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The activity of nybomycin against
Mycobacterium tuberculosis

by

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SUMMARY

Nybomycin was discovered in 1955, but was never developed for clinical use. The compound was noticed again in recent years when it displayed bactericidal activity against certain fluoroquinolone-resistant bacterial species. The work presented here aims chiefly at describing the effect of nybomycin on *Mycobacterium tuberculosis*. The study is made up of three parts.

In the first part, *in vitro* nybomycin susceptibility testing was conducted with various fluoroquinolone-susceptible and fluoroquinolone-resistant bacterial species. All *M. tuberculosis* isolates displayed low nybomycin inhibitory concentrations regardless of fluoroquinolone resistance. Similar susceptibility results were obtained for *N. gonorrhoeae* isolates, but results obtained with other bacterial species were less promising.

In the second part, *in silico* investigations were conducted to elucidate the mechanism of action of nybomycin in *M. tuberculosis*. Results show that nybomycin binds to *M. tuberculosis* gyrase enzyme with an affinity at least similar to that of fluoroquinolones. No clear differences in binding affinity were observed when *gyrA* mutations, commonly associated with fluoroquinolone resistance, were considered. The results suggest that the mechanism of action of nybomycin against *M. tuberculosis* involves inhibition of gyrase enzyme.

In the third part, *M. tuberculosis* mutants with increased nybomycin minimum inhibitory concentrations were selected and compared with the wild type organism

through whole genome sequencing. None of the isolates harbored any mutations commonly linked to known drug resistance mechanisms. This indicates that *M. tuberculosis* likely employs a novel mechanism of resistance against nybomycin. This may further signify that nybomycin has an additional mechanism of action against *M. tuberculosis*, besides the action on gyrase enzyme, as suggested by the *in silico* results from this study. Twenty-two genes were identified through whole genome sequencing that may potentially be linked to the mechanism of resistance and possibly an additional mechanism of action.

SUPERVISOR STATEMENT

As the candidate's supervisor, I agree to the submission of this thesis.

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Prof Prashini Moodley

As the candidate's co-supervisor, I agree to the submission of this thesis.

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AUTHOR'S DECLARATION

I, the undersigned hereby declare that:

This research has not been previously accepted for any degree and is not being currently considered for any other degree at any other university.

I declare that this thesis contains my own work except where specifically acknowledged.

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Date.....

ETHICS DECLARATION

The Biomedical Research Ethics Committee of KwaZulu-Natal (BE320/16) granted approval for this PhD project.

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LIST OF TABLES AND FIGURES

Table 3.1 Bacterial species and antimicrobial agents investigated using the multipoint inoculation method.

Figure 3.1 96-well test plate for pilot susceptibility testing of *M. tuberculosis* with highest and lowest final nybomycin concentrations of 100 µg/ml and 0.19531 µg/ml respectively.

Table 3.2 Characteristics of antibiotics used for susceptibility testing with the multipoint inoculator.

Table 3.3 WHO recommended critical concentrations used for determining drug resistance of *M. tuberculosis* isolates.

Table 3.4 Antimicrobial drug concentrations used for MIC (µg/ml) determination in *M. tuberculosis* isolates using the multipoint inoculator.

Table 3.5 CLSI susceptibility, intermediate and resistance breakpoints (µg/ml) for ciprofloxacin and nalidixic acid.

Table 3.6 Antimicrobial drug concentrations used for MIC (µg/ml) determination in *N. gonorrhoeae* using the multipoint inoculator.

Table 3.7 Antimicrobial drug concentrations used for MIC ($\mu\text{g/ml}$) determination in *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* isolates using the multipoint inoculator.

Table 3.8 Amount (gram) of antibiotic powder required to test all required drug concentrations once with the multipoint inoculator.

Figure 3.2 96-well test plate for susceptibility testing with highest and lowest final nybomycin concentrations of 32 $\mu\text{g/ml}$ and 0.01562 $\mu\text{g/ml}$ respectively.

Table 3.9 Primers used to PCR amplify a 762 base pair region of the *gyrA* gene.

Table 3.10 Master mix components for each 50 μl reaction.

Table 3.11 PCR cycling conditions.

Table 3.12 PCR conditions for amplification of tagmented DNA.

Table 4.1 Nybomycin minimum inhibitory concentrations ($\mu\text{g/ml}$) of fluoroquinolone-susceptible and fluoroquinolone-resistant *M. tuberculosis* isolates.

Figure 4.1 IS 6110 RFLP banding patterns, IS 6110 RFLP family name designations and dendrogram results.

Table 4.2 *M. tuberculosis* drug susceptibility results obtained with the multipoint inoculation method.

Table 4.3 MIC ($\mu\text{g/ml}$) values of *M. tuberculosis* isolates for nybomycin and a deoxynybomycin derivative (DNM-2) obtained with the MTT assay.

Table 4.4 MIC ($\mu\text{g/ml}$) values of *N. gonorrhoeae* isolates for ciprofloxacin, nalidixic acid, nybomycin, quinine, chloroquine, mefloquine and primaquine with the multipoint inoculation method.

Table 4.5 MIC ($\mu\text{g/ml}$) values of *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *S. aureus* and *E. faecalis* isolates for ciprofloxacin, nalidixic acid, nybomycin, quinine, chloroquine, mefloquine and primaquine with the multipoint inoculation method.

Table 4.6 DNA sequencing results of the *gyrA* QRDR from 32 *M. tuberculosis* isolates.

Table 4.7 Molecular docking scores and molecular weight of nybomycin, DNM-2, ciprofloxacin and moxifloxacin against wild type *M. tuberculosis* gyrase.

Table 4.8 Binding free energies of nybomycin, DNM-2 and ciprofloxacin against wild type and mutated *M. tuberculosis* gyrase.

Table 4.9 Per-residue interaction energy decomposition of *M. tuberculosis* gyrase in ligand binding for residue 90 and 94.

Table 4.10 Gene variants potentially responsible for phenotypic resistance to nybomycin.

B.1 MIC ($\mu\text{g/ml}$) results for *Mycobacterium tuberculosis* with multipoint inoculator

B.2 MIC ($\mu\text{g/ml}$) results for *Neisseria gonorrhoeae* with multipoint inoculator

B.3 MIC ($\mu\text{g/ml}$) results for *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Staphylococcus aureus* with multipoint inoculator.

B.4 MIC ($\mu\text{g/ml}$) results for *Mycobacterium tuberculosis* with MTT assay.

LIST OF ABBREVIATIONS AND ACRONYMS

3D	three-dimensional
°C	degrees Celsius
ABC	ATP binding cassette
ADMET	absorption, distribution, metabolism, excretion and toxicity
ATCC	American Type Culture Collection
bp	base pairs
cDNA	complementary deoxyribonucleic acid
CADD	computer-aided drug design
CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
CO ₂	carbon dioxide
CTAB	cetyltrimethylammoniumbromide
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNM-2	deoxynycomycin derivative
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
DST	drug susceptibility testing
EDTA	ethylene-diamine-tetra-acetic acid
gDNA	genomic DNA

GC	Neisseria gonorrhoeae
HCl	hydrochloric acid
HIV	Human immunodeficiency virus
HTS	high-throughput screening
ICU	intensive care unit
IS	insertion sequence
IU	international units
KZN	KwaZulu-Natal
MDR	multidrug-resistant
MIC	minimum inhibitory concentration
MOA	mechanism of action
MOR	mechanism of resistance
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NT	not tested
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NYC	New York City
OADC	oleic acid, albumin, dextrose and catalase
OD _{600nm}	optical density at 600 nm
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PE	genes that encode proteins whose N-termini contain the characteristic motifs Pro-Glu
PPE	genes that encode proteins whose N-termini contain the characteristic motifs Pro-Pro-Glu
PPT	polypropylene tube
QRDR	quinolone resistance-determining region
QSAR	quantitative structure-activity relationship
RFLP	restriction fragment length polymorphism
SA	South Africa
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
SSC	saline-sodium citrate
TB	tuberculosis
TBE	tris-borate-EDTA
TE	tris-EDTA
UK	United Kingdom
UKZN	University of KwaZulu-Natal
V	Volts
WGS	Whole genome sequencing
WHO	World Health Organization
x g	g-force
XDR	extensively drug-resistant

LIST OF APPENDICES

APPENDIX A – MEDIA AND REAGENTS

- A.1 Middlebrook 7H9 broth
- A.2 Chocolate agar plates
- A.3 Brain heart infusion broth
- A.4 Middlebrook 7H11 agar plates
- A.5 McFarland turbidity standards 0.5 and 1.0
- A.6 Phosphate buffered saline (PBS)
- A.7 Storage media for bacterial isolates (excluding *M. tuberculosis*)
- A.8 MTT Solution
- A.9 50% DMF solution
- A.10 SDS solutions
- A.11 1:1 SDS(20%)-DMF(50%) solution
- A.12 Middlebrook 7H10 agar
- A.13 GC agar for *N. gonorrhoeae* susceptibility testing
- A.14 Mueller Hinton agar
- A.15 10 mg/ml Proteinase K
- A.16 5M NaCl solution
- A.17 10% CTAB solution
- A.18 Chloroform:Isoamyl alcohol (24:1)
- A.19 TE Buffer
- A.20 1% Agarose gel
- A.21 TBE Buffer

- A.22 Gel loading dye for gel electrophoresis
- A.23 Gel loading dye for IS 6110 RFLP
- A.24 SSC solutions
- A.25 Soak I Solution
- A.26 Soak II Solution
- A.27 Primary Wash Buffer
- A.28 Hybridization buffer

APPENDIX B – RAW DATA (MINIMUM INHIBITORY CONCENTRATIONS)

- B.1 MIC ($\mu\text{g/ml}$) results for *Mycobacterium tuberculosis* with multipoint inoculator
- B.2 MIC ($\mu\text{g/ml}$) results for *Neisseria gonorrhoeae* with multipoint inoculator
- B.3 MIC ($\mu\text{g/ml}$) results for *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Staphylococcus aureus* with multipoint inoculator
- B.4 MIC ($\mu\text{g/ml}$) results for *M. tuberculosis* with MTT assay

TABLE OF CONTENTS

SUMMARY	i
SUPERVISOR STATEMENT	iii
AUTHOR'S DECLARATION	iv
ETHICS DECLARATION	v
ACKNOWLEDGEMENTS	vi
LIST OF TABLES AND FIGURES	viii
LIST OF ABBREVIATIONS AND ACRONYMS	xii
LIST OF APPENDICES	xv
CHAPTER 1 - INTRODUCTION	1
1.1 Background and research rationale	1
1.2 Aims	2
1.3 Objectives	2
CHAPTER 2 - LITERATURE REVIEW	4
2.1 A brief history of antibiotic discovery	4
2.2 A brief history of antimicrobial resistance	15
2.3 Background to the problem of <i>Mycobacterium tuberculosis</i>	22
2.4 A brief history of antimycobacterial discovery	23
2.5 Nybomycin	27
CHAPTER 3 – METHODS	34
3.1 Drug susceptibility testing	34
3.1.1 Isolate selection	34
3.1.2 Retrieval of isolates from storage.....	37

3.1.3	Preparation of working cultures.....	38
3.1.4	Screening with the MTT assay.....	40
3.1.5	Screening with the multipoint inoculator method.....	43
3.1.6	Further drug susceptibility testing with the MTT assay	54
3.2	DNA isolation	58
3.2.1	Bacterial cultures.....	58
3.2.2	DNA isolation with the cetyltrimethylammoniumbromide method	58
3.2.3	Estimation of DNA purity, concentration and quality	60
3.3	Genotyping using IS 6110 restriction fragment length polymorphism fingerprinting.....	62
3.3.1	Restriction	63
3.3.2	Gel electrophoresis	63
3.3.3	Southern blotting	64
3.3.4	Hybridization	65
3.3.5	Washing of membrane	66
3.3.6	Detection of banding patterns	66
3.3.7	Reading and interpretation of results	67
3.4	<i>gyrA</i> investigations	67
3.4.1	Polymerase chain reaction.....	68
3.4.2	Gel electrophoresis of PCR product.....	70
3.4.3	Gene sequencing, reading and interpretation of results	70
3.5	Computational investigations.....	71
3.5.1	Molecular docking investigations	71
3.5.2	Molecular dynamics simulations	72

