Determination of the mechanism of synergy of SQ109 with rifampicin and isoniazid in *Mycobacterium smegmatis*

Bayanika Manunu

Thesis submitted to the Faculty of Medicine and Health Sciences in fulfilment of the requirements for the degree of Master of Medical Science

Stellenbosch University

Promoter: Dr Monique Williams

Co-promoter: Dr Sven O Friedrich

March 2015

Declaration

I declare that the content of this thesis is my own, unaided work, and it has not previously submitted for any qualification or examination at any other University.

Bayanika Manunu

 $1^{\underline{st}}$ day of <u>December</u>, 2014

Copyright © 2015 Stellenbosch University

All rights reserved

Abstract

Multidrug resistance tuberculosis (MDR-TB) is a serious concern in the public health environment globally and the understanding of its mechanisms may help to prevent the emergence and spread of resistant strains of Mycobacterium tuberculosis (Mtb). Several compounds are being tested in clinical trials and SQ109 was identified as a promising new anti-TB drug because of its bactericidal activity against Mtb and demonstrated synergistic activity with the fist-line TB drugs. This study focussed on the mechanism of synergy of SQ109 with rifampicin (RIF) and isoniazid (INH) in Mycobacterium smegmatis (Msmeg). The influence of SQ109 on efflux in Msmeg was evaluated using two approaches. Firstly, accumulation and efflux of ethidium bromide (EtBr) was monitored using a semi-automated fluorometric assay and secondly efflux and accumulation of RIF in Msmeg was assessed using tandem mass spectrometry. Although SQ109 resulted in a slight decrease in EtBr efflux by Msmeg in some of the assays performed, this decrease was not consistently seen. SQ109 appeared to have no significant influence on the efflux or accumulation of RIF in Msmeg, suggesting that it does not act to inhibit efflux in this organism. Six spontaneous SQ109resistant mutants were generated in Msmeg and bactericidal activity of SQ109, RIF and INH against wild-type and mutant strains of Msmeg was assessed. The minimum inhibitory concentrations (MICs) for all three drugs increased in the mutant strains compared to the wild-type. Drug-drug interaction studies performed on one of the SQ109-resistant mutants revealed a change from synergy to additivity for both SQ109/RIF and SQ109/INH combinations, suggesting that identification of the genes harbouring mutations in these strains would shed light on the mechanism of synergy of SQ109 with RIF and INH. Sanger sequencing revealed that none of the SQ109-resistant mutants harboured mutations in MSMEG 0250 (mmpL3 homologue), a gene previously implicated in SQ109 resistance in M. tuberculosis. Preliminary whole genome sequencing data for six SQ109-resistant mutants identified SNPs in 10 genes, however the role of these genes in SQ109 resistance and synergy with RIF and INH in Msmeg remains to be verified.

Opsomming

Multi-middel weerstandige tuberkulose (MDR-TB) is 'n ernstige probleem in globale publieke gesondheid. Kennis van die meganisme van middelweerstandigheid kan help om die ontwikkeling en versprei van weerstandige Mycobacterium tuberculosis (Mtb) te voorkom. Verskeie middele word tans in kliniese proewe getoets. SQ109 is identifiseer as 'n belowende nuwe anti-TB middel as gevolg van die kiemdodende aktiwiteit wat dit teen Mtb toon en die sinergistiese aktiwiteit wat dit met die eerstelyn TB middele toon. Hierdie studie fokus op die meganisme van sinergie van SQ109 met rifampisien (RIF) en isoniasied (INH) in Mycobacterium smeqmatis (Msmeg). Twee benaderings is gebruik om die invloed van SQ109 op effluks in Msmeg te evalueer. Eerstens is opbou en effluks van ethidium bromied (EtBr) gemonitor deur van 'n semi-outomatiese fluorometriese toets gebruik te maak. Tweedens is effluks en opbou van rifampicin (RIF) in Msmeg ondersoek deur van tandem massaspektrometrie gebruik te maak. Alhoewel SQ109 'n effense afname in EtBr effluks in Msmeg in sommige van die eksperimente veroorsaak het, is die afname nie herhaaldelik deur al die eksperimente gesien nie. Dit het geblyk dat SQ109 geen beduidende invloed op effluks of opbou van RIF in Msmeg gehad het nie, wat daarop dui dat dit nie as 'n effluks inhibeerder in die organisme optree nie. Ses spontane SQ109 weerstandige mutante is in Msmeg gegenereer en die kiemdodende aktiwiteit van SQ109, RIF en INH teen die wildetipe en mutante is ondersoek. Die minimum inhiberende konsentrasie (MIC) vir al drie middels is verhoog in die mutante in vergelyking met die wilde-tipe. Middel-middel interaksie studies uitgevoer vir een van die SQ109 weerstandige mutante het getoon dat daar 'n verandering van sinergie to additiwiteit vir beide SQ109/RIF en SQ109/INH kombinasies was. Dit het voorgestel dat die identifisering van gene waarin mutasies voorkom in die SQ109 mutante kan lei tot die identifisering van die meganisme van sinergie van SQ109 met RIF en INH. Sanger DNA volgordebepaling het getoon dat geen van die SQ109 mutante mutasies in die MSMEG 0250 (mmpL3 homoloog), 'n geen wat voorheen geassosieer is met SQ109 weerstandigheid in M. tuberculosis, gehad het nie. Met voorlopige heel genoom volgorde bepaling vir die ses SQ109 mutante is SNPs in 10 gene identifiseer, maar die rol van die gene in SQ109 weerstandigheid en sinergie met RIF en INH in Msmeg moet verifieer word.

Acknowledgements

I would like to express my gratitude to the European & Developing Countries Clinical Trials Partnership (EDCTP) and Pan African Consortium for Evaluation of Anti-tuberculosis Antibiotics (PanACEA) for the financial support during my studies of Master of Science at Stellenbosch University.

I would like to extend my gratitude and appreciation to my supervisor, Dr Monique Williams for her guidance, availability and patience. Through your enthusiasm you have introduced me in the world of science. Also to my co-supervisor Dr Sven Friedrich for his motivation, helpful advice and research support. For this I will be forever grateful. I would like to thank Prof Rob Warren for giving me the opportunity to work in his laboratory and Prof Andreas Diacon for the opportunity to work on this project.

A special thanks to my wife Astrid Mwanzala Lupungi for her precious support, advice and encouragement. And to my family, I am especially thankful to my mother Mazangu Ndiatulu for her important advice.

Finally to my colleagues, at the Division of Molecular Biology and Human Genetics for their appreciative assistance. My special gratitude to Xavier Kayigire, Dolapo Awoniyi and Carine Kunsevi for their assistance, advice and friendship during this time.

Dedication

To my family for their support and strong advice

Table of contents

Decla	ration		l
Abstr	act		ii
Opso	mming		iii
Ackno	owledge	ments	iv
Dedic	ation		v
Table	of conte	ents	vi
Abbre	eviations	5	ix
List o	f figures		xi
List o	f tables		xii
Confe	rence/p	ooster presentations	xiii
Chap	ter 1: I	Literature review	1
1.1	Tuber	culosis	1
1.2	The na	atural history of tuberculosis infection	1
1.3	Treatr	ment of tuberculosis	2
1.4	Tuber	culosis drug resistance	3
1.5	Modu	lating intracellular drug concentration as mechanism	
	of alte	ering sensitivity	5
	1.5.1	The mycobacterium cell wall	5
	1.5.2	Transport across the mycobacterial cell wall	5
	1.5.3	Passive transport	6
	1.5.4	Active transport	7
		1.5.4.1 Influx transporters	7
		1.5.4.2 Efflux pumps	7
	1.5.5	Efflux pump inhibitors and their potential in TB treatment	10

1.6	New a	New anti-tuberculosis drug candidates					
1.7	SQ109	as potential anti-TB candidate	12				
	1.7.1	Mechanism of action of SQ109	13				
	1.7.2	Efflux as a mechanism of synergy for SQ109	14				
Chap	ter 2:	Materials and methods	16				
2.1	Bacte	rial strains, media and growth conditions	16				
2.2	Comp	ounds	17				
2.3	Deter	mination of the minimum inhibitory concentrations	17				
	2.3.1	Broth micro dilution method	18				
	2.3.2	MIC on solid madia	18				
2.4	Semi-	automated fluorometric accumulation and efflux assays	19				
	2.4.1	Ethidium bromide accumulation assay	20				
	2.4.2	Ethidium bromide efflux assay	20				
2.5	Rifam	picin accumulation in Msmeg	21				
	2.5.1	Rifampicin accumulation assay	21				
	2.5.2	Quantification of rifampicin concentration in cell lysates					
	using	LC-MS nethod	22				
	2.5.3	RCDC protein determination assay	22				
2.6	Isolati	Isolation of spontaneous SQ109-resistant mutants in Msmeg2					
	2.6.1	PCR and agarose gel electrophoresis	24				
	2.6.2	Sanger DNA sequencing	25				
2.7	Check	erboard drug interaction assays	26				
2.8	Whole	Whole genome sequencing of Msmeg SQ109-resistant mutants					
	2.8.1	Phenol-chloroform isoamyl alcohol (PCI) DNA extraction	28				
	2.8.1	Whole genome sequencing	29				

Chapt	ter 3: Results	.30			
3.1	Minimum inhibitory concentration determination	.30			
3.2	Assessment of the effect of SQ109 on EtBr accumulation in Msmeg	.30			
3.3	Assessment of the effect of SQ109 on RIF accumulation in Msmeg	.35			
3.4	Evaluation of growth of wild-type and SQ109-resistant strains of Msmeg				
	in liquid culture	.36			
3.5	Minimum inhibitory concentration determination for SQ109-resistant				
	Mutants	.37			
3.6	Assessment of drug-interactions in wild-type and SQ109-resistant				
	Mutants	.38			
3.7	Sanger sequencing of MSMEG_0250 in SQ109-resistant mutants	.41			
3.8	Identification of mutations in the genome of Msmeg SQ109-resistant				
	Mutants	.44			
Chapter 4: Discussion46					
Conclusion50					
Refer	ences	51			

Abbreviations

ABC ATP-binding cassette

AIDS Acquired Immune Deficiency syndrome

ATP Adenosine triphosphate

BCG Bacillus Calmette–Guérin

BSA Bovine serum albumin

CCCP Carbonyl cyanide m-chlorophenylhydrazone

CFU Colony forming unit

DMSO Dimethylsulphoxide

DNA Deoxyribonucleic acid

dNTPs Deoxynucleotide triphosphates

DOTS Directly Observed Therapy, Short Course

EDTA Ethylenediaminetetraacetic acid

EMB Ethambutol

EPI Efflux pump inhibitor

ETH Ethionamide

FIC Fractional inhibitory concentration

FICI Fractional inhibitory concentration index

FQ Fluoroquinolones

GS Glucose salt

HIV Human Immunodeficiency virus

INH Isoniazid

LC-MS Liquid chromatography tandem mass

spectrometry

LTBI Latent tuberculosis infection

MATE Multidrug and toxic compound extrusion

MDR Multidrug resistant

MFS Major facilitator superfamily

MIC Minimum inhibitory concentration

Mtb Mycobacterium tuberculosis

Msmeg Mycobacterium smegmatis

PBS Phosphate buffered saline

pH Potential of hydrogen

PZA Pyrazinamide

RCDC Reducing agent and detergent compatible

RIF Rifampicin

RNA Ribonucleic acid

RND Resistance nodulation division

SMR Small multidrug resistance

STR Streptomycin

TB Tuberculosis

TBE Tris base-boric acid-EDTA

XDR Extensively drug resistant

WHO World Health Organization

List of figures

Figure 1.1:	Chemical structure of SQ10913
Figure 3.1:	Accumulation of EtBr by Msmeg at increasing concentrations31
Figure 3.2:	Effect of verapamil and SQ109 on accumulation of
	EtBr by Msmeg33
Figure 3.3:	Effect of verapamil and SQ109 on efflux of EtBr by Msmeg35
Figure 3.4:	RIF intracellular concentration in Msmeg after 0, 5, 10 and
	20 minutes of exposure36
Figure 3.5:	Growth curves of Msmeg SQ109-resistant mutants
	and wild-type37
Figure 3.6 a:	Amplification of <i>MSMEG_0250</i> gene by primer set 142
Figure 3.6 b:	Amplification of <i>MSMEG_0250</i> gene by primer set 242
Figure 3.6 c:	Amplification of <i>MSMEG_0250</i> gene by primer set 343
Figure 3.6 d:	Amplification of <i>MSMEG_0250</i> gene by primer set 443
Figure 3.7:	Alignment of sequence obtained for each primer set
	with the gene sequence of MSMEG_025044

List of tables

Table 1.1:	Mode of action of TB drugs and the mechanisms of resistance
	present in mycobacteria4
Table 1.2:	Mtb and Msmeg efflux pumps associated with drug resistance11
Table 1.3:	New anti-TB drugs and their targets12
Table 2.1:	Compounds used
Table 2.2:	BSA standard curve preparation22
Table 2.3:	Primers used for <i>MSMEG_0250</i> gene amplification25
Table 2.4:	96 well plate design used for drug interaction assays27
Table 3.1:	MICs of various compounds for Msmeg in liquid medium30
Table 3.2:	MICs of RIF, INH and SQ109 for SQ109-resistant mutants of Msmeg38
Table 3.3:	Interaction between SQ109 and RIF against wild-type strain of Msmeg38
Table 3.4:	Interaction between SQ109 and INH against wild-type strain of Msmeg39
Table 3.5:	Interaction between SQ109 and RIF against SQ109-resistant strain
	of Msmeg39
Table 3.6:	Interaction between SQ109 and INH against SQ109-resistant strain
	of Msmeg40
Table 3.7:	Checkerboard synergy between SQ109/RIF and SQ109/INH Msmeg
	strains41
Table 3.8:	Genes involved in mutations of SQ109-resistant strains of Msmeg45

Conference/poster presentations

- Bayanika Manunu, Monique Williams, Sven O Friedrich, Andreas H Diacon.
 The effect of SQ109 on efflux in Mycobacterium smegmatis.
 - Poster presentation, Stellenbosch University Annual Academic Year Day. August 2012
- Bayanika Manunu, Monique Williams, Sven O Friedrich, Andreas H Diacon, Rob Warren.
 Isolation and characterization of SQ109-resistant mutants of Mycobacterium smegmatis.

