

Elucidating the antimycobacterial mechanisms of ciprofloxacin and decoquinatate derivative RMB041 on *Mycobacterium tuberculosis* using metabolomics

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**“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we can fear less.”**

~Marie Curie

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SUMMARY

Tuberculosis (TB) is amongst the leading causes of death brought about by a single infectious agent. Despite all the research done to date since the discovery of the infectious agent, *Mycobacterium tuberculosis* (*Mtb*) in 1882, its prevalence is still that of epidemic proportions. This can be ascribed to several factors, including, amongst others: the rapid development of anti-TB drug resistance, poor patient adherence, and inadequate treatment regimens. Subsequently, a better understanding of the mechanisms of action (MoA) of the new anti-TB drugs, as well as the possible mechanisms of resistance (MoR) of *Mtb* to these, is desperately needed. However, due to the many challenges associated with de novo drug development, including the high costs and the extremely long duration required for the completion of the mandatory clinical trials, the use of existing drugs for which safety and efficiency have already been verified, repurposed as anti-TB drugs, is considered a viable option for faster treatment availability. Drug discovery has also seen promising progress with a computational prediction of growth/replication targets, drug-host interactions, and pharmacokinetic properties.

To this end, we investigated and compared the whole metabolome of *Mtb* treated with ciprofloxacin and decoquinatone RMB041, respectively, to those of non-treated *Mtb* (control samples), by using two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) and various uni- and multivariate statistical methods. GCxGC-TOFMS is a powerful analytical technique that separates sample constituents at trace levels while providing accurate molecular structural information of the differentiated compounds. The identified metabolite markers characterizing the *Mtb* alterations induced by the presence of the drugs were used to elucidate the antimycobacterial MoA against *Mtb* and possible MoR by *Mtb* to the drug (in the case of the ciprofloxacin treatment). The treatment-induced metabolomic changes observed indicated that both ciprofloxacin and decoquinatone RMB041 cause inhibition/destruction of the cell wall and DNA damage. In the decoquinatone RMB041 treated group, inhibition of cell growth/replication and a reaction by *Mtb* going into a more pronounced state of dormancy, with a lack of DNA repair was additionally observed in response to the drug.

Considering that drugs commonly target multiple proteins, we investigated proteins possibly affected by decoquinatone RMB041 that would explain the altered metabolic pathways seen in the aforementioned experiments. For this, we used an *in silico* approach using various software and web servers. Interestingly, all of the identified protein targets identified via the *in silico*, were annotated to pathways, which also unanimously corresponded with our previously suggested MoA of decoquinatone derivative RMB04 determined by the metabolomics experiments. Furthermore, the target list included proteins that were previously suggested as promising targets against dormant *Mtb*. We additionally investigated the *in silico* safety profile and oral bioavailability, which also showed promising results.

In this pharmacometabolomics study, we identified the biochemical pathways that confirm the previously proposed MoA of ciprofloxacin and decoquinatone RMB041 against *Mtb*, identified previously unknown adaptive response mechanisms by *Mtb* to these anti-TB agents. We also prove the accuracy of using computational

software for determining/confirming the aforementioned activity of such novel repurposed anti-TB drugs and possible mechanisms of drug resistance by Mtb, and whether or not such medication can be used in combination with existing anti-TB drugs.

Key words: ciprofloxacin; decoquinat derivative; drug activity; drug resistance; *Mycobacterium tuberculosis*; metabolomics; computational drug design, virtual screening

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LIST OF ABBREVIATIONS AND SYMBOLS

<i>Abbreviation</i>	<i>Meaning</i>	<i>Abbreviation</i>	<i>Meaning</i>
ACP	Acyl carrier protein	AG	Arabinogalactan
AlrA	D-alanine racemase	ANOVA	Analysis of variance
API	Atmospheric pressure ionization	ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin	N,O- BSTFA	bis(trimethylsilyl)trifluoroacetamide
BSTFA	N,O- bis(trimethylsilyl)trifluoroacetamide	BTZ	Benzothiazinones
CE	Capillary electrophoresis	CFP-10	Culture filtrate protein 10
CHP	3-Cinnamoyl-4-hydroxy-pyran-2-one	CoA	Coenzyme A
DAG	Diacylglycerol	CYP450	Cytochrome P450
Ddl	D-alanine:d-alanine ligase	DAP	2,6-diaminopimelate
DMSO	Dimethyl sulfoxide	Ddn	Deazaflavin-dependant nitroreductase
DPA	Decaprenylphosphoryl arabinose	DNA	Deoxyribonucleic acid
DPR	Decaprenylphosphoryl ribose	EI	Electron ionization
eis	Enhanced intracellular survival	EMB	Ethambutol
ESAT-6	Early secreted antigenic target 6	ESI	Electrospray ionization
FQ	Fluoroquinolones	Fructose-5P	Fructose-6-phosphate
GC	Gas chromatography	GCxGC	Two-dimensional gas chromatography
Glucose-6P	Glucose-6-phosphate	Glycerol-3P	Glycerol-3-phosphate
GMs	Griselmycins	HIV	Human Immunodeficiency Virus
HP	High performance	HPLC	high-performance liquid chromatography
ICL	Isocitrate lyase	IFN-γ	Interferon gamma
INH	Isoniazid	KasA	Beta-ketoacyl carrier protein synthetase
KatG	Catalase-peroxidase enzyme	LAM	Lipoarabinomannans
LC	Liquid chromatography	LJ	Lowenstein-Jensen
MA	Mycolic acids	MAG	Monoacylglycerol
MAPc	Mycolyl arabinogalactan-peptidogalactan complex	MDR	Multidrug-resistant

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<i>Abbreviation</i>	<i>Meaning</i>	<i>Abbreviation</i>	<i>Meaning</i>
<i>MetPA</i>	<i>Metabolomics Pathway Analysis</i>	<i>MEX</i>	<i>Methoxamine hydrochloride</i>
<i>MEX</i>	<i>Methoxamine hydrochloride</i>	<i>MIC</i>	<i>Minimum inhibitory concentration</i>
<i>MoA</i>	<i>Mechanism of action</i>	<i>MoR</i>	<i>Mechanism of resistance</i>
<i>mRNA</i>	<i>Mitochondrial ribonucleic acid</i>	<i>MS</i>	<i>Mass spectrometry</i>
<i>MSⁿ</i>	<i>Sequential mass spectrometry</i>	<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
<i>MTBVAC</i>	<i>Live attenuated Mtb vaccine</i>	<i>NAA</i>	<i>Nucleic acid amplification</i>
<i>NAD⁺/H</i>	<i>Nicotinamide adenine dinucleotide/ with hydrogen</i>	<i>NADP⁺/H</i>	<i>Nicotinamide adenine dinucleotide phosphate/ with hydrogen</i>
<i>NRF</i>	<i>National Research Foundation</i>	<i>NSAIDs</i>	<i>Non-steroidal anti-inflammatory drugs</i>
<i>NTB</i>	<i>Nontuberculous mycobacteria</i>	<i>NWU</i>	<i>North-West University</i>
<i>OAA</i>	<i>Oxaloacetate</i>	<i>PA</i>	<i>Phosphatidate</i>
<i>PAMPs</i>	<i>pathogen-associated molecular patterns</i>	<i>PaMZ</i>	<i>Pretomanid, moxifloxacin, and pyrazinamide</i>
<i>PAS</i>	<i>Para-aminosalicylic acid</i>	<i>PBS</i>	<i>Phosphate-buffered saline</i>
<i>PCA</i>	<i>Principal component analysis</i>	<i>PG</i>	<i>Peptidoglycan</i>
<i>PCs</i>	<i>Principal components</i>	<i>PLS</i>	<i>Partial least squares regression</i>
<i>PIM</i>	<i>Phosphatidyl myo-inositol mannosides</i>	<i>PPD</i>	<i>Purified protein derivative</i>
<i>POA</i>	<i>Pyrazinoic acid</i>	<i>PRRs</i>	<i>pattern recognition receptors</i>
<i>PPP</i>	<i>Pentose phosphate pathway</i>	<i>QT</i>	<i>Cardiac contraction time intervals</i>
<i>PZA</i>	<i>Pyrazinamide</i>	<i>Ribose-5P</i>	<i>Ribose-5-phosphate</i>
<i>QRDR</i>	<i>Quinolone resistance-determining region</i>	<i>RNA</i>	<i>Ribonucleic acid</i>
<i>RIF</i>	<i>Rifampicin</i>	<i>rRNA</i>	<i>Ribosomal ribonucleic acid</i>
<i>ROS</i>	<i>Reactive Oxygen Species</i>	<i>SOS</i>	<i>Bacterial stress</i>
<i>SAM</i>	<i>S-Adenosyl methionine</i>	<i>TB</i>	<i>Tuberculosis</i>
<i>TAG</i>	<i>Triacylglycerol</i>	<i>TLC</i>	<i>Thin-layer chromatography</i>
<i>TCA</i>	<i>Tricarboxylic acid</i>	<i>TOF</i>	<i>Time-of-flight</i>
<i>TMSCI</i>	<i>Trimethylsilyl chloride</i>	<i>UV</i>	<i>Ultraviolet</i>
<i>TST</i>	<i>Tuberculin skin testing</i>	<i>XDR</i>	<i>Extensively drug-resistant</i>
<i>WHO</i>	<i>World Health Organization</i>	<i>ThyX</i>	<i>Thymidylate synthase</i>
<i>Xylulose-5P</i>	<i>Xylulose-5-phosphate</i>		

<i>Symbol</i>	<i>Meaning</i>	<i>Symbol</i>	<i>Meaning</i>
<	<i>Smaller than</i>	>	<i>Greater than</i>
↑	<i>Increased</i>	↓	<i>Decreased</i>
α	<i>Alpha</i>	β	<i>Beta</i>
γ	<i>Gamma</i>	°C	<i>Degrees Celsius</i>
g	<i>Gram</i>	mL	<i>Millilitre</i>
mg/g	<i>Milligram per gram</i>	mL/min	<i>Millilitre per minute</i>
$\mu\text{g/mL}$	<i>Microgram per millilitre</i>	m	<i>Metre</i>
mg/mL	<i>Milligram per millilitre</i>	μM	<i>Micromoles per liter</i>
CL_{int}	<i>Intrinsic clearance rate</i>	$\mu\text{L/min/mg}$	<i>Microliter per minute per milligram</i>
$t_{1/2}$	<i>Elimination half-life</i>	m/z	<i>Mass to charge ratio</i>

CHAPTER 1: PREFACE

1.1 BACKGROUND AND MOTIVATION

In 2018, the World Health Organization (WHO) underlined the need for a universal response to the global tuberculosis (TB) epidemic (Harding, 2020). In 2020, approximately 10 million people were diagnosed with TB, with more than 1.5 million fatalities (Harding, 2020), the latter of which was primarily due to multiple drug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* (*Mtb*), with treatment success rates of between 30% to 57%, of which over 10% resulted in relapse (WHO, 2020). The rapid development of drug resistance is largely attributable to poor patient adherence to anti-TB treatment due to serious adverse reactions, long treatment duration, and inaccessibility to treatment in many rural communities (Bradley *et al.*, 2015; Da Silva & Palomino, 2011; Holmes *et al.*, 2017). Even after treatment success, dormant *Mtb* in the host often survive (Peddireddy *et al.*, 2017). Further contributing to the increasing prevalence of resistant TB cases are inadequate immunization and diagnostic strategies and unsupervised therapies, which were exacerbated by the COVID-19 pandemic in 2020 (Harding, 2020; McQuaid *et al.*, 2020) and 2021. For the treatment of drug-resistant TB, only three drugs have received regulatory approval in the past 50 years, with resistance to these drugs also developing shortly thereafter (Harding, 2020; Nguyen *et al.*, 2018; Polsfuss *et al.*, 2019). Hence, in order to slow the spread of drug-resistant TB, there is an urgent need for the discovery of novel treatment strategies that would improve patient adherence, require less therapy supervision, and have better efficacy for killing drug-resistant *Mtb*. Part of the discovery of novel effective anti-TB drugs is determining the MoA of these drugs and *Mtb*'s response to treatment.

To this end, metabolomics and computational predictions could serve as useful tools to better understand the MoA or MoR of possible new anti-TB drugs (Aldred *et al.*, 2016), for which the minimum inhibitory concentrations (MIC) and intrinsic clearance rate (Cl_{int}), have already been assessed, and hence, potentially escalate the rate of discovery of new anti-TB drugs.

1.2 AIM AND OBJECTIVES

1.2.1 Aim

This research aims at using a well-described GCxGC-TOFMS metabolomics approach to elucidate the antimycobacterial MoA of the currently prescribed anti-TB drug, ciprofloxacin, and that of a new possible anti-TB drug candidate, decoquinatone RMB041, on laboratory cultured *Mtb* H37Rv. Additional *in silico* investigation of decoquinatone RMB041 aims to increase the understanding of the response of *Mtb* to this drug and evaluate its potential during host infection, as well as predict possible adverse effects. This study was done as part of a service agreement with the National Metabolomics Platform at the North-West University (NWU).

1.2.2 Objectives

The objectives of this study are as follows:

1. Culture *Mtb* in a predetermined sub-MIC (MIC_{50}) of
 - (a) Ciprofloxacin ($n=5$; $MIC_{50}=0.5\mu M$),
 - (b) Decoquinatone RMB041 ($n=5$; $MIC_{50}=10\mu M$) and
 - (c) without any anti-TB drug, i.e., control group ($n=5$).
2. Extract and prepare samples using the well described whole/total metabolome extraction and derivatization method and perform untargeted GCxGC-TOFMS analyses on these prepared sample extracts.
3. Validate the generated method and dataset by determining the repeatability of both the method and the analytical instrument used.
4. Statistically compare the data generated of the ciprofloxacin-treated group to the untreated group, and decoquinatone RMB041-treated group to the untreated group, in order to identify those metabolic markers best describing the observed metabolome changes induced by ciprofloxacin and decoquinatone RMB041 respectively. This information will then be interpreted in the light of existing literature to explain the MoA of these compounds.
5. Characterize absorption, distribution, metabolism, elimination, and toxicities of decoquinatone RMB041.
6. Identify decoquinatone RMB041 targets and investigate interactions between the drug and *Mtb* proteins, focusing on those relevant to drug resistance.

1.3 THESIS LAYOUT AND RESEARCH OUTPUTS

This thesis is written specifically to comply with the requirements of the NWU Potchefstroom Campus, South Africa, for the completion of the Doctoral Degree (Biochemistry) .

Chapter 1 (this chapter) is the preface consisting of a motivation, aims, objectives, the thesis layout, and the research and author contributions.

Chapter 2, the literature review, briefly discusses the prevalence and pathophysiology of TB, as well as previously published literature pertaining to the MoA and side effect of current (first-, second-, and third line) drugs, with emphasis on the 2 drugs used in this investigation, which is ciprofloxacin and decoquinat RMB041, in addition to other agents with newly discovered antimycobacterial potential. The reasons for using a GCxGC-TOFMS metabolomics research approach, instead of previously used omics technology, to elucidate the mechanisms, as well as identify metabolic biomarkers regarding TB, will be also fully explained. Parts of this chapter are published in a separate review paper.

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See Annexure A.

Chapter 3 entails a thorough description of the materials and methods used for the analytical processes, including *Mtb* cell culturing, metabolome extraction, derivatization, data processing, data clean-up, and statistical analysis. The repeatability regarding the use of the GCxGC-TOFMS, to order confirm the validity of the methods used, is also described.

Chapter 4 describes the antimycobacterial MoA of ciprofloxacin, by comparing the metabolomes of *Mtb* in the presence and absence of ciprofloxacin. This chapter has been published.

Citation: Knoll, K.E.; Lindeque, Z.; Adenijia, A.A.; Oosthuizen, C.B.; Lall, N.; Loots, D.T. (2021) Elucidating the Antimycobacterial Mechanism of Action of Ciprofloxacin Using Metabolomics. *Microorganisms*, 9(6), 1158. <https://doi.org/10.3390/microorganisms9061158>, impact factor: 4.2

See Annexure A.

Chapter 5 describes the antimycobacterial MoA of decoquinat derivative, by comparing the metabolomes of *Mtb* in the presence and absence of decoquinat derivative. This chapter has been published.

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See Annexure A.

Chapter 6 entails the in-silico investigation of decoquate RMB041, including pharmacokinetics and drug targets, as well as the potential of this drug against dormant *Mtb*.

Citation: Knoll, K.E.; Van der Walt, M.M.; Loots, D.T. (2021) *In silico* drug discovery strategies identified ADMET properties of decoquate RMB041 and its potential drug targets against *Mycobacterium Tuberculosis*. Accepted for publication in *Microbiology Spectrum*, Tracking number: Spectrum02315-21, impact factor: 7.2

See Annexure A.

Chapter 7 the conclusion of this investigation, restates the main argument, links the previous chapters to provide a global outcome with supporting evidence, and present possible future prospects.

1.4 AUTHOR CONTRIBUTIONS

The primary author of this thesis is Kirsten E. Knoll, who contributed to the conceptualizing, planning, sample analysis, statistical analysis, data interpretation and writing of the thesis and associated publications. The contributions of the co-authors, co-workers, and collaborators made towards this work, are summarised in *Table 1-1*.

The following is a statement from the study leader, the co-study leader, and the primary investigator, confirming their roles in the study and giving their permission that the data generated and conclusions made may form part of this thesis: I declare that my role in this study, as indicated in *Table 1-1*, is a representation of my actual contribution, and I hereby give my consent that this work may be published as part of the P.hD. thesis of Kirsten E. Knoll.



Prof. Du Toit Loots
Study Leader



Dr. Adetomiwa Adeniji
Co-study Leader



Kirsten Knoll
Primary Investigator

Table 1-1: Co-authors and their contributions.

Co-author/ Collaborator	Contribution
Prof. Du Toit Loots (Ph.D. Biochemistry)	Supervision, conceptualizing, planning, data interpretation, and guidance in writing of the thesis and associated publications.
Dr. Adetomiwa Adeniji (M.Sc. Microbiology; Ph.D. Biology)	Conceptualizing, planning, and guidance in writing of the thesis and associated publications.
Prof. Zander Lindeque (Ph.D. Biochemistry)	GCxGC-TOFMS sample analysis, statistical data analysis, interpretation, and guidance in writing of associated publications.
Prof. Carel Oosthuizen (Ph.D. Medicinal Plant Science)	Guidance in determining MIC values and <i>Mtb</i> cell culturing.
Prof. Dalene van der Walt (Ph.D. Biochemistry)	Guidance in conceptualizing, planning, and writing of the associated publication.
Prof. Namrita Lall (Ph.D. Medicinal Plant Science)	Guidance in determining MIC values.

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CHAPTER 2: LITERATURE REVIEW

Parts of this chapter were published:

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See Annexure A.

2.1 BACTERIOLOGY AND PATHOPHYSIOLOGY

Mtb, the causative agent for TB, was discovered by Robert Koch in 1882 (Koch, 1882). *Mtb* are rod-shaped, aerobic bacteria, with an extraordinary capability to survive and adapt to many environments (Talip *et al.*, 2013). This is largely due to the versatility of the complex cell wall (Knechel, 2009), rather than the release of specific toxins (Beste & McFadden, 2010; Luier & Loots, 2016). The unique cell wall, illustrated in Figure 2-1, consists of mycolic acids (MA), covalently attached to peptidoglycan (PG)-bound arabinogalactan (AG), to create an MA-PG-AG complex (MAPc) (Angala *et al.*, 2014; Seidel *et al.*, 2007). Interspersed within this complex are free MA, polypeptides, glycerolipids, lipomannan, and lipoarabinomannan (LAM), the latter being well known to facilitate *Mtb*'s survival in macrophages (Chatterjee *et al.*, 1991). Attached to the inner membrane of the cell wall are the phospholipids, phosphatidate (PA), and phosphatidyl *myo*-inositol mannosides (PIM), all of which play important roles in maintaining cell wall homeostasis (Salman *et al.*, 1999) and counteracting homeostatic disruptors (Bothra *et al.*, 2018; Santangelo *et al.*, 2016). This, along with the vast lipid barrier (Alderwick *et al.*, 2006), and the expression of efflux pumps (Angala *et al.*, 2014), gives the organism intrinsic drug resistance to several therapeutic drugs (Jarlier & Nikaido, 1994). The multifunctionality of the cell wall gained the interest of many researchers (Daniel *et al.*, 2014; Maitra *et al.*, 2019; Melly & Purdy, 2019), especially those pursuing anti-TB drug development (Gaur *et al.*, 2014; Knechel, 2009).

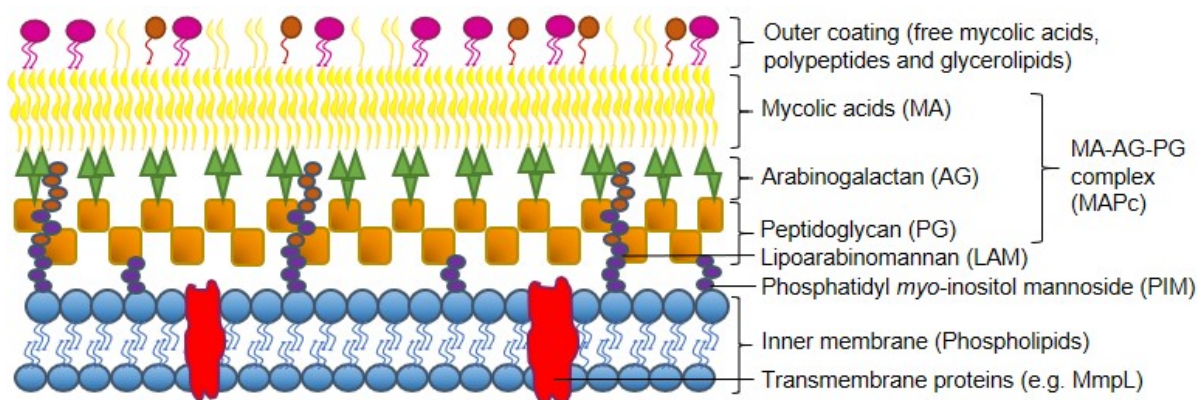


Figure 2-1: The *Mycobacterium tuberculosis* cell envelope. The mycolyl-arabinogalactan-peptidoglycan complex (MAPc), between the outer coating and the inner membrane.

Drug development has been hampered by the incomplete understanding of the physiology and metabolism of dormant *Mtb*, which can survive in a non-growing (or latent), and more drug-resistant state. This state can be activated in *Mtb* by a lack of nutrients, acidic stress, or hypoxia (Lenaerts *et al.*, 2015). Mycobacteria that enter into a dormant phase gradually shut down their respiration (even in the presence of oxygen), alter fatty acid and energy metabolism, decrease protein synthesis, increase mRNA stability and display increased stress resistance (Betts *et al.*, 2002). Several genes that are involved in the regulation of cell division, accumulation of polyphosphates, the transportation of peptides into the cell, and the homeostasis of redox stress, are differentially expressed in quiescent *Mtb* (Sureka *et al.*, 2008). There are some inconsistencies regarding the phenotype of dormant *Mtb*, likely due to variable effectors causing the stress and the ill-defined difference between the shift to dormancy versus complete quiescence (Peterson *et al.*, 2020). Key cellular activities that have been implicated during the adaption to growth-limiting conditions are the urea cycle, energy metabolism, fatty acid (FA) synthesis, and cell envelope lipid biosynthesis, which are all associated with remodelling of the tricarboxylic acid (TCA) cycle (Baker & Abramovitch, 2018; Deb *et al.*, 2009; Gengenbacher & Kaufmann, 2013). Some of these response mechanisms, especially those related to FA and cell membrane metabolism, have also been linked to polyamine synthesis (Majumdar *et al.*, 2016; Sakamoto *et al.*, 2020). The same cellular activities have been identified during the bacterial stress (SOS) response and involve enzymes such as isocitrate lyase (ICL) (which provides an energy-saving “short cut” via the glyoxylic shunt), KatG (responsible for regulating redox stress), and RSH (which produces hyperphosphorylated guanine nucleotides (p)ppGpp) (Dutta *et al.*, 2019; Ibeji *et al.*, 2020; Peterson *et al.*, 2020; Sarathy *et al.*, 2013; Shrivastava *et al.*, 2020). Enzymes that are associated with this stringent response in *Mtb*, are becoming attractive research options for identifying novel drug targets, and in the process, it was determined that drugs with higher lipophilicity are more active against dormant *Mtb* (Iacobino *et al.*, 2016; Yelamanchi & Surolia, 2020).

Transmission of TB occurs through small, *Mtb* infected airborne droplet nuclei that are released by individuals with active TB through verbal communication, sneezing, coughing, or singing (Holmes *et al.*, 2017; Sima *et al.*, 2019). The transmissibility depends on various factors, such as the bacilli load of the droplets, ventilation in the area, the virulence of the strain, and ultraviolet (UV) light (Knechel, 2009). When non-infected individuals inhale these droplet nuclei, the bacilli that do not get trapped by mucus and beat upwards for removal by cilia, traverse the respiratory tract and bronchi and reach the alveoli of the lungs, where pulmonary infection takes place (Abel *et al.*, 2014; Smith, 2003). Here, the lung's macrophages with pattern recognition receptors (PRRs) recognize the pathogen-associated molecular patterns (PAMPs) from *Mtb*, such as glycolipids, lipoproteins, and carbohydrates, engulf the droplets and initiate a cascade of down-stream immunological events, that determine whether the infection stays latent or progresses to active TB (Knechel, 2009). Vitamin D treatment is often co-administered, as this increases the function of the macrophages (Chai *et al.*, 2018). To eliminate or confine the slowly multiplying mycobacteria, macrophages release cytokines that attract T-lymphocytes to the infection site, constituting a cellular-mediated immune reaction that continues for 2-12 weeks (Kiran *et al.*, 2016). In individuals with an intact immune system, granuloma formation occurs. These are initiated by interferon- γ (IFN- γ) cytokines that are released by the activated T-lymphocytes (Fremond *et al.*, 2005; Naidoo *et al.*, 2019). Granulomas consist of a collection of macrophages encapsulated by fibroblasts, outside of which is a ring of lymphocytes (Martin *et al.*, 2016). At the centre of the granuloma, the caseum, a form of coagulative tissue necrosis, is replaced by fibrosis, which then undergoes calcification. These healed granulomas are known as “arrested lesions” and indicate a latent TB infection (Lenaerts *et al.*, 2015). When the immune system is compromised, for example by HIV, type 1 cytokine pathway defects, type 2 diabetes mellitus, or renal failure (Esmail *et al.*, 2014; Finlay *et al.*, 2012; S. K. Lau *et al.*, 2015), the coagulative tissue undergoes liquefactive necrosis (Martin *et al.*, 2016), which exposes extracellular bacteria and subsequently escalates the infection. This can happen at any point after the initial infection and is associated with active TB. Extrapulmonary TB arises when organisms then spread to other organs, such as the lymphatics (Ahluwalia, 2005), pleura, bones/joints (Sharma & Mohan, 2004), or meninges (Dhillon *et al.*, 2012). At a later stage of active TB disease progression, excessive immune reaction and the infection of the mentioned organs lead to clinical symptoms such as hemoptysis, coughing, night sweats, fever, chest pain, weight loss, dyspnoea, and weakness (Campbell & Bah-Sow, 2006; Knechel, 2009).

2.2 COMBATING TUBERCULOSIS

To reduce the global TB pandemic, several aspects need to be improved: (a) vaccination, (to better prevent active and recurrent TB), (b) diagnostics (for early identification of TB and specification of the *Mtb* strain to select accurate treatment), and (c) treatment regimens (to manage and eradicate both active and latent TB).

2.2.1 Vaccines

Vaccination primarily promotes the rapid onset of an adaptive immune response when infection occurs. This can be achieved by priming the host's immune response by non-virulent mycobacterial encounters to form memory T-cells, and hence an accelerated response when re-activated by *Mycobacteria* antigens on a second occasion (Andersen & Scriba, 2019). The Bacille Calmette-Guerin (BCG) vaccine, consisting of inactivated *Mycobacterium bovis* strains, has been used for over 80 years and is still currently the only available vaccine against TB (Borgers *et al.*, 2019). Although the BCG vaccine is considered adequate in protecting infants and children, its efficacy in adults is considerably lower and highly variable (Andersen & Scriba, 2019). Further limitations of the BCG vaccine include the lack of immunity against re-infection (Beste & McFadden, 2010), and adverse effects, such as lymphadenopathy and lymphadenitis (Zhu *et al.*, 2018).

Several new TB vaccines have been investigated of late, with a few currently undergoing clinical trials (Schrager *et al.*, 2018). The most clinically advanced candidate is the live attenuated *Mtb* vaccine, MTBVAC, which is based on genetically attenuated *Mtb* isolates that express the most human antigens to date (Gonzalo-Asensio *et al.*, 2017). Other popular choices for vaccines under investigation are those of inactivated whole-cell *M. vaccae* and *M. indicus pranii*, with the advantages of having overexpressed multistage immunodominant antigens and safe use in HIV-infected infants (Méndez-Samperio, 2018).

2.2.2 Diagnostics

The most common diagnostic methods for detecting TB infection/latent *Mtb*, are the tuberculin skin test (TST) and the interferon-gamma (IFN- γ) release assay. The TST uses purified protein derivative (PPD), a poorly defined mixture containing both secreted and somatic proteins of tuberculin antigens, obtained from heat-killed *Mtb* (Pai *et al.*, 2008), which is injected into the patient, usually on the ventral forearm. This diagnostic method is based on the acquired hypersensitivity reaction of the host, whereby T-lymphocytes release lymphokines, which result in thickening of the skin at the site of injection. IFN- γ release assays measure the antigen-stimulated IFN- γ in a patient's blood sample, in response to a combination of highly *Mtb*-specific antigens; culture filtrate protein 10 (CFP-10) and early secreted antigenic target 6 (ESAT-6) (Pai *et al.*, 2014). Unfortunately, both methods often present false-negative values in patients with a compromised immune system (Du Preez *et al.*, 2016; Samper & González-Martin, 2018), and are often unable to distinguish avirulent *M. bovis* strains and nontuberculous mycobacteria (NTB) from *Mtb* infection (Pai *et al.*, 2014; Van Pinxteren *et al.*, 2000).

The diagnosis of active TB is generally based on abnormalities on a chest radiograph of a patient showing abnormal respiratory symptoms. This can be followed by various methods including examining patient sputum (Knechel, 2009). The microscopic sputum smear requires heat-forced cell-wall permeation of a conventional dye and presents acid-fast bacilli as red (Costa *et al.*, 2008). This method is low-cost and can be used to monitor treatment progression as well. (Dhingra *et al.*, 2003). Bacteriological culture is the observation of the growth of

Mtb (obtained from sputum) in a culture media. This technique can be used to distinguish between various *Mycobacterium* species (Olivier & Loots, 2012) and, in the presence of antibiotics, detect drug resistance. Considering the slow growth rate of *Mtb*, bacteriological culture sampling is time-consuming and hence impractical as an initial diagnosis method, and although it is considered the “gold standard” for diagnosing TB, using this would prolong treatment onset (Moore & Curry, 1998).

As an optional follow-up to the sputum smear, nucleic acid amplification (NAA) methods can also be used. The fastest NAA technique commercially available is the GeneXpert MTB/RIF. This rapid molecular test detects *Mtb* DNA within 90 min and can pick up resistance to the most effective first-line drug, rifampicin (RIF), within two hours (Boehme *et al.*, 2011; Casela *et al.*, 2018; WHO, 2018). This test not only provides enough information to determine whether respiratory precautions should be maintained, the definite diagnosis of *Mtb*, as opposed to other mycobacteria, can also be done using such. Other methods for the identification of strains have also been described, such as using high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled with mass spectrometry (MS) (Dang *et al.*, 2013; Loots, 2016; Mason *et al.*, 2016). Delays in TB diagnosis frequently occur due to respiratory symptoms masking those of patients with HIV, the coexistence of chronic cough, or other lung diseases. Other factors involved in delayed TB diagnosis include false-negative sputum smears, old age, and alcohol or substance abuse (Chen *et al.*, 2011).

2.2.3 Treatment regimen

Antitubercular drugs are categorized based on their efficiency and the presence of adverse reactions. In this thesis, the U.S. guidelines were used (Brown, 2016), as some articles vary regarding the classification of second- and third-line drugs.

2.2.3.1 First-line regimen

Since the '90s, the standardized TB treatment involves the DOTS (Directly Observed Therapy, Short-course) regimen (Harding, 2020). This therapy approach includes a six-month course (Beste & McFadden, 2010) of a combination of four first-line drugs; a two-month intensive phase using RIF, isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA), followed by a four-month continuation phase using only INH and RIF (Borsari *et al.*, 2017). A summary of the adverse effects of these compounds are given in Table 2-1, and their respective chemical structures in Figure 2-2.

2.2.3.1.1 Rifamycins

Rifamycins, produced by *Streptomyces mediterranei*, have been used to treat TB since 1966 (Ginsberg & Spigelman, 2007). Previously, they have been considered sterilizing agents, meaning their purpose is to kill persistent *Mtb* associated with drug resistance (Arbex *et al.*, 2010; Prideaux *et al.*, 2015). Recently, however, it

was established that they are bacteriostatic, meaning that they mainly kill multiplying mycobacteria and tend to induce a state of dormancy in *Mtb* (Kaushik *et al.*, 2017). RIF is the most widely used rifamycin, especially for HIV-infected TB patients, due to weaker induction of hepatic enzymes and, hence, at lower risk of interaction with other co-administered antiretroviral agents (O'Brien & Nunn, 2001). Rifabutin, with a longer elimination half-life ($t_{1/2}$) than RIF (25 hours), is used in patients on protease inhibitors (Sekaggya-Wiltshire & Dooley, 2019), and has shown promise in cases involving RIF-resistant *Mtb* (Whitfield *et al.*, 2018). Rifapentine, although rarely used, is evaluated in combination with quinolones, in means of shortening the treatment regimen (Sekaggya-Wiltshire & Dooley, 2019). Pharmacokinetic properties of RIF include a minimum inhibitory concentration (MIC) of 0.5-1.25 $\mu\text{g/mL}$ (Kunin, 1996), a $t_{1/2}$ of up to 7 hours (Ji *et al.*, 1993; Lyons *et al.*, 2013), and a CL_{int} of $< 10 \mu\text{L/min/mg}$ (Lakshminarayana *et al.*, 2015). The low MIC values are partially due to its high lipophilicity, which enhances the ability to diffuse through the *Mtb* cell membrane. RIF is mainly metabolized in the liver to 25-desacetyl rifampicin and 3-formyl rifampicin (Loos *et al.*, 1985). This drug acts by blocking the β -subunit (RpoB) of the deoxyribonucleic acid (DNA)-dependant ribonucleic acid (RNA) polymerase (RNAP), ultimately inhibiting RNA transcription and protein synthesis (Arbex *et al.*, 2010). Other *Mtb* pathways found perturbed by RIF include those of glycerophospholipids, nucleotides, and amino acids (Zhao *et al.*, 2021).

RIF resistant mutant *Mtb* strains have been associated with altered energy metabolism and utilization of FA as the main source of carbon (Combrink & du Preez, 2020), and their survival response to this drug depends on the remodeling and maintaining of the cell wall (Du Preez & Loots, 2018). Over 90% of RIF resistant isolates are cross-resistant with INH and contain mutations in the *rpoB* gene (Farhat *et al.*, 2019; Goldstein, 2014; Kolyva & Karakousis, 2012; Palomino & Martin, 2014), with low-level resistance forming mutations in *rpoA* and *rpoC*, encoding the α and β' subunits of RNAP, respectively (Unissa & Hanna, 2017).

2.2.3.1.2 Isoniazid

Isoniazid (INH) has been used to treat TB since 1952 (Vilcheze & Jacobs, 2014). This drug has a MIC of 0.02-0.20 $\mu\text{g/mL}$ and is capable of eradicating 90% of *in vitro* mycobacteria within the first two days. However, resistance to INH has been reported almost immediately after its application, with an ever-increasing occurrence (Ginsberg & Spigelman, 2007; Kolyva & Karakousis, 2012; Sulis & Pai, 2020). INH is a pro-drug that requires oxidative activation by the *Mtb* catalase-peroxidase enzyme, KatG. The activated form of INH forms a complex with an NADH-dependant acyl carrier protein (ACP) reductase, InhA, and a beta-ketoacyl carrier protein synthetase, KasA, which are both essential for fatty acid elongation (Damntie *et al.*, 2014). By covalently binding to the nicotinamide ring of NAD^+ , bound within the active site of InhA, INH causes NADH to dissociate from InhA, thereby, inhibiting fatty acid elongation and MA synthesis (Bulatovic *et al.*, 2002; Chatterjee, 1997). Active INH metabolites were found to alter energy metabolism (Mehaffy *et al.*, 2018; Sriprakash & Ramakrishnan, 1970) and inhibit the biosynthesis of phospholipids (Wimpenny, 1963), proteins (Patil *et al.*,

2020), and nucleic acids (Timmins & Deretic, 2006). INH has a $t_{1/2}$ of 1.7 hours (Jayaram *et al.*, 2004) and a CL_{int} of $< 22 \mu\text{L}/\text{min}/\text{mg}$ (Lakshminarayana *et al.*, 2015).

The most observed mutations in INH-resistant clinical isolates are those in the *katG* gene of *Mtb* (Kolyva & Karakousis, 2012; Sulis & Pai, 2020). Low-level resistance may arise from mutations in *inhA* that result in reduced affinity of InhA enzymes, without affecting its enoyl reductase activity (Palomino & Martin, 2014; Sriprakash & Ramakrishnan, 1970). Other INH resistance-forming mutations appear in *ndh*, encoding NADH dehydrogenase, *kasA*, *ahpC*, and *rpoB* (Da Silva & Palomino, 2011; Damntie *et al.*, 2014). In previous studies, Loots (2014) reported that INH-resistant strains were less susceptible to INH by (a) upregulating the use of alkanes and fatty acids for energy and (b) synthesizing several compounds directly involved in counteracting oxidative stress, including those related to a novel ascorbic acid degradation pathway.

2.2.3.1.3 Ethambutol

EMB, of which the anti-TB activity was reported as early as 1961, primarily kills actively multiplying, rather than persistent, mycobacteria (Kolyva & Karakousis, 2012). EMB has a $t_{1/2}$ of 3 hours (Arbex *et al.*, 2010), with only 8-15% being metabolized by the liver, with the hydrophilic dicarboxylic acid as a known metabolite marker of this (Becker *et al.*, 2008; Kanaujia *et al.*, 2019). This drug functions by inhibiting mycobacterial membrane-associated arabinosyl transferases, which are involved in cell wall arabinan polymerization. This leads to the disruption of AG biosynthesis and a lack of arabinan receptors for MA (Da Silva & Palomino, 2011). The inhibited transfer of MA to the cell wall in turn results in the accumulation of trehalose mono- and dimycolate, and free MA, which subsequently alters the integrity of the cell wall envelope (Goude *et al.*, 2009). Disrupted cellular pathways include RNA/protein metabolism (Burke *et al.*, 2020; Forbes *et al.*, 1965), phospholipid synthesis, spermidine synthesis (Sareen & Khuller, 1988), and energy metabolism (increased NAD^+) (Sun *et al.*, 2019). Additionally, increased amounts of fatty acids and free mycolic acids have been identified in EMB-treated *Mtb* (Du Preez & Loots, 2018).

Resistance to EMB is usually associated with point mutations in the *embCAB* operon, coding for arabinosyl transferases (Kolyva & Karakousis, 2012). Most clinical isolated EMB-resistant organisms contain mutations in *embB*, of which mutations located in codon 306 are most prevalent (Damntie *et al.*, 2014; Ramaswamy *et al.*, 2000; Starks *et al.*, 2009; Streevatsan *et al.*, 1997). Mutations in Rv380c and RV3792, genes involved in decaprenylphosphoryl-B-D-arabinose biosynthesis and utilization, have also been found to significantly affect *Mtb* susceptibility to EMB (Palomino & Martin, 2014). Low-level resistance forming mutations involve those in genes *embR*, *rmlD*, *rmlA2*, Rv0340, and *panD* (Kolyva & Karakousis, 2012).

2.2.3.1.4 Pyrazinamide

Pyrazinamide (PZA), although discovered in 1952, has the least understood MoA of all the first-line anti-TB drugs (Kolyva & Karakousis, 2012). This drug kills dormant *Mtb* and contributes greatly to the reduction of the treatment duration of 9-12 months to 6 months. PZA is a prodrug that is converted to pyrazinoic acid (POA) by pyrazinamidase (Momekov *et al.*, 2014). The antimycobacterial activity of POA is attributed to exploiting *Mtb*'s weak acid features, rather than attacking a specific bacterial target. Because of an inefficient efflux system unique to *Mtb*, POA accumulates intracellularly, lowers intracellular pH, and, for reasons unknown, blocks the binding of coenzyme A (CoA) to aspartate decarboxylase (PanD) (Damntie *et al.*, 2014; Gopal *et al.*, 2019; Palomino & Martin, 2014), which subsequently inactivates a vital fatty acid synthase I (FAS I) (Ngo *et al.*, 2007). Furthermore, a protonated POA is proposed to de-energize the bacterial membrane by disrupting both the proton motive force and membrane transport (Da Silva & Palomino, 2011; Momekov *et al.*, 2014). This drug has a $t_{1/2}$ of 9 hours, and its activity can be enhanced by further reducing the pH (Fontes *et al.*, 2020).

Resistance to PZA is usually associated with *Mtb* point mutations in *pncA*, encoding for pyrazinamidase. (Damntie *et al.*, 2014). Most widely reported mutations are in a 561-bp region of the open reading frame or an 82-bp region of *pncA*'s presumed regulatory region (Palomino & Martin, 2014). Further resistance-associated mutations have been found in *rpsA*, the gene encoding 30 S ribosomal protein S1, involved in the tagging of proteins for degradation (Shi *et al.*, 2011), and *panD*, the gene encoding the aspartate 1-decarboxylase precursor, converting aspartic acid to alanine (Zhang *et al.*, 2013).

Table 2-1: Adverse effects of the first-line antitubercular drugs

First-line drug	Adverse effects	References
Rifamycins	Hypersensitivity reactions (including flu-like symptoms and urticaria), neurotoxicity (including headache, drowsiness, confusion, and muscular weakness), gastrointestinal abnormalities (including nausea, vomiting, anorexia, and diarrhoea), rashes, orange urine, sweat, tears, interstitial nephritis, acute tubular necrosis, and hematologic effects (including haemolysis, thrombocytopenia, and leukopenia).	(Combrink & du Preez, 2020; Rana, 2013)
Isoniazid (INH)	Hepatotoxicity, neurotoxicity (including confusion, depression, psychosis), hematologic abnormalities (including sideroblastic anemia, hemolytic anemia, thrombocytopenia, and neutropenia),	(Bangert & Hasbun, 2019; Combrink & du Preez, 2020)

increased urination, and gastrointestinal abnormalities (including nausea, vomiting, and diarrhea).

Ethambutol (EMB)	Retrobulbar neuritis, vision impairment (including loss of visual acuity and red/green color blindness), hyperuricaemia, rashes, dizziness, gastrointestinal abnormalities.	(Bangert & Hasbun, 2019; Lee & Nguyen, 2020)
Pyrazinamide (PZA)	Hepatotoxicity, drug induced fever, hyperuricemia, malaise, interstitial nephritis, pruritus, and gastrointestinal effects.	(Kwon <i>et al.</i> , 2020; Shih <i>et al.</i> , 2013)

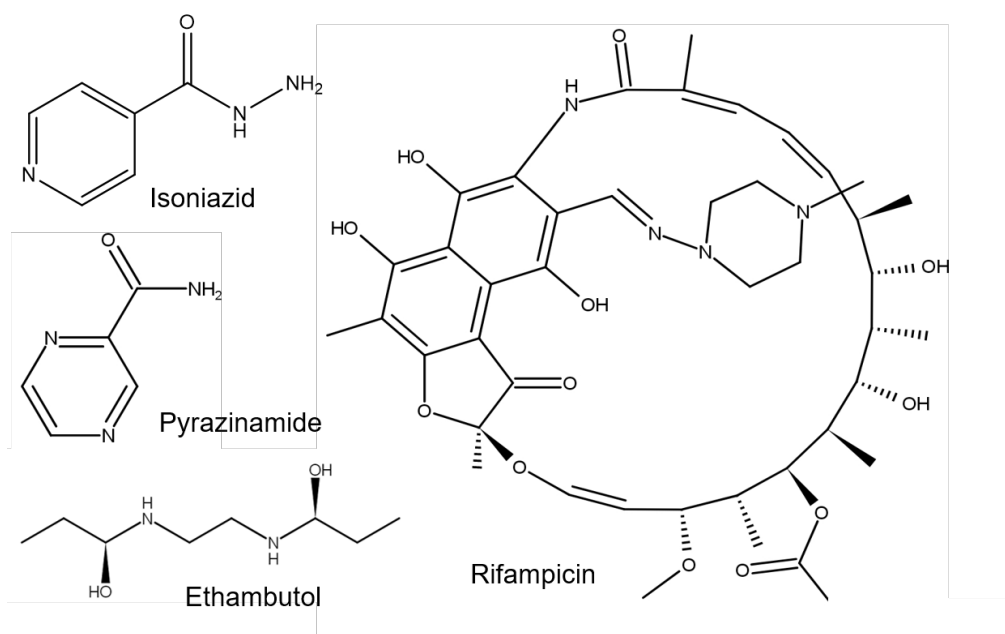


Figure 2-2: Structures of the first-line antitubercular drugs.

2.2.3.2 Second-line treatment

Second-line anti-TB drugs are typically used for the treatment of MDR-TB, which is diagnosed when resistance to rifampicin and/or isoniazid occurs. From 2018 to 2019, the WHO noted a 10% increase in the number of MDR-TB cases (WHO, 2020). The adverse effects of the second-line anti-TB drugs are summarized in Table 2-2, and their structures are presented in Figure 2-3.

2.2.3.2.1 Aminoglycosides

The aminoglycoside, streptomycin, was the first breakthrough in TB therapy in the early '40s and is currently an alternative first-line anti-TB agent, as recommended by WHO (WHO, 2019b). Its derivatives, however, amikacin and kanamycin, are currently used as second-line injectable medications (Dooley *et al.*, 2012; Kolyva & Karakousis, 2012). The antimycobacterial MoA of this group of compounds includes the irreversible inhibition of protein synthesis by binding to the 16S ribosomal RNA (rRNA) and S12 ribosomal protein, in the 30S ribosomal subunit (Da Silva & Palomino, 2011; Damntie *et al.*, 2014). This induces ribosomal changes, resulting in the misreading of mitochondrial RNA (mRNA) and subsequently the incorporation of incorrect amino acids into the peptide, resulting in a non-functional protein (Renko *et al.*, 2017).

High-level resistance has been associated with three mechanisms: (1) upregulation of the enhanced intracellular survival (*eis*) gene, which deactivates these compounds via *N*-acetylation (Houghton *et al.*, 2013), (2) mutations in the *rrs* gene, encoding 16S rRNA (Palomino & Martin, 2014), and (3) mutations in *rpsL*, encoding ribosomal protein S12 (Honore *et al.*, 1995; Meier *et al.*, 1996). Low-level resistance is usually characterized by a mutation in the *gidB* coding for a 16S rRNA methyltransferase, changes in cell envelope permeability, or diminished drug uptake (Wong *et al.*, 2011).

2.2.3.2.2 Fluoroquinolones

Fluoroquinolones (FQ's), first discovered in 1985, are continuously being used to develop newer with higher effectivity and improved pharmacokinetic properties (Aldred *et al.*, 2014; Arbex *et al.*, 2010; T. R. Blower *et al.*, 2016; Fan *et al.*, 2018). Their parent drugs, the quinolones; nalidixic acid, oxolinic acid, cinoxacin, and pipemidic acid, were first reported by George Leshner and colleagues in 1962 and used mainly to treat urinary tract infection, caused by *Enterobacteriaceae* (King *et al.*, 2000; Leshner *et al.*, 1962; Tillotson, 1996). Fluorination at the 6th carbon (C6) and modification of side-chains at position C1 and C8, resulted in the second-generation FQs; norfloxacin, enoxacin, ofloxacin, and ciprofloxacin, frequently used for ocular infectious diseases caused by *Streptococci* or *Staphylococci*, and the third-generation FQ's, including; levofloxacin, gatifloxacin and moxifloxacin, resulted in compounds with improved activity against both Gram-positive and Gram-negative bacteria and also improved absorption (Blondeau, 2004; Redgrave *et al.*, 2014). FQs have become one of the two most powerful classes of antimycobacterial drugs currently in routine use, and hence it is not surprising that these are also used for the treatment of MDR-TB (WHO, 2019a). One of the most potent FQ's, moxifloxacin, has shown promising results as a potential first-line agent as part of the PaMZ regimen (pretomanid, moxifloxacin, and pyrazinamide) (Dawson *et al.*, 2015; Tweed *et al.*, 2021). Ciprofloxacin and ofloxacin, are also frequently used in anti-TB regimens but have been poorly tolerated when used in combination with pyrazinamide (Alangaden & Lerner, 1997; Berning, 2001).

The high effectivity and broad antibacterial activity of ciprofloxacin has led to widespread use of this drug, which, unfortunately, has also resulted in significantly increased prevalence of resistance to this drug (Grimaldo *et al.*, 2001). Exposure to subinhibitory concentrations of ciprofloxacin, whether due to patient incompliance or an incorrect diagnosis, quickly results in resistance by the infective organism to the drug (Gumbo *et al.* (2005). Further investigations aiming to eradicate the rapid emergence of resistance would be beneficial, as ciprofloxacin offers several advantages compared to other FQs, such as a lower risk for hepatotoxicity, cardiovascular mortality, and ventricular arrhythmia (Gorelik *et al.*, 2019; Schloss *et al.*, 2018). Gatifloxacin on the other hand, although exhibiting generally good antimycobacterial activity (Hu *et al.*, 2003), was discontinued in the United States due to toxicity (Pranger *et al.*, 2019).

FQ's act by inhibiting enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, both of which are involved in bacterial DNA synthesis (Blondeau, 2004). DNA gyrase introduces negative superhelical twists in the bacterial DNA double helix that catalyze the separation of daughter chromosomes, whereas topoisomerase IV is responsible for the segregation into two daughter cells at the end of DNA replication, by removing the interlinking of daughter chromosomes (Blondeau, 2004). These interruptions cause DNA damage, and a subsequent SOS response follows. This response induces genes, primarily *recA* and *lexA*, and polymerase *dnaE2*, all involved in DNA repair, recombination, and mutagenesis (O'Sullivan *et al.*, 2008). Mycobacteria, however, only contain DNA gyrase, which takes on the functions of both DNA topoisomerases.

As such, resistance to FQ's by *Mtb* is rather prevalent (Aldred *et al.*, 2016; Palomino & Martin, 2014). Considering that resistance to FQ's is a criterion for defining XDR-TB, mutations to *Mtb*, and how this influences their susceptibility to resistance to FQ's, have been thoroughly investigated (Malik *et al.*, 2012). The most significant mutations are those in the conserved quinolone resistance-determining region (QRDR) of genes *gyrA* and *gyrB* (Da Silva & Palomino, 2011; Gibson *et al.*, 2018). Clinically relevant mutations at GyrA^{A90} and GyrA^{D94} have been found to cause quinolone resistance by disrupting the bridge-enzyme interaction, thereby decreasing drug affinity (Aldred *et al.*, 2016). GyrA^{D94} mutations include the replacement of aspartate with glycine, histidine, asparagine, tyrosine, or alanine. GyrA^{A90} mutations, on the other hand, include replacement of alanine with valine (Aldred *et al.*, 2014; Arjomandzadegan *et al.*, 2016; Aubry *et al.*, 2004; Chen *et al.*, 2012). While individual mutations in *gyrA* confer low-level resistance, high-level resistance usually requires multiple mutations in *gyrA*, or simultaneous mutations in *gyrA* and *gyrB* (Kolyva & Karakousis, 2012; Xu *et al.*, 1996). Low-level resistance has also been associated with the activation of efflux pumps (Chien *et al.*, 2017; Da Silva & Palomino, 2011). Interestingly, it has been reported that the simultaneous occurrence of mutations in T80A and A90G in *gyrA* led to hyper-susceptibility to several quinolones (Aubry *et al.*, 2006; Palomino & Martin, 2014). Exposure to FQ's before TB infection/diagnosis can also result in *Mtb* FQ resistance (Ahmad *et al.*, 2016).

2.2.3.2.3 Thioamides

Thioamide drugs, ethionamide, and prothionamide, are chemically related to INH and have been used as second-line anti-TB drugs since 1956 (Arbex *et al.*, 2010). Although thioamides, like INH, act by blocking mycolic acid synthesis (Palomino & Martin, 2014; Wang *et al.*, 2007), they are nicotinamide adenine dinucleotide phosphate (NADPH)-specific and need to be activated by the bacterial monooxygenase enzyme EthA (Kolyva & Karakousis, 2012). Both thioamides are rapidly eliminated from the body, with prothionamide having a slightly shorter $t_{1/2}$ (2.7 hours) than ethionamide (Jenner *et al.*, 1984; Lee *et al.*, 2009).

Thioamide resistance has been associated with mutations in the genes *ethA*, *inhA*, and Rv0565c (Dooley *et al.*, 2012; Hicks *et al.*, 2019).

2.2.3.2.4 Cycloserine/Terizidone

Cycloserine and terizidone, a combination of two cycloserine molecules, were synthesized in 1952 and are structural analogs of the amino acid d-alanine, an important bacterial cell wall component (Arbex *et al.*, 2010). They act by inhibiting the enzymes d-alanine racemase (AlrA) and d-alanine:d-alanine ligase (Ddl), which leads to the interruption of PG synthesis (Da Silva & Palomino, 2011; Kolyva & Karakousis, 2012).

Although the specific MoR of *Mtb* to these two drugs is unknown, resistance is suggested to be caused by the overexpression of AlrA and Ddl on a multicopy vector (Palomino & Martin, 2014).

2.2.3.2.5 Capreomycin

Capreomycin is a polypeptide antibiotic obtained from *Streptomyces capreolus*. Like STR, capreomycin acts by modifying ribosomal structures at the 16S rRNA, which leads to the inhibition of protein synthesis (Kolyva & Karakousis, 2012).

Its resistance is mostly conferred by mutations in *thyA* (Arbex *et al.*, 2010), the gene coding for an rRNA methyltransferase (Da Silva & Palomino, 2011), and aminoglycoside-resistance forming genes, *rrs* and *eis* (Houghton *et al.*, 2013; Palomino & Martin, 2014).

2.2.3.2.6 Para-aminosalicylic acid

Since 1946, para-aminosalicylic acid (PAS) has been used as an anti-XDR-TB drug (Arbex *et al.*, 2010). PAS inhibits tubercle bacilli by antagonizing bacterial folic acid synthesis and interfering with iron uptake (Minato *et al.*, 2015).

Although mutations in *thyA* (FX) account for up to 40% of phenotypic resistance and mutations in *folC*, encoding dihydrofolate synthase, has been associated with PAS-resistance (Palomino & Martin, 2014), the MoR is still mostly unknown (Dooley *et al.*, 2012).

2.2.3.2.7 Linezolid

Linezolid, the first oxazolidinone approved for clinical use, is not commonly recommended as an anti-TB drug due to its toxicities, particularly leukopenia, thrombocytopenia, and peripheral neuropathy (Kolyva & Karakousis, 2012). Linezolid exerts its activity by binding to the 23S rRNA in the ribosomal 50S subunit and consequently inhibiting an early step in protein synthesis (Da Silva & Palomino, 2011; Silva *et al.*, 2018).

Mutations at G2061T and G2576T in the 23S rRNA gene of *Mtb* are associated with *in vitro* high-level resistance (Kolyva & Karakousis, 2012). A more recent *in vitro* study found a mutation at T460C in *rplC*, the gene encoding the 50S ribosomal L3 protein, in linezolid-resistant mutants (Palomino & Martin, 2014).

Table 2-2: Adverse effects of the second-line antitubercular drugs.

Second-line drug	Adverse effects	References
Amikacin and kanamycin	Ototoxicity and nephrotoxicity, neurotoxicity (including neuromuscular blockade, tremor, lethargy, and, rarely, an organic brain syndrome).	(Bangert & Hasbun, 2019; Barnhill <i>et al.</i> , 2012)
Fluoroquinolones (FQ's)	Gastrointestinal abnormalities, neurotoxicity (dizziness, confusion, insomnia, depression, tremor, and agitation), and hypersensitivity reactions (including rash and urticaria, photosensitivity reactions, Stevens-Johnson syndrome, and anaphylaxis).	(Bangert & Hasbun, 2019; Barnhill <i>et al.</i> , 2012)
Thioamides	Gastrointestinal abnormalities, hepatotoxicity, neurotoxicity (including peripheral neuritis, diplopia, anxiety, depression, and hallucinations), endocrine abnormalities (including gynecomastia, alopecia, hypothyroidism, impotence, or menorrhagia), and mild skin reactions.	(Arbex <i>et al.</i> , 2010; Hsu <i>et al.</i> , 2010; Scardigli <i>et al.</i> , 2016; Thee <i>et al.</i> , 2016)
Cycloserine and terizidone	Hepatotoxicity, neurotoxicity (including peripheral neuropathy, confusion, depression, and psychosis), and hematological effects (including sideroblastic anemia, hemolytic anemia, thrombocytopenia, and neutropenia), Steven-Johnson syndrome, increased urination, and gastrointestinal discomfort.	(Arbex <i>et al.</i> , 2010; Van der Walt <i>et al.</i> , 2020)
Capreomycin	Nephrotoxicity and ototoxicity.	(Arnold <i>et al.</i> , 2017)
Para-aminosalicylic acid (PAS)	Gastrointestinal abnormalities (including diarrhea, nausea, and vomiting), and hypothyroidism	(Arbex <i>et al.</i> , 2010)

Linezolid	Hyperlactemia, myelosuppression, peripheral and optic neuropathy.	(Barnhill <i>et al.</i> , 2012; Nzila <i>et al.</i> , 2011; Sharma <i>et al.</i> , 2017)
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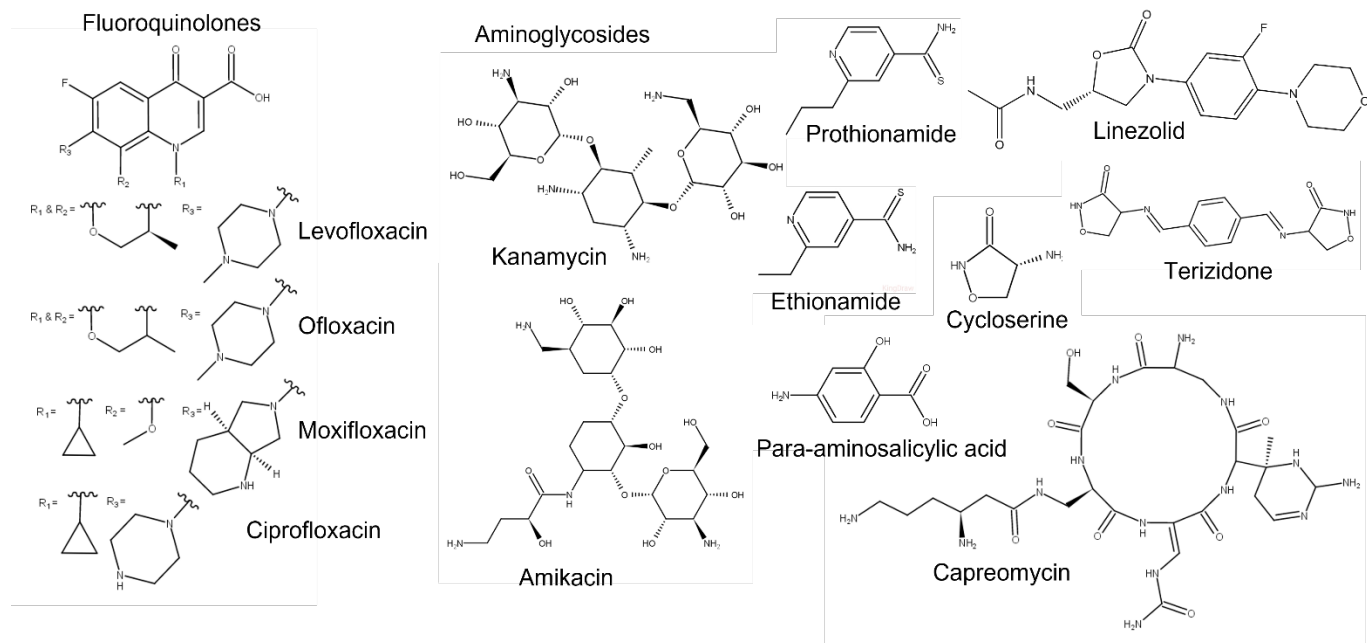


Figure 2-3: Structures of the second-line antitubercular drugs.

2.2.3.3 Third-line treatment

Third-line drugs are defined as drugs that may be useful but have doubtful or unproven efficacy. The drugs in the third-line treatment category are used for patients with XDR-TB, showing resistance to FQs and one of three injectable second-line drugs (capreomycin, kanamycin, or amikacin), in addition to INH and RIF (O'Brien & Nunn, 2001). Patients with drug-resistant TB are treated for up to 24 months with less effective, more toxic, and more expensive agents (Da Silva & Palomino, 2011; Mitchison & Davies, 2012). HIV co-infected patients treated with XDR-TB medication live an average of 25 days after diagnosis (Lawn & Wilkinson, 2006). The adverse effects of the third-line anti-TB medication are given in Table 2-3 and their chemical structures in Figure 2-4.

2.2.3.3.1 Clofazimine

Clofazimine, a riminophenazine, was developed in 1957 (Dey *et al.*, 2013) and only recently has been discovered to be effective against drug-resistant strains of *Mtb* (Xu *et al.*, 2012). Previously, it showed lower antimycobacterial activity against drug-susceptible TB but has successfully been used to treat leprosy, due to its

ability to concentrate in macrophages (Mafukidze *et al.*, 2016). This drug induces reactive oxygen species (ROS), which are reduced by NADH dehydrogenase and then spontaneously re-oxidized by O₂ (Hartkoorn *et al.*, 2014). The precise MoA yet remains unclear.