3.6 Selection and whole genome sequencing of <i>M. tuberculosis</i> mutants with increased nybomycin MICs	74
3.6.1 Selection of <i>M. tuberculosis</i> mutants with increased nybomycin MICs	74
3.6.2 Whole genome sequencing of <i>M. tuberculosis</i> mutants with increased nybomycin MICs	79
3.6.3 Bioinformatics analysis of whole genome sequencing results	85
CHAPTER 4 – RESULTS	87
4.1 Introduction	87
4.2 <i>In vitro</i> inhibitory effect of quinolones on various bacterial species	87
4.2.1 <i>M. tuberculosis</i>	88
4.2.2 <i>Neisseria gonorrhoeae</i> , <i>Escherichia coli</i> , <i>Klebsiella pneumoniae</i> , <i>Enterobacter cloacae</i> , <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter baumannii</i> , <i>Enterococcus faecalis</i> and <i>Staphylococcus aureus</i>	94
4.3 <i>In silico</i> effect of nybomycin and DNM-2 on <i>M. tuberculosis</i> gyrase enzyme	98
4.3.1 <i>gyrA</i> QRDR sequencing	98
4.3.2 Molecular docking investigations	100
4.3.3 Molecular dynamics simulations	101
4.4 Investigation of <i>M. tuberculosis</i> mutants with increased nybomycin MICs using whole genome sequencing	103
CHAPTER 5 – DISCUSSION AND CONCLUSION	109
5.1 <i>In vitro</i> inhibitory effect of quinolines on various bacterial species	109
5.1.1 <i>Mycobacterium tuberculosis</i>	109
5.1.2 <i>Neisseria gonorrhoeae</i>	112

5.1.3	Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus and Enterococcus faecalis	113
5.2	<i>In silico</i> effect of nybomycin and DNM-2 on <i>M. tuberculosis</i> gyrase enzyme.....	115
5.3	Investigation of <i>M. tuberculosis</i> mutants with increased nybomycin MICs using whole genome sequencing.....	120
5.4	Conclusion	131
REFERENCES		134
APPENDIX A – MEDIA AND REAGENTS		166
APPENDIX B – RAW DATA (MINIMUM INHIBITORY CONCENTRATIONS)...		178

CHAPTER 1 - INTRODUCTION

1.1 Background and research rationale

Mycobacterium tuberculosis has been destroying the lives of individuals and communities for millennia, its devastation unparalleled by any other microbe in known history. It has been advancing largely unabated despite humankind's best efforts to prevent, control and treat it. Notwithstanding some victories in richer countries over the previous millennium, disease due to *M. tuberculosis* escalated in poorer countries against a backdrop of emerging drug resistance and large portions of human populations co-infected with HIV. The development of new and effective antimicrobial remedies for treatment is of obvious importance, but novel antibiotics have not been very forthcoming since the "golden age" of antibiotic discovery more than 60 years ago. Drug discovery and development is a complicated and expensive enterprise, especially for antimicrobials. Researchers are increasingly beginning to pursue alternative avenues to add to our anti-tuberculosis armamentarium, including the re-evaluation of previously discovered but "forgotten" compounds. Nybomycin is such a compound that recently sparked the imagination of researchers when it was baptized the first "reverse antibiotic" because of its inhibitory *in vitro* effect on fluoroquinolone-resistant *Staphylococcus aureus* and *Enterococcus faecalis*, despite very poor activity against fluoroquinolone-susceptible isolates. It further exhibited some *in vitro* activity against drug-susceptible *M. tuberculosis*, thereby begging further investigation to determine its potential activity and mechanism of action against drug-resistant bacteria such as fluoroquinolone-resistant *M. tuberculosis*.

1.2 Aims

Aim 1

To investigate the *in vitro* inhibitory effect of nybomycin on common fluoroquinolone-resistant human bacterial pathogens, including *M. tuberculosis*, and to compare this with its effect on fluoroquinolone-susceptible isolates.

Aim 2

To examine the mechanism of action of nybomycin against *M. tuberculosis*.

Aim 3

To examine the mechanism of resistance *M. tuberculosis* against nybomycin.

1.3 Objectives

Objective 1

Determine and compare the *in vitro* minimum inhibitory concentrations of nybomycin in fluoroquinolone-resistant and fluoroquinolone-susceptible human bacterial pathogens, including *M. tuberculosis*.

Objective 2

Predict the mechanism of action of nybomycin on *M. tuberculosis* gyrase enzyme *in silico* with molecular docking investigations and molecular dynamics simulations.

Objective 3

Isolate *M. tuberculosis* mutants with increased nybomycin minimum inhibitory concentrations ($\mu\text{g/ml}$) and identify potential mechanism/s of resistance of *M. tuberculosis* to nybomycin through comparative whole genome sequencing and analysis.

CHAPTER 2 - LITERATURE REVIEW

2.1 A brief history of antibiotic discovery

In 1929 the bacteriologist Alexander Fleming reported his fortuitous discovery of penicillin¹. As there was no drug discovery platform available to systematically investigate drug leads, Fleming resorted to sending samples of his *Penicillium* to his collaborators and anyone who requested for it^{2,3}. Unfortunately, they all failed to isolate penicillin from the fungal cultures and interest in penicillin slowly waned^{2,3}. Ten years later in 1939, the pathologist Howard Walter Florey and biochemists Ernst Boris Chain and Norman Heatley secured funding to isolate penicillin and examine its biological characteristics^{2,3}. Within one year they managed to isolate pure penicillin from an extract of the *Penicillium* mold and used it to successfully conduct animal experiments²⁻⁴. In 1941, Albert Alexander became the first person to be treated with penicillin and by 1945, penicillin was made available to the public^{2,3,5}.

The drug discovery and development process surrounding penicillin took more than 10 years partly due to the absence of a methodical approach^{2,3}. Soil microbiologists Albert Schatz and Selman Waksman introduced the first reliable drug discovery platform with their discovery of streptomycin in 1944⁶⁻⁸. Pharmaceutical companies eagerly adopted this platform whereby they could systematically screen soil samples and fungal extracts for antimicrobials, thereby heralding the “Golden Age” of antibiotic discovery⁸⁻¹². Nearly all the antibiotic

classes in use today were discovered with the Waksman platform from the 1940s to the 1960s^{11,13–15}.

The Waksman platform is a simple and effective low-throughput screening system: soil samples are first cultured and screened for potential antimicrobial producing organisms such as *Actinomycetes*. Potential antibiotic-producing isolates are then inoculated into shake flasks and cultured using various combinations of culture media, temperatures and agitation speeds, to induce the production of antimicrobial substances. Next, possible antimicrobial substances are extracted from the cultured broths and deposited on filter paper disks that are thereafter placed onto agar plates with a suitable broth as the nutrient base, freshly seeded with a susceptible indicator organism. After overnight incubation, if the cultured broth contains any substance capable of killing the susceptible indicator microorganism, then there will be a growth-free area *i.e.* a zone around the paper disk where the indicator microorganism failed to grow. Purified products produced by *Actinomycetes* and other microbial species can then be further investigated for antimicrobial action first using *in vitro* and then with *in vivo* methods^{7,8,16–18}.

The first three decades of antimicrobial discovery mostly followed a paradigm of: (1.) phenotypic screening, (2.) isolation and structural characterization of the compound, (3.) establishing the mode of action, (4.) animal and human clinical trials, and finally (5.) introduction into the market⁸. The methodologies pioneered by Waksman *et al* to isolate organisms from the environment, to grow them in liquid culture and to extract and purify antimicrobials from these cultures have not changed significantly for many years. However, major improvements made with

nuclear magnetic resonance spectroscopy and mass-spectrometry translated into faster and easier compound structure determination and analysis. These technologies proved to be very useful at identifying already known substances and thereby avoiding expensive and time consuming re-investigation of previously discovered compounds^{7,8,16-18}.

The “Golden Age” of antibiotic discovery that took off at the end of the second world war lasted only little more than two decades^{6,9,10}. By the 1970s all the “low-hanging fruit” of easily discoverable natural products have been picked and antimicrobials were increasingly being re-discovered using the Waksman platform, making it an unattractive and costly endeavor for many pharmaceutical companies^{6,9,11,19}.

From the 1970s onwards, significant technological progress enabled investigators to (1.) determine the mechanism of action (MOA) of newly isolated compounds much faster, (2.) develop synthetic compounds and (3.) produce semi-synthetic analogues of existing compounds⁸. The preferred approach of pharmaceutical companies therefore soon shifted from the Waksman platform to medicinal chemistry methodologies whereby existing antibiotics and antibiotic scaffolds were being modified and improved through various means in an effort to create analogue compounds and derivatives with increased antimicrobial activity and fewer clinical side-effects^{8,14,15}. This approach yielded many new but similar compounds with often only marginal improvements compared to the original antimicrobials^{6,11,14,15}. The ensuing steady stream of analogues and derivatives of

previously discovered antimicrobial classes, that started in the 1970s, became a trickle by the late 1980s^{6,11,14,15}.

In the aftermath of the “Golden Age” of antimicrobial discovery, scientists also gradually began to employ a “reversed” approach to drug discovery by first identifying drug targets and then using various methods to devise a successful synthetic compound against the identified target¹². An ideal drug target is normally an enzyme that forms part of a critical or essential function in the microbe¹². These microbial enzymes should be absent or significantly different from those found in human cells, in order to avoid unwanted side-effects¹². Thus target-based screening was born⁸.

With the older drug-based approaches, such as the Waksman platform, the MOA is only determined once a compound has been identified to exert an inhibitory effect on the target organism⁸. Target-based screening on the other hand, is based on either a previously or a newly described or assumed drug target⁸. Potential drug targets *i.e.* essential and conserved microbial proteins are identified through techniques offered by cumulative technological advances in the fields of genetics, genomics, proteomics and biochemistry^{8,11,12}. A number of reviews have been published delineating different target-based drug screening approaches^{20–22}.

Rational drug design, a multi-disciplinary approach whereby several disciplines work together to design drug targets, slowly started to develop and mature over the ensuing decades²³. In order to find hit molecules, investigators can follow either one or both of the following two routes: (1.) design small molecules that will

bind to and inhibit the target molecule, or (2.) screen collections of chemicals, or partially purified extracts of natural products, with the help of biochemical assays that identify target inhibition^{8,12}. Once a positive interaction has been identified between the target and a test compound, then the test compound is further studied using *in vitro* and thereafter *in vivo* investigations^{8,11,12}.

The 1990s saw the advent of sophisticated genomics and a maturing field of biochemistry⁸. The development of combinatorial chemistry provided investigators with thousands of unique molecules derived from hundreds of chemical scaffolds⁸. Robotics together with various chemical assays enabled pharmaceutical companies to embark on high-throughput screening (HTS) programs pairing enormous libraries of synthetic chemicals against a growing number of potential drug targets delivered by genomic investigations^{8,11,12}. The much slower low-throughput screening for identifying useful natural compounds was therefore abandoned by almost all pharmaceutical companies in the period between the mid-1990s and mid-2000s⁸. This time period of roughly 10 years saw more compounds being screened than in the preceding 60 years put together⁸. The high-throughput biochemical assay-based platform proved very successful in identifying novel targets and in producing structurally optimized inhibitor molecules^{8,11}. Unfortunately, out of a number of hits only a few useful leads followed, but no broad-spectrum antibiotics reached the market using this approach^{11,12}. Bedaquiline, a narrow-spectrum antimicrobial discovered in 1997 and only effective against *Mycobacterium tuberculosis*, was as of 2017 the only antimicrobial with a truly novel mechanism of action, discovered through the HTS approach that has reached the market^{11,12}. The general failure of the HTS platform

is commonly ascribed to the fact that compounds identified this way were usually unsuccessful in adequately penetrating bacterial cell walls or cell membranes and therefore did not reach high enough concentrations at their intended target site^{11,12}. In recent years drug discovery efforts have thus started to move back to whole-cell screening, but now it is coupled with a strong emphasis on high target specificity¹². Delamanid is a good example of where HTS coupled with rational drug design lead to a drug that successfully reached the market²⁴. This narrow-spectrum drug is only active against *M. tuberculosis*²⁴. However, delamanid is a dihydro-nitroimidazooxazole derivative, related to metronidazole and is therefore not considered to have a completely novel mechanism of action^{25,26}. Bedaquiline (2012) and delamanid (2014) are the only new drugs that have been specifically developed against *M. tuberculosis* in almost 70 years that have reached the market²⁷.