 Poster presentation, Stellenbosch University Annual Academic Year Day. August 2013
- 3. **Bayanika Manunu,** Determination of the mechanism of action of SQ109 in *Mycobacterium tuberculosis*. Oral presentation, Annual PanACEA conference, Stellenbosch, October 2012

Chapter 1

Literature review

1.1 Tuberculosis

TB (tuberculosis) is one of the infectious diseases in the world that causes ill-health among millions of people each year and ranks as the second leading cause of death from an infectious disease worldwide, after the human immune-deficiency virus (WHO 2014). In 1882, Robert Koch identified *Mycobacterium tuberculosis* (Mtb) as the causative agent of tuberculosis by isolating it from infected individuals and visualised the bacilli microscopically using acid-fast staining.

Morbidity and mortality rates due to TB steadily dropped during the 20th century in the developed world, aided by better public health practices and widespread use of the *Mycobacterium bovis* BCG vaccine, as well as the development of antibiotics in the 1950s. This downward trend ended and the number of new cases started increasing in the mid-1980s. The major causes of this increase were homelessness and poverty in the developed world and the emergence of AIDS, with its destruction of the cell-mediated immune response in co-infected persons (Smith 2003).

Mtb belongs to the complex of mycobacteria that cause TB in either humans or animals. The Mtb complex consists of different species of mycobacteria including *M. tuberculosis, M. cannettii, M. bovis, M. africanum, M. microti, M. caprae*, and *M. pinnipedii. M. tuberculosis, M. africanum* and *M. cannettii* are human pathogens, while the rest of the species are pathogenic to animals (Smith 2003).

1.2 The natural history of tuberculosis infection

Tuberculosis is a communicable disease and is spread by airborne particles called droplets nuclei, which are particles of 1-5 microns in diameter.

The droplet nuclei can remain airborne for several minutes to hours after expectoration (Smith 2003; Ahmad 2010). Several factors determine the risk of infection such as the

immune status of the exposed individual, the bacillary load inhaled, the proximity, the frequency and the duration of exposure (Smith 2003).

When an inhaled droplet containing tubercle bacilli reach the alveoli of the lungs they are engulfed by alveolar macrophages where the majority of bacilli are killed. A small number of bacilli may replicate intracellularly, and are released when death of macrophages occurs (Korf *et al.et al.* 2005). These bacilli may cross the alveolar membrane to cause systemic dissemination and spread to more distant tissues and organs such as kidneys, brain, larynx, lymph node, lung, spine and bone (Schluger 2005).

In the majority of people infected with Mtb an effective cell-mediated immune response develops 2-8 weeks after infection, which stops further replication of the bacilli. The activated macrophages, T lymphocytes and other immune cells form a barrier shell called a granuloma that limits further multiplication and spread of the bacilli. Most of the Mtb are killed in the caseating granulomas, however the pathogen is not completely eliminated in some people but rather controlled by the immune system. This is the latent tuberculosis infection (LTBI), where individuals are asymptomatic (Ahmad 2010). Viable bacilli may persist in the necrotic material for years or even a lifetime, and if the immune system later becomes compromised, the bacilli begin to replicate rapidly and active tuberculosis develops (TB disease). The disease manifests mainly in the lungs, but the process can occur in other areas of the body (extra pulmonary TB). Several factors are involved in reactivation of latent infection, including uncontrolled diabetes mellitus, malnutrition, smoking, renal failure, organ transplantation, and therapy with immunosuppressive drugs. HIV infection causes depletion of CD4⁺ and CD8⁺ T-cells which provide protection (Walzl et al. 2011; Dartois 2014) against active TB by modulating phagocyte activity (Ahmad 2010; Walzl et al.et al. 2011), and is the most important factor for reactivation when co-infection occurs.

1.3 Treatment of Tuberculosis

Standard chemotherapy for drug sensitive TB consists of an intensive phase in which patients receive INH, RIF, PZA and EMB for two months, and a continuation phase of four months during which only isoniazid (INH) and rifampicin (RIF) are administered (WHO 2006). As the drugs have different targets within the bacilli, the combination of antibiotics

prevents the development of TB drug-resistance (Kremer and Besra 2002; Nikonenko *et al.* 2007; Palomino and Martin 2014)

In case of multidrug—resistant tuberculosis (MDR-TB), defined as resistance to both RIF and INH, the second-line drugs are used, namely the fluoroquinolones (ofloxacin, ciprofloxacin, levofloxacin or moxifloxacin) and the aminoglycosides (kanamycin, amikacin, capreomycin, ethionamide, and cycloserine). These drugs are generally less effective or more toxic (Blumberg *et al.* 2003). Extensively drug resistant tuberculosis (XDR-TB) is defined as MDR-TB plus resistance to fluoroquilones and at least to one of the aminoglycosides. XDR-TB is managed by using the third-line drugs such as linezolid, clofazimin, amoxicillin and clarithromycin (Prozorov *et al.* 2012). Recently some Mtb strains have been found resistant even to the third-line drugs or to all known TB drugs (Migliori *et al.* 2012).

1.4 Tuberculosis drug resistance

The emergence of drug resistance is one of the major problems for eradicating TB worldwide. Cases of MDR-TB and XDR-TB are being found in many countries and strategies such as utilising new drug combinations and the discovery of new drugs are required to ensure the future success of TB control programmes (Raman et al. 2008). The intrinsic resistance of Mtb to several antibiotics is a result of the low permeability of bacteria to different drugs, the stimulation of the efflux pumps and the inactivation of drugs by certain enzymes (Silva and Palomino 2011). Besides these, the acquisition of drug resistance occurs as a result of chromosomal mutations (Zhang and Yew 2009; Raja et al. 2011; Prozorov et al. 2012). These mutations result in resistance by preventing the binding of the drug to its specific target or drug-modifying enzyme due to a change in structure, or by altering the expression of the drug target or modifying enzyme (Prozorov et al. 2012). In the genome of Mtb, mutations occur spontaneously and frequencies have been estimated to be 3.5 x 10⁻⁶ for INH and 3.1 x 10⁻⁸ for RIF. The molecular mechanisms of resistance to TB drug is associated with gene mutations in specific regions. For example 95% of all RIF resistances are associated with mutations in an 81 bp region of the rpoB gene while 80% of mutations confer resistance to INH occur in codon 315 of the katG gene (Raman et al. 2008; Raja et al. 2011). The table 1.1 below shows current drugs used in TB treatment, their mechanisms of action and the genes involved in the mechanisms of resistance.

Table 1.1 Mode of action of TB drugs and the mechanisms of resistance present in mycobacteria

Drugs	Discovery	Mode of	Gene involved	Gene function	References
year		action and	in resistance		
		target			
Isoniazid	1952	Inhibits	KatG	Catalase peroxidase	(Zhang and
		mycolic acid	inhA	Enoyl ACP reductase	Yew 2009)
		synthesis			
Rifampicin	1966	Inhibits RNA	гроВ	B-subunit of RNA	(Prozorov et
		synthesis		polymerase.	al. 2012)
Ethambutol	1961	Inhibits arabi-	embB	Arabinosyl transferase	(Palomino
		nogalactan			and Martin
		synthesis			2014)
Pyrazinamide	1952	Depletion of	pncA	Pyrazinamidase	(Zhang and
		cell membrane			Yew 2009)
		potential			
Streptomycin	1944	Inhibits protein	rpsL,	S12 ribosomal protein	(Prozorov et
		synthesis	rrs,	16S rRNA	al. 2012)
			gidB	7-Methylguanosine	
				methyltransferase	
Fluoroquinolones	1963	Inhibits DNA	girA	DNA gyrase subunit A	(Silva and
		gyrase	girB	DNA gyrase subunit B	Palomino
					2011)
Aminoglycosides	1957	Inhibits protein	rrs	16S rRNA	(Palomino
		synthesis	rpsL	16S rRNA	and Martin
					2014)
Ethionamide	1956	Inhibits	ethA/EtaA	Flavin monooxygenase	(Silva and
		mycolic acid			Palomino
		synthesis			2011)
		synthesis			2011)

1.5 Modulating intracellular drug concentration as a mechanism of altering drug sensitivity

1.5.1 The mycobacterial cell wall

Mycobacteria are surrounded by a cell wall with the unique structural and functional characteristics and rich in lipid compounds. The mycobacterial cell wall is composed of a covalently associated complex of three structures: peptidoglycan, arabinogalactan, and mycolic acids and form the mycobacterial cell wall skeleton or the mycolyl-arabinogalactan-peptidoglycan complex (MAPc) (Crick *et al.* 2001). The MAPc is an ideal target for drug development and currently many compounds in use or in clinical trials inhibit the biosynthesis of cell wall structure (Crick *et al.* 2001; Hett and Rubin 2008).

The mycobacterial cell envelope can be divided into two main structural components, namely the cell membrane and cell wall. The outer leaflet of the cell wall is formed with the mycolic acids which are covalently attached to the arabinogalactan-peptidoglycan complex of the inner leaflet. The cell envelope of mycobacteria is unique in that besides the cell membrane and peptidoglycan layers, it also contains distinctive lipids and glycolipids that confer extreme hydrophobicity to the outer surface (Korf *et al.* 2005). Several cell wall components of Mtb have been identified as pathogen-associated molecular pattern (PAMP), including the glycolipid lipoarabinomannan (LAM) (Andries *et al.* 2005; Tahlan *et al.* 2012).

The unique structure of the cell wall plays a significant role in drug resistance as a barrier to the entry of drugs into the cell, which diminishes the accumulation of intracellular drugs while efflux mechanisms also contribute as an important factor in anti-TB resistance in Mtb such as fluoroquinolones, tetracyclines and amimoglygosides (Louw *et al.* 2009; Nikaido 2009).

1.5.2 Transport across the mycobacterial cell wall

Most molecules of biological origin are transported across the cellular membrane in a process that involves specific and specialised transport proteins such as porins, drug importers and efflux pumps (Hett and Rubin 2008; Grzegorzewicz *et al.* 2012; Chao *et al.* 2013). There are three types of transport operating across the cell envelope to move substances or chemotherapeutic agents in and out of the cell wall, namely passive

transport, facilitated transport and active transport. The first two processes of transport employ the power of diffusion and do not require any energy for transporting substances across the cell wall.

1.5.3 Passive transport

Passive transport is the moving of substances across cell membrane without the use of energy, this mechanism of transport includes simple diffusion, facilitated diffusion, and osmosis. Simple diffusion is the process by which small uncharged molecules, such as oxygen (O₂) and carbon dioxide (CO₂) easily permeate over the cell membrane from the higher concentration areas to the lower concentration areas. Osmosis is the diffusion of water through a semi-permeable membrane from the higher concentration areas to the lower concentration areas (Sarathy *et al.* 2012).

Facilitated transport is a form of diffusion that allows transport of substances or molecules through the cell membrane without requiring energy consumption. Substances or molecules across a membrane pass spontaneously through specific transmembrane transport proteins during this diffusion. This uptake pathway involves a limited range of compounds since channel diameters at the narrowest point define the exclusion limit, and other parameters such as the length of channels and the number of open pores that determine the speed of transport. A number of porins have been identified and studied in Gram-negative and Grampositive bacteria and two putative classes have been characterised in mycobacteria; MapAlike and OmpA-like pores in *M. smegmatis* and *M. tuberculosis* respectively (Sarathy *et al.* 2012).

MspA was the first porin to be identified in mycobacteria, previous studies have documented MspA-enabled transport of hydrophilic solutes and drug molecules across the cell membrane. The genome of *M. smegmatis* has revealed three more porin genes homologous to *mspA*, namely *mspB*, *mspC* and *mspD* and the studies showed that the deletion of porins is linked to increases in MICs of various antibiotics (Li and Nikaido 2009; Sarathy *et al.* 2012; Amaral *et al.* 2014). In several instances the increase in MICs has been associated with reduction in drug uptake in *M. smegmatis*.

OmpATb was the first porin-like identified in Mtb and this porin is encoded by the *Rv0899* gene. OmpATb plays a key role in conferring Mtb the ability to survive under acidic

environment. The deletion mutant in *OmpATb* exhibits a significant reduction in permeability to a number of hydrophilic molecules and impaired ability to grow at reduced pH.

1.5.4 Active transport

Active transport requires energy to transport substances or molecules across the cell membrane. The ATP used in active transportation may be used directly; when transporters bind ATP and use the energy of its hydrolysis to transport molecules against a concentration gradient, or indirectly, when ATP is used to generate a proton gradient. Active transport is further divided into two processes namely influx and efflux.

1.5.4.1 Influx transporters

In mycobacteria, the influx of toxic compounds is significantly restricted by the complex cell and lipid bilayer. Influx is the physiological mechanism that allows molecules to enter the cell by crossing the bacterial envelope. These influx transporters are proteins localized in the cell wall and selectively import molecules into the cell.

1.5.4.2 Efflux pumps

Bacterial efflux pumps are located in the cell membrane and are associated with antimicrobial resistance. Efflux pumps are transporter proteins that promote the extrusion of molecules out of the cell as they enter. The physiological role of these pumps is the extrusion of noxious agents from the cell, allowing the bacteria to survive in a hostile environment (Poole 2007). Recently it has been recognised that efflux pumps also play a role in altering the sensitivity of mycobacteria to drugs (Adams *et al.* 2011; Stephan *et al.* 2004). Genes encoding drug efflux transporters have been identified in the genome of several mycobacteria. These proteins have been implicated in the transport of a number of drugs such as tetracycline, fluoroquilones, aminoglycosides, rifampicin and isoniazid (De Rossi *et al.* 2006).

Efflux pumps in bacteria differ structurally and in their mode of action are classified into five families based on their bio energetic and structural characteristics, namely: the ATP-binding cassette (ABC) superfamily; the major facilitator superfamily (MFS); the multidrug and toxic compound extrusion (MATE) family; the small multidrug resistance (SMR) family; and the

resistance nodulation division (RND) family. Efflux pumps that belong to the ABC superfamily are considered primary active transporters because they utilize the free energy of ATP hydrolysis to extrude drugs out of the cell. The other families of efflux pumps are called secondary active transporters because they use the proton or sodium ions as a source of energy to extrude the drugs from the cell (Omote *et al.* 2006).

ATP –binding cassette (ABC) superfamily

The ABC transporters have an ATP hydrolysis mechanism involved in the extrusion of various molecules such as toxins, metabolites and drugs from the cell. ABC transporters appear to consist of four domains: two membrane-spanning domains (MSDs) and two nucleotide-binding domains (NBDs). The nucleotide binding domains are highly homologue and possess the walker A and walker B motifs which are common to all ATP-binding proteins. Genes encoding ABC transporters occupy 2.5% of the *M. tuberculosis* genome, based on structural similarities to the subunits of ABC transporters present in all living organisms, at least 37 complete and incomplete have been identified in *M. tuberculosis* (Braibant *et al.* 2000).