In vitro cross-resistance between clofazimine and bedaquiline is primarily caused by mutations in the transcriptional regulator Rv0678, related to upregulation of the multi-substrate efflux pump, MmpL5 (Hartkoorn *et al.*, 2014; Palomino & Martin, 2014).

2.2.3.3.2 Clarithromycin

Clarithromycin is a macrolide that exerts antimycobacterial activity by binding to the 50S ribosomal subunit and consequently inhibiting RNA-dependent protein synthesis (Kolyva & Karakousis, 2012). More specifically, the polypeptide exit tunnel is blocked, resulting in the dissociation of peptidyl-tRNA from the ribosome (Dinos, 2017).

Resistance to macrolides is associated with low cell wall permeability and expression of the *erm(37)* gene encoding 23S rRNA methyltransferase (Da Silva & Palomino, 2011; Kolyva & Karakousis, 2012).

2.2.3.3.3 Bedaquiline

The new diarylquinolone anti-TB drug, bedaquiline, has improved TB cure rates when added to the MDR-TB treatment regimen, with confirmed safety and efficacy (Pym *et al.*, 2016). Bedaquiline kills non-replicating mycobacteria (Robertson *et al.*, 2012), and is the only anti-TB drug that targets the mycobacterial energy metabolism (Pontali *et al.*, 2016), by inhibiting the proton pump of mycobacterial ATP synthase (Ginsberg & Spigelman, 2007; Silva *et al.*, 2018). A synergistic relationship between bedaquiline and PZA has been suggested (Kolyva & Karakousis, 2012). This use of this drug does face certain challenges, such as a doubled serum concentration when delivered in the fed compared to a fasted state, and reduced concentration when administered with rifampicin (Ginsberg & Spigelman, 2007).

The rapid emergence of resistance to bedaquiline has been reported in several cases (Veziris *et al.*, 2017). Aside from cross-resistance with clofazimine, due to the upregulated efflux pump MmpL, mutations in the *atpE* gene have been proposed to cause bedaquiline resistance (Da Silva & Palomino, 2011; Pontali *et al.*, 2016). Even so, the prevalence of mutant strains lacking mutations in this gene indicates an unspecified alternative MoR (Huitric *et al.*, 2010; Palomino & Martin, 2014).

2.2.3.3.4 Carbapenems (with beta-lactamase inhibitors)

Carbapenems imipenem and meropenem belong to a group of antibiotics known as beta-lactams. These drugs traverse through outer membrane proteins into the bacterial periplasmic space, where they bind to the penicillin-

binding proteins and consequently inhibit PG synthesis (Doi, 2020). Carbapenems are typically used in combination with beta-lactamase inhibitors to treat infection/disease caused by drug resistant *Mtb* strains. Beta-lactamase inhibitors, such as clavulanate and amoxicillin, prevent hydrolyzation of carbapenems (Gun *et al.*, 2020; Hugonnet *et al.*, 2009). Clavulanate seems to be more effective against MDR- and XDR-*Mtb* when combined with meropenem, when compared to a combination treatment with imipenem (Tiberi *et al.*, 2016).

Mtb reportedly develops resistance to carbapenems through increased efflux, beta-lactamase production, and impaired expression of outer membrane proteins (Doi, 2020).

2.2.3.3.5 Delamanid and Pretomanid

Delamanid and pretomanid (also known as PA-824) are bicyclic 4-nitroimidazoles, that represent one of the two novel antimycobacterial classes that were approved for the treatment of TB in the last 50 years (Rifat *et al.*, 2020). These nitroimidazoles are prodrugs that are reduced by a deazaflavin (F_{420})-dependant nitroreductase (Ddn) to a reactive nitro radical anion intermediate (Da Silva & Palomino, 2011). This reaction involves the oxidization of $F_{420}H_2$ to F_{420} by Fgd (F_{420} -dependant glucose-6-phosphate dehydrogenase). *In vitro*, delamanid seems to exhibit greater potency than pretomanid (Wen *et al.*, 2019). When added to a background treatment regimen, delamanid has repeatedly resulted in higher success rates (Scripconoka *et al.*, 2013). Delamanid has been conditionally approved for the treatment of XDR-TB in 2014 (Bloemberg *et al.*, 2015), but has had relatively limited clinical use to date, and is recommended to be used for less than six months (Gupta *et al.*, 2015). Delamanid and pretomanid inhibit the formation of mycobacterial cell wall components methoxy-MA and keto-MA, under aerobic conditions and induce respiratory poisoning in anaerobic conditions (Haver *et al.*, 2015; Xavier & Lakshmanan, 2014). An untargeted metabolomics study on pretomanid treated-*Mtb* indicated alterations in the pentose phosphate pathway (PPP), accumulation of hydroxymycolates, and inhibited protein synthesis (Baptista *et al.*, 2018; Nzila *et al.*, 2011).

$F_{420}H_2$ -deficient *Mtb* mutants showed hypersusceptibility to nitroimidazoles (Haver *et al.*, 2015; Wen *et al.*, 2019). Resistance is mainly associated with mutations in the gene *ddn*, coding for Ddn, and *fbtA-C*, involved in F_{420} synthesis (Bloemberg *et al.*, 2015). Low-level resistance forms by mutations in *fgd* coding for Fgd (Fujiwara *et al.*, 2018).

The combination of bedaquiline, linezolid, and pretomanid, known as Nix-TB, is currently under investigation as a potential anti-TB treatment regimen for MDR-TB (Cohen, 2017; Murray *et al.*, 2016), and shows good potential to treat MDR-TB within 6 to 9 months (Sloan & Lewis, 2016). The combination of pyrazinamide, moxifloxacin, and pretomanid, currently in phase 2 of clinical trials, has shown promising results for use as a second-line treatment regimen (Dawson *et al.*, 2015; Koser *et al.*, 2012; Williams *et al.*, 2012).

Table 2-3: Adverse effects of the third-line antitubercular drugs.

Third-line drug	Adverse effects	References
Clofazimine	Skin pigmentation and gastrointestinal disturbances.	(Dey <i>et al.</i> , 2013).
Clarithromycin	Prolonged cardiac contraction time intervals (QT) with cardiac arrhythmias and hepatotoxicity.	(Mafukidze <i>et al.</i> , 2016).
Delamanid	QT prolongation	(Xavier & Lakshmanan, 2014).
Bedaquiline	Gastrointestinal discomfort, arthralgia, headaches, and elevated transaminases.	(Pontali <i>et al.</i> , 2016)
Carbapenems	Neurotoxicity (delirium, myoclonia, confusion, seizures), skin rash, pruritus.	(Deshayes <i>et al.</i> , 2017; Lee & Bradley, 2019; Munoz-Gomez <i>et al.</i> , 2015)
Pretomanid (PA-824)	Hepatotoxicity, sinus tachycardia, mental status changes, and skin rash.	(Dawson <i>et al.</i> , 2015)

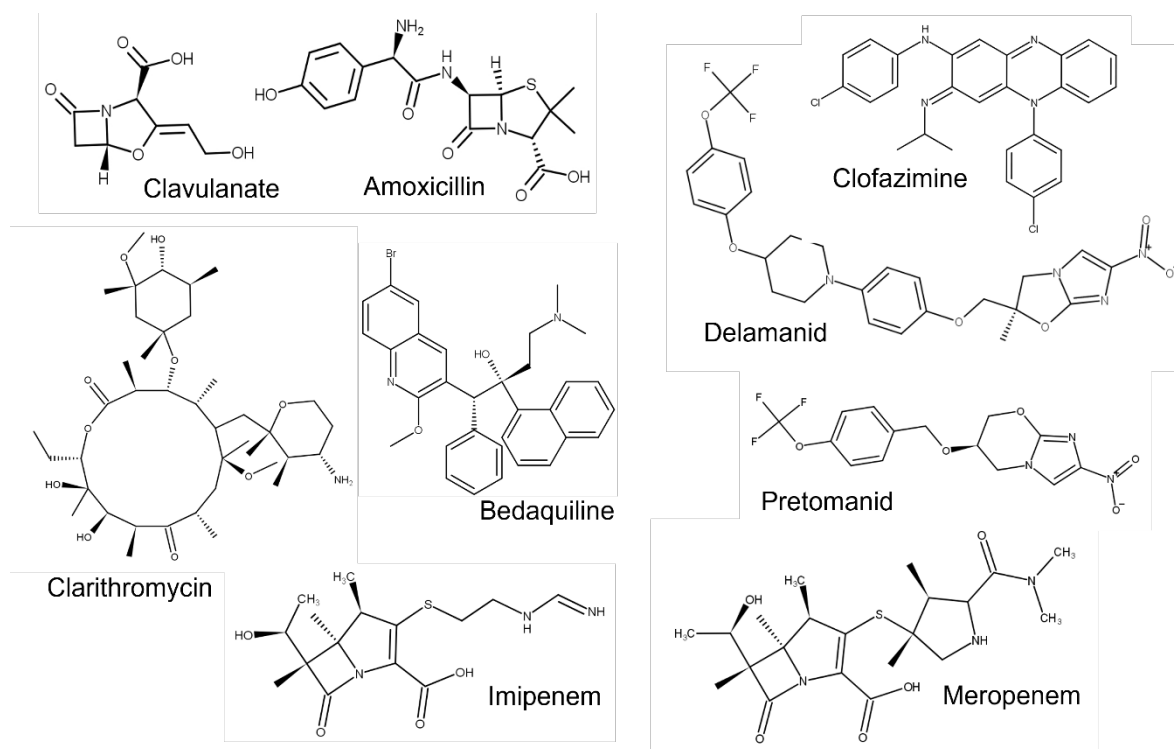


Figure 2-4: Structures of the third-line antitubercular drugs.

2.2.3.4 Investigational drugs

Due to the extremely high costs and long periods of time it takes for the completion of the mandatory clinical trials for new drug approval (Tiberi *et al.*, 2018), there is an increasing interest in the investigation of re-purposed drugs, of which the safety profiles have already been established (Brown, 2015; Dooley *et al.*, 2012). Several repurposed drugs have been derivatized into compounds that show a remarkable improvement of antimycobacterial effectivity, lower toxicity, and/or synergistic activity with other anti-TB drugs (Mafukidze *et al.*, 2016; Silva *et al.*, 2018). In this literature study, the most promising investigational derivatives discovered over the past five years are summarized. Amongst these, novel MoA includes disruption of: (a) cell wall biosynthesis, (b) energy production, (c) DNA metabolism, and (d) pyrimidine biosynthesis (Borsari *et al.*, 2017). Parts of this chapter has been published in the review by Adeniji *et al.* (2020) of which I was the second author and contributed equally to. This review paper also describes the investigation of antibiotics with little effect when used alone, but excellent synergistic activity with other anti-TB treatment regimens. Although in this literature study we summarised various sections of the review paper, we have attached this in Annexure A should you the reader be interested in greater detail on any of the below discussions. The structures of the parent compounds of the most promising investigational compounds discussed are illustrated in Figure 2-5.

2.2.3.4.1 Sulfadiazine

The successful use of sulfadiazine, more commonly used in the treatment of Leprosy, as an anti-MDR-TB and XDR-TB drugs has been reported (Brouqui *et al.*, 2017; Kamala, 1997; Sharma *et al.*, 2017). In a study by Bouzid *et al.* (2018), 92% of the tested clinical *Mtb* isolates, including 66 first-line anti-TB drug susceptible and 9 MDR isolates, were found to be susceptible to sulfadiazine, with MICs of 10-90 µg/mL. No synergistic effects between sulfadiazine and thrimetroprim, which are commonly prescribed together as cotrimoxazole (Ameen & Drancourt, 2013) were observed.

Further investigations are needed to establish the precise MoA of sulfadiazine and the resistance mechanism of *Mtb* to this drug.

2.2.3.4.2 Benzothiazinones

Benzothiazinones (BTZ), of which BTZ043 (2-[2-S-methyl-1,4-dioxo-8-azaspirodec8-yl]-8-nitro-6-(trifluoromethyl)-4H-1,3-benzothiazin-4-one) is the lead compound, is a newer class of antibiotic. BTZ043 is a prodrug, activated through the reduction of an essential nitro group to a nitroso derivative, which then reacts with the enzyme DprE1 (Kolyva & Karakousis, 2012; Palomino & Martin, 2014), and in turn inhibits the conversion of decaprenylphosphoryl ribose (DPR) to decaprenylphosphoryl arabinose (DPA), a precursor for AG synthesis (Borsari *et al.*, 2017). BTZ have *in vitro*, *ex vivo*, and *in vivo* antimycobacterial activity (Palomino & Martin, 2014), with synergistic activity when used together with bedaquiline (Hartkoorn *et al.*, 2014). Resistance to these has been associated with mutations in Rv3790, a gene involved in the epimerization of DPR to DPA, and the overexpression of a nitroreductase NfnB, which in turn results in the conversion of the essential nitro group to an amino group (Kolyva & Karakousis, 2012; Korkegian *et al.*, 2018; Palomino & Martin, 2014).

2.2.3.4.3 Nitrofurans derivatives

Based on the nitrofurans broad-spectrum antiparasitic and antibacterial activities, Krasavin *et al.* (2017) synthesized 3 nitrofurans analogues (10e, 10i, and 10n) with appreciable MICs (22–33 µM) against the *Mtb* H37Rv strain using isocyanide-based multicomponent chemistry. According to the authors, the 3 analogues displayed promising activity against MDR-*Mtb* isolates, poly-resistant patient-derived mutants, with acceptable cytotoxicity. From the several 5-nitro-2-furfurylidene N-aminolactams (10a–o) derivatives investigated, analogue 10n uniquely inhibited both MDR-*Mtb*: 2067 strain and the poly-resistant strain: 5307 with an improved MIC of 11 µM. Ran *et al.* (2016) had earlier reported the synthesis of 8 promising antitubercular agents (12e, 12k-m, 18a, 18d, 18e, and 18j), with compound 18e, exhibiting the best activity against *Mtb* H37Ra (MIC = 0.58 µM), with favorable cytotoxicity on Vero cells (IC₅₀ = 50.2 µM). Another group of pentacyclic nitrofurans analogues (9a/ Lee1106) with excellent nanomolar activity (1.9– 46 nM) against *Mtb* and safe

cytotoxicity profiles, was synthesized by Rakesh *et al.* (2014). From the compound set, 9a (MIC = 0.05 μ M) showed the best pharmacokinetics and also the most promise for further preclinical investigations, since it killed 99% of all hypoxic bacilli at a drug concentration of 18.94 μ M, during a rapid anaerobic dormancy evaluation (a model that mimics the nonreplicating-persister 2 conditions). Apart from 9a having a well-defined post-antibiotic effect and commendable synergistic properties with two other anti-TB agents, it was also active against pretomanid-resistant mutants.

2.2.3.4.4 Isatin derivatives

Isatin has a biologically versatile structure with many therapeutic applications (anti-bacterial, antiviral, antifungal, anti-tumor, analgesic, anti-convulsant, antioxidant, anti-inflammatory, anthelmintic, and anti-depressant). Furthermore, its stable indole nucleus enables the insertion of novel pharmacophore moieties, which has resulted in the discovery of many promising anti-TB agents (Fan *et al.*, 2018; Jiang *et al.*, 2018; Xu *et al.*, 2017; Xu; Zhao; Lv; *et al.*, 2019). Within various hybrid groups, including isatin-quinolone, isatin-thiazole, isoniazid-isatin, thiolactone-isatin, tetrahydropyrimidine-isatin, azetidinone-isatin, and chloroquinoline-isatin hybrids, a few derivatized compounds show promising anti-TB activity and moderate cytotoxicity (Xu *et al.*, 2017 Zhao *et al.*, 2019).

2.2.3.4.5 Quinoxaline derivatives

Another class of antimycobacterial recently showing promise for use against *Mtb* is the quinoxaline-1,4-di-N-oxide derivatives (Keri *et al.*, 2018). The quinoxaline ring in these compounds has a heterocyclic bioactive scaffold known for its isomeric promiscuity, and hence, a number of analogues of these, including various quinoxaline-1,4-di-N-oxide derivatives (quinoxaline-2-carbonitrile-1,4-di-N-oxide; quinoxaline-2-carboxylate-1,4-di-N-oxide; 1,4-di-N-oxide-3-methylquinoxaline-2-carboxylic acid aryl amide; ethyl quinoxaline-2-carboxylates; quinoxaline-1,4-di-N-oxide with fluoroquinolone and chalcone; 2-acetyl-3-methylquinoxaline-1,4-di-N-oxide; 1,4-di-N-oxidequinoxaline-2-ylmethylene isonicotinic acid hydrazide; 3methylquinoxaline-2-carboxylate-1,4-dioxide, and quinoxalinecarbonitrile-1,4-di-N-oxide) have been reported to exert significant anti-TB properties *in vitro* (MIC = 0.32–20.49 μ M) (Keri *et al.*, 2018).

2.2.3.4.6 Spectinomycin, pyridomycins, and caprazamycin derivatives

Spectinamides have significant narrow-spectrum activity against non-replicating TB, MDR-TB, and XDR-TB, while showing no obvious cytotoxicity, good pharmacokinetics, and *in vivo* efficacy (Bruhn *et al.*, 2015; Herrmann *et al.*, 2017; Horlacher *et al.*, 2013; Lee *et al.*, 2014). Spectinamides act by inhibiting protein synthesis, whereby some, like spectinamide 1599 (MIC = 0.61–3.86 μ M), are also reported to evade the Rv1258c

efflux pump innate resistance mechanism of *Mtb*. According to the same authors, a pyridomycin derivative dihydropyridomycin (mycolic acid synthesis inhibitor at MIC = 0.78 μ M) and caprazamycin (CPZ)-derivative caprazene (CPZEN-45; MIC = 1.81–2.91 μ M) also exhibit better activity against *Mtb* when compared with that of their parent compounds. Interestingly, dihydropyridomycin not only retains the parent compound (pyridomycin) characteristic MoA by directly binding to inhA, but unlike pyridomycin, it lacks the enol ester moiety in its scaffold, which favorably enables it to be more easily synthesized (Hartkoorn *et al.*, 2012). Possessing an isopropyl moiety also enables dihydropyridomycin to prevent the occurrence of mutations in KatG, the inhA-resistance-activating enzyme (Dong *et al.*, 2017). CPZEN-45, on the other hand, displayed comparatively better solubility in water, better activity against MDR-*Mtb*, XDR-*Mtb*, and many CPZ-resistant isolates. It was furthermore determined that it exhibits antibiosis by inhibition of the translocase I (MraY) and GlcNAc-1-phosphate transferase (WecA) and subsequently inhibiting mycolyl-AG synthesis (Dong *et al.*, 2017; Herrmann *et al.*, 2017).

2.2.3.4.7 Griselimycin derivatives

Griselimycins (GMs), are cyclic depsidecapeptide agents isolated from the *Streptomyces* strain DSM 40835 with anti-TB action, albeit with poor pharmacokinetics. However, a GM derivative, cyclohexylgriselimycin, shows far better anti-TB action, with a MIC of 0.05 μ M against drug-susceptible *Mtb* H37Rv and a MIC of 0.17 μ M for the same strain encapsulated in macrophage-like (RAW264.7) cells (Kling *et al.*, 2015). Cyclohexylgriselimycin acts against *Mtb* by targeting its DnaN (the DNA polymerase sliding clamp that anchors DNA polymerase) and subsequently inhibits mycobacterial DNA replication and repair (Holzgrabe, 2015; Kling *et al.*, 2015). It has additionally been shown to exhibit low cytotoxicity (Dong *et al.*, 2017) and high activity in acute and chronic TB mouse models, showing promising synergistic properties with RIF and PZA and the possibility of shortening TB treatment duration (Herrmann *et al.*, 2017).

2.2.3.4.8 Napthoquinone derivatives

Napthoquinones exhibit broad-spectrum antimicrobial activity (Uc-Cachón *et al.*, 2014), including inhibition of thymidylate synthase (ThyX) and DNA gyrase, both essential for DNA synthesis, and possible induction of apoptosis as a result of ROS (Karkare *et al.*, 2018; Pereyra *et al.*, 2019). Halicki *et al.* (2018) evaluated the anti-TB potential of six 1,4-napthoquinones derivatives against 3 *Mtb* strains (H37Rv (ATCC 27294); isoniazid mono-resistant strain (ATCC 35822); RIF mono-resistant strain (ATCC 35838)), concluding appreciable MICs (12.5 – 206.6 μ M). Two of the analogues showed favourable interactions with INH and RIF. The J774A.1 (ATCC TIB-67) macrophage cytotoxicity assay of the compounds showed that the compounds with lower MIC values exhibited low cytotoxicity.

2.2.3.4.9 Coumarin derivatives

Reports on the antimicrobial activity of coumarin-based compounds have been increasing over the years and recently included Mtb inhibition (Abdizadeh *et al.*, 2017; Jeyachandran *et al.*, 2012). Based on the parent compound's (coumarin: 1,2-benzopyrone) structural versatility and scaffolding ability (Kapp *et al.*, 2017), newer derivatives having potential anti-TB activity have been synthesized and investigated (Reddy *et al.*, 2018). Recently, Mangasuli *et al.* (2018) and Reddy *et al.* (2018) reported the synthesis of 2 specific coumarin derivatives, (1) a coumarin-theophylline hybrid (3a) and (2) a coumarin-oxime ether derivative (1 h), which both exhibited excellent anti-TB activity of MIC 0.32 μ M and 0.12 μ M respectively—MIC ranges close to the first-line anti-TB drug INH. Additionally, the majority of the derivatives in the study of Reddy *et al.* (2018) exhibited MICs in the range of 0.12– 16.51 μ M while also projecting good cytotoxicity profiles against Vero cells and excellent DNA cleaving properties (depicting nuclease activity). According to Mangasuli *et al.* (2018), 3a also showed good binding interactions with the Mtb 4decoquinatase enzyme and possible broad-spectrum antimicrobial properties (inhibiting *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*). Possible anti-TB MoA exhibited by coumarin derivatives includes disruption of cytochrome synthesis, inhibition of cell proliferation, activation of macrophages and inhibition of kinase (Kawate *et al.*, 2013; Niu *et al.*, 2017).

2.2.3.4.10 3-Cinnamoyl-4-hydroxy-pyran-2-one (CHP) derivatives

The synthesis and use as an anti-TB drug, of 25 different scaffolds of 3-cinnamoyl-4-hydroxy-pyran-2-one (CHP; Figure 2-5), with previously determined anti-HIV and anticancer potential, was done by et . Two of the CHP derivatives (2a and 2u) showed promising anti-Mtb activity (MIC = 14.03 and 12.5 μ M, respectively) with no apparent toxicity against 4 human cell lines (human kidney (HEK-293), colon cancer (HCT-116), prostate cancer (PC-3) and breast cancer (MCF-7)). The two CHP derivatives, moreover, displayed unique specificity for Mtb inhibition, in that they were ineffective against *M. smegmatis* and various other gram-negative and gram-positive bacteria, suggesting that the two CHP-based anti-TB compounds, would most likely not disrupt the human microbiome.

2.2.3.4.11 Isoniazid derivatives

Castelo-Branco *et al.* (2018) identified a few isoniazid hydrazides with striking anti-Mtb potency (MIC = 0.44 - 0.47 μ M), and lower mutagenicity and hepatotoxicity (against HepG2 cells) than their parent compounds. Interestingly, they also concluded that (a) their activities are dependent on the isonicotyl moiety but may be independent of the INH moiety and hydrolysis thereof, and (b) *Mtb* strains that exhibit both INH KatG mutation and RIF rpoB mutation were highly susceptible to these anti-TB compounds.

2.2.3.4.12 Quinolone derivatives (including decoquinatone RMB041)

Nalidixic acid, the first quinolone developed by George Lesher and colleagues in 1962, had limited effect against Gram-negative bacteria and was used mainly for urinary tract infection caused by *Enterobacteriaceae*. Since then, the study of quinolone structure-activity relationships has enabled researchers to modify quinolone structure, to lessen their toxicity while improving the spectrum of use and efficacy (Fan *et al.*, 2018; Taira *et al.*, 1993; Tillotson, 1996). One such derivative, bedaquiline (a diarylquinoline; MIC = 0.05–0.22 μ M) (Andries *et al.*, 2005), which was approved as an anti-TB agent in 2012 for its characteristic non-cross-resistance in MDR and XDR-Mtb strains (Kaur *et al.*, 2015), has spurred the investigating of various other quinolone derivatives as explorative scaffolds for the synthesis of similar compounds with enhanced anti-TB action.

Amongst the investigational fluoroquinolones, the 8-methoxy-derivatives of gatifloxacin and moxifloxacin, show the highest potency against *Mtb* (Aldred *et al.*, 2014; T. R. Blower *et al.*, 2016; Ginsberg & Spigelman, 2007). Pavić *et al.* (2018), investigated primaquine derivatives with amides, ureas, and semicarbazides, of which the two urea derivatives exhibited promising anti-TB activity (MICs = 4.29 μ M) and low cytotoxicity (128.30 - 321.88 μ M).

Decoquinatone is a quinolone derivative currently used as an inexpensive coccidiostat, active against a broad spectrum of species and strains of coccidia in poultry (McLoughlin & Chute, 1970). This drug displays an excellent safety profile and pharmacokinetic properties (T. Nam *et al.*, 2011), speculatively due to its long lipophilic side-chain, resulting in improved cell envelope permeability (Beteck *et al.*, 2016). This characteristic should allow excellent intracellular penetration and enhanced activity against *Mtb* encapsulated in macrophages and granulomatous lesions, and possibly result in significantly shorter treatment durations (Aljayyousi *et al.* 2017). Unfortunately, its poor solubility in both aqueous and organic solvents, complicates decoquinatone drug development. Decoquinatone targets the ubiquinol-binding pocket, QcrB, of *P. falciparum* cytochrome *b*, subsequently inhibiting the electron transport chain (ETC) (Nam *et al.*, 2011). Decoquinatone has also been investigated in the treatment for malaria, TB, and toxoplasmosis (Beteck *et al.*, 2016). The N-alkyl decoquinatone RMB041, has to date shown the highest activity against *Mtb*, with MIC₉₀, similar to that of ciprofloxacin (1.25 μ M vs. 1.5–12 μ M) (Beteck *et al.*, 2018; Ginsburg *et al.*, 2003). Follow-up *in vitro* and murine (C57BL/6 male mice) studies evaluating multiple parameters (e.g., *in vitro* microsomal stability, kinetic solubility, lipophilicity, and pharmacokinetic parameters) of these decoquinatone derivatives further showed promising anti-TB drug profiles (Tanner *et al.*, 2019, 2020). This includes low cytotoxicity against, amongst others, human fetal lung fibroblast WI-38 cells, with an IC₅₀ value of over 30 μ M. This drug seems to primarily target the cell wall metabolism on *Mtb*, rather than QcrB, with inhibition of DNA metabolism identified as a secondary target (Beteck *et al.*, 2018). The specific antimycobacterial MoA is yet to be determined.

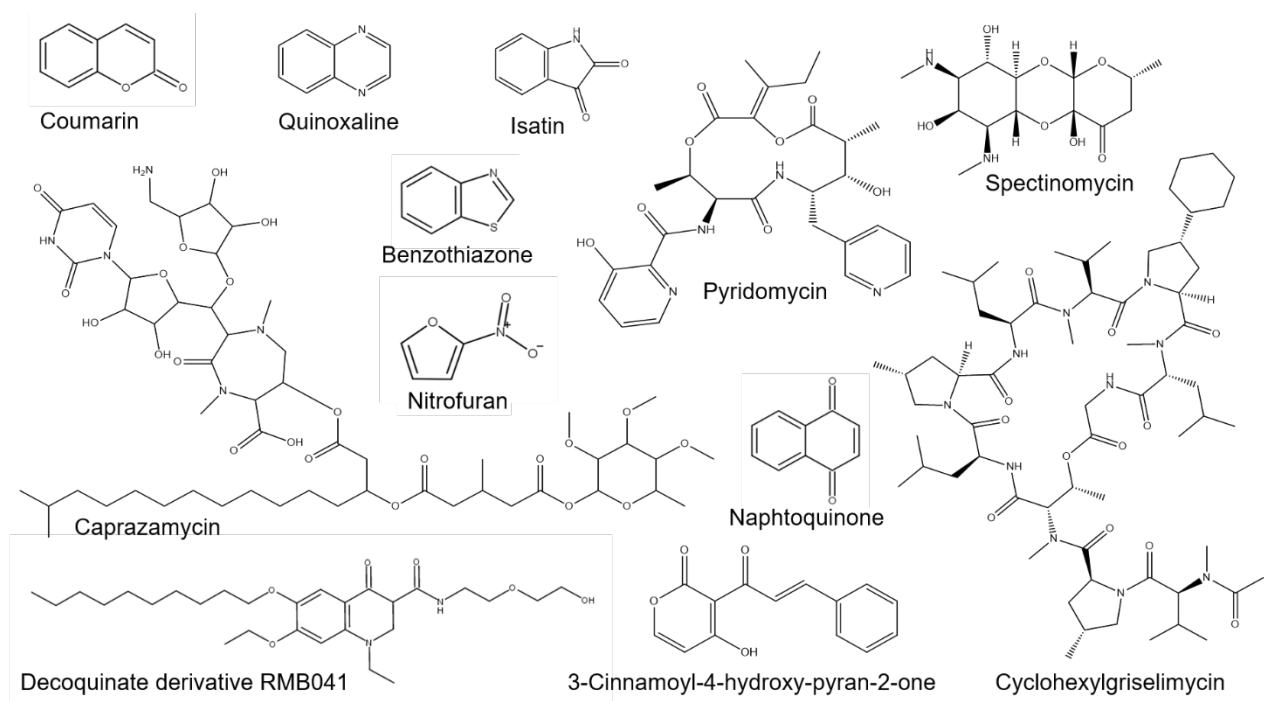


Figure 2-5: Structures of the most promising investigational antitubercular compounds.

2.3 METABOLOMICS

Current diagnostic and therapeutic strategies to date have mostly been developed using genomics, transcriptomics, and proteomics (Papaventsis *et al.*, 2017; Walker *et al.*, 2015). In recent years, the use of metabolomics has become an increasingly popular research approach and used as a standalone technology or in combination with the other aforementioned omics strategies (Ma *et al.*, 2018). As per definition, metabolomics is the unbiased identification and quantification of all metabolites in a biological system (cell, tissue, or organism) (Du Preez & Loots, 2013; Du Preez *et al.*, 2016), with metabolites representing the intermediate- and ultimate downstream products of all cellular processes (Lau *et al.*, 2015). Considering that upstream processes are impacted by a variety of genetic factors (e.g., gender and intra-individual gene mutations), environmental perturbations (e.g., diets, air pollution, and water impurities) (Li *et al.*, 2021), and habits (e.g., physical activity, smoking, etc.) (Koser *et al.*, 2012), the metabolome presents the dynamic response to all biological stimuli (Kell, 2006; Verpoorte *et al.*, 2008; Wang *et al.*, 2019). Metabolomics, therefore, prevents the limited focus on individual parameters in isolation, which can lead to an overestimation of the importance of these factors, and rather creates a type of snapshot of the entire phenotype (Beste & McFadden, 2010; Lu & Chen, 2017; Luies *et al.*, 2017).

2.3.1 A brief history of “omics” sciences

A century after the recognition of “genetics” in the 1920s, the Nobel Prize geneticists stressed how much more knowledge was needed before technology was possible and that it would be “hardly available in the 21st century, if ever” (Bashford & Levine, 2010). Yet, in the 1940s, Felix Bloch and Edward Millis Purcell invented nuclear magnetic resonance (NMR), for chemical compound separation and identification (Kleinberg & Jackson, 2001). In 1953, Francis Crick, James Watson, and Rosalind Franklin used X-ray crystallography to decipher the DNA structure (Rich & Stevens, 2004), and then 20 years later, Horning and Horning (1971) used gas chromatography-mass spectrometry (GC-MS) for the metabolic profiling of urine. As science, along with technology, advanced through the 20th century, it became clear that genes led to transcripts, which then translate into proteins. These proteins are regulated by multitudes of small molecules, termed metabolites, which include amino acids, fatty acids, nucleic acids, sugars, enzymes, vitamins, amines, and co-factors (Doerr, 2018). With “-omics” being used as a collective term for characterizing and quantifying biological molecules that translate into structure and function (Wishart, 2007), the sciences of “proteomics” (Wilkins, 2009), “transcriptomics” (Zhou *et al.*, 1998), and “metabolomics” (Brindle & Haggie, 2000) originated.

2.3.2 The role of metabolomics in human health research

Metabolomics offers various advantages when used for medicinal and health research purposes. Concerning TB, the investigation of intra-host changes and host-pathogen interactions during latent, active, and TB-treatment can be used to improve vaccination strategies (Betts, 2002; Díaz *et al.*, 2019; Koeken *et al.*, 2020), predict disease prognosis (Daniels & Hill, 1952; Meissner-Roloff *et al.*, 2012; Weiner *et al.*, 2018) and predict treatment outcomes (Das *et al.*, 2016; Du Preez & Loots, 2011; L Luies *et al.*, 2017; Mason *et al.*, 2016). During these investigations, it is important to keep in mind that varying genetic, environmental and habitual factors, result individual variation to the disease progression and treatment thereof, in each patient (Feng *et al.*, 2015), some of which negatively influences the efficacy of the treatment strategy and subsequently contributes to the development of drug resistance and treatment failure (Stein, 2011).

Metabolomics has not only been applied to direct characterization of patient collected diagnostic material, but also to investigating *Mtb* cultures directly, gaining insights into improved diagnostics (Du Preez *et al.*, 2016; Loots, 2016; Zhang *et al.*, 2018), to uncover novel drug targets (Borsari *et al.*, 2017; Chu *et al.*, 2015; Lu & Chen, 2017; Luier & Loots, 2016; Warner, 2014; Yeung, 2018), to elucidate the MoA of anti-TB drugs (R. Baptista *et al.*, 2018; Koen *et al.*, 2018a, 2018b), and identify various resistance mechanisms of *Mtb* (Loots, 2014; Palomino & Martin, 2014). The toxicity of drugs can also be determined by studying mammalian cells in the presence of and absence of anti-TB medication (Bartel *et al.*, 2013).

2.3.3 Sample selection and preparation

A variety of sample material can be used for TB metabolomic studies, including lung tissue (Che *et al.*, 2013), spleen tissue (Fremond *et al.*, 2005), liver tissue (Berning, 2001), sputum (Du Preez & Loots, 2013; Mourão *et al.*, 2016), blood (plasma/ serum) (Feng *et al.*, 2015; Zhou *et al.*, 2013), urine (Mason *et al.*, 2016), breath (Kolk *et al.*, 2012; Nizio, 2016; Phillips *et al.*, 2010) and cerebrospinal fluid (Mason *et al.*, 2017; Zhang *et al.*, 2019). The choice of sample is typically determined by the goal of the study. Urinary profiles, for instance, represent unchanged or end products as excreted, mostly hydrophilic, derived from endogenous and environmental sources, whereas blood-related samples are used to study a wider range of intermediate and dynamic variations of the endogenous metabolism, in response to changes in endogenous and exogenous gene expression (Doerr, 2018; van Holland *et al.*, 2012). Before collecting the predetermined sample material for a metabolomics investigation, it is important to decide the most appropriate time and method that will be used, as diurnal and dietary influences can change metabolome composition, and hence could greatly influence the reproducibility of the analytical sample (Dunn & Ellis, 2005). Fully elaborated advantages and disadvantages of various sample matrices can be found in a review by du Preez *et al.* (2019).

Appropriate selection of the sample preparation method, prior to metabolomics analyses, depends on the analytical equipment selected, the type of sample used, and whether the analyses will be done in an untargeted or targeted manner. For instance, various hyphenated MS techniques are considered less robust than NMR spectrometry techniques, and require more complex sample preparation prior to analyses, however are far more sensitive in comparison (Du Preez *et al.*, 2016). Sputum samples for instance, due to their high viscosity and uneven consistency, require additional sample homogenization prior to performing the standardised metabolomics extraction (Kolk *et al.*, 2012). The best described untargeted metabolomics approach, uses what is termed the total metabolome extraction, and aims to extract all metabolites in the analyzed biological system, including; alkenes, alkanes, lipids, amino acids, alcohols, organic acids, monosaccharides, purines, pyrimidines, etc. (Beukes *et al.* (2019). Various targeted metabolomic approaches can also be used, which focus on a smaller fragment of the metabolome, such as exclusively the lipids or organic acids, representing specific metabolomic pathways that are known to play a significant role in the examined biochemical setting (Bartel *et al.*, 2013; Cha *et al.*, 2009).

Sample preparation is typically divided into two parts: extraction and derivatization. Extraction reagents are typically selected based on which types of compounds one wishes to extract and analyse. Multiple extraction reagents can also be used in various ratios, to broaden the spectrum of metabolites one wishes to investigate. Various ratios of methanol and water are typically used to successfully extract semi-polar compounds, and chloroform for extracting apolar compounds (Moco *et al.*, 2007). The aforementioned “total metabolome extraction method”, hence typically employs a combination of all three; chloroform, methanol, and water (Beukes *et al.*, 2019). Derivatization during a GC based untargeted metabolomics experiment also occurs in 2 steps: (1) methoximation of oximes, steroids, and ketoacids, followed by (2) the

trimethylsilylation of fatty acids (Moros *et al.*, 2017). Some techniques however, such as NMR, although comparatively less sensitive, require no sample preparation beforehand.

2.3.4 Analytical approaches

The most commonly used analytical approaches for metabolomics include NMR spectrometry (Pan & Raftery, 2007; Zhang *et al.*, 2019; Zhou *et al.*, 2013) and GC, or liquid chromatography (LC) combined with MS (Dang *et al.*, 2013; Du Preez & Loots, 2012; Mason *et al.*, 2017; Mourão *et al.*, 2016; Schoeman & Loots, 2011). The latter techniques have comparatively higher sensitivity, higher metabolite specificity, and comprehensive metabolome coverage (Beste & McFadden, 2010; Ma *et al.*, 2018). Other metabolomics approaches include thin-layer chromatography (TLC), capillary electrophoresis (CE)-MS, and sequential MS (MSⁿ) (Verpoorte *et al.*, 2008). When selecting the most appropriate analytical approach for metabolomics research, the advantages and limitations of each should be considered, prior to selection.

GC-MS is a relatively affordable method and has a good dynamic range. This technique has comparatively high sensitivity, mass resolution, and accuracy, which all add up to impressive reproducibility (Luies *et al.*, 2017; Ma *et al.*, 2018). During GC, the injected samples are vaporized and separated based on their partitioning between the mobile and the stationary phase by a carrier gas, usually helium (or nitrogen, which is slower) (Lei *et al.*, 2011). The separated compounds then pass a metallic filament to which voltage is applied. This filament streams electrons to ionize the constituents that are moving freely in high vacuum. This concept is known as electron ionization (EI), which is considered a hard ionization technique, meaning a high quantity of residual energy is bestowed on the molecule to invoke large degrees of fragmentation (Wang *et al.*, 2018). The ionized fragments are identified by their unique mass to charge ratio (m/z) (Eiceman, 2000). When coupled with TOF-MS, higher mass accuracy and scan rates are obtained, which lead to an increased number of peaks and further improved metabolite identification (Moco *et al.*, 2007; Verpoorte *et al.*, 2008). During the TOF-MS technique, ion populations are accelerated by an electric field through constant kinetic energy, after which the detector measures the “time of flight” of the separated metabolites and indicates this as the square root of m/z (velocity). The distribution of velocities is then used to identify the metabolites (Guilhaus, 1995) in combination to the mass fragment patterns generated by the MS. A two-dimensional GC (GCxGC) enables further separation, by allowing the metabolites eluting from the first column, to undergo a second dimensional separation on a second column according to different compound characteristics (e.g., polarity) to the first separation. This provides improved sensitivity with larger dimensionality and dynamic range, ultimately presenting approximately triple the number of observable raw peaks as would a conventional GC-MS allow for (Kell, 2006; Loots, 2014; Winnike *et al.*, 2015). Due to the high temperatures applied during GC-related techniques, compound derivatization is required for the analysis of non-volatile compounds in the mixture, as is the case when using an untargeted total metabolome extraction (Dunn & Ellis, 2005).

LC-MS differs from GC-MS, in that the mobile phase is liquid, usually water or organic solvents, instead of gas. This technique uses soft-ionization methods such as electrospray ionization (ESI) or, less commonly so, atmospheric pressure ionization (API), which bestows far less amounts of residual energy onto the molecules, and subsequently invokes very little / even no fragmentation of the eluting compounds (Wang *et al.*, 2018). These methods can operate in both positive and negative ionization modes, which identifies compounds that are either easily protonated (such as acylcarnitines, phenols, and alcohols) or deprotonated (such as fatty acids), respectively (Calderón-Santiago *et al.*, 2016). Semi-polar compounds are generally measured with reverse-phase columns, while polar compounds are measured with hydrophilic columns (Moco *et al.*, 2007). These analyses are performed at lower temperatures, meaning the samples need not be volatile and require less complicated sample preparation (Dunn & Ellis, 2005). Compared to GC-MS, LC-MS has lower chromatographic resolution and higher signal-to-noise ratios (Luies *et al.*, 2017).

NMR-spectrometry for metabolomics applications is also a popular method due to its high selectivity, reproducibility, and the fact that it requires little sample preparation (Verpoorte *et al.*, 2008). In addition, it does not destruct the analyzed samples (Emwas *et al.*, 2019; Zhang *et al.*, 2019). This analytical approach is based on the identification of signals produced by the proton content of extracts, after strong magnetic fields and radio frequency pulses are applied (Dunn & Ellis, 2005). NMR-spectrometry is a good choice for broad-based analysis, as its sensitivity does not depend on metabolite acidity or hydrophobicity (Pan & Raftery, 2007). Unlike MS, which measures relative values of the individual compounds by making use of calibration curves of all individual compounds, the signal intensity of NMR depends only on the molar concentration of the solution (Verpoorte *et al.*, 2008). Due to its far inferior sensitivity however compared to that of LC or GC-MS, this technique identifies fewer metabolites and requires large sample volumes (L Luies *et al.*, 2017; Ma *et al.*, 2018; Pan & Raftery, 2007).

The data acquired by the analytical techniques are presented as complex chromatograms of the separated compounds. These are simplified by apparatus-specific software (such as AMDIS, MetAlignTM, or MET-IDEA) (Dang *et al.*, 2013). GCxGC-TOFMS techniques generally use the deconvolution software, LECO ChromaTOF (Cheng *et al.*, 2013). These software packages aim to reduce ‘noise’, correct baseline imperfections, and align peaks for maximum identification and quantification of compounds (Kolk *et al.*, 2012).

2.3.5 Statistical approaches

Various uni- and multivariate statistical methods can be used to interpret the data. These statistical methods can be executed with web-based software such as MetaboAnalyst, Agilent MassHunterQualitative Analysis, and XCMS, all of which operate in R (providing a wide variety of analytical and graphical techniques) (Xia *et al.*, 2009). Univariate techniques, e.g., t-test, effect size, analysis of variance (ANOVA) and the Wilcoxon rank-sum test, observe a single statistical outcome, namely: the metabolites that show the strongest response under the

investigated condition (Castagné & Chadeau-Hyam, 2019). Multivariate techniques, such as cluster analysis, principal component analysis (PCA) and partial least squares and discriminant analysis (PLS-DA), capture changes of multiple metabolite variables and utilize dependency structures (Kalivodová *et al.*, 2015). PCA and cluster analysis are examples of “unsupervised” methods, meaning they do not take the labelling of the groups into account. Cluster analysis is achieved with self-organizing maps, hierarchical clustering (e.g., agglomerative), or partitioned clustering (e.g. k-means) (Goodacre *et al.*, 2007). PCA is a linear mixture model that calculates the variance of the metabolites and decorrelates them into principal components (PCs), which are then sorted by the amount of variance that each PC explains (Broadhurst & Kell, 2006). The linear mixture model: PLS-DA, can be thought of as a “supervised” version of PCA, meaning it achieves dimensionality reduction but with full awareness of the group labels (Goodacre *et al.*, 2007; Ruiz-Perez *et al.*, 2020). A combination of statistical methods is typically applied to these large metabolomics data sets, to calculate the differences, as well as determine the significance of the differences, and in so doing to obtain substantial and trustworthy metabolite markers of the perturbation investigated (Bartel *et al.*, 2013). These metabolites can be compared to those in databases with known metabolic pathway maps (METLIN, Human Metabolome, MassBank, LipidMaps, KEGG, etc.) and to the published literature, thereby to identify potential biomarker functionality (Kell, 2006; Lau *et al.*, 2015) and subsequently gain a better understanding of the perturbation.

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CHAPTER 3: METABOLOMIC METHODOLOGY

3.1 EXPERIMENTAL DESIGN

In essence the experimental design comprised of *in vitro* bacterial cell culturing, experimental (drug) interventions and measurement of resulting metabolomic alterations. To determine the repeatability (of both the analyst and the apparatus) of the procedure used in this experiment, cultured *Escherichia coli* samples were used. After the repeatability of the methodology was confirmed, the procedure was applied to 15 cultured *Mtb* samples, either treated with ciprofloxacin (n=5), decoquinatone RMB041 (n=5), or without any treatment (n=5), as represented in Figure 3-1.

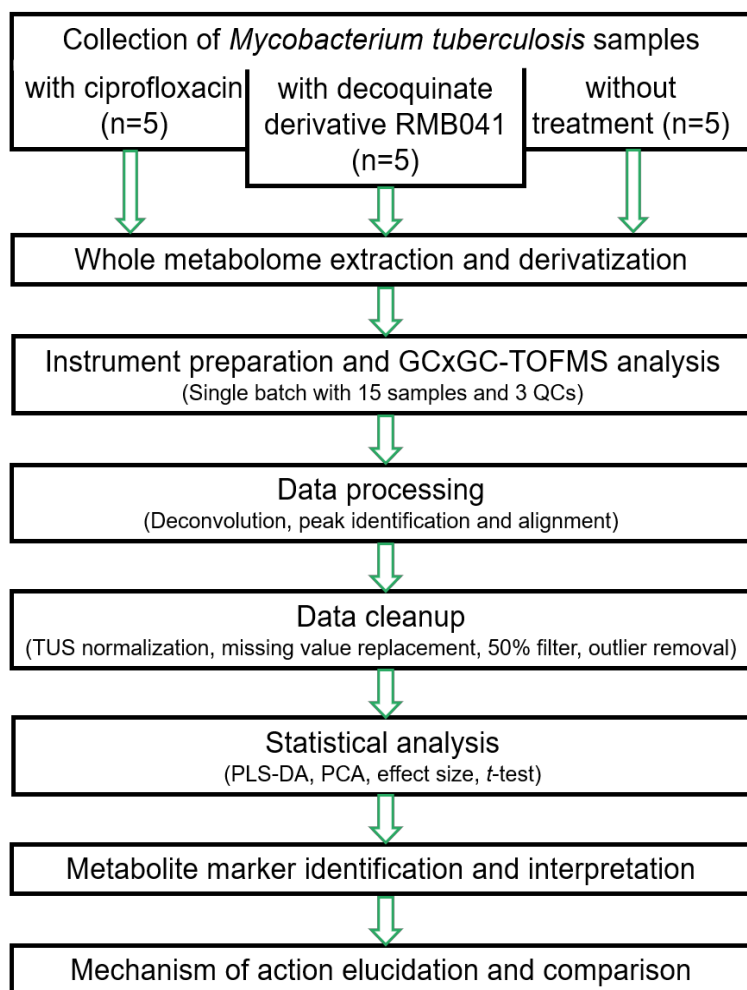


Figure 3-1: Experimental design. A schematic representation of the procedures followed during this metabolomic investigation.

Each of the aforementioned steps will be discussed in greater detail in the remaining sections of this chapter

3.2 MATERIALS AND METHOD

3.2.1 Determining the minimum inhibitory concentrations of ciprofloxacin and decoquinate RMB041

The antimycobacterial MIC and sub-MIC (IC₅₀) values of ciprofloxacin and decoquinate RMB041 were determined prior to our analysis, using the 96-well microtitre PrestoBlue (Thermo Fisher, Carlsbad, CA, USA) assay as previously described (Franzblau *et al.*, 1998). This method is based on the color change brought about by viable mycobacteria, whereby rezasurin is reduced to rasofurin. MIC values are represented by the lowest concentrations where no color change occurred from blue to pink. The absorbance was read with a BioTekPowerWave™ XS microplate reader, after which quantitative cell viability analysis was performed using KC Junior™ at a wavelength of 570 nm. GraphPad Prism version 4.0.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com) was then used to determine the IC₅₀ values.

3.2.2 Sample collection and preparation

Individually cultured *Mtb* H37Rv ATCC 27294 (obtained from the University of Pretoria, Gauteng, South Africa) were prepared in the absence (n = 5) and presence of ciprofloxacin (n = 5) and decoquinate RMB041 (n = 5), respectively (Figure 3-1).

Mtb H37Rv ATCC 27294 (obtained from the University of Pretoria, Gauteng, South Africa) were cultured and maintained on Lowenstein Jensen (LJ) Slants for 4 weeks. The bacterial inoculum was prepared to a concentration relating to 1 McFarland standard (3.0×10^8 cfu/mL) in 4 mL of Middlebrook 7H9 broth (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 10% Middlebrook OADC (oleic acid, albumin, dextrose, and catalase) (Becton, Dickinson, UK). To assure a pure culture and prevent contamination, 2% PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) (Becton, Dickinson, UK) was added, which has negligible impact on *Mtb* growth (Merkal & Richards, 1972 ; Palomino & Portaels, 1998). In Eppendorf tubes, ciprofloxacin and decoquinate RMB041 were dissolved in 0.2% dimethyl sulfoxide (DMSO) to yield final concentrations of 0.3 µM and 10 µM, respectively (as determined by the MIC experiment before this, described above), to which 1mL of the prepared inoculum was added to yield final assay volumes of 5 mL, with bacterial test concentrations of 6×10^7 CFU per 1 mL. The *Mtb* control samples were prepared similarly, by adding 4mL Middlebrook 7H9 with 0.2 % DMSO to the bacterial inoculum. After 5 days of incubation at 37° C, the samples were centrifuged at 4500 rpm for 15 min, washed twice with phosphate-buffered saline (PBS) (GE Life Sciences, Massachusetts, USA), and re-centrifuged again to pellet the cells and remove the PBS. Finally, the PBS was removed, and the samples were stored immediately at -80° C.

3.2.3 Whole-cell extraction and derivatization

The samples were extracted and derivatized as previously described by Meissner-Roloff *et al.* (2012), with a few minor modifications. An accurate measure of 7 mg wet cell mass of each of the aforementioned individually

cultured samples was weighed into Eppendorf tubes. For the quality control (QC) samples, the remaining wet cell mass of each of the 15 samples was combined in one tube, vortexed for 1 minute, and divided into 3 Eppendorf tubes, with equally distributed masses. All samples were extracted after the addition of 50 μ L of the internal standard (3-Phenylbutyric acid (0.132 mg/mL H₂O) (Sigma-Aldrich, # S36478V)), using 1 mL of chloroform, methanol, and water mixture (1:3:1 ratio), via shaking in a vibration mill at 30 Hz for 5 min with a 3 mm carbide tungsten bead. The samples were then centrifuged at 12 000 rpm for 5 min. The extract was decanted from the pellet into glass GC sample vials and dried under a nitrogen stream until evaporated at 40° C. After adding 50 μ L of a 98% methoxamine hydrochloride (MEX) (Sigma-Aldrich, # 226904) reconstituted in 99.8% pyridine anhydrous (# 270970)) to a concentration of 15 mg/mL, the glass vials were heated at 50°C for 90 min, followed by the addition of 40 μ L N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylsilyl chloride (TMSCI) (# 43928), and heating again for 60 min at 50° C. Lastly, each extract was transferred to a 0.1 mL vial insert in a GC sample vial and injected into GCxGC-TOFMS.

3.2.4 GCxGX-TOFMS analyses

A 4D Pegasus GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA) was used for the analysis. The samples were analyzed in random sequence, in a 1:10-split ratio. Quality control (QC) samples were added at regular intervals, to monitor the analytical performance throughout the entire analysis. The processed samples were injected into Rxi-5Sil MS primary capillary column (28.8 m x 0.25 mm internal diameter, 0.25 μ m film thickness, Restec), and a Rxi-17 secondary capillary column (1.2 m x 0.25 mm internal diameter, 0.25 μ m film thickness), for GC compound separation. The primary GC oven temperature was set at 70° C for 2 min and then increased at a rate of 4° C/min to a final temperature of 300° C, at which it was maintained for a further 2 min. The secondary oven was set at 85° C for 2 min, increased at 4.5° C/min, to a final temperature of 300° C, at which it was maintained for further 4.5 min. Helium, set to a column flow rate of 1 mL/min, was used as a carrier gas. The front inlet was held at a constant temperature of 270° C. Mass spectrometric data acquisition was carried out at -70 eV, with a solution delay of 350 sec. Mass spectrometric detection of eluting masses in the range (50-800 m/z) was achieved at a rate of 50 spectra/sec.

3.2.5 Data processing and cleaning

ChromaTof software (version 4.32) was used for mass spectral deconvolution (at a signal-to-noise ratio of 20), peak alignment, and peak identification on the collected mass spectra. Metabolites were identified by comparing their mass fragment patterns and retention times with those of commercially available libraries assembled from previously injected standards. After data processing, a standard metabolomics data clean-up

procedure was carried out. Briefly, each detected compound was normalized by dividing its relative concentration by the total useful signal (TUS) of the sample (the average of all compounds that are detected in all samples). By incorporating the weights of each sample, the final concentrations were converted to ug/mL. All zero values, as they are mostly due to low abundance rather than being absent, were replaced by a value calculated as 20% of the detection limit of the entire dataset. Compounds that were undetected in more than 80% of samples per group were removed. We transformed the data to a different scale by using centring, scaling, and log transformation. This gives weight to less abundant compounds to prevent these from being overwhelmed by highly abundant compounds.

The data were then analysed with a variety of uni- and multivariate statistical methods. The multivariate statistical approaches included both unsupervised PCA and supervised PLS-DA, which were processed by MetaboAnalyst (a web-based software package based on the statistical package “R”, version 2.10.0). PCA is based on an algorithm that identifies certain trends, regardless of the labelling, and compresses a large number of possibly related variables into a smaller number of unrelated variables. These are known as PCs, which are ranked according to the highest variance explained for and presented as a score plot with one PC per axis. Each metabolite is assigned a modelling power that represents its contribution to this differentiation. In contrast to the unsupervised method, PLS-DA takes group labelling into account and utilizes linear regression to identify the variables that best describe the differentiated sample groups. Here, the metabolites are ranked according to the variables’ influence on the projection (VIP) parameter, reflecting their importance to the group.

Uni-variate analysis included (1) t-test, calculated with the formula:

$$t = \frac{x_1 - x_2}{\sqrt{s^2 \left(\frac{1}{n_1} + \frac{1}{n_2} \right)}}$$

where t is the t-test value, x_1 and x_2 are the means of the group being compared, s is the standard error of the mean (measuring how far the average of the treatment group is likely to be from the control group), and n_1 and n_2 are the number of subjects in each sample,

and (2) effect size, calculated using the formula:

$$\text{Cohen's } d = \frac{M_1 - M_2}{SD}$$

where Cohen’s d is the effect size value, M_1 is the mean of the treatment group, M_2 is of the mean of the control group, and SD is the pooled standard deviation of the groups.

3.2.6 Repeatability

To address the third objective of this study (section 1.2.2), the repeatability of the GCxGC-TOFMS apparatus (machine repeatability) and that of the extraction/derivatization methodology (analyst repeatability), were determined. Repeatability can be defined as the likelihood of achieving the same results when the experimental method is repeated (Hu *et al.*, 2001). Repeatability assessments not only ensure the reliability and integrity of data, but also serves as a means of evaluating the suitability of the methodology (Broadhurst *et al.*, 2018), and hence it is a key aspect in any scientific analysis.

In this study, machine repeatability was determined by repeatedly injecting the same *Escherichia coli* (*E. coli*) sample extract (extracted and derivatized using the exact same protocol as described above) repeatedly (n=3) into the GCxGC-TOFMS, to exclusively consider variation caused by the apparatus only. Analyst repeatability was determined by injecting individually extracted *E. coli* samples (n=3) into the GCxGC-TOFMS, taking into account the analyst variation in sample preparation. After processing the resulting data (as described in section 4.3.4), the CV values of all the detected compounds were calculated and the ten most abundant compounds, that were positively identified, selected for further descriptions. The CV value of a compound is used to measure its repeatability and is calculated using the following formula:

$$CV = (\text{Standard deviation} \div \text{Average concentration}) \times 100$$

CV values are however also influenced by the detected compound's concentration, i.e., low concentrations are expected to result in higher CV values, and vice versa. The FDA recommends a hypothetical CV cut-off value of 20% for GC-MS analysis (FDA, 2001). However, metabolomics, by definition, aims to consider all compounds in an unbiased manner, even those occurring in extremely low concentrations, to answer biochemical questions as accurately as possible. In this instance, a CV value of 50% was previously shown optimal for metabolomic investigations (Schoeman *et al.*, 2012).

When visualizing the machine repeatability graphically (Figure 3-2), approximately 95% of all the compounds identified (n=143) had a value below a CV of 50%. Interestingly, almost 80% of all compounds detected had CV values under 20%. This confirms the excellent repeatability of the GCxGC-TOFMS apparatus. When evaluating the analyst repeatability, approximately 75% of the detected metabolites fall below a CV of 50%, and over half of all detected metabolites had CV values below 20%. Since each step of the extraction procedure results in possible sources of variation, higher CV values are expected in the analyst repeatability test. It is also important to keep in mind, that due to the slow growth of the *Mtb* H37Rv cells used for the study, the extraction method was adjusted for low cell masses, also contributing to an additional variation. Considering this, the analytical method and analyst show excellent repeatability for the experiment to follow.

To include a more traditional report of the repeatability, and considering the influence of compound concentrations on CVs, the ten compounds with the highest concentrations throughout the analysis were identified and evaluated. These compounds, together with their relative concentrations and CV values, for both the machine and analyst repeatability evaluation, are listed in Table 3-1. It is apparent, that all of the compounds listed fall well within the FDA-recommended cut-off value of 20% (FDA, 2001) further confirming the validity of the method for our applications.

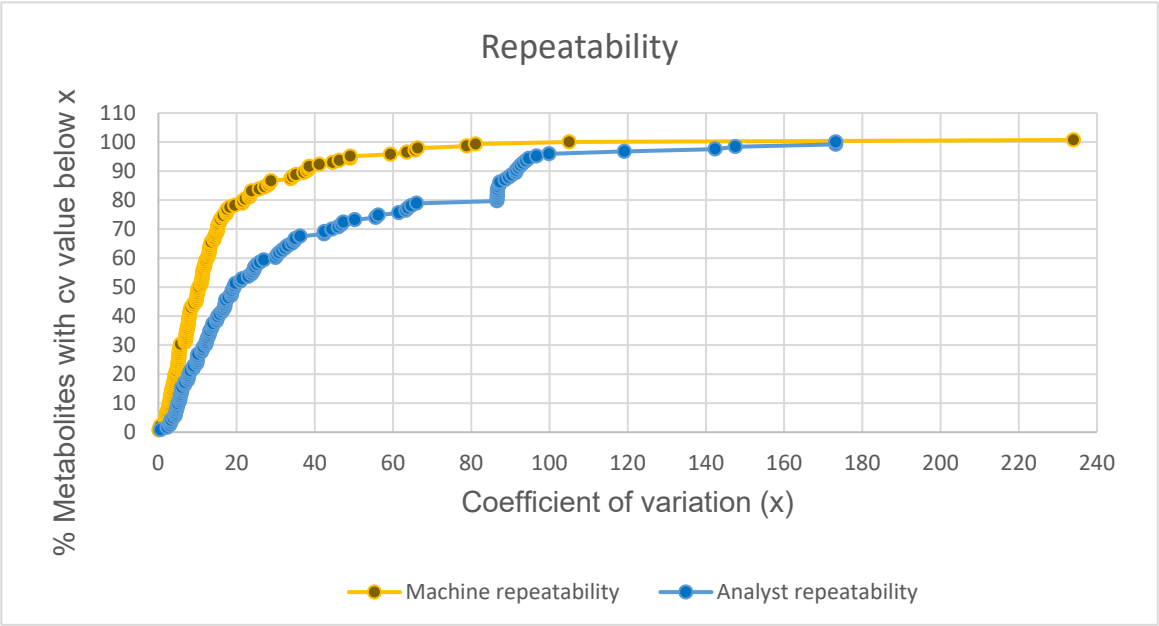


Figure 3-2: Distribution of the coefficient of variation (CV) values for all detected compounds of the machine and analyst repeatability.

Table 3-1: The ten positively identified compounds with the highest concentrations throughout the entire GCxGC-TOFMS analysis, with their average concentrations and CV values of the machine and analyst repeatability.

Metabolite name	Average concentration (µg/g creatinine) (standard error of the mean)	Machine repeatability (CV value)	Analyst repeatability (CV value)
2,3-Dihydroxyhexadecanoic acid	13.674 (1.232)	3.099	9.006
D-Glucose	28.462 (4.860)	2.928	17.073
Dibutyl phthalate	23.375 (3.089)	5.278	13.217
Dodecanoic acid	10.708 (0.967)	2.026	9.031
D-Glucitol-6-Phosphate	7.821 (0.340)	8.299	4.344
Hexadecanoic acid-TMS	40.640 (2.279)	2.232	5.609
Octadecanoic acid	22.837 (2.279)	8.093	9.979
9-Octadecenoic acid	37.529 (0.843)	3.087	2.249

Malic acid	6.993 (0.223)	3.184	8.967
Tetradecanoic acid	12.350 (0.957)	0.542	7.749

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CHAPTER 4: THE ANTIMYCOBACTERIAL MECHANISM OF CIPROFLOXACIN

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See Annexure A

4.1 ABSTRACT

In the interest of developing more effective and safer anti-tuberculosis drugs, we used a GCxGC-TOF-MS metabolomics research approach to investigate and compare the metabolic profiles of *Mtb* in the presence and absence of ciprofloxacin. The metabolites that best describe the differences between the compared groups were identified as markers characterizing the changes induced by ciprofloxacin. Malic acid was ranked as the most significantly altered metabolite marker induced by ciprofloxacin, indicative of an inhibition of the tricarboxylic acid (TCA) and glyoxylate cycle of *Mtb*. The altered fatty acid, *myo*-inositol, and triacylglycerol metabolism seen in this group supports previous observations of ciprofloxacin action on the *Mtb* cell wall. Furthermore, the altered pentose phosphate intermediates, glycerol metabolism markers, glucose accumulation, as well as the reduction in the glucogenic amino acids specifically, indicate a flux toward DNA (as well as cell wall) repair, also supporting previous findings of DNA damage caused by ciprofloxacin. This study further provides insights useful for designing network whole-system strategies for the identification of possible modes of action of various drugs and possibly adaptations by *Mtb* resulting in resistance.