Since the 1980s there was a steady decline in the approval of new antibiotics by the United States Food and Drug Administration¹³. Excluding drugs against *M. tuberculosis*, the last 20 years saw only two novel classes of systemic antibiotics reach the market, the oxazolidinones (linezolid in 2000) and cyclic lipopeptides (daptomycin in 2003)^{13,28}. Both drugs are only useful against Gram-positive bacteria³⁰. For Gram-negative bacteria, there have been no novel antibiotic classes discovered since nalidixic acid and the fluoroquinolones in the 1960s²⁸.

Advances in genomics and the dire global need for novel antibiotics led to the re-evaluation of the “old-fashioned” whole-cell or drug-based approach to the

screening of natural compounds. Investigation of the *Streptomyces* genome in the early 2000s revealed surprisingly large numbers of gene clusters coding for secondary metabolites not previously found to be expressed during laboratory culture methods⁸. It was subsequently estimated that less than 10% of these genes are expressed in sufficient amounts using the current culture conditions applied in drug screening programs⁸. Investigators are now pursuing various new culture and genetic manipulations to entice the organisms to reveal their “hidden” metabolites⁸. Natural product screening and drug-based antimicrobial design recently gained attention through a new innovation, called the iChip^{14,31}. Historically 99% of all microbial species on our planet could not be cultured using current methodologies^{11,32}. This greatly limits the number of bacteria that can be investigated for antibiotic production^{14,31,32}. Some of these previously “non-culturable” bacteria have recently been grown inside diffusion chambers that were incubated in the natural environment of soil bacteria^{11,14,31,32}. This approach has been improved and resulted in the development of the iChip, a device that allows for high-throughput cultivation of several bacterial species at the same time^{14,31,32}. Teixobactin was the first novel natural-product antibiotic in nearly thirty years. It was discovered in 2015 using the iChip method and has subsequently shown great promise against *S. aureus* and *M. tuberculosis*³²⁻³⁴.

Although not very successful, drug modifications and the screening of synthetic compound libraries remained the principal platforms for discovering new antibiotics for many years^{6,11,14}. Fortunately, scientific and technological advances during the last 20 years provided researchers with a greatly enhanced toolbox with which to approach antimicrobial discovery^{14,35,36}. Alongside the return of compound-based

screening methodologies and HTS, there also evolved a myriad of new and non-traditional methodologies and screening systems. These methodologies have been employed with varying degrees of success^{9,14,15}. This includes (1.) discovery of novel cellular functions, (2.) targeting of critical survival functions *in vivo*, (3.) targeting of different physiological states of bacteria, (4.) regulation of host cell functions, and (5.) investigations to identify effective drug combinations³⁶.

The role of computational methods in antimicrobial design and discovery has been increasing over the last three decades, and various *in silico* methodologies have been developed that now form an integral part of the drug discovery and development landscape³⁶⁻³⁸. Computer-aided drug design (CADD) specifically assists with the identification of potentially effective compounds, predict their pharmacological actions and side effects, and enhances their bioavailability. It so promises to reduce both the cost of drug discovery and the time for new drugs to reach the clinic³⁹. CADD potentially plays three leading parts in the antimicrobial discovery process: (1.) virtual screening to reduce the number of compounds earmarked for *in vitro* and *in vivo* testing, (2.) evaluation and optimization of the absorption, distribution, metabolism and excretion and toxic characteristics (ADMET) of lead compounds and (3.) designing of effective derivatives and novel compounds^{39,40}.

Target identification can be either ligand-based or structure-based. Ligand-based CADD is an indirect approach that utilizes current knowledge of known active and inactive compounds. Pharmacophore modeling and quantitative structure-activity relationship (QSAR) modeling are two of the most popular ligand-based

approaches used by CADD specialists today^{39,40}. Pharmacophore modeling allows description of the crucial features of a ligand that are responsible for a particular biological interaction when interacting with another molecule³⁹. The pharmacophore model can also be used for lead optimization, virtual screening approaches and *de novo* drug design³⁹. QSAR modeling provides a mathematical association between the structural characteristics and the target response of a collection of molecules³⁹.

Structure-based CADD involves the construction and investigation of three-dimensional (3D) structures of molecules and exploits this knowledge about the target protein structure to compute the interaction energies between the protein target and test compounds³⁹. The premise of this approach is that the ability of a molecule to interact with a protein and exhibit the intended biological effect, is dependent on its potential to appropriately interact with a specific binding site on the target protein in the first place³⁹. Information about target protein structures is typically obtained from X-ray crystallography or magnetic resonance investigations³⁹. In the absence of these techniques, *in silico* methods such as homology modelling can sometimes be employed to predict the target's 3D structure³⁹. Molecular docking simulations that have been in use since the 1980s, as well as *de novo* ligand design are two widely used structure-based CADD approaches^{39,40}.

Molecular docking is used to predict the energy interactions within ligand-receptor complexes^{39,40}. Molecular docking experiments usually involve first sampling and then the assessment of the binding affinity using a predetermined scoring

system^{39,40}. During the sampling stage, the ligand conformation, pose, position and orientation are predicted^{39,40}. This process generates a large variety of target-ligand complex conformations and orientations that may include many inappropriate poses and inactive molecules^{39,40}. To make sense of the data, a predetermined scoring system is used to estimate and rank the target-ligand complex conformations and this can be done according to empirical scoring functions, knowledge-based scoring functions, consensus scoring functions and molecular mechanics based functions, also called force-field based functions^{39,40}. Molecular docking can be done blindly i.e. without knowledge of the potential binding sites, but knowing beforehand where the potential binding site might be markedly improves docking efficiency⁴⁰. For blind docking, potential binding sites may be identified with the help of cavity detection software or online servers such as PASS, Metapocket and POOL⁴¹⁻⁴³.

Molecular dynamics simulations are used to gain insight into the potential conformational changes and fluctuations during ligand-target protein interactions; as well as to investigate the adaptability and variability potentials of the target protein^{39,40}. Commonly used software packages for molecular dynamics simulation investigations include AMBER, NAMD and GROMACS^{39,43}. Molecular dynamics simulations performed on high-speed supercomputers are usually completed within a few nanoseconds to microseconds³⁹.

Computational techniques are also increasingly being used to predict a pharmacokinetic i.e. ADMET profile of potential compounds and therefore provide

a useful early screening tool³⁹. The *in silico* obtained ADMET profiles can also be used to optimize test compounds before further investigations are executed³⁹.

Several drug discovery approaches have been attempted through the years, with varying degrees of success^{6,10,27,35,44–52}. Some recent non-traditional approaches include:

Human microbiome. The human microbiome is investigated for commensal antibiotic-producing organisms that help one commensal to compete with other commensals^{14,53}. An example of where this bared fruit was when Zipperer *et al* discovered that *Staphylococcus lugdunensis* produces a potent antibiotic compound called lugdunin, that is active against *Staphylococcus aureus*^{14,53}. Both these organisms are commensals of the anterior nares of humans^{14,53}.

Bacteriocins. Bacteriocins are peptides produced by bacteria that are active against other bacteria but not against its producer¹⁴. Theoretically, bacteriocins could help wild type bacteria replace its drug-resistant counterparts without negatively affecting other commensals¹⁴. To date, very little *in vivo* data have been published that successfully demonstrate this effect¹⁴.

Phages and phage components. Bacteriophages and phage lysins are interesting avenues that have been explored in recent years, but published evidence is still sparse and unconvincing^{14,54,55}. Tailocins are tail structures from defective phages that display antimicrobial properties^{14,56}. Gebhart *et al* isolated tailocins from *Clostridium difficile* isolates by inducing the bacterium's SOS response^{57,58}.

These tailocins were modified by Kirk *et al* and produced promising results against *Clostridium difficile* in mice^{14,59}.

Bacteriovores. A fascinating concept that has lately received attention is that of bacteriovores¹⁴. Bacteriovores such as *Bdellovibrio bacterivorus* and *Micavibrio aeruginasavorus* consume other bacteria and have shown some encouraging results both with *in vitro* investigations and with *in vivo* investigations using animal models^{60–64}.

Despite major scientific advances and a plethora of new technologies at our disposal, the majority of antibiotics in use today have been derived from natural products and target the bacterial cell wall, DNA or ribosomes^{8,15}. The role of natural products as a source of novel antimicrobials cannot be overstated.

2.2 A brief history of antimicrobial resistance

With the start of the “Golden Age” of antibiotic discovery, humanity finally had at its disposal real weapons with which to address the scourge of microbial infections^{11,29}. It is believed that antibiotics have increased average human life expectancy by 10 to 20 years²⁹. Unfortunately, our microbial adversaries very soon started to respond to our new wonder weapons with a myriad of clever resistance mechanisms^{11,29}. The discovery and development of new antibiotics have not been keeping track with the rate at which drug-resistant bacteria advance⁶.

As microbes replicate they naturally produce numerous small subpopulations with various combinations of genomic mutations. If the microbial population is exposed to any external threat such as an antibiotic, there may very well be a subpopulation of microbes that is already resistant to this new threat. The antibiotic may therefore kill off the susceptible microbes, but not the drug-resistant subpopulation. This drug-resistant subpopulation consequently gets “selected” by the antibiotic and can continue to proliferate and spread. The resistance-conferring genes are then vertically passed on to the next generation of microbial offspring. More frighteningly is the fact that such resistance mechanisms can spread horizontally between members of the same species, and even to other species. This is accomplished through various mobile genetic elements that transfer resistance genes between microbes by mechanisms known as transformation, conjugation and transduction⁶⁵⁻⁶⁷. Apart from the mechanism whereby use of antimicrobials select drug-resistant subpopulations, there is also emerging evidence suggesting a more direct correlation between antimicrobial use and resistance^{68,69}. One possible mechanism is the induction of mutations by sub-inhibitory concentrations of antimicrobials^{68,69}.

The resistance-acquiring capabilities and activities of microbes are part of their natural survival living processes and have been at work long before the introduction of antimicrobials in modern medicine^{6,69-71}. It is therefore not surprising that resistance was reported to sulfonamides within two years after humans started administering these compounds to patients. These sulfonamides were the first effective antimicrobials that reached the market in 1937^{69,72}. This pattern of the introduction of a new antimicrobial followed by microbial resistance

to the antimicrobial soon thereafter has been repeated ever since, without any exceptions^{15,69,70,73}. In fact, many antibiotics that have reached the market, did so with the scientific community's knowledge that bacteria already displayed natural resistance to it¹¹.

Infections due to antimicrobial resistant pathogens have a significant clinical and economic impact that is of growing global concern^{13,74}. High mortality rates have been reported from hospitals across the globe for infections caused by antibiotic resistant bacteria, especially in intensive care units (ICU)⁷⁴.

In the United States alone, the Centers for Disease Control and Prevention estimated in 2013 that two million individuals contract infections due to drug-resistant bacteria every year, 23 000 of which are fatal^{13,69}. The estimated annual direct and indirect cost to the country was estimated at \$55 billion^{13,69}. In the European Union, antimicrobial resistance is held responsible for the death of approximately 25 000 humans and an annual monetary loss of approximately €1.5 billion⁷⁴⁻⁷⁶. This is excluding the indirect costs related to antimicrobial resistance. For developing countries, good quality data on the clinical and economic impact of antimicrobial resistance are lacking but the situation is likely at least similar if not worse than for developed countries^{28,69,74,77}.