Major facilitator superfamily (MFS)

The MFS is a large superfamily of membrane transporters and are present in all organisms. MFS possess 12 or 14 putative transmembrane segments and are involved in the transport of many different compounds such as simple sugars, oligosaccharides, amino acids, drugs, nucleosides and Krebs cycle metabolites. The MFS contains several important efflux pumps, like QacA and QacB of *S. aureus* and EmrB of *E.coli*. Bioinformatic analysis of *M. tuberculosis* genome has identified 16 open reading frames that encodes for putative drug pumps that belong to the MFS (De Rossi *et al.* 2006). In mycobacteria, most of the efflux pumps belong to this superfamily.

In 1996, LfrA was discovered as the first multidrug efflux pump in *M. smegmatis*. LfrA belongs to MFS class, and is responsible for the intrinsic resistance to fluoroquinolones and tetracycline. The deletion of *lfrA* gene in different studies results in the increased susceptibility to a number of antimicrobials such as fluoroquinlones, ethidium bromide and acriflavine (Li *et al.* 2004). Several drug efflux pumps have subsequently been identified in

other mycobacteria and implicated in the mechanisms of drug resistance (Table 2.2) (Li *et al.* 2004).

Multidrug and toxic compound extrusion (MATE) family

The MATE transporters have been originally described in *Vibrio parahaemolyticus* (NorM), *Vibrio cholera* (VcrM; VcmA), *E.coli* (YdhE), *Pseudomonas aeruginosa* (PmpM), and *Clostridium difficile* (CdeA) (Omote *et al.* 2006). Most MATE transporters consist of 400 – 550 residues with 12 transmembrane helices and they confer resistance to multiple toxic cationic agents, such as ethidium bromide, berberine, acriflavine and norfloxacin, using sodium ion gradient force across the plasma membrane. The use of sodium motive force as the driving force for drug extrusion to distinguish these efflux pumps to other secondary transporter families (Piddock 2006).

o Small multidrug resistance (SMR) family

The smallest efflux pumps belong to the SMR family. These transporter proteins have typically 100-120 amino acids and contain four membrane-spanning helices. SMR family pumps confer resistance to various compounds such as methyl viologen, benzalkonium, ethidium bromide, acriflavine, cetylpyridinium and proflavin. One of these pumps, EmrE, was cloned from *E. coli* and confers resistance to ethidium bromide and methyl viologen (Poulsen *et al.* 2011). The Mmr protein from *M. tuberculosis* was recently discovered, it confers resistance to ethidium bromide, acriflavine and methyl viologen. The purified Mmr protein had also demonstrated to function as a proton/drug antiporter *in vitro* (Nikaido 2009).

Resistance nodulation division (RND) family

The RND transporters are proteins with 12 transmembrane domains and include a number of multi-drug efflux proteins. Most RND transporters have been studied in Gram-negative bacteria and confer resistance to antibiotics in these microorganisms. The most studied examples of the RND transporters are the AcrAB-Tolc of *E. coli* and MexABOprM of *Pseudomonas aeruginosa* that catalyse the efflux of a number of antimicrobial agents (Piddock 2006; Poole 2007).

In mycobacteria, the identified drug efflux pumps are located in the cytoplasmic membrane. The genome of *M. tuberculosis* contains 13 genes that encode RND proteins designated MmpL (Mycobacterial membrane protein Large) (Domenech *et al.* 2005). The MmpL proteins are similar to each other in both sequence and structure; they each comprise 950 amino acids residues and predicted to contain 12 membrane-spanning helices (De Rossi *et al.* 2006).

1.5.5 Efflux pump inhibitors and their potential in TB treatment.

Efflux pump inhibitors (EPIs) are a group of compounds that play a role in increasing the activity of antibiotics by limiting the function of efflux pumps. EPIs have the potential to contribute as antimicrobial agents in the treatment of TB, specifically in treating drug resistant TB (Dutta *et al.* 2010).

Previous studies have demonstrated that EPIs such as thioridazine, carbonyl cyanide m-chlorophenylhydrazone (CCCP), chlorpromazine, reserpine and verapamil have an inhibition effect against efflux pumps in mycobacteria (Paixão *et al.* 2009; Jin *et al.* 2010; Rodrigues *et al.* 2013). The combination of these EPIs with anti-TB drugs *in vitro* were shown to decrease the level of TB drug resistance in Mtb (Louw *et al.* 2009; Dutta *et al.* 2010). In mice infected with MDR strains, verapamil was able to restore susceptibility of the strains to first-line drugs (Louw *et al.* 2009). Another study has reported that a decrease in MIC and an increase in intracellular accumulation of ciprofloxacin in ciprofloxacin-susceptible and resistant strains of Mtb were related to the presence of reserpine (Huang *et al.* 2013).

Table 1.2: Mtb and Msmeg efflux pumps associated with drug resistance

Mycobacteria	Efflux pump	Gene	Family	Inhibitor	Drug resistance	References
Mtb	EfpA	efpA	MFS	-	INH	(De Rossi <i>et</i>
						al. 2006)
Mtb	MmpL7	mmpL7	RND	Reserpine	INH	(Sarathy et
				СССР		al. 2012)
Mtb	-	Rv2686c,	ABC	Verapamil	FQ	(Pasca et al.
		Rv2687c,		Reserpine		2004)
		Rv2688c		СССР		
Mtb	DrrAB	drrA	ABC	Verapamil	Doxorubicin	(Louw et al.
		drrB		Reserpine		2009)
Mtb	P55 ^b	Rv1410c	MFS	Valinomycin	RIF	(Sarathy et
				СССР	Aminoglycosides	al. 2012)
					Tetraclycline	
Msmeg	LfrA	lfrA	MFS	СССР	FQ	(Li et al.
					Doxorubicin	2004)
Mtb	Mmr	Rv3065	SMR	СССР	Erythromycin	(Rodrigues
						et al. 2013)
Msmeg	Tet(V)	tet(V)	MFS	СССР	Tetracycline	(De Rossi <i>et</i>
						al. 2006)
Mtb	JefA	Rv2459	MFS	СССР	INH	(Sarathy et
				Valinomycin	ЕМВ	al. 2012)

1.6 New anti-tuberculosis drug candidates

A number of compounds have been screened for TB drug development and many of these are already being tested in different phases of clinical trials. New anti-TB drugs should either reduce the length of TB treatment or minimise the doses administered under DOTS or be effective against MDR/XDR-TB or be able to respond in TB/HIV co-infection treatment.

In line with these requirements, new anti-TB drugs should have divergent mechanisms of action by binding to targets that differ from those of old anti-TB drugs (Kremer and Besra 2002). For some of these new drugs the mechanisms of resistance have already been described before entering into clinical trials (Rivers and Mancera 2008; Silva and Palomino

2011). Table 1.3 details the most promising new anti-TB drugs already in clinical trials and their targets.

Table 1.3: New anti-TB drugs and their targets

Drug	Mode of action	Target	Gene	References
TMC207	Inhibition of ATP	ATP synthase	atpE	(Diacon <i>et al.</i>
(diarylquinoline)	synthesis			2009; Matteelli <i>et</i>
				al. 2010)
PA-824	Inhibition of protein	Nitro reductase	Rv0407	(Rivers and
(nitroimidazooxazine)	and cell wall lipids		Rv3547	Mancera 2008;
	syntheses.		Rv3261	Kolyva and
			Rv3262	Karakousis 2012)
OPC-67683	Inhibition of mycolic	Nitro reductase	Rv3547	(Matsumoto et al.
(nitrodihydro-	acid and cell wall lipid			2006)
imidazooxazole	syntheses			
derivative)				
SQ109 (diamine)	Inhibition of cell wall	MmpL3	mmpL3	(Tahlan <i>et al.</i>
	synthesis			2012)
Linezolid	Inhibition of protein	50S ribosomal subunit	rpIC	(Livermore 2003;
	biosynthesis			Escribano <i>et al</i> .
				2007)
AZD5847	Inhibition of protein	50S ribosomal subunit	rrl; rplC	(Balasubramanian
	biosynthesis			et al. 2014)
BTZ043	Inhibition of	DprE1 subunit of	Rv3790	(Makarov et al.
(nitrobenzothiazinone)	arabinane synthesis	decaprenylphosphoryl-		2009)
		β-o-ribose 2'-		
		epimerase		

1.7 SQ109 as potential anti-TB candidate

SQ109 (1, 2-ethylenediamine) is one of the most promising new anti-TB drugs. This compound is an analogue of EMB, and is currently being evaluated against Mtb in clinical trials (Sacksteder *et al.* 2012). SQ109 was chosen as a new anti-TB candidate, after the synthesis and screening of 63,238 compounds from a chemical library of 1, 2-

ethylenediamine pharmacophores of EMB and entered pharmacological and toxicological tests in rats, dogs and monkeys before beginning its clinical phases (Sacksteder *et al.* 2012).

SQ109 has shown activity *in vitro* and *in vivo* against both susceptible and resistant Mtb strains (Protopopova *et al.* 2005) and demonstrated synergy with current first-line drugs, RIF and INH, and an additive effect with streptomycin (Onajole *et al.* 2010; Sacksteder *et al.* 2012).

Figure 1 1: Chemical structure of SQ109

1.7.1 Mechanism of action of SQ109

SQ109 is proposed to function by interfering with the assembly of mycolic acids into the cell wall of Mtb resulting in accumulation of trehalose monomycolate (TMM), which is the precursor of the trehalose dimycolate (TDM) (Tahlan *et al.* 2012). Kapil Tahlan *et al.* were unable to generate spontaneous SQ109-resistant mutants in Mtb, but they observed that mutants resistant to SQ109 analogues were also cross-resistant to SQ109. Whole-genome sequencing showed mutations in *mmpL3* gene, which encodes a transporter of TMM, this implies that MmpL3 is the targets of SQ109 (Tahlan *et al.* 2012). Deletion of the homologue of MmpL3 in Msmeg, *msmeg_0250* resulted in the intracellular accumulation of TMM, confirming its role in cell wall synthesis (Varela *et al.* 2012).

MmpL3 is one of the MmpL proteins belonging to the resistance, nodulation, and cell division (RND) protein family. The RND proteins are a family of multidrug resistance pumps, specialised in transporting different molecules across the cell wall including drugs, fatty acids aliphatic and aromatics solvents (Domenech *et al.* 2005) as described in 2.5.3.2. MmpL proteins are present in both slow and fast growing mycobacteria and each protein transports specific molecules. For example in Mtb, MmpL7 is responsible for transporting phthiocero dimycocerostate (PDIM), while MmpL8 is required in sulfolipid 1 synthesis by

carrying a precursor of this substance (Pasca *et al.* 2005; Varela *et al.* 2012). A recent study reported that SQ109 is able to dissolve the transmembrane electrochemical proton gradient, suggesting that SQ109 acts on other essential processes in the cell beyond TMM transportation process (Li *et al.* 2014).

1.7.2 Efflux as a mechanism of synergy for SQ109

Several studies have demonstrated *in vitro* synergistic effect between the new anti-TB drug SQ109 and the front-line anti-TB drugs (Chen 2006; Onajole *et al.* 2010). In the case of RIF, the synergistic interaction occurs in both directions, i.e. SQ109 increases the activity of RIF and vice versa. A decrease in the MIC for RIF was observed in the presence of SQ109 in RIF resistant Mtb strains (Chen 2006). The mechanism of synergy between SQ109 and RIF and INH is not currently understood. Given that SQ109-resistant mutants were found to harbour mutations in the *mmpL3* gene, which encodes a transporter, it is possible that SQ109 may exert its synergistic effect by modulating the transport of RIF and INH across the mycobacterial cell envelope.

Hypothesis: SQ109 exerts its synergistic effect with RIF and INH by inhibiting drug efflux and increasing the intracellular drug concentration in mycobacterial cells.

Aims: This study aimed to determine the influence of SQ109 exposure on efflux in Msmeg and to investigate the mechanism of synergy of SQ109 with RIF and INH in Msmeg by generating SQ109-resistant mutants.

Objectives:

- 1. To determine the influence of SQ109 on accumulation and efflux of EtBr in Msmeg using a semi-automated fluorometric assay.
- 2. To determine the influence of SQ109 on rifampicin accumulation and efflux in Msmeg using a mass-spectrometry-based assay.
- 3. To generate SQ109-resistant mutants of Msmeg and determine the susceptibility of these mutants to SQ109, rifampicin and isoniazid.
- 4. To investigate the drug-drug interactions between SQ109 and rifampicin or isoniazid in SQ109-resistant mutants.
- 5. To identify the chromosomal mutation responsible for SQ109 resistance in Msmeg.

Msmeg is a non-pathogenic, fast-growing organism which is closely related to Mtb and shares more than 2000 homologues with Mtb including the same unusual cell wall structure. Both, Msmeg and Mtb show synergy with rifampicin and isoniazid, therefore we hypothesize that the mechanism is the same in both species. The EtBr efflux assay used in this study could not be performed in the BSL3 facility since the RotoGene[™] 6000 instrument required is not present in this facility. The assay could therefore only be performed in a BSL2 laboratory. Msmeg was therefore used as a model organism in this study to investigate efflux as the mechanism of synergy of SQ109 with rifampicin and isoniazid in mycobacteria (Kang *et al.* 2008; Chao *et al.* 2013).

Chapter 2

Materials and methods

2.1 Bacterial strains, media and growth conditions

Mycobacterium smegmatis mc²155 (Msmeg) was obtained from the Division of Molecular Biology and Human Genetics at Stellenbosch University (South Africa) and maintained as a frozen glycerol stock at –80°C for all experiments done in this project. Liquid cultures of Msmeg were grown in Middlebrook 7H9 medium (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 2% glucose, 0.85% sodium chloride (NaCl) and 0.05% Tween 80 (7H9 glucose-salt), and incubated at 37°C in a shaking incubator at 200 rpm. Solid cultures were maintained on 7H10 agar (Becton Dickinson) supplemented with 2% glucose, 0.85% sodium chloride (NaCl) and incubated at 37°C.

Growth curves were performed by inoculating a 1 ml glycerol stock into 50 ml 7H9 glucose-salt and incubated for 12 hours. This starter culture was then used to inoculate a volume of 0.5 ml in liquid culture, which was subsequently incubated for 27 hours, and the growth monitored by transferring 1 ml of each culture into a cuvette and measuring the OD_{600nm} at intervals of 3 hours. Cultures with OD_{600nm} readings above 1 were diluted appropriately.

2.2 Compounds

The compounds listed in table 2.1 were used in this project.