4.2 INTRODUCTION

TB, caused by *Mtb*, remains one of the leading causes of death globally from a single infectious agent (MacNeil *et al.*, 2019), resulting in a mortality rate of 1.5 million and an infection rate of about 10 million annually (Harding, 2020). Furthermore, the prevalence of drug resistant TB is also increasing, primarily due to poor adherence to the drug regimen in patients (Vernon *et al.*, 2019), as a result of the many side effects experienced by patients being treated with first-line anti TB medication, accompanied by the long treatment duration required (Da Silva & Palomino, 2011; Holmes *et al.*, 2017). Further contributing factors to developing drug resistant TB include inaccurate diagnosis, unsupervised treatment, poor economic status (Migliori *et al.*,

2020), and a further exacerbation in 2020 by the COVID-19 pandemic (Harding, 2020; McQuaid *et al.*, 2020). Currently, the WHO's approved first-line therapy for patients with active TB is a 6-month "directly observed treatment short-course" (DOTS) regimen consisting of isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and rifampicin (RIF) (Luies *et al.*, 2017; WHO, 2019b). Infection with multi-drug resistant (MDR)-TB and extensively drug resistant (XDR)-TB requires treatment using various second-line antibiotics that are expensive, have far more side effects due to their higher toxicity, and need to be consumed for even a longer duration (Ahmad *et al.*, 2016; Harding, 2020). The only newly approved drugs for TB over the past 50 years are the second-line drugs for treating MDR-TB—linezolid, bedaquiline and delamanid—but not long after, resistance followed. Considering this, there is an urgent need for well-tolerated and effective treatments for TB using drugs with novel modes of action against the infectious organism.

A suggested approach for avoiding the long drug trial phases usually required for approving new drug candidates is further investigation of already existing drugs, repurposed for use in treating TB/MDR-TB, (Adeniji *et al.*, 2020; Brown, 2015). In order for a drug to be selected for possible repurposing applications, it should preferably be affordable, easily available, and show good pharmacokinetic/pharmacodynamic properties. For these reasons, several fluoroquinolones (FQs) are being intensively investigated for use as anti-TB therapy (Pranger *et al.*, 2019). FQs, originally used to treat urinary tract infection (Domagala, 1994), were first shown to be effective against *Mtb* in 1984, and have since gained continuous interest for such applications (Berning, 2001; Jabeen *et al.*, 2015; O'Sullivan *et al.*, 2008). Currently, they are among the most frequently prescribed drugs (Chen *et al.*, 2011) and are considered the backbone of MDR-TB treatment (Ahmad *et al.*, 2016; Raman *et al.*, 2005). FQs target two *Mtb* topoisomerase deoxyribonucleic acid (DNA) enzymes, DNA gyrase and topoisomerase IV (Drlica & Zhao, 1997). The first introduces negative super helical twists in the bacterial DNA double helix and catalyzes the separation of daughter chromosomes (Blondeau, 2004), whereas the latter is responsible for the segregation into two daughter cells at the end of DNA replication (Zawadzki *et al.*, 2015). Earlier generations, ciprofloxacin and levofloxacin, exhibit greater activity against Gram-negative bacteria (and some Gram-positive bacteria) and target mainly DNA gyrase (Chatterji *et al.*, 2001; Collin *et al.*, 2011; Walker, 1999). FQs inhibit DNA gyrase by binding to the enzyme and DNA, which leads to double-stranded DNA breaks (Aldred *et al.*, 2016).

The innate resistance mechanism of *Mtb* to many anti-TB drugs can be attributed to its complex cell wall (Jarlier & Nikaido, 1994); however, the specific, highly lipophilic characteristics of FQs (Arjomandzadegan *et al.*, 2016) provide great permeability over this (Angala *et al.*, 2014). These antibiotics chelate with Mg^{2+} cations and electrostatically interact with membrane phosphodiesterases, subsequently traversing the *Mtb* cell wall (Fresta *et al.*, 2002). FQs are not exempt from resistance, however, of which the best described are mutations in genes *gyrA* and *gyrB*, encoding subunit GyrA and GyrB of DNA gyrase (Blower *et al.*, 2016; Chen *et al.*, 2012; Disratthakit *et al.*, 2016). Furthermore, several resistance-forming proteins have also been identified, including

the efflux pumps, LfrA (Aldred *et al.*, 2014; Rossi *et al.*, 2006), and MmpL (mycobacterial membrane protein large) (Radhakrishnan *et al.*, 2014), the target protection proteins, MfpA and MfpB (*Mycobacterium* fluoroquinolone resistance proteins A and B) (Montero *et al.*, 2001; Redgrave *et al.*, 2014), and the cell-survival promotor, HtrA2 (high temperature requirement A) (Daisy *et al.*, 2012). DNA repair and mutations by the SOS regulon have also been described (O'Sullivan *et al.*, 2008).

Although interactions between DNA gyrase and FQs have been thoroughly investigated (Tim R Blower *et al.*, 2016; Drlica & Zhao, 1997; Palomino & Martin, 2014), little is known about their biochemical mechanisms of action against *Mtb* specifically, or *Mtb* drug resistance to these (Ahmad *et al.*, 2016; Jabeen *et al.*, 2015). The results published thus far are somewhat contradictory. In a study by Verma *et al.* (1999), the macromolecular composition of the *M. smegmatis* cell wall after sub-MIC ciprofloxacin treatment indicated a significant decrease in the total lipids, phospholipids, and sugars, suggesting ciprofloxacin-induced alterations of the cell wall. In contrast, Halouska *et al.* (2012) indicated ciprofloxacin-induced inhibition of transcription, translation, and DNA supercoiling, without changes to the cell wall. While most data suggest cell death due to the inhibition of DNA replication (Ginsburg *et al.*, 2003), altered DNA biosynthesis could set in motion secondary events contributing to ciprofloxacin's bacteriostatic or bactericidal effects. Furthermore, it is important to remember that stronger target activity does not predict better antimycobacterial activity. This is perfectly demonstrated by ciprofloxacin dimers, which show enhanced DNA gyrase inhibition, while less effectively killing *Mtb* (Ross *et al.*, 2015). This is thought to be due to a stronger cleavage of FQ to the DNA-enzyme complex, which results in less single-strand DNA fragments, and subsequently prevents RecA from recognizing damaged DNA and inducing the SOS regulon (Pham *et al.*, 2019). The SOS response assists in killing by releasing ROS (Podlesek & Zgur Bertok, 2020; Tyagi *et al.*, 2015), yet simultaneously activates DNA repair and creates a dormancy state, ultimately leading to resistance. Before this SOS response can be used to advantage as a possible mode of action against *Mtb*, this phenomenon, and how it connects to the mechanism of ciprofloxacin, still needs to be elucidated.

The selection of ciprofloxacin as our investigational compound was predominantly based on its safety profile. Although less potent than moxifloxacin (MIC 0.12–0.5 µg/mL), and levofloxacin (MIC 1 µg/mL), ciprofloxacin (MIC 0.5–4.0 µg/mL) (Arbex *et al.*, 2010) has the lowest risk for causing serious ventricular arrhythmia, cardiovascular mortality, and hepatotoxicity (Gorelik *et al.*, 2019; Schloss *et al.*, 2018). Furthermore, ciprofloxacin demonstrates the highest clearance rate of all FQs (Aminimanizani *et al.*, 2001) and is thus the preferred option for the treatment of renally impaired patients (Berning, 2001). The levels of ciprofloxacin in cerebrospinal fluid can be as high as 40–90% compared to that in plasma (Arbex *et al.*, 2010), which offers further advantages for its use in the treatment of tuberculous meningitis. Adverse drug reactions (ADRs) are usually minimal (5% or less), and the most common ADRs are usually gastrointestinal in nature (nausea, vomiting, diarrhea, and abdominal pain) (Ball, 1986; Xu; Zhao; Deng; *et al.*, 2019). Previous studies

demonstrated that mitochondrial topoisomerases bear less than 30% homology to their prokaryotic counterparts and are not inhibited (Barnhill *et al.*, 2012; Blondeau, 2004), but it has been reported that ciprofloxacin does affect mitochondrial DNA synthesis (Hangas *et al.*, 2018). The pharmacological advantages of ciprofloxacin have not gone unnoticed, as stated by the World Health Organization (WHO, 2019a), which has included ciprofloxacin as a critically important antibiotic.

Most of the evidence brought to light thus far has been generated using genomics, transcriptomics, or proteomics (Correia *et al.*, 2017). Metabolomics, the latest addition to “omics” technologies, identifies the down-stream metabolites of altered pathways and therefore presents a more sensitive level of organization, from which up-stream deductions can be made (Lau *et al.*, 2015; Luies *et al.*, 2017). We identified the metabolite markers best differentiating *Mtb* with and without ciprofloxacin, using a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS) metabolomics approach, combined with universally connected metabolic libraries and advanced statistical analysis, in order to better elucidate its MoA.

4.3 MATERIALS AND METHODS

4.3.1 Cell culture samples

Antimycobacterial MICs and sub-MIC (50% inhibitory concentrations (IC₅₀)) of ciprofloxacin were determined via the Alamar Blue assay (Franzblau *et al.*, 1998). The cell cultures (5 individually cultured samples per group) were prepared as previously described (Van Breda *et al.*, 2015), in the presence and absence of ciprofloxacin. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA unless otherwise stated. Briefly, *Mtb* H37Rv ATCC 27294 (kindly obtained from the Medical Research Council, Pretoria, Gauteng, South Africa) was cultured and maintained for 4 weeks on LJ slants. The bacterial inoculum was prepared to a McFarland standard of 1 (approximately 3×10^8 colony-forming units/mL) in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson, UK) and 2% PANTA (polymyxin B, amphotericin B, nalidixic acid and trimethoprim, azlocillin) (Becton, Dickinson, UK). PANTA was added for the prevention of contamination with negligible impact on *Mtb*'s growth (Palomino & Portaels, 1998). Ciprofloxacin was dissolved in DMSO (150 µM) and diluted into Middlebrook 7H9 broth to a final concentration of 0.3 µM (0.12 µg/mL) (0.2 % DMSO). One millilitre of the prepared inoculum was added to yield a final assay volume of 5 mL, with a bacterial test concentration of 6×10^7 CFU/mL ciprofloxacin. For the untreated *Mtb* control samples, 4 mL of Middlebrook 7H9 broth (0.2 % DMSO) was added to each replicate culture, followed by the addition of the bacterial inoculum as described above. The DMSO solvent was kept constant throughout the assay. After 5 days of incubation at 37 °C, the samples were centrifuged to pellet the bacteria at 4500 rpm for 15 min. The pellets were washed with 1 mL of phosphate-buffered saline (PBS) and

pelleted again under the same conditions. Finally, the PBS was aspirated from the samples and the pellets were stored immediately at -80 °C until further testing.

4.3.2 Whole metabolome extraction and derivatization procedure

The metabolites were extracted from the samples and derivatized as previously described by Beukes *et al.* (2019), with slight modifications. Briefly, 8 mg of each of the individually cultured samples were weighed out into an Eppendorf tube, followed by the addition of 50 µL 3-phenylbutyric acid (0.13 mg/ml H₂O) (Sigma-Aldrich, Lot#536478V) as internal standard. One milliliter of chloroform: methanol: water (1:3:1 ratio) solution was added, after which the Eppendorf tubes were shaken in a vibration mill at 30 Hz for 5 min, with a 3 mm carbide tungsten bead in each. The samples were centrifuged at 12 000 rpm for 5 min and the supernatant was transferred to a GC glass vial. The extracts were dried under a nitrogen stream, followed by the addition of 50 µL MEX (Sigma-Aldrich, Lot#BCBP2843V) in pyridine (Lot#S2BC335SV) at a concentration of 15 mg/mL. The glass vials were heated at 50°C for 90 min. Following, methoximation, 40 µL BSTFA with 1% TMSCI (Lot#BCBW2670) was added, and vials were heated again for 60 min at 50° C. Each extract was then transferred to a 0.1 mL vial insert in a GC sample vial and injected into GCxGC-TOF-MS.

4.3.3 GCxGC-TOFMS analysis

A 4D Pegasus GCxGC-TOF-MS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA) coupled to TOF-MS (LECO Africa) was used for the analysis. The samples were analyzed in random sequence, in a split-less ratio. To monitor the analytical performance throughout the entire analysis, a quality control (QC) sample was analyzed at regular intervals. The processed samples were injected into Rxi-5Sil MSprimary capillary column (28.8 m x 0.25 mm internal diameter, 0.25 µm film thickness, Restec), and a Rxi-17 secondary capillary column (1.2 m x 0.25 mm internal diameter, 0.25 µm film thickness), for GC compound separation. The primary GC oven temperature was set at 70°C for 2 min and then increased at a rate of 4°C/min to a final temperature of 300°C, at which it was maintained for an additional 2 min. The secondary oven was set at 85°C for 2 min, increased at 4.5°C/min, to a final temperature of 300°C, at which it was maintained for 4.5 min. Helium, set to a column flow rate of 1 mL/min, was used as a carrier gas and held at a constant temperature of 270°C. Mass spectrometric data acquisition was carried out at -70 eV, with a solution delay of 350 sec, and a mass range of 50-800 m/z was scanned with a rate of 200 spectra/sec.

4.3.4 Data processing, clean-up, and statistics

ChromaTof software (version 4.32) was used for mass spectral deconvolution (at a signal-to-noise ratio of 20), peak alignment, and peak identification on the obtained mass spectra. Metabolites were identified by

comparing their mass fragment patterns to that of compounds in commercially available databases containing previously injected standards. For normalization and assessment of data quality, the data were pretreated using a standardized metabolomics data clean-up procedure (Beukes *et al.*, 2019). Each detected compound was normalized using MS TUS, which is based on a factor calculated from the sum of all metabolites identified in all samples, and by calculating the relative concentration of each by using the internal standard. All missing/zero values were replaced by a value calculated as 20% of the minimum detection limit of the entire dataset, as these are most likely present in sub-minimum concentrations rather than being completely absent (Gromski *et al.*, 2014). An 80% data filter was then applied to eliminate compounds with more than 80% zero values within both groups (Wei *et al.*, 2018). To provide a balanced representation of all metabolites, log transformation and auto-scaling (mean-centered and divided by the standard deviation of each variable) were applied. This prevents compounds with minor concentrations to be overlooked, due to the domination of compounds with higher concentrations (Shen & Zhu, 2019). Making use of MetaboAnalyst (Version 5.0) (Xia & Wishart, 2011), multivariate statistical methods in the form of unsupervised PCA and supervised PLS-DA were applied (Meissner-Roloff *et al.*, 2012). Subsequently, univariate analysis was done by calculating t-test and effect size values (Sullivan & Feinn, 2012).

Relationships between the selected metabolites were mapped using the KEGG, MetaCyc, and BioCyc databases, in addition to intensive research of the previously published literature on the topic.

4.4 RESULTS

4.4.1 Data overview

When visualizing the analytical technique's repeatability graphically Figure 4-1, approximately 86% of all the compounds identified (n=260) had a CV value under 50%, of which 70% had CV values under the 20%. The analytical technique used during this analysis thus proves to be highly repeatable and can be trusted to provide reliable results. PCA was initially used to get an overview of the natural grouping of metabolic data (Figure 4-2). The total variance between the groups, described by the first two PCs, was 57.1%, of which PC1 and PC2 accounted for 32.1% and 25%, respectively. The PCA scores plot of the metabolite data analyzed by GCxGC-TOF-MS shows clear clustering between *Mtb* in the presence and absence of ciprofloxacin, as represented in Figure 4-2.

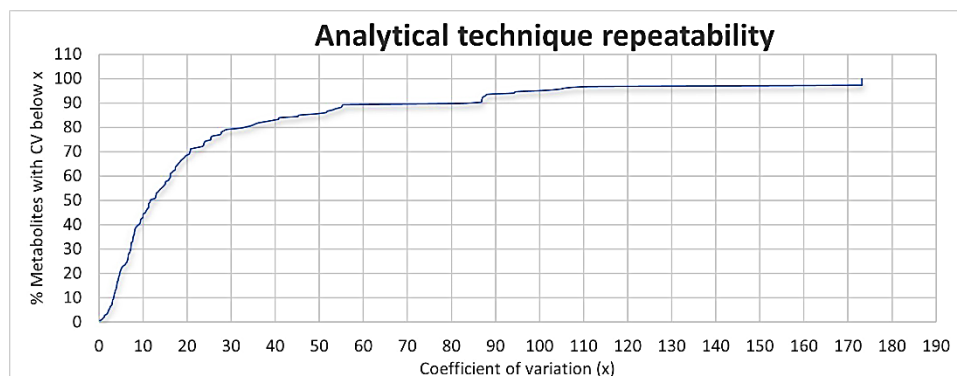


Figure 4-1: Distribution of coefficients of variation values for technical repeatability.

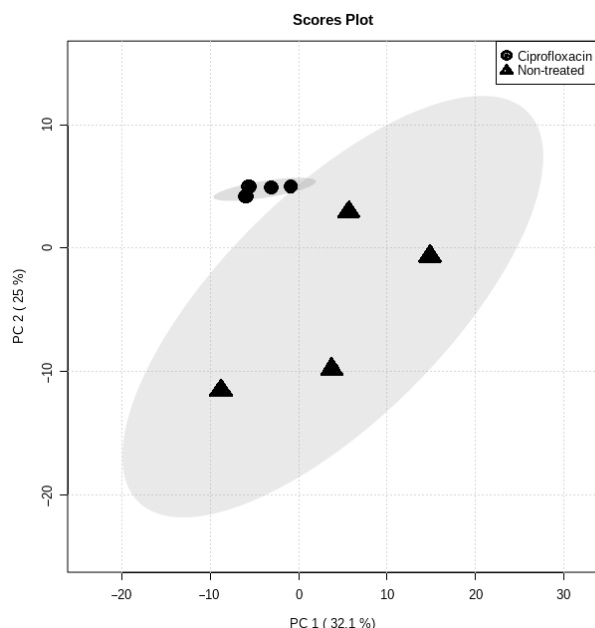


Figure 4-2: PCA scores plot obtained from GCxGC-TOFMS whole metabolome analysis of *Mtb* samples in the presence and absence of ciprofloxacin. The variances accounted for are indicated in parenthesis.

4.4.2 Marker selection

The metabolite markers (n=26) best describing the differences between the ciprofloxacin and control samples were selected based on compliance with the following criteria: a PLS-DA VIP value > 1 (Cho *et al.*, 2008), a t-test P-value < 0.05 (Byliński *et al.*, 2018) or an effect size > 0.8 (Coe, 2002) (Figure 4-3).

The selected metabolites markers are listed according to their PLS-DA VIP values in Table 4-1, along with their respective average concentrations and univariate test outcomes. Of the total, 61.5 % (16/26) markers were elevated, of which most include fatty acids. The most differentiating marker was malic acid, with an exceptionally high d-value of 6.621 and a low p-value of > 0.0001 .

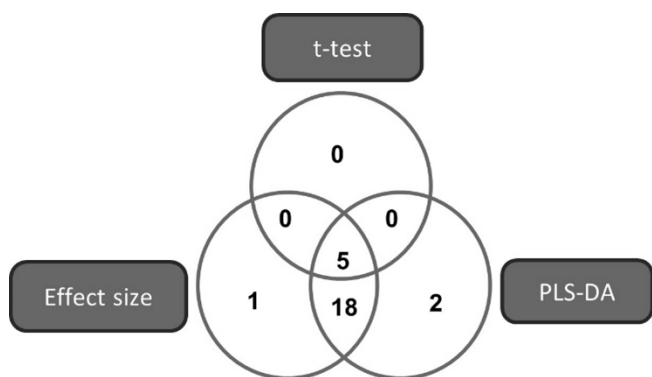


Figure 4-3: Venn diagram illustrating the multi-statistical approach for selecting the metabolites that best describe the variation detected in the metabolome of *Mtb* cultured with and without ciprofloxacin.

Table 4-1: Metabolite markers best describing the variance between the individually cultured *Mtb* samples in the absence (*Mtb* controls) and presence of ciprofloxacin.

Metabolite name (ChEBI ID)	Average concentration (mg/g cell mass) (Standard deviation)		t-test (<i>P</i> - value)	Effect size (d-value)	PLS-DA (VIP)	Fold Change (log ₂)
	<i>Mtb with</i> ciprofloxacin	<i>Mtb controls</i>				
Malic acid (6650)	0.033 (0.002)	0.054 (0.004)	0.000	6.621	2.012	-0.39
Aspartic acid (17053)	0.007 (0.001)	0.013 (0.004)	0.008	2.236	1.732	-0.46
Glycerol (17754)	0.497 (0.033)	0.633 (0.031)	0.013	1.932	1.678	-0.21
5-Oxoproline (17203)	0.069 (0.014)	0.099 (0.020)	0.014	1.962	1.665	-0.30
Xylofuranose (46432)	0.075 (0.037)	0.034 (0.008)	0.037	1.376	1.511	1.21
Myo-inositol-1-phosphate (18297)	0.004 (0.001)	0.003 (0.001)	0.051	1.637	1.445	0.33
9-Hexadecenoic acid (28716)	0.005 (0.000)	0.004 (0.001)	0.091	1.329	1.299	0.25
Nonadecanoic acid	0.067 (0.011)	0.055 (0.008)	0.094	1.151	1.292	0.22
Heptadecanoic acid (32365)	0.015 (0.003)	0.011 (0.002)	0.095	1.355	1.288	0.36
Octadecanoic acid (28842)	0.582 (0.164)	0.371 (0.141)	0.096	1.241	1.287	0.57
Valine (16414)	0.017 (0.004)	0.022 (0.005)	0.106	1.117	1.255	-0.23
β-Aminoisobutanoic acid (33094)	0.011 (0.002)	0.014 (0.004)	0.121	1.256	1.216	-0.21
Glutamic acid (16015)	0.010 (0.001)	0.015 (0.006)	0.124	0.960	1.209	-0.33
Hexadecanoic acid (15756)	0.575 (0.051)	0.480 (0.077)	0.128	0.969	1.198	0.20
9-Octadecenoic acid (36021)	0.679 (0.060)	0.600 (0.041)	0.132	1.089	1.187	0.13
Tetradecanoic acid (28875)	0.089 (0.010)	0.071 (0.017)	0.141	0.934	1.167	0.25
Eicosanoic acid (28822)	0.005 (0.001)	0.004 (0.000)	0.146	0.991	1.154	0.25
Glucose (17234)	0.045 (0.006)	0.037 (0.005)	0.152	0.934	1.140	0.22

Urea (16199)	0.005 (0.002)	0.002 (0.002)	0.171	0.816	1.099	1.50
N-Acetyl-L-Lysine (64859)	0.002 (0.001)	0.004 (0.003)	0.175	0.884	1.090	-0.50
2-Monopalmitin (75455)	0.011 (0.002)	0.009 (0.001)	0.175	0.907	1.089	0.22
1-Monomyristin (75562)	0.007 (0.003)	0.005 (0.001)	0.175	0.882	1.089	0.40
Pentadecanoic acid (42504)	0.005 (0.001)	0.004 (0.001)	0.181	1.021	1.077	0.25
Glycerol 3-phosphate (15978)	0.005 (0.001)	0.007 (0.002)	0.186	0.908	1.067	-0.29
1-Monoheptadecanoin (144339)	0.010 (0.002)	0.007 (0.001)	0.186	0.947	1.066	0.43
Erythritol (17113)	0.021 (0.002)	0.023 (0.001)	0.202	0.856	1.034	-0.09

4.5 DISCUSSION

In this study, we identified several significantly altered metabolites induced by the administration of ciprofloxacin to *Mtb* culture, which when interpreted, in the light of known metabolism and previous ciprofloxacin findings, better elucidate its mechanisms of action against *Mtb*, as shown in Figure 4-4. The most prominently altered pathways included gluconeogenesis, fatty acid metabolism, amino acid metabolism, the PPP, and the urea cycle.

Of note, was the elevated levels of various even and odd chain saturated fatty acids of between 14 to 20 carbons (C14:0-C20:0) in length, in the *Mtb* treated with ciprofloxacin. This was also true for two Δ^9 -unsaturated fatty acids; 9-hexadecenoic (Δ^9 C16:1) and 9-octadecenoic (Δ^9 C18:1) acids. These indicate a strongly upregulated synthesis towards cell wall repair, supporting previous evidence associating ciprofloxacin with cell wall damage (Verma *et al.*, 1999). Simplified, lying outside of the cytoplasmic membrane, a PG layer is covalently attached to AG, which itself attaches to MA, to form the MAPc (Chatterjee *et al.*, 1991; Vijay *et al.*, 2017). Interspersed within the MAPc, are the glycerolipids, PIM, and LAM (Jankute *et al.*, 2015). PIM is a crucial part of the membrane structure and serves as a precursor of LAM (Chiaradia *et al.*, 2017). The saturated fatty acid markers in this study are produced by FAS I. FAS I generates 16 to 26 carbon length fatty acyl-CoAs (Abrahams & Besra, 2016), which are fed into FAS II for elongation. FAS I and FAS II provide acyl-groups for the synthesis of all cell envelope components, except for AG (Black & DiRusso, 2003; Santangelo *et al.*, 2016). Δ^9 C16:1 and Δ^9 C18:1, and their precursors, hexadecanoic (C16:0) and octadecanoic (C18:0) acid, respectively, are considered major fatty acids of glycerolipids and mycolic acids (Nigou *et al.*, 2003; Škovierová *et al.*, 2009; Vilchèze & Jacobs, 2007). Δ^9 C16:1 and Δ^9 C18:1 are reduced from C16:0 and C18:0, in the presence of Fe²⁺, a flavin, NADPH, and O₂ (Fulco & Bloch, 1964; Fulco *et al.*, 1964). Interestingly, some *mmpL* genes, encoding fatty acid transporter protein MmpL, are reportedly repressed when their transcriptional regulator proteins bind to C:16 fatty acids and monoacylglycerols (MAG) (Delmar *et al.*, 2015; Melly & Purdy, 2019). Even so, further research is needed to establish the possible activity of different fatty acids on different MmpL regulator proteins.

It is, however, important to note, that the damage that ciprofloxacin administration induces to the cell wall may be direct, but is most likely indirect by inhibition of other energy-producing mechanisms or simply by inducing the SOS response in *Mtb*. This may be shifting energy production away from glucose, toward using preferably fatty acids (Rhee *et al.*, 2011), and hence less of these fatty acids are now available to cell wall synthesis. Increased FA synthesis and/or the accumulation of cell wall components have also been identified in *Mtb* treated with EMB, INH, PZA, RIF, and pretomanid (Baptista *et al.*, 2018; Loots, 2014; Rosen *et al.*, 2017; Xiang *et al.*, 2021; Zhao *et al.*, 2021).

The dramatically elevated synthesis of intracellular fatty acids would be expected to consume a considerable amount of carbon, which can be supplied from various sources (Rhee *et al.*, 2011). However, as will be explained, glucose and glycerol seem to be the major suppliers for such, and for the various components required for DNA repair. This is supported by previous findings indicating ROS, produced during the FQ-induced SOS-response, causes oxidative stress, which in turn activates utilization of triacylglycerol (TAG) and cell wall lipids for energy (Bothra *et al.*, 2018; Crellin *et al.*, 2011; Zerbini *et al.*, 1997), as is generally the case during the non-replicative phase of *Mtb* (Kumar *et al.*, 2011; Kumar & Sanyal, 2012). These results are also supported by previous findings showing reduced concentrations of phospholipids and mycolic acids in the cell wall macromolecules of sub-MIC ciprofloxacin-treated *M. smegmatis* (Verma *et al.*, 1999), since these are now being preferentially used for energy production, with glucose supplying the necessary carbon substrates for the continued synthesis of these much-needed fatty acids which are now preferentially used for energy production.

A shift in energy supply to β -oxidation of fatty acids, as opposed to the TCA cycle, reserves NAD^+ , in addition to CO_2 , for *de novo* synthesis of nucleotides (for DNA repair and fatty acids). NADH and NADPH released during fatty acid metabolism, fuels the upregulated non-oxidative PPP and glycerolipid metabolic pathway. In this study, the downregulated TCA cycle is indicated by a reduction in malic acid and aspartic acid, which according to the uni-and multivariate statistics, were ranked as the two most important metabolite markers altered by ciprofloxacin (Table 4-1). Aspartic acid is considered a validated reporter of oxaloacetate (OAA) (Erental *et al.*, 2014), and together with malic acid, support TCA cycle inhibition. Although oxidative stress is normally associated with an increase in glyoxylate shunt activity, in this study, the glyoxylate shunt is downregulated (Figure 4-4). This indicates a greater need for carbon flux through gluconeogenesis towards the PPP and glucose, for subsequent fatty acid and nucleotide synthesis (Horecker, 2002; Lucarelli *et al.*, 2010). Reduced aspartic acid concentrations were also previously found in a study investigating phenotypic antibacterial persistence in *Mtb* (Larrouy-Maumus *et al.*, 2016). These results support the SOS response-induced decrease in oxidative phosphorylation and increase in energy reserves for DNA repair, in response to FQs (Erental *et al.*, 2014; Pham *et al.*, 2019). It is noteworthy to mention that the ATPase activity of DNA gyrase (29), shows reduced adenosine triphosphate (ATP) conversion in the presence of ciprofloxacin (Kampranis &

Maxwell, 1998), which most likely is to reserve ATP for other energy-consuming pathways, such as gluconeogenesis and DNA repair (Villela *et al.*, 2011).

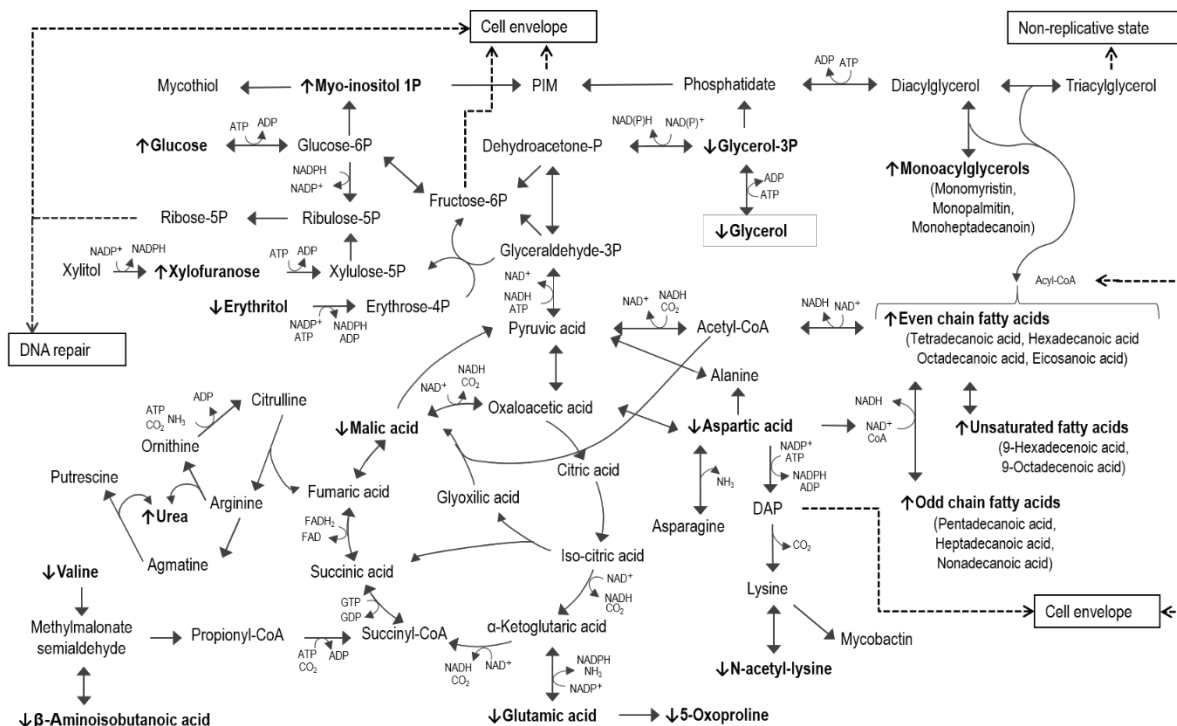


Figure 4-4: Metabolomic pathway map of *Mtb* treated with ciprofloxacin. The metabolite markers best describing the variation in the metabolome compared to that of untreated *Mtb* are represented in bold text with up or down arrows indicating elevated or reduced concentrations, respectively.

Various PPP intermediates were detected to be altered in the ciprofloxacin treated *Mtb*, which contribute to DNA and cell wall synthesis (Crellin *et al.*, 2011; Maitra *et al.*, 2019; Miggiano *et al.*, 2020). Under normal circumstances, erythrose-4-phosphate and glyceraldehyde-3-phosphate produce xylulose-5-phosphate (xylulose-5P) and fructose-6-phosphate (fructose-6P) (Rhee *et al.*, 2011). The latter is converted to glucose-6-phosphate, for subsequent PIM synthesis, via the myo-inositol pathway (Haite *et al.*, 2005), and to glucose-N-acyl 6-phosphate (GlcN6P), for subsequent PG synthesis (Heijenoort, 2001). Xylose-5P is the precursor of ribose-5-phosphate (ribose-5P), which, in the presence of ATP, is converted to 5-phosphoribosyl-1-pyrophosphate (pRpp) (Horecker, 2002; Titgemeyer *et al.*, 2007). PRpp is the branch point intermediate of decaprenyl-phospho-arabinofuranose (DPA) (Alderwick *et al.*, 2011), the only donor of arabinose to AG and LAM (Klutts *et al.*, 2002; Lucarelli *et al.*, 2010) and nucleotide synthesis. In this investigation, the upregulated non-oxidative PPP in the ciprofloxacin treated *Mtb*, is supported by the elevated concentrations of xylofuranose and reduced erythritol (Figure 4-4). Furthermore, it is well known, that PPP metabolism is fueled by glucose and the glucogenic amino acids; valine, aspartic acid, and glutamic acid (Sheppard *et al.*, 2008), all of which

were specifically and significantly reduced in the ciprofloxacin-treated *Mtb*, in addition to their degradation products; β -aminoisobutanoic acid (Kudo *et al.*, 2014), 5-oxoproline (Rieck *et al.*, 2017) and N-acetyl-lysine (Hasenoehrl *et al.*, 2019), respectively (Figure 4-4). Furthermore, the reduced levels of glutamic acid and aspartic acid in our investigation, support an oxidative state in the ciprofloxacin treated group (Rizvi *et al.*, 2019). The above-mentioned amino acids also serve as precursors of cell wall related intermediates. Alanine, derived from aspartic acid, and valine combined, produce CoA, which is used for converting fatty acids into cell wall lipids (Daniel *et al.*, 2014; Genschel, 2004; Leonardi & Jackowski, 2007). Valine also serves as a precursor of propionyl-CoA (Kudo *et al.*, 2014; Massey *et al.*, 1976), and is used for the elongation of odd chain fatty acids (Surger *et al.*, 2018). Aspartic acid additionally is the precursor of NAD⁺, 2,6-diaminopimelate (DAP) (Rodionova *et al.*, 2014; Zhao *et al.*, 2018), and S-adenosyl methionine (SAM) (Ramakrishnan *et al.*, 1972). Considering this NAD⁺ is subsequently an important cofactor for FAS I (Heath *et al.*, 1999), and SAM is required for the methylation of cell-wall fatty acids (Martin & McMillan, 2002). Furthermore, the aforementioned DAP, along with alanine and glutamic acid, serve as substrates for PG (Crick *et al.*, 2001). Lastly, the observed flux through aspartic acid can be supported by the elevated levels of urea in *Mtb* treated with ciprofloxacin (Agapova *et al.*, 2019), and considering that aspartic acid is one of the top 3 metabolite markers identified (Table 4-1), it is possibly utilized contributing to many of the cell-envelope changes observed.

The FQ-induced SOS response is known to cause cell growth arrest (Iacobino *et al.*, 2021), and in this study, inhibited cell growth is evident by the increased TAG metabolism and reduced TCA activity, in addition to the inhibited protein synthesis observed in the ciprofloxacin treated *Mtb*. Protein synthesis requires ATP-dependent activation of glutamate and aspartate, followed by amination by ammonia, to form aminoacyl-tRNA (Sheppard *et al.*, 2008). However, in this study, urea was found elevated in *Mtb* treated with ciprofloxacin, indicating accumulation of ammonia and reduced recycling of nitrogen via glutamine and proline metabolism. This, in addition to a decreased degradation of urea by urease, also linked to the *Mtb* stress-response (Petridis *et al.*, 2016), retains ammonia from protein synthesis (Gouzy *et al.*, 2014). Inhibited protein formation has also been suggested in a proteomics study of *Mtb* treated with ciprofloxacin (Stallings *et al.*, 2009), as well as a metabolomics study of *M. smegmatis* treated with ciprofloxacin (Halouska *et al.*, 2012). Disruption of protein synthesis has also been reported in studies investigating the response of *Mtb* to pretomanid, streptomycin, EMB, RIF, PZA, INH (Rafael Baptista *et al.*, 2018; Burke *et al.*, 2020; Khan *et al.*, 2018; Patil *et al.*, 2020). The inhibited protein synthesis observed, would in turn disrupt the functionality of membrane proteins, subsequently inhibiting nutrient uptake as well as fatty acid transport to the cell wall (Radhakrishnan *et al.*, 2014). Additionally, urea acts as an osmolyte preventing dehydration or water loss as a result of the seemingly damaged cell wall (Yancey, 2001). Increased urea could also be indicative of polyamine synthesis (Figure 4-4) (Majumdar *et al.*, 2016). Polyamines, such as putrescine, have been reported to reduce the accumulation of trans-membrane proteins (Jain & Tyagi, 1987) and contribute to the phenotypic drug resistance to FQs (Sarathy *et al.*, 2013).

Changes to the MAG; 1-monomyristin, 2-monopalmitin, and 1-monoheptadecanoin observed, are indicative of cell envelope changes via the glycerolipid and triacylglycerol (TAG) pathways. TAG in the cell wall (Ortalo-Magne *et al.*, 1996) can be metabolized during the stress-induced transition to the non-replicated phase (Singh *et al.*, 2009) and infection (Muñoz-Eliás *et al.*, 2006). *Mtb*'s adaption during these circumstances involves the use of lipids as the main energy reserves as previously mentioned (Daniel *et al.*, 2004). During the deacetylation of TAG to diacylglycerol (DAG), and from DAG to MAG, acyl-CoA's are released, which are either directed towards glycerolipid synthesis (Figure 4-4) or energy production (Crellin *et al.*, 2013; Deb *et al.*, 2006). Glycerolipid and TAG metabolism share an important intermediate at the branch point, 1,2-diacyl-*sn*-glycerol 3-phosphate, commonly known as PA (Figure 4-4) (Barksdale & Kim, 1977). PA is synthesized via 2 pathways: 1) the phosphorylation of DAG, by DAG kinase (Athenstaedt & Daum, 1999) or 2) the acylation of glycerol-3-phosphate (glycerol-3P) by glycerol phosphate- and acyl glycerol phosphate acyltransferase (Rottig & Steinbuchel, 2013). In this study, the concentrations of glycerol-3-phosphate, and its precursor, glycerol, were decreased, supporting a MAG metabolic flux towards TAG and PIM in the ciprofloxacin treated *Mtb* group. Glycerol-3P appears to recycle the transport of membrane lipids (Aschauer *et al.*, 2018) and can thus be expected to be the rate limiting step, which would explain the accumulation of the fatty acids and the accompanied reduction in glycerol levels. The synthesis of glycerolipids also requires myo-inositol, which is produced via glucose-6-phosphate (glucose-6P) (Clarke *et al.*, 2015). Firstly, myo-inositol 3-phosphate synthase converts glucose-6P into myo-inositol 3-phosphate, which in turn is dephosphorylated by several myo-inositol monophosphates to produce myo-inositol (Nigou *et al.*, 2003). The elevated levels of myo-inositol monophosphates in the ciprofloxacin-treated *Mtb*, hence further support the aforementioned flux towards glycerolipids (Salman *et al.*, 1999). Furthermore, myo-inositol-1-phosphate is converted by a glycosyltransferase, Msh, mycothiol. Mycothiol is also considered an important antioxidant required for balancing the cytosolic NAD⁺/NADH ratio (Newton *et al.*, 2006). In addition to the abovementioned inhibition of protein synthesis and enhanced fatty acid synthesis, altered energy metabolism has also consistently been identified in response to the first-line drugs, INH (Mehaffy *et al.*, 2018), EMB (Sun *et al.*, 2019), PZA (Khan *et al.*, 2019) and RIF (Combrink & du Preez, 2020), supporting these results. In summary, the mycobacterial cell wall metabolism is visibly linked to the SOS response, which has frequently been proposed to cause resistance towards ciprofloxacin and challenge the otherwise impressive bactericidal activity of this drug (O'Sullivan *et al.*, 2008; Qin *et al.*, 2015; Tian *et al.*, 2005; Torres-Barceló *et al.*, 2015).

4.6 CONCLUDING REMARKS

In this study, we investigated the metabolic changes to *Mtb* induced by sub-MIC of ciprofloxacin, to better understand its MoA and the resultant adaptations of *Mtb*. Previous studies have identified alterations in transcription, translation, and cell wall synthesis, as part of the MoA of ciprofloxacin against *Mtb*. Our

metabolomics study identified metabolite markers that support previous results, as indicated by the drastic accumulation of metabolites associated with the cell wall and DNA repair. Moreover, many of these markers indicate an SOS-induced shift to the non-replicative phase which is a key mechanism determining *Mtb* persistence and tolerance to various anti-TB drugs. This study not only gives a better understanding of ciprofloxacin's mode of action but provides helpful insight for further investigation of antibiotic-induced resistance by *Mtb*, and perhaps the use of ciprofloxacin, in combination with existing anti-TB drugs.

4.7 REFERENCES

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CHAPTER 5: THE ANTIMYCOBACTERIAL MECHANISM OF DECOQUINATE RMB041

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See Annexure A.

5.1 ABSTRACT

TB, caused by *Mtb*, still remains one of the leading causes of death from a single infectious agent worldwide. The high prevalence of this disease is mostly ascribed to the rapid development of drug resistance to the current anti-TB drugs, exacerbated by lack of patient adherence due to drug toxicity. The aforementioned highlights the urgent need for new anti-TB compounds with different antimycobacterial mechanisms of action to those currently being used. A N-alkyl quinolone; decoquinat RMB041, has recently shown promising antimicrobial activity against *Mtb*, while also exhibiting low cytotoxicity and excellent pharmacokinetic characteristics. Its exact MoA, however, is still unknown. Considering this, we used GCxGC-TOFMS and well described metabolomic approaches, to analyze and compare the metabolic alterations of *Mtb* treated with decoquinat RMB041 by comparison to non-treated *Mtb* controls. The most significantly altered pathways in *Mtb* treated with this drug include fatty acid metabolism, amino acid metabolism, glycerol metabolism, and the urea cycle. These changes support previous findings suggesting this drug acts primarily on the cell wall and secondarily on the DNA metabolism of *Mtb*. Additionally, we identified the inhibition of protein synthesis and a state of dormancy.

5.2 INTRODUCTION

TB, caused by *Mtb*, is currently considered the deadliest infectious disease worldwide (Harding, 2020). TB causes about 1.3 million deaths annually, in addition to approximately 300 000 deaths of patients coinfectd with the human immunodeficiency virus (HIV), while newly infecting 10 million people per annum (Harding, 2020; Tanner *et al.*, 2019). Furthermore, the prevalence of MDR- and XDR-TB is also on a rise (Nguyen *et al.*, 2018; Polsfuss *et al.*, 2019). The urgency for developing new anti-TB drugs that are less expensive, less cytotoxic, and more efficient, as well as being readily co-administered with HIV treatment, is indisputable.

Currently, the first-line treatment for TB entails a six-month DOTS regimen, including isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and rifampicin (RIF) (WHO, 2019b). Infection with MDR- and XDR-TB requires more expensive treatment for longer treatment duration, using drugs that have a higher risk of adverse effects (Da Silva & Palomino, 2011; Holmes *et al.*, 2017), which in turn leads to poorer patient compliance, and subsequently an escalated increase of drug-resistant TB (Vernon *et al.*, 2019). Further contributing factors to the development of drug-resistant TB include; inaccurate diagnosis, unsupervised treatment protocols, and poor economic status (Duarte *et al.*, 2018; Migliori *et al.*, 2020; Watermeyer & Penn, 2019), and in 2020, this was further exacerbated by the COVID-19 pandemic (Harding, 2020; McQuaid *et al.*, 2020). Over the last 50 years, the only new antimycobacterial compounds to be approved, as last-resort options, are the anti-TB drugs; pretomanid, bedaquiline, and delamanid (Nguyen *et al.*, 2018; Polsfuss *et al.*, 2019; WHO, 2019a, 2019b). Recently, the WHO and the UN held their first high-level meeting aimed at initiating an urgent global response to end the TB epidemic by 2030 (Harding, 2020). However, certain objectives need to be met before this goal can be achieved. Most importantly, elucidating the antimycobacterial mechanisms of action of drugs is considered essential and a means towards finding novel *Mtb* drug compounds and targets (Sloan & Lewis, 2016). Furthermore, the mechanisms of drug resistance also need to be fully understood (Kolyva & Karakousis, 2012). Equally important is economic efficiency, particularly in high-burden countries with insufficient funding for extremely long, yet mandatory, clinical trials. It is thus beneficial to further investigate already approved drugs, with established pharmacokinetic properties and safety profiles (Ginsberg & Spigelman, 2007).

Decoquinatone, an anticoccidial quinolone used as a broad-spectrum antibiotic, has gained interest for its efficacy against malaria, toxoplasmosis, and tuberculosis (Beteck *et al.*, 2019), accompanied by an excellent safety profile (McLoughlin & Chute, 1970), high permeability into the cells and its relatively low costs (Beteck *et al.*, 2016). Recently, a N-alkyl decoquinatone derivative (RMB041) showed high activity against *Mtb* ($MIC_{90} = 1.61 \mu M$), with similar *in vitro* potency to that of ciprofloxacin ($1.5\text{--}12 \mu M$), gatifloxacin ($0.66\text{--}1.3 \mu M$), and moxifloxacin ($0.62\text{--}1.3 \mu M$) (Ginsburg *et al.*, 2003). Its permeability ($\text{LogP}_{app} = -4.8$, where compounds with values > -5 are considered highly permeable (Wohnsland & Faller, 2001)) confers a benefit for drug diffusion through the lipid cell wall (Nam *et al.*, 2011), and penetration of infected macrophages and granulomatous lesions in the lungs (Lenaerts *et al.*, 2015; Tanner *et al.*, 2020). This drug also shows low cytotoxicity in human fetal lung fibroblasts (Beteck *et al.*, 2018), as well as promising pharmacokinetic properties, including the intravenous elimination half-life ($t_{1/2}$) of 62.3 hours in murine models, and a low human CL_{int} ($16 \mu L/\text{min}/\text{mg}$) (Tanner *et al.*, 2019). The $t_{1/2}$ is relatively long when compared to that of other well-known anti-TB drugs such as RIF (7.19 h) (Ji *et al.*, 1993; Lyons *et al.*, 2013), EMB (3h) (Arbex *et al.*, 2010), INH (1.7 h) (Jayaram *et al.*, 2004) and PZA (1.05 h) (Grosset & Ji, 1998), whereas the CL_{int} performs well compared to that of EMB ($>75 \mu L/\text{min}/\text{mg}$) and INH ($<22 \mu L/\text{min}/\text{mg}$) but less so in comparison to RIF ($<10 \mu L/\text{min}/\text{mg}$) (Lakshminarayana

et al., 2015). Drugs with lower CL_{int} and longer $t_{1/2}$ require less frequent administration/dosing, which could improve patient compliance and contribute to lowering the prevalence of drug resistance (Dheda *et al.*, 2014). Furthermore, decoquinatone RMB041 could hold the key to shortening the duration of TB treatment and reduce the current costs of therapy (Warner & Mizrahi, 2014). Considering this, decoquinatone RMB041 is a promising anti-TB candidate. However, little is known about its antimycobacterial MoA. Contrary to decoquinatone, which primarily targets the ubiquinol-binding pocket, the RMB041 derivative rather shows activity against the cell wall and DNA metabolism of *Mtb* as its primary and secondary targets, respectively (Beteck *et al.*, 2018). The exact underlying MoA, however, is yet to be determined.

In this study, two-dimensional gas chromatography coupled with a time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach, combined with universally connected metabolic libraries and advanced statistical analysis, was used to identify the metabolite markers best differentiating *Mtb* treated with and without decoquinatone RMB041.

5.3 MATERIALS AND METHODS

5.3.1 Cell culture

The reagents used for this investigation were purchased from Sigma-Aldrich, St. Louis, MO, USA unless specified otherwise. The microtiter Alamar Blue assay was used to determine the antimycobacterial sub-minimum inhibitory concentration (IC_{50}) of decoquinatone RMB041 [35]. Briefly, cell cultures were prepared as delineated by Van Breda *et al.* (2015), with minor modifications. *Mtb* H37Rv ATCC 27294, obtained from the Medical Research Council (Pretoria, Gauteng, South Africa), was cultured and maintained on LJ slants. After four weeks of incubation, a bacterial inoculum of McFarland 1 (approximately 3×10^8 colony-forming units/mL) was prepared in Middlebrook 7H9 broth containing 10% OADC (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson, UK) and 2% PANTA (polymyxin B, amphotericin B, nalidixic acid and trimethoprim, azlocillin) (Becton, Dickinson, UK). The latter shows a negligible impact on *Mtb* growth and was added to prevent contamination (Palomino & Portaels, 1998). In five of the ten aliquots, 4mL decoquinatone RMB041 was dissolved in DMSO (150 μ M) and further diluted in Middlebrook 7H9 broth to yield a final concentration of 10 μ M (0.2 % DMSO). One milliliter of the prepared inoculum was added for a final assay volume of 5 mL, with a bacterial test concentration of 6×10^7 CFU/mL decoquinatone RMB041. The untreated *Mtb* control samples were prepared in the remaining five aliquots, by adding the bacterial inoculum, as described above, in 4 mL of Middlebrook 7H9 broth (0.2 % DMSO). After 5 days of incubation at 37 °C, we generated mycobacteria wet cell pellets by centrifuging the samples at 4500 rpm for 15 min. These were then washed with 1 mL of PBS and re-pelleted as described above. Lastly, the PBS was aspirated, and the samples and the samples were stored at -80 °C until further testing.

5.3.2 Whole metabolome extraction and derivatization procedure

The extraction and derivatization procedures were carried out as previously described by Beukes *et al.* (2019), with slight modifications. Briefly, 8 mg of each of the ten individually cultured samples were weighed into an Eppendorf tube. As an internal standard, 50 μ L 3-phenylbutyric acid (0.13125 mg/ml H₂O) (Sigma-Aldrich, Lot#536478V) was added, and a two-phase extraction was performed with chloroform: methanol: water in a 1:3:1 ration (1 mL in each sample). The Eppendorf tubes, containing a 3mm carbide tungsten bead in each, were shaken in a vibration mill at 30 Hz for 5 min, followed by centrifugation at 12 000 rpm for 5 min. After transferring each extract to a GC glass vial, the samples were dried under a nitrogen stream. Derivatization was achieved by firstly adding 50 μ L MEX (Sigma-Aldrich, Lot#BCBP2843V) in pyridine (Lot#S2BC335SV) (at a final concentration of 15mg/mL), to the glass vials, and heating at 50°C for 90 min, followed by the addition of 40 μ L BSTFA with 1% TMSCI (Lot#BCBW2670) and again heating for 60 min at 50°C. Lastly, each extract was transferred to a 0.1 mL vial insert, which was placed into their respective GC sample vial prior to injection into the GCxGC-TOFMS.

5.3.3 GCxGC-TOFMS analysis

The metabolomics analysis was performed using a 4D Pegasus GCxGC-TOFMS (LECO Africa (Pty) Ltd, Johannesburg, South Africa), equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA) coupled to TOFMS (LECO Africa). The GC-MS parameters were set as previously described (Beukes *et al.*, 2019). A Rxi-5Sil MS capillary column was used as the primary column (28.8 m x 0.25 mm internal diameter, 0.25 μ m film thickness, Restec), and a Rxi-17 capillary column (1.2 m x 0.25 mm internal diameter, 0.25 μ m film thickness). The primary GC oven temperature was set at 70°C for 2 min and increased at a rate of 4°C/min to a final temperature of 300°C, at which it was maintained for an additional 2 min. The secondary oven was set at 85°C for 2 min, increased at 4.5°C/min, to a final temperature of 300°C, at which it was maintained for 4.5 min. Helium, used as a carrier gas, was set to a column flow rate of 1 mL/min and held at a constant temperature of 270°C. Mass spectrometric data acquisition was carried out using a filament bias of -70 eV, with a 350 s solvent delay, and an acquisition scanned mass range of 50-800 m/z at 200 spectra/s. To ensure high internal validity, the samples were analyzed in random sequence. The analytical performance of GCxGC-TOFMS throughout the analysis was monitored by adding QC samples at the start, in the middle, and at the end of the sequence.

5.3.4 Data processing, clean-up, and statistics

Mass spectral deconvolution (at a signal-to-noise ratio of 20), peak alignment, and peak identification was done with ChromaTof software (version 4.32, Leco Corporation, St. Joseph, MI, USA). For the identification of

metabolites, their mass fragment patterns were compared to those of compounds in commercially available databases containing previously injected standards. The quality of the data was evaluated by pretreating the data using a standardized metabolomics data clean-up procedure (Beukes *et al.*, 2019). MS total useful signal was used for data normalization. Considering that the zero values are most likely present in sub-minimum concentrations rather than being completely absent, they were replaced by a value calculated as 20% of the minimum detection limit of the entire dataset (Gromski *et al.*, 2014). An 80% data filter was then applied to remove compounds that were only detected in two (or less) samples per group (Wei *et al.*, 2018). Log transformation and auto-scaling were applied to balance metabolite representations. This prevents the domination of compounds with higher concentrations over compounds with lower concentrations (Shen & Zhu, 2019).

Using MetaboAnalyst (Version 5.0, <https://www.metaboanalyst.ca>), two multivariate statistic methods were applied: (1) an PCA and (2) PLS-DA (Meissner-Roloff *et al.*, 2012). Additional univariate statistics were also calculated via (1) *t*-test and (2) effect size values (Sullivan & Feinn, 2012).

5.4 RESULTS

5.4.1 Data overview

Principal component analysis (PCA) was initially used to get an overview of the natural grouping of metabolic data (Figure 1). The total variance described by the first two principal components (PCs) was 52%, of which PC1 accounted for 30.6% and PC2 for 21.4%, respectively. The PCA scores plot of the metabolite data analyzed by GCxGC-TOFMS shows clear clustering between *Mtb* treated with DQ derivative RMB041 and those cultures that were not (Figure 5-1). The scores plot also shows that the variance of the treated *Mtb* group is greater than the controls, as a result of greater variance in the metabolite concentrations.

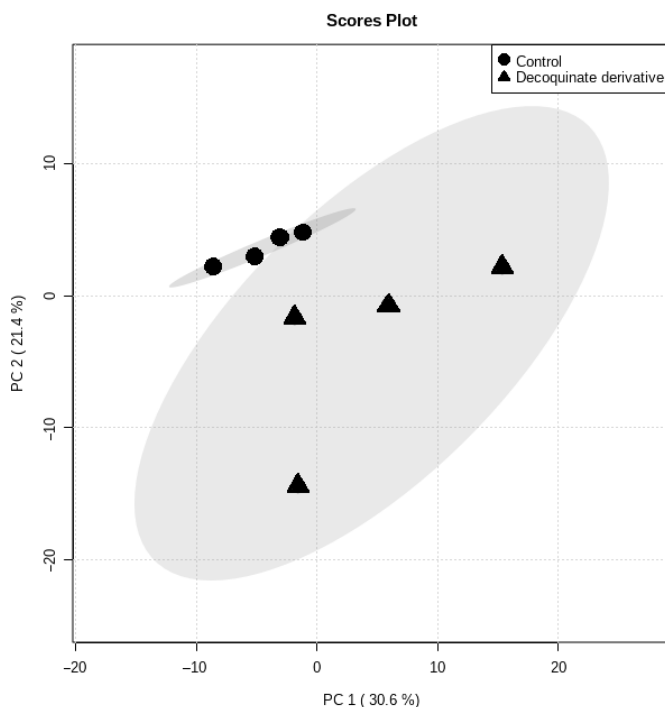


Figure 5-1: PCA scores plot obtained from GCxGC-TOFMS whole metabolome analysis of *Mtb* samples in the presence and absence of decoquinat RMB041.

5.4.2 Marker selection

Based on compliance to the following criteria: a PLS-DA VIP value > 1 (Cho *et al.*, 2008), a t-test P-value < 0.05 (Byliński *et al.*, 2018) or an effect size > 0.8 (Sullivan & Feinn, 2012), 36 metabolites were selected as markers best describing the difference between *Mtb* treated with decoquinat RMB041 and *Mtb* control samples (Figure 5-2). The metabolites are listed in Table 5-1, according to their VIP values.

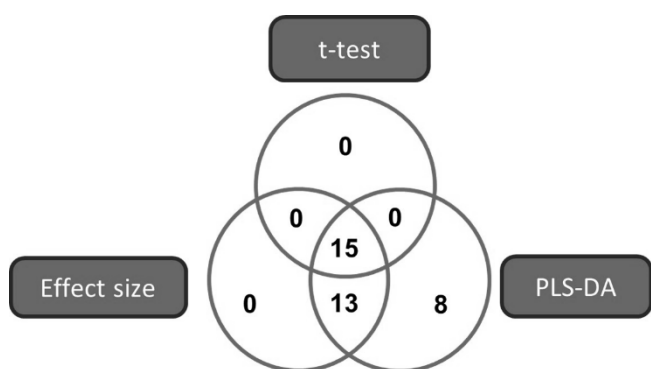


Figure 5-2: Venn diagram illustrating the multi-statistical approach for selecting the metabolites that best describe the variation detected in the metabolome of *Mtb* cultured with and without decoquinat RMB041.

Table 5-1: The selected metabolite markers best describing the variance between the metabolome of *Mtb* cultured in the absence (*Mtb* controls) and the presence of decoquinat RMB041.

Metabolite name (CHEBI ID)	Average concentration (mg/g cells) (standard deviation)		<i>t</i> -test (P- value)	Effect size (d-value)	PLS- DA (VIP)	Fold Change (Log ₂)
	<i>Mtb</i> decoquinat RMB041	with <i>Mtb</i> controls				
9-Octadecenoic acid (36021)	0.967 (0.192)	0.560 (0.041)	0.000	34.47	2.59	0.73
Myo-Inositol (17268)	0.024 (0.032)	0.020 (0.002)	0.000	5.298	1.99	0.20
Ribitol (15963)	0.318 (0.513)	0.100 (0.005)	0.006	2.098	1.98	2.18
Arabitol (18403)	0.877 (0.962)	0.790 (0.199)	0.010	2.206	1.98	0.11
Eicosanoic acid (28822)	0.018 (0.025)	0.004 (0.001)	0.000	3.968	1.94	3.50
Erythritol (17113)	0.023 (0.027)	0.028 (0.001)	0.001	3.176	1.92	-0.18
N-Acetyl-Lysine (35704)	0.000 (0.000)	0.004 (0.003)	0.009	1.938	1.81	-1.00
9,12-Octadecadienoic acid (17351)	0.578 (0.827)	0.111 (0.008)	0.016	1.835	1.67	4.21
Valine (16414)	0.039 (0.063)	0.022 (0.005)	0.019	1.908	1.65	0.77
Aspartic acid (17053)	0.018 (0.033)	0.013 (0.004)	0.038	1.341	1.65	0.38
Pentadecanoic acid (42504)	0.017 (0.023)	0.004 (0.001)	0.021	1.648	1.64	3.25
Galactose (28260)	0.004 (0.002)	0.011 (0.001)	0.185	0.747	1.57	-0.64
Glutamic acid (16015)	0.024 (0.043)	0.015 (0.006)	0.032	1.549	1.56	0.60
Lysine (18019)	0.016 (0.030)	0.009 (0.006)	0.047	1.291	1.56	0.78
2-Hydroxyglutaric acid (32796)	0.003 (0.004)	0.002 (0.001)	0.037	1.384	1.53	0.50
Proline (26271)	0.022 (0.041)	0.016 (0.013)	0.060	1.202	1.50	0.38

Glycerol-3-phosphate (15978)	0.012 (0.016)	0.007 (0.002)	0.049	1.308	1.47	0.71
Malonic acid (30794)	0.173 (0.213)	0.039 (0.045)	0.249	0.684	1.46	3.44
Glycerol (17754)	0.994 (1.45)	0.633 (0.031)	0.057	1.171	1.44	0.57
Nonadecanoic acid (NSC11914)	0.499 (0.731)	0.055 (0.009)	0.065	1.127	1.41	8.07
Alanine (16977)	0.321 (0.621)	0.207 (0.152)	0.067	1.156	1.40	0.55
Octadecanoic acid (28842)	0.027 (0.034)	0.005 (0.000)	0.068	1.150	1.40	4.40
β -Aminoisobutanoic acid (33094)	0.035 (0.057)	0.014 (0.004)	0.073	1.310	1.38	1.50
γ -Aminobutyric acid (16865)	0.011 (0.018)	0.016 (0.006)	0.085	1.315	1.34	-0.31
Tyrosine (17895)	0.006 (0.012)	0.003 (0.001)	0.147	0.843	1.32	1.00
9-Hexadecenoic acid (59265)	0.105 (0.153)	0.018 (0.003)	0.099	0.975	1.29	4.83
Heptadecanoic acid (32365)	0.052 (0.069)	0.011 (0.002)	0.103	0.975	1.29	3.73
Ribofuranose (33942)	0.003 (0.003)	0.007 (0.002)	0.239	0.653	1.28	-0.57
Glucose (17243)	0.045 (0.031)	0.037 (0.005)	0.174	0.770	1.24	0.22
Isoleucine (17191)	0.018 (0.032)	0.008 (0.008)	0.216	0.702	1.21	1.25
9Z-Octadec-1-ol (73504)	0.026 (0.036)	0.004 (0.003)	0.276	0.601	1.17	5.50
Decanoic acid (30813)	0.008 (0.009)	0.005 (0.001)	0.157	0.836	1.18	0.60
Malic acid (6650)	0.084 (0.127)	0.054 (0.004)	0.172	0.775	1.11	0.56
Ornithine (15729)	0.029 (0.054)	0.025 (0.015)	0.191	0.841	1.09	0.16
Serine (17822)	0.011 (0.021)	0.004 (0.002)	0.151	0.850	1.06	1.75
Threonine (16857)	0.049 (0.088)	0.016 (0.008)	0.173	0.791	0.93	2.06

5.5 DISCUSSION

In this study we identified a number of significantly altered metabolites induced by the administration of DQ derivative RMB041 to *Mtb* culture. In the light of known metabolism and previous DQ derivative RMB041 findings, the interpretation of these selected metabolite markers, and their associated metabolic pathways, lead to better elucidation of the antimycobacterial MoA of this drug. The most prominently altered pathways (amino acid metabolism, fatty acid metabolism, pentose phosphate pathway (PPP), and the urea cycle) are mapped in Figure 5-3.