Current estimates for annual global deaths attributable to antimicrobial resistance sits at approximately 700 000 individuals and is predicted to increase to 10 million individuals annually by 2050, surpassing the number of deaths due to cancer.

The collective cost that antimicrobial resistance will pose to the world economy is estimated to be \$100 trillion per year^{13,19,29,77}.

Enterococcus spp, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp* are collectively represented by the widely used acronym ESKAPE and are globally recognized as the most common antimicrobial-resistant bacterial species associated with infections that carry a higher risk of mortality^{13,74}. Multidrug-resistant (MDR) ESKAPE organisms are associated with an increased length of hospital stay and higher health care costs compared to infections with their drug-susceptible counterparts^{13,74}. In 2017 the World Health Organization (WHO) went further and published a list of the priority antimicrobial resistant pathogens requiring intensive drug discovery research efforts to combat infections due to these organisms^{28,78}. Addressing *M. tuberculosis* separately, other organisms were grouped into different prioritized categories^{28,78}. The “critical priority” category consisted of *A. baumannii* (carbapenem-resistant), *P. aeruginosa* (carbapenem-resistant) and Enterobacteriaceae (carbapenem-resistant and/or third generation cephalosporin-resistant)^{28,78}. Almost one third of hospital acquired infections in acute care settings, and over 40% in ICUs are caused by these “critical priority” pathogens⁷⁹. A previous report by the WHO stated that infections due to antimicrobial resistant *K. pneumoniae* (third generation cephalosporin-resistant and carbapenem-resistant) and *Escherichia coli* (third generation cephalosporin-resistant, including extended spectrum beta-lactamase positive and fluoroquinolone-resistant) lead to a significant increase in the 30-day as well as all-cause mortality of hospitalized patients⁸⁰. The WHO’s “high priority category” was made up by *E. faecium*

(vancomycin-resistant), *S. aureus* (vancomycin-resistant and/or methicillin-resistant), *Helicobacter pylori* (clarithromycin-resistant), *Campylobacter spp* (fluoroquinolone-resistant), *Salmonella spp* (fluoroquinolone-resistant) and *Neisseria gonorrhoeae* (third generation cephalosporin-resistant and/or fluoroquinolone-resistant)^{28,78}. Drug-resistant *S. aureus* and *E. faecium* are the two most important Gram-positive bacteria on the WHO list; and although there are significantly more drugs available to treat infections caused by them, as well as compounds in the drug-development pipeline compared to the Gram-negative bacteria, they are still considered highly problematic^{28,78}. Infections due to methicillin-resistant *S. aureus* compared to methicillin-susceptible *S. aureus* are responsible for substantial increases in all-cause mortality, *S. aureus* attributable mortality, ICU mortality, septic shock and length of hospital stay^{69,81}. Enterococci resistant to vancomycin ranks as the second most common cause of nosocomial infections in the United States, but data from developing countries and particularly Africa is limited⁸²⁻⁸⁵. Drug-resistant *M. tuberculosis* is also a global threat which will be discussed in section 2.3.

Several generic principles have been proposed over the years to curb the problem of antimicrobial resistance and maximize the lifespan usefulness of the currently available antimicrobials, for example optimal diagnosis, prescription and administration practices, avoidance of use of antimicrobials in agriculture, development of new and novel antimicrobials, quality assurance of antimicrobials and enhanced surveillance systems⁶⁷. Programs and interventions employing these principles are generally referred to as antimicrobial stewardship practices and are encountered in diverse contexts such as human health, animal health and

the environment⁸⁶. Faced with divergent definitions of antimicrobial stewardship, Dyar *et al* recently proposed the following simple definition: “A coherent set of actions which promote using antimicrobials responsibly”⁸⁶. In the context of human healthcare, the generic principles mentioned above have been expounded upon in detailed guidelines and academic publications by prominent advisory bodies such as the Infectious Diseases Society of America (IDSA), the Society of Healthcare Epidemiology of America (SHEA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID)^{67,86–90}. In their updated antimicrobial stewardship guidelines of 2016, IDSA identified “preauthorization of antimicrobial prescriptions and/or prospective audit and feedback” as the backbone of any healthcare antimicrobial stewardship program⁸⁹. The only other interventions that received strong recommendations in this guideline were: (1.) interventions that promote the appropriate use of oral antibiotics for initial therapy as well as switching from intra-venous to oral therapy, (2.) use of antimicrobials for the shortest effective time possible, (3.) pharmacokinetics monitoring and adjustment of aminoglycoside therapy and (4.) avoidance of antimicrobials with a high risk for *Clostridium difficile* infections⁸⁹.

Antimicrobial resistance has been rightfully identified as a serious threat to public health as early as the 1990s^{91,92}. During the G8 Summit of 2013, antimicrobial resistance was further described as the “major health security challenge of the 21st century” that demands intensive global collaboration³⁴. Despite these and many other warnings, our predicament just keep getting worse due to the “selective pressures” effected by our misuse and overuse of antimicrobials^{34,69}. The world may soon have to face a “post-antibiotic” era due to our inability to change the

trajectory of our actions^{15,34,93}. The WHO Director, Margaret Chan ominously stated in 2012 that the post-antibiotic era may very well mean an end to modern medicine as we know it^{34,94}. In this tug-of-war between humans and microbes it currently seems like the microbes will have the final say and that the prophesized dawn of the “post-antibiotic” era is upon us^{11,29,95}.

The discovery of new antibiotics is therefore of obvious importance to the continued availability of drugs effective against the rising wave of drug-resistant bacteria¹⁴. Unfortunately, discovering and developing new antibiotics is not seen as an economically lucrative use of scarce resources by pharmaceutical companies, especially when natural product screening is involved¹⁴. The discovery and development of new antibiotics is a complex, expensive and time-consuming process¹². Newly introduced antibiotics are usually subjected to restrictive regulatory constraints resulting in limited use and therefore low sale profits¹⁴. Discouragingly the development of a new drug, from concept to market, currently takes up to 15 years and is estimated to cost more than \$1 billion or even \$2.5 billion^{9,12,29}. Adding to this economic hurdle the numerous regulatory and scientific hindrances, and it becomes clear why 10 of the 15 largest pharmaceutical companies have since the turn of the century abandoned or drastically reduced their antibiotic research efforts^{28,34,96}. However, the recent discovery that *Actinomycetes* may be able to produce a much larger number of active compounds than previously believed, has now opened up again the avenue for natural product discovery and a focus on natural product discovery is strongly encouraged⁹⁷.

Another serious consideration is the re-investigation of natural compounds that were previously discovered, but that were not considered high-quality leads at the time and consequently never reached the market. Three good examples of this are fidaxomicin, linezolid and streptogramin B, discovered in 1948, 1955 and 1963; that respectively only reached the market much later in 1998, 2000 and 2011¹¹.

2.3 Background to the problem of *Mycobacterium tuberculosis*

M. tuberculosis is a causative agent of tuberculosis (TB), a leading cause of mortality worldwide, especially when associated with HIV⁹⁸. During 2017 approximately 10 million individuals were infected with *M. tuberculosis* and 1.6 million succumbed to this infection⁹⁸. *M. tuberculosis* and HIV act synergistically and is particularly lethal when combined with antimicrobial resistance^{99,100}. MDR TB is defined as TB with *in vitro* resistance to first-line drugs isoniazid and rifampicin¹⁰¹⁻¹⁰³. MDR TB accounted for roughly 510 000 cases and 230 000 deaths during 2017⁹⁸. Extensively drug-resistant (XDR) TB is MDR TB with *M. tuberculosis* with additional *in vitro* resistance to fluoroquinolones and at least one of the second-line injectable agents¹⁰¹⁻¹⁰³. Roughly 8.5% of individuals with resistance to isoniazid and rifampicin have XDR TB⁹⁸. With a worldwide mortality of at least 30% and a South African mortality rate of 47% in 2012, XDR TB presents a serious threat to public health, especially in populations with a high prevalence of HIV^{98,102}. New treatment options are urgently needed to address the growing problem of drug-resistant tuberculosis.

2.4 A brief history of antimycobacterial discovery

The first antibiotic found to be effective against *M. tuberculosis* was streptomycin, discovered by Albert Schatz, Elizabeth Bugie and Selman Waksman in 1943 from the screening of soil samples containing *Actinomyces* bacteria^{7,104}. This was soon followed by para-aminosalicylic acid (1946)¹⁰⁵, isoniazid (1951)¹⁰⁴, pyrazinamide (1952)¹⁰⁶, cycloserine (1952)^{107–109}, ethionamide (1956)^{110,111}, kanamycin (1957)^{104,112}, the rifamycins (1957)^{104,113–115}, capreomycin (1960)^{104,116} and ethambutol (1961)^{104,117} by various different investigators. These drugs are still in use today and many of them continue to form the backbone of tuberculosis treatment programs worldwide^{98,101}. After the initial flurry of drug discoveries and the subsequent treatment successes, new drug discoveries effectively came to a standstill^{6,104}. Unfortunately, during the 1980's and 1990's, drug-resistant *M. tuberculosis* started to raise its ugly head and authorities realized that our treatment arsenal needs to be expanded^{104,118}. To this end several known drugs were reinvestigated, with varying degrees of success, for potential use as antimycobacterial agents, including the rifamycins, fluoroquinolones, linezolid and clofazimine¹⁰⁴. To date, the only new drugs developed specifically against drug-resistant *M. tuberculosis* that reached the market since the 1960's were bedaquiline and delamanid^{50,78,104}. Disappointingly, limited access and drug resistance have already undermined the expected success of both drugs^{24,27,50,119}. Towards the end of 2017 the WHO reported on the current drug development pipeline, including drugs against *M. tuberculosis*^{28,78}. Only one drug is currently in phase three clinical trials (pretomanid), two in phase two trials (delpazolid, SQ-109) and four in phase one trials (GSK-3036656, Q-203, PBTZ-169 and

OPC-167832)^{28,78}. Two of these drugs, pretomanid and delamanid are closely related to currently available drugs, and cross-resistance may therefore markedly reduce their anticipated usefulness^{28,78}. Despite the progress made since the early 2000s, when no new drugs were in clinical development, the global plea for novel, safe and effective antimycobacterials remains as urgent as ever^{27,28,46,78,120}.

Strategies for the screening and discovery of new antimycobacterial drugs are generally classified as either “target-to-drug” (or “target-based”) or “drug-to-target” (or cell-based or phenotypic) approaches, but oftentimes investigators make use of a combination of these two strategies^{48,52}.

Drug-to-target approach. The starting point of this approach is typically the *in vitro* exposure of live cultures of *M. tuberculosis* to various concentrations of potentially active compounds usually obtained from specific compound collections or libraries^{11,35,48}. HTS approaches are used to screen large numbers of compounds without necessarily having knowledge about its molecular structure or what the molecular target might be^{11,35,48}. *In vitro* bactericidal activity usually implies that the compound can cross the bacterial cell wall and reach a sufficient concentration to exert a killing effect^{11,35,48}. Once this effect has been evaluated, then the MOA should be determined^{11,48}. A whole plethora of methodologies are available making use of genomics, transcriptomics, proteomics, metabolomics, live-cell imaging and electron microscopy^{11,48,121,122}. It is important to identify the MOA and the drug target to enable researchers to improve the primary compound, for example improving its bactericidal capability or water solubility^{11,35,48,123}. Deciphering the MOA can however be a very challenging endeavour^{35,48}.

Whole genome sequencing (WGS) plays a central role in elucidating the drug target: drug-resistant mutants are selected by exposing wild type *M. tuberculosis* to various concentrations of the testing compound^{124–129}. By comparing the genome of the wild type and the mutant with each other, the mechanism of resistance (MOR) may be elucidated^{126,130–132}. This knowledge is then used to extrapolate the MOA through recombineering and complementation techniques^{48,124,126,133}. A common approach to elucidate the MOA is to “knock-out” the implicated gene in a wild type organism and then compare its *in vitro* susceptibility to the testing compound, with that of the wild type organism^{125,132,134,135}. Unfortunately there may be a mismatch between the MOR and the MOA and additional investigations are usually required⁴⁸. Furthermore, many antimycobacterial drugs function as prodrugs and comparative genomics may rather identify mutations in genes coding for non-essential enzymes responsible for activating the prodrug instead of the genes coding for the essential target⁴⁸.