Table 2.1: Compounds used

Compounds	Supplier	Stock solution concentration,		
		diluent and storage conditions		
Ethidium bromide (EtBr)	Sigma-Aldrich,	10 mg/ml in distilled sterile		
	Johannesburg, South Africa	water, stored at room		
		temperature.		
Rifampicin (RIF)	Sigma-Aldrich,	4 mg/ml in DMSO/water mixture		
	Johannesburg, South Africa	(9:1) stored as frozen aliquots at		
		-80°C.		
Isoniazid (INH)	Sigma-Aldrich,	10 mg/ml in distilled sterile		
	Johannesburg, South Africa	water. Stored as frozen aliquots		
		at -80°C.		
SQ109	Sequella, Rockville, MD,	10 mg/ml in distilled sterile		
	USA	water. Stored as frozen aliquots		
		at-80°C.		
Verapamil (VP)	Sigma-Aldrich,	0.4 mg/ml in distilled sterile		
	Johannesburg, South Africa	water. Stored as frozen aliquots		
		at -80°C.		

All working solutions were prepared from defrosted aliquots in distilled water to obtain the required working concentration.

2.3 Determination of the Minimum Inhibitory Concentrations

The minimum inhibitory concentration (MIC) of a compound is defined as the lowest concentration of the compound that inhibits the visible growth of a microorganism. MICs of all compounds were determined by broth micro-dilution method in 96-well micro titre plates (Greiner bio one, Frickenhausen, Germany) and after incubation period, results were visually read and recorded (Jenkins and Schuetz 2012).

2.3.1 Broth micro dilution method

The broth micro dilution method allows the testing of a range of compound concentrations on a single 96-well micro titre plate (Greiner bio one, Frickenhausen, Germany) for MIC determination (Andrews 2001; Luber *et al.* 2003; Wiegand *et al.* 2008). Briefly, a 20 ml starter culture was inoculated using 1 ml of an Msmeg glycerol stock and grown overnight at 37° C in the shaking incubator at 200 rpm to an OD_{600nm} of 0.8. A volume of 1 ml was then sub-cultured into 20 ml of 7H9 glucose-salt and grown to an OD_{600nm} of 0.8. The culture was then diluted 1:100 in 7H9 broth containing glucose-salt.

A 96-well plate with 12 rows containing 8 wells each was prepared by adding 50 μ l of 7H9 glucose-salt to each well in Row 2 to 12. Row 1 was loaded with 100 μ l of each drug, diluent and media as follows:

100 μΙ	100 μΙ	100 μΙ	100 μΙ	100 μΙ	100 μΙ	100 μΙ	100 μΙ
media	compound	compound A	Compound A	compound B	compound B	compound	media
	diluent	at 4 X maximum	diluent				
		concentration	concentration	concentration	concentration		

A 1 in 2 serial dilution was performed by transferring 50 μ l of the solutions from Row 1 to Row 2 using a multichannel pipette. The solutions from Row 2 were mixed and then 50 μ l were transferred to Row 3, and so on, until Row 12 was reached. The last 50 μ l were discarded to bring the volume down to 50 μ l. Finally, 50 μ l of diluted Msmeg culture was added to each well from Row 2 to 12. The micro titre plate was sealed with sealing film, placed back into the original plastic bag and incubated at 37°C for 3 to 4 days. After the incubation period, wells which had visible growth were scored and the MIC was defined as the concentration range between the highest concentration of the compound at which growth was observed and the lowest concentration that inhibited visible growth.

2.3.2 MIC on solid media

The MIC of SQ109 for Msmeg was determined in duplicate on 7H10 agar (Becton Dickinson) supplemented with 2% glucose and 0.85% sodium chloride (NaCl). Middlebrook 7H10 agar quadrant plates (8 divisions) were prepared to contain the following concentrations of SQ109: 5.0, 10, 15, 25 and 50 μ g/ml. Msmeg was cultured overnight at 37°C in the shaking

incubator at 200 rpm to an OD_{600nm} of 0.8. A 10-fold serial dilution of the culture from 10^{-1} up to 10^{-8} was prepared and a volume of $10\,\mu l$ of each dilution was spread onto the corresponding dilution quadrant of 7H10 agar plates containing different drug concentrations. The plates were incubated at $37^{\circ}C$ for 3 to 4 days (Heifets and Lindholm-Levy 1989). The concentration that resulted in the complete inhibition of mycobacterial growth after incubation was recorded as the MIC of the drug. Plates with no drug were used as controls.

2.4 Semi-automated fluorometric accumulation and efflux assays

Mycobacteria are resistant to most of the commonly used antimicrobial agents due to the structure of their cell wall which is rich in lipid composition and plays a role of a barrier to noxious compounds and contributes to the slow drug uptake (Rodrigues *et al.* 2011).

Active efflux pump systems extrude noxious compounds and antibiotics from the cell reducing their intracellular concentration (Rodrigues *et al.* 2011), therefore reduced permeability of mycobacterial cell wall and active efflux systems are two main mechanisms that contribute to mycobacterial intrinsic resistance to a number of antibiotics (Louw *et al.* 2009).

Several methods have been used to study the balance between entry and extrusion of a given compound. These include the measurement of radiolabelled or metal-labelled substrate. The semi-automated fluorometric method has been developed using a real-time thermocycler to assess accumulation and efflux by measuring the fluorescence of EtBr (Paixão *et al.* 2009; Machado *et al.* 2012).

EtBr is a membrane penetrating dye which has a low fluorescence outside the cell. Once EtBr is inside the cell its fluorescence increases because it binds to the DNA. In a semi-automated assay the fluorescence of EtBr is read and quantified using the Rotor-GeneTM 6000 (Corbett Research, Sidney, Australia) which allows multiple samples to be monitored simultaneously in a temperature-controlled environment.

2.4.1 Ethidium bromide accumulation assay

Msmeg was cultured in 20 ml of 7H9 glucose-salt until it reached a mid-log phase corresponding to an OD_{600nm} of 0.8. The culture was then centrifuged at 13,000 rpm for 3 minutes. After discarding the supernatant, the pellet was washed twice with an equal volume of phosphate buffered saline (PBS), re-suspended in PBS, and adjusted to an OD_{600nm} of 0.4. The accumulation assays were performed in 200 μ l PCR micro tubes with 100 μ l as a final volume of solutions. In order to determine the optimum EtBr concentration for the assay, 10 μ l of EtBr solutions with different concentrations were added to 90 μ l of mycobacterial culture. The final concentrations of EtBr in each mixture were as follows: 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 μ g/ml. Micro tubes were placed into a 36-well rotor in the Rotor-GeneTM 6000 and the fluorescence of EtBr was determined each minute for the first 60 minutes at 25°C.

To determine the inhibitory effect of SQ109 on EtBr accumulation in Msmeg, mycobacterial cells were prepared as described above. After adjusting the OD to 0.4, EtBr was added into a cell suspension to a final concentration of 0.5 μ g/ml (Concentration that resulted to a minimal accumulation of EtBr). Volumes of 95 μ l were transferred into micro tubes and 5 μ l of verapamil or SQ109 at their half MICs (100 μ g/ml and 1.0 μ g/ml respectively) or water (micro tubes served as controls) were added (Jin *et al.* 2010; Rodrigues *et al.* 2011). Verapamil is a known efflux inhibitor and therefore served as a positive control. Micro tubes were placed into a 36-well rotor and the fluorescence of EtBr was acquired as described above. Every experiment was performed in triplicate.

2.4.2 Ethidium bromide efflux assay

Mycobacterial cells were loaded with EtBr by incubating a cell suspension prepared as described in 3.4.1 with 3.125 μ g/ml EtBr and 100 μ g/ml verapamil at 25°C (concentrations correspond to half of the MIC of both compounds) for 60 minutes to ensure a maximum accumulation of EtBr without compromising the cellular viability (Paixão *et al.* 2009; Rodrigues *et al.* 2011).

The EtBr loaded cells were centrifuged at 13,000 rpm for 3 minutes and the pellet was washed with PBS. The washed cells were then re-suspended in EtBr-free PBS and the

OD_{600nm} was adjusted to 0.4. Volumes of 95 μ l were transferred into micro tubes and 5 μ l of verapamil, SQ109 or water was added as required. The efflux of EtBr was determined by its fluorescence as described 3.4.1 (Rodrigues *et al.* 2011).

2.5 Rifampicin accumulation in Msmeg

RIF is one of the first anti-TB drugs and previous studies have suggested that efflux of RIF plays a role in determining the level of resistance of mycobacteria to this drug. We have utilised a mass spectrometry-based assay to determine the intracellular concentration of RIF in the presence of SQ109 and a known efflux pump inhibitor, verapamil.

2.5.1 Rifampicin accumulation assay

An Msmeg starter culture was grown as described in 3.1 and 5 ml of this was used to inoculate 150 ml of 7H9 broth. This subculture was grown to an OD_{600nm} of 0.8 and the dividing mycobacteria were harvested by centrifugation for 10 minutes at 3,200 x g at room temperature. The supernatant was discarded, the pellet re-suspended in 10 ml PBS and the culture adjusted to an OD_{600nm} of 4.0.

The uptake of RIF was performed by exposing mycobacteria to RIF with a concentration of 8.23 µg/ml for 20 minutes. Three aliquots of 500 µl each were removed after 0, 5, 10 and 20 minutes and loaded onto a 0.22 µm micro centrifugal filter (Millipore Millex-GV polyvinylidene difluoride PVDF membrane; ultra-free MC Dura 0.22/µm pore size, Merck, Darmstadt, Germany) The samples were centrifuged at 4° C for 1 minute at 13,000 x g and the flow-through was discarded. The cells retained on the membrane were re-suspended in 500 µl ice-cold PBS, spun down again (13,000 x g for 1 minute at 4° C) to wash the cells. The pellet was re-suspended in 500 µl sterile water. The washed mycobacteria were then disrupted by probe sonication three times for 15 seconds at 4° C (amplitude: 20; energy: 30 J). The lysates were cleared by centrifugation at 4° C for 5 minutes at 13,000 x g and 4° C then filtered through a sterile membrane as above. Finally, 130 µl of methanol and 65 µl of acetonitrile were added to the flow-through and the samples were stored at -20°C until analysis.

To investigate the effect of verapamil or SQ109 on RIF accumulation, cells were preincubated with either compound at a concentration of half their MIC for 3 minutes prior to the addition of RIF.

2.5.2 Quantification of rifampicin concentration in cell lysates using LC-MS method

Samples containing RIF were sent to the central analytical facilities (Mass Spectrometry unit) at Stellenbosch University for RIF concentration determination by liquid chromatography-tandem mass spectrometry. The analysis was performed with a Thermo Scientific Easy-nLC II system connected to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany).

2.5.3 RCDC protein determination assay

The protein content of the lysates was determined in order to normalise the RIF concentration obtained to total protein. This was determined by RCDC (*Reducing Agent and Detergent Compatible*) method as follows:

A protein standard curve was prepared from a 1.5 mg/ml Bovine Serum Albumin (BSA) stock solution (Bio-Rad Laboratories, Hercules, CA) as indicated in Table 2.2.

Table 2.2: BSA standard curve preparation

BSA concentration (mg/ml)	1.5 mg/ml BSA stock (μl)	Sterile water (µl)
0	0	25
0.1	1.67	23.33
0.2	3.33	21.67
0.5	8.33	16.67
1.0	16.67	8.33
1.5	25	0

The determination was conducted according to the instructions of the manufacturer of the protein assay kit (Bio-Rad Laboratories, Hercules, CA) with each lysate and BSA standard curve sample. Briefly, aliquots of $25\,\mu l$ from each sample were transferred into $1.5\,m l$ Eppendorf tubes and $125\,\mu l$ of buffer R1 was added. All specimens were mixed well by

vortexing for 20 seconds, a volume of 125 μ l buffer R2 was added and each sample vortexed again for 20 seconds. All samples were centrifuged for 5 minutes at 15,000 X g and 4°C, the supernatant removed and the pellets were left to dry for 5 minutes. After this, a mixture of buffer A and buffer S (125 μ l of A + 2.5 μ l of S per sample) was prepared and 127 μ l were added to each sample. A volume of 1 ml of reagent B was added to each tube and all were left at room temperature for 15 minutes. The mixture was then transferred into a plastic cuvette and the OD_{595nm} was determined using a spectrophotometer. A protein standard curve was plotted using Excel software and used to determine the protein concentration in each sample. When appropriate, dilutions of the samples were made.

2.6 Isolation of spontaneous SQ109-resistant mutants in Msmeg

Genetic mutations confer resistance to many antibiotics in bacteria. Mutations may occur spontaneously in the chromosomal genes or through gene transfer of plasmids between bacteria by either conjugation, transduction or transfection via bacteriophages (Pope *et al.* 2008). *In vitro*, the selection of spontaneous mutations that result in resistance to a drug can be done by culturing bacterial strains in the presence of a concentration above the MIC for that drug (Morlock *et al.* 2000; Boshoff *et al.* 2003). The mutation rate, which is the chance of a mutation to occur per cell generation, and the mutation frequency, which is defined as the proportion of mutants per culture, can be determined experimentally (Morlock *et al.* 2000; Wang *et al.* 2001; Pope *et al.* 2008).

SQ109 resistant mutants were generated by culturing Msmeg in 20 ml of 7H9 glucose-salt at 37° C overnight to an OD_{600nm} of 0.8. The culture was diluted and inoculated into 50 ml of 7H9 broth corresponding to approximately 10^{6} CFU/ml. Aliquots of 5 ml were dispensed into 10 culture tubes and the tubes were incubated at 37° C for 3 days in a shaking incubator at 200 rpm (Morlock *et al.* 2000; Boshoff *et al.* 2003). The cells were pelleted by centrifugation at $1,811 \times g$ for 10 minutes at room temperature and the supernatant was discarded. The bacterial pellet was re-suspended in 1 ml water with 0.5% Tween 80, centrifuged again and the supernatant aspirated, leaving a small amount of liquid. The pellet was then resuspended, and the cultures were spread in duplicate onto Middlebrook 7H10 solid media with glucose-salt supplement and SQ109 at either of the following concentrations: 10, 25 or $50 \, \mu g/ml$. One of the 10 tubes was used to determine the CFU/ml by plating serial dilutions

 $(10^{-5} - 10^{-10})$ of the culture on 7H10 glucose-salt without drug. All plates were incubated for 10 days at 37°C.

2.6.1 PCR and agarose gel electrophoresis

The extraction of the DNA template from Msmeg wild-type and mutants for PCR was started by heating the cells in order to disrupt the cell envelope. A volume of 300 μ l from Msmeg wild-type or mutant culture was boiled at 95°C in a heating block for 10 minutes. After cooling to room temperature, the samples were centrifuged at 13,000 rpm for 5 minutes and the supernatant was immediately processed or kept in the freezer at -20°C as DNA template.