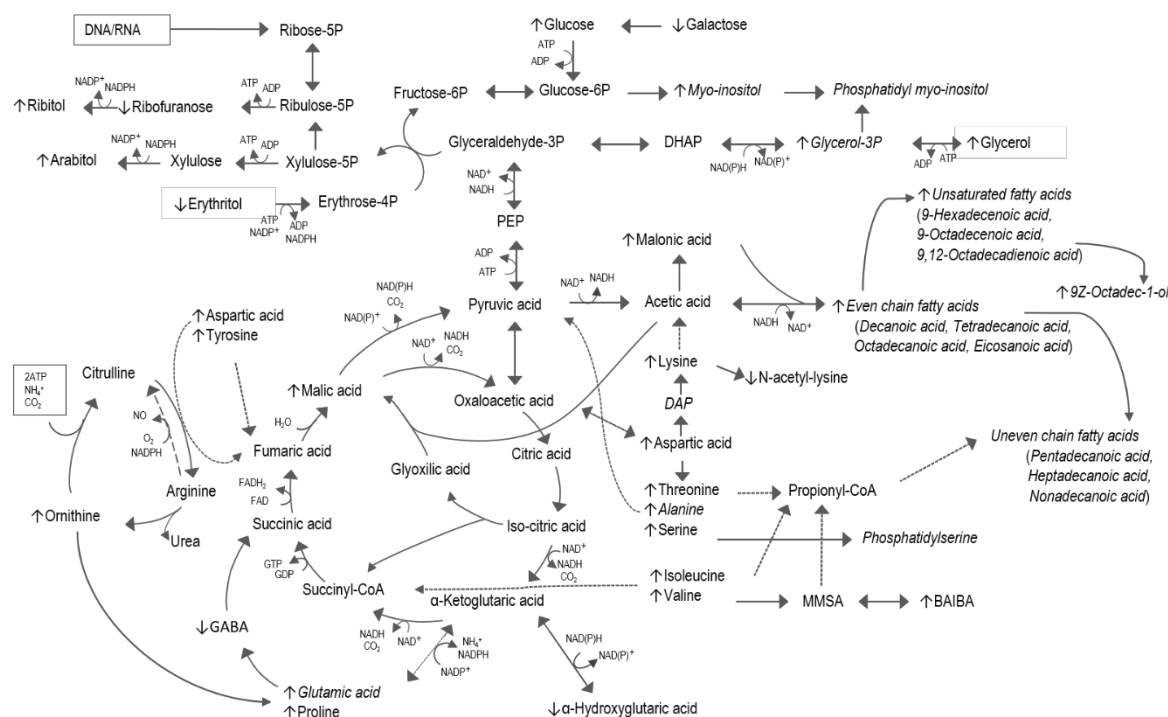


Figure 5-3: Metabolomic pathway map of decoquinat RMB041-treated *Mtb*. The metabolite markers best describing the variation in the metabolome compared to that of untreated *Mtb* are represented with up or down arrows indicating elevated or reduced concentrations, respectively. The dotted lines represent the flux of amino acids from nucleotide degradation into the central carbon metabolism. Metabolites that are incorporated into the cell wall are indicated in *italic*.

Preeminent in the *Mtb* treated with decoquinat RMB041 is the elevation of many of the even and odd saturated fatty acids of between 10 to 20 carbons (C10:0-C20:0). This was also true for the Δ^9 -mono-unsaturated fatty acids; Δ^9 C18:1, as previously mentioned, and 9-hexadecenoic (Δ^9 C16:1), in addition to the $\Delta^{9,12}$ -poly-unsaturated fatty acid; 9,12-octadecenoic ($\Delta^{9,12}$ C18:2). These indicate a strongly upregulated synthesis towards cell wall repair (Verma *et al.*, 1999), and an accumulation thereof due to decoquinat RMB041 inhibiting this process, which confirms previous findings suggesting the antimycobacterial activity of decoquinat RMB041 targeting the cell wall (Beteck *et al.*, 2018). The distinctly multilaminar cell wall of *Mtb* consists of a PG layer covalently attached to AG (Chatterjee *et al.*, 1991), which itself attaches to mycolic acids. Interspersed, is the mycobacterial plasma membrane consisting of glycerophospholipids and the glycerolipids, PIM, LM, and LAM (Jankute *et al.*, 2015), all of which are considered critical for maintaining cell wall integrity (Fukuda *et al.*, 2013), and PIM to the low permeability of the cell envelope and intrinsic tolerance to antibiotics (Bansal-Mutalik & Nikaido, 2014). The synthesis of PIM requires myo-inositol and glycerol-3-phosphate (glycerol-3P), which were also elevated in this study. Myo-inositol is produced via the conversion of glucose-6-phosphate (glucose-

6P) to myo-inositol 1-phosphate, before dephosphorylation by myo-inositol monophosphate phosphatase (Movahedzadeh *et al.*, 2010). Glycerol-3P is acetylated to produce PA (Rottig & Steinbuchel, 2013), which serves as the precursor for all glycerolipids (Barksdale & Kim, 1977). The saturated fatty acids identified in this study are synthesized by FAS I and provide fatty acyl-CoA's to FAS II for elongation (Abrahams & Besra, 2016). FAS I and FAS II provide acyl-groups for the synthesis of all cell wall components except for AG (Chatterjee *et al.*, 1991; Clarke *et al.*, 2015; Crellin *et al.*, 2013). C14:0, C16:0, C18:0, Δ^9 C16:1, and Δ^9 C18:1, are considered major fatty acids of the glycerolipids and mycolic acids (Ramakrishnan *et al.*, 1972; Salman *et al.*, 1999; Vilcheze *et al.*, 2013). C16:0 and C18:0 are oxidized, in the presence of Fe^{2+} , a flavin, NADPH, and O_2 to produce Δ^9 C16:1 and Δ^9 C18:1, respectively (Fulco & Bloch, 1964; Fulco *et al.*, 1964).

The mycobacterial cell envelope also plays an important role during the survival of dormant, non-replicating *Mtb* (Antonova *et al.*, 2018; Zhang & Rock, 2008). Mycobacterial membrane proteins recognize stress conditions (Wong *et al.*, 1999) and release acyl-CoA's for rearrangement of the cell wall components (Bothra *et al.*, 2018) and/or energy metabolism (Antonova *et al.*, 2018). The expression of the stress response operon requires continuous energy (Wakamoto *et al.*, 2013), which is mostly derived from NADH and NADPH released during fatty acid metabolism (Gago *et al.*, 2018). Furthermore, an upregulated glyoxylate shunt, as opposed to the oxidative half of the TCA cycle, along with reduced glycolysis and altered glycerol metabolism, is associated with *Mtb* in a state of dormancy (Dhar & McKinney, 2010; Gomez & McKinney, 2004; Kumar & Sanyal, 2012). In our investigation, the elevated levels of malic acid, glucose, sugar alcohols (except for erythritol), glycerol-3P, and glycerol (Figure 5-3) suggest that the same dormancy occurs in *Mtb* treated with the decoquinatone RMB041. Elevated levels of ribitol and arabinol, in the PPP, indicate a metabolic flux of glucose metabolism towards the PPP, and not towards energy production via glycolysis (Horecker, 2002). Furthermore, the decreased concentrations of erythritol confirm the flux towards PPP, and also the aforementioned PIM synthesis via fructose-6-phosphate (fructose-6P) (Figure 5-3). From the results it is evident, that fatty acids are most likely the preferred energy source, further confirmed by the elevated levels of short-chain fatty acids (C10:0-C14:0) and malonic acid, which are derived via β -oxidation of FAS I and FAS II products, in the presence of NAD^+ (Boshoff *et al.*, 2008), in the decoquinatone RMB041 treated *Mtb*. Regeneration of NAD^+ is maintained by the increased expression of isocitrate lyase, leading to enhanced flux through the glyoxylate shunt (Wayne, 1994). This also reduces the release of CO_2 , resulting in a direct carbon flux towards FAS I and FAS II (Rhee *et al.*, 2011), for subsequent energy production (Antonova *et al.*, 2018) and cell wall remodeling (Bothra *et al.*, 2018). In the presence of ATP, the resultant CO_2 , together with NH_4 released during amino acid metabolism, are incorporated into the urea cycle via ornithine (which is elevated in the RMB041 treated *Mtb*, indicating an upregulated urea cycle) (Figure 5-3) (Solomon *et al.*, 2010). Ornithine serves as the precursor of polyamines, which previously have been associated with antibiotic-induced mutations (Majumdar *et al.*, 2016). Furthermore, ornithine contributes to the reclamation of carbon to the TCA cycle via proline and glutamic acid. This can be

obtained via γ -aminobutyric acid (GABA), or via α -ketoglutaric acid (Figure 5-3) (Lee *et al.*, 2018). Notably, decoquinatone RMB041-treated *Mtb* prefers the latter, which could be explained by the necessity of α -ketoglutaric acid to incorporate amino acids such as aspartic acid into the TCA cycle (Yelamanchi & Surolia, 2020). Moreover, NAD(P)H and α -ketoglutaric acid are converted to NAD(P)⁺ and α -hydroxyglutaric acid (Quartararo *et al.*, 2013), which, too, was found to be elevated in this study. This might further indicate an imbalanced NAD(P)⁺/NAD(P)H ratio, supporting need for NAD(P)⁺ for survival on fatty acids as the main energy source, and/or disruption of the membrane, leading to a disturbed electron transport chain (ETC) and, subsequently, accumulation of NADH (Baker *et al.*, 2019).

The *Mtb* non-replicative phase is also associated with a reduction/stagnation of DNA, RNA, and protein synthesis (Gopinath *et al.*, 2015; Hu *et al.*, 1998). The accumulation of urea (0.012 vs 0.002 mg/g; P = 0.63) in the *Mtb* treated with decoquinatone RMB041, although not selected as a metabolite marker using the statistical selection process described above, indicates an accumulation of nitrogen species, indicative of this (Sheppard *et al.*, 2008). Urea could also serve as an opposing force to prevent osmotic lysis (Yancey, 2001) brought about by dehydration as a result of altered cell envelope integrity. Aspartic acid, along with ten other amino acids (Table 5-1), were found elevated in this study. When considering this in the light of the elevated levels of sugar alcohols detected in the decoquinatone RMB041 treated *Mtb* group, degradation of the nucleic acids, to purine and pyrimidines is evident (Villela *et al.*, 2011; Vogels & Van der Drift, 1976). Speculatively, the direct activity of decoquinatone RMB041 on DNA would most likely result in an attempt towards DNA repair, as was previously indicated by reduced sugar alcohols in *Mtb* treated with ciprofloxacin (Knoll *et al.*, 2021). In this case, however, we see an accumulation of both amino acids and sugar alcohols, suggesting DNA replication was not yet inhibited or repair has not yet been activated at the time the cells were harvested. This may however occur at a later stage, or at higher decoquinatone RMB041 concentrations perhaps, but in the present study, the *Mtb* treated with decoquinatone RMB041, are most likely still in a non-replicative state after the irreversible cell envelope damage, which is the primary target of decoquinatone RMB041 (Beteck *et al.*, 2018). This is also supported by previous findings suggesting DNA replication is a secondary target and cell wall metabolism as a primary target of decoquinatone RMB041 (Beteck *et al.*, 2018).

In a study by Rizvi *et al.* (2019), oxidative stress, inherent with the shift to a dormant state in *Mtb*, resulted in the statistically significant accumulation of the same six (aspartic acid, valine, threonine, alanine, isoleucine, and lysine) of the ten amino acids identified as metabolite markers in the decoquinatone RMB041 treated *Mtb* in our study. The flux of lysine towards acetic acid, and subsequent fatty acid synthesis, is supported by decreased levels of its break-down product, N-acetyl-lysine (Hasenoehrl *et al.*, 2019). Furthermore, the branched-chain amino acids; threonine, isoleucine, and valine, serve as precursors of propionyl-CoA (Kudo *et al.*, 2014) for elongation of odd chain fatty acids (Surger *et al.*, 2018). Flux towards propionyl-CoA from valine is furthermore supported by the increased levels of β -aminoisobutanoic acid (BAIBA) in the decoquinatone RMB041 treated *Mtb*,

whereby the intermediate, methyl-malonyl semialdehyde (MMSA), is the direct precursor of propionyl-CoA (Serafini *et al.*, 2019).

Serine, one of the elevated amino acid markers in the decoquinatone RMB041 treated *Mtb* group, also serves as a precursor of phosphatidylserine (Cassilly & Reynolds, 2018). Translocation of phosphatidylserine on the outer membrane of *Mtb* has been associated with cell death induced by ROS (Rachman *et al.*, 2006). ROS are released during the antibiotic-induced stress response and accumulate when DNA repair is unsuccessful, which in turn results in the aforementioned dormant phase (Hong *et al.*, 2019; Iacobino *et al.*, 2021). Altered PG metabolism, as is brought about by cell wall active antibiotics, has also been linked to changes in the growth and division of bacterial cells (Mueller & Levin, 2020). This further suggests irreversible inhibition by decoquinatone RMB041 to the cell envelope, which would result in continuous activation of the stress response operon, requiring non-stop energy, supplied by fatty acid metabolism, and, partially, the TCA cycle, followed by the induction of a dormant state, whereby cell growth and division is ultimately inhibited.

In summary, this metabolomics approach better characterized the antimycobacterial mechanism of decoquinatone RMB041, which not only confirmed previously proposed mechanisms of action but also suggests possible new mechanisms, which could be further investigated and confirmed using alternative strategies. A possible limitation, however, is the degradation of thermolabile metabolites into smaller unidentifiable “unknown” compounds (Fiehn, 2017).

5.6 CONCLUDING REMARKS

Decoquinatone RMB041 has been previously proposed to act against *Mtb*, primarily via inhibition/destruction of its cell wall, and inhibition/destruction of DNA as a secondary target (Beteck *et al.*, 2018). This metabolomic investigation indicated an altered metabolite profile (drastically elevated levels of various fatty acids and glycerolipid precursors, amino acids, and urea cycle intermediates) confirming the aforementioned observations in addition to an inhibition of protein synthesis and a state of dormancy. This study not only confirms or improves upon the existing knowledge pool of novel antimycobacterial mechanisms of actions but also provides a useful tool for investigating repurposed and/or adjunctive drugs against *Mtb*. Furthermore, a better understanding of the dormancy process as indicated here could lead to new treatment regimens that reduce the emergence of relapse and resistance (Caño-Muñoz *et al.*, 2018).

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CHAPTER 6: *IN SILICO* DRUG DISCOVERY STRATEGIES IDENTIFIED ADMET PROPERTIES OF DECOQUINATE RMB041 AND ITS POTENTIAL DRUG TARGETS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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See Annexure A.

6.1 ABSTRACT

The highly adaptive cellular response of *Mtb* to various antibiotics and the high costs for clinical trials, hampers the development of novel antimicrobial agents with improved efficacy and safety. Subsequently, *in silico* drug screening methods are more commonly being used for the discovery and development of drugs, and have been proven useful for predicting the pharmacokinetics, toxicities, and targets, of prospective new antimicrobial agents. In this investigation we used a reversed target fishing approach to determine potential hit targets and their possible interactions between *Mtb* and decoquinat RMB041, a propitious new antituberculosis compound. Two of the thirteen identified targets, Cyp130 and BlaI, were strongly proposed as optimal drug-targets for dormant *Mtb*, of which the first showed the highest comparative binding affinity to decoquinat RMB041. The metabolic pathways associated to the selected target proteins were compared to previously published molecular mechanisms of decoquinat RMB041 against *Mtb*, whereby we confirmed disrupted metabolism of proteins, cell wall components, and DNA. We also described the steps within these pathways that are inhibited and elaborated on decoquinat RMB041's activity against dormant *Mtb*. This compound has previously showed promising *in vitro* safety and good oral bioavailability, which were both supported by this *in silico* study. The pharmacokinetic properties and toxicity of this compound were predicted and investigated using the online tools pkCSM and SwissADME, and Discovery Studio software, which furthermore supports previous safety and bioavailability characteristics of decoquinat RMB041 for use as an antimycobacterial medication.

6.2 INTRODUCTION

TB, caused by *Mtb*, remains one of the leading causes of death by a single infectious agent (WHO, 2020). Despite the many efforts to search for novel anti-TB treatment, only three drugs have been approved and released into the pharmaceutical market over the last 50 years (WHO, 2019b). The inefficiency of many of the existing anti-TB medication, can be attributed to the rapid development of drug resistance and poor understanding of the preceding cellular transition to dormancy (Martinez-Jimenez *et al.*, 2013). Research on compounds effective against active *Mtb* have shed light on the importance of only a few genes/proteins required for its non-replicative survival, enabling mycobacteria to redirect energy sources for overcoming stress (deprivation of nutrients, acidic environments, and exposure to reactive oxygen species, and various Anti-TB drugs) that resulted in the transition to dormancy and the subsequent anti-TB drug resistance (Mueller & Levin, 2020). The increasing worldwide prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB cases has also highlighted *Mtb*'s phenotypic plasticity during latency (Andryukov *et al.*, 2019; Gample *et al.*, 2019; Stelitano *et al.*, 2020). Furthermore, drugs that are effective against non-replicating *Mtb* are still lacking. Additional factors preventing the development of successful anti-TB drugs are the long duration and high costs of novel drug discovery and as the onset of unexpected adverse reactions during clinical trials.

New anti-TB drug discovery is complexed and requires a comprehensive understanding of a drug's functionality and its target proteins in both *Mtb* and the host. Fortunately, the ongoing progress of high-throughput laboratory and computational technology has served to promote exponential growth in both data volume and the variety of data sources, which has enabled researchers to create and apply computational prediction techniques, aimed at reducing the lengthiness and cost of all phases of drug discovery (Campbell *et al.*, 2014). Computer-aided drug design entails webserver and software that identify hit targets, analyze target-protein interactions, annotate cellular functionalities of proteins, and anticipate possible side-effects. All have proven helpful during drug development of potent anti-tuberculous compounds or the prevention of the inclusion of toxic drugs in clinical trials (Hurle *et al.*, 2013; Xu *et al.*, 2018).

Decoquinatone RMB041 (see structure in Figure 6-1) is an inexpensive compound with promising *in vitro* antimycobacterial activity (Beteck *et al.*, 2018; Knoll *et al.*, 2021), and some good *in vitro* ADMET (absorption, distribution, metabolism, elimination, and toxicity) properties (Tanner *et al.*, 2019). To identify and characterize decoquinatone RMB041's hit targets, we combined several functions offered by a variety of software and online webserver; reverse pharmacophore docking, also known as target fishing, by PharmMapper (Wang *et al.*, 2017), prioritization of targets by TDR Targets, identification of correlated proteins by TBDB, mapping of co-expressed proteins by STRING, annotation of metabolomic pathways by KEGG, virtual screening with Discovery Studio (DS) Visualizer, and docking with AutoDock Vina. Virtual docking has presented some difficulties of late, during the analysis of best binding poses (defined as binding affinities) for lead optimization, which is not surprising, considering that there are many factors that must be considered that may influence the

binding of molecules (Vieira & Sousa, 2019). Hence, for the purpose of this study, estimated binding affinities were determined (Ali *et al.*, 2018; Dar & Mir, 2017; Pagadala *et al.*, 2017), also considering entropic and enthalpic influences, the mobility of ligands and proteins, the charge distribution over a ligand, surrounding water molecules, and the various possible conformations of the compound of interest (Pantsar & Poso, 2018).

Previously, Beteck *et al.* (2018), proved low cytotoxicity of decoquinat RMB041 against WI-38 human fetal lung fibroblasts. In the light of this, we predicted the toxicity of decoquinat RMB041, by investigating those properties generally known to significantly induce toxicity of well-known drugs currently available. Additionally, further properties attributing to absorption, distribution, metabolism, and elimination were determined and evaluated using DS Tools, SwissADME, and pkCSM (Macalino *et al.*, 2020). A previous metabolomic GCxGC-TOFMS investigation of decoquinat RMB041 on *Mtb*, indicated a mechanism of action by perturbation of the mycobacterial cell wall and DNA synthesis, and also a proposed mechanism by which *Mtb* might develop a resistance to decoquinat RMB041. In this study, we further contribute to this knowledge

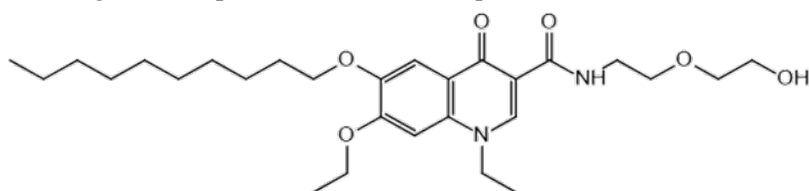


Figure 6-1: Structure of decoquinat RMB041.

by using an *in silico* approach to confirm and build on previous knowledge related to decoquinat RMB041 mechanism of action, toxicity and possible *Mtb* resistance.

6.3 MATERIAL AND METHODS

To ensure the probability of high effectivity and low toxicity of decoquinat RMB041, several tools were employed. The procedure followed during this study is illustrated in Figure 6-2.

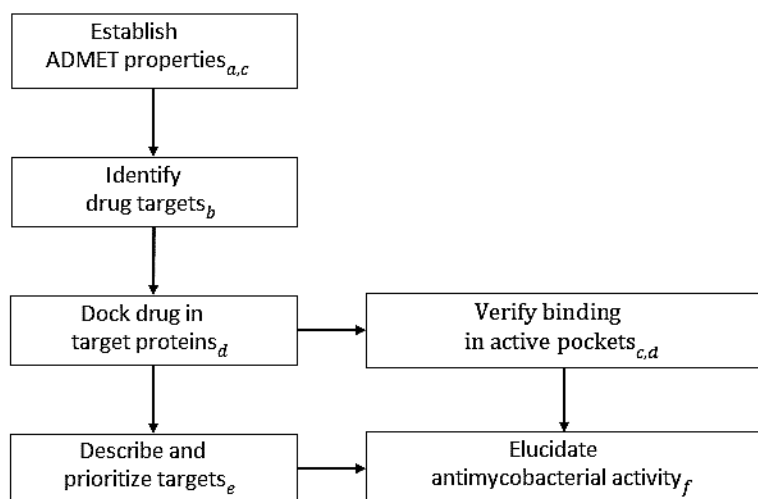


Figure 6-2: The workflow followed to elucidate the potential antimycobacterial mechanism of decoquinatone RMB041. The tools used during each step: a) pkCSM and SwissADME, b) PharmMapper, c) Discovery Studio, d) AutoDock Vina, e) TDR Targets, f) KEGG and STRING.

6.3.1 Evaluation of molecular and pharmacokinetic properties

The chemical 2D structure of the ligand was drawn with ACD/ChemSketch (commercial version) and converted to a 3D (pdb) file with DS v.4.5. ADMET properties of decoquinatone RMB041 were identified by using the ADMET descriptors algorithm and toxicity prediction extensible protocol of BIOVIA DS Visualizer v.4.5 (Accelrys) (Software Inc., San Diego, CA) and the web servers Molinspiration (<http://molinspiration.com/cgi-bin/properties>), SwissADME (<http://www.swissadme.ch/>) and pkCSM (<http://biosig.unimelb.edu.au/>). The results are documented in Figure 6-3. Although sharing similar functions, these tools apply different calculating algorithms, and can be used in combination to ensure improved precision of elucidation. SwissADME and DS also provided the molecular properties, which are reported in Table 6-1. The structure of decoquinatone RMB041 was also evaluated with respect to pharmacokinetic rules frequently applied during drug manufacturing using this methodology Table 6-2.

6.3.2 Computational target fishing

Pharmacophore screening

PharmMapper (<http://www.lilab-ecust.cn/pharmmapper/>) was chosen in this study for the identification of potential protein targets, due to its wide popularity of use in applications of drug discovery. PharmMapper analyzes the spatial arrangement of key functional groups of a molecule and assigns pairwise fit scores according to the matching of pharmacophores between the ligand (being the drug) and the protein (Wang *et al.*, 2017). For

this study, protein targets with fitness scores of ≥ 4 were chosen to filter out insignificant pharmacophore models. The pharmacophores are collected in an internal database, PharmTargetDB, annotated from continuously updated databases, DrugBank, BindingDB, PDTD, TargetBank and Protein Database Bank (PDB). This software uses Cavity to identify all potential binding sites in proteins. The identification codes were retrieved from UniProt (<https://www.uniprot.org/uniprot/>) and the PDB (<https://www.rcsb.org/>) database. Only predicted protein targets relevant to *Mtb* were included for evaluation and deemed of importance of the study, and those in humans and other bacteria were excluded. The results are documented in Table 6-4.

Reverse Docking

To validate the identified drug targets, decoquinatone RMB041 was docked in the active cavity of each of PharmMapper's predicted target proteins deemed valuable for the treatment of TB. The targets were then arranged according to their estimated binding strengths. Results documented in Table 6-4.

Protein and ligand preparation

PDB codes provided by PharmMapper were searched in PDB (<http://www.pdb.org>), where their three-dimensional structures with their co-crystallized ligands (solved at 0.90-1.8 Å resolution²) were then downloaded. Manual preparation was done with AutoDock v.4.2.6, which included the removal of water, deletion of all hetero atoms, assignment of atom types, addition of polar hydrogen atoms, merging of non-polar hydrogen atoms, and finally, the addition of Kollmann charges. Although docking programs account for flexibility of ligands, a remaining challenge is the flexibility of the entire protein. To minimize standard errors, the proteins were prepared as rigid structures (Trott & Olson, 2010). A pdbqt file of each of the target proteins were subsequently prepared with AutoDock Tools v.1.5.6 (Huey *et al.*, 2012). Hydrogen and partial charge in the molecular system were assigned using AMBER force field.

For the preparation of decoquinatone RMB041, its geometry force field was minimized using YASARA (<http://www.yasara.org/>) (Land & Humble, 2018), prior to manual preparation involving the addition of polar hydrogens atoms, merging of non-polar hydrogens atoms, and addition of Gasteiger charges. PDBQT files were saved for docking.

Grid box preparation and docking

File conversions (mol2 to pdb to pdbqt) required for the separate steps of docking were performed with the open-source toolbox Open Babel v. 2.3.2 (O'Boyle *et al.*, 2011). To ensure inclusion of all active sites, the entire macromolecule was selected as a search space for binding site cavities, with spacing's set at 0.90-1 Å, and saved as grid box parameters. Molecular docking scores of the compound to each protein were calculated by AutoDock

Vina v.1.1.2, for which a Lamarckian Genetic Algorithm was used, and expressed as free binding energies (ΔG kcal·mol⁻¹) (Sandeep *et al.*, 2011). Nine different orientations of decoquinatone RMB041 were searched per protein and ranked according to the binding free energies. To identify configurations that bind to active sites of their respective proteins, each binding pose was visually inspected in AutoDock, and those that interact with active site residues were saved as a pdbqt file and analyzed in DS, where the residues were then labeled. To validate interactions of decoquinatone RMB041 within active cavity sites, the target residues were compared to those of the original co-crystallized ligands, which, if PDB cavity site records were available, were either retrieved with DS Tools v.1.5.6 or obtained from previous literature. Cavity sites were provided with the name of the specific amino acid, the respective chain, and the number of the residues, e.g., Lys A:123.

6.3.3 Prioritization of protein targets

To identify proteins that are most likely essential during infection, i.e., important during the *M tuberculosis* transition to dormancy/latency, the structure of the compound was uploaded to the webserver TDR Targets (<https://tdrtargets.org>). TDR Targets, first introduced in 2008, has since been a reliable open-access resource for finding and prioritizing novel protein targets (Landaburu *et al.*, 2020). This webserver integrates genomes from EupathDB, GenBank, GenoList and Mycobrowser and isolates proteins based on assay space and literature reviews on essential proteins during survival during dormancy/latency (e.g., by lack of oxygen, lack of nutrients, ROS, acidity).

6.3.4 KEGG, GO, and network analysis

Annotations of the target proteins were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG)(<https://www.genome.jp/kegg/>). A literature search of the identified target proteins of both active and dormant *Mtb* was done. Further, to include up to date information on dormant *Mtb*, articles published within the past 5 years were selected with the exclusion of preprints, articles citing original articles, and studies on vaccinations and diagnostics.

6.3.5 Co-expression

Proteins that are negatively and positively correlated to any of the selected hit targets (Pearson correlation coefficient < -0.6 and > 0.6) (Akoglu, 2018), were obtained from TBDB (<http://tbdb.bu.edu/>). Proteins without known functions or assigned COG (Clusters of Orthologous Groups) categories were excluded. Target proteins were entered into STRING (<https://string-db.org/>), which provided a network with protein-protein interactions (medium confidence score of 0.4) in *Mtb* H37Rv.

6.4 RESULTS AND DISCUSSION

General molecular properties

Aside from the number of hydrogen acceptors and the number of atoms – the latter because pkCSM counts only heavy atoms, while DS counts all atoms of the molecule – in our study, SwissADME and DS agreed on all decoquinatate RMB041's molecular properties (Table 6-1).

Table 6-1: Molecular properties of decoquinatate RMB041, as provided by SwissADME and Discovery Studio.

Molecular properties	SwissADME	Discovery Studio
Canonical SMILES	<chem>CCCCCCCCCOc1cc2c(cc1OCC)n(CC)cc(c2=O)C(=O)NCCOC</chem>	-0
Formula	C28H44N2O6	C28H44N2O6
MW	504.66	504.67
#Atoms	36	80
TPSA	99.02	98.37
Molecular Refractivity	145.00	-
#Rotatable bonds	19	19
#H-bond acceptors	6	8
#H-bond donors	2	2

Likeness of oral administration

Pharmaceutical companies generally apply one or more regulations that assist in determining the drug likeness of oral administration, of which Lipinski's rule of five is the most common criteria (Mishra *et al.*, 2009). The number of violations of the rules of Lipinski, Veber, Egan, and Muegge, provided by SwissADME, along with the molecular properties breaching these rules, are presented in Table 6-2. With regards to the applied pharmaceutical regulations, only the molecular weight (MW) and the amount of rotatable bonds breach the rules. These broken rules do not necessarily indicate a lack of drug efficacy (Mullard, 2018). All in all, the results presented here indicate promising effectivity of decoquinatate RMB041 after oral administration.

Table 6-2: Number of violations of commonly applied pharmaceutical rules of drugability, as provided by SwissADME.

Pharmaceutical test	SwissADME	Rule violated	Reference
Lipinski #violations	1/5	MW > 500	Lipinski (2000)
Veber #violations	1/2	#Rotatable bond > 10	Veber <i>et al.</i> (2002)
Egan #violations	0/2	-	Egan <i>et al.</i> (2000)
Muegge #violations	2/8	MW > 300 #Rotatable bond > 15	Muegge <i>et al.</i> (2001)

Absorption, distribution, metabolism, elimination, and toxicity

In earlier studies, decoquinatone RMB041 ($\text{MIC}_{90} = 1.25 \mu\text{M}$) has shown to have promising bioavailability and distribution characteristics (Tanner *et al.*, 2019). Additional positive ADMET-related properties were also indicated by DS and prediction webserver used in this study. ADMET influencing factors from these tools and previous literature (Beteck *et al.*, 2018; Tanner *et al.*, 2019) are presented in Table 6-3.

Table 6-3: ADMET pharmacokinetic properties of decoquinatone RMB041, provided by computational prediction methods and previous literature.

Absorption	SwissADME	pkCSM	DiscoveryStudio	Previous literature
Lipophilicity	4.63 (iLogP)		4.80 (AlogP98)	4.90 (cLogP)
Aqueous Solubility (log mol/L)	-5.68	-5.82	-3.60	-
Solubility	Moderate	-	Good	-
Caco2 permeability (log P_{app})	-	0.662	-	-
GI absorption	High	82.14%	Moderate	-
Bioavailability	0.55	-	-	21%

Pgp substrate	No	Yes	-	-
Pgp I & II inhibitor	-	Yes	-	-
Distribution				
BBB permeant (log)	No	-0.741	Very low	-
CNS permeant (log)	-	-3.671	-	-
VD _{ss} (human)	0.32 log L/kg	-	-	-
Metabolism				
Plasma Protein Binding	-	-	No	-
Plasma Binding (Fu)	-	0.06 (human)	-	0.1 (mouse)
Microsomal Binding (Fu)	-	-	-	0.06 (mouse)
CYP1A2 inhibitor	No	No	-	-
CYP2C19 inhibitor	No	Yes	-	-
CYP2C9 inhibitor	No	Yes	-	-
CYP2D6 inhibitor	No	No	No	-
CYP3A4 inhibitor	Yes	Yes	-	-
Elimination				
CL _{int}	-	-	-	16 mL/min/kg
E _H	< 0.43	-	-	-
CL _{tot}	-	19 mL/min/kg	-	-
t _{1/2}	-	-	-	23.4h
Toxicity				
AMES toxicity	NP	No	Non-Mutagen	NP
Max tolerated dose	NP	799 mg/kg (Human)	90 mg/kg (Rat)	NP
hERG I & II inhibitor	NP	No	-	NP
Hepatotoxicity	NP	Yes	No	NP
Carcinogen (standard FDA test)	NP	NP	Non-carcinogen	NP
Aerobic Biodegradability	NP	NP	Degradable	NP

NP, none predicted; P_{app} , apparent permeability coefficient; GI, gastrointestinal; Pgp, P-glycoprotein; BBB; blood brain barrier; CNS, central nervous system; VD_{ss} , volume of distribution; Fu, fraction unbound; CL_{int} ,

intrinsic clearance; E_H , hepatic elimination; CL_{tot} , total clearance; $t_{1/2}$, half-life; AMES, assay of the ability of a chemical compound to induce mutations in DNA. Previous literature data are from Beteck (et al., 2018) and Tanner (et.al., 2019)

Considering all parameter related to decoquinatone RMB041 absorption, the high lipophilicity ($\text{LogP} = 4.63$ to 4.90 ; $\text{LogD} = 4.80$) of decoquinatone RMB041 in our investigation, suggests excellent permeation through the mycobacterial cell wall and into macrophages, and higher likelihood of being effective against dormant *Mtb* (Iacobino et al., 2017). Moderate to good aqueous solubility Table 6-3 indicates its practicality during drug formulation and gastrointestinal (GI) absorption (Bergström & Avdeef, 2019; Tihanyi & Vastag, 2011). A bioavailability score of 0.55 additionally confirms good absorption after oral administration (Martin YC 2005). However, the low Caco-2 cell permeability (human model for GI absorption; High when $\text{LogP}_{app} > 0.9$) indicates that further improvement of the solubility might be necessary. Another important influencer on absorbance is the amount of efflux from cellular tissue, especially by p-glycoprotein (Pgp). The contradictory results (SwissADME vs pkCSM) regarding the binding of decoquinatone RMB041 to Pgp Table 6-3 suggests further experimentation regarding this would be necessary. In either case however, whether it is not a substrate for Pgp (as predicted by pkCSM), or whether it is a substrate, and inhibits Pgp I and II, the results indicate a lack of export, meaning decoquinatone RMB041 is likely to accumulate in the target organs.

When considering the distribution criteria in this investigation, all three prediction methods state unlikely penetration of decoquinatone RMB041 through the blood-brain barrier (BBB) Table 6-3 (Das & Chakraborty, 2015). This may indicate a low probability of neurotoxic effects, such as confusion, depression, psychosis, or muscular weakness, which appear in response to various other current anti-TB medications (Bangert & Hasbun, 2019; Rana, 2013). It may also indicate however, that this compound would likely be of little use for the treatment of TB meningitis. Blood plasma volume of distribution of decoquinatone RMB041 ($\log V_{Dss} > 0.32$) Table 6-3 is on the higher end of the spectrum ($\log V_{Dss} < -0.15$ is low; $\log V_{Dss} > 0.45$ is high), suggesting moderate delivery to infected areas (Smith et al., 2015; Tanner et al., 2020).

Considering the metabolism of the decoquinatone RMB041, the cytochrome P450's, the most significant metabolizing enzymes to be considered during drug metabolism, oxidize xenobiotics to facilitate their excretion (Glue & Clement, 1999). The inhibition of these enzymes by an antibiotic indicates the likelihood of toxic accumulation, as well as possible interference with the pharmacokinetics of co-administered antibiotics. Despite differing conclusions about CYP2C9 and CYP2C19 inhibition by decoquinatone RMB041, pkCSM's and SwissADME's calculations presented in this study regarding the two main cytochrome isoforms, 2D6 and 3A4, are in agreement Table 6-3. CYP2C9 was recognized by PharmMapper as a potential protein target, supporting the results by pkCSM. Although no inhibition of CYP2D6 is expected, inhibition of CYP3A4 suggests that care should be taken when co-administering decoquinatone RMB041 with other antimycobacterial compounds, in order to prevent toxic accumulation of

either or both drugs.

When evaluating the elimination characteristics of decoquinatone RMB041 using the data generated by pkCSM, SwissADME and previous experimentation results by Tanner *et al.* (2019) and colleagues, we retrieved estimated values for the total clearance rate ($CL_{tot} = 19 \text{ mL/min/kg}$), intrinsic clearance (reflecting hepatic and biliary excretion) ($CL_{int} = 16 \text{ mL/min/kg}$), hepatic excretion ($E_H < 0.43$), and the elimination half-time ($t_{1/2} = 23.4 \text{ hours}$) Table 6-3. The $t_{1/2}$ is far superior to those of the other first-line drugs rifampicin ($t_{1/2} = 7 \text{ hours}$) (Ji *et al.*, 1993), isoniazid ($t_{1/2} = 1.7 \text{ hours}$) (Jayaram *et al.*, 2004), and ethambutol ($t_{1/2} = 3 \text{ hours}$) (Arbex *et al.*, 2010), and indicates less frequent drug administration would be required, which is beneficial for patient coherence and possibly decreasing the consequential occurrence of drug resistance.

Decoquinatone RMB041 has previously shown little cytotoxicity against WI-38 human fetal lung fibroblasts ($IC_{50} = 56.2 \text{ }\mu\text{M}$) (Beteck *et al.*, 2018). According to pkCSM and DS results in this study, this compound is neither carcinogenic, nor mutagenic, nor likely to interfere with heart rhythms (Table 6-3). Furthermore, the odds of having neurotoxic and hepatotoxic properties are also low. The positive hepatotoxicity by pkCSM is only based on the similarity of structural features to compounds with liver-associated adverse effects, whereas DS, which showed no possible hepatotoxicity, uses additional information related to dose concentrations to establish the probability of hepatotoxicity. Furthermore, our investigation indicated that decoquinatone RMB041 shows a promising safety profile compared to that of other first- and second line antitubercular drugs which exhibit cardiotoxic, hepatotoxic and/or neurotoxic effects (Bangert & Hasbun, 2019; Kwon *et al.*, 2020; Lee & Nguyen, 2020; Ray *et al.*, 2017). That said, the contradictory results by pkCSM and DS concerning hepatotoxicity necessitate further experimentation. Aerobic biodegradability, a trait that is often underestimated and missed during drug development, is important for the prevention of wastewater pollution, which would else cause serious harm to the aquatic ecosystems and increase antibiotic resistance in humans (Polianciuc *et al.*, 2020). The degradation of this compound therefore suggests that it is safe in the face of environmental pollution also, just as a matter of interest.

Identification and prioritization of drug targets.

It is widely known that drugs commonly target several proteins (as opposed to only one particular protein) (Hopkins, 2007). PharmMapper, as used in this study, compares the pharmacophores of the investigated drug compound, to those derived from ligands in complex crystal-structures and provides predicted corresponding protein targets (Wang *et al.*, 2017). A total of twelve *Mtb* proteins were identified by PharmMapper as potential targets of decoquinatone RMB041. These are listed according to their docking scores in Table 6-4, along with their respective PharmMapper fit scores, UniProt and PDB accession codes, and associated KEGG reactions. A limitation one should keep in mind about current computer-aided drug design,

is that only the known active sites in databases can be used for identifying suitable ligand scaffolds. Further research on active sites of proteins would increase the accuracy of the elucidated mechanism of action, which will undoubtedly improve as more data becomes available regarding this.

The docking affinities calculated by AutoDock Vina ranged between -7.5 to -5.3 kcal/mol and indicated strong binding to the target proteins Table 6-4. Several other studies have ranked targets by their importance in *Mtb*, focusing mainly on drugability and essentiality *in vitro* survival (Anand & Chandra, 2014; Melak & Gakkhar, 2015). However, it is also important to consider the various conditions that *Mtb* are exposed to in the host during infection or disease, such as acidity, reactive oxygen species, nutrient restriction, and various antibiotics in the case of a treated TB patient. During previous genomic, proteomic, and metabolomic investigations of *Mtb* exposed to each of the aforementioned conditions, it has become apparent that, although the initial response mechanism may differ, *Mtb* resilience ultimately depends on its ability to survive in a non-replicative/latent TB state, which is the case in most individuals infected with TB, which is for one third of the global population currently (WHO, 2020), and hence, it would be of value to investigate targets that would shorten the treatment duration of latent *Mtb*. One should also consider the neighboring network of the target gene/protein, i.e., targeting a gene/protein associated/interacting with many other gene/proteins might prove more successful than targeting one with fewer interacting genes/proteins. Lastly, the exclusivity of a target gene/protein to *Mtb*, is also an extremely sought after characteristic in drug design, and additionally, if other genes/proteins within in *Mtb* have a similar function, its less likely to disarray essential cellular processes.

In order to predict the decoquinatone RMB041's drug targets, we determined their ranking by TDR Targets, which, with the help of integrated databases, allows users to prioritize genes and their annotated proteins based on various filtering criteria and criteria-specific weighting (Landaburu *et al.*, 2020). We filtered non-human homologs and looked at three ranking orders: (1) with respect to homology to gut flora, similarity to essential genes, and maintaining of persistence (Hasan *et al.*, 2006), (2) upregulation of genes during dormancy and transition to dormancy (Murphy & Brown, 2007), and (3) ortholog-based inference of essentiality of genes during experimental conditions relevant to drug discovery. Based on the first ranking order, 313 protein targets annotated to highly prioritized genes were provided, of which only one, Cyp130, was also identified by PharmMapper as a target of decoquinatone RMB041. The second-ranking order provided a list of 420 proteins, of which only one, BlaI, is a decoquinatone RMB041 target. According to the third-ranking criteria, both Cyp130 and BlaI (2 out of 397 proteins) are essential targets of decoquinatone RMB041. *FolP1* and *lysA* were also number one and number three, respectively, on a list of 4000 genes that were ranked based on their metabolic uniqueness in a study by Hasan *et al.* (2006), further indicating the importance of their encoded proteins.

Table 6-4: The targets identified by PharmMapper, along with their respective identification codes, fit scores, binding free energies, and residues that interact with both, their respective co-crystallized ligands and decoquinate RMB041.

Target protein	PDB ID	UniProt ID	Fit score	Binding free energy (kcal/mol)	Overlapping residues
Cyp130 (Rv1256c)	2UVN	P9WPN5	4.36	-7.5	Leu A:71, Pro A:87, Pro A:88, Phe A:236, Thr A:239, Met A:240, Thr A:247, Pro A:289, Val A:290, Phe A:347, Cys A:354, Leu A:355, Gly A:356, Ala A:359, Ala A:360, Val A:393
FbpB (Rv1886)	1F0N	P9WQP1	4.30	-7.4	Asp A:40, Leu A:42, Arg A:43, Ser A:126, Leu A:152, Leu A:163, Leu A:229, Phe A:232, His A:262, Trp A:264, Trp A:267
LysA (Rv1293)	2O0T	P9WIU7	5.70	-7.0	Cys A:375, Glu A:376, Ser A:377, His B:213, Arg A:303, Tyr B:405
AdoK (Rv2202c)	2PKF	P9WID5	4.24	-6.9	Val A:49, Gln A:172, Asn A:195, Thr A:223, Val A:255, Asp A:257, Phe A:259, Ser A:281, Leu A:288
SecA1 (Rv3240)	1NKT	P9WGP5	4.90	-6.6	Gln A:80, Phe A:83, Gln A:86, Lys A:107, Leu A:109, Arg A:137, Trp A:141, Asp A:493, Asn A:499, Asp A:501, Arg A:573
GlnA1 (Rv2220)	2bvc	P9WN39	4.71	-6.5	Glu A:133, Glu A:214, Lys A:215, Glu A:227, His A:276, His A:278, Arg A:347, Arg A:352, Arg A:364, Glu A:366, Arg A:368

LpdA (Rv3303c)	1XDI	P9WHH7	4.11	-6.3	Cys A:48, Lys A:52, Tyr A:450
LppX (Rv2945)	2BYO	P9WK65	4.75	-5.9	Val A:45, Leu A:54, Leu A:55, Ile A:57, Ala A:60, Phe A:85, Ile A:92, Ile A:106, Leu A:109, Ser A:110, Arg A:113, Met A:158
GlcB (Rv1837c)	1N8W	P9WK17	6.48	-5.9	Leu A:117, Val A:118, Val A:119, Pro A:120, Phe A:126, Asn A:129, Ala A:130, Ser A:275, Arg A:312, Pro A:543, Ser A:544, Pro A:545, Cys A:619, Ser A:620, Lys A:621, Met A:631, Phe B:310
FabD (Rv2243)	2QC3	P9WNG5	4.46	-5.5	His A:90, Ser A:91, Asn A:155
BlaI (Rv1846c)	2G9W	P9WMJ5	4.63	-5.3	Lys B:3, Arg A:6, Arg B:6
FolP1 (Rv3608c)	1EYE	P9WND1	4.45	-5.3	Asp A:21, Gly A:181, Phe A:182, Lys A:213

Cellular mechanisms. In this study, the KEGG database BRITE hierarchy files annotated ten of the twelve targets, of which all are involved in *Mtb* energy metabolism, protein synthesis, fatty acid synthesis, cell wall synthesis, TCA cycle activity, and/or amino acid synthesis. All of the aforementioned pathways were found to be altered in our previous study (Knoll *et al.*, 2021), possibly indicating the imbuing of adaptations by *Mtb* in attempt to counteract or adapt to the disruption of the primary targets identified in this study. Correlations of *Mtb* genes have been extensively studied and are still barely understood. Aside from the very many genes in *M. tuberculosis* (Papakonstantinou *et al.*, 2021), the complexity of interdependent intracellular genetic expressions makes it almost impossible to predict the complete cellular response to external stimuli accurately. For instance, even if the expressed proteins show a strong positive correlation to each other, it is impossible to tell if the inhibition of one would lead to the inhibition of the other, without extensive experimentation of each interaction separately. However, it can indicate the metabolic pathways that are likely to be altered in the inhibition process and give information on which drugs might work well in conjunction with decoquinatone RMB041. With help of

STRING and TBDB, in this study we identified protein targets and analyzed direct correlations between these (Figure 6-3). TBDB links *Mtb* gene-expression microarray data to their encoded proteins and provides the strength of the correlation between proteins (Galagan *et al.*, 2010). Of a total of 144 correlated proteins, only ten were negatively correlated. If one were to assume that inhibition of the protein targets would influence the expression of the correlated proteins, it would mean that 134 other proteins might be expressed to a lesser degree, while ten proteins might be expressed to a larger extent.

Beteck, Seldon (13) and colleagues established that decoquinatone RMB041 primarily acts on the cell wall and, secondly, inhibits DNA synthesis. Our findings during an earlier metabolomic investigation confirmed this and also suggested that decoquinatone RMB041 inhibits protein synthesis, and pathways associated with *Mtb* in the non-replicating state (Knoll *et al.*, 2021). In the current study, we examined each drug target to better understand the perturbations caused by decoquinatone RMB041 and explore the cellular response by which *Mtb* counteracts these, such as those leading to the metabolomic changes revealed in our previous investigation and will be discussed below.

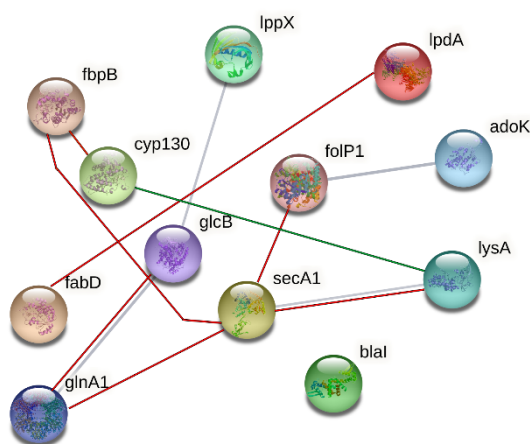


Figure 6-3: Interconnected network provided by STRING (grey), with additional interactions retrieved from TBDB; Positively correlated interactions (red) and negatively correlated interactions (green).

BlaI controls the expression of the Rv1864c regulon, which comprises genes involved in drug transport, detoxification, and ATP synthesis (Mishra *et al.*, 2017). Dissociation of this enzyme from its operator site allows the transcription of the genes belonging to this regulon, including *blaI*, encoding itself, and *blaC*, encoding beta-lactamase (BlaC) (Sala *et al.*, 2009), which causes resistance to beta-lactams (Black *et al.*, 2014). Depending on the ratio of BlaI inhibition versus *blaI* transcription and the mycobacterial degradation of the inhibitor, it could either enhance efflux, ATP synthesis and tolerance to stress, or repress the regulon, leading to opposite effects. Either way, it would disrupt the NAD(P)H: NAD(P)⁺ ratio. Considering that previous studies indicated elevated NAD(P)⁺ in the presence of decoquinatone RMB041 (Knoll *et al.*, 2021), it is tempting to assume that hydrogen ions are lacking, meaning ATP synthase is inhibited, of which one could deduct that the entire regulon is being

repressed. Considering that TDR Targets also deduces its answers based on orthologous experimental results of studies on other bacteria, such as *Eschericia coli* or staphylococci, the prioritization of BlaI as a drug target is a promising outcome. Nonetheless, further experimentation would bring clarity to what the consequences of BlaI inhibition would be. The DNA binding site of BlaI shares three residues with that of decoquinatone RMB041 (based on the AutoDock Vina results), and the interaction between the drug and BlaI involves five strong hydrogen bonds.

Cyp130 is one of twenty cytochrome P450 enzymes responsible for the incorporation/reduction of molecular oxygen, and functions in conjunction with Tap (Rv1258c), for the export of drug molecules (Podust *et al.*, 2009). Furthermore, it is well known that Cyp130 is important for growth and virulence of *Mtb*, and the inhibition of Cyp130, effective against MDR- *Mtb* strains (49). In the aforementioned study, Cyp130 was also indicated with a high priority as an anti-tuberculous target in two of the selected TDR Target ranking orders, and reportedly inhibited by a drug group called azoles, with econazole showing the most potent activity (49). Interestingly, our study shows that the binding cavity of econazole includes sixteen residues that also interact with decoquinatone RMB041 (Ouellet *et al.*, 2008) (Table 6-4; Figure 6-4). Cyp130 shows the highest comparative binding affinity to decoquinatone RMB041, mostly via hydrophobic interactions and two strong hydrogen bonds.

LpdA is a NAD(P)H-requiring flavoprotein disulfide reductase that has been found to be significantly upregulated during the transition of *M. tuberculosis* towards a state of dormancy (Argyrou *et al.*, 2004; Bacon *et al.*, 2014). In this investigation we see, within the active cavity of LpdA, bound to both, FAD and NADP⁺, three residues also interact with decoquinatone RMB041 (Figure 6-4). Inhibition of this enzyme would prevent the conversion from 2,6-dimethyl-1,4-benzoquinone to 5-hydroxy-1,4-naphraquinone and a subsequent accumulation of NAD(P)H. Elevated NAD(P)H levels in turn, would induce counteractive pathways, for instance the NAD(P)H dependent production of α -hydroxyglutaric acid, fatty acids, and sugar alcohols. All three pathways have been found to be altered in our previous metabolomics study (Knoll *et al.*, 2021), further supporting decoquinatone RMB041's inhibition of LpdA and, accordingly, a promising activity against dormant/latent *Mtb*.

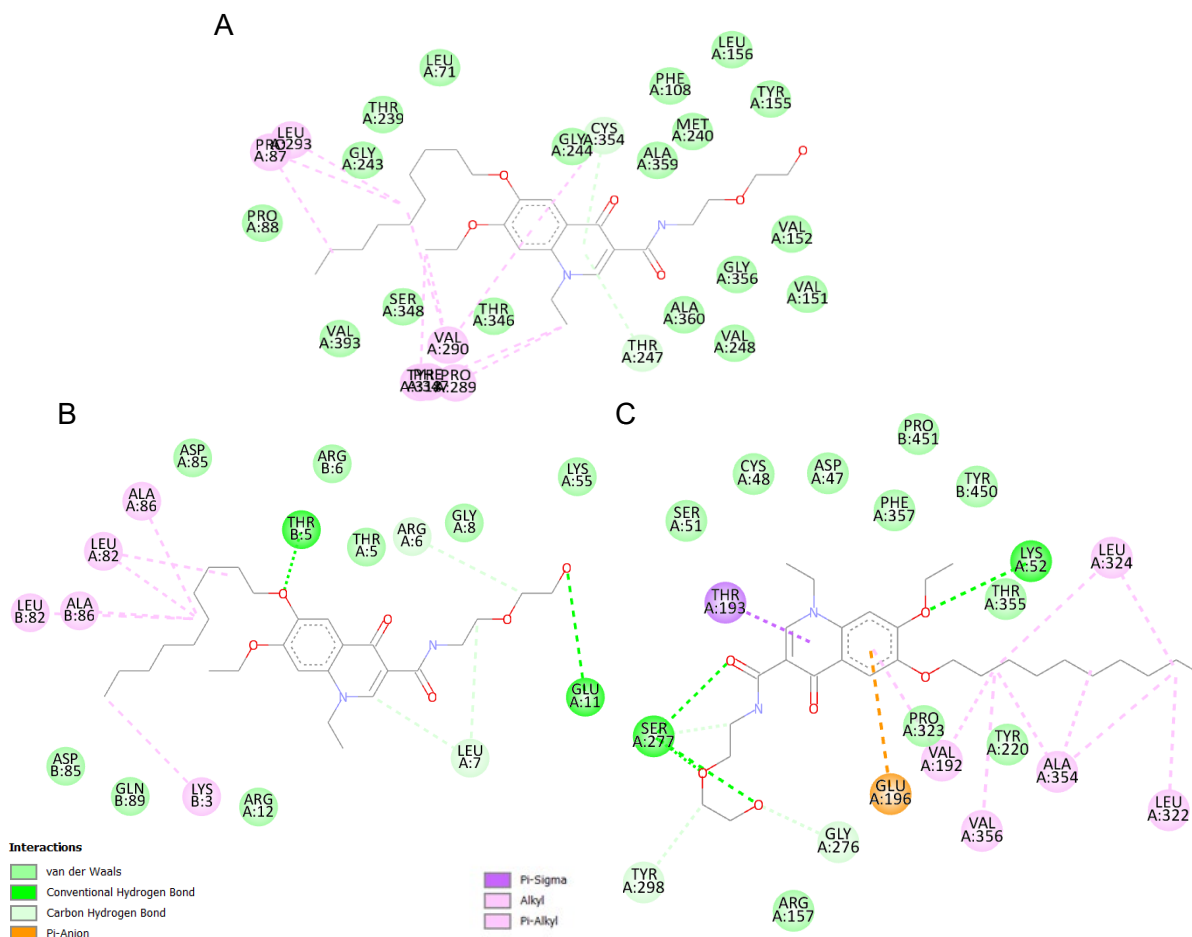


Figure 6-4: Interactions between (A) Cyp130 and decoquinatone RMB041, (C) BlaI and decoquinatone RMB041, and (D) LpdA and decoquinatone RMB041.

Two of the identified target proteins in our investigation, SecA1 and LppX (Figure 6-5), are transmembrane transporters. SecA1 is ATPase coupled and responsible for the transport of the majority of proteins, including those suppressing phagocyte maturation (Zhai *et al.*, 2019). Its previously demonstrated active site, when bound with adenosine diphosphate (ADP)- β -S, shares eleven residues with the binding site of decoquinatone RMB041 (Sharma *et al.*, 2003). Inhibition of SecA1 results in an elevated ATP:ADP ratio, i.e., which would disrupt intracellular energy metabolism, in addition to prevent the export of proteins required for cell wall biosynthesis (Duong, 2003; Hou *et al.*, 2008). The assimilation of these proteins would in turn induce protein degradation, especially during nutrient starvation (Muller & Weber-Ban, 2019), to provide proteinogenic amino acids as a source of nitrogen and carbon. This response is supported in our previous metabolomics study, which indicated elevated levels of proteinogenic amino acids in decoquinatone RMB041 treated *Mtb* (Knoll *et al.*, 2021). SecA1 has been referred to as an optimal co-target, with “co-target” defined as a protein whose inhibition, in addition to that of the primary target, would hinder the development of resistance (Raman & Chandra, 2008). The other

transmembrane transporter, LppX, functions by carrying lipophilic molecules across the mycobacterial membrane. Its encoding gene, *lppX*, is upregulated during host-infection (Schwab *et al.*, 2009) and enhances the bacilli's capability to escape the host's immune response (Sulzenbacher *et al.*, 2006). Decoquinatone RMB041 and vaccenic acid, share nineteen residues within the active cavity site with which they interact (Sulzenbacher *et al.*, 2006). Compared to vaccenic acid, decoquinatone RMB041 forms several more hydrophobic interactions, indicating a stronger competition. Inhibition of LppX, would likely cause accumulation of fatty acids, confirmed by the drastically elevated levels of these compounds in our previous metabolomics study (Nicoara *et al.*, 2013).

FabD, the malonyl transacylase, is the first of five enzymes (*fabD*, *acpM*, *kasA*, *kasB*, and *accD6*) responsible for fatty acid elongation (Crellin *et al.*, 2013; Slayden & Barry, 2002). The role of the encoding gene, *fabD*, during the stress response is unclear, as it was underrepresented after exposure to rifampicin (Meneguello *et al.*, 2020), upregulated in the presence of isoniazid (Jagadeb *et al.*, 2019), and overexpressed after the removal of a stressor (Nieto *et al.*, 2017). Nonetheless, it has frequently been associated with drug resistance (Jagadeb *et al.*, 2019; Kumari *et al.*, 2013), indicating its importance for survival after exposure to antibiotics. FabD is a target of the Pup-proteasome degradation (Festa *et al.*, 2010), which could mean that the protein is readily marked for destruction, followed by the regeneration of mutated FabD. When FabD is inhibited, malonyl-CoA accumulates (12), and also preferentially used as a substrate for mycolic acid synthesis, an important cell wall component for survival during dormancy (Chakraborty & Kumar, 2019). Despite this however, no shortage of elongated fatty acids occurs, indicating the existence of similarly functional proteins, and/or the break-down of cell wall components into fatty acids, that are considered less vital for survival under conditions of stress, which in this case were trehalose dimycolates. This enzyme's lack in unique functionality, would also explain why it was not considered as an important target by TDR Targets. The active site of the original crystal structure of FabD contains acetic acid (Li *et al.*, 2007), of which three interactive residues also bond with decoquinatone RMB041 (Figure 6-5), as indicated in this investigation. FabD is positively correlated to KasA, an enzyme involved in the inhibitory activity of isoniazid on fatty acid elongation, suggesting possible synergistic activity of decoquinatone RMB041 with isoniazid.