Advantageously, the drug-to-target approach lends itself to the early discovery of additional target and pathways, as well as the possibility of finding valuable prodrugs⁵². Initial screening sometimes includes experiments where *M. tuberculosis* is cultured and exposed to the investigating compound inside macrophages or lung fibroblast cells^{48,134,136}. This provides further evidence that the compound might have *in vivo* effectivity^{48,134,136}. Together with positive *in vitro* screening outcomes and identification of the drug target, it is usually necessary to determine the cytotoxic and apoptotic potential of the testing compound^{48,134,137}.

Target-to-drug approach. Investigators exploit our growing body of scientific knowledge, especially in the fields of chemistry and molecular biology, to identify potential drug targets^{11,35,48}. These hypotheses are then used to engineer large libraries of compounds that may act on these potential drug targets^{11,35,48}. Several HTS methods can then be utilized to screen such compound libraries to identify possible hits for further investigation^{11,35,48}. Target-based HTS has become widespread for *M. tuberculosis* since the early 2000s, but has had limited success when used alone^{48,52}. Probable reasons for its disappointing performance include: (1.) deficient information on target susceptibility, (2.) poor drug penetration into microbe or surrounding tissue, (3.) drug metabolism problems and (4.) efflux of drugs out of microbes⁴⁸. Target-based hits must therefore always be followed up with selected whole-cell phenotypic investigations before *in vivo* testing can be pursued^{10,11,48,105}.

The drug-to-target approach is considered by some as more successful than the target-to-drug approach with both bedaquiline and delamanid having been discovered this way^{48,138}. However, this approach has several limitations: (1.) it is often very difficult to unravel the molecular target of the compound, (2.) selection and genomic investigation of phenotypic drug-resistant mutants in order to describe the MOR often does not help to identify the MOA, (3.) relatively high rate of discovering pro-dugs and (4.) many compounds end up being highly promiscuous in their target binding^{48,139–141}.

In the overlap between the drug-to-target and target-to-drug approaches, several disciplines have made integral contributions in recent years and include amongst

others: (1.) comparative genomics to identify genes that correspond with phenotypic resistance, (2.) structural genomics to generate 3D structures of gene products that inform our understanding of protein folding, binding and functioning, (3.) protein crystallization and X-ray data provides 3D structures of important proteins that can be used for *in silico* screening, molecular docking investigations and to expand our knowledge of binding-site interactions and specifically ligand-receptor binding^{51,123,142}.

2.5 Nybomycin

Nybomycin is a naturally occurring compound that was discovered in the 1950s during the “Golden Age” of antibiotic discovery, but was never developed for clinical use^{143–147}. Asheshov *et al* first reported on the broad anti-phage spectrum of activity of culture liquids from a streptomycete, designated A 717, found in Missouri soil¹⁴⁴. A colorless crystalline compound was subsequently isolated from this culture liquid and mycelia and it was named nybomycin^{145,147}. On further investigation nybomycin displayed antibacterial activity as well¹⁴⁵. Strelitz *et al* described the antibacterial activity of nybomycin in a small collection of bacterial isolates using disk diffusion and broth dilution methods in parallel¹⁴⁵. The two methods were in agreement and included seven *E. coli* isolates, four *S. aureus* isolates and one isolate each of *K. pneumoniae*, *P. aeruginosa*, *Mycobacterium smegmatis*, *Staphylococcus muscae*, *Bacillus subtilis*, *B. cereus* and *B. mycoides*¹⁴⁵. Nybomycin had an observable inhibitory effect on most isolates, but noticeably had no effect on the majority of *E. coli* isolates, two out of the four *S. aureus* isolates and the one *P. aeruginosa* isolate¹⁴⁵. Susceptibility of

Gram-negative bacteria to nybomycin could not be reproduced during more recent studies^{143,148}. The same investigators also performed preliminary toxicity studies in mice and reported that the mice tolerated intra-peritoneal nybomycin doses of 250 mg/kg¹⁴⁵. In 1961, Rinehardt *et al* described the chemical structure of nybomycin as well as one of its degradation products, deoxynybomycin¹⁴⁹. A separate group of investigators isolated deoxynybomycin from *Streptomyces hyalinum* in 1970 and reported a higher bactericidal activity than with nybomycin itself¹⁵⁰. In the same year Rinehardt *et al* published their revised structure of nybomycin¹⁵¹. The synthesis of nybomycin was further described and refined during the 1970s^{152–155}. During this time nybomycin was classified as a pyridoquinoline compound^{152,156}. Interestingly, Rinehardt *et al* also synthesized anthraquinone during their early work with nybomycin^{149,157}. This compound was subsequently renamed to deoxynyboquinone and is described as a potent antineoplastic agent with a currently unknown mechanism of action^{157,158}. The quinoline ring that is found in nybomycin's structure is also found in fluoroquinolone antibiotics, certain anti-malarial drugs and some anticancer drugs^{152,156,159}. This may indicate similar mechanisms of actions shared between these drugs and a self-evident starting point for further investigations to unravel nybomycin's mechanism of action^{152,156,160}. The quinoline ring itself has been described as a promising scaffold for future anti-tuberculosis drug development^{161,162}.

More recently in 2011, nybomycin was noticed again after Chinese investigators isolated deoxynybomycin from *Pseudonocardia* SCSIO 01299, a marine actinomycete found in a deep-sea sediment collected from the South China Sea^{163,164}. Two groups from Japan also isolated nybomycin in recent years^{143,165}.

The first group while screening soil samples for antibacterial activity against *S. aureus* and the second group from *Streptomyces spp* MS44 found in marine sediment collected in 2000 at Maizuru, Japan^{143,165}.

In 2012, Hiramatsu *et al* examined the *in vitro* effect of nybomycin on *S. aureus* (including methicillin-resistant *S. aureus*) and *Enterococcus faecalis* (including vancomycin-resistant *Enterococci spp*); and found fluoroquinolone-resistant bacteria to be susceptible to nybomycin and fluoroquinolone-susceptible bacteria to be resistant to nybomycin¹⁴³. This unexpected inverse relationship was further investigated through the selection of nybomycin-resistant mutants¹⁴³. Nine independent fluoroquinolone-resistant *S. aureus* isolates were methodically exposed to nybomycin so that nybomycin resistant-mutants could be selected out^{143,166}. These nybomycin-resistant *S. aureus* mutants were found to be fluoroquinolone-susceptible and DNA sequencing confirmed that their *gyrA* genes mutated back to the original wild type^{143,166}.

Nybomycin therefore possibly has a novel mechanism of action whereby it specifically inhibits mutated DNA gyrase in *S. aureus*, but not the wild type enzyme^{143,166}. Hiramatsu *et al* coined the term “reverse antibiotic” to refer to compounds that exhibit this phenomenon and categorized nybomycin as the first antibiotic in this new class¹⁴³. Flavones such as apigenin are natural antibiotics produced by plants and is the second group of compounds to have been designated reverse antibiotics in relation to fluoroquinolones^{166–168}. It is further postulated that the alternation of an antibiotic with its specific reverse antibiotic when treating an infection, may possibly keep the offending organism susceptible

to either one of the two antimicrobials, thereby overcoming the problem of accumulating resistance and multidrug-resistant bacteria altogether^{143,166}.

Quinolone compounds, some of which are antibacterial, are naturally produced by some animals, plants and bacteria^{169,170}. It is known that certain environmental bacteria are resistant to fluoroquinolones, often due to mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene^{143,171}. For example, fluoroquinolone antibiotics cannot stably bind to the quinolone binding pocket of staphylococcal GyrA when a S84L mutation is present, and such isolates are therefore resistant to fluoroquinolones¹⁶⁶. Docking simulation studies by Hiramatsu *et al* showed that the loss of the serine residue in position 84 of GyrA of *S. aureus* does not negatively affect stable binding of nybomycin to the mutated binding pocket¹⁶⁶. It is suggested that because quinolone antibiotics have been present in nature before the introduction of synthetic fluoroquinolones, that certain bacteria started to produce nybomycin in order to compete with fluoroquinolone-resistant bacteria^{143,171}.

Fluoroquinolone resistance in most clinically significant bacteria is the result of a large variety of potential mutations in genes coding for both subunits of the gyrase enzyme, in addition to other resistance mechanisms such as efflux pumps^{160,172,173}. This is in contrast to *M. tuberculosis* where fluoroquinolone resistance is very seldom due to mutations in *gyrB* and almost exclusively confined to a small number of mutations in codons 90, 91 and 94 of *gyrA*^{160,173–175}. Globally the most commonly identified *gyrA* QRDR amino acid substitutions are that of alanine to valine in codon 90 and aspartic acid to glycine in

codon 94^{160,173,174}. Mutations in codons 90 and 94 correlate well with increased minimum inhibitory concentrations (MIC) to ciprofloxacin, ofloxacin and moxifloxacin^{173,174,176}. In the KwaZulu-Natal province of South Africa, the A90V amino acid substitution was identified as the dominant *gyrA* mutation in XDR TB isolates^{120,160,174}.

DNA is naturally in a supercoiled state that hinders DNA transcription and replication¹⁷². DNA gyrase briefly reverses this ATP-dependent supercoiling by catalyzing double-stranded DNA (dsDNA) breaks¹⁷². Fluoroquinolones disrupt bacterial growth by forming covalent, but reversible gyrase-DNA adducts, that block the resealing of these dsDNA breaks¹⁷⁷. In most bacterial species, fluoroquinolones bind via a hydrated magnesium ion bridge to the serine residue located in the second position of the helix $\alpha 4$ of the GyrA subunit of gyrase¹⁷⁷. However, for wild type *M. tuberculosis* this amino acid is alanine and corresponds to codon 90 of the QRDR of GyrA¹⁷⁵. This difference translates into a markedly naturally lower binding affinity between fluoroquinolones and *M. tuberculosis* GyrA as compared to other bacterial species¹⁷⁷. Recent investigations by Aldred and Blower *et al* emphasized this point by showing how fluoroquinolone-gyrase binding was improved when the naturally occurring alanine was replaced with a serine^{175,177}. They further described how fluoroquinolones form weaker non-anchoring bonds with Ala90 and stronger anchoring bonds with downstream Asp94, but that the alanine at codon 90 is essential for facilitating Asp94 to anchor the drug-GyrA bridge^{175,177}.

Morimoto *et al* (2013) investigated the effect of nybomycin on *E. coli*¹⁴⁸. All 14 isolates tested resistant to nybomycin, irrespective of the fluoroquinolone susceptibility status¹⁴⁸. However, all isolates had markedly improved nybomycin activity (MIC \leq 1 μ g/ml) when tested in the presence of an efflux pump inhibitor Phe-Arg beta-naphtylamide (PA β N)¹⁴⁸. This suggests that efflux pumps may be an important nybomycin resistance mechanism in *E. coli* and possibly other Gram-negative bacteria¹⁴⁸.

Arai *et al* investigated the antimicrobial effect of nybomycin on *M. smegmatis* and *M. bovis* BCG¹⁶⁵. Both organisms displayed a minimum inhibitory concentration of 1.0 μ g/ml during active (aerobic) growth as well as in hypoxia-induced dormant growth states¹⁶⁵. They further observed morphological changes similar to those found with mutations in *pknA*, *pknB*, *ftsZ* and *whmD* genes¹⁶⁵. The investigators proceeded to perform WGS on spontaneously nybomycin-resistant *M. smegmatis*, but did not identify any mutations in the *pknA*, *pknB*, *ftsZ*, *whmD*, *gyrA* and *gyrB* genes¹⁶⁵. They did however identify two amino acid level mutations: L59P in *tetR* family transcriptional repressor and A23V in glycine/D-amino acid oxidase¹⁶⁵. Nybomycin was further found to have moderately low MICs against fluoroquinolone-susceptible *M. tuberculosis*, but drug-resistant isolates were not investigated¹⁶⁵. Their results suggest that nybomycin directly binds to various areas of mycobacterial DNA rather than any one specific gene or protein¹⁶⁵. This binding of nybomycin hypothetically leads to the inhibition of DNA replication and transcription¹⁶⁵. Their proposed mechanism of action is in keeping with the proposed mechanisms of action of pyridoquinoline-related compounds used against malaria such as quinine, chloroquine, mefloquine and primaquine¹⁷⁸.