To amplify the MSMEG 0250 gene (3042 bp), four sets of primers were designed using primer3 program (primer3.ut.ee) version 4.0 as shown in Table 2.3. The PCR reaction contained 10 x PCR Buffer with 2 mM MgCl₂, 1 mM 5 X GC-RICH solution, 200 µM of each dNTP, 0.5 μM of each primer, 2 U/μl of FastStart Taq DNA polymerase and 1 μl DNA template and nuclease-free water to a final volume of 50 µl. The PCR amplification was carried out as follows: 2 minutes initial denaturation at 95°C, 35 cycles with 30 seconds denaturation at 95°C, 30 seconds annealing at 55°C and 1.5 minutes elongation at 72°C, followed by 7 minutes of final elongation at 72°C. The PCR products were separated on a 1% agarose gel using the TBE buffer system (0.089 M Tris base, 0.089 M Boric acid and 2 mM EDTA as final concentration in the 1 x TBE buffer with a pH of 8) with 5 μl of Ethidium bromide (final concentration of 0.5 μ g/ml). A volume of 10 μ l of 6 x DNA loading buffer (Thermo scientific) was added to each 50 µl PCR product and 10 µl of the sample was then loaded onto the gel. The DNA electrophoresis was performed at 104 volts for 1 hour in 1 X TBE buffer. A 1 kb DNA marker (Thermo scientific) was used as comparison for the sizes of the DNA fragments and the bands were visualised with the Gel Doc Imaging System (Bio-Rad Laboratories, Johannesburg, South Africa).

Table 2.3: Primers used for MSMEG_0250 gene amplification

Primer name	Sequence
Fo1	5'- GGT CGG ACC GTG TAC CAG - 3'
Re1	5'- GTG CAC GGG GGT GAA CTC - 3'
Fo2	5'- ATC GGC GAG GAC CAG AAG - 3'
Re2	5'- GGC AGG TAT TTC TCG CTG A - 3'
Fo3	5'- GGC GGT ATC AGC GAG AAA TA - 3'
Re3	5'- AGA CCC AGC TTC TCC TGC AC - 3'
Fo4	5'- GTG CAG GAG AAG CTG GGT CT - 3'
Re4	5'- GCT TGG TCT CCG GAT CCT C - 3'

2.6 2. Sanger DNA sequencing

Sanger's method is a DNA sequencing procedure developed by Frederick Sanger and colleagues in 1977 (Sanger and Coulson 1975). The method is based on the addition of chain-terminating dideoxynucleotides (ddNTP's) to the normal nucleotides (NTP's) by DNA polymerase during replication of DNA (Sanger and Coulson 1975). Dideoxynucleotides differ to the normal nucleotides by the presence of a hydrogen group on the 3' carbon instead of a hydroxyl group (OH) (Murphy et al. 2005). The incorporation of these modified nucleotides into the sequence prevents the integration of normal nucleotides because the phosphodiester bond cannot be established between the dideoxynucleotide and the next nucleotide. This results in the termination of the DNA chain (Murphy et al. 2005). For each piece of DNA to be sequenced, four sequencing reactions are required. Each reaction contains all four dNTPs and one of the four modified nucleotides (ddATP, ddGTP, ddCTP and ddTTP). Following amplification, each of the four reactions is run in a separate lane on a polyacrylamide gel in order to visualise the different sized DNA bands, each terminated at a different position in the sequence where the specific dideoxynucleotide has been incorporated (Murphy et al. 2005; Morozova and Marra 2008). PCR products which corresponded to the correct amplicon size were sent to the central analytical facilities (DNA

Stellenbosch University https://scholar.sun.ac.za

sequencing unit) at Stellenbosch University for Sanger DNA sequencing. Data were analysed using the DNA sequence analysis software, sequencer version 5.1.

2.7 Checkerboard drug-interaction assays

Testing of interactions between drugs is an important step in the development of new anti-TB drugs since any new drug would have to be included into the existing regimen. The micro-dilution checkerboard method is one of the methods used in the laboratory for drug interaction assessment. The method uses 96-well micro titre plates containing serial dilutions of drug concentrations alone and in combination (Jenkins and Schuetz 2012). The effect occurring during drug interactions may be synergistic, additive, indifferent or antagonistic (Sopirala *et al.* 2010; Tan *et al.* 2011; Jenkins and Schuetz 2012) and this is determined by calculating the fractional inhibitory concentration index (FICI) as follows:

FICI = FIC of drug A + FIC of drug B

$$FIC of drug A = \frac{MIC of drug A in combination}{MIC of drug A alone} FIC of drug B = \frac{MIC of drug B in combination}{MIC of drug B alone}$$

The FICI is interpreted as follows:

FICI ≤ 0.5 : defined as synergy FICI $> 0.5 - \leq 1$: defined as additivity

FICI $> 1 - \le 4$: defined as indifference FICI > 4: defined as antagonism

Experiments on drug interactions were determined by checkerboard titration and performed using the broth micro dilution method as described in 3.3.1. For this, each well of the 96-well plate contained 50 μ l of solution with combinations of drugs. The dilution of the first drug was done from Column 1 to Column 11, skipping Column 2 while the second drug was diluted from Row A to Row H, skipping row B of the 96-well plate. Row B was used for the MIC determination of the first drug alone and Column 2 for the MIC of the second drug alone. Column 12 was inoculated with media and fresh culture only, and served as a drug free control, 50 μ l of freshly grown and diluted culture was added to each well containing drugs in different concentrations. Plates were incubated and analysed as described above for broth micro dilution assay.

The table 2.4 represents the design of a 96 well plate that was used for drug interaction determination.

Table 2.4: 96 well plate design used for drug interaction assays

		Drug B at 4 X max conc. Without diluted culture									
		(100 μl in each well)									
	\times	Drug A MIC testing									
ture		A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	
Cult		B/2	B/2	B/2	B/2	B/2	B/2	B/2	B/2	B/2	
uted		A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	
t dii		B/4	B/4	B/4	B/4	B/4	B/4	B/4	B/4	B/4	
hou		A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	
Wit		B/8	B/8	B/8	B/8	B/8	B/8	B/8	B/8	B/8	
onc.		A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	
ax c	testing	B/16	B/16	B/16	B/16	B/16	B/16	B/16	B/16	B/16	lo.
x m ach		A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	ontr
Drug A at 8 x max conc. Without diluted culture (100 μl in each well)	MIC	B/32	B/32	B/32	B/32	B/32	B/32	B/32	B/32	B/32	Drug-free control
IBA O µl	В	A/2	A/4	A/8	A/16	A/32	A/64	A/128	A/256	A/512	ıg-fr
Drug (100	Drug	B/64	B/64	B/64	B/64	B/64	B/64	B/64	B/64	B/64	Dr

Drug interaction wells

2.8 Whole genome sequencing of Msmeg SQ109-resistant mutants

Next Generation Sequencing (NGS) methods have been developed to sequence an entire genome from a single sample in a short time, and this technology is used for sequencing large genomes (human genomes) or small genomes such as viral and bacterial genomes (Grada and Weinbrecht 2013). Several potential applications can be run by NGS technologies such as whole genome sequencing (WGS) and whole exon sequencing (WES) for mutation detection, transcriptome sequencing for gene expression determination, targeted sequencing for mutation validation and epigenetic markers confirmation for disease diagnosis (Liu *et al.* 2012; Xuan *et al.* 2013).

Different NGS platforms have emerged with ultra-high-throughput and low-cost effective interests. The Illumina sequencing system is one of the most commonly used NGS platform and can process 35-bp reads and produce about 1 Giga base (Gb) of good quality sequence per run within 2 to 3 days. The Illumina technology uses the sequencing by synthesis (SBS) method (Ansorge 2009).

2.8.1 Phenol-chloroform isoamyl alcohol (PCI) DNA extraction

To detect the mutations in the whole genomic DNA of Msmeg SQ109-resistant mutants, the DNA was extracted using the Phenol-Chloroform Isoamyl Alcohol (PCI) method. Briefly, colonies were grown in liquid medium as described in 3.1 and 100 μ l of each culture was plated out onto 7H10 agar (Becton Dickinson) supplemented with glucose-salt. The plates were incubated for 5 days at 37°C and then placed in an oven for one hour at 80°C to inactivate the mycobacteria. The confluent growth was harvested using a sterile loop and transferred into a 50 ml Falcon tube containing 6 ml of DNA extraction buffer (pH 7.4) and glass beads (4 mm, filled up to 3 ml in Falcon tube). The bacteria were vortexed for 2 minutes, 100 μ g/ml of lysozyme and 10 μ g/ml of RNAse A were added and the mixtures incubated for 2 hours at 37°C. Finally, 600 μ l of proteinase K buffer and 300 μ l of proteinase K enzyme were added to the tubes which were incubated for 16 hours at 45°C.

A volume of 5 ml of Phenol-Chloroform Isoamyl Alcohol was added to the tubes, which were then incubated for 2 hours at room temperature, with inversion every 30 minutes. The tubes were centrifuged at 1,811 x g for 20 minutes at room temperature and three separate layers were obtained, namely an aqueous top phase containing DNA, an interphase and an organic phase. The top phases were carefully removed and transferred into new 50 ml Falcon tubes each containing 5 ml of Chloroform Isoamyl Alcohol and a second centrifugation step was performed at 1,811 x g for 20 minutes at room temperature.

The supernatants with DNA were carefully removed and transferred into new 50 ml Falcon tubes containing $600 \, \mu l$ of sodium acetate. The tubes were gently inverted for 5 minutes followed by an addition of 7 ml ice-cold isopropanol.

The tubes were again gently inverted and the DNA was fished using glass rods. The glass rods were placed for 10 minutes in 1.5 ml Eppendorf tubes with 1 ml 70% ethanol, then transferred to a clean Eppendorf and allowed to air dry for 2 to 3 hours at room temperature. A volume of 300 μ l of TE buffer was added to each tube with a glass rod to dissolve the DNA. All samples were stored at -20°C prior to whole genome sequencing.

2.8.2 Whole genome sequencing

DNA samples were sent to the University of the Western Cape for whole genome sequencing. The analysis of WGS data was performed by Dr M de Vos as follows:

The analysis pipeline includes the use of three different aligners (BWA, Novoalign and SMALT) to map the sequencing reads for each isolate to the reference Msmeg genome. The Genome Analysis ToolKit (GATK) was then used to identify variants (SNPs and Indels) from each aligner file for each sequenced isolate relative to the reference Msmeg genome. A variant was considered to be high confidence if it was identified in all three alignment files and only these were used for subsequent analysis. Due to the overall low coverage of the sequencing data, a variant was only considered for future analysis if it was covered by at least 10 reads.

To recognise variants that were acquired in the mutant isolates, the high confidence variants identified for the wild type isolate were compared to the variants identified for each of the mutant isolates. The unique variants for each mutant isolate were manually inspected using GenomeView to ensure that the variant considered unique to one isolate was not present in the wild-type isolate. The read coverage of each variant was also used to classify each variant as fixed (the variant allele occurs in more than 80 % of the reads) or heterogeneous (the variant allele occurs in between 20-80 % of the reads).

Chapter 3

Results

3.1 Minimum inhibitory concentration determination

The MICs of the following compounds against wild-type Msmeg were determined by broth micro dilution in 7H9: EtBr, verapamil, RIF, INH, SQ109 and values used in subsequent experiments (Andrews 2001; Paixão *et al.* 2009). Compounds were tested in the following ranges: $200 - 0.195 \,\mu\text{g/ml}$ (EtBr), $6,400 - 6.25 \,\mu\text{g/ml}$ (verapamil) and $64 - 0.0625 \,\mu\text{g/ml}$ for the three antibiotics. The MIC was defined as the median between the highest concentration of the compound at which growth was observed and the lowest concentration that inhibited visible growth. The MIC results of all compounds are shown in Table 3.1.

Table 3.1: MICs of various compounds for Msmeg in liquid medium

Compound	Range (μg/ml)	MIC (μg/ml)
EtBr	200 – 0.195	6.25 - 12.5
Verapamil	6400 – 6.25	200 - 400
SQ109	64 – 0.0625	2.0 – 4.0
RIF	64 – 0.0625	0.5 – 1.0
INH	64 – 0.0625	4.0 - 8.0

The MIC of SQ109 was also determined for Msmeg on solid media. 10 μ g/ml was chosen as the MIC of SQ109 for Msmeg on solid medium. In comparison to liquid medium, the MIC of SQ109 was five times higher on agar than in broth.

3.2 Assessment of the effect of SQ109 on EtBr accumulation in Msmeg

The effect of SQ109 on efflux pump activity in Msmeg was assessed by monitoring the rate of accumulation or efflux of EtBr. In order to determine the optimum EtBr concentration for these assays, the level of accumulation of different concentrations of EtBr by Msmeg was

assessed (Figure 3.1). As expected, an increase in fluorescence was observed as the concentration of EtBr was increased in the assay.

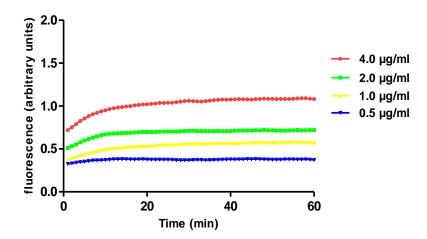
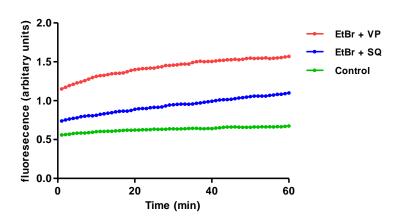


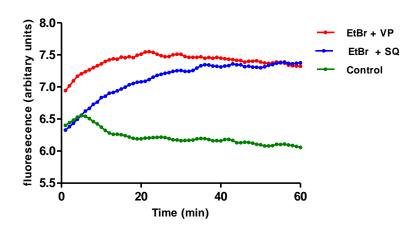
Figure 3.1: Accumulation of EtBr by Msmeg at increasing concentrations. Mycobacterial cells were exposed to different concentrations of EtBr ranging from 0.125 to 4 μ g/ml. The fluorescence of EtBr was monitored at 25°C every minute for a period of 60 minutes.

EtBr enters the cell by diffusion, but must be actively pumped out of the cell by efflux pumps. At $0.5~\mu g/ml$, very little increase in fluorescence is observed, suggesting that the efflux pumps are able to pump out the EtBr before it is able to interact with DNA and proteins within the cells. As the EtBr concentration is increased however, the efflux pumps are not able to extrude the EtBr at a high enough rate and this allows EtBr to bind to intracellular components, causing an increase in fluorescence. An EtBr concentration of $0.5~\mu g/ml$ was therefore selected for subsequent efflux experiments as the effect of a compound that inhibited efflux would be more easily detected using this concentration. Verapamil was previously demonstrated to inhibit efflux in mycobacteria and was therefore used as a positive control for this assay. Addition of verapamil to the efflux assay resulted in an increase in EtBr accumulation as previously reported (Figure 3.2). Performing the efflux assay in the presence of SQ109 appeared to inhibit EtBr efflux in some experiments, but this result was not reproducible (Fig 3.2 9 (c) and (d)).