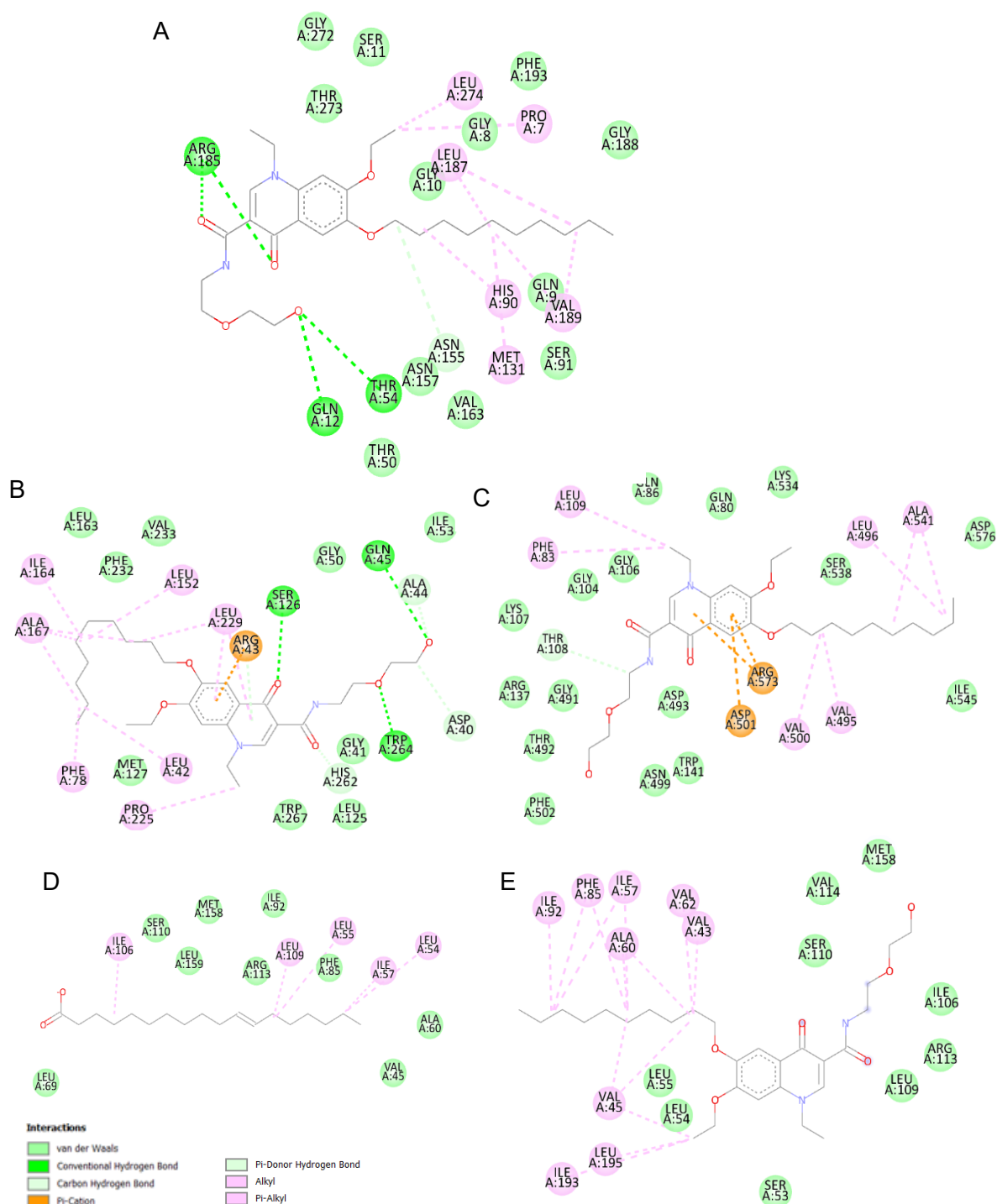


Figure 6-5: Interactions between decoquinone RMB041 and (A) *FabD*, (B) *FbpB* and (C) *SecA1*, as well as those between *LppX* and (D) vaccenic acid and (E) decoquinone RMB041.

FbpB, also known as antigen 85B, catalyzes the biogenesis of trehalose dimycolates (TDM) (catalo 19) and is the most abundant of all mycoyl transferases. TDM accumulate outside the cell wall and desensitize the cell to antibiotics (Trutneva *et al.*, 2020). *FbpB* is overexpressed during early infection of macrophages, suggesting

it to be important for the shift to *Mtb* dormancy/latency (Wilkinson *et al.*, 2001). Inhibition of FbpB would result in the accumulation of TDM, followed by its break-down into mycolic acids, which can either be used to synthesize other cell wall components or be broken down further into long-chain fatty acids. Although FbpB is highly expressed during early infection of macrophages (Anderson *et al.*, 2001), its encoding gene, *fbpB*, has been seen to be downregulated during hypoxia (Betts *et al.*, 2002), indicating little importance of FbpB during dormancy. Nonetheless, FbpB inhibition might serve useful to prevent desensitization of co-administered antimycobacterial agents. Within this study, we found that the binding of decoquinatone RMB041 to FbpB (Figure 6-5) is verified by eleven interacting residues that also non-covalently bind to trehalose (Anderson *et al.*, 2001). Interactions between decoquinatone RMB041 and FbpB, showing the second highest binding affinity, include six strong hydrogen bonds.

Malate synthase (GlcB) is responsible for the synthesis of malic acid via the glyoxylic shunt, a key pathway during *Mtb* dormancy, that incorporates even chain fatty acids into the TCA cycle (Huang *et al.*, 2016). GlcB upregulates the dormancy regulator (DosR) regulon and increases tolerance to stress (Hamidieh *et al.*, 2021). Inhibition of GlcB in this investigation would explain the accumulation of the even-chain fatty acids shown in our previous metabolomics study on decoquinatone RMB041 treated *Mtb*, although levels of malic acid were increased, likely due to the incorporation of proteinogenic amino acids (Knoll *et al.*, 2021). Although GlcB was noted as necessary during *Mtb* dormancy/latency, its absence in the prioritizing lists of TDR Targets in our study indicates that *Mtb* possesses several enzymes controlling malic acid. The latter could be expected, considering that it is a primary element within the central carbon flux. Furthermore, our results indicate GlcB's natural ligand, acetyl coenzyme A, shares seventeen interacting residues with decoquinatone RMB041 (Figure 6-6) (Smith *et al.*, 2003). According to the TBDB results, GlcB is positively correlated to rifampicin targets, RpoB and RpoC, suggesting possible synergistic activity of this drug with decoquinatone RMB041.

Meso-diaminopimelate decarboxylase (LysA) catalyzes the final step of lysine biosynthesis. Its encoding gene, *lysA*, is regulated by LysR, which is repressed in the presence of lysine (Rodionov *et al.*, 2003). Upregulation of *lysA* has been noted in immunocompromised mice (23), and the highly conserved nature of this gene indicates that it is mainly associated with a *Mtb* stress responses (Papakonstantinou *et al.*, 2021). Under normal circumstances, inhibition of LysA would lower lysine levels and LysR would keep inducing *lysA* expression, creating a metabolic loop and preventing protein synthesis. However, concurrent protein degradation would provide sufficient lysine for the organism, as shown in our previous metabolomics study, which would in turn repress LysR. In our study, inhibition of LysA would, in this case, not provide much assistance in eliminating mycobacteria. The targeting of this enzyme is supported by six residues that interact with both

decoquinatone RMB041 and lysine (Figure 6-6) (Gokulan *et al.*, 2003).

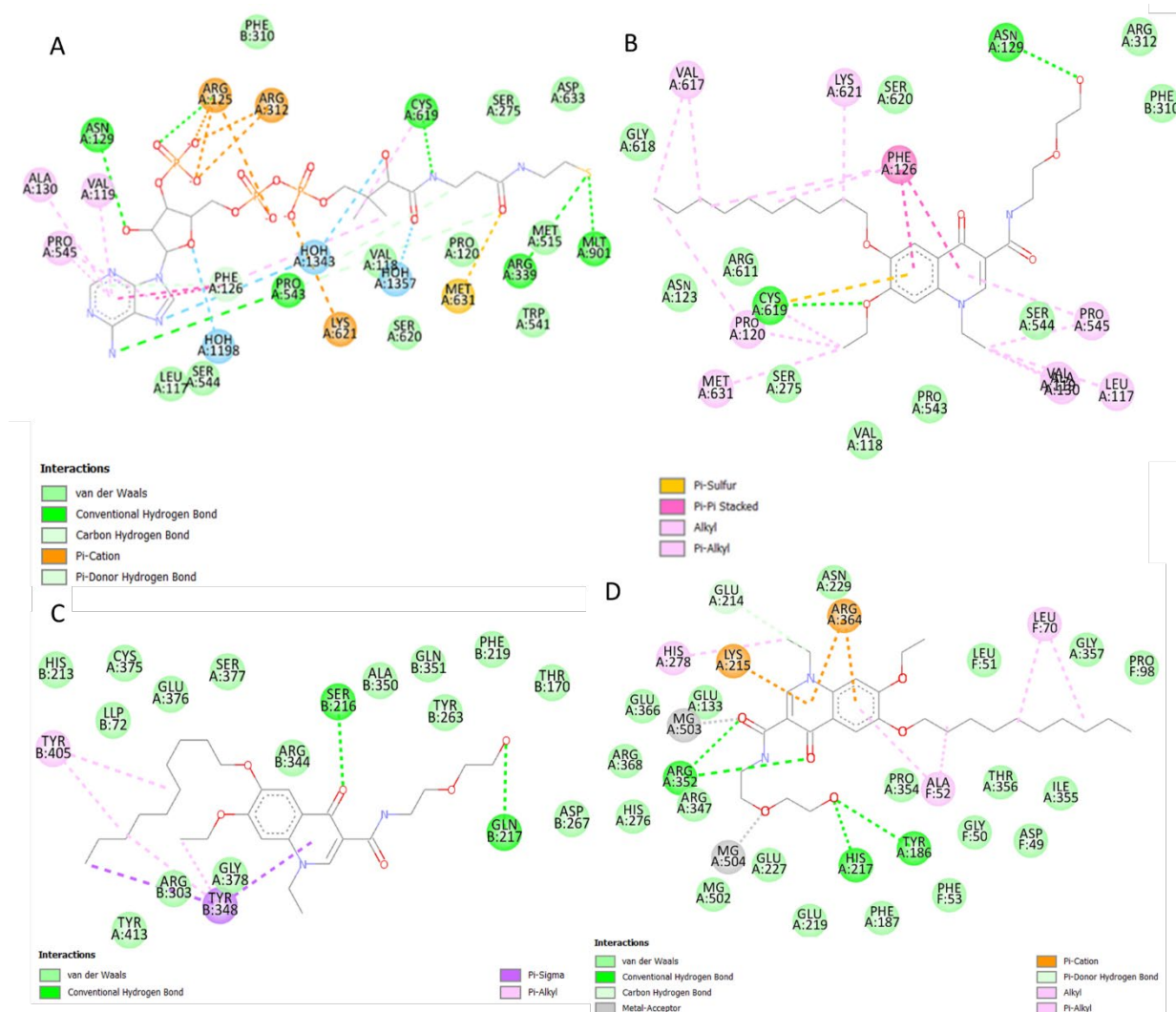


Figure 6-6: Interactions between (A) GlcB and acetyl-coenzyme-A, (B) GlcB and decoquinatone RMB041, (C) LysA and decoquinatone RMB041, and (D) GlnA1 and decoquinatone RMB041.

Glutamine synthetase (GlnA1) is a key enzyme used during nitrogen metabolism and cell wall synthesis (Moreira *et al.*, 2016). It catalyzes ATP-dependent ammonium condensation with glutamate to form glutamine, adenosine diphosphate, and phosphate. The crystal structure of GlnA1 was investigated in a complex with methionine sulfoximine phosphate, a product of methionine sulfoximine phosphorylation, and includes eleven residues in its active binding site (Figure 6-6) (Krajewski *et al.*, 2005). As indicated by our study, these residues also appear in the binding pocket of decoquinatone RMB041 (Figure 6-6). Inhibition of this enzyme would explain the previously identified elevation of glutamate levels (Knoll *et al.*, 2021). GlnA1 has been proposed as a promising anti-TB target with high importance during dormancy (Agapova *et al.*, 2019; Trutneva *et al.*, 2020) and mycobacterial growth in macrophages (Tullius *et al.*, 2003). According to the TBDB results, GlnA1 is

positively correlated to four proteins that are targeted by other anti-TB drugs. RpsL, EmbA, RpsA, and AtpE are drug targets of streptomycin (protein synthesis inhibitor), ethambutol (mycolic acid transfer inhibitor), pyrazinamide (fatty acid synthesis inhibitor), and bedaquiline (ATP synthase inhibitor), respectively. Hence, further experimentation of decoquinatone RMB041 with one or more of these drugs might prove synergistic activity against *Mtb*.

Adenosine kinase (AdoK) catalyzes the phosphorylation of adenosine to adenosine monophosphate (AMP). This enzyme participates in the purine salvage pathway and plays a crucial role in nucleotide synthesis during *Mtb* persistence (Long *et al.*, 2003). The active binding site was previously illustrated with adenosine (Reddy *et al.*, 2007), and involves nine residues that also interact with decoquinatone RMB041 (Figure 6-7), as indicated in our investigation. Inhibition of this enzyme would be expected to prevent the formation of DNA during the shift to dormancy (as opposed to acutely disrupt DNA synthesis when administered). This “belated” disruption of DNA formation by decoquinatone RMB0041, was also confirmed in two previous studies (Beteck *et al.*, 2018; Knoll *et al.*, 2021).

Inhibition of folic acid synthase (FolP1) would also lead to a disrupted DNA synthesis in *Mtb*, as proven by previous experiments on mycobacteria in the presence of sulfamethoxazole and trimethoprim (Liu *et al.*, 2015). FolP1 catalyzes the synthesis of the 6-hydroxymethyl-dihydropteroate and pyrophosphate, from the substrates 6-hydroxymethyl-7,8-dihydropterin-pyrophosphate and *para*-aminobenzoic acid, and has previously been proposed to be a promising drug target against *Mtb* (Liu *et al.*, 2015). Its encoding gene, *folP1*, is upregulated during the early and late dormancy (Bacon *et al.*, 2014), indicating its importance in maintaining *Mtb* during the non-replicative state. In our study, the crystal structure of FolP1 in complex with 6-hydroxymethylpterin monophosphate (Baca *et al.*, 2000) shares four interacting residues with decoquinatone RMB041 (Figure 6-7).

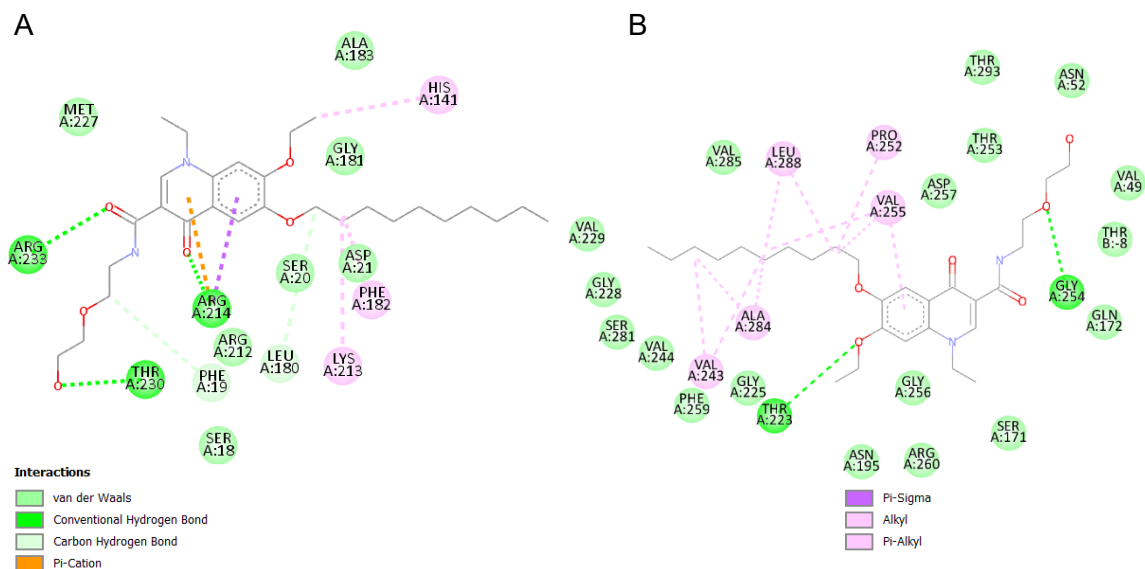


Figure 6-7: Interactions between decoquinatone RMB041 and (A) AdoK and (B) FolP1.

6.5 CONCLUSION

Various *in silico* drug discovery strategies have been implemented during this study to identify potential drug targets for decoquinatone RMB041 against *Mtb*. Furthermore, we generate additional support for the previously determined metabolic pathways that were disrupted by decoquinatone RMB041 and indicate at which enzymes the particular disruptions may occur. In addition, feasible synergistic activity of decoquinatone RMB041 with other antimicrobials was shown. In this study, we also elaborated on the possible activity of decoquinatone RMB041 against dormant *Mtb* and identified the drug targets for such. Lastly, the importance of including *in silico* drug discovery strategies and how they can be used as a complementary tool to other research approaches (*in vivo* and *in vitro* techniques) is highlighted. Additional wet-lab experiments determining the effectivity of decoquinatone RMB041 against various clinical isolates and other resistant strains of *Mtb* would strengthen our knowledge of the scope of efficacy of this antitubercular drug.

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CHAPTER 7: CONCLUSION AND FINAL REMARKS

7.1 INTRODUCTION

The history of *Mtb*, anti-TB drugs, metabolomics, and its role in finding solutions towards combating TB (Chapter 2), the metabolomic methods used to identify metabolite markers characterizing the changes induced by ciprofloxacin and decoquinatone RMB041 to *Mtb* (Chapter 3), the interpretation of these to better describe the antimycobacterial MoA of these two drugs (Chapter 4, 5, and 6), and the safety profile / pharmacokinetic properties of decoquinatone RMB041 (Chapter 6), have been thoroughly discussed in this thesis. This final chapter will discuss the practicality of a metabolomic GCxGC-TOFMS approach for the purpose of elucidating the antimycobacterial mechanisms of anti-TB drugs, discuss the MoA of ciprofloxacin and decoquinatone derivative RMB041 comparatively, and make suggestions for future research.

7.2 CONCLUSION

The differences between the metabolome of untreated *Mtb*, compared to that of *Mtb* treated with ciprofloxacin and decoquinatone derivative RMB041, respectively, were explored using a metabolomic GCxGC-TOFMS technique and validated statistical methods. This approach successfully measured the metabolic changes resulting from all up-stream mechanisms (transcriptional, proteomic, and genetic) affected by the two respective drugs, and provided an arguably accurate representation of the *Mtb* phenotype in the presence of these drugs, since many of our findings are supported by previous genomics, proteomics, and transcriptomics data.

The metabolomic analysis of *Mtb* treated with ciprofloxacin (Chapter 4) and decoquinatone RMB041 (Chapter 5) revealed several significantly altered pathways, summarized in Table 7-1 below. The results in Chapter 4 supports previous studies of the MoA of ciprofloxacin, which reported changes in transcription, translation, and cell wall synthesis. This study strengthens previous reports on the involvement of an altered polyamine synthesis pathway (contributing to drug resistance to FQs), and identified additionally altered pathways associated with the SOS response, elevated FA synthesis as a substrate for energy and cell wall repair, alterations in the cell wall structure, activated TAG synthesis, inhibited protein synthesis, and altered energy metabolism.

The metabolomic changes discussed in Chapter 5, confirmed previous observations of the MoA of decoquinatone RMB041 on the *Mtb* cell wall (as primary target) and DNA (as secondary target) inhibition.

Table 7-1: Comparison of the altered pathways identified in ciprofloxacin and decoquinatone RMB041 treated *Mycobacterium tuberculosis*. Differences are indicated in bold.

ALTERED PATHWAY	CIPROFLOXACIN TREATED <i>MTB</i>	DECOQUINATE RMB041 TREATED <i>MTB</i>
Pentose phosphate pathway	↑	↓
Glycolysis	↓	↓
Lipid storage (TAG) metabolism	↑	-
Cell envelope metabolism	↑	↑
Fatty acid metabolism	↑	↑
Nucleotide synthesis	↑	↓
Protein synthesis	↓	↓
Glyoxylate shunt	↓	↑
Urea cycle	↑	↑
Antioxidant defence	↑	↑

Comparatively, both the ciprofloxacin and decoquinatone RMB041 treated *Mtb* groups showed a response to drug induced oxidative stress and a subsequent SOS response-induced transition to the dormant phase. The metabolomic differences between the two groups observed in Table 7-1, appear to be because of the lacking DNA repair capacity in the *Mtb* treated with decoquinatone RMB041, compared to activated DNA repair mechanisms induced in response to ciprofloxacin in *Mtb*. As mentioned in Chapter 4, *Mtb* treated with ciprofloxacin displays an upregulated synthesis of fatty acids in order to fuel the upregulated PPP and gluconeogenesis for subsequent nucleotide synthesis for DNA repair, and additionally for energy production and cell wall repair. Furthermore, the metabolomics results also elucidated a possible mechanism of *Mtb* resistance to the ciprofloxacin treatment. The direct activity of decoquinatone RMB041 on DNA would also most likely result in an attempt towards DNA repair, as was previously indicated by reduced sugar alcohols in *Mtb* treated with ciprofloxacin, accompanied by elevated nucleotide synthesis. In the decoquinatone RMB041 treated group however, we saw an accumulation of both amino acids and sugar alcohols, and a subsequent reduction in nucleotide synthesis, suggesting DNA repair is inhibited or repair mechanisms have not yet been activated at the time the cells were harvested. This may however still possibly occur at a later stage, or at higher decoquinatone RMB041 concentrations, but in the present study, the *Mtb* treated with decoquinatone RMB041, are most likely in a more pronounced non-replicative state after the irreversible cell envelope damage, which is the primary target of the decoquinatone derivative RMB041 as explained in Chapter 5. Further support for a higher state of dormancy in the decoquinatone RMB041 treated *Mtb*, is the more pronounced inhibition of protein synthesis and metabolism associated with inhibition of cell growth / replication.

Similar to that seen with the decoquinatone RMB041 and ciprofloxacin, all first-line anti-TB drugs induce pathways associated with the transition to dormant *Mtb*. It seems reasonable to presume that, regardless of the

primary target or complicated upstream mechanisms, the resistance of *Mtb* to anti-TB drugs ultimately depends on its ability to survive in quiescence, while attempting to alter the affected pathways and adapting upstream proteins and genes.

The results of our *in silico* study (Chapter 6.) support the previously proposed MoA of decoquinatone RMB041 and indicate the target proteins / genes involved. As described in Chapter 6, the consequences of interference with these targets also concur with the metabolic perturbations identified in the metabolomics investigation of Chapter 5. Two of these target proteins, Cyp130 and BlaI, are highly associated with the inhibition of dormant *Mtb*. This, and the seemingly irreversible damage on DNA (Chapter 5.), indicate the potential of decoquinatone RMB041, in combination with other antimycobacterial drugs, in order to eradicate TB.

According to various ADMET docking approaches, decoquinatone RMB041 also exhibits an impressive safety profile, which, along with the long $t_{1/2}$, benefits patient compliance and consequently resistance development.

7.3 FUTURE RESEARCH PROSPECTS

The variant used in this study was selected due to its common use in *Mtb* susceptibility studies and does not represent resistant strains. Therefore, the conclusions are not necessarily applicable to circulating strains. This study highlights the need for selecting and investigating a few highly promising compounds with good safety profiles and potent activity on resistant / quiescent *Mtb*. *In vitro* investigations of decoquinatone RMB041-induced inhibition of Cyp130 and BlaI, in dormant *Mtb* cells, would further confirm the reliability of *in silico* results of this study. Further investigations of this compound, in combination with drugs that target pathways that are upregulated during the shift dormancy, such as PZA, would also be useful to establish its efficacy on quiescent *Mtb* cells. Should this prove to be successful, it would improve the efficacy of current anti-TB regimens, reduce the risks of adverse side effects, and consequently shorten TB treatment.

Furthermore, the rapid spread of drug resistance, may be reduced by the co-administration of various non-antibiotic drugs, such as metformin and / or non-steroidal anti-inflammatory drugs (NSAIDs), which could possibly assist in treating the comorbid illnesses and ensure optimal strength of the patient's immune system, while being treated with the current anti-TB regimens.

Further applications of metabolomics and the identification of the treatment induced metabolites changes to a TB infected patient during the course of treatment, could also be useful for developing better methods for treatment monitoring, and would enable more frequent testing of the blood / urine to determine drug effectivity, coherence of the treated patients to the treatment protocol, and additionally perhaps even, to predict treatment outcome earlier.

ANNEXURE A

The following is a list of publications emanating from this study:

- Adeniji, A.A., Knoll, K.E., Loots. (2020) Potential anti-TB investigational compounds and drugs with repurposing potential in TB therapy: A conspectus. *Applied microbiology and biotechnology*. 104, 5633-5662, <https://doi.org/10.1007/s00253-020-10606-y>. Impact factor: 4.8
- Knoll, K.E.; Lindeque, Z.; Adenijia, A.A.; Oosthuizen, C.B.; Lall, N.; Loots, D.T. (2021) Elucidating the Antimycobacterial Mechanism of Action of Ciprofloxacin Using Metabolomics. *Microorganisms*, 9(6), 1158. <https://doi.org/10.3390/microorganisms9061158>. Impact factor: 4.2
- Knoll, K.E.; Lindeque, Z.; Adenijia, A.A.; Oosthuizen, C.B.; Lall, N.; Loots, D.T. (2021) Elucidating the Antimycobacterial Mechanism of Action of Decoquinat RMB041 Using Metabolomics. *Antibiotics*, 10(6), 693. <https://doi.org/10.3390/antibiotics10060693>. Impact factor: 4.6
- Knoll, K.E.; Mietha M. van der Walt, Du Toit Loots. (2021) *In silico* drug discovery strategies identified ADMET properties of decoquinat RMB041 and its potential drug targets against *Mycobacterium Tuberculosis*. Accepted for publication in *Microbiology Spectrum*. Impact factor: 7.2



Potential anti-TB investigational compounds and drugs with repurposing potential in TB therapy: a conspectus

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Abstract

The latest WHO report estimates about 1.6 million global deaths annually from TB, which is further exacerbated by drug-resistant (DR) TB and comorbidities with diabetes and HIV. Exiguous dosing, incomplete treatment course, and the ability of the tuberculosis bacilli to tolerate and survive current first-line and second-line anti-TB drugs, in either their latent state or active state, has resulted in an increased prevalence of multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant TB (TDR-TB). Although a better understanding of the TB microanatomy, genome, transcriptome, proteome, and metabolome, has resulted in the discovery of a few novel promising anti-TB drug targets and diagnostic biomarkers of late, no new anti-TB drug candidates have been approved for routine therapy in over 50 years, with only bedaquiline, delamanid, and pretomanid recently receiving tentative regulatory approval. Considering this, alternative approaches for identifying possible new anti-TB drug candidates, for effectively eradicating both replicating and non-replicating *Mycobacterium tuberculosis*, are still urgently required. Subsequently, several antibiotic and non-antibiotic drugs with known treatment indications (TB targeted and non-TB targeted) are now being repurposed and/or derivatized as novel antibiotics for possible use in TB therapy. Insights gathered here reveal that more studies focused on drug-drug interactions between licensed and potential lead anti-TB drug candidates need to be prioritized. This write-up encapsulates the most recent findings regarding investigational compounds with promising anti-TB potential and drugs with repurposing potential in TB therapy.

Keywords Adjunct drugs · Anti-tuberculosis therapy · Drug resistance · Monotherapy · *Mycobacterium tuberculosis* · Prodrugs

Introduction

Tuberculosis (TB), caused by the organism *Mycobacterium tuberculosis*, is considered to be the most prevalent cause of death in humans from a single infectious agent. The latest WHO report estimates 10.0 million people globally with active TB, resulting in 1.6 million deaths (300,000 of which are co-infected with HIV) (WHO 2018). This high incidence of TB is ascribed to several factors including (1) the difficulties/complications in totally eradicating *M. tuberculosis* (Mtb) from its diseased host, due to the physiological competence (persistence) of the infectious agent (Cambier et al. 2014; Mishra and Surolia 2018), (2) its tolerance to current anti-

TB drugs/antibiotics (Antonova et al. 2018; Lee et al. 2019; Liu et al. 2016), and (3) the compromised immune status of the infected host (Bonds and Sampson 2018; Campanico et al. 2018). This is further exacerbated by the increased prevalence of drug-resistant (DR), multi-drug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) TB causing strains, in many populations (Jansen and Rhee 2017; Kolyva and Karakousis 2012).

Even though TB is generally considered to be a curable disease (for most drug-sensitive clinical TB cases), the current first-line anti-TB drugs (isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB)) given as part of the Directly Observed Therapy Short Course (DOTS), are inadequate for totally eradicating TB (Chetty et al. 2017; Evans and Mizrahi 2018). This is also partially because other socioeconomic and demographic factors like poor hygiene, income constraints, malnutrition, overcrowding and poor ventilation, lack of social support, and stigmatization also significantly contribute to the continued communicability and persistence of TB (Creswell et al. 2011; Duarte et al. 2018; Ivanovs et al. 2016; Lönnroth et al. 2010).

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Although an improved understanding of TB and the Mtb biosystem, using a variety of omics research strategies, has to date resulted in the discovery of various novel and promising anti-TB drug targets over the years (Gibson et al. 2018; Haas et al. 2016; Loots 2016; Luies et al. 2017; Swanepoel and Loots 2014), new treatment strategies are still urgently needed to alleviate the TB burden, and especially so considering the recent rise in DR-strains of Mtb, and subsequently the need for faster treatment strategies, and better patient compliance to such agents (Dong et al. 2017; Lin et al. 2018; Mori et al. 2017). New anti-TB drug discovery, is now more than ever, focused on finding new anti-TB agents, which exhibit lower toxicity, improved efficacy, and prevent or even cures MDR-TB (Castelo-Branco et al. 2018).

Over these past 4 decades, there has been little in terms of novel anti-TB drug discoveries, which is most likely attributed to extremely high costs typically required for developing and testing such, exacerbated by the low-profit margins when such eventually reaches the market (Tacconelli et al. 2018). According to recent reports, the cost of developing totally novel drugs has escalated to about \$2.6 billion per drug and to the duration of development up to 15 years, before a potential novel drug is eventually commercialized (Ramón-García et al. 2016). Subsequently, big-pharma and other academic or commercial research groups no longer find this approach to drug discovery an attractive business option (Deshpande et al. 2017). Besides, there is now also the requirement, as part of the drug development pipeline, to determine synergistic efficacy and safety, with other therapeutic agents, and this further hampers new drug discovery in terms of development time and costs (Evans and Mizrahi 2018). For these reasons, repurposing existing drugs has also become a more popular option in recent years (Ramón-García et al. 2016; Savoia 2016).

Reports from over four decades indicate that about 23 candidate anti-TB agents (antibiotic and non-antibiotic) are undergoing different stages of clinical trials. Table 1 highlights candidate anti-TB agents (antibiotic and non-antibiotic) that are associated with clinical trials. From the 8 candidates (a subset of the 23 candidates) that were previously undergoing either phase 1, 2, or 3 clinical trials (Ryan and Lo 2014), 3 candidates (bedaquiline (BDQ), delamanid (DLM), and pretomanid (PMD)) have received expedited and/or conditional regulatory approval (Keam 2019; WHO 2014). Interestingly, 13 of the drugs presently recommended by WHO for MDR-TB therapy neither were developed as anti-TB drugs nor were they licensed for TB treatment in most countries of the world (Lange et al. 2019); 7 of the aforementioned new anti-TB drug candidates were classified as repurposed drugs (Fan et al. 2018a).

Before this review, several articles (including reviews) on the newly approved regulatory anti-TB agents (BDQ, DLM, and PMD) for routine therapy (Keam 2019; Lakshmanan and Xavier 2013; Tiberi et al. 2018) and other anti-TB drug

candidates with promising activity against different Mtb strains (susceptible, resistant, and latent) at various stages of investigation (in vitro, in silico, murine, and clinical trial) exist (Campanico et al. 2018; Dong et al. 2017; Evans and Mizrahi 2018; Kumar et al. 2018; Surineni et al. 2018). Several reports also exist on novel anti-TB drug targets and mechanisms of action (MOA) (Chiarelli et al. 2018; Cleghorn et al. 2018; El Asrar et al. 2017; Gibson et al. 2018; Korkegian et al. 2018; Singh and Mizrahi 2017; Wang et al. 2018; Yang et al. 2018; Zhang et al. 2018). As newer information on the derivatives of old TB (antibiotic and non-antibiotic) drugs and previously repurposed (successful and unsuccessful; antibiotic and non-antibiotic) TB drugs are becoming available, this review encapsulates the most recent reports (including review articles) on investigational compounds (associated with old therapeutic agents) and drugs with repositioning potential for anti-TB therapy accumulated over the last 6 years. The review articles were included because of insights provided by the authors as regards the topic of discussion. Currently, information on newly synthesized compounds either related to old therapeutic agents (or non-related) and those non-related to repurposed drugs in any way is numerous and inexhaustible. This paper, therefore, only provides a quick go-to for readers interested in anti-TB drug discovery and development, relative to both old therapeutic agents and drugs with repositioning potentials (antibiotic and non-antibiotic).

Information search approach

A step-wise search strategy was employed to identify and collect articles reporting: (i) in vitro evaluations, (ii) murine investigations, and (iii) Clinical trials from different “open access” databases (Google Scholar, [PubMed.gov](https://pubmed.ncbi.nlm.nih.gov/), and Web of Science) while emphasizing the keywords investigational anti-Mtb compounds, anti-TB lead drugs, anti-TB drug candidates, derivatives of old anti-TB drugs, repurposed MDR-TB, XDR-TB drugs, antibiotic and non-antibiotic TB drugs, and novel TB compounds related to old TB drugs. The [ClinicalTrials.gov](https://clinicaltrials.gov/) website was employed to identify relevant clinical trials. For the purpose of this review, manuscript collection was limited to publications from 2015 to 2020 (except where additional information from a previous report pertaining to the specific compound of interest was required).

Investigational compounds with promising anti-TB potential

At present, the WHO anti-tuberculosis essential medications list include EMB, INH, RIF, PZA, rifabutin, amikacin, amoxicillin-clavulanic acid (AMC), BDQ, clofazimine (CFZ), cycloserine, DLM, ethionamide (ETH), levofloxacin

Table 1 Candidate anti-TB agents (antibiotic and non-antibiotic) associated with clinical trials

Anti-TB agent	Clinical trials		Application; mechanism of action
	Recruiting	Active	Completed
Amikacin ^{OL} and ^R (antibiotic)	Phase 4: (NCT03237182 2020)	—	—
Azithromycin ^A (antibiotic)	Phase 3: (NCT03545373 2020)	—	—
	Phase 2: (NCT03160638 2020)	—	—
	Phase 3: (NCT04062201 2020); Phase 2 and 3: (NCT03338621 2020); Phase 2 and 3: (NCT02589782 2020)	Phase 3: (NCT02409290 2020); Phase 2 and 3: (NCT02454205 2020) Phase 3—active: (NCT02276755 2020)	Phase 2: (NCT02365623 2020); Phase 3: (NCT02333799 2020)
Bedaquiline ^A (antibiotic)	Phase 3: (NCT04062201 2020); Phase 2 and 3: (NCT03338621 2020); Phase 2 and 3: (NCT02589782 2020)	Phase 3: (NCT02409290 2020); Phase 2 and 3: (NCT02454205 2020) Phase 3—active: (NCT02276755 2020)	Phase 2: (NCT02365623 2020); Phase 3: (NCT02333799 2020)
Cholecalciferol ^R (vitamin D3 - secosteroid dietary supplement)	—	—	—
Clarithromycin ^R (antibiotic)	—	—	—
Clofazimine ^{OL} and ^R ; (antibiotic)	Phase 2: (NCT02589782 2020)	Phase 3: (NCT02409290 2020)	Phase 4: (NCT01521364 2020)
Contezolid (antibiotic)	Phase 3: (NCT04062201 2020)	—	—
—	Phase 3: (NCT02754765 2020)	—	—
Etoricoxib (cyclooxygenase-2 (COX-2) inhibitor)	—	Phase 2: (NCT02503839 2020)	Phase 1: (NCT03033329 2020)
Delamanid ^A (antibiotic);	Phase 3: (NCT04062201 2020)	—	—
Doxycycline ^R (antibiotic)	—	—	Phase 3: (NCT01424670 2020)
Ibuprofen ^R (nonselective (COX-2) inhibitor)	—	—	Phase 2: (NCT02774993 2020)
Kanamycin ^{OL} and ^R (antibiotic)	—	Phase 2 and 3: (NCT02454205 2020)	Phase 2: (NCT02781909 2020)
Levofloxacin ^{OL} and ^R (antibiotic)	Phase 3: (NCT04062201 2020)	Phase 3: (NCT02409290 2020); Phase 2 and 3: (NCT02454205 2020)	Phase 4: (NCT00495339 2020) Completed: (NCT02169141 2020)
Linezolid ^{OL} and ^R (antibiotic)	Phase 3: (NCT04062201 2020) Phase 2 and 3: (NCT02589782 2020)	Phase 2 and 3: (NCT02454205 2020)	Phase 2: (NCT02279875 2020) Phase 4: (NCT01521364 2020)

Table 1 (continued)

Anti-TB agent	Clinical trials		Application; mechanism of action	
	Recruiting	Active	Completed	
Meropene-amoxicillin-clavulanic acid ^A and ^R (antibiotic)	Phase 2: (NCT03174184 2020)	—	Phase 3: (NCT02333799 2020)	Anti-Mtb combinatorial agents; inhibition of mycobacterial L,D-transpeptidases
Metronidazole ^R	—	—	Phase 2: (NCT00425113 2020)	In anti-TB regimen; inhibition of bacterial nucleic acid synthesis
Moxifloxacin ^{OL} and ^R (antibiotic)	Phase 2 and 3: (NCT03338621 2020)	Phase 3: (NCT02409290 2020)	Phase 2: (NCT00164463 2020)	In anti-TB regimen; inhibition of DNA gyrase
Nitazoxanide ^R (antibiotic)	Phase 2 and 3: (NCT02589782 2020) Phase 2 and 3: (NCT03338621 2020)	Phase 2 and 3: (NCT02454205 2020)	Phase 1 and 2: (NCT02114684 2020)	
	—	—	Phase 2: (NCT02684240 2020)	Anti-Mtb monotherapy; disruption of Mtb membrane potential and pH homeostasis and stimulation of host macrophage autophagy in TB patient
Prednisolone ^R (Corticosteroid)	—	—	Phase 3: (NCT01924286 2020)	Adjunctive host-directed therapy; anti-inflammatory
Pretomanid ^A (antibiotic)	Phase 2 and 3: (NCT02589782 2020) Phase 2 and 3: (NCT03338621 2020)	—	Phase 3: (NCT02333799 2020)	In anti-TB regimen; possibly inhibition of cell wall mycolic acid synthesis
Rifabutin ^R modified adjunct therapy: (i. ^R everolimus; ii. ^R auranofin; and iii. ^R cholecalciferol)	—	Phase 2: (NCT02968927 2020)	—	Adjunctive host-directed therapy
Sutezolid ^R (antibiotic)	—	—	Phase 2: (NCT01225640 2020)	In anti-TB regimen prodrg; inhibition of bacterial protein synthesis
Telacebec (Q203)	—	—	Phase 2: (NCT03199313 2020)	In anti-TB regimen; inhibition of Mtb growth by targeting the respiratory chain cytochrome bcc complex
TBI-223	Phase 1: (NCT03758612 2020)	—	Phase 2: (NCT03563599 2020)	Unspecified
TBA-7371	—	—	Phase 1: (NCT03199339 2020)	Unspecified
Trimethoprim-sulfamethoxazole ^{OL} and ^R (antibiotic)	—	—	Phase 2: (NCT01832987 2020)	Anti-Mtb; inhibition of tetrahydrofolic acid synthesis

A, approved; *OL*, off-label; *R*, undergoing repositioning

(LFX), linezolid (LZD), meropenem, moxifloxacin (MXF), p-aminosalicylic acid (PAS), streptomycin, and rifapentine (RPT) (WHO 2015, 2019). The 3 recently approved (BDQ, DLM, and PMD) medications have adverse side effects even when co-administered and are only administered under stringent conditions where treatment options are limited. New anti-TB drugs are still insufficient and the numbers of investigational compounds undergoing human clinical trials are limited. Therefore, the need for continuous discovery of anti-TB compounds with new action mechanism and reduced treatment duration, lower toxicity and higher potency (including latent TB stages), low production and purchase cost, easy accessibility, improved drug-drug interaction, and their subsequent inclusion in TB therapy cannot be overemphasized. In this regard, we will discuss a few of the investigational compounds (related to old antimicrobial agents) recently reported to show promising anti-TB potential below with highlights in Table 2. Figure 1 also shows the chemical structures of these investigational compounds.

Decoquinolate derivatives

The multi-therapeutic efficacy of decoquinolate (DQ; Fig. 1), an anticoccidial drug used in poultry and livestock farming, was evaluated by Beteck et al. (2018). In their study, the authors converted DQ via divergent chemistry, into several analogs of N-alkylated quinolone amides, three of which showed remarkable activity against Mtb. Furthermore, due to DQ's lipophilic decyl side chain, active DQ analogs will most likely diffuse passively over the Mtb mycolic acid cell wall. This characteristic should allow excellent intracellular penetration and enhanced activity against Mtb encapsulated in macrophages and granulomatous lesions, and possibly shorten treatment duration remarkably (Aljayyousi et al. 2017). This observation is corroborated by the Rosenthal et al. (2012) investigation, whereby upon exposure of BALB/c mice to an INH-PZA-RIF regimen instead of INH-PZA-RPT, approximately 50% reduction in TB treatment duration was recorded. The reduction was attributed to the increased daily dose of RPT instead of rifampin (10 mg/kg of RIF was replaced with 7.5–10 mg/kg of RPT) which resulted in greater exposure of the mice to the former.

Additionally, the N-alkylated amide DQs expressed anti-Mtb MIC₉₀ of 1.25–3.64 µM, caused by possible cell wall homeostatic disruptions (Beteck et al. 2018). A follow-up in vitro and murine (C57BL/6 male mice) study by Tanner et al. (2019) evaluating multiple parameters (e.g., in vitro microsomal stability, kinetic solubility, lipophilicity, and pharmacokinetic parameters) of these DQs further showed promising anti-TB drug profiles. The addition of the DQ analogs in TB therapy will, however, require further anti-Mtb murine investigations and clinical trials to ascertain its pharmacokinetics/pharmacodynamics relative to humans.

Primaquine derivatives

The 2012 approval of the anti-TB compound BDQ (a diarylquinoline derivative; MIC = 0.05–0.22 µM) (Andries et al. 2005), and its characteristic non-cross-resistance to MDR and XDR-Mtb strains (Kaur et al. 2015), recently led to investigating the use of various quinolone derivatives, such as the antimalarial drug primaquine (PQ; Fig. 1), for use as explorative scaffolds for the synthesis of similar compounds with anti-TB action. Pavić et al. (2018) reported the anti-Mtb activity of both urea 2 m and bis-urea 4u, 2 primaquine derivatives as (MIC = 4.29 µM). The 2 PQ analogs, from “4 series of PQ derivatives” (amides 1a-k; ureas 2a-s; semicarbazides 3a-c; and bis-ureas 4a-u), displayed favorably low cytotoxicity (2 m (321.88 µM) and 4u (128.30 µM)), while also exhibiting a better anti-Mtb activity than the reference drug ciprofloxacin in the study.

Coumarin derivatives

Report on the antimicrobial activity of coumarin-based compounds has been increasing over the years. Coumarins possess a variety of biological properties that includes Mtb inhibition (Abdizadeh et al. 2017; Basanagouda et al. 2014; Jeyachandran et al. 2012; Kawate et al. 2013; Niu et al. 2017), and based on the parent compound's (coumarin: 1,2-benzopyrone (Fig. 1)) structural versatility and scaffolding ability (Bansal et al. 2013; Kapp et al. 2017), newer derivatives having potential anti-TB activity have been synthesized and investigated in vitro Reddy (Reddy et al. 2015a; Reddy et al. 2015b). Recently, Mangasuli et al. (2018) and Reddy et al. (2018) reported the synthesis of 2 specific coumarin derivatives, (1) a coumarin-theophylline hybrid (3a) and (2) a coumarin-oxime ether derivative (1 h), which both exhibited excellent anti-TB activity of MIC 0.32 µM and 0.12 µM respectively—MIC ranges close to the first-line anti-TB drug INH. Additionally, the majority of the derivatives in the study of Reddy et al. (2018) exhibited MICs in the range of 0.12–16.51 µM—while also projecting good cytotoxicity profiles against Vero cells and excellent DNA cleaving properties (depicting nuclease activity). According to Mangasuli et al. (2018), 3a also showed good binding interactions with the Mtb 4DQU enzyme and possible broad-spectrum antimicrobial properties (inhibiting *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi*). Possible anti-TB MOA exhibited by coumarin derivatives includes disruption of cytochrome synthesis; inhibition of cell proliferation; activation of macrophages; kinase inhibitor. The inclusion of other compound moieties (of interest to TB drug development) (e.g., oxime and heterocyclic moieties) in the coumarin nucleus could provide avenues for synthesizing potential new anti-TB lead drugs.

Table 2 Recent investigational compounds with promising anti-TB potential

Parent candidate agent/compound	Previous application	Target/mechanism of action for TB	Method of investigation	Reference
			Anti-mycobacterial evaluation and MIC values	Cytotoxicity evaluation
Decoquinone derivatives (N-Alkyl-decoquinone quinolone amides)	Coccidiostat (anticoccidial parasites)	Possible cell wall disruption and inhibition of DNA gyrase	Broth microdilution (7H9 and GAST/Fe media) and quantitative fluorescence assay; MIC for Mtb	Sulforhodamine B assay; IC ₅₀ for fetal lung fibroblast cells = 40.1–56.2 μ M (Beteck et al. 2018; Tanner et al. 2019)
Primaquine derivatives (2 m and 4u)	Antimalarial drug	Inhibition of proliferation	H37Rv = 1.25–3.64 μ M Broth microdilution (7H9GC medium); MIC of 2 m and 4u for (i. Mtb H37Ra = 4.29 and 3.15 μ M; ii. <i>M. avium</i> complex CTT19/06 = 4.29 and 25.15 μ M; and iii. <i>M. avium</i> subsp. <i>paratuberculosis</i> = 4.29 and 6.29 μ M respectively	MABA (RPMI 1640 medium); IC ₅₀ of 2 m and 4u for L-6 cells from rat skeletal myoblasts = 321.88 and 128.30 μ M respectively (Pavić et al. 2018)
Coumarin derivatives: 1 h: coumarin-oxime-ether derivative 3a: theophylline hybrid	Broad-spectrum therapeutic activity (anti-allergic, anti-bacterial, antifungal, anti-inflammatory, anti-thrombotic, anti-ringworm, antiplatelet and antiviral)	Possible disruption of cytochrome synthesis; inhibition of cell proliferation; activation of macrophages; kinase inhibitor	MABA (7H9 broth); MIC of 1 h and 3a for Mtb H37Rv = 0.12 and 0.32 μ M	MTT assay (DMEM); IC ₅₀ of 1 h for vero cells = 5.39 μ M and 3a = NA (Reddy et al. 2018) (Mangasuli et al. 2018)
Quinoxaline derivatives ((35a (1,4-di-N-oxide-quinoxaline-2-ylmethylene isonicotinic acid hydrazide)); 37d (3-methylquinoxaline-2-carboxylate-1,4-dioxide); and 41a and b (3-methylquinoxaline-2-carboxylate-1,4-dioxides)) Santivañez-Veliz et al. 2016)	Anti-candida, anti-inflammatory/-antioxidant, anti-tumor, anti-trypanosomal, antibiotic (against multidrug-resistant Gram-positive bacteria)	Possibly inhibits bacterial nucleic acid synthesis	MABA; MIC of 35a, 37d, 41a, and 41b for Mtb H37Rv = 0.5, 0.31, 1.19, and 1.20 μ M respectively;	Non-radioactive Cell Proliferation and MTT Assay; IC ₅₀ of 35a, 37d, 41a, and 41b for vero cells = 123, 145, 140, and 153 μ M respectively (Jaso et al. 2005; Kert et al. 2018; Pan et al. 2016;
Nitrofurans analogs (a. 10e, 10i, 10n; b. 18e; and c. 9a)	Anti-bacterial and antiparasitic	Possible production of toxic free radical species	a. REMA; MIC of 10e, 10i, and 10n for Mtb H37Rv = 33, 32, and 22 μ M; and on mutant isolates = 17–66, 16–63 and 11–44 μ M respectively; b. MIC of 18e for Mtb H37Ra = 0.58 μ M (assay/-medium unspecified); c. Broth microdilution; MIC of 9a for Mtb H37Rv = 0.05 μ M	a. WST-1 assay; IC ₅₀ of 10e, 10i, and 10n for (i. immortalized cancer cells FS4-LTM = all > 100 μ M; ii. L929 cells = 50, 50, and > 100 μ M; iii. KB-3-1 cells = 61, 16, and 27 μ M and iv. MCF-7 cells = 19, 9, and 12 μ M respectively); b. MTT assay; IC ₅₀ of 18e for vero cells = 50.2 μ M; c. Viability assay—CellTiter-GloH Luminescent Cell; IC ₅₀ of 9a for kidney epithelial cells = 36.74 μ M (Krasavin et al. 2017); b. 18e (Ran et al. 2016); c. 9a (Rakesh et al. 2014)

Table 2 (continued)

Parent candidate agent/compound	Previous application	Target/mechanism of action for TB	Method of investigation	Reference
			Anti-mycobacterial evaluation and Cytotoxicity evaluation MIC values	
Spectinomycin derivative (spectinamide 1599)	Broad-spectrum anti-bacterial (second-line anti-gonococci)	Possible inhibition of ribosomal translocation and consequent protein synthesis	Broth microdilution (7H10 medium) and MGIT 960 assay; MIC for H37Rv = 3.25 μ M and various resistant isolates = 0.61–3.86 μ M	(Herrmann et al. 2017; Hoagland et al. 2016; Lee et al. 2014a)
Pyridomycin: didydropyridomycin derivative (I)	Anti-mycobacterial	Inhibition of mycolic acid synthesis and consequent cell wall synthesis	REMA (7H9 medium); MIC for Mtb H37Rv 0.78 μ M	(Horlacher et al. 2013)
Caprazamycin (caprezone 4-buylamide: (CPZEN-45))	Antimycobacterial	Inhibiting enzyme WeeA and translocase I (MraY)/peptidoglycan synthesis/cell wall mycolylarabinogalactan	Broth microdilution (7H10 medium); MIC for Mtb H37Rv = 1.81 μ M and for <i>M. bovis</i> = 2.91 μ M	(Chetty et al. 2017; Dong et al. 2017; Salomon et al. 2013)
Griselimycin derivatives (cyclohexylgriselimycin)	Anti-mycobacterial	Inhibits DNA replication and repair	MABA (7H9 medium); MIC for (i. Mtb H37Rv = 0.05 μ M and ii. Mtb infected macrophage-like RAW264.7 cells = 0.17 μ M)	(Dong et al. 2017; Kling et al. 2015)
3-Cinnamoyl-4-hydroxy-pyran-2-one (CHP) analogs 2a and 2u	Anti-HIV and anticancer agent	Disruption of mitochondrial membrane potential, increased ROS induction, caspase-3 activation, and Mtb cell wall disruption	Broth microdilution assay (7H9 medium); MIC of 2a and 2u for Mtb H37Rv = 14.03 and 12.5 μ M	MTT assay; IC ₅₀ of 2a and 2u for (i. human kidney (HEK-293); ii. colon cancer (HCT-116); iii. Prostate cancer (PC-3); and iv. breast cancer (MCF-7) cells = 80 and > 100 μ M respectively for all cell lines) (Bhat et al. 2018; Lan et al. 2015)
Naphthoquinone derivatives a. 6 (2-(Phenylamino) naphthalene-1,4-dione); b. E1 ((2-chloro-3-(4-methanesulfonylpiperazin-1-yl)-1,4-dihydronaphthalene-1,4-dione) and idebenone	Broad-spectrum (antibiotic, antiviral, antifungal, and antiparasitic), anti-inflammatory, and anti-proliferative	Possibly induction of apoptosis (ROS production) and inhibition of the enzymes ThyX and DNA gyrase	a. REMA (7H9 medium); MIC of 6 for (i. Mtb H37Rv = 100.4 μ M; ii. INHR Mtb = 100.4 μ M; and iii. RIF-R Mtb = 12.5 μ M); b. MABA (7H9 medium); MICs of E1 and idebenone for Mtb H37Rv = 46 and 93 μ M respectively	a. 6 (Halicki et al. 2018); b. E1 and idebenone (Djaout et al. 2016)
Isatin derivatives: a. 1 (ciprofloxacin methylene isatin); b. 4 (balofloxacin ethylene isatin);	Broad-spectrum (antiviral, antifungal, antibiotic, and anti-helminthic),	DNA gyrase inhibitor	MABA (a–h); a–c. (MIC of 1, 4, 10b, and 11i for Mtb	a–f. (Xu et al. 2017b)

Table 2 (continued)

Parent candidate agent/compound	Previous application	Target/mechanism of action for TB	Method of investigation	Reference
			Anti-mycobacterial evaluation and Cytotoxicity evaluation MIC values	
c. 10b and 11i (isatin-thiazoles); d. 15c and 15 l (tetrahydropyrimidine-isatins); e. 17b (thiolactone-isatin hybrids); f. 20a-c and 21a-d (thiacetazone-isatins); g. 22 (azetidinone-isatin derivatives) h. 24f and 24j (chloroquinoline-isatin hybrids)	anti-inflammatory, anti-tumor, antioxidant, anti-convulsant, and anti-depressant		H37Rv = 1.39 nM, 0.38, 21.4, and 0.39 μ M respectively) d. Complete inhibition of Mtb H37Rv by 15c and 15 l at DC of 7.47 and 7.45 mM; e. MIC of 17b for Mtb H37Rv = 63.7 μ M; f. MIC of 20a, 20b, 20c and 21a–d for Mtb H37Rv = 63.94, 29.34, 13.27, and 10 μ M respectively; g. 90%–96% inhibition of Mtb H37Rv by all 22 hybrids at DC of 0.57–0.77 mM h. MIC of 24f and 24j on Mtb H37Rv < 10.12 μ M	g. (Deng et al. 2018)
Isoniazid derivatives (acylhydrazides 14, 15 and 16)	Antimycobacterial	Inhibitor of mycolic acid synthesis; inhibition of cell growth	MABA and Broth microdilution (7H9 medium); MICs of 14, 15, and 16 for (i. Mtb H37Rv = 0.47, 0.45, and 0.43 μ M; ii INHR Mtb = 38.3, 36.7, and 69.4 μ M; and iii. INH/RIF-R Mtb = 38.3, 36.7, 34, and 34.7 μ M respectively)	REMA; cell viability of 14, 15, and 16 tested at 10 μ M for macrophage cell line J774A.1 = 97.3%, 98.2%, and 98.8%; WST-1 assay; LC ₅₀ of 14, 15 and 16 for HepG2 hepatocarcinoma cells = 31.5, > 100, and 110 μ M (Castelo-Branco et al. 2018)

DMEM, Dulbecco Modified Eagle Medium; MGIT, mycobacteria growth indicator tube; MABA, Microplate Alamar Blue Assay; REMA, resazurin reduction microplate assay; WST-1, water soluble tetrazolium; NA, not applicable; DC, drug concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LC₅₀, concentration required to kill 50% of the tested population

Quinoxaline derivatives

Another class of antimycobacterial recently showing promise for use against Mtb is the quinoxaline-1,4-di-*N*-oxide derivatives (Keri et al. 2018). The quinoxaline ring (Fig. 1) in these compounds has a heterocyclic bioactive scaffold known for its isomeric promiscuity, and hence, a number of analogs of these, including various quinoxaline-1,4-di-*N*-oxide derivatives (quinoxaline-2-carbonitrile-1,4-di-*N*-oxide; quinoxaline-2-carboxylate-1,4-di-*N*-oxide; 1,4-di-*N*-oxide-3-methylquinoxaline-2-carboxylic acid aryl amide; ethyl quinoxaline-2-carboxylates; quinoxaline-1,4-di-*N*-oxide with fluoroquinolone and chalcone; 2-acetyl-3-methylquinoxaline-1,4-di-*N*-oxide; 1,4-di-*N*-oxide-quinoxaline-2-ylmethylene isonicotinic acid hydrazide; 3-methylquinoxaline-2-carboxylate-1,4-dioxide, and quinoxaline-carbonitrile-1,4-di-*N*-oxide) have been reported to exert significant anti-TB properties in vitro (MIC = 0.32–20.49 μ M (Keri et al. 2018). Considering the efficacy of these compounds, it is highly likely that these quinoxaline-1, 4-di-*N*-oxide derivatives will progress into preclinical evaluation in the near future.

Nitrofuran derivatives

Previous concerns about the potential toxicity accompanied by a poor understanding of the exact mode of actions of nitrofurans

(nifuroxazide, nitrofurazone, and nitrofurantoin; Fig. 1) have limited their use in therapeutic applications (Budha et al. 2008; Krasavin et al. 2017). New efforts have been made towards the synthesis of novel nitrofuran derivatives with enhanced antimicrobial activity and lower cytotoxicity. Based on the nitrofurans broad-spectrum antiparasitic and antibacterial activities, Krasavin et al. (2017) synthesized 3 nitrofuran analogs (10e, 10i and 10n) with appreciable MICs (22–33 μ M) against the Mtb H37Rv strain using isocyanide-based multicomponent chemistry. According to the authors, the 3 analogs displayed promising activity against MDR-Mtb isolates, poly-resistant patient-derived mutants, and acceptable cytotoxicity. From the several 5-niro-2-furfurylidene *N*-aminolactams (10a–o) derivatives investigated, analog 10n uniquely inhibited both MDR-Mtb: 2067 strain and the poly-resistant strain: 5307 with an improved MIC of 11 μ M. Ran et al. (2016) had earlier reported the synthesis of 8 promising antitubercular agents (12e, 12 k, 12 l, 12 m, 18a, 18d, 18e, and 18j). Compound 18e exhibited the best activity against Mtb H37Ra (MIC = 0.58 μ M), with favorable cytotoxicity property on Vero cells (IC₅₀ = 50.2 μ M). Another group of pentacyclic nitrofuran analogs (9a/Leel106) with excellent nanomolar activity (1.9–46 nM) against Mtb and safe cytotoxicity profiles was synthesized by Rakesh et al. (2014). From the compound set, 9a (MIC = 0.05 μ M) with the best

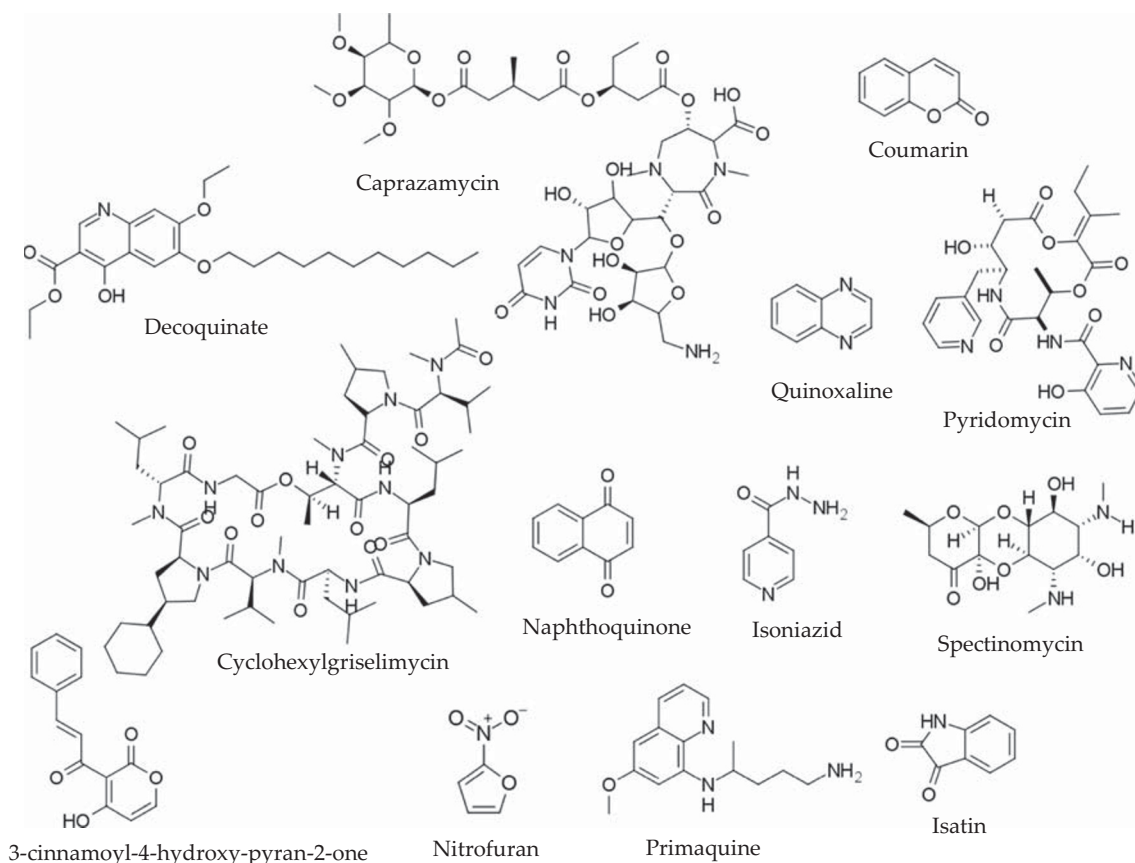


Fig. 1 Chemical structures of the parent drug/molecule of investigational compounds with promising anti-TB potential

pharmacokinetics showed promise for further preclinical investigation, as it killed 99% of hypoxic bacilli at drug concentration (DC) of 18.94 μM during a rapid anaerobic dormancy evaluation (a model that mimics the non-replicating-persisters 2 conditions). Apart from 9a having a well-defined post-antibiotic effect and commendable synergistic properties with 2 other anti-TB agents, it was also active against PMD-resistant mutants.

Spectinomycin, pyridomycins, and caprazamycin derivatives

Spectinamides (analogs of spectinomycin; Fig. 1) have significant narrow-spectrum activity against non-replicating TB, MDR-TB, and XDR-TB, while showing no obvious cytotoxicity, and good pharmacokinetics and in vivo efficacy (Bruhn et al. 2015; Herrmann et al. 2017; Horlacher et al. 2013; Lee et al. 2014b). Spectinamides (protein synthesis inhibitors), like spectinamide 1599 (MIC = 0.61–3.86 μM), are also reported to evade the Rv1258c efflux pump innate resistance mechanism of Mtb. According to the same authors, a pyridomycin-derivative dihydropyridomycin (mycolic acid synthesis inhibitor at MIC = 0.78 μM) and caprazamycin (CPZ)-derivative caprazene (CPZEN-45 = 1.81–2.91 μM) also exhibit better activity against Mtb when compared with that of their parent compounds. Interestingly, the aforementioned dihydropyridomycin not only retains the parent compound (pyridomycin) characteristic MOA by directly binding to *inhA*, but unlike pyridomycin, it lacks the enol ester moiety in its scaffold, which favorably enables it to be more easily synthesized (Hartkoorn et al. 2012). Possessing an isopropyl moiety also enables dihydropyridomycin to prevent the occurrence of mutations in *KatG*, the *inhA*-resistance-activating enzyme (Dong et al. 2017). CPZEN-45, on the other hand, displayed comparatively better solubility in water, better activity against MDR-Mtb, XDR-Mtb, and even many CPZ-resistant isolates. It was furthermore determined that it exhibits antibiosis by inhibition of the translocase I (MraY) and GlcNAc-1-phosphate transferase (WecA) and subsequently inhibiting mycolylarabinogalactan synthesis (Dong et al. 2017; Herrmann et al. 2017).