Deoxynybomycin has also been investigated in recent years^{143,166,179}. Due to the insolubility of deoxynybomycin in aqueous solutions, Parkinson and Hergenrother *et al* synthesized and evaluated a collection of deoxynybomycin derivatives¹⁷⁹. The most promising of their derivatives, designated DNM-2, produced very favorable *in vivo* results when treating sepsis in mice due to fluoroquinolone-resistant *S. aureus*¹⁷⁹. Seventy-two minutes after administration of an oral dose of 50 mg/kg, a peak serum concentration (C_{max}) of 12.8 µg/ml was recorded¹⁷⁹. DNM-2 further exhibited low minimum inhibitory concentrations (MIC) against fluoroquinolone-resistant *S. aureus* and was able to effectively clear an infection with this organism from mice¹⁷⁹. There is currently no published literature on the effect of deoxynybomycin against *M. tuberculosis*.

CHAPTER 3 – METHODS

3.1 Drug susceptibility testing

Three *Mycobacterium tuberculosis* isolates were exposed to nybomycin using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results thereof prompted the screening of a much larger collection of *M. tuberculosis* isolates, as well as other commonly isolated human bacterial pathogens, by using Clinical and Laboratory Standards Institute (CLSI) agar dilution methodology with a multipoint inoculator. The susceptibility of *Neisseria gonorrhoeae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Enterococcus faecalis* isolates to nybomycin, quinine, chloroquine, mefloquine and primaquine were determined using the same multipoint inoculation methodology. Unexpected results obtained with nybomycin and *M. tuberculosis* were confirmed with a second round of testing using the MTT assay and a subset of *M. tuberculosis* isolates. Susceptibility of *M. tuberculosis* to a deoxynybomycin derivative (DNM-2) was likewise determined using the same subset of isolates and MTT testing methodology.

3.1.1 Isolate selection

The initial screening investigation was performed using three *M. tuberculosis* isolates from the Department of Infection Prevention and Control, School of Laboratory Medicine and Medical Sciences, College of Health Sciences,

University of KwaZulu-Natal, South Africa. The susceptibility of two fluoroquinolone-susceptible and one fluoroquinolone-resistant isolate to nybomycin was investigated using the MTT assay. Results from the screening investigation prompted further investigation using a larger selection of 32 *M. tuberculosis* isolates. Seventeen *M. tuberculosis* isolates were obtained from the culture collection of the Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa. A further 14 clinical isolates were included from the culture collection of the Department of Infection Prevention and Control, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, South Africa. Routine drug susceptibility testing using the 1% proportion method and IS 6110 fingerprinting have been previously performed for all isolates. The results of these tests were taken into consideration during the isolate selection process, to assure that a diverse collection of *M. tuberculosis* isolates was studied. The final collection for testing using multipoint inoculation included 12 fluoroquinolone-susceptible and 20 fluoroquinolone-resistant *M. tuberculosis* isolates. Susceptibility of *M. tuberculosis* isolates to isoniazid, rifampicin, amikacin and ofloxacin were also determined using the same multipoint inoculation methodology. This was done in order to both confirm the validity of the method and to verify the previously determined drug resistance profiles. The antimicrobial agents tested using the multipoint inoculator are presented in table 3.1.

Table 3.1 Bacterial species and antimicrobial agents investigated using the multipoint inoculation method.

Bacterial species	Number of isolates*	Drugs investigated
<ul style="list-style-type: none"> • <i>M. tuberculosis</i> 	<ul style="list-style-type: none"> • 32 (12, 20) 	Nybomycin Isoniazid Rifampicin Amikacin Ofloxacin
<ul style="list-style-type: none"> • <i>N. gonorrhoeae</i> • <i>E. coli</i> • <i>K. pneumoniae</i> • <i>E. cloacae</i> • <i>P. aeruginosa</i> • <i>A. baumannii</i> • <i>S. aureus</i> • <i>E. faecalis</i> 	<ul style="list-style-type: none"> • 30 (7, 8, 15) • 6 (2, 2, 2) • 4 (2, 1, 1) • 4 (1, 0, 3) • 7 (2, 0, 5) • 5 (1, 1, 3) • 4 (2, 1, 1) • 4 (1, 1, 2) 	Nybomycin Ciprofloxacin Nalidixic Acid** Quinine Chloroquine Mefloquine Primaquine

Fluoroquinolone-susceptible control strains included in the above: *M. tuberculosis* H37Rv (ATCC 27294), *N. gonorrhoeae* ATCC 49226 and *E. coli* ATCC 25922.

*The total number of isolates tested is followed by a breakdown according to fluoroquinolone resistance: (susceptible, resistant) or (susceptible, intermediate, resistant).

** Susceptibility of *S. aureus* and *E. faecalis* to nalidixic acid was not investigated, as there are currently no minimum inhibitory concentration (MIC) breakpoints published for nalidixic acid and these two bacterial species.

Unexpected results obtained with nybomycin and *M. tuberculosis* were confirmed with a second round of testing using the MTT assay. Due to financial constraints, a subset of 10 fluoroquinolone-susceptible and 13 fluoroquinolone-resistant

M. tuberculosis isolates were selected for confirmatory testing. DNM-2 was acquired at this stage and bacterial susceptibility to DNM-2 was determined using the same subset of 23 *M. tuberculosis* isolates and MTT testing methodology.

All *N. gonorrhoeae* isolates (n=30) studied were obtained from the culture collection of the Department of Infection Prevention and Control, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, South Africa. *E. coli* (n=6), *K. pneumoniae* (n=4), *E. cloacae* (n=4), *P. aeruginosa* (n=7), *A. baumannii* (n=5), *S. aureus* (n=4) and *E. faecalis* (n=4) isolates were obtained from the culture collection of the Medical Microbiology Laboratory, National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital, Durban, South Africa. Results of routinely performed drug susceptibility testing were taken into consideration to ensure that both fluoroquinolone-susceptible and fluoroquinolone-resistant isolates were studied for all species. Susceptibility of isolates to ciprofloxacin and nalidixic acid were evaluated concurrently. The antimicrobial agents tested using the multipoint inoculator are presented in table 3.1.

3.1.2 Retrieval of isolates from storage

M. tuberculosis isolates stored at -70 °C were removed from the freezer and thawed at room temperature. Once thawed, 100 µl of each isolate was transferred into a container (Sterilin Polypropylene 30 ml Universal Container, Thermo Fisher Scientific, Massachusetts, USA) containing three milliliters of sterile Middlebrook 7H9 broth (Appendix A.1). Inoculated broths were then incubated in an upright

position at 37 °C in a shaking incubator (New Brunswick I26 Incubator Shaker, Eppendorf, Germany) for up to four weeks until growth was observed.

All other bacterial species were recovered from -70 °C storage by inoculating one bead for each isolate onto a separate chocolate agar plate (Appendix A.2). Inoculated plates were incubated for 24 to 48 hours at 37 °C in 5% CO₂ (Shel Lab CO₂ Incubator SC031, Sheldon Manufacturing, Cornelius, Oregon, USA) until growth was observed. Some of the *N. gonorrhoeae* isolates failed to grow with this approach and these were first cultured in brain heart infusion broth (Appendix A.3) before being subcultured onto chocolate agar plates.

3.1.3 Preparation of working cultures

3.1.3.1 *M. tuberculosis*

After vortexing (Vortex Mixer VM-300, Axiom Solutions, Bürstadt, Germany) for two minutes, 200 µl of each broth culture was aspirated and then spread out evenly across a Middlebrook 7H11 agar plate (Appendix A.3), for single colony growth. A chocolate agar plate (Appendix A.2) was inoculated to exclude potential contamination. The chocolate agar plates were incubated in 5% CO₂ at 37 °C for two days and then inspected for the presence of any growth. The inoculated Middlebrook 7H11 plates were heat-sealed in gas permeable bags and incubated in 5% CO₂ at 37 °C for seven days, after which they were transferred to the hot room (ambient air, 37 °C) for another five weeks. After a total of six weeks' incubation, a single colony from each *M. tuberculosis* isolate was picked off and

inoculated into 5 ml Middlebrook 7H9 broth with Tween-80 and sterile glass beads (Appendix A.1). Tween-80 has a surfactant action and its purpose was thus to help prevent bacterial clumps from forming¹³⁵. Sterile glass beads served to help break down bacterial clumps during vortexing steps. Single colonies were used in order to avoid inadvertently using mixed or contaminated cultures in subsequent investigations. The inoculated broths were then incubated at 37 °C in a New Brunswick I26 Incubator Shaker for two to three weeks, until a turbidity of approximately 1.0 McFarland was reached (Appendix A.5). *M. tuberculosis* cultures with this turbidity contain approximately $2-3 \times 10^8$ colony forming units per milliliter (CFU/ml) and are considered to be in the log phase of growth. Throughout the incubation period, the screw-caps were left slightly loose to allow gas diffusion and thereby avoiding the formation of an anaerobic environment. Each container was also mixed with the vortex mixer for one minute on alternate days to help avoid bacterial clumping. After the desired turbidity was reached, the vortex mixer was used for two minutes for each culture to break down bacterial clumps after which each broth culture was passed four times through a 25-gauge needle using a syringe. Each culture was also sonicated twice (Misonix Sonicator s-4000, QSonica, Connecticut, USA) for ten seconds at an amplitude of 10%, reaching a power of 10 Watt. A ten second pause was allowed in-between the two 10 second sonication periods so that the container could be tilted three times to mix its contents. Thereafter, the cultures were left undisturbed for 20 to 30 minutes so that any residual clumps could sink to the bottom of the container. This comprehensive attempt at reducing bacterial clumping was embarked upon in order to minimize the chances of drug susceptibility testing results indicating false antibiotic resistance. A second sterility check was performed at this stage by

culturing some of the supernatant on chocolate agar plates. The supernatant was then aspirated and transferred to a sterile 30 ml universal container where its turbidity was adjusted to a McFarland standard of 0.5 (Appendix A.5). This turbidity indicates the presence of approximately 1.5×10^8 CFU/ml. The adjusted supernatants i.e. working culture solutions were used for all subsequent laboratory investigations and small volumes thereof were also dispensed into cryovials and stored at $-70\text{ }^{\circ}\text{C}$ for later use.

3.1.3.2 Other bacteria

Single colonies were picked off and again cultured for 24 to 48 hours on chocolate agar plates (Appendix A.2). This was done to avoid contaminants and to make sure pure cultures were being used. From pure subcultures, suspensions with a McFarland of 0.5 were made in phosphate buffered saline (PBS) (Appendix A.6). Each sample was homogenized with a vortex mixer for 30 seconds and its turbidity adjusted to a McFarland of 0.5. The adjusted bacterial suspensions i.e. working cultures were used for all subsequent susceptibility testing. Colonies were also suspended in storage medium and stored at $-70\text{ }^{\circ}\text{C}$ for later investigations (Appendix A.7).

3.1.4 Screening with the MTT assay

The MTT assay was performed as described previously¹⁸⁰. This established methodology compares well with reference susceptibility testing methods^{180,181}. Testing was done in triplicate.