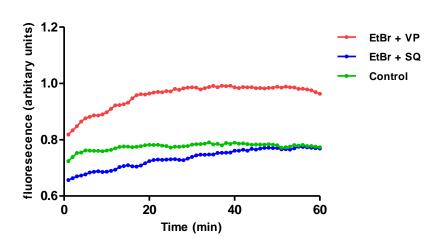
(a)



(b)



(c)



(d)

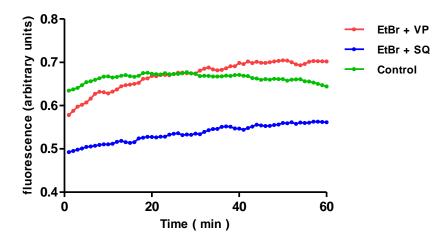
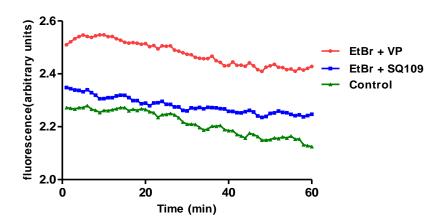


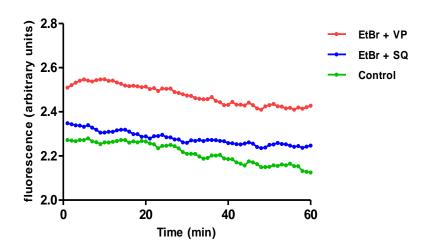
Figure 3.2: Effect of verapamil and SQ109 on accumulation of EtBr by Msmeg. Mycobacterial cells were loaded with $0.5 \mu g/ml$ of EtBr in presence of verapamil or SQ109. The fluorescence was assessed at 25°C every minute for a period of 60 minutes. Results from 4 experiments (a), (b), (c) and (d) are shown.

As an alternative for measuring the effect of SQ109 on efflux in Msmeg, an assay measuring the rate of efflux from cells pre-loaded with EtBr was performed. Once again, the rate of efflux was decrease in the presence of verapamil (Figure 3.3). The rate of efflux in the presence of SQ109 appeared to be marginally lower, however as with the previous assay, these results were not definitive because of the variation observed between the experiments.

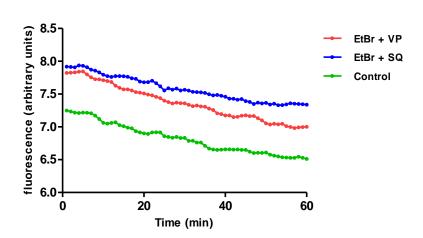
(a)



(b)



(c)



(d)

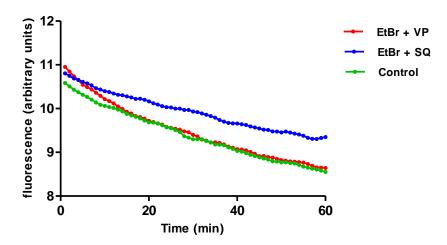


Figure 3.3: Effect of verapamil and SQ109 on the efflux of EtBr in Msmeg. Mycobacterial cells were preloaded with EtBr in the presence of verapamil for 60 minutes to reach the maximun accumulation. The fluorescence was assessed at 25°C every minute for a period of 60 minutes after the addition of verapamil or SQ109. Results from 4 experiments (a), (b), (c) and (d) are shown.

Since the EtBr accumulation and efflux assays did not yield definitive results, an alternative assay to test the effect of SQ109 on RIF accumulation by quantifying the concentrations of RIF in cell lysates using liquid chromatography-tandem mass spectrometry was employed.

3.3 Assessment of the effect of SQ109 on RIF accumulation in Msmeg

Previous studies have demonstrated that RIF is extruded from mycobacteria by efflux pumps (Piddock *et al.* 2000; Poole 2007; Louw *et al.* 2009; Balganesh *et al.* 2012). In order to determine the effect of SQ109 on the accumulation of RIF in Msmeg cells, the intracellular RIF concentration of cells was measured by LC-MS/MS over time following the addition of RIF to a cell suspension (Figure 3.4). The addition of verapamil resulted in an increased RIF accumulation, while SQ109 appeared to have no significant influence on RIF accumulation.

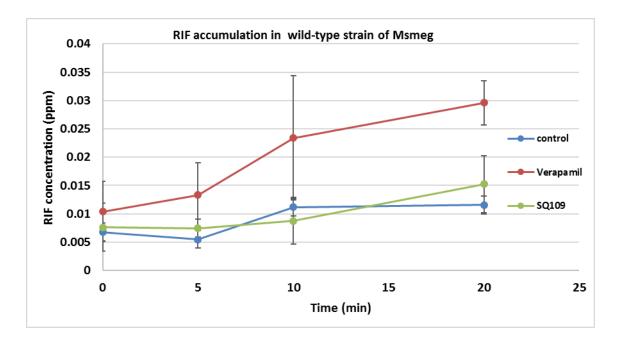


Figure 3.4: RIF intracellular concentration measurements in Msmeg after 0, 5, 10 and 20 minutes of exposure.

3.4 Evaluation of growth of wild-type and SQ109-resistant strains of Msmeg in liquid culture

SQ109-resistant mutants of Msmeg were selected on plates containing 50 μ g/ml of SQ109. Six SQ109-resistant mutants were selected for further characterisation. The growth of the 6 mutants and the wild type parental strain was assessed in complete media over 72 hours (Figure 3.5). No difference in the growth kinetics between the wild type and the mutant strains were observed.

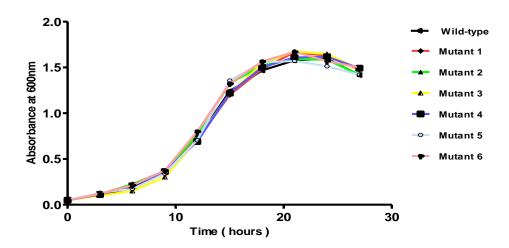


Figure 3.5: Growth curves of Msmeg SQ109-resistant mutants and wild-type

This indicates that the acquisition of resistance to SQ109 does not reduce the fitness of Msmeg under the conditions tested (Figure 3.5).

3.5 Minimum inhibition concentration determination for SQ109-resistant mutants

The MICs of RIF, INH and SQ109 for each of the 6 mutants were determined by broth micro-dilution (Table 3.2). The MICs of RIF and INH were increased 4-fold compared to wild-type while the MIC of SQ109 showed an increase of 20-fold. There was no difference in MICs between the mutants for RIF and INH, whereas the value for SQ109 of mutant 6 was between $40-50 \,\mu\text{g/ml}$ compared to the lower MIC 35-45 $\,\mu\text{g/ml}$ (Table 3.1 and 3.2).

Table 3.2: MICs of RIF, INH and SQ109 for SQ109-resistant mutants of Msmeg

Msmeg SQ109	Rifampicin (µg/ml)	Isoniazid (µg/ml)	SQ109 (μg/ml)
resistant- mutants			
Mutant 1	2.0 – 4.0	16 - 32	35 – 45
Mutant 2	2.0 – 4.0	16 - 32	35 – 45
Mutant 3	2.0 – 4.0	16 - 32	35 – 45
Mutant 4	2.0 – 4.0	16 - 32	35 – 45
Mutant 5	2.0 – 4.0	16 - 32	35 – 45
Mutant 6	2.0 – 4.0	16 - 32	40 – 50

3.6 Assessment of drug-interactions in wild type and SQ109-resistant mutants

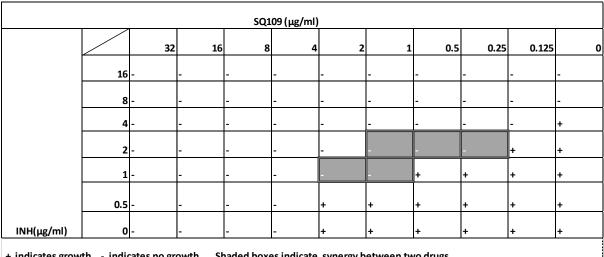
Previous studies have demonstrated that SQ109 and the first-line drugs RIF and INH act synergistically against Msmeg (Chen 2006; Reddy *et al.* 2010). The combined activity of SQ109 with either RIF or INH against wild-type and SQ109-resistant strains of Msmeg was therefore assessed.

Table 3.3: Interaction between SQ109 and RIF against wild type strain of Msmeg

	SQ109 (μg/ml)										
		32	16	8	4	2	1	0.5	0.25	0.125	
	4	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-
	1	-	-	-	-	-	-	-	-	-	-
	0.5	-	-	-	-	-	-	-	-	+	+
	0.25	-	-	-	-	-	-	-	+	+	+
	0.125	-	-	-	-	+	+	+	+	+	+
RIF(µg/ml)	0	-	-	-	-	+	+	+	+	+	+

38

Table 3.4: Interaction between SQ109 and INH against wild type strain of Msmeg



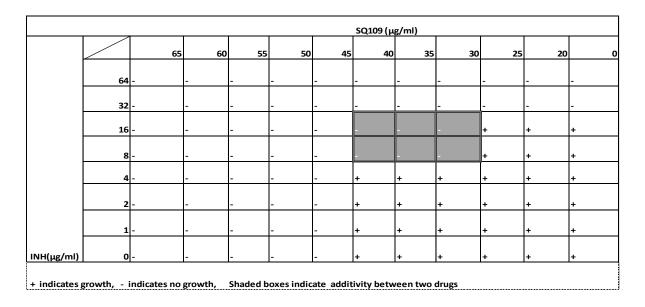
+ indicates growth, - indicates no growth, Shaded boxes indicate synergy between two drugs

Table 3.5: Interaction between SQ109 and RIF against SQ109-resistant strain of Msmeg

	SQ109 (µg/ml)											
		65	60	55	50	45			30	25	20	0
	16	-		_	_	_	_	_	-	_	_	_
	8		_	_	_	_	_	_	_	_	-	_
	4		-	-	_	_	_	_	_	_	_	_
	2		_	_		_				+	+	+
	1	_	_	-	_	_			+	+	+	+
	0.5		_				+	+	+	+	+	+
			-	-								
	0.25	-	-	-	-	-	+	+	+	+	+	+
RIF(µg/ml)	0	-	-	-	-	-	+	+	+	+	+	+

+ indicates growth, - indicates no growth, Shaded boxes indicate additivity between two drugs

Table 3.6: Interaction between SQ109 and INH against SQ109-resistant strain of Msmeg



The combination of SQ109/RIF against Msmeg wild-type, decreased the MIC of SQ109 16-fold in the presence of RIF, while the MIC of RIF was decreased 4-fold in the presence of SQ109 (Table 3.3). For the combination of SQ109/INH, the MIC of SQ109 was decreased 16-fold in the presence of INH while the MIC of INH was decreased 8-fold in the presence of SQ109 (Table 3.4).

The decrease in MIC of SQ109 was 1.5-fold and 4-fold for RIF in the SQ109 mutant (Table 3.5) similarly, the combination of SQ109/INH decreased the MIC of SQ109 1.5-fold while the MIC of INH decreased 4-fold (Table 3.6). The interaction study was performed on the SQ109-resistant mutant 1 only.

The FICI was calculated by using the median between the highest concentration of the drug at which growth was observed and the lowest concentration at which growth was inhibited.

Table 3.7: Checkerboard synergy between SQ109/RIF and SQ109/INH Msmeg strains

Msmeg	Compound	MIC alone	MIC in	FIC	FICI	Activity
		(μg/ml)	combination			
			(μg/ml)			
Wild-type	SQ109	2.0 – 4.0	0.125 - 0.25	0.0625	0.3125	Synergistic
	RIF	0.5 – 1.0	0.125 – 0.25	0.25		
	SQ109	2.0 – 4.0	0.125 - 0.25	0.0625	0.1875	Synergistic
	INH	4.0 – 8.0	0.5 – 1.0	0.125		
Mutant	SQ109	35 – 45	25 – 30	0.647	0.897	Additive
	RIF	2.0 – 4.0	0.5 – 1.0	0.25		
	SQ109	35 – 45	25 – 30	0.647	0.897	Additive
	INH	16 - 32	4.0 – 8.0	0.25		

Synergistic effects were observed for SQ109/RIF and SQ109/INH combinations against Msmeg wild-type respectively, while the effect was additive for both SQ109/RIF and SQ109/INH combinations on SQ109-resistant mutant (Table 3.7). The synergistic effect between SQ109/RIF and SQ109/INH against Msmeg was also observed in a previous study (Reddy *et al.* 2010).

3.7 Sanger sequencing of MSMEG_0250 in SQ109-resistant mutants

A previous study demonstrated that mutations in *mmpL3* of Mtb are associated with resistance to SQ109 (Tahlan *et al.* 2012). Msmeg contains a homologue of *mmpL3*, namely *MSEG_0250*. In order to determine if mutations in this gene were responsible for the resistance phenotypes of the 6 Msmeg mutants, four primer sets (Table 2.3) were designed to perform direct sequencing of this gene. A successful amplification was confirmed by agarose gel electrophoresis (Figure 6), and the PCR products were submitted for Sanger sequencing. The alignment of the sequence obtained for each PCR product with the sequence of *MSMEG_0250* is shown in Figure 7.

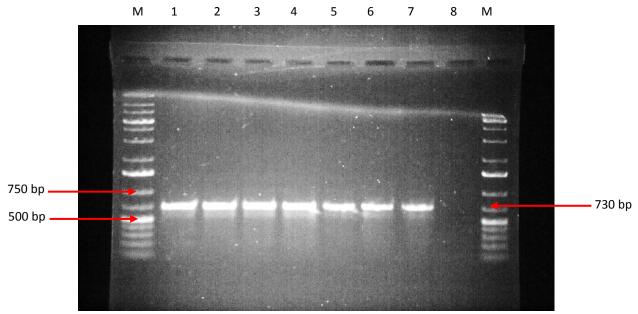


Figure 3.6 a: Amplification of $MSMEG_0250$ gene by primer set 1. Lanes 1-6 contain product from DNA of six colonies, Lane 7 represents wild-type DNA performed as a positive control, Lane 8 is the negative control and M represents a 1 kb DNA marker.