Griselimycin derivatives

Griselimycins (GMs), are cyclic depsidecapeptide agents isolated from the *Streptomyces* strain DSM 40835 with anti-TB action, albeit with poor pharmacokinetics. However, a GM derivative, cyclohexylgriselimycin (CGM; Fig. 1), shows far better anti-TB action, with a MIC of 0.05 μM against drug-susceptible Mtb H37Rv and a MIC of 0.17 μM for the same strain encapsulated in macrophage-like (RAW264.7) cells

Kling et al. (2015). Cyclohexylgriselimycins acts against Mtb by targeting its DnaN (the DNA polymerase sliding clamp that anchors the DNA to DNA polymerase) and subsequently inhibits mycobacterial DNA replication and repair, (Holzgrave 2015; Kling et al. 2015). It has additionally been shown to exhibit high activity in acute and chronic TB mouse models, showing promising synergistic properties with RIF and PZA and the possibility of shortening TB treatment duration (Herrmann et al. 2017).

3-Cinnamoyl-4-hydroxy-pyran-2-one (CHP) derivatives

The synthesis and use as an anti-TB drug, of 25 different scaffolds of 3-cinnamoyl-4-hydroxy-pyran-2-one (CHP; Fig. 1), with previously determined anti-HIV and anticancer potential, was done by Bhat et al. (2018). Two of the CHP derivatives (2a and 2u) showed promising anti-Mtb activity (MIC = 14.03 and 12.5 μM , respectively) with no apparent toxicity against 4 human cell lines (human kidney (HEK-293), colon cancer (HCT-116), prostate cancer (PC-3) and breast cancer (MCF-7)). The two CHP derivatives, moreover, displayed unique specificity for Mtb inhibition, in that they were ineffective against *M. smegmatis* and various other gram-negative and gram-positive bacteria, suggesting that the two CHP-based anti-TB compounds, would most likely not disrupt the human microbiome.

Naphthoquinone derivatives

Halicki et al. (2018), evaluated the anti-TB potential of six 1,4-naphthoquinones derivatives against 3 Mtb strains (H37Rv (ATCC 27294); isoniazid mono-resistant strain (ATCC 35822); RIF mono-resistant strain (RMPR strain; ATCC 35838). Overall, the 6 naphthoquinones showed appreciable MIC (206.6 and 12.5 μM) against the Mtb strains (compound 1 - H37Rv = 110.6 μM , INHR = 110.6 μM , RMPR = 110.6 μM ; compound 2 - H37Rv = 103.3 μM , INHR = 206.6 μM , RMPR = 206.6 μM ; compound 3 - H37Rv = 54.8 μM , INHR = 54.8 μM , RMPR = 54.8 μM ; compound 4 - H37Rv = 54.8 μM , INHR = 234 μM , RMPR = 234 μM ; compound 5 - H37Rv = 72.2 μM , INHR = 36.1 μM , RMPR = 36.1 μM ; and compound 6 - H37Rv = 100.4 μM , INHR = 100.4 μM , RMPR = 12.5 μM). The J774A.1 (ATCC TIB-67) macrophage cytotoxicity assay of the compounds showed that the compounds with lower MIC values exhibited low cytotoxicity. Two of the analogs (compounds 5 and 6), showed favorable interactions with INH and RIF. Remarkably, analogue 6 inhibited the RMPR strain (MIC = 12.5 μM), at a concentration 8x less than the inhibitory concentration against H37Rv and INHR strains (MIC = 110.4 μM); the analogue also inhibited the RMPR strain (MIC = 12.5 μM) at a concentration 50x less than the inhibitory

concentration of RIF (MIC = 622.2 μ M) against the RMPR strain. Employing a Bayesian pharmacophore modeling (to search for and collect new potent anti-Mtb), an in vitro target-based whole-cell assay and a machine learning approach, two new naphthoquinones derivatives (2-chloro-3-(4-methanesulfonylpiperazin-1-yl)-1,4-dihydronaphthalene-1,4-dione (MIC₉₀ = 62.5 μ M) and idebenone (MIC₉₀ = 125 μ M)) inhibiting Mtb H37Rv strain (moderately) and the Mtb ThyX enzyme were also identified by Djaout et al. (2016). Naphthoquinones (Fig. 1), exhibit broad-spectrum antimicrobial activity (Coelho et al. 2010; Fiorito et al. 2014; Mata-Santos et al. 2015; Neto et al. 2014; Uc-Cachón et al. 2014), that includes potent Mtb antagonism, with a possible MOA related to the induction of apoptosis (as result of reactive oxygen species (ROS) production), ThyX inhibition (thymidylate synthase (TS) enzyme essential for DNA synthesis) and DNA gyrase inhibition (Da Silva et al. 2003; Djaout et al. 2016; Karkare et al. 2013; Pereyra et al. 2019).

Isatin derivatives

Recent detailed reviews by Fan et al. (2018b); Xu et al. (2017b); Xu et al. (2019), and Jiang et al. (2018), discussed the anti-TB activity of isatin and various isatin derivatives. Isatin (Fig. 1), has a versatile structure biologically and subsequently, many therapeutic applications (anti-bacterial, antiviral, antifungal, anti-tumor, analgesic, anti-convulsant, antioxidant, anti-inflammatory, anthelmintic and anti-depressant). Furthermore, due to its stable indole nucleus, insertion of novel pharmacophore moieties is possible, which has resulted in the discovery of many promising anti-TB agents, including, a. various isatin-quinolone/fluoroquinolone hybrids: gatifloxacin (GTFX) (MIC = 0.50 μ M against H37Rv), ciprofloxacin methylene isatin (MIC = 1.39 nM) against Mtb H37Rv; b. FQ ethylene isatin hybrids: balofloxacin (BLFX) ethylene isatin (MIC = 0.38 μ M against Mtb H37Rv ATCC 27294); c. Isatin-thiazole hybrids: 10b (MIC = 21.4 μ M); 11i (MIC = 0.39 μ M for Mtb H37Rv; d. tetrahydropyrimidine-isatin hybrids: 15c and 15 l (complete inhibition at DC of (7.47 and 7.45 μ M); e. Thiolactone-isatin hybrids: 17b (MIC = 63.7 μ M) f. Isoniazid-isatin hybrids: 20a (MIC = 63.94 μ M), 20b (MIC = 29.34 μ M), 20c (MIC = 13.27 μ M) 21a-d (MIC = 10 μ M); g. azetidinone-isatin hybrids: 22 (90–96% inhibition at DC of 0.57–0.77 μ M); h. chloroquinoline-isatin hybrids: 24f and 24j (MIC < 10.12 μ M)), all with promising antimycobacterial capacity (via inhibiting Mtb DNA gyrase), and moderate cytotoxicity (Xu et al. 2017b; Xu et al. 2019). Because of its broad hybridizable (biological activity) potential, isatin remains a viable precursor in drug design and medicinal chemistry. Deng et al. (2018), also synthesized and tested the in vitro anti-Mtb and anti-MDR-Mtb capacity of ten new homonuclear and heteronuclear bis-isatins

derivatized with ethylene (5-fluoroisatin dimers 4a–) with MIC ranging between 63.2 and 673.68 μ M. The analog 4a was the most potent of these with a MIC of between 63.2–80.8 μ M against the Mtb H37Rv and INH, RIF, and EMB MDR-TB strains.

Isoniazid derivatives

Isoniazid (INH; Fig. 1), one of the first, first-line anti-TB drugs developed more than 40 years ago, acts by inhibiting Mtb mycolic acid synthesis. Despite its low cost and high efficacy for treating active TB, the production of the inactive *N*-acetyl isoniazid, stemming from INH *N*-acetylation, limits its applications. This *N*-acetylation is activated by the enzyme *N*-acetyltransferase (NAT), present in both humans and mycobacteria, and responsible for the mutations in the INH target sites. Some isoniazid hydrazides labeled 14–16, with striking anti-Mtb potency and reduced hepatocytotoxicity (lower toxicity to HepG2 cells) than their parent compounds were identified by Castelo-Branco et al. (2018). The conclusions made by the researchers that further necessitates investigating is that (a) the Mtb strains that exhibited both INH KatG mutation (SR 2571, SR 0215, and T113) and RIF rpoB mutation (T09) were highly susceptible to these anti-TB compounds, (b) the activities of compounds 14–16 (MIC = 0.47 μ M, 0.45 μ M, and 0.43 μ M respectively) are dependent on the isonicotyl moiety but may be independent of the INH moiety and hydrolysis thereof, and (c) the analogs only showed mutagenicity at concentrations higher than 500 μ M in comparison with INH which was mutagenic at 50 μ M, under the same assay conditions. Studies on drugs targeting KatG mutation metabolic pathways and biomarkers have provided insights that may assist in the discovery of new INH derivatives. Loots (2014) reported that isoniazid-resistant strains were less susceptible to INH by (a) upregulating the use of alkanes and fatty acids (as carbon and energy source) and (b) synthesizing several compounds directly involved in alleviating oxidative stress, including those related to a novel ascorbic acid degradation pathway.

The need for repurposing chemotherapeutic agents for treating TB

Several well-known and/or abandoned antibiotics/therapeutic agents, currently or previously used for the treatment of other communicable (e.g., HIV, cancer, and malaria) and noncommunicable diseases (e.g., hypertension and diabetes) are now being repurposed/reformulated/repositioned and tested for possible application in the treatment of drug-sensitive MDR-, XDR-, and TDR-TB (Mishra et al. 2018). The popularity in rescuing/rediscovering old and/or abandoned drugs, previously approved for use via preclinical or full clinical

trials, for reuse as antimicrobial agents against infectious diseases, or infectious agents other than their original targets, has grown considerably over recent years (Gns et al. 2019). The advantages of this become apparent when one considers that prior approval of these agents, although previously for other purposes, could serve to expedite regulatory approval for their later use in the treatment of other diseases, saving on the development costs and time (Sharma et al. 2017; Tiberi et al. 2018).

The relevance and topicality of this theme is confirmed by a review article published in 2016 by Mafukidze et al. (2016), which discusses the then ongoing or scheduled drug trials for compounds targeted for use against MDR-TB, using various repurposed compounds, including STREAM (a trial evaluating the Standard Treatment Regimen of Anti-TB Drugs for Patients with MDR-TB (StopTbPartnership 2020a, b, c)); endTB (aimed at expanding new drug markets for TB and evaluating newly approved anti-MDR-TB compounds); TB PRACTECAL (Pragmatic Clinical Trial for a More Effective Concise and Less Toxic MDR-TB Treatment Regimen(s) (StopTbPartnership, 2020a, b), RESIST-TB (RESIST-TB 2020), and many other such registered clinical investigations. As of 2016, the major drugs still undergoing official clinical investigations for repurposing (either monotherapeutically or combinatorially) are CFZ, LFX, MFX, LZD, imipenem, meropenem, and amoxi-CLV (Mafukidze et al. 2016). Although most of the repurposed anti-TB agents recently described have been investigated as one would a monotherapeutic agent, most of these would likely be implemented as a combination therapy, as suggested by many research groups, e.g., colistin sulfate (CST) (Koen et al. 2018a) and colistin methanesulfonate (CMS) (Koen et al. 2018b).

The group of repositioned drugs currently optimized and approved for off-label use in several TB therapy combinations, especially life-threatening situations where limited drug options exist and the drug is likely to be of benefit to the patients, includes (i) *the injectables* (amikacin and kanamycin) (Arnold et al. 2017; FDA 2018; Modongo et al. 2014); (ii) carbapenems-AMC (C-AMC) (Mishra and Caminero 2018; Tiberi et al. 2016), CFZ (Ammerman et al. 2018; Tyagi et al. 2015); (iii) *fluoroquinolones* (MFX: (NCT00164463 2020; NCT02114684 2020; Perumal et al. 2019; Weiner et al. 2018)); LFX: (Ghimire et al. 2016; NCT00495339 2020; NCT02169141 2020; NCT03000517 2020; van't Bovenind-Vrubleuskaya et al. 2017), OFX, LZD (<https://www.tballiance.org/news/fda-approves-new-treatment-highly-drug-resistant-forms-tuberculosis>, and *sulfamethoxazole/trimethoprim* (Alsaad et al. 2016; NCT01832987 2020) (see Table 1). Some of the repurposed drugs (e.g., LZD and CFZ) are also considered prodrugs that could be used synergistically with a variety of other anti-TB antibiotics, including the first liners: ETH and PAS, and the

new entries: BDQ, DLM, PMD, and SQ109 (Kroesen et al. 2017; Tiberi et al. 2018; Zumla et al. 2014). Considering this, it is recommended that multiple drug combinations are also evaluated as part of the standard protocols of preclinical or clinical drug trials (Aguilar-Ayala et al. 2018), in order to determine possible synergy, cross-reactivity, and cross-resistance.

For instance, upon completion of the phase 3 clinical trials for the use of LZD (300–600 mg daily) in combination with BDQ and/or PMD for treating different forms of DR-TB, LZD despite its previously reported cytotoxic demerits (Agyeman and Ofori-Asenso 2016; Harausz et al. 2017) is now included in the BDQ, LZD and PMD shorter regimen for treating various kinds of DR-TB (Alliance). On the downside, however, Zong et al. (2018) had previously reported a possible LZD resistance in *Mtb* due to mutation in the *rplD* region which merits further investigation.

Also reports on CFZ (originally used in the treatment of leprosy) show CFZ has proven activity (MIC = 0.13–4.22 μ M) against MDR, XDR, slowly replicating and non-replicating *Mtb* strains in vitro (Xu et al. 2012). Some murine model second-line regimen assessments have shown that regimen containing CFZ enhanced the delay of *Mtb* regrowth with high susceptibility patterns (Grosset et al. 2013; Swanson et al. 2016; Tyagi et al. 2015). The drug's advantages include its lipophilic characteristics, its affordability, and its promise of shortening MDR-TB drug regimens when used synergistically with other second-line anti-TB drugs (Duan et al. 2019; Lange et al. 2019; Tyagi et al. 2015). CFZ supposedly functions by inhibiting the *Mtb* respiratory chain and ion transporters, subsequently resulting in the production of ROS (Lechartier and Cole 2015). Ammerman et al. (2018) indicated that CFZ increases the efficacy of standard first-line drugs, allowing for smaller dosages of the latter to be administered with improved efficacy in the mouse model. Additionally, Mirnejad et al. (2018) recently published a review article, describing in detail CFZ's synergistic reactions with other lead compounds including PMD, MFX, benzothiazinones, and MmpL3 protein inhibitors.

Furthermore, in line with studies regarding multidrug combination therapy involving repurposed drugs, some carbapenem derivatives were investigated along with some B-lactams groups. Diacon et al. (2016), reported the inhibition of *Mtb* β -lactamase hydrolytic activity when a combination of meropenem-AMC was administered during a clinical trial, with little cytotoxicity. The idea of this combinatory approach is that the β -lactamase inhibitor would enhance the potency of the β -lactam antibiotics. According to Dhar et al. (2015), faropenem (MIC = 4.55 μ M) showed in vitro activity against *Mtb* cells and potentiates as an alternative drug for drug-resistant TB in comparison with meropenem (MIC = 1.69 μ M). The whole-blood bactericidal activity (WBA) trial by Gurumurthy et al. (2017), however, indicated the inactivity

of the drug monotherapeutically against Mtb but showed promising pharmacokinetics with rifampicin/faropenem/AMC groups. Kaushik et al. (2017) also indicated that biapenem (a carbapenem derivative; with MIC of 5.71 to 45.71 μM) has appreciable pharmacokinetics and is highly active against three different *M. tuberculosis* strains (115R: low-level RIF resistance; 124R: high-level RIF resistance, and the H37Rv: drug-susceptible parent strain) when administered alone and in combination with RIF, in TB diseased mice. Although in vitro synergy between biapenem and RIF against two of the aforementioned Mtb strains (115R and H37Rv) were observed, the two drugs lacked synergistic activity against the 124R strain. Considering the unique MOA of carbapenems (inhibition of mycobacterial L, D-transpeptidases) (Kaushik et al. 2017), and the fact that biapenem more specifically resists hydrolysis, and also inhibits human renal dehydropeptidase-I (DHP-I), it is considered a highly attractive option in anti-Mtb drug regimen. A recent article by Mishra and Caminero (2018) reported the successful inclusion of low-dose AMC co-administered with meropenem in the treatment of pre-extensive DR pulmonary TB. Other drug candidates with potential for repositioning for anti-TB therapy are further discussed below and are shown in Table 3.

Candidate drugs with potential for reformulation and inclusion in TB therapy

Avibactam investigated with β -lactams

A recent study by Deshpande et al. (2017) investigated the efficacy of using a ceftazidime and avibactam (a non- β -lactam β -lactamase inhibitor; Fig. 2) combination treatment strategy, against semi-dormant and intracellular MDR and XDR clinical isolates of Mtb using the hollow fiber system model of TB (HFS-TB) and several DC. In another follow-up study by Deshpande et al. (2018a), the first-generation penicillins benzylpenicillin or phenoxymethylpenicillin (penicillin V) showed promising antimycobacterial activity when used alone and synergistically with β -lactamase inhibitors. Benzylpenicillin, in combination with avibactam, for instance, displayed high activity against intracellular Mtb in the HFS-TB model, with a MIC of 2.99 μM . Deshpande et al. (2018a) concluded that the investigated penicillin combinations (penicillin-avibactam and or penicillin-ceftazidime-avibactam) had bactericidal activity against MDR-TB, incurable clinical strains of Mtb, and non-replicating bacilli, with a promising tendency to also shorten treatment duration among patients harboring MDR and XDR-TB strains. Accordingly, the benzylpenicillin-avibactam combination also exhibited lower cidal potential in comparison with ceftazidime-avibactam-penicillin combination. The tolerable cytotoxic profiles

recorded for the drug combinations in both studies could additionally make them promising additions to other recently approved medications (BDQ, DLM, and PMD), especially in the treatment of vulnerable populations such as neonates, pregnant women, and HIV-AIDS patients.

Diosmin

Another drug with potential for reuse demonstrating synergistic benefits particularly with AMC is diosmin (DIO; Fig. 2). Diosmin (transformed to diosmetin after oral administration) is an antioxidant, antihyperglycemic, antimutagenic, anti-inflammatory, and antiulcer agent, currently used for venous disorders and as a bioflavonoid dietary supplement (Arab et al. 2015; Dung et al. 2012; Feldo et al. 2018; Liu et al. 2014; Srinivasan and Pari 2012). Many of the proposed mechanisms of action DIO include (i) protein phosphatase 2A (PP2A) activation (Dung et al. 2012), (ii) inhibition of NF- κ B pathways activation with subsequent inhibition of the expression of proinflammatory cytokines and T cell receptors reduction (Feldo et al. 2018), and (iii) activation of the Janus tyrosine kinase-2 (JAK2) signal transducer and activator of transcription-3 (STAT3) pathways (Liu et al. 2014). Based on the fact that L,D-transpeptidase enzymes (Ldt_{Mm1} and Ldt_{Mm2}), involved in cell wall biosynthesis, cause resistance to β -lactams and that sufficient inhibition of Ldt_{Mm2} and subsequent destruction of the 3 $\rightarrow$ 3 and 4 $\rightarrow$ 3 transpeptide linkages (peptidoglycan peptide chains) may lead to total Mtb killing (Gupta et al. 2010; Pushkaran et al. 2019), Pushkaran et al. (2019) performed a molecular simulation study in which the structures of Ldt_{Mm1} and Ldt_{Mm2} of *Mycobacterium marinum* (81.7–82.2% identical to those of Mtb) were used, to identify FDA-approved compounds with the highest binding affinity. Both diosin and diosmetin were chosen for further in vivo investigation in *M. marinum* cells and *M. marinum*-infected *Drosophila melanogaster* model based on good bioavailability properties. Although separately the compounds did not show significant inhibition, when combined with AMC they reduced the mycobactericidal concentration by half (from 21.89 μM to 10.96 μM). The authors reported no cross-reaction between AMC and DIO, and with the combination showing moderate in vitro activity against 2 Mtb strains (H37Ra and another MDR clinical strain), a TB regimen having a β -lactam and β -lactamase—L,D-transpeptidase inhibitors will be of significant addition to the currently available anti-TB therapy.

Rimonabant

Ramesh et al. (2016) and Gajbhiye et al. (2015) recently reported that the anti-obesity agent rimonabant (MIC of 91.91 μM) and several of its analogs exhibited moderate anti-Mtb action. Rimonabant is structurally similar to

BM212 (an anti-Mtb MmpL3 inhibitor) and functions by disrupting the Mtb cell wall. Rimonabant (Fig. 2) can also permeate the blood-brain-barrier and additionally targets the expression of the cannabinoid receptor-1 (CB1) in the central and peripheral nervous system (Ramesh et al. 2016). Additionally, the Gajbhiye et al. (2015) study reported the synthesis of a potential lead anti-TB rimonabant analog (JMG-14) with an in vitro MIC of 7.84 μ M). Subsequently, three other rimonabant derivatives (12f: MIC = 2.44 μ M; 18a: MIC = 0.07 μ M; and 18e: MIC = 0.88 μ M) with far better MICs against Mtb than the parent molecule were reported by Ramesh et al. (2016), based on data from an in vitro study. The latter investigation was targeted at the possible synthesis of rimonabant analogs with potential use in brain TB therapy; while cytotoxic evaluation of the compounds (12f, 18a, and 18e) had an excellent inhibitory effect on Mtb in the cell lines (human hepatoma cells (HepG2), lung epithelial cells (A549), RAW macrophages, and THP-1 monocytes), cytotoxicity was, however, evident. The four promising anti-TB rimonabant derivatives (12f, 18, 18e, and JMG-14) are investigational compounds that require further in vivo investigation.

Nitazoxanide

Nitazoxanide (NTZ; Fig. 2), a broad-spectrum anti-infective drug, mostly acts by inhibiting the pyruvate ferredoxin/flavodoxin oxidoreductase (PFOR) of pathogenic anaerobe - impairing metabolic activity. However, in Mtb, low levels (DC = 52.07 μ M) of NTZ and its metabolite tizoxanide (TIZ) kill replicating and non-replicating bacilli by disrupting the pathogen's pH balance and membrane potential, suppressing its chaperone-usher (CU) pathway, additionally resulting in host macrophage autophagy in the TB patients (de Carvalho et al. 2011; de Carvalho et al. 2009; Lam et al. 2012; Shakya et al. 2018). As was also reported by Harausz et al. (2016), NTZ's and TIZ's lowest concentration of activity against Mtb H37Rv in vitro and whole blood assay were 52.07 and 416.56 μ M respectively. An undergoing phase 2 clinical trials of NTZ as a prospective drug for treating drug-susceptible patients with uncomplicated pulmonary TB was recently completed (NCT02684240 2020; StopTBPartnership 2020a, b).

Lansoprazole analogs

Rybniker et al. (2015) discovered that the benzimidazole drug lansoprazole (an inhibitor of the gastric proton pump in humans (Fig. 2)), when metabolized in the host cell from lansoprazole (LPZ) to lansoprazole sulfide (LPZS) via a sulfide reduction, has in vivo activity against intracellular DR-Mtb strains (MIC range = 0.49–1.78 μ M) and acts by

inhibition of the Mtb's cytochrome Bc1 complex. LPZS, however, does not retain the proton pump inhibition capacity of the parent compound LPZ and since LPZS appears to be non-cytotoxic and is easily commercially available, its direct use as an Mtb cytochrome b enzyme complex inhibitor was suggested for future research investigations (Herrmann et al. 2017; Mori et al. 2017; Rybniker et al. 2015; Yates et al. 2017). With the current low prevalence of cytochrome *bc1*-resistant mutations in the genome of Mtb complex (MTC) clinical isolates (Rybniker et al. (2019)), the future clinical application of LPZS shows great promise.

Oxazolidinones

Several oxazolidinones (bacterial translation and protein synthesis inhibitors) currently used against MDR gram-positive bacteria (e.g., linezolid (MRX-1), tedizolid, and tedizolid (Fig. 2)) have recently been investigated for use in TB chemotherapy. As of 2016, MRX-1 (MIC = 0.5 μ M) and its prodrug MRX4 were reportedly undergoing phase 1 and 3 clinical trials in the USA and China (MicruRX) (NCT03033329 2020). Recent clinical trial reports of tedizolid, a linezolid analog with unique bactericidal activity against Mtb, targeting improved treatment outcomes in drug-resistant TB patients (monotherapeutically and combinatorially), are also available (NCT01225640 2020; NCT03199313 2020; Wallis et al. 2014). Both tedizolid and tedizolid phosphate have been investigated as possible alternatives to LZD in anti-TB treatment. Deshpande et al. (2018b) indicated that tedizolid has comparatively better efficacy than LZD in infants, without exhibiting mitochondrial gene toxicity or the cytotoxicity induced by LZD treatment. Deshpande et al. (2018b) also indicated a significant bacterial killing capacity using a combination treatment strategy of tedizolid and moxifloxacin. Of all the oxazolidinones investigated to date, TZD has comparatively the best MIC of 0.33 μ M (Shoen et al. (2018). Tedizolid's prodrug tedizolid phosphate (next-generation oxazolidinone) is metabolized in vivo to microbiologically active tedizolid by plasma phosphatases (Molina-Torres et al. 2014) and in this active state, tedizolid phosphate inhibits bacterial translation and protein synthesis of various gram-positive organisms (e.g., linezolid-resistant staphylococci, methicillin-resistant *S. aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA), coagulase-negative staphylococci, *Streptococcus agalactiae*, vancomycin-resistant *Enterococcus* spp. and *Streptococcus pyogenes*) (Lee and Caffrey 2018).

Tetracyclines

Over the last 40 years, doxycycline (Fig. 2) has been investigated and its cytotoxicity, MOA, resistance profiles, and market availability merits are well described. In 2017, a phase 2

Table 3 Drugs with repositioning potential for anti-TB therapy

Drug	Previous application	Target/mechanism of action for TB	Method of investigation	Antimycobacterial evaluation and MIC values	Cytotoxicity evaluation	Reference
β	-lactams-penicillin combination (a. CAV; b. pen-avi; c. pen-ceft-avi)	Anti-multidrug-resistant Gram-negative bacteria	β -lactamase cell wall synthesis inhibition	Broth microdilution and BACTEC MGIT assay; a. MIC of CAV for Mtb H37Ra and H37Ra = 19.72 μ M; b. median MIC of pen-avi for MDR-TB strains = 2.99 μ M and MIC for Mtb H37Ra infected THP-1 human-derived monocytes in the HFS-TB = 11.97 μ M (pen); MIC of ceft-avi-pen is unspecified	Clinical trial dosage of ceft-avi simulation = 50 mg/kg; Clinical trial dosage of pen-avi simulation in neonates = 10,000 U/kg/h and in adults = 25,000 U/kg/h; pen-ceft-avi = NA	
(Deshpande et al. 2017); (Deshpande et al. 2018a)						
Diosmin (DIO) with amoxicillin (AMC)	Antioxidant, antihyperglycemic, antitumorigenic, anti-inflammatory, and antiulcer agent	L,D-transpeptidase enzymes inhibition	BACTEC MGIT 960 assay (7H9-OADC and 7H11; Mtb H37Ra and MDR clinical strain - complete growth inhibition at combined DC (AMC: $\geq$ 54.73 μ M and DIO: 821.69 μ M); and DC of 2.73 mM AMC and 3.29 mM DIO lead to 66% increased fly survival in <i>M. marinum</i> infected <i>D. melanogaster</i> fly model;	NA		(Pushkaran et al. 2019)
Rimonabant silicon analogs (12f, 18 and 18e)	anti-obesity treatment (cannabinoid receptor-1 (CB1) inhibitor)	Cell wall disruption	MABA (7H9-OADC); MIC of 12f, 18 and 18e for Mtb H37Rv = 2.44, 0.07 and 0.88 μ M	MTT assay; IC ₅₀ for lung epithelial cells (A549) and human hepatoma cells (HepG2) = $\geq$ 3 μ M		(Ramesh et al. 2016)
Nitrothiazole (Nitazoxanide)	Anti-protozoal; anti-bacterial (specifically anaerobic bacteria)	Disruption of Mtb membrane potential and pH homeostasis and stimulation of host macrophage autophagy in TB patient; non-competitive inhibition of the PFOR in anaerobic bacteria	BACTEC MGIT assay (7H9 medium); MIC for Mtb H37Rv = $\geq$ 52.07 μ M; WBA (RPMI 1640 medium); MIC for Mtb H37Rv = $\geq$ 416.56 μ M	NA		(de Carvalho et al. 2009; Harausz et al. 2016; Maitra et al. 2015)
Lansoprazole analogue (lansoprazole sulfide (LPZS))	Treatment of peptic ulcer, gastroesophageal reflux, and Zollinger–Ellison syndrome (proton pump inhibitor (PPI))	Cytochrome <i>bc</i> ₁ complex inhibition	REMA assay (7H9 medium); MIC for Mtb H37Rv and 6 INH/RIF-R strains = 0.49–1.78 μ M;	High-Throughput drug screen assay; IC ₅₀ for Mtb Erdman infected MRC-5 human lung fibroblasts and RAW264.7 macrophages = 0.59 μ M; Broth		(Rybníček et al. 2015)

Table 3 (continued)

Drug	Previous application	Target/mechanism of action for TB	Method of investigation	Antimycobacterial evaluation and MIC values	Cytotoxicity evaluation	Reference
Oxazolidinone (Tedizolid)	Antibiotic for	multidrug/vancomycin-resistant Gram-positive bacteria	Inhibition of bacterial protein synthesis/bacterial translation/ bacterial ribosome	Micro- and macro-broth dilution and MGIT assay (7H9-OADC media); MIC for Mtb H37Rv, H37Ra, CDC1551, HN878, and 18b = 0.33–1.35 μ M	microdilution ((7H10 medium); Mtb H37Rv infected female BALB/c mice treated with a DC of 300 mg/kg (no signs of toxicity)	HFS-TB (RPMI 1640 medium); Mtb H37Rv-infected THP-1 cells treated with the DC of 200 mg/day (no influence on mitochondrial enzyme inhibition)
(Deshpande et al. 2018b; Molina-Torres et al. 2014; Shoen et al. 2018; Srivastava et al. 2018)						
Tetracycline derivative (Doxycycline)	Anti-respiratory tract infection	Inhibition of bacterial protein synthesis and inhibit matrix metalloproteinase (MMP)	BACTEC MGIT assay (7H9-OADC media); MIC for various Mtb clinical strains = 18–36 μ M; Luminex and REMA Assay (7H9 and 7H11 media); MIC for H37Rv = 5.6 and 11.25 μ M	NA		(Gonzalo et al. 2015); (Walker et al. 2012)
Nitroimidazoles: Bicyclic 4-nitroimidazole derivative (metronidazole)	Broad-spectrum antimicrobial activity; treatment of trichomoniasis, ulcerative gingivitis, amoebic dysentery, and Chagas disease	Inhibition of bacterial nucleic acid synthesis via ferredoxins non-enzymatic reduction	REMA and Broth microdilution (7H9-OADC media); H37Rv and 55 MDR-Mtb strains (94.5% were susceptible when tested at a DC of 187.13 μ M)	NA		(Fan et al. 2018a; Imperiale et al. 2017; Lewis and Sloan 2015)
Polymyxin: colistin methanesulfonate and colistin)	Broad-spectrum antibiotic (treatment of cystic fibrosis and ventilator associated pneumonia)	Disruption of cell wall	MABA (7H10 media and Sauton media); MIC of colistin for Mtb H37Ra and Mtb H37Rv = 1.73 μ M; MIC of CMS not specified	NA		(Koen et al. 2018a; Koen et al. 2018b; Van Breda et al. 2015); (Bax et al. 2015)

HFS-TB, hollow fiber system model of TB; MGIT, mycobacteria growth indicator tube; MABA, Microplate Alamar Blue Assay; REMA, resazurin reduction microplate assay; NA, not applicable; WBA, whole-blood assay; DC, drug concentration; CAV, ceftazidime-avibactam; Pen-avi, penicillin-avibactam; Pen-ceft-avi, penicillin-ceftazidime-avibactam

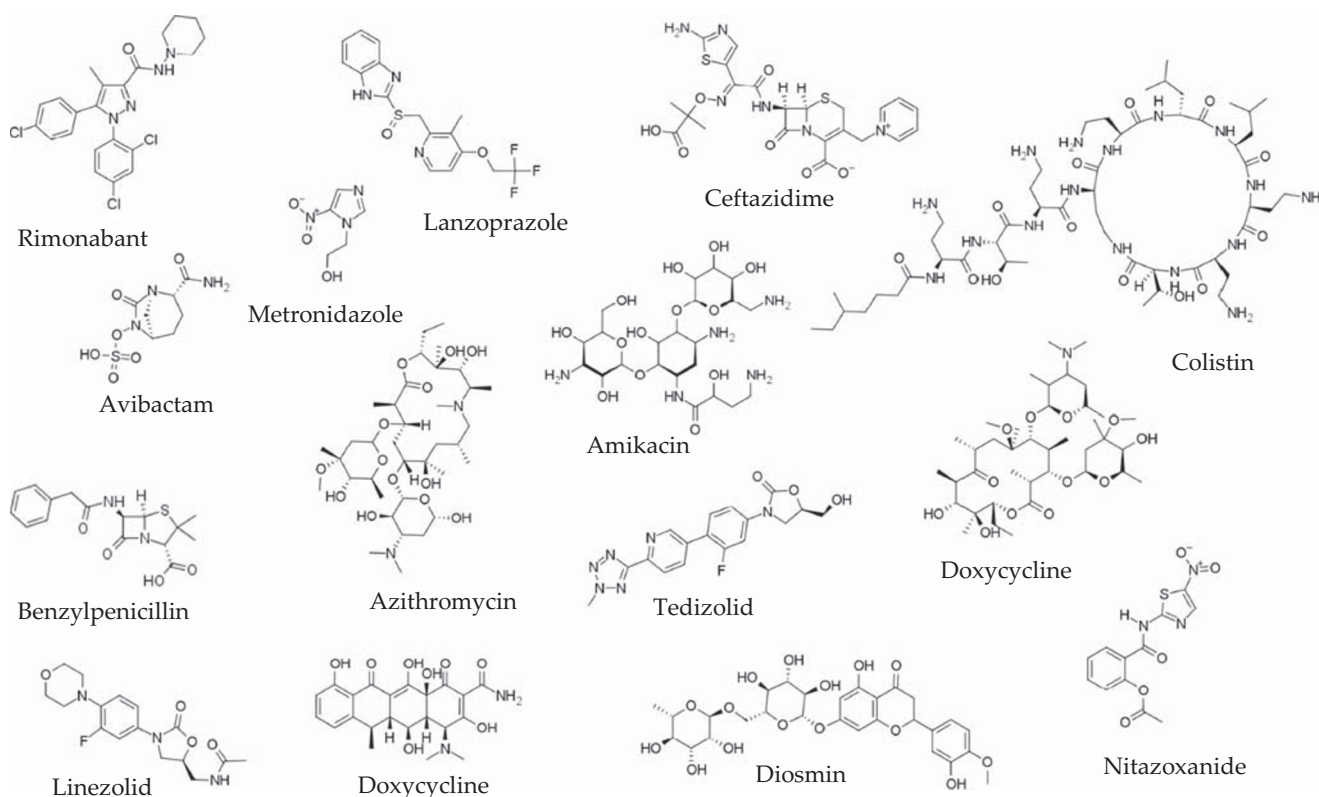


Fig. 2 Chemical structures of the drugs with potential for reformulation and inclusion in TB therapy

clinical trial that commenced in 2015 to determine doxycycline's efficacy in host immunomodulation in pulmonary TB patients was completed. Data and outcomes for this study are, however, presently unavailable (NCT02774993 2020). Also, Gonzalo et al. (2015) assessed the synergistic, indifference, or antagonistic activities of amikacin (semi-synthetic aminoglycoside antibiotic) and doxycycline against 17 MDR-, 1 RIF-resistant-, 3 XDR-, 1 poly-resistant (streptomycin/isoniazid)-, 1 isoniazid-resistant-, 5 fully susceptible-, and H37Rv-Mtb strains. A synergistic complementation between amikacin ($MIC_{50} = 0.85 \mu M$ and $MIC_{90} = 1.71 \mu M$) and doxycycline ($MIC_{50} = 18 \mu M$ and $MIC_{90} = 36 \mu M$) against 18 of the 29 strains tested was observed without negative drug-drug interaction. To circumvent Mtb's doxycycline efflux-mediated resistance, further investigation into this synergistic mechanism is required considering the proposed adjunctive use of matrix metalloproteinases (MMPs) inhibitors (Sabir et al. 2019; Walker et al. 2012).

Metronidazole

Driven by the approval of the bicyclic 4-nitroimidazoles anti-TB prodrugs DLM ($MIC = 0.011\text{--}0.044 \mu M$) and PMD ($MIC = 0.04\text{--}0.70 \mu M$) (Keam 2019), new efforts have been made towards the synthesis of novel nitroimidazole derivatives with improved efficacy against Mtb, and less

toxicity to the patient (Alliance 2020; NCT01424670 2020; NCT03086486 2020). However, it is significant to mention the phase 3 clinical trial of metronidazole for combinatorial TB therapy completed in 2013 (NCT00425113 2020). Metronidazole is a broad-spectrum antibiotic (orally administered 250–750 mg/day (Fig. 2)) that inhibits nucleic acid synthesis via ferredoxins non-enzymatic reduction. A concise review by Fan et al. (2018a), for further reading on this compound class, discusses the recent discoveries regarding imidazole-containing derivatives with potential anti-Mtb properties.

Polymyxins

Polymyxins (e.g., polymyxin B/colistin) and CST are typically used to treat DR gram-negative bacterial infections; however, their application for use as anti-TB drugs, initially indicated unfavorable pharmacodynamics and cytotoxicity when administered alone. Recent studies, however, suggest that the inclusion of colistin and its analog CMS, in combination with other first- or second-line anti-TB drugs, improves on their pharmacokinetics and pharmacodynamics (Bax et al. 2015). More specifically, when amikacin and INH were administered with colistin, they showed improved antimycobacterial activity, especially in highly metabolically active Mtb populations, and additionally, this combination treatment approach

prevented Mtb from developing resistance to the amikacin and INH (Bax et al. 2015). Colistin (Fig. 2) acts by disrupting the Mtb cell wall making it more permeable to other drugs (Koen et al. 2018a; Koen et al. 2018b; Van Breda et al. 2015), and the reduced toxicity of CMS makes it more attractive for use in combinational anti-TB drug regimens.

Macrolides

The macrolides azithromycin (AZM; Fig. 2) and clarithromycin (CLR) are traditionally anti-inflammatory agents, which also exhibit broad-spectrum antibiotic properties via the inhibition of bacterial protein synthesis. In 2013, a phase 4 clinical trial investigating the benefits of combining AZM and LZD in treating MDR- and XDR-TB patients commenced and was recently completed. (NCT01521364 2020). A phase 2 clinical trial investigating the possible use of azithromycin for host-directed therapy in pulmonary TB equally also commenced in 2018 and aims to determine the immunomodulating activity of AZM in adult pulmonary TB patients undergoing TB first-line drug therapy (NCT03160638 2020). Another randomized phase 3 trial involving AZM, evaluating the merits of employing response to broad-spectrum antibiotics as an exclusion diagnostics for TB in adult patients against the risk of antimicrobial resistance (AMR) is currently recruiting (NCT03545373 2020).

The possibility of using CLR as an anti-TB agent is still not known since its monotherapeutic activity against Mtb is only weakly bacteriostatic. However, it has been reported to augment the activities of the INH, ETH, RIF, LZD, and spectinomycin in vitro, while also exhibiting immunomodulatory effects similar to that of azithromycin (Bolhuis et al. 2013; NCT01521364 2020; van der Paardt et al. 2015; Van der Paardt et al. 2017). Furthermore, a recent report by Van der Paardt et al. (2017) indicated that the prolonged use of CLR with 4 other anti-TB agents (MFX, AMC, cycloserine, para-aminosalicylic acid, and prothionamide), in patients diagnosed with MDR-TB, further supports the tolerability and safety of macrolides for possible prolonged clinical use. Considering the above, both drugs are potentially important anti-TB drug candidates that should be further investigated for their possible applications in synergistic/combinational anti-TB drug treatment options (especially for MDR/XDR pulmonary TB). Figure 2 highlights the chemical structures of these reusable drugs in TB treatment.

Adjunct drugs with potential for reuse in anti-TB therapy

Apart from the aforementioned potential anti-TB agents being investigated for use as monotherapeutic agents and/or in a combinatory fashion with other known anti-TB agents, and/

or as prodrugs for treating TB, there are also other well-known compounds currently being considered for use in treating various other symptoms/diseases associated with TB (Haraus et al. 2017). Various TB-associated infections or comorbidities occurs as a result of the immunocompromised state of the TB patient, which makes them easily susceptible to other infections/diseases including malaria, HIV, TB meningitis, hepatitis, nosocomial infections, and diabetes (Caraffa et al. 2018; Keddie et al. 2018). Subsequently, host-directed drug therapy approaches (in combination with classical traditional antimicrobial strategies) have been investigated of late (Kroesen et al. 2017; Rayasam and Balganesch 2015). To this end, several compounds or drugs used for the treatment of other TB-associated diseases (Table 4) have been investigated and shown to function complementary to the existing anti-TB drugs, improving the efficacy of the latter (Ordóñez et al. 2014; Zumla et al. 2014). Since many of these compounds or drugs function by improving/altering the host's metabolism and immune action, they become less prone to Mtb pathogen resistance. The chemical structures of these adjunctive group of drugs with potential for reuse in anti-TB therapy are shown in Fig. 3.

Metformin, for instance, is a popular anti-diabetic drug, but recent reports have indicated that metformin inhibits the growth of mycobacteria intracellularly (Wallis et al. 2016), via the induction of reactive oxygen species, while also enhancing the activity of other anti-TB drugs (Gns et al. 2019; Mishra et al. 2018). Several studies have also shown that efflux pump inhibitors, exert promising activities against both drug-susceptible and DR-Mtb isolates, and also augment the efficacy of anti-TB drugs when administered as a co-treatment option (Chen et al. 2018; Chien et al. 2017; Gupta et al. 2013; Jaiswal et al. 2017; Jang et al. 2017; Pieterman et al. 2018; Pule et al. 2015). Verapamil (40–240 mg/day (oral standard)) and reserpine (0.1–0.25 mg/day (oral standard)) (traditionally used for treating high blood pressure, angina, and supraventricular tachycardia) are known efflux pump inhibitors and when used in conjunction with the anti-TB agents CFZ and BDQ dramatically increased drug efficacy of the two latter anti-TB agents in vitro (as determined by a marked decrease in the MIC of the two drugs to Mtb) but not for CFZ in vivo (Gupta et al. 2015; Xu et al. 2017a). When verapamil and reserpine have additionally been shown to reduce macrophage-induced drug tolerance (Mishra et al. 2018), indicating their capacity to be used to potentially shorten treatment duration. The synergistic activity of verapamil, with for example BDQ in vitro and murine models, however, was reported to be inconsistent (Field 2015). According to Huang et al. (2013), reserpine decreased the MIC of ciprofloxacin in 30% of ciprofloxacin-susceptible Mtb strains tested, and in 50% of ciprofloxacin-resistant Mtb efflux pump carriers tested, while further exhibiting the capacity to subvert the mechanisms of resistance of a few other clinically relevant Mtb

Table 4 Adjunct drugs with potential for reuse in anti-TB therapy

Drug	Previous application	Application for TB (target/mechanism of action)	Antimycobacterial activity outcome/evaluation method/trial phase	Dosages currently used	Reference
Metformin	anti-diabetic drug; inhibition of mitochondrial respiration (AMP-activated protein kinase (AMPK))	Induction of intracellular ROS and activation of AMPK	Reduced risk of subsequent active TB; cohort study	500–1000 mg/day (orally)	(Lee et al. 2018; Rena et al. 2017; Tseng 2018)
Efflux pump inhibitors a. verapamil b. reserpine and derivatives c. carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	a. and b. antihypertensive drug; c. antipsychotics; insecticide; anti-helminthic	a., b., and c. inhibition of efflux system and increasing serum concentrations of companion drugs	a. MIC for Mtb H37Rv = 393.95 µM; and increased oral bioavailability of bedaquiline on BALB/c mice; MABA b. 50–82% inhibition of Mtb H37Rv at a DC of 6.25 µg/mL; Broth microdilution and MABA; c. 2–8-fold reduction of the MIC of RIF for 7 MDR-Mtb isolates treated with a DC of 2 µg/ml 7H9 broth microdilution:	a. 40–240 mg/day (orally); b. 0.1–0.25 mg/day (orally); and c. NA	a. (Gupta et al. 2013; Xu et al. 2017a); (DB00661 2020); b. (Begum et al. 2012; DB00206 2020); and c. (Kardan-Yamchi et al. 2019)
Cilostazol (phosphodiesterase inhibitors)	Antiplatelets, vasodilator, anti-hypertension, and anti-erectile dysfunction	Cellular phosphodiesterase inhibition	Decreased time to bacterial clearance with standard TB treatment tested on Mtb H37Rv infected BALB/c and C3HeB/FeJ mice treated with a DC of 36.94 µg/ml; 7h11 broth microdilution	10 mg/kg or 50–100 mg/day (orally)	(DB01166 2020; Maiga et al. 2012);
Cholecalciferol (vitamin D3; secosteroid dietary supplement)	Treatment and prevention of bone disorders;	Reduced risk of developing active TB	Phase 3 clinical trial	Variable	(NCT02276755 2020)
Ibuprofen (cyclooxygenase (COX) inhibitor)	An anti-inflammatory for treating juvenile rheumatoid arthritis and osteoarthritis	Improve immune-mediated host clearance of Mtb; nonselective inhibitor of cyclooxygenase (COX)	Reduction of lesions and bacillary load in lungs of Mtb H37Rv infected C3HeB/FeJ mice treated with a DC of 80 mg/kg daily; 7H11 broth dilution; phase 2 clinical trial	100–800 mg/day (orally)	(Vilaplana et al. 2013); (NCT02781909 2020)
Etoricoxib (cyclooxygenase-2 (COX-2) inhibitor)	Treatment of osteoarthritis, ankylosing spondylitis, rheumatoid arthritis;	Improve immune-mediated host clearance of Mtb; anti-inflammatory	Phase 1 clinical trial	unspecified	(NCT02503839 2020); (DB01628 2020)
Prednisolone (phospholipase A2 inhibitor)	Adjunctive anti-inflammatory and immunosuppressant therapeutic agent	Anti-pericarditis; inhibition of phospholipase A2	Lowered incidence of immune reconstitution inflammatory syndrome; phase 3 clinical trial completed	20–40 mg/day (orally)	(Meinijes et al. 2018); (NCT01924286 2020)
Levamisole	Broad-spectrum medication (anti-helminthic; anti-rheumatic; antiparasitic)	Immune modulation	Decreased tuberculin reaction in patients with initial Mantoux ≥ 20 mm; human preclinical trial	150 mg/day (orally)	(Shamkuwar et al. 2017)
Rifabutin modified therapy (a. everolimus (mTOR inhibitor); b. auranofin; c. cholecalciferol (vitamin D3; secosteroid dietary supplement); and d. CC-11050)	a. Immunosuppressant in cancer therapy; b. Anti-inflammatory in rheumatoid arthritis therapy (possible inhibitor of kappa-B kinase and thioredoxin reductase); and c.	Shorten treatment duration and/or prevent permanent lung damage	Phase 2 clinical trial	a. 2.5–10 mg/day (orally); b. 3 mg/day (orally); c.	(NCT02968927 2020)

Table 4 (continued)

Drug	Previous application	Application for TB (target/mechanism of action)	Antimycobacterial activity outcome/evaluation method/trial phase	Dosages currently used	Reference
	Treatment and prevention of bone disorders			variable; and d. unspecified	
<i>MABA</i> , Microplate Alamar Blue Assay; <i>DC</i> , drug concentration; <i>ROS</i> , reactive oxygen species					

strains (for example, D94A *gyrA* and D94G *gyrA* Mtb mutants).

The effluxes carbonyl cyanide 3-chlorophenylhydrazone (CCCP), piperine, and verapamil have the potential to improve the potency of telacebec-Q203 at non-cytotoxic concentrations (Jang et al. 2017). Q203 is a recently discovered imidazopyridine amide Mtb growth inhibitor that targets the respiratory chain cytochrome bcc complex (Jang et al. 2017; Pethe et al. 2013). In the Jang et al. (2017) study, verapamil also increased the potency of Q203 (16.5–3.5 nM) and BDQ (168.6–17.3 nM) as opposed to MOX (0.3–0.29 μ M). The augmentation of colistin's bioactivity against MDR *Enterobacteriaceae* and MDR-/XDR- and RIF-resistant Mtb isolates, using another efflux pump carrier, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 0.05–0.25 mg/day), has also been reported (Kardan-Yamchi et al. 2019; Osei Sekyere and Amoako 2017). Sinha et al. (2017) additionally indicated the synergistic efficacy of using CCCP in combination with a novel bisbenzimidazole, 3'H-2,5'-bibenzimidazole (PPEF) (a topoisomerase IA poison inhibitor and non-gyrase inhibitor), against various other XDR bacterial strains (apart from that of Mtb). Considering the above, the use of efflux pump inhibitors in combination with anti-TB drugs shows very real promise.

Zumla et al. (2014) and Zumla et al. (2015) indicated that some non-steroidal anti-inflammatory drugs (NSAIDs) also have anti-Mtb properties and the capacity to reduce Mtb load and subsequently minimize lung damage in mice. For example, two phosphodiesterase inhibitors, cilostazol (an antiplatelet agent usually administered at 50–100 mg/day) and sildenafil (an anti-hypertension and anti-erectile dysfunction medication usually administered at 20–100 mg/day), when used in combination with the standard first-line anti-TB treatment regimen, were shown to reduce tissue damage and speed up mycobacterial clearance and the time required for lung sterilization, by 1 month (Maiga et al. 2012; Maiga et al. 2013; Rayasam and Balganes 2015). Likewise, ibuprofen, a cyclooxygenase (COX) inhibitor, administered at a dose of 100–800 mg, increased the efficacy of those drugs being used to treat XDR-TB (Vilaplana et al. 2013). On the other hand, however, according to Naftalin et al. (2018), celecoxib (also a COX inhibitor), neither displayed activity when used alone or in combination with standard anti-TB drugs (RIF or PZA), as assessed using a whole-blood bactericidal (WBA) assay. The outcome of the in vivo clinical trials currently being conducted on the use of etoricoxib another COX inhibitor as adjunct anti-TB drugs (NCT02503839 2020) should give more definitive answers.

Using isoprinosine to activate the host humoral and cell-mediated immune response (including that associated interleukin-2 (IL-2), interleukin-4 (IL-4), tumor necrosis factor-alpha (TNF- α), tumor necrosis factor-beta (TNF- β), and interferon-gamma (IFN- γ)) was recently reiterated by

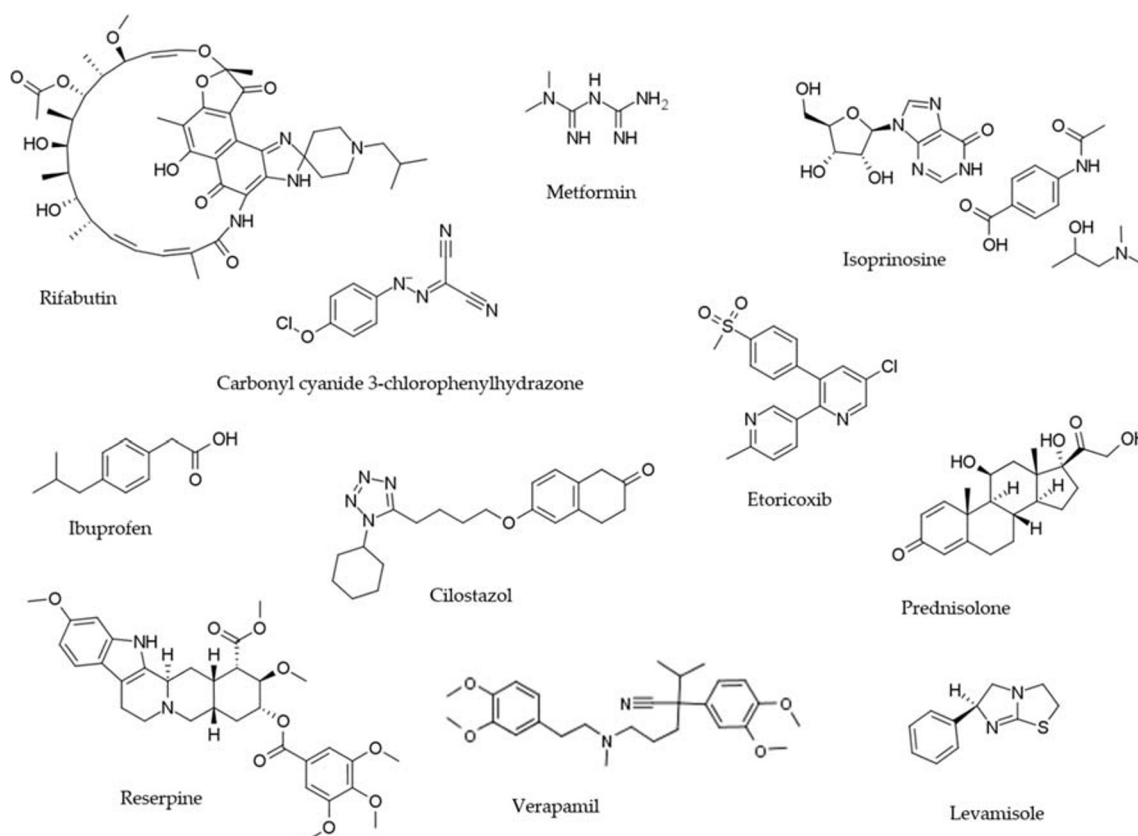


Fig. 3 Chemical structures of drugs with adjunctive potential in anti-TB treatment

Mishra et al. (2018). The authors suggest that immune augmentation by isoprinosine could most likely enhance the expulsion of *Mtb* in the host, thereby further improving the patient's response to the anti-*Mtb* chemotherapy. Isoprinosine is an approved antiviral drug (typically administered at a dosage of 500 mg/day) with known toxicity and pharmacokinetics; hence, further research into its adjunctive synergistic role in anti-TB regimen is worth pursuing. Another human clinical trial by Shamkuwar et al. (2017) proved that the immunomodulatory adjuvant levamisole (administered at a dosage of 150 mg/day) reduced the treatment duration of standard anti-TB drugs, in the absence of any serious side effects. Complementary to these findings, Caraffa et al. (2018) indicated that despite an early diagnosis of tuberculous meningitis (TBM), immediately followed by standard anti-TB treatment, in conjunction with adjuvant corticosteroid therapies, a positive treatment outcome was only achieved after the addition of the administration of the adjuvant immunomodulatory drug thalidomide.

Corticosteroid (e.g., prednisolone) usage during TB treatment (along with standard TB regimen) has been long reported to reduce the effect of drug toxicity, and several other disease complications. Mayosi et al. (2014) previously reported that although the administration prednisolone (anti-inflammatory/immunosuppressant) correlates with a reduced incidence for developing constrictive pericarditis in tuberculous

pericarditis patients, it showed no significant effect in reducing mortality rates due to tuberculous constrictive pericarditis and cardiac tamponade. The researchers further associated an increased incidence of cancer in patients using prednisolone. Based on the clinical trial that was completed in 2017 (NCT01924286 2020), Meintjes et al. (2018), however, reported a reduction in the incidence of paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) due to prednisolone administration, without an increased incidence of developing cancers and or an increased susceptibility for acquiring severe infections. The use of prednisolone administered at a dose of 5–60 mg/day for alleviating TB comorbidities has also recently been revisited (Walker et al. 2018). In light of the aforementioned contradictions, future studies are necessary to achieve a more definitive insight into prednisolone's TB immunotherapeutic efficacy (e.g., its use clinically for preventing TB-IRIS). Clinical evaluations of the adjunctive role of other corticosteroids to reduce TB mortality, or accelerate TB patient recovery are also still needed.

An interventional phase two clinical evaluating the safety, efficacy and biomarker response of host-directed drugs (i) everolimus: (immunodepressant—mTOR (mammalian target of rapamycin) inhibitor); (ii) auranofin: (anti-inflammation—possible Kappa-B kinase and thioredoxin reductase inhibitors); (iii) cholecalciferol: (secosteroid dietary supplement—

regulation of gene transcription); and (iv) an unknown compound (CC-11050), in combination with rifabutin in adults with drug-sensitive smear-positive pulmonary TB has been ongoing since 2016 (NCT02968927 2020). The rifabutin-multiple host-directed drug trial seeks to determine the potential of the regimen to shorten TB treatment duration and/or avert perpetual lung damage. Another clinical trial evaluating cholecalciferol's (vitamin D3) role in lowering the risk of contracting latent TB in school-age Mongolian children has also been ongoing since 2014 (NCT02276755 2020). Since the focus of host-directed anti-TB therapies is to facilitate the clearance of the mycobacteria, improve patient compliance and shorten treatment duration, studies on the above adjuvants as regards TB regimen remain an important future research domain. As a final note, we recommend the review paper by Kroesen et al. (2017), highlighting the importance of adjunct therapies for enhancing the potency of various monotherapeutic anti-TB agents, for further reading on the topic.

Final remarks

Several investigational compounds have the potential for further development as additional parts of the anti-TB regimen, as previously mentioned. Their inclusion in TB therapy will not only lead to shortened treatment by adding synergistic effects (such as griselimycin with RIF and PZA) but can also lead to higher success rates by improving effectivity against resistant *Mtb* strains (such as nitazoxanide, nitrofurantoin, and isoniazid hydrazides) or preventing certain resistance mechanisms (such as dihydropyridomycin). Further proving the importance of building on investigational compounds is the probability to improve patient adherence by reducing drug complications and adverse effects due to lower cytotoxicity and hepatotoxicity (as indicated by primaquine derivatives and isoniazid hydrazides).

Several factors can hinder the advancement of research involving drug repurposing—including legislative, organizational, regulatory, and technical factors that cannot be overlooked (Pushpakom et al. 2019). The success of drug repositioning leverages on the facts that (1) common biomolecular pathways are associated with different diseases mechanisms and that (2) previously approved drugs for other diseases are an important consideration for treating such since they have already been determined to have favorable human pharmacokinetic properties, the latter of which often helps circumvent the early phase clinical trials, and subsequently reduces the costs and time to bring such drugs to market. Selecting such, however, requires a thorough search of the literature housed in the drug repositories and databases (e.g., Therapeutic Target Database (TTD), Pubchem, Pathway Interaction Database (PID), Search Tool for Retrieval of

Interacting Genes/Proteins (STRING), Clinical trials and Adverse Effect data and Side Effect Resource (SIDER)), as well as an in-depth understanding of a particular disease and the mechanisms by which successful drug candidates already function in alleviating such disease, to predict which other compounds, already available for the treatment of other diseases, may be investigated for treating these (Gns et al. 2019).

It is encouraging to see a vast amount of possible new candidate compounds, suggested to be used as either monotherapeutic or combined therapy options, particularly towards eradicating TB. It is however imperative that the many possible drug candidates with direct or indirect anti-TB activity (e.g., cilostazol, sildenafil, NTZ, mefloquine, tizoxanide, metronidazole, entacapone, ibuprofen, and tolcapone), previously documented to have extremely promising monotherapeutic or adjunctive activity against *Mtb* in vitro (Harausz et al. 2016; Vilaplana et al. 2013), be tested in vivo, and possibly later in human clinical trials. Insights gathered here reveal that the focus of recent anti-TB drug development program is centered around developing drug combinations or regimens able to shorten treatment durations instead of monotherapeutic anti-TB agents; therefore, more studies focused on drug-drug interactions (licensed and candidate anti-TB drugs) need to be prioritized.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies involving human participants or animals.

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Article

Elucidating the Antimycobacterial Mechanism of Action of Ciprofloxacin Using Metabolomics

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Abstract: In the interest of developing more effective and safer anti-tuberculosis drugs, we used a GCxGC-TOF-MS metabolomics research approach to investigate and compare the metabolic profiles of *Mtb* in the presence and absence of ciprofloxacin. The metabolites that best describe the differences between the compared groups were identified as markers characterizing the changes induced by ciprofloxacin. Malic acid was ranked as the most significantly altered metabolite marker induced by ciprofloxacin, indicative of an inhibition of the tricarboxylic acid (TCA) and glyoxylate cycle of *Mtb*. The altered fatty acid, *myo*-inositol, and triacylglycerol metabolism seen in this group supports previous observations of ciprofloxacin action on the *Mtb* cell wall. Furthermore, the altered pentose phosphate intermediates, glycerol metabolism markers, glucose accumulation, as well as the reduction in the glucogenic amino acids specifically, indicate a flux toward DNA (as well as cell wall) repair, also supporting previous findings of DNA damage caused by ciprofloxacin. This study further provides insights useful for designing network whole-system strategies for the identification of possible modes of action of various drugs and possibly adaptations by *Mtb* resulting in resistance.

Keywords: fluoroquinolones; ciprofloxacin; untargeted metabolomics; *Mycobacterium tuberculosis*; tuberculosis; GCxGC-TOFMS



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1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), remains one of the leading causes of death globally from a single infectious agent [1], resulting in a mortality rate of 1.5 million and an infection rate of about 10 million annually [2]. Furthermore, the prevalence of drug resistant TB is also increasing, primarily due to poor adherence to the drug regimen in patients [3], as a result of the many side effects experienced by patients being treated with first-line anti TB medication, accompanied by the long treatment duration required [4,5]. Further contributing factors to developing drug resistant TB include inaccurate diagnosis, unsupervised treatment, poor economic status [6], and a further exacerbation in 2020 by the COVID-19 pandemic [2,7]. Currently, the WHO's approved first-line therapy for patients with active TB is a 6-month "directly observed treatment short-course" (DOTS) regimen consisting of isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and rifampicin (RIF) [8,9]. Infection with multi-drug resistant (MDR)-TB and extensively drug resistant (XDR)-TB requires treatment using various second-line antibiotics that are expensive, have far more side effects due to their higher toxicity, and need to be consumed for even a longer duration [2,10]. The only newly approved drugs for TB over the past 50 years are the second-line drugs for treating MDR-TB—linezolid, bedaquiline and delamanid—but not long after, resistance followed [9,11,12].