3.1.4.1 Preparation of nybomycin working solution and 96-well test plate

Nybomycin powder with a purity of 99% was procured from SantaCruz Biotechnology, Dallas, Texas, USA. A small amount of the antibiotic powder was dissolved in DMSO and further diluted with sterile PBS to a final concentration of 200 µg/ml with 4% DMSO. This was then used for two-fold serial dilutions with Middlebrook 7H9 broth in a sterile 96-well flat-bottom plate. Ten two-fold dilutions, from 200 µg/ml to 0.39063 µg/ml were prepared. Figure 3.1 illustrates how the 96-well plate was set up.

Figure 3.1 96-well test plate for pilot susceptibility testing of *M. tuberculosis* with highest and lowest final nybomycin concentrations of 100 µg/ml and 0.19531 µg/ml respectively.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	A
B	dH ₂ O	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.39063	0.19531	dH ₂ O	B
C	dH ₂ O	100	50	25	12.5	6.25	3.125	1.5625	0.78125	0.39063	0.19531	dH ₂ O	C
D	dH ₂ O	100	50	25	12.5	6.26	3.125	1.5625	0.78125	0.39063	0.19531	dH ₂ O	D
E	dH ₂ O	Positive control	Positive control	Positive control	Negative control	Negative control	Negative control	empty	empty	empty	empty	dH ₂ O	E
F	dH ₂ O	Positive control	Positive control	Positive control	Negative control	Negative control	Negative control	empty	empty	empty	empty	dH ₂ O	F
G	dH ₂ O	Positive control	Positive control	Positive control	Negative control	Negative control	Negative control	empty	empty	empty	empty	dH ₂ O	G
H	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Values displayed are the final drug concentrations ($\mu\text{g/ml}$) after 1:2 dilution with bacterial inoculum.

Negative control: 200 μl sterile Middlebrook 7H9 broth.

Positive control: 100 μl bacterial inoculum with 100 μl sterile Middlebrook 7H9 broth.

3.1.4.2 Preparation of inoculum and inoculation of test plate

The turbidity of previously prepared bacterial working cultures was adjusted to 0.5 McFarland and then further diluted 1:100 to provide a bacterial suspension with 1.5×10^6 CFU/ml for each *M. tuberculosis* isolate. One hundred microliters of each bacterial suspension were added to each drug-containing well and the positive control wells. Contents were mixed by pipetting up and down three times. The final *M. tuberculosis* concentration in each well was therefore 7.5×10^5 CFU/ml.

3.1.4.3 Culture, incubation and reading of results

The plates were heat-sealed in gas-permeable bags and incubated at 37 °C in ambient air for seven days. After seven days of incubation, 15 μl of the 5 mg/ml MTT solution (Appendix A.8) was added to one positive control and one negative control well, mixed by pipetting, sealed as described before and incubated overnight. After overnight incubation 50 μl SDS-DMF solution (A.11) was added to the same two control wells. The plate was placed back in the incubator for two to three hours before the color reaction was read. A color change from yellow to purple indicated the presence of actively metabolizing cells and a yellow color

indicated negative growth. If no growth was observed in the positive control well, then the process was repeated with another positive control well, on the same day. Once growth has been observed in the positive control well, and the negative control showed no growth, then 15 μ l MTT solution was added to all the other bacteria-containing wells and another negative control well. The MIC (μ g/ml) was then read the next day after the addition of SDS-DMF and the incubation step, as described. The MIC was determined as the lowest drug concentration where a yellow colour was visible with the naked eye.

3.1.5 Screening with the multipoint inoculator method

The CLSI recommended agar dilution methodology was used for all drug-bug combinations¹⁸²⁻¹⁸⁵. Drug suppliers, purity and solvent particulars of each compound tested are presented in table 3.2. Antibiotic stock solutions, bacterial inoculums and all testing were performed in triplicate to ensure accuracy and reproducibility.

Table 3.2 Characteristics of antibiotics used for susceptibility testing with the multipoint inoculator.

Antibiotic	Supplier*	Purity (%)	Solvent
Isoniazid	Sigma-Aldrich	≥ 99	Distilled water
Rifampicin	Sigma-Aldrich	≥ 97	Methanol
Ofloxacin	Sigma-Aldrich	98	Acetic acid
Amikacin	Sigma-Aldrich	98	Distilled water
Quinine sulfate	Sigma-Aldrich	≥ 98	Distilled water
Chloroquine diphosphate	Sigma-Aldrich	≥ 98	Distilled water
Mefloquine hydrochloride	Sigma-Aldrich	> 98	DMSO
Primaquine biphosphate	Sigma-Aldrich	98	Distilled water
Ciprofloxacin	Sigma-Aldrich	≥ 98	Distilled water
Nalidixic Acid	Sigma-Aldrich	≥ 98	Ethanol
Nybomycin	BioAustralis	> 95	DMSO

*Sigma-Aldrich, South Africa

*BioAustralis, Smithfield, New South Wales, Australia

All compounds were stored at temperatures recommended by the supplier.

For *M. tuberculosis*, drug susceptibility testing with isoniazid, rifampicin, ofloxacin and amikacin served three purposes. Firstly, it confirmed the previously performed phenotypic classification of *M. tuberculosis* isolates with the 1% proportion method. Secondly, it served as an internal confirmation of the accuracy of the multipoint inoculator method as it was used in this study. Thirdly, it provided a quantitative means of comparing inhibitory drug levels of ofloxacin with that of nybomycin in order to identify a possible reverse antibiotic effect with nybomycin.

For the other bacterial species, drug susceptibility testing with ciprofloxacin and nalidixic acid served similar purposes. Additionally it also provided a quantitative means of comparing inhibitory drug levels of ciprofloxacin with that of nybomycin,

quinine, chloroquine, mefloquine and primaquine in order to identify a possible reverse antibiotic effect with nybomycin with the other quinoline-containing drugs.

3.1.5.1 Determining drug concentration testing ranges and preparation of antibiotic stock solutions

There are no MIC breakpoints ($\mu\text{g/ml}$) available for testing bacteria with nybomycin, quinine, chloroquine, mefloquine and primaquine. Hiramatsu *et al* reported the minimum concentration of nybomycin to inhibit wild type *S. aureus* to be $\geq 64 \mu\text{g/ml}$, while isolates with phenotypic fluoroquinolone resistance, required nybomycin concentrations ranging from ≤ 0.06 to $1.0 \mu\text{g/ml}$ ^{143,166}. Three drug-susceptible *M. tuberculosis* isolates investigated by Arai *et al* required at least 4.2, 5.2 and $6.3 \mu\text{g}$ nybomycin per milliliter respectively for visual inhibition of growth¹⁶⁵. Due to the limited data available and the high cost of nybomycin antibiotic powder, it was decided to test twelve two-fold dilutions of nybomycin from 32 to $0.0156 \mu\text{g/ml}$ for all isolates.

Critical drug concentrations for the anti-tuberculosis drugs tested in this study have been previously published by the World Health Organization (WHO) and are presented in table 3.3¹⁸⁶. For this study, these same drug concentrations were used as MIC resistance breakpoints ($\mu\text{g/ml}$). The full range of drug concentrations tested with each antimicrobial are presented in table 3.4.

Table 3.3 WHO recommended critical concentrations used for determining drug resistance of *M. tuberculosis* isolates¹⁸⁶.

Antibiotic	Critical concentration (µg/ml)
Isoniazid	0.2
Rifampicin	1.0
Amikacin	4.0
Ofloxacin	2.0

These critical concentrations (µg/ml) are specifically intended for use with the 1% proportion method on Middlebrook 7H10 agar¹⁸⁶.

Table 3.4 Antimicrobial drug concentrations used for MIC (µg/ml) determination in *M. tuberculosis* isolates using the multipoint inoculator.

Antibiotic	Concentrations (µg/ml)												
Isoniazid	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
Rifampicin	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
Amikacin	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
Ofloxacin	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64
Nydomycin	0.0156	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	-

CLSI recommended susceptibility, intermediate and resistance breakpoints (µg/ml) were used with ciprofloxacin and nalidixic acid against the other bacterial isolates as presented in table 3.5.

Table 3.5 CLSI susceptibility, intermediate and resistance breakpoints ($\mu\text{g/ml}$) for ciprofloxacin and nalidixic acid.

Antibiotic	Test organism/s	MIC ($\mu\text{g/ml}$) breakpoints		
		Susceptible	Intermediate	Resistant
Ciprofloxacin	<ul style="list-style-type: none"> • <i>N. gonorrhoeae</i> 	≤ 0.06	0.12 - 0.5	≥ 1
	<ul style="list-style-type: none"> • <i>E. coli</i> • <i>K. pneumoniae</i> • <i>E. cloacae</i> • <i>P. aeruginosa</i> • <i>A. baumannii</i> • <i>E. faecalis</i> • <i>S. aureus</i> 	≤ 1	2	≥ 4
Nalidixic Acid*	<ul style="list-style-type: none"> • <i>E. coli</i> • <i>K. pneumoniae</i> • <i>E. cloacae</i> • <i>P. aeruginosa</i> • <i>A. baumannii</i> 	≤ 16	-	≥ 32

*Gram-positive bacteria are intrinsically resistant to nalidixic acid.

The full range of drug concentrations tested with *N. gonorrhoeae*, *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* are presented in table 3.6 and table 3.7.

Table 3.6 Antimicrobial drug concentrations used for MIC ($\mu\text{g/ml}$) determination in *N. gonorrhoeae* using the multipoint inoculator.

Antibiotic	Concentrations ($\mu\text{g/ml}$)														
Ciprofloxacin	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Nalidixic acid	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Nydomycin	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	-	-	-
Quinine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Chloroquine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Mefloquine	-	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	-
Primaquine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128

Table 3.7 Antimicrobial drug concentrations used for MIC ($\mu\text{g/ml}$) determination in *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* isolates using the multipoint inoculator.

Antibiotic	Concentrations ($\mu\text{g/ml}$)														
Ciprofloxacin	-	-	-	-	0.125	0.25	0.5	1	2	4	8	16	32	-	-
Nalidixic acid	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Nyobomycin	0.0078	0.0156	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	-	-	-
Quinine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Chloroquine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128
Mefloquine	-	-	0.0313	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	-
Primaquine	-	-	-	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128

*Gram-positive bacteria are intrinsically resistant to Nalidixic Acid

Antibiotic stock solutions were prepared by first weighing out and adding the required amount of antibiotic powder to sterile 30 ml universal containers. The equation below was used to calculate the amount of each drug required for one round of drug susceptibility testing, and the calculation results for all drugs are presented in table 3.8.

$$\begin{aligned}
 \text{Drug required (gram)} &= \text{HDC} \times \text{AV} \times \text{DDF} \times \text{CF} \times 100/\text{P} \\
 &= \text{HDC} \times 20 \times 2 \times 10^{-6} \times 100/\text{P} \\
 &= \text{HDC}/\text{P} \times 0.004
 \end{aligned}$$

- HDC = Highest drug concentration to be tested ($\mu\text{g/ml}$)
- AV = Agar volume in each plate

- DDF = Double dilution factor. This was 2 for all drugs, because a double amount of drug was required in the first plate from where 1:2 dilutions were made to the subsequent plates
- CF = Conversion factor i.e. 10^{-6} to convert microgram to gram
- P = % purity of antimicrobial powder, as indicated by the manufacturer

Table 3.8 Amount (gram) of antibiotic powder required to test all required drug concentrations once with the multipoint inoculator.

Antibiotic	Highest concentration tested ($\mu\text{g/ml}$)	<i>M. tuberculosis</i>	<i>N. gonorrhoeae</i>	<ul style="list-style-type: none"> • <i>E. coli</i> • <i>K. pneumoniae</i> • <i>E. cloacae</i> • <i>P. aeruginosa</i> • <i>A. baumannii</i> • <i>E. faecalis</i> • <i>S. aureus</i>
Isoniazid	64	0.00259 g	-	-
Rifampicin	64	0.00264 g	-	-
Amikacin	64	0.00261 g	-	-
Ofloxacin	64	0.00261 g	-	-
Nybomycin	32	0.00135 g	-	-
	16	-	0.00067 g	0.00067 g
Ciprofloxacin	128	-	0.00522 g	-
	32	-	-	0.00131 g
Nalidixic Acid	128	-	0.00522 g	0.00522 g
Quinine sulfate	128	-	0.00522 g	0.00522 g
Chloroquine diphosphate	128	-	0.00522 g	0.00522 g
Mefloquine hydrochloride	64	-	0.00261 g	0.00261 g
Primaquine biphosphate	128	-	0.00522 g	0.00522 g

Double amounts of the calculated minimum required antibiotic presented in table 3.8 were weighed out and used. This was necessary to account for some losses during the subsequent filtering of stock solutions aimed at removing bacterial contaminants.