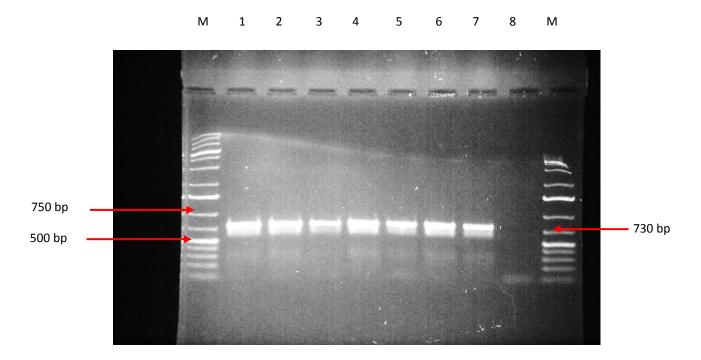


Figure 3.6 b: Amplification of *MSMEG_0250* **gene by primer set 2**. Lanes 1 – 6 contain product from DNA of six colonies, Lane 7 represents wild-type DNA performed as a positive control, Lane 8 is the negative control (no DNA) and M represents a 1 kb DNA marker.

5

Μ

Μ

1

2

3

Figure 3.6 c: Amplification of *MSMEG_0250* gene by primer set 3. Lanes 1 – 6 contain product from DNA of six colonies, Lane 7 represents wild-type DNA performed as a positive control, Lane 8 is the negative control and M represents a 1 kb DNA marker.

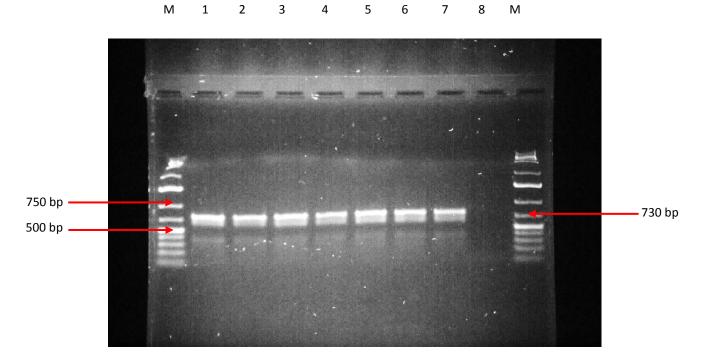


Figure 3.6 d: Amplification of *MSMEG_0250* **gene by primer set 4**. Lanes 1 – 6 contain product from DNA of six colonies, Lane 7 represents wild-type DNA performed as a positive control, Lane 8 is the negative control and M represents a 1 kb DNA marker.



Figure 3.7: Alignment of sequence obtained for each primer set with the gene sequence of MSMEG_0250

The sequence analysis of all PCR products of six isolated mutant strains did not reveal any mutation, therefore it is likely that other genes in Msmeg are involved in the resistance to SQ109. Whole genome sequencing was therefore performed to identify the mutations that confer this resistance.

3.8 Identification of mutations in the genome of Msmeg SQ109-resistant mutants

To determine genes that are involved in mutants of Msmeg being resistant to SQ109, genomic DNA was extracted from the six colonies and sent for sequencing. A number of single nucleotide polymorphisms (SNPs) were identified for each mutant.

Table 3.8: Genes involved in mutations of SQ109-resistant strains of Msmeg

Mutant	SNPs genes	Product	Indels genes	Product
Mutant 1	MSMEG_4010	Glyoxalase family protein		
Mutant 2	MSMEG_5880	Nicotine dehydrogenase		
Mutant 3	MSMEG_4421	Conserved hypothetical protein		
	MSMEG_6381	Conserved hypothetical protein		
	MSMEG_6672	Type I phosphodiesterase		
Mutant 4	MSMEG_3152	Ferrochelatase	Intergenic region MSMEG_3111	Extracellular
	MSMEG_4010	Glyoxalase family protein		solute-binding protein, family
	MSMEG_5855	Conserved hypothetical protein		protein 1
	MSMEG_6220 Intergenic region	Lipoprotein		
Mutant 5			MSMEG_5639	Enoyl-CoA hydratase
Mutant 6	Intergenic region			

The identification of genes were done by mapping the sequencing reads of each mutant to the reference Msmeg genome and from there single-nucleotide polymorphisms (SNPs) and small insertions and deletions (indels) were identified by Genome Analysis ToolKit (GATK). No SNPs or indels were identified in the intergenic region for Mutant 6 (Table 3.8).

Chapter 4

Discussion

SQ109 is a promising new anti-TB drug that has shown synergistic activity with the current TB drugs, specifically with RIF and INH (Onajole *et al.* 2010). In this project, the mechanism of synergy of SQ109 with RIF and INH was studied in Msmeg. We hypothesised that the synergistic activity of SQ109 was due to inhibition of efflux in mycobacteria. Efflux systems are physiological mechanisms used by mycobacteria to defend themselves against toxic substances, including drugs, by extruding these compounds from the cells. These systems decrease the concentration of drugs in the cells and prevent them from reaching their targets. Inhibition of these efflux pumps increases the concentration of drugs in mycobacterial cells, thereby decreasing the concentration of drug required to kill them. This effect results in an apparent decrease in the MIC of the drug.

The inhibitory concentrations of EtBr, verapamil, SQ109, RIF and INH for Msmeg were determined. MICs of EtBr, verapamil and SQ109 were in the same range as in the previous studies (Table 3.1) (Rodrigues et al. 2008, 2011; Reddy et al. 2010; Gupta and Bhakta 2012). For RIF and INH, the MIC values were different from those previously reported. The MIC of RIF in this study was determined to be between $0.5 - 1.0 \mu g/ml$. In previous studies the MIC for RIF was reported to be 8.0 μg/ml or 1.0 μg/ml and the MIC for INH was reported to be 8.0 µg/ml as well (Teng and Dick 2003; Reddy et al. 2010). The difference may be influenced by the media. Liquid culture was performed in 7H9 media supplemented with glucose-salt and in a previous study, 7H9 liquid medium was supplemented with albumin-dextrosecatalase (ADC); this may affect the activity of the compounds against Msmeg. Drug-drug interaction studies in wild-type Msmeg confirmed the synergy previously reported between SQ109 and RIF or SQ109 and INH (Reddy et al. 2010). The fractional inhibitory concentration index (FICI) for the SQ109/RIF combination against Msmeg wild-type was higher compared to the literature, although it was still below the cut-off value of 0.5 for synergy (Table 3.7) (Reddy et al. 2010). The reason for this difference could be the time of exposure of mycobacterial cells to the drugs by incubating the plate for longer or shorter periods. During drug interactions the plates were incubated for 6 to 7 days, while in the previous study the plates were read for 3 to 4 days.

The effect of SQ109 on the EtBr efflux system was assessed by monitoring the rate of accumulation and efflux of EtBr in Msmeg. For the accumulation assay, an increase in fluorescence was observed with increasing EtBr concentrations. This is consistent with a previous study using a similar range of EtBr concentrations (Rodrigues et al. 2008; Jin et al. 2010). Verapamil was found to increase EtBr accumulation in Msmeg (Figure 3.2 and 3.3), as observed previously (Jin et al. 2010; Rodrigues et al. 2011). This effect is thought to be caused by the inhibition of efflux pumps, although the target(s) of verapamil in mycobacteria are not known. Verapamil was used in our assays as a positive control compared to SQ109. The effect of SQ109 on EtBr accumulation and efflux in Msmeg produced inconsistent results. In some assays, SQ109 increase EtBr accumulation, to a lesser extent than verapamil, however this was not consistently observed (Figure 3.2 and 3.3). The effect of SQ109 was also assessed on RIF accumulation by measuring the concentrations of RIF in Msmeg. A significant increase of concentration of RIF in Msmeg was observed in the presence of verapamil, while in the presence of SQ109, the accumulation of RIF was similar to untreated cells (Figure 3.4). SQ109 therefore does not appear to influence RIF accumulation in Msmeg, however its effect on RIF accumulation in other mycobacteria cannot be inferred from this result. This therefore requires further investigation. et al.A previous study reported that Mtb strains harbouring mutations in mmpL3 were crossresistant to SQ109, suggesting that this transporter may be the target of SQ109 (Tahlan et al. 2012). MmpL3 is involved in transporting cell wall precursors across the cell membrane. It was therefore hypothesised that SQ109 increases the permeability of the cell wall allowing drugs to enter the cell more easily. This could account for the increase of RIF activity in the presence of SQ109 in Mtb shown in the literature (Daffé and Etienne 1999; Nikaido 2001).

Spontaneous SQ109-resistant mutants of Msmeg were generated by selection on plates containing lethal concentrations of the drug. The Sanger DNA sequencing did not identify any mutations in *MSMEG_0250* gene (*mmpL3* homologue) (Figure 3.7), and this suggests that other genes may be involved in the resistance to SQ109 in Msmeg. In comparison to a previous work, spontaneous SQ109-resistant mutants could not be isolated in Mtb (Tahlan

et al. 2012). However, the generation of resistant mutants of Mtb to analogues of SQ109 showed cross-resistance to SQ109 and the whole genome sequencing of these mutants identified mutations in then *mmpL3* gene (Li et al. 2014). This suggests that the mechanism of resistance is different in the two species. This notion is supported by a recent study which reported that MmpL3 may not be the only target for SQ109 in Mtb (Li et al. 2014).

An increase in MICs for SQ109, RIF and INH was demonstrated in an SQ109-resistant mutant of Msmeg. MIC values increased compared to the wild-type and this increase was 4-fold for RIF and INH and 20-fold for SQ109 (Table 3.2). For SQ109 the significant increase of MIC value may be a result of a decrease of drug affinity to its target, due to mutations that changed the structure of this target. The growth of each SQ109-resistant mutant strain of Msmeg was characterised in liquid medium and compared to the wild-type strain. No significant differences between the wild-type and mutant strains were observed (Figure 3.5), suggesting that the mutations present in these strains have no influence on the fitness of the bacteria under the conditions tested.

Drug-drug interaction studies between SQ109, RIF or INH were conducted for SQ109-resistant mutant 1 using the checkerboard method (K H Rand 1993; Reddy *et al.* 2010). The activity of two drugs in combination was assessed as synergistic, additive, indifferent or antagonistic. Our study demonstrated synergy between SQ109/RIF or SQ109/INH against wild-type of Msmeg with the fractional inhibitory concentration index (FICI) of 0.3125 and 0.1875 respectively, and an additive activity in SQ109-resistant mutant strain with FICI value 0.897 for both combinations (Table 3.7). The additive activity found in SQ109/RIF and SQ109/INH combinations against SQ109-resistant mutant suggests that SQ109 may have multiple targets within Msmeg. The mutation(s) present in SQ109-resistant mutant 1 has abolished the synergy of SQ109 with RIF and INH. However, the fact that additivity is now observed suggests that SQ109 has residual activity, which acts on another target.

The whole genome sequencing of the six mutants identified genes that may be responsible for their resistance phenotype (Table 3.8). We did not investigate the link between these genes and mutations that confer resistance to SQ109 in Msmeg. However, further investigation will follow to confirm these mutations within these genes by Sanger DNA

sequencing and their role in SQ109 resistance and synergy with RIF and INH will be further investigated.

Conclusion

This study investigated the influence of SQ109 on efflux in Msmeg, as a mechanism of its synergy with RIF and INH. Limitations associated with the methodology used to investigate the effect of SQ109 on efflux in Msmeg prevented us from drawing definitive conclusions from these experiments.. SQ109-resistant mutants of Msmeg did not harbour mutations in the *MSMEG_0250* gene, suggesting that MmpL3 is not the target of SQ109 in Msmeg and that the mechanism of resistance is different from Mtb. The identification of SNPs and Indels in the genome of SQ109-resistant mutants suggests that SQ109 may have multiple targets in Msmeg, although the exact role of these SNPs and indels in resistance to SQ109 is yet to be determined. The knowledge of efflux systems, drug interactions and the molecular mechanism of resistance are important for the development of new drugs to treat MDR-TB and XDR-TB.

References

- Adams, KN, Takaki, K, Connolly, LE, et al. (2011). Drug Tolerance in Replicating Mycobacteria Mediated by a Macrophage-Induced Efflux Mechanism. *Cell* 145: 39–53.
- Ahmad, S (2010). Pathogenesis, Immunology, and Diagnosis of Latent *Mycobacterium* tuberculosis Infection. *J Immunol Res* 2011: e814943.
- Amaral, L, Martins, A, Spengler, G, et al. (2014). Efflux pumps of Gram-negative bacteria: what they do, how they do it, with what and how to deal with them. Exp Pharmacol Drug Discov 4: 168.
- Andrews, JM (2001). Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* 48: 5–16.
- Andries, K, Verhasselt, P, Guillemont, J, et al. (2005). A diarylquinoline drug active on the ATP synthase of Mycobacterium tuberculosis. *Science* 307: 223–7.
- Ansorge, WJ (2009). Next-generation DNA sequencing techniques. *New Biotechnol* 25: 195–203.
- Balasubramanian, V, Solapure, S, Iyer, H, et al. (2014). Bactericidal activity and mechanism of action of AZD5847, a novel oxazolidinone for treatment of tuberculosis.

 Antimicrob Agents Chemother 58: 495–502.
- Balganesh, M, Dinesh, N, Sharma, S, et al. (2012). Efflux Pumps of Mycobacterium tuberculosis Play a Significant Role in Antituberculosis Activity of Potential Drug Candidates. *Antimicrob Agents Chemother* 56: 2643–51.

- Blumberg, HM, Burman, WJ, Chaisson, RE, et al. (2003). American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: treatment of tuberculosis. Am J Respir Crit Care Med 167: 603–62.
- Boshoff, HIM, Reed, MB, Barry, CE, et al. (2003). DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. *Cell* 113: 183–93.
- Braibant, M, Gilot, P, Content, J (2000). The ATP binding cassette (ABC) transport systems of Mycobacterium tuberculosis. *FEMS Microbiol Rev* 24: 449–67.
- Chao, MC, Kieser, KJ, Minami, S, et al. (2013). Protein complexes and proteolytic activation of the cell wall hydrolase RipA regulate septal resolution in mycobacteria. *PLoS Pathog* 9: e1003197.
- Chen, P (2006). Synergistic interactions of SQ109, a new ethylene diamine, with front-line antitubercular drugs in vitro. *J Antimicrob Chemother* 58: 332–7.
- Crick, DC, Mahapatra, S, Brennan, PJ (2001). Biosynthesis of the arabinogalactanpeptidoglycan complex of Mycobacterium tuberculosis. *Glycobiology* 11: 107R – 118R.
- Daffé, M, Etienne, G (1999). The capsule of Mycobacterium tuberculosis and its implications for pathogenicity. *Tuber Lung Dis Off J Int Union Tuberc Lung Dis* 79: 153–69.
- Dartois, V (2014). The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. *Nat Rev Microbiol* 12: 159–67.
- Diacon, AH, Pym, A, Grobusch, M, et al. (2009). The diarylquinoline TMC207 for multidrugresistant tuberculosis. *N Engl J Med* 360: 2397–405.
- Domenech, P, Reed, MB, Barry, CE (2005). Contribution of the Mycobacterium tuberculosis

 MmpL Protein Family to Virulence and Drug Resistance. *Infect Immun* 73: 3492–501.