Considering this, there is an urgent need for well-tolerated and effective treatments for TB using drugs with novel modes of action against the infectious organism.

A suggested approach for avoiding the long drug trial phases usually required for approving new drug candidates is further investigation of already existing drugs, repurposed for use in treating TB/MDR-TB, [13,14]. In order for a drug to be selected for possible repurposing applications, it should preferably be affordable, easily available, and show good pharmacokinetic/pharmacodynamic properties. For these reasons, several fluoroquinolones (FQs) are being intensively investigated for use as anti-TB therapy [15]. FQs, originally used to treat urinary tract infection [16], were first shown to be effective against *Mtb* in 1984, and have since gained continuous interest for such applications [17–19]. Currently, they are among the most frequently prescribed drugs [20] and are considered the backbone of MDR-TB treatment [10,21]. FQs target two *Mtb* topoisomerase deoxyribonucleic acid (DNA) enzymes, DNA gyrase and topoisomerase IV [22]. The first introduces negative super helical twists in the bacterial DNA double helix and catalyzes the separation of daughter chromosomes [23], whereas the latter is responsible for the segregation into two daughter cells at the end of DNA replication [24]. Earlier generations, ciprofloxacin and levofloxacin, exhibit greater activity against Gram-negative bacteria (and some Gram-positive bacteria) and target mainly DNA gyrase [25–27]. FQs inhibit DNA gyrase by binding to the enzyme and DNA, which leads to double-stranded DNA breaks [28–32].

The innate resistance mechanism of *Mtb* to many anti-TB drugs can be attributed to its complex cell wall [33]; however, the specific, highly lipophilic characteristics of FQs [34] provide great permeability over this [35]. These antibiotics chelate with Mg^{2+} cations and electrostatically interact with membrane phosphodiester, subsequently traversing the *Mtb* cell wall [36]. FQs are not exempt from resistance, however, of which the best described are mutations in genes *gyrA* and *gyrB*, encoding subunit GyrA and GyrB of DNA gyrase [37–39]. Furthermore, several resistance-forming proteins have also been identified, including the efflux pumps, LfrA [40,41], and MmpL (mycobacterial membrane protein large) [42], the target protection proteins, MfpA and MfpB (*Mycobacterium* fluoroquinolone resistance proteins A and B) [43,44], and the cell-survival promotor, HtrA2 (high temperature requirement A) [45]. DNA repair and mutations by the SOS regulon have also been described [18].

Although interactions between DNA gyrase and FQs have been thoroughly investigated [22,38,46], little is known about their biochemical mechanisms of action against *Mtb* specifically, or *Mtb* drug resistance to these [10,19]. The results published thus far are somewhat contradictory. In a study by Verma et al. [47], the macromolecular composition of the *M. smegmatis* cell wall after sub-MIC ciprofloxacin treatment indicated a significant decrease in the total lipids, phospholipids, and sugars, suggesting ciprofloxacin-induced alterations of the cell wall. In contrast, Halouska et al. [48] indicated ciprofloxacin-induced inhibition of transcription, translation, and DNA supercoiling, without changes to the cell wall. While most data suggest cell death due to the inhibition of DNA replication [49], altered DNA biosynthesis could set in motion secondary events contributing to ciprofloxacin's bacteriostatic or bactericidal effects. Furthermore, it is important to remember that stronger target activity does not predict better antimycobacterial activity. This is perfectly demonstrated by ciprofloxacin dimers, which show enhanced DNA gyrase inhibition, while less effectively killing *Mtb* [50]. This is thought to be due to a stronger cleavage of FQ to the DNA-enzyme complex, which results in less single-strand DNA fragments, and subsequently prevents RecA from recognizing damaged DNA and inducing the SOS regulon [32]. The SOS response assists in killing by releasing ROS [51,52], yet simultaneously activates DNA repair and creates a dormancy state, ultimately leading to resistance. Before this SOS response can be used to advantage as a possible mode of action against *Mtb*, this phenomenon, and how it connects to the mechanism of ciprofloxacin, still needs to be elucidated.

The selection of ciprofloxacin as our investigational compound was predominantly based on its safety profile. Although less potent than moxifloxacin (MIC 0.12–0.5 $\mu\text{g/mL}$), and levofloxacin (MIC 1 $\mu\text{g/mL}$), ciprofloxacin (MIC 0.5–4.0 $\mu\text{g/mL}$) [53] has the lowest

risk for causing serious ventricular arrhythmia, cardiovascular mortality, and hepatotoxicity [54,55]. Furthermore, ciprofloxacin demonstrates the highest clearance rate of all FQs [56] and is thus the preferred option for the treatment of renally impaired patients [17]. The levels of ciprofloxacin in cerebrospinal fluid can be as high as 40–90% compared to that in plasma [53], which offers further advantages for its use in the treatment of tuberculous meningitis. Adverse drug reactions (ADRs) are usually minimal (5% or less), and the most common ADRs are usually gastrointestinal in nature (nausea, vomiting, diarrhea, and abdominal pain) [57,58]. Previous studies demonstrated that mitochondrial topoisomerases bear less than 30% homology to their prokaryotic counterparts and are not inhibited [23,59], but it has been reported that ciprofloxacin does affect mitochondrial DNA synthesis [60]. The pharmacological advantages of ciprofloxacin have not gone unnoticed, as stated by the World Health Organization [61], which has included ciprofloxacin as a critically important antibiotic.

Most of the evidence brought to light thus far has been generated using genomics, transcriptomics, or proteomics [62]. Metabolomics, the latest addition to “omics” technologies, identifies the down-stream metabolites of altered pathways and therefor presents a more sensitive level of organization, from which up-stream deductions can be made [8,63]. We identified the metabolite markers best differentiating *Mtb* with and without ciprofloxacin, using a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOF-MS) metabolomics approach, combined with universally connected metabolic libraries and advanced statistical analysis, in order to better elucidate its mechanism of action.

2. Materials and Methods

2.1. Bacterial Culture

Antimycobacterial minimum inhibitory concentration (MIC) and sub-MIC (50% inhibitory concentrations (MIC₅₀)) of ciprofloxacin were determined via the Alamar Blue assay [64]. The cell cultures (5 individually cultured samples per group) were prepared as previously described [65], in the presence and absence of ciprofloxacin. All reagents were purchased from Sigma-Aldrich, St. Louis, MO, USA, unless otherwise stated. Briefly, *Mtb* H37Rv ATCC 27294 (kindly obtained from the Medical Research Council, Pretoria, Gauteng, South Africa) was cultured and maintained for 4 weeks on Lowenstein Jensen (LJ) slants. The bacterial inoculum was prepared to a McFarland standard of 1 (approximately 3×10^8 colony-forming units/mL) in Middlebrook 7H9 broth supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson, UK) and 2% PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) (Becton, Dickinson, UK). PANTA was added for the prevention of contamination with negligible impact on *Mtb*'s growth [66]. Ciprofloxacin was dissolved in DMSO (150 µM), added as a vehicle control, and diluted into Middlebrook 7H9 broth to a final concentration of 0.3 µM (0.12 µg/mL) (0.2% DMSO). One milliliter of the prepared inoculum was added to yield a final assay volume of 5 mL, with a bacterial test concentration of 6×10^7 CFU per 1 mL of ciprofloxacin. For the untreated *Mtb* control samples, 4 mL of Middlebrook 7H9 broth (0.2% DMSO) was added to each replicate culture, followed by the addition of the bacterial inoculum as described above. The DMSO solvent was kept constant throughout the assay. After 5 days of incubation at 37 °C, the samples were centrifuged to pellet the bacteria at 4500 rpm for 15 min. The pellets were washed with 1 mL of PBS and pelleted again under the same conditions. Finally, the PBS was aspirated from the samples and the pellets were stored immediately at −80 °C until further testing.

2.2. Whole Metabolome Extraction Procedure and Derivatization

The metabolites were extracted from the samples and derivatized as previously described by Beukes, et al. [67], with slight modifications. Briefly, 8 mg of each of the individually cultured samples were weighed out into an Eppendorf tube, followed by the addition of 50 µL 3-phenylbutyric acid (0.13 mg/mL H₂O) (Sigma-Aldrich, Lot#536478V) as

internal standard. One milliliter of a chloroform: methanol: water (1:3:1 ratio) solution was added, after which the Eppendorf tubes were shaken in a vibration mill at 30 Hz for 5 min, with a 3 mm carbide tungsten bead in each. The samples were centrifuged at 12,000 rpm for 5 min and the supernatant was transferred to a GC glass vial. The extracts were dried under a nitrogen stream, followed by the addition of 50 μ L methoxamine hydrochloride (Sigma-Aldrich, Lot#BCBP2843V) in pyridine (Lot#S2BC335SV) at a concentration of 15 mg/mL. The glass vials were heated at 50 °C for 90 min. Following, methoximation, 40 μ L *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride (Lot#BCBW2670) was added, and vials were heated again for 60 min at 50 °C. Each extract was then transferred to a 0.1 mL vial insert in a GC sample vial and injected into GCxGC-TOF-MS.

2.3. GCxGC-TOFMS Analysis

A 4D Pegasus GCxGC-TOF-MS (LECO Africa (Pty) Ltd., Johannesburg, South Africa) equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, USA) coupled to TOF-MS (LECO Africa) were used for the analysis. The samples were analyzed in random sequence, with split-less injection. To monitor the analytical performance throughout the entire analysis, a quality control (QC) sample was analyzed at regular intervals. The processed samples were injected into Rxi-5Sil MS primary capillary column (28.8 m $\times$ 0.25 mm internal diameter, 0.25 μ m film thickness, Restec), and a Rxi-17 secondary capillary column (1.2 m $\times$ 0.25 mm internal diameter, 0.25 μ m film thickness), for GC compound separation. The primary GC oven temperature was set at 70 °C for 2 min, and then increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for an additional 2 min. The secondary oven was set at 85 °C for 2 min, increased at 4.5 °C/min, to a final temperature of 300 °C, at which it was maintained for 4.5 min. Helium, set to a column flow rate of 1 mL/min, was used as a carrier gas, and held at a constant temperature of 270 °C. Mass spectrometric data acquisition was carried out at -70 eV, with a solution delay of 350 sec, and a mass range of 50–800 m/z was scanned with a rate of 200 spectra/sec.

2.4. Data Processing, Clean-Up, and Statistics

ChromaTof software (version 4.32) was used for mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment, and peak identification on the obtained mass spectra. Metabolites were identified by comparing their mass fragment patterns to those of compounds in commercially available databases containing previously injected standards. For normalization and assessment of data quality, the data were pretreated using a standardized metabolomics data clean-up procedure [67]. Each detected compound was normalized using MS total useful signal (TUS), which is based on a factor calculated from the sum of all metabolites identified in all samples, and by calculating the relative concentration of each by using the internal standard. All missing/zero values were replaced by a value calculated as 20% of the minimum detection limit of the entire dataset, as these are most likely present in sub-minimum concentrations rather than being completely absent [68]. An 80% data filter was then applied to eliminate compounds with more than 80% zero values within both groups [69]. To provide a balanced representation of all metabolites, log transformation and auto-scaling (mean-centered and divided by the standard deviation of each variable) were applied. This prevents compounds with minor concentrations from being overlooked due to the domination of compounds with higher concentrations [70]. Making use of MetaboAnalyst (Version 5.0) [71], multivariate statistical methods in the form of unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) were applied [72]. Subsequently, uni-variate analysis was performed by calculating t-test and effect size values [73].

Relationships between the selected metabolites were mapped using the KEGG, MetaCyc, and BioCyc databases, in addition to intensive research of the previously published literature on the topic.

3. Results

3.1. Data Overview

When visualizing the analytical technique's repeatability graphically (Figure 1), approximately 86% of all the compounds identified ($n = 260$) had a coefficient of variation (CV) value under 50%, while 70% had CV values under 20%. The analytical technique used during this analysis thus proved to be highly repeatable and can be trusted to provide reliable results. PCA was initially used to obtain an overview of the natural grouping of metabolic data (Figure 2). The total variance between the groups, described by the first two principal components (PCs), was 57.1%, of which PC1 and PC2 accounted for 32.1% and 25%, respectively. The PCA scores plot of the metabolite data analyzed by GCxGC-TOF-MS shows clear clustering between *Mtb* in the presence and absence of ciprofloxacin, as represented in Figure 2.

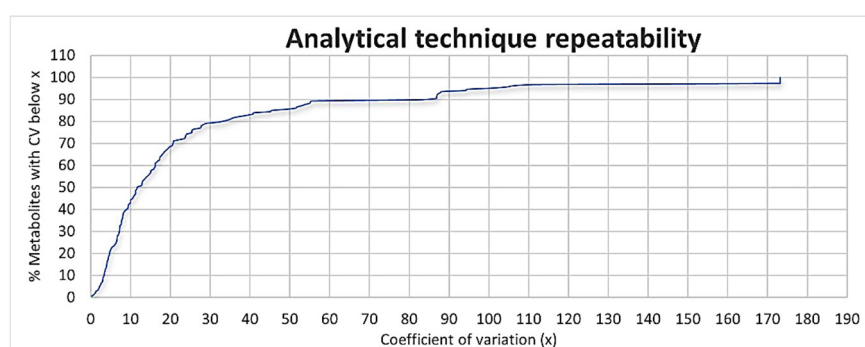


Figure 1. Distribution of the coefficients of variation values for technical repeatability.

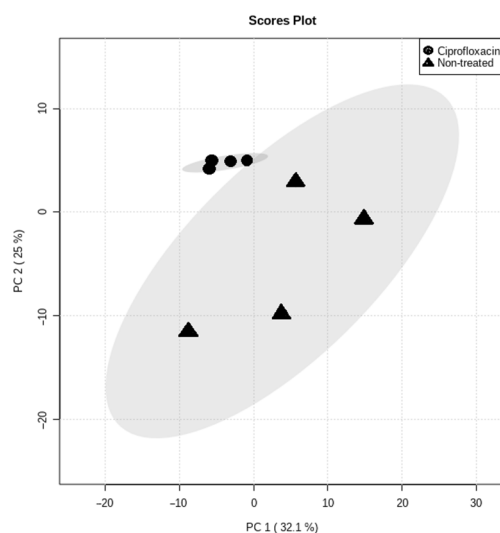


Figure 2. PCA scores plot obtained from GCxGC-TOFMS whole metabolome analysis of *Mtb* samples in the presence and absence of ciprofloxacin. The variances accounted for are indicated in parentheses.

3.2. Marker Selection

The metabolite markers ($n = 26$) best describing the differences between the ciprofloxacin and control samples were selected based on compliance with the following criteria: a PLS-DA VIP value > 1 [74], a t -test p -value < 0.05 [75], or an effect size > 0.8 [76] (Figure 3).

The selected metabolite markers are listed according to their PLS-DA VIP values in Table 1, along with their respective average concentrations and univariate test outcomes. Of the total, 61.5% (16/26) of the markers were elevated; most of these were fatty acids. The most differentiating marker was malic acid, with an exceptionally high d -value of 6.621 and low p -value of > 0.0001 .

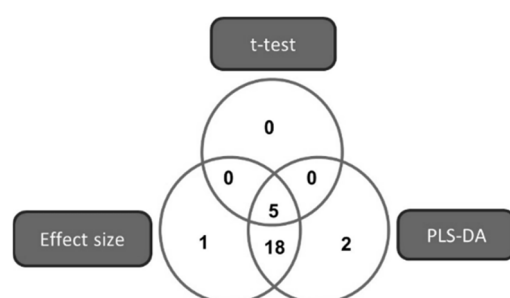


Figure 3. Venn diagram illustrating the multi-statistical approach for selecting the metabolites that best describe the variation detected in the metabolome of *Mtb* cultured with and without ciprofloxacin.

Table 1. Metabolite markers best describing the variance between the individually cultured *Mtb* samples in the absence (*Mtb* controls) and presence of ciprofloxacin.

Metabolite Name (ChEBI ID)	Average Concentration (mg/g Cell Mass) (Standard Deviation)		<i>t</i> -Test (<i>p</i> -Value)	Effect Size (<i>d</i> -Value)	PLS-DA (VIP)	Fold Change (log ₂)
	<i>Mtb</i> with Ciprofloxacin	<i>Mtb</i> Controls				
Malic acid (6650)	0.033 (0.002)	0.054 (0.004)	0.000	6.621	2.012	−0.39
Aspartic acid (17053)	0.007 (0.001)	0.013 (0.004)	0.008	2.236	1.732	−0.46
Glycerol (17754)	0.497 (0.033)	0.633 (0.031)	0.013	1.932	1.678	−0.21
5-Oxoproline (17203)	0.069 (0.014)	0.099 (0.020)	0.014	1.962	1.665	−0.30
Xylofuranose (46432)	0.075 (0.037)	0.034 (0.008)	0.037	1.376	1.511	1.21
Myo-inositol-1-phosphate (18297)	0.004 (0.001)	0.003 (0.001)	0.051	1.637	1.445	0.33
9-Hexadecenoic acid (28716)	0.005 (0.000)	0.004 (0.001)	0.091	1.329	1.299	0.25
Nonadecanoic acid	0.067 (0.011)	0.055 (0.008)	0.094	1.151	1.292	0.22
Heptadecanoic acid (32365)	0.015 (0.003)	0.011 (0.002)	0.095	1.355	1.288	0.36
Octadecanoic acid (28842)	0.582 (0.164)	0.371 (0.141)	0.096	1.241	1.287	0.57
Valine (16414)	0.017 (0.004)	0.022 (0.005)	0.106	1.117	1.255	−0.23
β-Aminoisobutanoic acid (33094)	0.011 (0.002)	0.014 (0.004)	0.121	1.256	1.216	−0.21
Glutamic acid (16015)	0.010 (0.001)	0.015 (0.006)	0.124	0.960	1.209	−0.33
Hexadecanoic acid (15756)	0.575 (0.051)	0.480 (0.077)	0.128	0.969	1.198	0.20
9-Octadecenoic acid (36021)	0.679 (0.060)	0.600 (0.041)	0.132	1.089	1.187	0.13
Tetradecanoic acid (28875)	0.089 (0.010)	0.071 (0.017)	0.141	0.934	1.167	0.25
Eicosanoic acid (28822)	0.005 (0.001)	0.004 (0.000)	0.146	0.991	1.154	0.25
Glucose (17234)	0.045 (0.006)	0.037 (0.005)	0.152	0.934	1.140	0.22
Urea (16199)	0.005 (0.002)	0.002 (0.002)	0.171	0.816	1.099	1.50
<i>N</i> -Acetyl-L-Lysine (64859)	0.002 (0.001)	0.004 (0.003)	0.175	0.884	1.090	−0.50
2-Monopalmitin (75455)	0.011 (0.002)	0.009 (0.001)	0.175	0.907	1.089	0.22
1-Monomyristin (75562)	0.007 (0.003)	0.005 (0.001)	0.175	0.882	1.089	0.40
Pentadecanoic acid (42504)	0.005 (0.001)	0.004 (0.001)	0.181	1.021	1.077	0.25
Glycerol 3-phosphate (15978)	0.005 (0.001)	0.007 (0.002)	0.186	0.908	1.067	−0.29
1-Monoheptadecanoin (144339)	0.010 (0.002)	0.007 (0.001)	0.186	0.947	1.066	0.43
Erythritol (17113)	0.021 (0.002)	0.023 (0.001)	0.202	0.856	1.034	−0.09

4. Discussion

In this study, we identified a number of significantly altered metabolites induced by the administration of ciprofloxacin to *Mtb* culture, which when interpreted, in the light of known metabolism and previous ciprofloxacin findings, better elucidate its mechanisms of action against *Mtb*, as shown in Figure 4. The most prominently altered pathways included gluconeogenesis, fatty acid metabolism, amino acid metabolism, the pentose phosphate pathway (PPP), and the urea cycle.

Of note were the elevated levels of various even- and odd-chain saturated fatty acids of between 14 to 20 carbons (C14:0–C20:0) in length, in the *Mtb* treated with ciprofloxacin. This was also true for two Δ^9 -unsaturated fatty acids; 9-hexadecenoic (Δ^9 C16:1), and 9-octadecenoic (Δ^9 C18:1) acids. These indicate a strongly upregulated synthesis toward cell wall repair, supporting previous evidence associating ciprofloxacin with cell wall damage [47]. Simplified, lying outside of the cytoplasmic membrane, a peptidoglycan (PG) layer is covalently attached to arabinogalactan (AG), which itself attaches to mycolic acids (MA) to form the MA-AG-PG complex (MAPc) [77,78]. Interspersed within the MAPc, are the glycerolipids, phosphatidyl myo-inositol mannosides (PIM) and lipoarabinoman-

nans (LAM) [79]. PIM is a crucial part of the membrane structure and serves as a precursor of LAM [80]. The saturated fatty acid markers in this study are produced by fatty acid synthase type I (FAS I). FAS I generates 16 to 26 carbon length fatty acyl-coenzyme A (CoA) [81], which are fed into FAS II for elongation. FAS I and FAS II provide acyl groups for the synthesis of all cell envelope components, except for AG [82,83]. Δ^9 C16:1 and Δ^9 C18:1 and their precursors, hexadecanoic (C16:0) and octadecanoic (C18:0) acid, respectively, are considered major fatty acids of glycerolipids and mycolic acids [84–86]. Δ^9 C16:1 and Δ^9 C18:1 are reduced from C16:0 and C18:0, in the presence of Fe^{2+} , a flavin, NADPH, and O_2 [87,88]. Interestingly, some *mmpL* genes, encoding fatty acid transporter protein MmpL have been shown to be repressed when their transcriptional regulator proteins bind to C:16 fatty acids and monoacylglycerols (MAG) [89,90]. Even so, further research is needed to establish possible activity of different fatty acids on different MmpL regulator proteins. It is, however, important to note that the damage that ciprofloxacin administration induces to the cell wall may be direct, but is most likely indirect, by inhibition of other energy-producing mechanisms or simply by induction of the SOS response in *Mtb*, shifting energy production away from glucose toward the preferential use of fatty acids [91]; hence, less of these fatty acids are now available to cell wall synthesis. Increased FA synthesis or the accumulation of cell wall components has also been identified in *Mtb* treated with EMB, INH, PZA, RIF, and pretomanid [92–96].

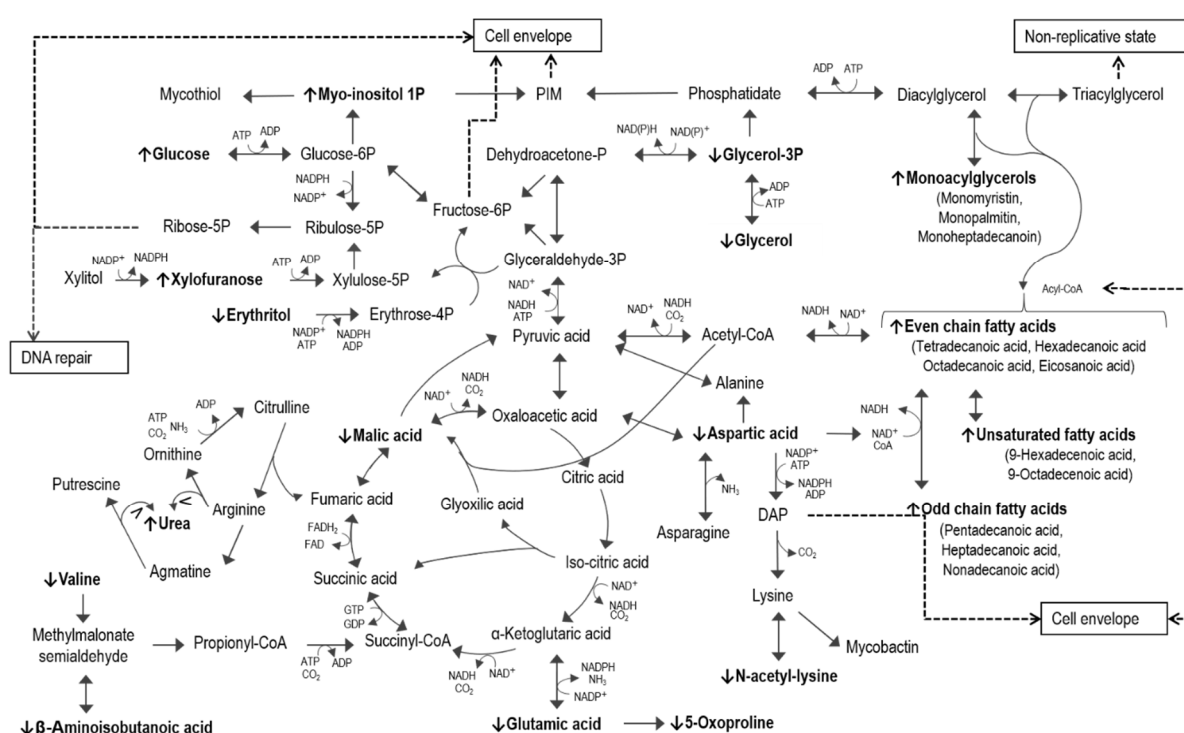


Figure 4. Metabolomic pathway map of *Mtb* treated with ciprofloxacin. The metabolite markers best describing the variation in the metabolome compared to those of untreated *Mtb* are represented in bold text with up or down arrows indicating elevated or reduced concentrations, respectively.

The dramatically elevated synthesis of intracellular fatty acids would be expected to consume a considerable amount of carbon, which can be supplied from various sources [91]. However, as will be explained, glucose and glycerol seem to be the major suppliers for such, and for the various components required for DNA repair. This is supported by previous findings indicating ROS, produced during the FQ-induced SOS-response, causes oxidative stress, which in turn activates utilization of triacylglycerol (TAG) and cell wall lipids for energy [97–99], as is generally the case during the non-replicative phase of *Mtb* [100,101]. These results are also supported by previous findings showing reduced concentrations of

phospholipids and mycolic acids in the cell wall macromolecules of sub-MIC ciprofloxacin-treated *M. smegmatis* [47], because these are now being preferentially used for energy production, with glucose supplying the necessary carbon substrates for the continued synthesis of these much-needed fatty acids, which are now preferentially used for energy production.

A shift in energy supply to β -oxidation of fatty acids, as opposed to the TCA cycle, reserves NAD^+ , in addition to CO_2 , for *de novo* synthesis of nucleotides (for DNA repair and fatty acids). NADH and NADPH released during fatty acid metabolism fuel the upregulated non-oxidative PPP and glycerolipid metabolic pathway. In this study, the downregulated TCA cycle was indicated by a reduction in malic acid and aspartic acid, which according to the uni- and multivariate statistics, were ranked as the two most important metabolite markers altered by ciprofloxacin (Table 1). Aspartic acid is considered a validated reporter of oxaloacetate (OAA) [102], and together with malic acid, supports TCA cycle inhibition. Although oxidative stress is normally associated with an increase in glyoxylate shunt activity, in this study the glyoxylate shunt was clearly downregulated. This indicates a greater need of carbon flux through gluconeogenesis toward the PPP and glucose, for subsequent fatty acid and nucleotide synthesis [103,104]. Reduced aspartic acid concentrations were also previously found in a study investigating phenotypic antibacterial persistence in *Mtb* [105]. These results support the SOS response-induced decrease in oxidative phosphorylation and increase in energy reserves for DNA repair in response to FQs [32,102]. It is noteworthy to mention that the ATPase activity of DNA gyrase (29), shows reduced adenosine triphosphate (ATP) conversion in the presence of ciprofloxacin [106], which most likely is for the purpose of reserving ATP for other energy consuming pathways, such as gluconeogenesis and DNA repair [107].

Various PPP intermediates that contribute to DNA and cell wall synthesis were detected to be altered in the ciprofloxacin-treated *Mtb*, [99,108,109]. Under normal circumstances, erythrose-4-phosphate and glyceraldehyde-3-phosphate produce xylulose-5-phosphate (xylulose-5P) and fructose-6-phosphate (fructose-6P) [91]. The latter is converted to glucose-6-phosphate, for subsequent PIM synthesis, via the myo-inositol pathway [110], and to glucose-*N*-acyl 6-phosphate (GlcN6P), for subsequent PG synthesis [111]. Xylose-5P is the precursor of ribose-5-phosphate (ribose-5P), which, in the presence of ATP, is converted to 5-phosphoribosyl-1-pyrophosphate (pRpp) [103,112]. pRpp is the branch point intermediate of decaprenyl-phospho-arabinofuranose (DPA) [113], the only donor of arabinose to AG and LAM [104,114] and nucleotide synthesis. In this investigation, the upregulated non-oxidative PPP in the ciprofloxacin-treated *Mtb* was supported by the elevated concentrations of xylofuranose and reduced erythritol (Figure 4). Furthermore, it is well known that PPP metabolism is fueled by glucose and the glucogenic amino acids valine, aspartic acid, and glutamic acid [115], all of which were specifically and significantly reduced in the ciprofloxacin-treated *Mtb*, in addition to their degradation products β -aminoisobutanoic acid [116], 5-oxoproline [117], and *N*-acetyl-lysine [118], respectively (Figure 4). Furthermore, the reduced levels of glutamic acid and aspartic acid in our investigation supported an oxidative state in the ciprofloxacin-treated group [119]. The above-mentioned amino acids also serve as precursors of cell wall related intermediates. Alanine derived from aspartic acid combines with valine to produce CoA, which is used for converting fatty acids into cell wall lipids [120–122]. Valine also serves as a precursor of propionyl-CoA [116,123] and is used for the elongation of odd-chain fatty acids [124]. Aspartic acid additionally is the precursor of NAD^+ , 2,6-diaminopimelate (DAP) [125,126], and *S*-adenosyl methionine (SAM) [127]. Considering this, NAD^+ is subsequently an important cofactor for FAS I [128], and SAM is required for the methylation of cell-wall fatty acids [129]. Furthermore, the aforementioned DAP, along with alanine and glutamic acid, serve as substrates for PG [130]. Lastly, the observed flux through aspartic acid can be supported by the elevated levels of urea in *Mtb* treated with ciprofloxacin [131], and considering that aspartic acid is one of the top three metabolite markers identified (Table 1), it is possibly utilized, contributing to many of the cell-envelope changes observed.

The FQ-induced SOS response is known to cause cell growth arrest [132], and in this study, inhibited cell growth is evident by the increased TAG metabolism and reduced TCA activity, in addition to the inhibited protein synthesis observed in the ciprofloxacin-treated *Mtb*. Protein synthesis requires ATP-dependent activation of glutamate and aspartate, followed by amination by ammonia, to form aminoacyl-tRNA [115]. However, in this study, urea was found to be elevated in *Mtb* treated with ciprofloxacin, indicating accumulation of ammonia and reduced recycling of nitrogen via glutamine and proline metabolism. This, in addition to a decreased degradation of urea by urease, also linked to the *Mtb* stress response [133], retains ammonia from protein synthesis [134]. Inhibited protein formation has also been suggested in a proteomics study of *Mtb* treated with ciprofloxacin [135], as well as a metabolomics study of *M. smegmatis* treated with ciprofloxacin [48]. Disruption of protein synthesis has also been reported in studies investigating the response of *Mtb* to pretomanid, streptomycin, EMB, RIF, PZA, and INH [95,136–138]. The inhibited protein synthesis observed would in turn disrupt the functionality of membrane proteins, subsequently inhibiting nutrient uptake as well as fatty acid transport to the cell wall [42]. Additionally, urea acts as an osmolyte, preventing dehydration or water loss as a result of the seemingly damaged cell wall [139]. Increased urea could also be indicative of polyamine synthesis (Figure 4) [140]. Polyamines, such as putrescine, have been reported to reduce accumulation of trans-membrane proteins [141] and contribute to the phenotypic drug resistance to FQs [142].

Observed changes to the monoacylglycerols (MAG) 1-monomyristin, 2-monopalmitin, and 1-monoheptadecanoin are indicative of cell envelope changes via the glycerolipid and triacylglycerol (TAG) pathways. TAG in the cell wall [143] can be metabolized during the stress-induced transition to the non-replicated phase [144] and during infection [145]. *Mtb*'s adaptation during these circumstances involves the use of lipids as main energy reserves, as previously mentioned [146]. During the deacetylation of TAG to diacylglycerol (DAG), and from DAG to MAG, acyl-CoAs are released, which are either directed toward glycerolipid synthesis (Figure 4) or energy production [147,148]. Glycerolipid and TAG metabolism share an important intermediate at the branch point, 1,2-diacyl-*sn*-glycerol 3-phosphate, commonly known as phosphatidate (PA) (Figure 4) [149]. PA is synthesized via two pathways: 1) the phosphorylation of DAG by DAG kinase [150] or 2) the acylation of glycerol-3-phosphate (glycerol-3P) by glycerol phosphate and acyl glycerol phosphate acyltransferase [151]. In this study, the concentrations of glycerol-3-phosphate and its precursor glycerol were decreased, supporting a MAG metabolic flux toward TAG and PIM in the ciprofloxacin-treated *Mtb* group. Glycerol-3P appears to recycle the transport of membrane lipids [152] and can thus be expected to be the rate-limiting step, which would explain the accumulation of the fatty acids and the accompanying reduction in glycerol levels. The synthesis of glycerolipids also requires myo-inositol, which is produced via glucose-6-phosphate (glucose-6P) [153]. Firstly, myo-inositol 3-phosphate synthase converts glucose-6P into myo-inositol 3-phosphate, which in turn is dephosphorylated by several myo-inositol monophosphates to produce myo-inositol [84]. The elevated levels of myo-inositol monophosphates in the ciprofloxacin-treated *Mtb* hence further support the aforementioned flux toward glycerolipids [154]. Furthermore, myo-inositol-1-phosphate is converted by a glycosyltransferase, mycothiol (Msh). Mycothiol is also considered an important antioxidant required for balancing the cytosolic NAD⁺/NADH ratio [155]. In addition to the abovementioned inhibition of protein synthesis and enhanced fatty acid synthesis, altered energy metabolism has also consistently been identified in response to the first-line drugs INH [156], EMB [157], PZA [158], and RIF [159], supporting these results. In summary, the mycobacterial cell wall metabolism is visibly linked to the SOS response, which has frequently been proposed to cause resistance toward ciprofloxacin and challenge the otherwise impressive bactericidal activity of this drug [18,160–162].

5. Conclusions

In this study, we investigated the metabolic changes to *Mtb* induced by sub-MIC of ciprofloxacin, in order to better understand its mechanism of action and the resultant adaptations of *Mtb*. Previous studies have identified alterations in transcription, translation, and cell wall synthesis as part of the mechanism of action of ciprofloxacin against *Mtb* (51, 52, 135). Our metabolomics study identified metabolite markers that support previous results, as indicated by the drastic accumulation of metabolites associated with cell wall and DNA repair. Moreover, many of these markers indicate an SOS-induced shift to the non-replicative phase, which is a key mechanism determining *Mtb* persistence and tolerance to various anti-TB drugs. This study not only gives a better understanding of ciprofloxacin's mode of action but provides helpful insight for further investigation of antibiotic-induced resistance by *Mtb*, and also perhaps the use of ciprofloxacin in combination with existing anti-TB drugs.

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Article

Elucidating the Antimycobacterial Mechanism of Action of Decoquinolate Derivative RMB041 Using Metabolomics

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Abstract: Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), still remains one of the leading causes of death from a single infectious agent worldwide. The high prevalence of this disease is mostly ascribed to the rapid development of drug resistance to the current anti-TB drugs, exacerbated by lack of patient adherence due to drug toxicity. The aforementioned highlights the urgent need for new anti-TB compounds with different antimycobacterial mechanisms of action to those currently being used. An *N*-alkyl quinolone; decoquinolate derivative RMB041, has recently shown promising antimicrobial activity against *Mtb*, while also exhibiting low cytotoxicity and excellent pharmacokinetic characteristics. Its exact mechanism of action, however, is still unknown. Considering this, we used GCxGC-TOFMS and well described metabolomic approaches to analyze and compare the metabolic alterations of *Mtb* treated with decoquinolate derivative RMB041 by comparison to non-treated *Mtb* controls. The most significantly altered pathways in *Mtb* treated with this drug include fatty acid metabolism, amino acid metabolism, glycerol metabolism, and the urea cycle. These changes support previous findings suggesting this drug acts primarily on the cell wall and secondarily on the DNA metabolism of *Mtb*. Additionally, we identified metabolic changes suggesting inhibition of protein synthesis and a state of dormancy.

Keywords: decoquinolate derivative RMB041; *Mycobacterium tuberculosis*; tuberculosis; metabolomics; GCxGC-TOFMS; mechanism of action



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1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), is currently considered the deadliest infectious disease worldwide [1]. TB causes about 1.3 million deaths annually, in addition to approximately 300,000 deaths of patients coinfecting with the human immunodeficiency virus (HIV), while newly infecting 10 million people per annum [1,2]. Furthermore, the prevalence of multiple-drug resistant (MDR) and extensively drug resistant (XDR) TB is also on the rise [3,4]. The urgency for developing new anti-TB drugs that are less expensive, less cytotoxic, and more efficient, as well as being readily co-administered with HIV treatment, is indisputable. Currently, the first-line treatment for TB entails a six-month “directly observed treatment short-course” (DOTS), which includes using isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), and rifampicin (RIF) in combination [5]. Infection with MDR- and XDR-TB requires more expensive treatment approaches, for a longer treatment duration, using drugs that have a higher risk of adverse side effects [6,7], which in turn leads to poorer patient compliance and subsequently an escalated increase of drug resistant TB [8]. Further contributing factors to the development of drug resistant TB include inaccurate diagnosis, unsupervised treatment protocols, and

poor economic status [9–11], and in 2020, this was further exacerbated by the COVID-19 pandemic [1,12]. Over the last 50 years, the only new antimycobacterial compounds to be approved, as last-resort options, are the anti-TB drugs linezolid, bedaquiline, and delamanid [3–5,13]. Recently, the WHO and the UN held its first high-level meeting aimed at initiating an urgent global response to end the TB epidemic by 2030 [1]. However, certain objectives need to be met before this goal can be achieved. Most importantly, elucidating the antimycobacterial mechanisms of action of drugs is considered essential and a means towards finding novel *Mtb* drug compounds and targets [14]. Furthermore, the mechanisms of drug resistance also need to be fully understood [15]. Equally important is economic viability, particularly in high-burden countries with insufficient funding for extremely long, yet mandatory, clinical trials. It is thus beneficial to further investigate already approved drugs with established pharmacokinetic properties and safety profiles [16].

Decoquinatone (DQ), an anticoccidial quinolone used as a broad-spectrum antibiotic, has gained interest for its efficacy against malaria, toxoplasmosis, and tuberculosis [17], accompanied by an excellent safety profile [18], high permeability into the cells, and its relatively low costs [19]. Recently, an *N*-alkyl DQ derivative (RMB041) showed high activity against *Mtb* ($MIC_{90} = 1.61 \mu M$), with similar in vitro potency to that of ciprofloxacin ($1.5\text{--}12 \mu M$), gatifloxacin ($0.66\text{--}1.3 \mu M$), and moxifloxacin ($0.62\text{--}1.3 \mu M$) [20]. Its permeability ($\text{LogP}_{app} = -4.8$, where compounds with values > -5 are considered highly permeable [21]) confers a benefit for drug diffusion through the lipid cell wall [22] and penetration of infected macrophages and granulomatous lesions in the lungs [23,24]. This drug also shows low cytotoxicity in human fetal lung fibroblasts [25], as well as promising pharmacokinetic properties, including an intravenous elimination half-life ($t_{1/2}$) of 62.3 h in murine models and a low human intrinsic clearance rate (CL_{int}) ($16 \mu L/\text{min}/\text{mg}$) [2]. The $t_{1/2}$ is relatively long when compared to that of other well-known anti-TB drugs such as RIF (7.19 h) [26,27], EMB (3 h) [28], INH (1.7 h) [29], and PZA (1.05 h) [30], whereas the CL_{int} performs well compared to that of EMB ($>75 \mu L/\text{min}/\text{mg}$) and INH ($<22 \mu L/\text{min}/\text{mg}$) but less so in comparison to RIF ($<10 \mu L/\text{min}/\text{mg}$) [31]. Drugs with lower CL_{int} and longer $t_{1/2}$ require less frequent administration/dosing, which could improve patient compliance and contribute to lowering the prevalence of drug resistance [32]. Furthermore, DQ derivative RMB041 could hold the key to shortening the duration of TB treatment and reduce the current costs of therapy [33]. Considering this, DQ derivative RMB041 is a promising anti-TB candidate. However, little is known about its antimycobacterial mechanism of action. Contrary to DQ, which primarily targets the ubiquinol-binding pocket, the RMB041 derivative rather shows activity against the cell wall and DNA metabolism of *Mtb* as its primary and secondary targets, respectively [25]. The exact underlying mechanism of action, however, is yet to be determined.

In this study, a two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach, combined with universally connected metabolic libraries and advanced statistical analysis, was used to identify the metabolite markers best differentiating *Mtb* treated with and without DQ derivative RMB041.

2. Results

2.1. Data Overview

Principal component analysis (PCA) was initially used to get an overview of the natural grouping of metabolic data (Figure 1). The total variance described by the first two principal components (PCs) was 52%, of which PC1 accounted for 30.6% and PC2 for 21.4%, respectively. The PCA scores plot of the metabolite data analyzed by GCxGC-TOFMS shows clear clustering between *Mtb* treated with DQ derivative RMB041 and those cultures that were not (Figure 1). The scores plot also shows that the variance of the treated *Mtb* group is clearly greater than the controls, as a result of greater variance in the metabolite concentrations.

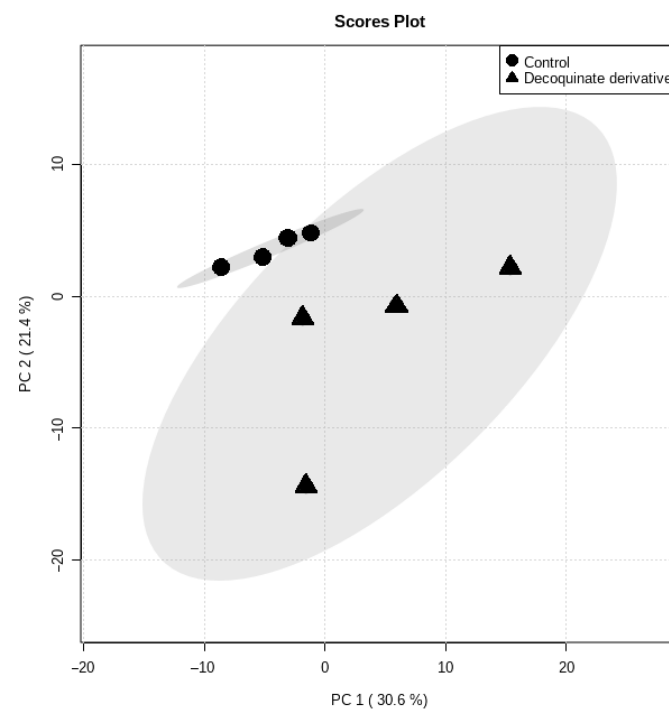


Figure 1. PCA scores plot obtained from GCxGC-TOFMS whole metabolome analysis of *Mtb* samples in the presence and absence of DQ derivative RMB041.

2.2. Marker Selection

Based on compliance to the following criteria: a PLS-DA VIP value > 1 [34], a *t*-test *p*-value < 0.05 [35] or an effect size > 0.8 [36], 36 metabolites were selected as markers best describing the difference between *Mtb* treated with DQ derivative RMB041 and untreated *Mtb* control samples (Figure 2). The metabolites are listed in Table 1, according to their VIP values.

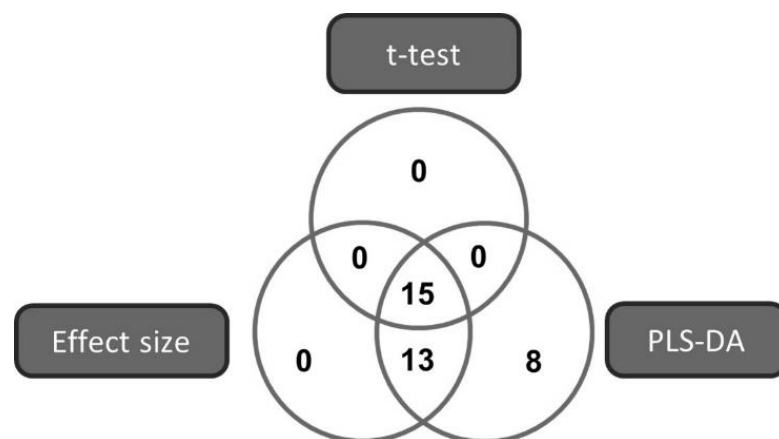


Figure 2. Venn diagram illustrating the multi-statistical approach for selecting the metabolites that best describe the variation detected in the metabolome of *Mtb* cultured with and without DQ derivative RMB041.

Table 1. The selected metabolite markers best describing the variance between the metabolome of *Mtb* cultured in the absence (*Mtb* controls) and presence of DQ derivative RMB041.

Metabolite Name (CHEBI ID)	Average Concentration (mg/g Cells) (Standard Deviation)		<i>t</i> -Test (<i>p</i> -Value)	Effect Size (<i>d</i> -Value)	PLS-DA (VIP)	Fold Change (Log ₂)
	<i>Mtb</i> with DQ derivative RMB041	<i>Mtb</i> controls				
9-Octadecenoic acid (36021)	0.967 (0.192)	0.560 (0.041)	0.000	34.47	2.59	0.73
Myo-Inositol (17268)	0.024 (0.032)	0.020 (0.002)	0.000	5.298	1.99	0.20
Ribitol (15963)	0.318 (0.513)	0.100 (0.005)	0.006	2.098	1.98	2.18
Arabitol (18403)	0.877 (0.962)	0.790 (0.199)	0.010	2.206	1.98	0.11
Eicosanoic acid (28822)	0.018 (0.025)	0.004 (0.001)	0.000	3.968	1.94	3.50
Erythritol (17113)	0.023 (0.027)	0.028 (0.001)	0.001	3.176	1.92	−0.18
N-Acetyl-Lysine (35704)	0.000 (0.000)	0.004 (0.003)	0.009	1.938	1.81	−1.00
9,12-Octadecadienoic acid (17351)	0.578 (0.827)	0.111 (0.008)	0.016	1.835	1.67	4.21
Valine (16414)	0.039 (0.063)	0.022 (0.005)	0.019	1.908	1.65	0.77
Aspartic acid (17053)	0.018 (0.033)	0.013 (0.004)	0.038	1.341	1.65	0.38
Pentadecanoic acid (42504)	0.017 (0.023)	0.004 (0.001)	0.021	1.648	1.64	3.25
Galactose (28260)	0.004 (0.002)	0.011 (0.001)	0.185	0.747	1.57	−0.64
Glutamic acid (16015)	0.024 (0.043)	0.015 (0.006)	0.032	1.549	1.56	0.60
Lysine (18019)	0.016 (0.030)	0.009 (0.006)	0.047	1.291	1.56	0.78
2-Hydroxyglutaric acid (32796)	0.003 (0.004)	0.002 (0.001)	0.037	1.384	1.53	0.50
Proline (26271)	0.022 (0.041)	0.016 (0.013)	0.060	1.202	1.50	0.38
Glycerol-3-phosphate (15978)	0.012 (0.016)	0.007 (0.002)	0.049	1.308	1.47	0.71
Malonic acid (30794)	0.173 (0.213)	0.039 (0.045)	0.249	0.684	1.46	3.44
Glycerol (17754)	0.994 (1.45)	0.633 (0.031)	0.057	1.171	1.44	0.57
Nonadecanoic acid (NSC11914)	0.499 (0.731)	0.055 (0.009)	0.065	1.127	1.41	8.07
Alanine (16977)	0.321 (0.621)	0.207 (0.152)	0.067	1.156	1.40	0.55
Octadecanoic acid (28842)	0.027 (0.034)	0.005 (0.000)	0.068	1.150	1.40	4.40
β-Aminoisobutanoic acid (33094)	0.035 (0.057)	0.014 (0.004)	0.073	1.310	1.38	1.50
γ-Aminobutyric acid (16865)	0.011 (0.018)	0.016 (0.006)	0.085	1.315	1.34	−0.31
Tyrosine (17895)	0.006 (0.012)	0.003 (0.001)	0.147	0.843	1.32	1.00
9-Hexadecenoic acid (59265)	0.105 (0.153)	0.018 (0.003)	0.099	0.975	1.29	4.83
Heptadecanoic acid (32365)	0.052 (0.069)	0.011 (0.002)	0.103	0.975	1.29	3.73
Ribofuranose (33942)	0.003 (0.003)	0.007 (0.002)	0.239	0.653	1.28	−0.57
Glucose (17243)	0.045 (0.031)	0.037 (0.005)	0.174	0.770	1.24	0.22
Isoleucine (17191)	0.018 (0.032)	0.008 (0.008)	0.216	0.702	1.21	1.25
9Z-Octadec-1-ol (73504)	0.026 (0.036)	0.004 (0.003)	0.276	0.601	1.17	5.50
Decanoic acid (30813)	0.008 (0.009)	0.005 (0.001)	0.157	0.836	1.18	0.60
Malic acid (6650)	0.084 (0.127)	0.054 (0.004)	0.172	0.775	1.11	0.56
Ornithine (15729)	0.029 (0.054)	0.025 (0.015)	0.191	0.841	1.09	0.16
Serine (17822)	0.011 (0.021)	0.004 (0.002)	0.151	0.850	1.06	1.75
Threonine (16857)	0.049 (0.088)	0.016 (0.008)	0.173	0.791	0.93	2.06

3. Discussion

In this study we identified a number of significantly altered metabolites induced by the administration of DQ derivative RMB041 to *Mtb* culture. In the light of known metabolism and previous DQ derivative RMB041 findings, the interpretation of these selected metabolite markers, and their associated metabolic pathways, lead to better elucidation of the antimycobacterial mechanism of action of this drug. The most prominently altered pathways (amino acid metabolism, fatty acid metabolism, pentose phosphate pathway (PPP), and the urea cycle) are mapped in Figure 3.

Preeminent in the *Mtb* treated with DQ derivative RMB041 is the elevation of many of the even and odd saturated fatty acids of between 10 to 20 carbons (C10:0–C20:0). This was also true for the Δ^9 -mono-unsaturated fatty acids (Δ^9 C18:1), as previously mentioned, and 9-hexadecenoic (Δ^9 C16:1), in addition to the $\Delta^{9,12}$ -poly-unsaturated fatty acid 9,12-octadecenoic ($\Delta^{9,12}$ C18:2). These indicate a strong upregulated synthesis towards cell wall repair [37] and an accumulation thereof due to DQ derivative RMB041 inhibiting this process, which strengthens previous findings, suggesting the antimycobacterial activity of DQ derivative RMB041 targeting the cell wall [25]. The distinctly multilaminate cell wall of *Mtb* consists of a peptidoglycan (PG) layer covalently attached to arabinogalactan (AG) [38], which itself attaches to mycolic acids. Interspersed is the mycobacterial plasma membrane, consisting of glycerophospholipids and glycerolipids, phosphatidyl myo-inositol mannosides (PIM), lipomannans (LM), and lipoarabinomannans (LAM) [39], all of which are considered critical for maintaining cell wall integrity [40], and PIM, which contributes to the low permeability of the cell envelope and intrinsic tolerance to antibiotics [41]. The synthesis of PIM requires myo-inositol and glycerol-3-phosphate (glycerol-3P), which were also

through the glyoxylate shunt [64]. This also reduces the release of CO₂, resulting in a direct carbon flux towards FAS I and FAS II [65], for subsequent energy production [54] and cell wall remodeling [56]. In the presence of ATP, the resultant CO₂, together with NH₄ released during amino acid metabolism, are incorporated into the urea cycle via ornithine (which is elevated in the RMB041 treated *Mtb*, indicating an upregulated urea cycle) (Figure 3) [66]. Ornithine serves as the precursor of polyamines, which have previously been associated with antibiotic-induced mutations [67]. Furthermore, ornithine contributes to the reclamation of carbon to the TCA cycle via proline and glutamic acid. This can be obtained via γ -aminobutyric acid (GABA) or via α -ketoglutaric acid (Figure 3) [68]. Notably, DQ derivative RMB041-treated *Mtb* prefers the latter, which could be explained by the necessity of α -ketoglutaric acid to incorporate amino acids, such as aspartic acid, into the TCA cycle [69]. Moreover, NAD(P)H and α -ketoglutaric acid are converted to NAD(P)⁺ and α -hydroxyglutaric acid [70], which was also found to be elevated in this study. This might further indicate an imbalanced NAD(P)⁺/NAD(P)H ratio, supporting the need for NAD(P)⁺ for survival of fatty acids as the main energy source, and/or disruption of the membrane, leading to a disturbed electron transport chain (ETC) and, subsequently accumulation of NADH [71].

The *Mtb* non-replicative phase is also associated with a reduction/stagnation of DNA, RNA, and protein synthesis [72,73]. The accumulation of urea (0.012 vs. 0.002 mg/g; $p = 0.63$) in the *Mtb* treated with DQ derivative RMB041, although not selected as a metabolite marker through the statistical selection process described above, indicates the conservation of nitrogen from protein synthesis [74]. Inhibition of protein synthesis would subsequently disrupt the functionality of membrane proteins and result in an accumulation of fatty acids as a result of inhibition of cell wall synthesis [75]. Urea could also serve as an opposing force to prevent osmotic lysis [76] brought about by dehydration as a result of an altered cell envelope integrity. Aspartic acid, along with ten other amino acids (Table 1), were found elevated in this study. When considering this in light of the elevated levels of sugar alcohols detected in the DQ derivative RMB041 treated *Mtb* group, degradation of the nucleic acids to purine and pyrimidines is evident [77,78]. Speculatively, the direct activity of DQ derivative RMB041 on DNA would most likely result in an attempt towards DNA repair, as was previously indicated by reduced sugar alcohols in *Mtb* treated with ciprofloxacin [79]. In this case, however, we saw an accumulation of both amino acids and sugar alcohols, suggesting DNA replication has not yet been inhibited or repair mechanisms have not yet been activated at the time the cells were harvested. This may, however, occur at a later stage, or at higher DQ derivative RMB041 concentrations perhaps, but in the present study, the *Mtb* treated with DQ derivative RMB041 are most likely still in a non-replicative state after the irreversible cell envelope damage, which is the primary target of DQ derivative RMB041 [25]. This is also supported by previous findings suggesting DNA replication as a secondary target and cell wall metabolism as a primary target of DQ derivative RMB041 [25].

In a study by Rizvi, et al. [80], oxidative stress, inherent with the shift to a dormant state in *Mtb*, resulted in the statistically significant accumulation of the same six (aspartic acid, valine, threonine, alanine, isoleucine, and lysine) of the ten amino acids identified as metabolite markers in the DQ derivative RMB041 treated *Mtb* in our study. The flux of lysine towards acetic acid, and subsequent fatty acid synthesis, is supported by decreased levels of its break-down product, N-acetyl-lysine [81]. Furthermore, the branched chain amino acids, threonine, isoleucine and valine, serve as precursors of propionyl-CoA [82] for elongation of odd chain fatty acids [83]. Flux towards propionyl-CoA from valine is furthermore supported by the increased levels of β -aminoisobutanoic acid (BAIBA) in the DQ derivative RMB041 treated *Mtb*, whereby the intermediate methyl-malonyl semialdehyde (MMSA) is the direct precursor of propionyl-CoA [84].

Serine, one of the elevated amino acid markers in the DQ derivative RMB041 treated *Mtb* group, also serves as a precursor of phosphatidylserine [85]. Translocation of phosphatidylserine on the outer membrane of *Mtb* has been associated with cell death induced

by reactive oxygen species (ROS) [86]. ROS are released during the antibiotic-induced stress response and accumulate when DNA repair is unsuccessful, which in turn results in the aforementioned dormant phase [87,88]. Altered PG metabolism, as is brought about by cell wall active antibiotics, has also been linked to changes of growth and division of bacterial cells [89]. This further suggests irreversible inhibition by DQ derivative RMB041 to the cell envelope, which would result in a continuous activation of the stress response operon, requiring non-stop energy, supplied by fatty acid metabolism, and, partially, the TCA cycle, followed by the induction of a dormant state, whereby cell growth and division is ultimately inhibited.

In summary, this metabolomics approach better characterized the antimycobacterial mechanism of decoquinone RMB041, which not only confirmed previously proposed mechanisms of action but also suggests possible new mechanisms, which could be further investigated and confirmed using alternative strategies. A possible limitation, however, is the degradation of thermolabile metabolites into smaller unidentifiable “unknown” compounds [90].

4. Materials and Methods

4.1. Cell Culture

The reagents used for this investigation were purchased from Sigma–Aldrich, St. Louis, MO, USA, unless specified otherwise. The microtiter Alamar Blue assay was used to determine the antimycobacterial sub-minimum inhibitory concentration (IC₅₀) of DQ derivative RMB041 [91]. Cell cultures were prepared as delineated by Van Breda et al. [92], with minor modifications. Briefly, *Mtb* H37Rv ATCC 27294, obtained from the Medical Research Council (Pretoria, Gauteng, South Africa), was cultured and maintained on Lowenstein-Jensen (LJ) slants. After four weeks of incubation, a bacterial inoculum of McFarland 1 (approximately 3×10^8 colony-forming units/mL) was prepared in Middlebrook 7H9 broth containing 10% OADC (oleic acid, albumin, dextrose, catalase) (Becton, Dickinson, UK) and 2% PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) (Becton, Dickinson, UK). The latter shows a negligible impact on *Mtb* growth and was added to prevent contamination [93]. In five of the ten aliquots, 4 mL DQ derivative RMB041 was dissolved in DMSO (150 µM) and further diluted in Middlebrook 7H9 broth to yield a final concentration of 10 µM (0.2% DMSO). A volume of 1 mL of the prepared inoculum was added to reach a final assay volume of 5 mL, with a bacterial test concentration of 6×10^7 CFU/mL DQ derivative RMB041. The untreated *Mtb* control samples were prepared in the remaining five aliquots by adding the bacterial inoculum, as described above, in 4 mL of Middlebrook 7H9 broth (0.2% DMSO). After 5 days of incubation at 37 °C, the mycobacteria cultures were pelleted by centrifuging the samples at 4500 rpm for 15 min. These were then washed with 1 mL of PBS and re-pelleted, as described above. Lastly, the PBS was carefully aspirated, and the samples and the samples were stored at −80 °C until further testing.

4.2. Whole Metabolome Extraction Procedure and Derivatization

The extraction and derivatization procedures were carried out as previously described by Beukes et al. [94], with slight modifications. Briefly, 8 mg of each of the ten individually cultured samples were weighed into an Eppendorf tube. As internal standard, 50 µL 3-phenylbutyric acid (0.13125 mg/mL H₂O) (Sigma–Aldrich, Lot#536478V) was added, and a two-phase extraction was performed with chloroform: methanol: water in a 1:3:1 ratio (1 mL in each sample). The Eppendorf tubes, containing a 3 mm carbide tungsten bead in each, were shaken in a vibration mill at 30 Hz for 5 min, followed by centrifugation at 12,000 rpm for 5 min. After transferring each extract to a GC glass vial, the samples were dried under a nitrogen stream. Derivatization was achieved by firstly adding 50 µL methoxamine hydrochloride (Sigma–Aldrich, Lot#BCBP2843V) in pyridine (Lot#S2BC335SV) (at a final concentration of 15 mg/mL) to the glass vials and heating at 50 °C for 90 min, followed by the addition of 40 µL N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with

1% trimethylsilyl chloride (TMSCI) (Lot#BCBW2670) and again heating for 60 min at 50 °C. Lastly, each extract was transferred to a 0.1 mL vial insert, which were placed into their respective GC sample vial prior to injection into the GCxGC-TOFMS.

4.3. GCxGC-TOFMS Analysis

The metabolomics analysis was performed using a 4D Pegasus GCxGC-TOFMS (LECO Africa (Pty) Ltd., Johannesburg, South Africa), equipped with a Gerstel Multi-Purpose Sampler (Gerstel GmbH and Co. KG, Mülheim an der Ruhr, Germany) and an Agilent 7890 gas chromatograph (Agilent, Atlanta, GA, USA) coupled to TOFMS (LECO Africa). The GC-MS parameters were set as previously described [94]. A Rxi-5Sil MS capillary column was used as the primary column (28.8 m × 0.25 mm internal diameter, 0.25 µm film thickness, Restec), along with a Rxi-17 capillary column (1.2 m × 0.25 mm internal diameter, 0.25 µm film thickness). The primary GC oven temperature was set at 70 °C for 2 min, and increased at a rate of 4 °C/min to a final temperature of 300 °C, at which it was maintained for an additional 2 min. The secondary oven was set at 85 °C for 2 min, increased at 4.5 °C/min, to a final temperature of 300 °C, at which it was maintained for 4.5 min. Helium, used as a carrier gas, was set to a column flow rate of 1 mL/min and held at a constant temperature of 270 °C. Mass spectrometric data acquisition was carried out using a filament bias of −70 eV, with a 350 s solvent delay, and an acquisition scanned mass range of 50–800 *m/z* at 200 spectra/s. To ensure high internal validity, the samples were analyzed in random sequence. The analytical performance of GCxGC-TOFMS throughout the analysis was monitored by adding quality control (QC) samples at the start, in the middle, and at the end of the sequence.

4.4. Data Processing, Clean-Up, and Statistics

Mass spectral deconvolution (at a signal to noise ratio of 20), peak alignment, and peak identification was done with ChromaTof software (version 4.32, Leco Corporation, St. Joseph, MI, USA). For the identification of metabolites, their mass fragment patterns were compared to those of compounds in the commercially available database, NIST 11, and an in-house created Organic Acids library, containing previously injected standards. The quality of the data was evaluated by pretreating the data using a standardized metabolomics data clean-up procedure [94]. MS total useful signal was used for data normalization. Considering that the zero values are most likely present in sub-minimum concentrations rather than being completely absent, they were replaced by a value calculated as 20% of the minimum detection limit of the entire dataset [95]. An 80% data filter was then applied to remove compounds that were only detected in two (or less) samples per group [96]. Log transformation and auto-scaling was applied to balance metabolite representations. This prevented domination of compounds with higher concentrations over compounds with lower concentrations [97].

