anticancer and antioxidant activities of 2,4,5-trimethoxy chalcones and analogues from asaronaldehyde: structure-activity relationship. Eur J Med Chem 62:435-442

Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE 3rd, Stover CK (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science 272:1641-1643

Shi W, Zhang X, Jiang X, Yuan H, Lee JS, Barry CE 3rd, Wang H, Zhang W, Zhang Y (2011) Pyrazinamide inhibits trans-translation in *Mycobacterium tuberculosis*: a potential mechanism for shortening the duration of tuberculosis chemotherapy. Science 333:1630-1632

Siddiqi N, Das R, Pathak N, Banerjee S, Ahmed N, Katoch VM, Hasnain SE (2004) *Mycobacterium tuberculosis* isolate with a distinct genomic identity overexpresses a tap-like efflux pump. Infection 32:109–111

Singh R, Manjunatha U, Boshoff HI, Ha YH, Niyomrattanakit P, Ledwidge R, Dowd CS, Lee Y, Kim P, Zhang L, Kang S, Keller TH, Jiricek J, Barry CE 3rd (2008) PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. Science 322:1392-1395

Singh M, Jadaun GP, Ramdas, Srivastava K, Chauhan V, Mishra R, Gupta K, Nair S, Chauhan DS, Sharma VD, Venkatesan K, Katoch VM (2011) Effect of efflux pump inhibitors on drug susceptibility of ofloxacin resistant *Mycobacterium tuberculosis* isolates. Indian J Med Res 133:535–540

Sivakumar PM, Seenivasan SP, Kumar V, Doble M (2007) Synthesis, antimycobacterial activity evaluation, and QSAR studies of chalcone derivatives. Bioorg Med Chem Lett 17:1695-1700

Sivakumar PM, Muthu Kumar T, Doble M (2009) Antifungal activity, mechanism and QSAR studies on chalcones. Chem Biol Drug Des 74:68-79

Sivakumar PM, Ganesan S, Veluchamy P, Doble M (2010) Novel chalcones and 1,3,5-triphenyl-2-pyrazoline derivatives as antibacterial agents. Chem Biol Drug Des 76:407-411

Smart BE (2001) Fluorine substituent effects (on bioactivity). J Fluorine Chem 109:3-11

Smit FJ, N'da DD (2014) Synthesis, *in vitro* antimalarial activity and cytotoxicity of novel 4-aminoquinolinyl-chalcone amides. Bioorg Med Chem 22:1128-1138

Smith I (2003) *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 16:463-496

Solomon VR, Lee H (2012) Anti-breast cancer activity of heteroaryl chalcone derivatives. Biomed Pharmacother 66:213-220

Spagnolo L, Törö I, D'orazio M, O'Neill P, Pedersen JZ, Carugo O, Rotilioe G, Battistoni A, Djinović-Carugo, K (2004) Unique features of the *sodC*-encoded superoxide dismutase from *Mycobacterium tuberculosis*, a fully functional copper-containing enzyme lacking zinc in the active site. J Biol Chem 279:33447-33455

Spies FS, da Silva PE, Ribeiro MO, Rossetti ML, Zaha A (2008) Identification of mutations related to streptomycin resistance in clinical isolates of *Mycobacterium tuberculosis* and possible involvement of efflux mechanism. Antimicrob Agents Chemother 52:2947–2949

Stehr M, Elamin AA, Singh M (2014) Filling the Pipeline-New Drugs for an Old Disease. Curr Top Med Chem 14:110-129

Stover CK, Warrener P, Van Devanter DR, Sherman DR, Arain TM, Langhorne MH, Anderson SW, Towell JA, Yuan Y, McMurray DN, Kreiswirth BN, Barry CE III, Baker WR (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature 405:962-966

Tahlan K, Wilson R, Kastrinsky DB, Arora K, Nair V, Fischer E, Barnes SW, Walker JR, Alland D, Barry CE III, Boshoff HI (2012) SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 56:1797-1809

Taniguchi H, Chang B, Abe C, Nikaido Y, Mizuguchi Y, Yoshida SI (1997) Molecular analysis of kanamycin and viomycin resistance in *Mycobacterium smegmatis* by use of the conjugation system. J bacteriol 179:4795-4801

Telenti A, Imboden P, Marchesi F, Matter L, Schopfer K, Bodmer T, Lowrie D, Colston MJ, Cole S (1993) Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 341:647-651

Teriete P, Yao Y, Kolodzik A, Yu J, Song H, Niederweis M, Marassi FM (2010) *Mycobacterium tuberculosis* Rv0899 adopts a mixed alpha/beta-structure and does not form a transmembrane beta-barrel. Biochemistry 49:2768-2777

Thomas JP, Baughn CO, Wilkinson RG, Shepherd RG (1961) A new synthetic compound with antituberculous activity in mice: ethambutol (dextro-2,2'-(ethylenediimino)-di-l-butanol). Am Rev Respir Dis 83:891-893

Timmerman P, Zhu M, Lee E, Williams P, de Chaffoy D, Huitric E, Hoffner S, Cambau E, Truffot-Pernot C, Lounis N, Jarlier V (2005) A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science 307:223–227

Tran TD, Nguyen TT, Do TH, Huynh TN, Tran CD, Thai KM (2012) Synthesis and antibacterial activity of some heterocyclic chalcone analogues alone and in combination with antibiotics. Molecules 17:6684-6696

Trivedi AR, Dodiya DK, Ravat NR, Shah VH (2008) Synthesis and biological evaluation of some new pyrimidines via a novel chalcone series. Arkivoc 11:131-141

Tuberculosis Drug Screening Program (2001) Search for new drugs for treatment of tuberculosis. Antimicrob Agents Chemother 45:1943-1946