- Dutta, NK, Mehra, S, Kaushal, D (2010). A Mycobacterium tuberculosis Sigma Factor

 Network Responds to Cell-Envelope Damage by the Promising Anti-Mycobacterial

 Thioridazine. *PLoS ONE* 5: e10069.
- Escribano, I, Rodríguez, JC, Llorca, B, et al. (2007). Importance of the efflux pump systems in the resistance of Mycobacterium tuberculosis to fluoroquinolones and linezolid.

 Chemotherapy 53: 397–401.
- Grada, A, Weinbrecht, K (2013). Next-Generation Sequencing: Methodology and Application. *J Invest Dermatol* 133: e11.
- Grzegorzewicz, AE, Pham, H, Gundi, VAKB, et al. (2012). Inhibition of mycolic acid transport across the Mycobacterium tuberculosis plasma membrane. *Nat Chem Biol* 8: 334–41.
- Gupta, A, Bhakta, S (2012). An integrated surrogate model for screening of drugs against Mycobacterium tuberculosis. *J Antimicrob Chemother* dks056.
- Heifets, L, Lindholm-Levy, P (1989). Comparison of bactericidal activities of streptomycin, amikacin, kanamycin, and capreomycin against Mycobacterium avium and M. tuberculosis. *Antimicrob Agents Chemother* 33: 1298–301.
- Hett, EC, Rubin, EJ (2008). Bacterial growth and cell division: a mycobacterial perspective. *Microbiol Mol Biol Rev MMBR* 72: 126–56, table of contents.
- Huang, T-S, Kunin, CM, Wang, H-M, et al. (2013). Inhibition of the Mycobacterium tuberculosis reserpine-sensitive efflux pump augments intracellular concentrations of ciprofloxacin and enhances susceptibility of some clinical isolates. *J Formos Med Assoc* 112: 789–94.
- Jenkins, SG, Schuetz, AN (2012). Current Concepts in Laboratory Testing to Guide

 Antimicrobial Therapy. *Mayo Clin Proc* 87: 290–308.

- Jin, J, Zhang, J-Y, Guo, N, et al. (2010). Farnesol, a potential efflux pump inhibitor in Mycobacterium smegmatis. *Mol Basel Switz* 15: 7750–62.
- Kang, C-M, Nyayapathy, S, Lee, J-Y, et al. (2008). Wag31, a homologue of the cell division protein DivIVA, regulates growth, morphology and polar cell wall synthesis in mycobacteria. *Microbiol Read Engl* 154: 725–35.
- K H Rand, HJH (1993). Reproducibility of the microdilution checkerboard method for antibiotic synergy. *Antimicrob Agents Chemother* 37: 613–5.
- Kolyva, AS, Karakousis, PC (2012). Old and New TB Drugs: Mechanisms of Action and Resistance. InTech Open Access Publisher. ISBN: 9533079487.
- Korf, J, Stoltz, A, Verschoor, J, et al. (2005). TheMycobacterium tuberculosis cell wall component mycolic acid elicits pathogen-associated host innate immune responses.

 Eur J Immunol 35: 890–900.
- Kremer, LS, Besra, GS (2002). Current status and future development of antitubercular chemotherapy. *Expert Opin Investig Drugs* 11: 1033–49.
- Liu, L, Li, Y, Li, S, et al. (2012). Comparison of Next-Generation Sequencing Systems. *BioMed Res Int* 2012: e251364.
- Livermore, DM (2003). Linezolid in vitro: mechanism and antibacterial spectrum. *J*Antimicrob Chemother 51 Suppl 2: ii9–16.
- Li, W, Upadhyay, A, Fontes, FL, et al. (2014). Novel Insights into the Mechanism of Inhibition of MmpL3, a Target of Multiple Pharmacophores in Mycobacterium tuberculosis.

 Antimicrob Agents Chemother 58: 6413–23.
- Li, X-Z, Nikaido, H (2009). Efflux-Mediated Drug Resistance in Bacteria: an Update. *Drugs* 69: 1555–623.

- Li, X-Z, Zhang, L, Nikaido, H (2004). Efflux pump-mediated intrinsic drug resistance in Mycobacterium smegmatis. *Antimicrob Agents Chemother* 48: 2415–23.
- Louw, GE, Warren, RM, Pittius, NCG van, et al. (2009). A Balancing Act: Efflux/Influx in Mycobacterial Drug Resistance. *Antimicrob Agents Chemother* 53: 3181–9.
- Luber, P, Bartelt, E, Genschow, E, et al. (2003). Comparison of Broth Microdilution, E Test, and Agar Dilution Methods for Antibiotic Susceptibility Testing of Campylobacter jejuni and Campylobacter coli. *J Clin Microbiol* 41: 1062–8.
- Machado, D, Couto, I, Perdigão, J, et al. (2012). Contribution of Efflux to the Emergence of Isoniazid and Multidrug Resistance in Mycobacterium tuberculosis. *PLoS ONE* 7: e34538.
- Makarov, V, Manina, G, Mikusova, K, et al. (2009). Benzothiazinones kill Mycobacterium tuberculosis by blocking arabinan synthesis. *Science* 324: 801–4.
- Matsumoto, M, Hashizume, H, Tomishige, T, et al. (2006). OPC-67683, a Nitro-Dihydro-Imidazooxazole Derivative with Promising Action against Tuberculosis In Vitro and In Mice. *PLoS Med* 3: e466.
- Matteelli, A, Carvalho, AC, Dooley, KE, et al. (2010). TMC207: the first compound of a new class of potent anti-tuberculosis drugs. Future Microbiol 5: 849–58.
- Migliori, GB, Centis, R, D'Ambrosio, L, *et al.* (2012). Totally Drug-Resistant and Extremely Drug-Resistant Tuberculosis: The Same Disease? *Clin Infect Dis* 54: 1379–80.
- Morlock, GP, Plikaytis, BB, Crawford, JT (2000). Characterization of Spontaneous, In Vitro-Selected, Rifampin-Resistant Mutants of Mycobacterium tuberculosisStrain H37Rv.

 Antimicrob Agents Chemother 44: 3298–301.
- Morozova, O, Marra, MA (2008). Applications of next-generation sequencing technologies in functional genomics. *Genomics* 92: 255–64.

- Murphy, KM, Berg, KD, Eshleman, JR (2005). Sequencing of genomic DNA by combined amplification and cycle sequencing reaction. *Clin Chem* 51: 35–9.
- Nikaido, H (2001). Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol* 12: 215–23.
- Nikaido, H (2009). Multidrug Resistance in Bacteria. Annu Rev Biochem 78: 119–46.
- Nikonenko, BV, Protopopova, M, Samala, R, et al. (2007). Drug Therapy of Experimental

 Tuberculosis (TB): Improved Outcome by Combining SQ109, a New Diamine

 Antibiotic, with Existing TB Drugs. Antimicrob Agents Chemother 51: 1563–5.
- Omote, H, Hiasa, M, Matsumoto, T, et al. (2006). The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations. *Trends Pharmacol Sci* 27: 587–93.
- Onajole, OK, Govender, P, Helden, PD van, et al. (2010). Synthesis and evaluation of SQ109 analogues as potential anti-tuberculosis candidates. *Eur J Med Chem* 45: 2075–9.
- Paixão, L, Rodrigues, L, Couto, I, *et al.* (2009). Fluorometric determination of ethidium bromide efflux kinetics in Escherichia coli. *J Biol Eng* 3: 18.
- Palomino, JC, Martin, A (2014). Drug Resistance Mechanisms in Mycobacterium tuberculosis. *Antibiotics* 3: 317–40.
- Pasca, MR, Guglierame, P, Arcesi, F, et al. (2004). Rv2686c-Rv2687c-Rv2688c, an ABC

 Fluoroquinolone Efflux Pump in Mycobacterium tuberculosis. *Antimicrob Agents*Chemother 48: 3175–8.
- Pasca, MR, Guglierame, P, De Rossi, E, et al. (2005). mmpL7 Gene of Mycobacterium tuberculosis Is Responsible for Isoniazid Efflux in Mycobacterium smegmatis.

 Antimicrob Agents Chemother 49: 4775–7.

- Piddock, LJV (2006). Multidrug-resistance efflux pumps? Not just for resistance. *Nat Rev Microbiol* 4: 629–36.
- Piddock, LJV, Williams, KJ, Ricci, V (2000). Accumulation of rifampicin by Mycobacterium aurum, Mycobacterium smegmatis and Mycobacterium tuberculosis. *J Antimicrob Chemother* 45: 159–65.
- Poole, K (2007). Efflux pumps as antimicrobial resistance mechanisms. Ann Med 39: 162–76.
- Pope, CF, O'Sullivan, DM, McHugh, TD, et al. (2008). A Practical Guide to Measuring

 Mutation Rates in Antibiotic Resistance. *Antimicrob Agents Chemother* 52: 1209–14.
- Poulsen, BE, Cunningham, F, Lee, KKY, et al. (2011). Modulation of Substrate Efflux in

 Bacterial Small Multidrug Resistance Proteins by Mutations at the Dimer Interface

 J. Bacteriol 193: 5929–35.
- Protopopova, M, Hanrahan, C, Nikonenko, B, et al. (2005). Identification of a new antitubercular drug candidate, SQ109, from a combinatorial library of 1, 2-ethylenediamines. *J Antimicrob Chemother* 56: 968–74.
- Prozorov, AA, Zaĭchikova, MV, Danilenko, VN (2012). [Mycobacterium tuberculosis mutants with multidrug resistance: history of origin, genetic and molecular mechanisms of resistance, and emerging challenges]. *Genetika* 48: 5–20.
- Raja, A, Prabakaran, P, Gajalakshm, P (2011). Drug Resistant Tuberculosis and its Survielance. *Curr Res Tuberc* 3: 1–8.
- Raman, K, Yeturu, K, Chandra, N (2008). targetTB: A target identification pipeline for Mycobacterium tuberculosis through an interactome, reactome and genome-scale structural analysis. *BMC Syst Biol* 2: 109.

- Reddy, VM, Einck, L, Andries, K, et al. (2010). In Vitro Interactions between New

 Antitubercular Drug Candidates SQ109 and TMC207. Antimicrob Agents Chemother

 54: 2840–6.
- Rivers, EC, Mancera, RL (2008). New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. *Drug Discov Today* 13: 1090–8.
- Rodrigues, L, Ramos, J, Couto, I, et al. (2011). Ethidium bromide transport across

 Mycobacterium smegmatis cell-wall: correlation with antibiotic resistance. BMC

 Microbiol 11: 35.
- Rodrigues, L, Villellas, C, Bailo, R, et al. (2013). Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 57: 751–7.
- Rodrigues, L, Wagner, D, Viveiros, M, et al. (2008). Thioridazine and chlorpromazine inhibition of ethidium bromide efflux in Mycobacterium avium and Mycobacterium smegmatis. *J Antimicrob Chemother* 61: 1076–82.
- De Rossi, E, Aínsa, JA, Riccardi, G (2006). Role of mycobacterial efflux transporters in drug resistance: an unresolved question. *FEMS Microbiol Rev* 30: 36–52.
- Sacksteder, KA, Protopopova, M, Barry, CE, et al. (2012). Discovery and development of SQ109: a new antitubercular drug with a novel mechanism of action. Future

 Microbiol 7: 823–37.
- Sanger, F, Coulson, AR (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Biol* 94: 441–8.
- Sarathy, JP, Dartois, V, Lee, EJD (2012). The Role of Transport Mechanisms in Mycobacterium Tuberculosis Drug Resistance and Tolerance. *Pharmaceuticals* 5: 1210–35.
- Schluger, NW (2005). The Pathogenesis of Tuberculosis. Am J Respir Cell Mol Biol 32: 251–6.

- Silva, PEAD, Palomino, JC (2011). Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: classical and new drugs. *J Antimicrob Chemother* dkr173.
- Smith, I (2003). Mycobacterium tuberculosis pathogenesis and molecular determinants of virulence. *Clin Microbiol Rev* 16: 463–96.
- Sopirala, MM, Mangino, JE, Gebreyes, WA, et al. (2010). Synergy testing by Etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant Acinetobacter baumannii. *Antimicrob Agents Chemother* 54: 4678–83.
- Stephan, J, Mailaender, C, Etienne, G, et al. (2004). Multidrug Resistance of a Porin Deletion

 Mutant of Mycobacterium smegmatis. Antimicrob Agents Chemother 48: 4163-70
- Tahlan, K, Wilson, R, Kastrinsky, DB, et al. (2012). SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of Mycobacterium tuberculosis. *Antimicrob Agents Chemother* 56: 1797–809.
- Tan, TY, Lim, TP, Lee, WHL, et al. (2011). In Vitro Antibiotic Synergy in Extensively Drug-Resistant Acinetobacter baumannii: the Effect of Testing by Time-Kill, Checkerboard, and Etest Methods. *Antimicrob Agents Chemother* 55: 436–8.
- Teng, R, Dick, T (2003). Isoniazid resistance of exponentially growing Mycobacterium smegmatis biofilm culture. *FEMS Microbiol Lett* 227: 171–4.
- Varela, C, Rittmann, D, Singh, A, et al. (2012). MmpL genes are associated with mycolic acid metabolism in mycobacteria and corynebacteria. *Chem Biol* 19: 498–506.
- Walzl, G, Ronacher, K, Hanekom, W, et al. (2011). Immunological biomarkers of tuberculosis.

 Nat Rev Immunol 11: 343–54.

- Wang, G, Wilson, TJM, Jiang, Q, et al. (2001). Spontaneous Mutations That Confer Antibiotic

 Resistance in Helicobacter pylori. Antimicrob Agents Chemother 45: 727–33.
- WHO (2014). Global tuberculosis report 2013. http://www.who.int/tb/publications/global_report/en/
- WHO (2006). International Standards for Tuberculosis Care.

 http://www.who.int/tb/publiations/2006/istc_report.pdf
- Wiegand, I, Hilpert, K, Hancock, REW (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 3: 163–75.
- Xuan, J, Yu, Y, Qing, T, et al. (2013). Next-generation sequencing in the clinic: promises and challenges. *Cancer Lett* 340: 284–95.
- Zhang, Y, Yew, WW (2009). Mechanisms of drug resistance in Mycobacterium tuberculosis.

 Int J Tuberc Lung Dis Off J Int Union Tuberc Lung Dis 13: 1320–30.