Using MetaboAnalyst (Version 5.0, <https://www.metaboanalyst.ca>, accessed on 3 May 2021) [98], two multivariate statistic methods were applied: (1) an unsupervised principal component analysis (PCA) and (2) a supervised partial least squares-discriminant analysis (PLS-DA) [99]. Additional univariate statistics were also calculated via (1) *t*-test and (2) effect size values [36].

5. Conclusions

DQ derivative RMB041 has been previously proposed to act against *Mtb*, primarily via inhibition/destruction of its cell wall and inhibition/destruction of DNA as a secondary target [25]. This metabolomic investigation indicated an altered metabolite profile (drastically elevated levels of various fatty acids and glycerolipid precursors, amino acids, and urea cycle intermediates) confirming the aforementioned observations in addition to an inhibition of protein synthesis and a state of dormancy. This study not only confirms or improves upon the existing knowledge pool of novel antimycobacterial mechanisms of actions, but also provides a useful tool for investigating repurposed and/or adjunctive

drugs against *Mtb*. Furthermore, a better understanding of the dormancy process as indicated here and could lead to new treatment regimens that reduce the emergence of relapse and resistance [100].

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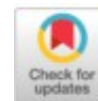
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In Silico Drug Discovery Strategies Identified ADMET Properties of Decoquinatone RMB041 and Its Potential Drug Targets against Mycobacterium tuberculosis

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ABSTRACT The highly adaptive cellular response of Mycobacterium tuberculosis to various antibiotics and the high costs for clinical trials, hampers the development of novel antimicrobial agents with improved efficacy and safety. Subsequently, in silico drug screening methods are more commonly being used for the discovery and development of drugs, and have been proven useful for predicting the pharmacokinetics, toxicities, and targets, of prospective new antimicrobial agents. In this investigation we used a reversed target fishing approach to determine potential hit targets and their possible interactions between M. tuberculosis and decoquinatone RMB041, a propitious new antituberculosis compound. Two of the 13 identified targets, Cyp130 and Blal, were strongly proposed as optimal drug-targets for dormant M. tuberculosis, of which the first showed the highest comparative binding affinity to decoquinatone RMB041. The metabolic pathways associated with the selected target proteins were compared to previously published molecular mechanisms of decoquinatone RMB041 against M. tuberculosis, whereby we confirmed disrupted metabolism of proteins, cell wall components, and DNA. We also described the steps within these pathways that are inhibited and elaborated on decoquinatone RMB041's activity against dormant M. tuberculosis. This compound has previously showed promising in vitro safety and good oral bioavailability, which were both supported by this in silico study. The pharmacokinetic properties and toxicity of this compound were predicted and investigated using the online tools pkCSM and SwissADME, and Discovery Studio software, which furthermore supports previous safety and bioavailability characteristics of decoquinatone RMB041 for use as an antimycobacterial medication.

IMPORTANCE This article elaborates on the mechanism of action of a novel antibiotic compound against both, active and dormant Mycobacterium tuberculosis and describes its pharmacokinetics (including oral bioavailability and toxicity). Information provided in this article serves useful during the search for drugs that shorten the treatment regimen for Tuberculosis and cause minimal adverse effects.

KEYWORDS in silico, decoquinatone RMB041, virtual docking, Mycobacterium tuberculosis, pharmacokinetics

Tuberculosis (TB), caused by Mycobacterium tuberculosis, remains one of the leading causes of death by a single infectious agent (1). Despite the many efforts to search

for novel anti-TB treatment, only three drugs have been approved and released into the pharmaceutical market over the last 50 years (2). The inefficiency of many of the existing anti-TB medication, can be attributed to the rapid development of drug resistance and poor understanding of the preceding cellular transition to dormancy (3). Research on compounds effective against active M. tuberculosis have shed light on the importance of only a few genes/proteins required for its nonreplicative survival, enabling mycobacteria to redirect energy sources for overcoming stress (deprivation of

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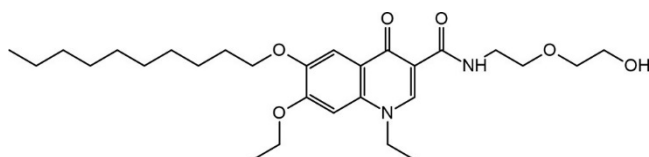


FIG 1 Structure of decoquinat RMB041.

nutrients, acidic environments, and exposure to reactive oxygen species, and various Anti-TB drugs) that resulted in the transition to dormancy and the subsequent anti-TB drug resistance (4). The increasing worldwide prevalence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB cases has also highlighted *M. tuberculosis*'s phenotypic plasticity during latency (5-7). Furthermore, drugs that are effective against nonreplicating *M. tuberculosis* are still lacking. Additional factors preventing the development of successful anti-TB drugs are the long duration and high costs of novel drug discovery and as the onset of unexpected adverse reactions during clinical trials.

New anti-TB drug discovery is complexed and requires a comprehensive understanding of a drug's functionality and its target proteins in both *M. tuberculosis* and the host. Fortunately, the ongoing progress of high-throughput laboratory and computational technology has served to promote exponential growth in both data volume and the variety of data sources, which has enabled researchers to create and apply computational prediction techniques, aimed at reducing the lengthiness and cost of all phases of drug discovery (8). Computer-aided drug design entails web servers and software that identify hit targets, analyze target-protein interactions, annotate cellular functionalities of proteins, and anticipate possible side effects. All have proven helpful during drug development of potent anti-TB compounds or the prevention of the inclusion of toxic drugs in clinical trials (9, 10).

F1 Decoquinat RMB041 (see structure in Fig. 1) is an inexpensive compound with promising in vitro antimycobacterial activity (11, 12), and some good in vitro ADMET (absorption, distribution, metabolism, elimination, and toxicity) properties (13). To identify and characterize decoquinat RMB041's hit targets, we combined several functions offered by a variety of software and online web servers; reverse pharmacophore docking, also known as target fishing, by PharmMapper (14), prioritization of targets by TDR Targets, identification of correlated proteins by TBDB, mapping of coexpressed proteins by STRING, annotation of metabolomic pathways by KEGG, virtual screening with Discovery Studio (DS) Visualizer, and docking with AutoDock Vina. Virtual docking has presented some difficulties of late, during the analysis of best binding poses (defined as binding affinities) for lead optimization, which is not surprising, considering that there are many factors that must be considered that may influence the binding of molecules (15). Hence, for the purpose of this study, estimated binding affinities were determined (16-18), also considering entropic and enthalpic influences, the mobility of ligands and proteins, the charge distribution over a ligand, surrounding water molecules, and the various possible conformations of the compound of interest (19).

Previously, Beteck, Seldon (12), indicated low cytotoxicity of decoquinat RMB041 against WI-38 human fetal lung fibroblasts. In the light of this, we predicted the toxicity of decoquinat RMB041, by investigating those properties generally known to significantly induce toxicity of well-known drugs currently available. Additionally, further properties attributing to absorption, distribution, metabolism, and elimination were determined and evaluated using DS Tools, SwissADME, and pkCSM (20). A previous metabolomic GCxGC-TOFMS investigation of decoquinat RMB041 on *M. tuberculosis*, indicated a mechanism of action by perturbation of the mycobacterial cell wall and DNA synthesis and also a proposed mechanism by which *M. tuberculosis* might develop a resistance to decoquinat RMB041. In this study, we further contribute to this knowledge by using an in silico approach to confirm and build on previous knowledge related to decoquinat RMB041 mechanism of action, toxicity and possible *M. tuberculosis* resistance.

TABLE 1 Molecular properties of decoquinat RMB041, as provided by SWISSadme and Discovery Studio

Molecular properties	SWISSadme	Discovery studio
Canonical SMILES	<chem>CCCCCCCCCOC1C2C(C1OCC)N(C2)C(=O)C(=O)NCCOCCO</chem>	-0
Formula	C ₂₈ H ₄₄ N ₂ O ₆	C ₂₈ H ₄₄ N ₂ O ₆
MW	504.66	504.67
Number Atoms	36	80
TPSA	99.02	98.37
Molecular Refractivity	145.00	-
Number Rotatable bonds	19	19
Number H-bond acceptors	6	8
Number H-bond donors	2	2

RESULTS AND DISCUSSION

General molecular properties. Aside from the number of hydrogen acceptors and the number of atoms—the latter because pkCSM counts only heavy atoms, while DS counts all atoms of the molecule—in our study SWISSadme and DS agreed on all decoquinat RMB041's molecular properties (Table 1).

Likeness of oral administration. Pharmaceutical companies generally apply one or more regulations that assist in determining the drug likeness of oral administration, of which Lipinski's rule of five is the most common criteria (21). The number of violations of the rules of Lipinski, Veber, Egan, and Muegge, provided by SWISSadme, along with the molecular properties breaching these rules, are presented in Table 2. In regard to the applied pharmaceutical regulations, only the molecular weight (MW) and the amount of rotatable bonds breach the rules. These broken rules do not necessarily indicate a lack of drug efficacy (22). All in all, the results presented here indicate promising effectivity of decoquinat RMB041 after oral administration.

Absorption, distribution, metabolism, elimination, and toxicity. In earlier studies, decoquinat RMB041 (MIC₉₀ = 1.25 mM) has shown to have promising bioavailability and distribution characteristics (13). Additional positive ADMET-related properties were also indicated by DS and prediction webserver used in this study. ADMET influencing factors from these tools and previous literature (12, 13) are presented in Table 3.

Considering all parameters related to decoquinat RMB041 absorption, the high lipophilicity (LogP = 4.63 to 4.90; LogD = 4.80) of decoquinat RMB041 in our investigation, suggests excellent permeation through the mycobacterial cell wall and into macrophages, and higher likelihood of being effective against dormant *M. tuberculosis* (23). Moderate to good aqueous solubility (Table 3) indicates its practicality during drug formulation and gastrointestinal (GI) absorption (24, 25). A bioavailability score of 0.55 additionally confirms good absorption after oral administration (Martin YC 2005). However, the low Caco-2 cell permeability (human model for GI absorption; High when LogP_{app} > 0.9) indicates that further improvement of the solubility might be necessary. Another important influencer on absorbance is the amount of efflux from cellular tissue, especially by p-glycoprotein (Pgp). The contradictory results (SWISSadme versus pkCSM) regarding the binding of decoquinat RMB041 to Pgp (Table 3) suggests further experimentation regarding this would be necessary. In either case however, whether it is not a substrate for Pgp (as predicted by

TABLE 2 Number of violations of commonly applied pharmaceutical rules of drugability, as provided by SWISSadme

Pharmaceutical test	SWISSadme	Rule violated	Reference
Lipinski number violations	1/5	MW > 500	Lipinski (80)
Veber number violations	1/2	Number Rotatable bond > 10	Veber, Johnson (96)
Egan number violations	0/2	-	Egan, Merz (97)
Muegge number violations	2/8	MW > 300 Number Rotatable bond > 15	Muegge, Heald (98)

TABLE 3 ADMET pharmacokinetic properties of decoquinat RMB041, provided by computational prediction methods and previous literature

Absorption	SWISSadme	pkCSM	DiscoveryStudio	Previous literature
Lipophilicity	4.63 (iLogP)		4.80 (AlogP98)	4.90 (cLogP)
Aqueous Solubility (log mol/L)	25.68	25.82	23.60	-
Solubility	Moderate	-	Good	-
Caco2 permeability (log P _{app})	-	0.662	-	-
GI absorption	High	82.14%	Moderate	-
Bioavailability	0.55	-	-	21%
Pgp substrate	No	Yes	-	-
Pgp I & II inhibitor	-	Yes	-	-
Distribution				
BBB permeant (log)	No	20.741	Very low	-
CNS permeant (log)	-	23.671	-	-
VD _{ss} (human)	0.32 log L/kg	-	-	-
Metabolism				
Plasma Protein Binding	-	-	No	-
Plasma Binding (Fu)	-	0.06 (human)	-	0.1 (mouse)
Microsomal Binding (Fu)	-	-	-	0.06 (mouse)
CYP1A2 inhibitor	No	No	-	-
CYP2C19 inhibitor	No	Yes	-	-
CYP2C9 inhibitor	No	Yes	-	-
CYP2D6 inhibitor	No	No	No	-
CYP3A4 inhibitor	Yes	Yes	-	-
Elimination				
CL _{int}	-	-	-	16 mL/min/kg
E _H	0.43	-	-	-
CL _{tot}	-	19 mL/min/kg	-	-
t _{1/2}	-	-	-	23.4h
Toxicity				
AMES toxicity	NP	No	Non-Mutagen	NP
Max tolerated dose	NP	799 mg/kg (Human)	90 mg/kg (Rat)	NP
hERG I & II inhibitor	NP	No	-	NP
Hepatotoxicity	NP	Yes	No	NP
Carcinogen (standard FDA test)	NP	NP	Noncarcinogen	NP
Aerobic Biodegradability	NP	NP	Degradable	NP

NP, none predicted; P_{app}, apparent permeability coefficient; GI, gastrointestinal; Pgp, P-glycoprotein; BBB, blood brain barrier; CNS, central nervous system; VD_{ss}, volume of distribution; Fu, fraction unbound; CL_{int}, intrinsic clearance; E_H, hepatic elimination; CL_{tot}, total clearance; t_{1/2}, half-life; AMES, assay of the ability of a chemical compound to induce mutations in DNA.

pkCSM), or whether it is a substrate, and inhibits Pgp I and II, the results indicate a lack of export, meaning decoquinat RMB041 is likely to accumulate in the target organs.

When considering the distribution criteria in this investigation, all three prediction methods state unlikely penetration of decoquinat RMB041 through the blood-brain barrier (BBB) (Table 3) (26). This may indicate a low probability of neurotoxic effects, such as confusion, depression, psychosis, or muscular weakness, which appear in response to various other current anti-TB medications (27, 28). It may also indicate however, that this compound would likely be of little use for the treatment of TB meningitis. Blood plasma volume of distribution of decoquinat RMB041 (log VD_{ss} . 0.32) (Table 3) is on the higher end of the spectrum (log VD_{ss} , 20.15 is low; log VD_{ss} .0.45 is high), suggesting moderate delivery to infected areas (29, 30).

Considering the metabolism of the decoquinat RMB041, the cytochrome P450's, the most significant metabolizing enzymes to be considered during drug metabolism, oxidize xenobiotics to facilitate their excretion (31). The inhibition of these enzymes by an antibiotic indicates the likelihood of toxic accumulation, as well as possible interference with the pharmacokinetics of coadministered antibiotics. Despite differing conclusions about CYP2C9 and CYP2C19 inhibition by decoquinat RMB041, the pkCSM's and SWISSadme's calculations presented in this study regarding the two main cytochrome isoforms, 2D6 and 3A4, are in agreement (Table 3). CYP2C9 was recognized by

TABLE 4 The targets identified by PharmMapper, along with their respective identification codes, fit scores, binding free energies, and residues that interact with both, their respective cocrystallized ligands and decoquinat RMB041

Target protein	PDB iD	UniProt ID	Fit score	Binding free energy (kcal/mol)	Overlapping residues
Cyp130 (Rv1256c)	2UVN	P9WPN5	4.36	27.5	Leu A:71, Pro A:87, Pro A:88, Phe A:236, Thr A:239, Met A:240, Thr A:247, Pro A:289, Val A:290, Phe A:347, Cys A:354, Leu A:355, Gly A:356, Ala A:359, Ala A:360, Val A:393
FbpB (Rv1886)	1F0N	P9WQP1	4.30	27.4	Asp A:40, Leu A:42, Arg A:43, Ser A:126, Leu A:152, Leu A:163, Leu A:229, Phe A:232, His A:262, Trp A:264, Trp A:267
LysA (Rv1293)	2O0T	P9WIU7	5.70	27.0	Cys A:375, Glu A:376, Ser A:377, His B:213, Arg A:303, Tyr B:405
AdoK (Rv2202c)	2PKF	P9WID5	4.24	26.9	Val A:49, Gln A:172, Asn A:195, Thr A:223, Val A:255, Asp A:257, Phe A:259, Ser A:281, Leu A:288
SecA1 (Rv3240)	1NKT	P9WGP5	4.90	26.6	Gln A:80, Phe A:83, Gln A:86, Lys A:107, Leu A:109, Arg A:137, Trp A:141, Asp A:493, Asn A:499, Asp A:501, Arg A:573
GlnA1 (Rv2220)	2bvc	P9WN39	4.71	26.5	Glu A:133, Glu A:214, Lys A:215, Glu A:227, His A:276, His A:278, Arg A:347, Arg A:352, Arg A:364, Glu A:366, Arg A:368
LpdA (Rv3303c)	1XDI	P9WHH7	4.11	26.3	Cys A:48, Lys A:52, Tyr A:450
LppX (Rv2945)	2BYO	P9WK65	4.75	25.9	Val A:45, Leu A:54, Leu A:55, Ile A:57, Ala A:60, Phe A:85, Ile A:92, Ile A:106, Leu A:109, Ser A:110, Arg A:113, Met A:158
GlcB (Rv1837c)	1N8W	P9WK17	6.48	25.9	Leu A:117, Val A:118, Val A:119, Pro A:120, Phe A:126, Asn A:129, Ala A:130, Ser A:275, Arg A:312, Pro A:543, Ser A:544, Pro A:545, Cys A:619, Ser A:620, Lys A:621, Met A:631, Phe B:310
FabD (Rv2243)	2QC3	P9WNG5	4.46	25.5	His A:90, Ser A:91, Asn A:155
Blal (Rv1846c)	2G9W	P9WMJ5	4.63	25.3	Lys B:3, Arg A:6, Arg B:6
FolP1 (Rv3608c)	1EYE	P9WND1	4.45	25.3	Asp A:21, Gly A:181, Phe A:182, Lys A:213

PharmMapper as a potential protein target, supporting the results by pkCSM. Although no inhibition of CYP2D6 is expected, inhibition of CYP3A4 suggests that care should be taken when coadministering decoquinat RMB041 with other antimycobacterial compounds, in order to prevent toxic accumulation of either or both drugs.

When evaluating the elimination characteristics of decoquinat RMB041 using the data generated by pkCSM, SWISSadme, and previous experimentation results by Tanner, Haynes (13) and colleagues, we determined estimated values for the total clearance rate ($CL_{tot} = 19$ mL/min/kg), intrinsic clearance (reflecting hepatic and biliary excretion) ($CL_{int} = 16$ mL/min/kg), hepatic excretion (E_H , 0.43), and the elimination half-time ($t_{1/2} = 23.4$ h) (Table 3). The $t_{1/2}$ is far superior to those of the other first-line drugs rifampicin ($t_{1/2} = 7$ h) (32), isoniazid ($t_{1/2} = 1.7$ h) (33), and ethambutol ($t_{1/2} = 3$ h) (34), and indicates less frequent drug administration would be required, which is beneficial for patient coherence and possibly decreasing the consequential occurrence of drug resistance.

Decoquinat RMB041 has previously shown little cytotoxicity against WI-38 human fetal lung fibroblasts ($IC_{50} = 56.2$ mM) (12). According to the pkCSM and DS results in this study, this compound is neither carcinogenic, nor mutagenic, nor likely to interfere with heart rhythms (Table 4). Furthermore, the odds of having neurotoxic and hepatotoxic properties are also low. The positive hepatotoxicity by pkCSM is only based on the similarity of structural features to compounds with liver-associated adverse effects, whereas DS, which showed no possible hepatotoxicity, uses additional information related to dose concentrations to establish the probability of hepatotoxicity. Furthermore, our investigation indicated that decoquinat RMB041 shows a promising safety profile compared to that of other first- and second line antitubercular drugs which exhibit cardiotoxic, hepatotoxic and/or neurotoxic effects (28, 35-37). That said, the contradictory results by pkCSM and DS concerning hepatotoxicity necessitates further experimentation. Aerobic

T4

biodegradability, a trait that is often underestimated and missed during drug development, is important for the prevention of wastewater pollution, which would else cause serious harm to the aquatic ecosystems and increase antibiotic resistance in humans (38). The degradation of this compound therefore suggests that it is safe in the face of environmental pollution also, just as a matter of interest.

Identification and prioritization of drug targets. It is widely known that drugs commonly target several proteins (as opposed to only one particular protein) (39). PharmMapper, as used in this study, compares the pharmacophores of the investigated drug compound, to those derived from ligands in complex crystal-structures and provides predicted corresponding protein targets (14). A total of 12 *M. tuberculosis* proteins were identified by PharmMapper as potential targets of decoquinat RMB041. These are listed according to their docking scores in Table 4, along with their respective PharmMapper fit scores, UniProt and PDB accession codes, and associated KEGG reactions. A limitation one should keep in mind about current computer-aided drug design, is that only the known active sites in databases can be used for identifying suitable ligand scaffolds. Further research on active sites of proteins would increase the accuracy of the elucidated mechanism of action, which will undoubtedly improve as more data becomes available regarding this.

The docking affinities calculated by AutoDock Vina ranged between 27.5 to 25.3 kcal/mol and indicated strong binding to the target proteins (Table 4). Several other studies have ranked targets by their importance in *M. tuberculosis*, focusing mainly on drugability and essentiality in vitro survival (40, 41). However, it is also important to consider the various conditions that *M. tuberculosis* are exposed to in the host during infection or disease, such as acidity, reactive oxygen species, nutrient restriction, and various antibiotics in the case of a treated TB patient. During previous genomic, proteomic, and metabolomic investigations of *M. tuberculosis* exposed to each of the aforementioned conditions, it has become apparent that, although the initial response mechanism may differ, *M. tuberculosis* resilience ultimately depends on its ability to survive in a nonreplicative/latent TB state, which is the case in most individuals infected with TB, which is for one third of the global population currently (1) and hence, it would be of value to investigate targets that would shorten the treatment duration of latent *M. tuberculosis*. One should also consider the neighboring network of the target gene/protein, i.e., targeting a gene/protein associated with many other gene/proteins might prove more successful than targeting one with fewer interacting genes/proteins. Lastly, the exclusivity of a target gene/protein to *M. tuberculosis*, is also an extremely sought-after characteristic in drug design, and additionally, if other genes/proteins within in *M. tuberculosis* have a similar function, its less likely to disarray essential cellular processes.

To predict the decoquinat RMB041's drug targets, we determined their ranking by TDR Targets, which, with the help of integrated databases, allows users to prioritize genes and their annotated proteins based on various filtering criteria and criteria-specific weighting (42). We filtered nonhuman homologs and looked at three ranking orders: (a) with respect to homology to gut flora, similarity to essential genes, and maintaining of persistence (43), (b) upregulation of genes during dormancy and transition to dormancy (44), and (c) ortholog-based inference of essentiality of genes during experimental conditions relevant to drug discovery. Based on the first ranking order, 313 protein targets annotated to highly prioritized genes were provided, of which only one, Cyp130, was also identified by PharmMapper as a target of decoquinat RMB041. The second-ranking order provided a list of 420 proteins, of which only one, Blal, is a decoquinat RMB041 target. According to the third-ranking criteria, both Cyp130 and Blal (2 out of 397 proteins) are essential targets of decoquinat RMB041. FolP1 and lysA were also number one and number three, respectively, on a list of 4000 genes that were ranked based on their metabolic uniqueness in a study by Hasan, Daugelat (43), further indicating the importance of their encoded proteins.

Cellular mechanisms. In this study, the KEGG database BRITE hierarchy files annotated 10 of the 12 targets, of which all are involved in *M. tuberculosis* energy metabolism,

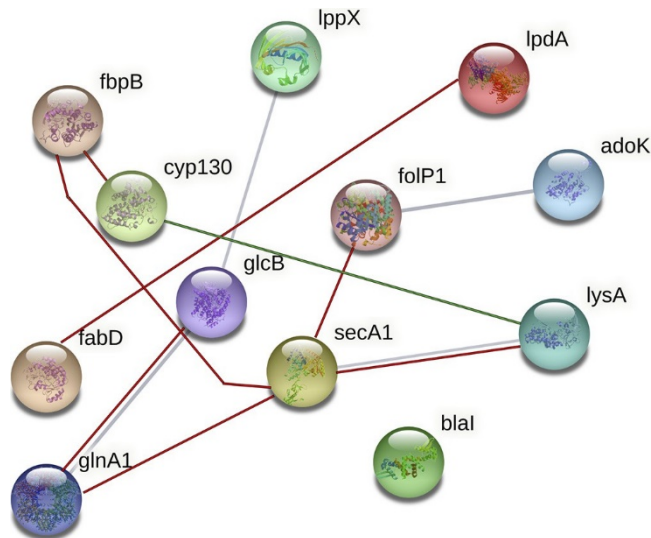


FIG 2 Interconnected network provided by STRING (gray), with additional interactions retrieved from TBDB; Positively correlated interactions (red) and negatively correlated interactions (green).

protein synthesis, fatty acid synthesis, cell wall synthesis, TCA cycle activity, and/or amino acid synthesis. All of the aforementioned pathways were found to be altered in our previous study (11), possibly indicating the imbuing of adaptations by *M. tuberculosis* in an attempt to counteract or adapt to the disruption of the primary drug targets identified in this study. Correlations of *M. tuberculosis* genes have been extensively studied and are still barely understood. Aside from the very many genes in *M. tuberculosis* (45), the complexity of interdependent intracellular genetic expressions makes it almost impossible to predict the complete cellular response to external stimuli accurately. For instance, even if the expressed proteins show a strong positive correlation to each other, it is impossible to tell if the inhibition of one would lead to the inhibition of the other, without extensive experimentation of each interaction separately. However, it can indicate the metabolic pathways that are likely to be altered in the inhibition process and give information on which drugs might work well in conjunction with decoquinat RMB041. With help of STRING and TBDB, in this study we identified protein targets and analyzed direct correlations between these (Fig. 2). TBDB links *M. tuberculosis* gene-expression microarray data to their encoded proteins and provides the strength of the correlation between proteins (46). Of a total of 144 correlated proteins, only 10 were negatively correlated. If one were to assume that inhibition of the protein targets would influence the expression of the correlated proteins, it would mean that 134 other proteins might be expressed to a lesser degree, while 10 proteins might be expressed to a larger extent.

Beteck, Seldon (13) and colleagues established that decoquinat RMB041 primarily acts on the cell wall and, second, inhibits DNA synthesis. Our findings during an earlier metabolomic investigation confirmed this and also suggested that decoquinat RMB041 inhibits protein synthesis, and pathways associated with *M. tuberculosis* in the nonreplicating state (11). In the current study, we examined each drug target to better understand the perturbations caused by decoquinat RMB041 and explore the cellular response by which *M. tuberculosis* counteracts these, such as those leading to the metabolomic changes revealed in our previous investigation and will be discussed below.

Blal controls the expression of the Rv1864c regulon, which comprises genes involved in drug transport, detoxification, and ATP synthesis (47). Dissociation of this enzyme from its operator site allows the transcription of the genes belonging to this regulon, including blal, encoding itself, and blaC, encoding beta-lactamase (BlaC) (48), which causes resistance to beta-lactams (49). Depending on the ratio of Blal inhibition

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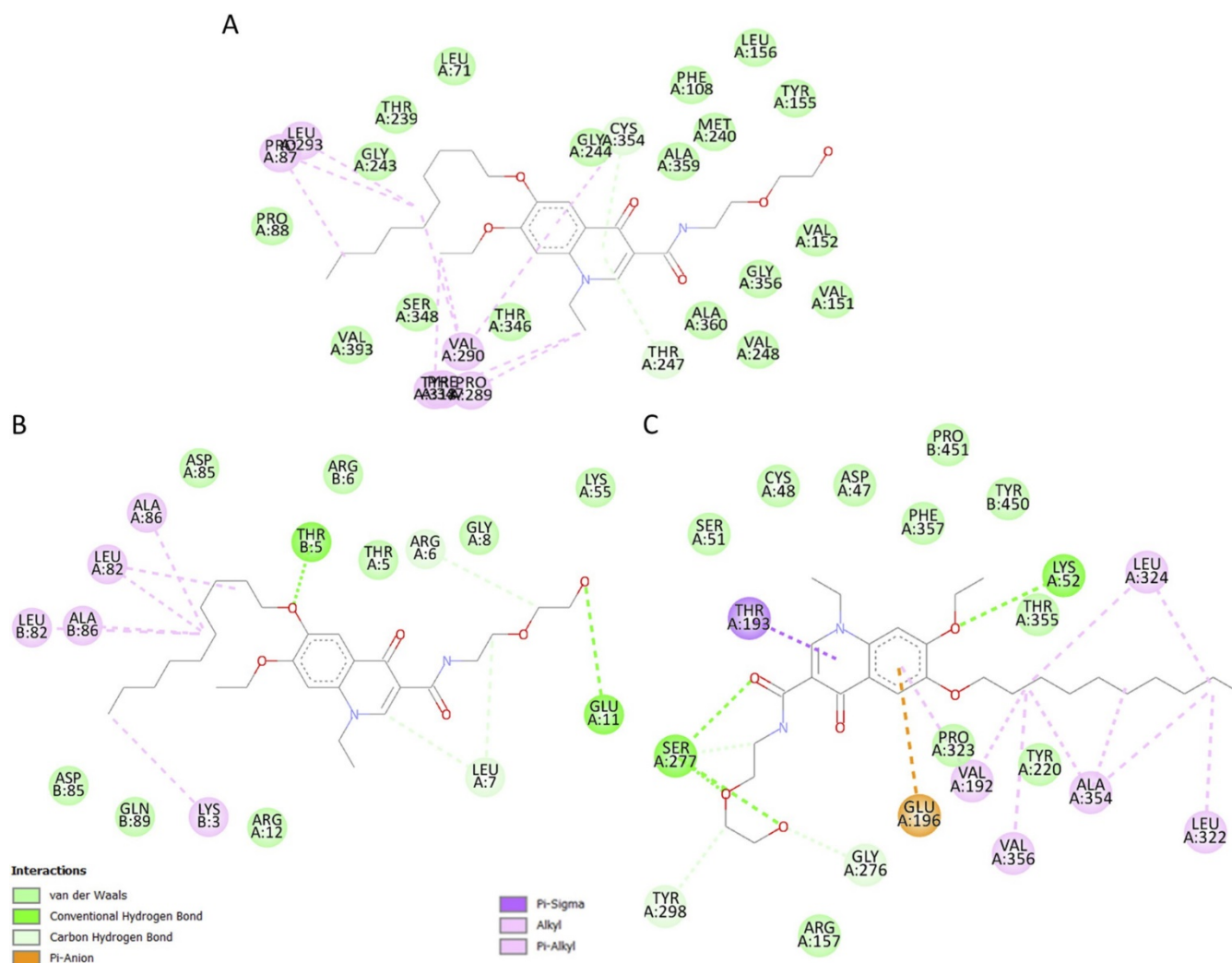


FIG 3 Interactions between (A) Cyp130 and decoquinone RMB041, (C) Blal and decoquinone RMB041, and (D) LpdA and decoquinone RMB041.

versus blal transcription and the mycobacterial degradation of the inhibitor, it could either enhance efflux, ATP synthesis and tolerance to stress, or repress the regulon, leading to opposite effects. Either way, it would disrupt the $\text{NAD(P)H}:\text{NAD(P)}^+$ ratio. Considering that previous studies indicated elevated NAD(P)^+ in the presence of decoquinone RMB041 (11), it is tempting to assume that hydrogen ions are lacking, meaning ATP synthase is inhibited, of which one could deduce that the entire regulon is being repressed. Considering that TDR Targets also deduces its answers based on orthologous experimental results of studies on other bacteria, such as *Escherichia coli* or *Staphylococci*, the prioritization of Blal as a drug target is a promising outcome. Nonetheless, further experimentation would bring clarity to what the consequences of Blal inhibition would be. The DNA binding site of Blal shares three residues with that of decoquinone RMB041 (based on the AutoDock Vina results), and the interaction between the drug and Blal involves five strong hydrogen bonds (Fig. 3).

Cyp130 is one of 20 cytochrome P450 enzymes responsible for the incorporation/reduction of molecular oxygen, and functions in conjunction with Tap (Rv1258c), for the export of drug molecules (50). Furthermore, it is well known that Cyp130 is important for growth and virulence of *M. tuberculosis*, and hence the inhibition of Cyp130, could be a valuable target for the treatment of infection/disease caused by *M. tuberculosis* (45). In the aforementioned study, Cyp130 was also indicated with a high priority as an anti-tuberculous target in two of the selected TDR Target ranking orders, and

reportedly inhibited by a drug group called azoles, with econazole showing the most potent activity (45). Interestingly, our study shows the binding cavity of econazole includes 16 residues that also interact with decoquinat RMB041 (51) (Fig. 3). Cyp130 shows the highest comparative binding affinity to decoquinat RMB041, mostly via hydrophobic interactions and two strong hydrogen bonds.

LpdA is a NAD(P)H-requiring flavoprotein disulfide reductase that has been found to be significantly upregulated during the transition of *M. tuberculosis* toward a state of dormancy (52, 53). In this investigation we see, within the active cavity of LpdA, bound to both FAD and NADP1, three residues also interact with decoquinat RMB041 (Fig. 3). Inhibition of this enzyme would prevent the conversion from 2,6-dimethyl-1,4-benzoquinone to 5-hydroxy-1,4-naphraquinone and a subsequent accumulation of NAD(P)H. Elevated NAD(P)H levels in turn, would induce counteractive pathways, for instance the NAD(P)H dependent production of α -hydroxyglutaric acid, fatty acids, and sugar alcohols. All three pathways have been found to be altered in our previous metabolomics study (11), further supporting decoquinat RMB041's inhibition of LpdA and, accordingly, a promising activity against dormant/latent *M. tuberculosis*.

F4

Two of the identified target proteins in our investigation, SecA1 and LppX (Fig. 4), are transmembrane transporters. SecA1 is ATPase coupled and responsible for the transport of the majority of proteins, including those suppressing phagocyte maturation (54). Its previously demonstrated active site, when bound with ADP (ADP)-b-S, shares 11 residues with the binding site of decoquinat RMB041 (55). Inhibition of SecA1 results in an elevated ATP:ADP ratio, i.e., which would disrupt intracellular energy metabolism, in addition to prevent the export of proteins required for cell wall biosynthesis (56, 57). The assimilation of these proteins would in turn induce protein degradation, especially during nutrient starvation (58), to provide proteinogenic amino acids as a source of nitrogen and carbon. This response is supported in our previous metabolomics study, which indicated elevated levels of proteinogenic amino acids in decoquinat RMB041 treated *M. tuberculosis* (11). SecA1 has been referred to as an optimal cotarget, with "cotarget" defined as a protein whose inhibition, in addition to that of the primary target, would hinder the development of resistance (59). The other transmembrane transporter, LppX, functions by carrying lipophilic molecules across the mycobacterial membrane. Its encoding gene, *lppX*, is upregulated during host-infection (60) and enhances the bacilli's capability to escape the host's immune response (61). Decoquinat RMB041 and vaccenic acid, share 19 residues within the active cavity site with which they interact (61). Compared to vaccenic acid, decoquinat RMB041 forms several more hydrophobic interactions, indicating a stronger competition. Inhibition of LppX, would likely cause accumulation of fatty acids, confirmed by the drastically elevated levels of these compounds in our previous metabolomics study (62).

FabD, the malonyl transacylase, is the first of five enzymes (*fabD*, *acpM*, *kasA*, *kasB*, and *accD6*) responsible for fatty acid elongation (63, 64). The role of the encoding gene, *fabD*, during the stress response is unclear, as it was underrepresented after exposure to rifampicin (65), upregulated in the presence of isoniazid (66), and overexpressed after the removal of a stressor (67). Nonetheless, it has frequently been associated with drug resistance (66, 68), indicating its importance for survival after exposure to antibiotics. FabD is a target of the Pup-proteasome degradation (69), which could mean that the protein is readily marked for destruction, followed by the regeneration of mutated FabD. When FabD is inhibited, malonyl-CoA accumulates (12), and also preferentially used as a substrate for mycolic acid synthesis, an important cell wall component for survival during dormancy (70). Despite this however, no shortage of elongated fatty acids occurs, indicating the existence of similarly functional proteins, and/or the break-down of cell wall components into fatty acids, that are considered less vital for survival under conditions of stress, which in this case were trehalose dimycolates. This enzyme's lack in unique functionality, would also explain why it was not considered an important target by TDR Targets. The active site of the original crystal structure of FabD contains acetic acid (71),

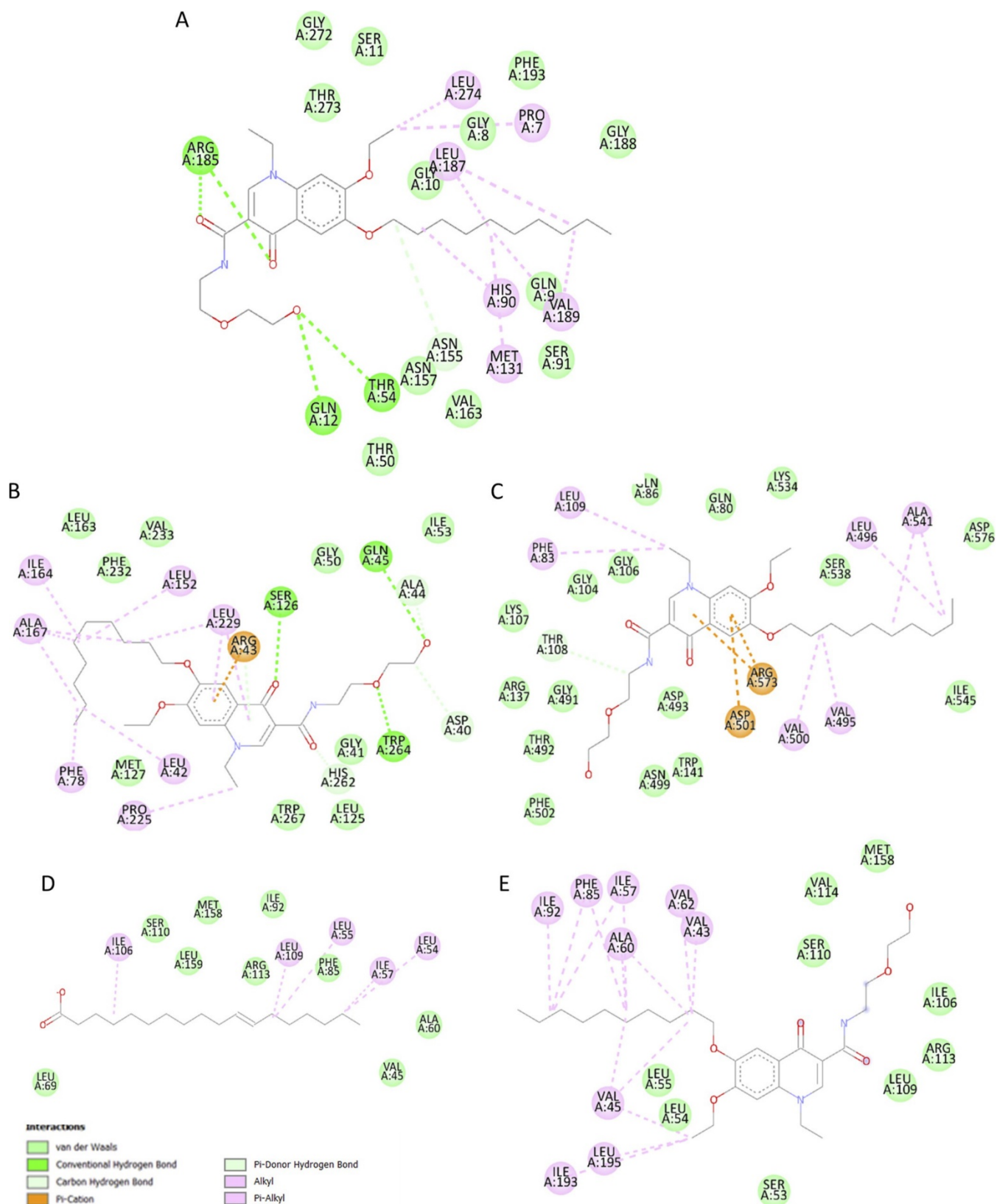


FIG 4 Interactions between decoquinate RMB041 and (A) FabD, (B) FbpB and (C) SecA1, as well as those between LppX and (D) vaccenic acid and (E) decoquinate RMB041.

of which three interactive residues also bond with decoquinat RMB041 (Fig. 4), as indicated in this investigation. FabD is positively correlated with KasA, an enzyme involved in the inhibitory activity of isoniazid on fatty acid elongation, suggesting possible synergistic activity of decoquinat RMB041 with isoniazid.

FbpB, also known as antigen 85B, catalyzes the biogenesis of trehalose dimycolates (TDM) and is the most abundant of all mycoyl transferases. TDM accumulate outside the cell wall and desensitize the cell to antibiotics (72). FbpB is overexpressed during early infection of macrophages, suggesting it to be important for the shift to *M. tuberculosis* dormancy/latency (73). Inhibition of FbpB would result in the accumulation of TDM, followed by its break-down into mycolic acids, which can either be used to synthesize other cell wall components or be broken down further into long-chain fatty acids. Although FbpB is highly expressed during early infection of macrophages (74), its encoding gene, *fbpB*, has been seen to be downregulated during hypoxia (75), indicating little importance of FbpB during dormancy. Nonetheless, FbpB inhibition might serve useful to prevent desensitization of coadministered antimycobacterial agents. In this study, we found that the binding of decoquinat RMB041 to FbpB (Fig. 4) is verified by 11 interacting residues that also noncovalently bind to trehalose (74). Interactions between decoquinat RMB041 and FbpB, showing the second highest binding affinity, include six strong hydrogen bonds.

Malate synthase (GlcB) is responsible for the synthesis of malic acid via the glyoxylic shunt, a key pathway during *M. tuberculosis* dormancy, that incorporates even chain fatty acids into the TCA cycle (76). GlcB upregulates the dormancy regulator (DosR) regulon and increases tolerance to stress (77). Inhibition of GlcB in this investigation would explain the accumulation of the even-chain fatty acids shown in our previous metabolomics study on decoquinat RMB041 treated *M. tuberculosis*, although levels of malic acid were increased, likely due to the incorporation of proteinogenic amino acids (11). Although GlcB was noted as necessary during *M. tuberculosis* dormancy/latency, its absence in the prioritizing lists of TDR Targets in our study indicates that *M. tuberculosis* possesses several enzymes controlling malic acid. The latter could be expected, considering that it is a primary element within the central carbon flux. Furthermore, our results indicate GlcB's natural ligand, acetyl coenzyme A, shares 17 interacting residues with decoquinat RMB041 (Fig. 5) (78). According to the TBDB results, GlcB is positively correlated with rifampicin targets, RpoB and RpoC, suggesting possible synergistic activity of this drug with decoquinat RMB041.

Meso-diaminopimelate decarboxylase (LysA) catalyzes the final step of lysine biosynthesis. Its encoding gene, *lysA*, is regulated by LysR, which is repressed in the presence of lysine (79). Upregulation of *lysA* has been noted in immunocompromised mice (80), and the highly conserved nature of this gene indicates that it is mainly associated with *M. tuberculosis* stress responses (45). Under normal circumstances, inhibition of LysA would lower lysine levels and LysR would keep inducing *lysA* expression, creating a metabolic loop and preventing protein synthesis. However, concurrent protein degradation would provide sufficient lysine for the organism, as shown in our previous metabolomics study, which would in turn repress LysR. In our study, inhibition of LysA would, in this case, not provide much assistance in eliminating mycobacteria. The targeting of this enzyme is supported by six residues that interact with both decoquinat RMB041 and lysine (Fig. 5) (81).

Glutamine synthetase (GlnA1) is a key enzyme used during nitrogen metabolism and cell wall synthesis (82). It catalyzes ATP-dependent ammonium condensation with glutamate to form glutamine, ADP, and phosphate. The crystal structure of GlnA1 was investigated in a complex with methionine sulfoximine phosphate, a product of methionine sulfoximine phosphorylation, and includes 11 residues in its active binding site (83). As indicated by our study, these residues also appear in the binding pocket of decoquinat RMB041 (Fig. 5). Inhibition of this enzyme would explain the previously identified elevation of glutamate levels (11). GlnA1 has been proposed as a promising anti-TB target with high importance during dormancy (72, 84) and mycobacterial

F5

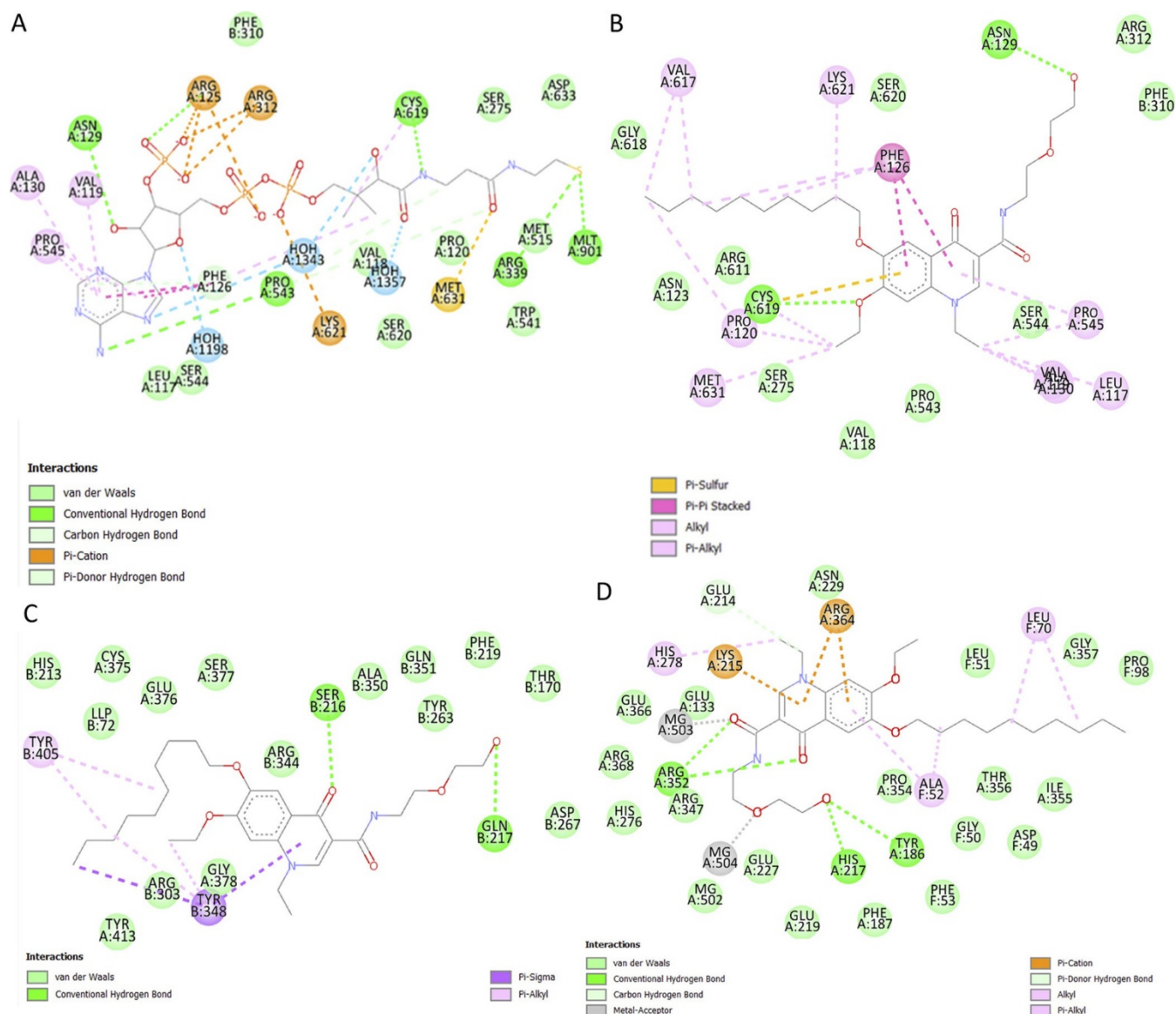


FIG 5 Interactions between (A) GlcB and acetyl-coenzyme-A, (B) GlcB and decoquinat RMB041, (C) LysA and decoquinat RMB041, and (D) GlnA1 and decoquinat RMB041.

growth in macrophages (85). According to the TBDB results, GlnA1 is positively correlated with four proteins that are targeted by other anti-TB drugs. RpsL, EmbA, RpsA, and AtpE are drug targets of streptomycin (protein synthesis inhibitor), ethambutol (mycolic acid transfer inhibitor), pyrazinamide (fatty acid synthesis inhibitor), and bedaquiline (ATP synthase inhibitor), respectively. Hence, further experimentation of decoquinat RMB041 with one or more of these drugs might prove synergistic activity against *M. tuberculosis*.

Adenosine kinase (AdoK) catalyzes the phosphorylation of adenosine to AMP (AMP). This enzyme participates in the purine salvage pathway and plays a crucial role in nucleotide synthesis during *M. tuberculosis* persistence (86). The active binding site was previously illustrated with adenosine (87) and involves nine residues that interact with decoquinat RMB041 (Fig. 6), as indicated in our investigation. Inhibition of this enzyme would be expected to prevent the formation of DNA during the shift to dormancy (as opposed to acutely disrupt DNA synthesis when administered). This “be-lated” disruption of DNA formation by decoquinat RMB041, was also confirmed in two previous studies (11, 12).

F6

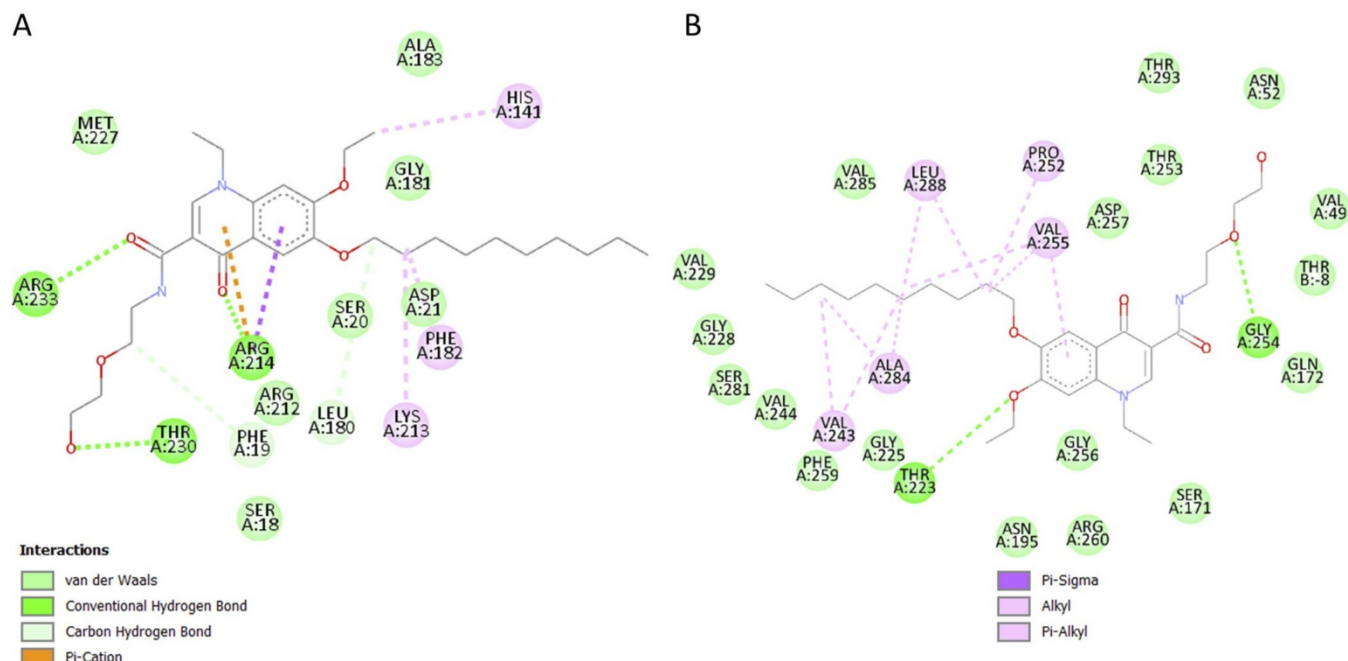


FIG 6 Interactions between decoquinat RMB041 and (A) AdoK and (B) FolP1.

Inhibition of folic acid synthase (FolP1) would also lead to disrupted DNA synthesis in *M. tuberculosis*, as proven by previous experiments on mycobacteria in the presence of sulfamethoxazole and trimethoprim (88). FolP1 catalyzes the synthesis of the 6-hydroxymethyl-dihydropteroate and pyrophosphate, from the substrates 6-hydroxymethyl-7,8-dihydropterin-pyrophosphate and para-aminobenzoic acid and has previously been proposed to be a promising drug target against *M. tuberculosis* (88). Its encoding gene, folP1, is upregulated during the early and late dormancy (52), indicating its importance in maintaining *M. tuberculosis* during the nonreplicative state. In our study, the crystal structure of FolP1 in complex with 6-hydroxymethylpterin monophosphate (89) shares four interacting residues with decoquinat RMB041 (Fig. 6).

Conclusion. Various in silico drug discovery strategies have been implemented during this study to identify potential drug targets for decoquinat RMB041 against *M. tuberculosis*. Furthermore, we generate additional support for the previously determined metabolic pathways that were disrupted by decoquinat RMB041 and indicate at which enzymes the particular disruptions may occur. In addition, feasible synergistic activity of decoquinat RMB041 with other antimicrobials was shown. In this study, we also elaborated on the possible activity of decoquinat RMB041 against dormant *M. tuberculosis* and identified the drug targets for such. Lastly, the importance of including in silico drug discovery strategies and how they can be used as a complementary tool to other research approaches (in vivo and in vitro techniques) is highlighted. Additional wet-lab experiments determining the effectivity of decoquinat RMB041 against various clinical isolates and other resistant strains of *M. tuberculosis*, would strengthen our knowledge of the scope of efficacy of this antitubercular drug.

MATERIALS AND METHODS

To ensure the probability of high effectivity and low toxicity of decoquinat RMB041, several tools were employed. The procedure followed during this study is illustrated in Fig. 7.

Evaluation of molecular and pharmacokinetic properties. The chemical 2D structure of the ligand was drawn with ACD/ChemSketch (commercial version) and converted to a 3D (pdb) file with DS v.4.5. ADMET properties of decoquinat RMB041 were identified by using the ADMET descriptors algorithm and toxicity prediction extensible protocol of BIOVIA DS Visualizer v.4.5 (Accelrys) (Software Inc., San Diego, CA) and the web servers Molinspiration (<http://www.molinspiration.com/cgi-bin/properties>), SwissADME

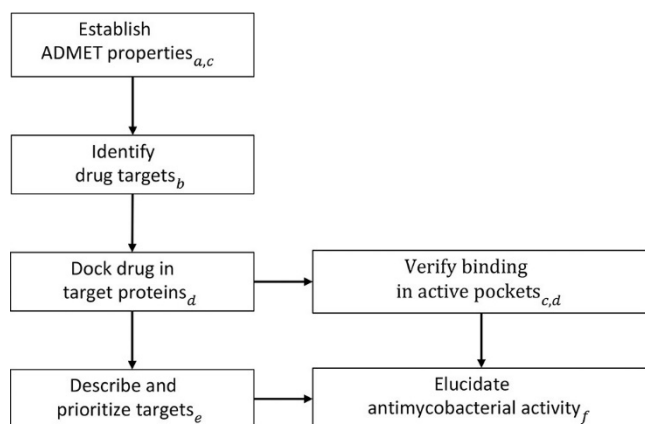


FIG 7 The workflow followed to elucidate the potential antimycobacterial mechanism of decoquinat derivative RMB041. The tools used during each step: a) pkCSM and SWISSadme, b) PharmMapper, c) Discovery Studio, d) AutoDock Vina, e) TDR Targets, f) KEGG and STRING.

(<http://www.swissadme.ch/>) and pkCSM (<http://biosig.unimelb.edu.au/>). The results are documented in Table 3. Although sharing similar functions, these tools apply different calculating algorithms, and can be used in combination to ensure improved precision of elucidation. SwissADME and DS also provided the molecular properties, which are reported in Table 1. The structure of decoquinat RMB041 was also evaluated with respect to pharmacokinetic rules frequently applied during drug manufacturing using this methodology (Table 2).

Computational target fishing. Pharmacophore screening: PharmMapper (<http://www.ilab-ecust.cn/pharmmapper/>) was chosen in this study for the identification of potential protein targets, due to its wide popularity of use in applications of drug discovery. PharmMapper analyzes the spatial arrangement of key functional groups of a molecule and assigns pairwise fit scores according to the matching of pharmacophores between the ligand (being the drug) and the protein (14). For this study, protein targets with fitness scores of \$4 were chosen to filter out insignificant pharmacophore models. The pharmacophores are collected in an internal database, PharmTargetDB, annotated from continuously updated databases, DrugBank, BindingDB, PDTD, TargetBank and Protein Database Bank (PDB). This software uses Cavity to identify all potential binding sites in proteins. The identification codes were retrieved from UniProt (<https://www.uniprot.org/uniprot/>) and the PDB (<https://www.rcsb.org/>) database. Only predicted protein targets relevant to *M. tuberculosis* were included for evaluation and deemed of importance of the study, and those in humans and other bacteria were excluded. The results are documented in Table 4.

Reverse Docking. To validate the identified drug targets, decoquinat RMB041 was docked in the active cavity of each of PharmMapper's predicted target proteins deemed valuable for the treatment of TB. The targets were then arranged according to their estimated binding strengths. Results documented in Table 4.

Protein and ligand preparation: PDB codes provided by PharmMapper were searched in PDB (<http://www.pdb.org>), where their three-dimensional structures with their cocrystallized ligands (solved at 0.90–1.8 Å resolution²) were then downloaded. Manual preparation was done with AutoDock v.4.2.6, which included the removal of water, deletion of all hetero atoms, assignment of atom types, addition of polar hydrogen atoms, merging of nonpolar hydrogen atoms, and finally, the addition of Kollmann charges. Although docking programs account for flexibility of ligands, a remaining challenge is the flexibility of the entire protein. To minimize standard errors, the proteins were prepared as rigid structures (90). A pdbqt file of each of the target proteins were subsequently prepared with AutoDock Tools v.1.5.6 (91). Hydrogen and partial charge in the molecular system were assigned using AMBER force field.

For the preparation of decoquinat RMB041, its geometry force field was minimized using YASARA (<http://www.yasara.org/>) (92), prior to manual preparation involving the addition of polar hydrogens atoms, merging of nonpolar hydrogens atoms, and addition of Gasteiger charges. PDBQT files were saved for docking.

Grid box preparation and docking: File conversions (mol2 to pdb to pdbqt) required for the separate steps of docking were performed with the open-source toolbox Open Babel v. 2.3.2 (93). To ensure inclusion of all active sites, the entire macromolecule was selected as a search space for binding site cavities, with spacing's set at 0.90–1 Å and saved as grid box parameters. Molecular docking scores of the compound to each protein were calculated by AutoDock Vina v.1.1.2, for which a Lamarckian Genetic Algorithm was used, and expressed as free binding energies (DG kcal mol^{−1}) (94). Nine different orientations of decoquinat RMB041 were searched per protein and ranked according to the binding free energies. To identify configurations that bind to active sites of their respective proteins, each binding pose was visually inspected in AutoDock, and those that interact with active site residues were saved as a pdbqt file and analyzed in DS, where the residues were then labeled. To validate interactions of decoquinat RMB041 within active cavity sites, the target residues were compared to those of the original cocrystallized ligands, which, if PDB cavity

site records were available, were either retrieved with DS Tools v.1.5.6 or obtained from previous literature. Cavity sites were provided with the name of the specific amino acid, the respective chain, and the number of the residues, e.g., Lys A:123.

Prioritization of protein targets. To identify proteins that are most likely essential during infection, i.e., important during the *M. tuberculosis* transition to dormancy/latency, the structure of the compound was uploaded to the webserver TDR Targets (<https://tdrtargets.org>). TDR Targets, first introduced in 2008, has since been a reliable open-access resource for finding and prioritizing novel protein targets (42). This webserver integrates genomes from EupathDB, GenBank, GenoList, and Mycobrowser and isolates proteins based on assay space and literature reviews on essential proteins during survival during dormancy/latency (e.g., by lack of oxygen, lack of nutrients, ROS, acidity).

KEGG, GO, and network analysis. Annotations of the target proteins were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG) (<https://www.genome.jp/kegg/>). A literature search of the identified target proteins of both active and dormant *M. tuberculosis* was done. Further, to include up to date information on dormant *M. tuberculosis*, articles published within the past 5 years were selected with the exclusion of preprints, articles citing original articles, and studies on vaccinations and diagnostics.

Coexpression. Proteins that are negatively and positively correlated with any of the selected hit targets (Pearson correlation coefficient, 20.6 and 0.6) (95), were obtained from TBDB (<http://tbdb.bu.edu/>). Proteins without known functions or assigned COG (Clusters of Orthologous Groups) categories were excluded. Target proteins were entered into STRING (<https://string-db.org/>), which provided a network with protein-protein interactions (medium confidence score of 0.4) in *M. tuberculosis* H37Rv.

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ANNEXURE B

The following is the ethics exempt letter for this study.

Prof DT Loots
Biochemistry
Focus Area Human Metabolomics

17 September 2019

Dear Prof Loots

PROOF THAT THE FOLLOWING STUDY DOES NOT REQUIRE ETHICAL APPROVAL

Study title: Elucidating the antimicrobial mechanism of ciprofloxacin and a new decoquinolate derivative on *Mycobacterium tuberculosis* using metabolomics

Study leader: Prof DT Loots

Student: KE Knoll - 25773267

On 10/04/2019, the scientific committee, Focus Area Human Metabolomics Scientific Committee, reviewed the aforementioned study and recommended to the North-West University Health Research Ethics Committee (NWU-HREC), on the required template, that no ethical approval was required as this study does not:

- Involve any human participants or their data/information
- Involve any human samples
- Involve any animals
- Involve any animal samples
- Have any possible environmental impact

The study involves laboratory work and the possible risks to researchers are covered by NWU-HREC approved Standard Operating Procedures for:

- Good laboratory practice
- Appropriate waste management
- Appropriate safety measures and protection of laboratory workers

Following review of this recommendation by the scientific committee, the NWU-HREC is in agreement that the aforementioned study does not require ethical approval.

Yours sincerely

NWU-HREC Chairperson

Head of Health Sciences Ethics
Office for Research, Training and
Support