Valdameri G, Gauthier C, Terreux R, Kachadourian R, Day BJ, Winnischofer SM, Rocha ME, Frachet V, Ronot X, Di Pietro A, Boumendjel A (2012) Investigation of chalcones as selective inhibitors of the breast cancer resistance protein: critical role of methoxylation in both inhibition potency and cytotoxicity. J Med Chem 55:3193-3200

van Rie A, Victor TC, Richardson M, Johnson R, van der Spuy GD, Murray EJ, Beyers N, Pittius NC, van Helden PD, Warren RM (2005) Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Resp Crit Care 172:636

Velayati AA, Farnia P, Ibrahim TA, Haroun RZ, Kuan HO, Ghanavi J, Farnia P, Kabarei AN, Tabarsi P, Omar AR, Varahram M, Masjedi MR (2009) Differences in cell wall thickness between resistant

and nonresistant strains of *Mycobacterium tuberculosis*: using transmission electron microscopy. Chemotherapy 55:303-307

Victor TC, van Rie A, Jordaan AM, Richardson M, van der Spuy GD, Beyers N, van Helden PD, Warren R (2001) Sequence Polymorphism in the *rrs* gene of *Mycobacterium tuberculosis* is deeply rooted within an evolutionary clade and is not associated with streptomycin resistance. J Clin Microbiol 39:4184-4186

Vilkas E, Amar C, Markovits J, Vliegenthart JFG, Kamerling JP (1973) Occurrence of a galactofuranose disaccharide in immunoadjuvant fractions of *Mycobacterium tuberculosis* (cell walls and wax D). Biochim Biophys Acta 297:423-435

Wade MM, Zhang Y (2004) Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Front Biosci 9:975-994

Wang L, Slayden RA, Barry CE III, Liu J (2000) Cell wall structure of a mutant of *Mycobacterium smegmatis* defective in the biosynthesis of mycolic acids. J Biol Chem 275:7224-7229

Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, van Pittius NCG, van Helden PD (2004) Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Resp Crit Care 169:610-614

Watanabe S, Zimmermann M, Goodwin MB, Sauer U, Barry 3rd CE, Boshoff HI (2011) Fumarate reductase activity maintains an energized membrane in anaerobic *Mycobacterium tuberculosis*. PLoS Pathog 7:e1002287

Wehrli W, Staehelin M (1971) Actions of the rifamycins. Bacteriol Rev 35:290-309

Williams KN, Brickner SJ, Stover CK, Zhu T, Ogden A, Tasneen R, Tyagi S, Grosset JH, Nuermberger EL (2009) Addition of PNU-100480 to first-line drugs shortens the time needed to cure murine tuberculosis. Am J Respir Crit Care Med 180:371-376

Wilson M, DeRisi J, Kristensen HH, Imboden P, Rane S, Brown PO, Schoolnik GK (1999) Exploring drug-induced alterations in gene expression in *Mycobacterium tuberculosis* by microarray hybridization. Proc Natl Acad Sci USA 96:12833-12838

Winder FG, Collins PB (1970) Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J Gen Microbiol 63:41-48

Wong SY, Lee JS, Kwak HK, Via LE, Boshoff HI, Barry CE III (2011) Mutations in *gidB* confer low-level streptomycin resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 55:2515-2522

Working group on new TB drugs (2013) Global TB Drug pipeline. http://www.newtbdrugs.org/pipeline-discovery.php. Accessed 24 January 2014

World Health Organization (2010) Treatment of tuberculosis: guidelines for national programmes. http://whqlibdoc.who.int/publications/2010/9789241547833_eng.pdf. Accessed 5 January 2014

World Health Organization (2013a) Global Tuberculosis Report 2013. http://apps.who.int/iris/bitstream/10665/75938/1/9789241564502_eng.pdf. Accessed 14 January 2014

World Health Organization (2013b) Definitions and reporting framework for tuberculosis- 2013 revision. http://apps.who.int/iris/bitstream/10665/79199/1/9789241505345_eng.pdf. Accessed 3 January 2014

Wu JH, Wang XH, Yi YH, Lee KH (2003) Anti-AIDS agents 54. A potent anti- HIV chalcone and flavonoids from genus desmos. J Bioorgan Med Chem 13:1813-1815

Wu MH, Yang XH, Zou WD, Liu WJ, Li C (2006) Refinement of the crystal structure of (E)-1,3-diphenyl-2-propen-1-one, C15H12O. Z Kristallogr 221:323-324

Yadav N, Dixit SK, Bhattacharya A, Mishra LC, Sharma M, Awasthi SK, Bhasin VK (2012) Antimalarial activity of newly synthesized chalcone derivatives *in vitro*. Chem Biol Drug Des 80:340-347

Yajko DM, Madej JJ, Lancaster MV, Sanders CA, Cawthon VL, Gee B, Babst A, Hadley WK (1995) Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*. J Clin Microbiol 33:2324–2327

Yeager RL, Munroe WG, Dessau FI (1952) Pyrazinamide (aldinamide*) in the treatment of pulmonary Tuberculosis. Trans Annu Meet Natl Tuberc Assoc 48:178-201

Zager EM, McNerney R (2008) Multidrug-resistant tuberculosis. BMC Infect Dis 8:10

Zhang Y, Heym B, Allen B, Young D, Cole S (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358:591-593

Zhang Y, Mitchison D (2003) The curious characteristics of pyrazinamide: a review. Int J Tuberc Lung Dis 7:6-21

Zhang Y (2005) The magic bullets and tuberculosis drug targets. Annu Rev Pharmacol Toxicol 45:529-564

Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, Jang J, Nam J, Dick T, Walker JR, Pethe K, Camacho LR (2013) para-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. J Biol Chem. 288:23447-23456