Each antibiotic stock solution was therefore prepared by adding 0.8 ml solvent (table 3.2) to the weighed out antibiotic powder. The antibiotic powder was carefully mixed with the solvent by pipetting up and down five to ten times. A vortex mixer was also used to make sure the antibiotic powder is thoroughly mixed and in solution. Using no more than 0.8 ml of solvent assured that the bacteria were not exposed to more than 1% solvent, as higher concentrations of solvents may adversely affect bacterial growth.

Next a 1:10 dilution was made by adding 7.2 ml sterile PBS to the antibiotic suspension. It was then mixed using the vortex mixer and the resultant 8 ml antibiotic stock solution was filter sterilized through a 0.22 micron Millipore filter (Merck Millipore, SA). The final product was then either used immediately, or stored in cryovials at -70 °C until further use.

The preparation of nybomycin stock solution deviated from the above-explained procedure. Due to the high cost of nybomycin, only small amounts could be procured and double volumes could not be used as for the other antibiotics. The weighed-out amount of antibiotic was therefore dissolved in 0.4 ml solvent and thereafter diluted with 3.6 ml sterile of PBS to give a final volume of 4 ml. As some antibiotic stock solution usually inadvertently get left behind in the 0.22 micron

Millipore filter during filter sterilization, this step was omitted. Great care was thus taken to ensure the nystomycin powder were not contaminated during the handling thereof. All antibiotic stock and working solutions were only frozen and thawed once, after which they were discarded.

3.1.5.2 Preparation of antibiotic working solutions and culture media

Either freshly prepared or stored antibiotic stock solution were retrieved from the -70 °C freezer and left to thaw at room temperature. In preparation for agar plate pouring, 17 sterile 30 ml universal containers were positioned in a row on a tube rack. Two milliliters PBS were added to all containers, except the first one. Four milliliters stock solution were added to the first container and two-fold serial dilutions performed by removing 2 ml from the first container and adding it to the second container. It was then thoroughly mixed by pipetting up-and-down five times and thereafter 2 ml were transferred to the third container. This mixing and transferring of 2 ml of antibiotic solution from one container to the next was repeated up to the twelfth tube. Two milliliters antibiotic solution was discarded from the twelfth container so that only 2 ml fluid remained in it. The first 12 containers represented the 12 antibiotic concentrations that were tested for each antibiotic and the last five containers were used as drug-free controls.

Eighteen milliliters of freshly prepared and cooled down Middlebrook 7H10 agar (Appendix A.12), maintained in its liquid state in a water bath set at 45 °C, were next added to each 2 ml of antibiotic working solution in the 30 ml container. For *Neisseria gonorrhoeae*, GC agar (Appendix A.13) was used and for the other

bacterial species Mueller-Hinton agar (Appendix A.14). In the same way, media was added to the five containers containing 2 ml of PBS without any antibiotic. Immediately after 18 ml of media was added to any particular container, its screw-cap was tightly closed and the container carefully tilted three times to mix its contents. The cap was then removed and the full 20 ml amount of media promptly poured into an empty 90 mm Petri dish before the agar had time to solidify. The agar plates were closed and left on the benchtop until the agar solidified, after which the plates were sealed in plastic bags and stored at 4-8 °C until further use. Plates were used within one week of preparation.

3.1.5.3 Preparation of inoculum and inoculation of solid agar plates

A Steers-type multipoint inoculator known as the Cathra replicator was used to inoculate all bacterial isolates onto the surfaces of agar plates containing various concentrations of the different test antibiotics^{182,187,188}. The replicator seed tray is made up of 37 individual wells that each can accommodate 0.5 ml of a separate bacterial isolate suspension. Crystal violet was added to the first well on the seed tray in order to assist the correct marking and numbering of each isolate after the inoculation process was complete. Each floating pin of the instrument has a diameter of 3 mm and is designed to pick up and transfer a single drop with a volume of 2 µl, from the replicator seed tray to each of 37 separate spots on the agar surface of a single solid agar plate^{182,184,189,190}.

To ensure that approximately 10^4 CFU were delivered to each 2 µl spot, the bacterial working solution was first diluted 1:10 in sterile PBS containing

1% Tween-80 (Appendix A.6) to obtain a concentration of 10^7 CFU/ml^{183,184,189}. Each well of the Cathra replicator seed tray was loaded with 0.5 ml of inoculum of a different isolate¹⁸⁴. Inoculation with the Cathra multipoint inoculator proceeded in a systematic fashion starting with the plates containing the lowest drug concentration and ending with the plates containing the highest drug concentration. Additionally, there was a drug-free plate included before the first and last drug-containing plates, as well as after every third drug-containing plate. These measures served as (1.) antibiotic-free controls, (2.) contamination controls as well as (3.) to reduce the potential carry-over of antibiotic to the seed wells and subsequent agar plates.

3.1.5.4 Culture, incubation and reading of results

After inoculation, plates with *M. tuberculosis* were left in the biosafety cabinet for up to an hour for the inoculums to dry. Next the plates were heat-sealed in gas-permeable plastic bags and incubated at 37 °C in 5% CO₂. After one week incubation, the cultures were transferred to a hot room with a temperature of 37 °C and ambient air for an additional 2 to 4 weeks of incubation. Plates were read after 3 to 4 weeks' incubation, or later depending on when the positive controls indicated visible growth. For the other bacterial species, the plates were incubated for 24 hours at 37 °C in 5% CO₂. Incubation was extended for an additional 24 hours for the *N. gonorrhoeae* isolates.

The multipoint plate reader was used to inspect agar plate surfaces for any signs of growth. The MIC ($\mu\text{g/ml}$) was determined as the lowest concentration of drug

where no bacterial growth could be observed.

3.1.6 Further drug susceptibility testing with the MTT assay

In order to confirm the susceptibility results obtained with the multipoint inoculator, the MTT assay was used with a subset of 23 *M. tuberculosis* isolates. This well-established method compares closely with reference susceptibility testing methods^{180,181}. DNM-2 was likewise evaluated against the same subset of 23 *M. tuberculosis* isolates.

3.1.6.1 Preparation of antimicrobial working solutions and 96-well test plate

As stated before, nybomycin was procured from BioAustralis (Smithfield, New South Wales, Australia) and DNM-2 was donated by Paul Hergenrother¹⁷⁹.

There is currently no published data for laboratory investigations performed with the deoxynybomycin-derivative in *M. tuberculosis* and therefore also no MIC breakpoints ($\mu\text{g/ml}$) to adopt. Parkinson *et al* reported MICs with DNM-2 of between 0.0625 and 4 $\mu\text{g/ml}$ for methicillin-resistant *S. aureus* and between 0.25 and 8 $\mu\text{g/ml}$ for vancomycin-resistant *E. faecalis* for nybomycin. With only one published paper on the deoxynybomycin-derivative as guidance, it was decided to investigate *M. tuberculosis* susceptibility to 12 different DNM-2 concentrations i.e. 12 $\mu\text{g/ml}$ and eleven two-fold dilutions thereof. A limited amount of the DNM-2 powder necessitated the use of micro broth dilution methodology for susceptibility testing. *M. tuberculosis* susceptibility to nybomybcin were determined alongside

DNM-2 using the same micro broth dilution methodology and the same drug concentrations that were used with the solid agar dilution method. Nybomycin susceptibility testing was repeated using this methodology in order to confirm the results obtained with the solid agar method. The same nybomycin concentrations were investigated than were tested with the multipoint inoculator.

Nybomycin. The supplied nybomycin antibiotic powder had a purity of >95%. Therefore every 1.05263 µg of the powder represented 1 µg pure nybomycin and this was taken into account for all the subsequent calculations. In order to ensure that bacteria will not be exposed to more than 1% DMSO in the final culture, 1.052 mg nybomycin was weighed out and added to 625 µl DMSO. This was thoroughly mixed and dissolved by pipetting up-and-down and with the vortex mixer and provided an effective nybomycin stock solution with a concentration of 1600 µg/ml. Antibiotic working solutions were prepared by diluting the stock solution 1:50 i.e. adding 40 µl of stock solution to 1960 µl Middlebrook 7H9 broth whereupon a final concentration of 32 µg/ml was reached. Two hundred microliters of this antibiotic working solution were added to the first well and two-fold serial dilutions performed by transferring 100 µl from the first well to the second well prefilled with 100 µl Middlebrook 7H9 broth. The resultant 200 µl was mixed by pipetting up and down three times and thereafter 100 µl was aspirated and transferred to the third well. This two-fold serial dilution process was continued until the last well was reached, after which 100 µl was discarded so that the last well remained with 100 µl antibiotic solution. The remaining antibiotic stock solution was stored in cryovials at -70 °C for future use. Antibiotic solutions were only frozen and thawed once, after which they were discarded.

DNM-2. Stock solutions were prepared by dissolving 0.72 mg antibiotic powder in 0.6 ml DMSO, followed by extensive mixing with the vortex mixer for 15 minutes until it was fully dissolved, to give a final concentration of 1200 µg/ml. The antibiotic stock solution was diluted 1:50 by adding it to 29.4 ml Middlebrook 7H9 broth, in order give the highest testable antibiotic concentration of 12 µg/ml, as well as a maximum DMSO concentration of 1%. Higher drug concentrations could unfortunately not be tested due to the poor solubility of DNM-2. Antibiotic solutions were only frozen and thawed once, after which they were discarded.

Preparation of 96-well test plate. Testing was conducted in a sterile 96-well, flat-bottomed microtitre plate (Porvair, WhiteSci, SA). As illustrated in figure 3.2, the outside wells of the plate were filled with 100 µl sterile distilled water to minimize any effect that evaporation might have during incubation. All wells earmarked for the different concentrations of antibiotic as well as nine positive control wells each received 100 µl Middlebrook 7H9 broth, the three wells identified to receive the highest antibiotic concentration were left empty. Nine negative control wells were filled with 200 µl Middlebrook 7H9 broth. Two hundred microliters of the antibiotic working solution were added to each of the three wells reserved for the highest concentration of antibiotic. Antibiotic two-fold serial dilutions were performed by removing 100 µl from the highest antibiotic concentration well and transferring it to the second highest antibiotic concentration well that already contain 100 µl of 7H9 broth, followed by pipetting up and down five times to ensure adequate mixing and homogeneity. One hundred microliters were then removed from the second well and transferred to the third well. This process of transferring 100 µl between wells together with mixing by using the pipette, was repeated until the last well

representing the lowest antibiotic concentration, where the final 100 µl was discarded and the well left with 100 µl antibiotic solution. This was completed immediately before the addition of microbial inoculum.

Figure 3.2 96-well test plate for susceptibility testing with highest and lowest final nymbomycin concentrations of 32 µg/ml and 0.01562 µg/ml respectively.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	A
B	dH ₂ O	32	16	8	4	2	1	dH ₂ O	Positive control	Positive control	Positive control	dH ₂ O	B
C	dH ₂ O	32	16	8	4	2	1	dH ₂ O	Positive control	Positive control	Positive control	dH ₂ O	C
D	dH ₂ O	32	16	8	4	2	1	dH ₂ O	Positive control	Positive control	Positive control	dH ₂ O	D
E	dH ₂ O	0.5	0.25	0.125	0.0625	0.03125	0.015625	dH ₂ O	Negative control	Negative control	Negative control	dH ₂ O	E
F	dH ₂ O	0.5	0.25	0.125	0.0625	0.03125	0.015625	dH ₂ O	Negative control	Negative control	Negative control	dH ₂ O	F
G	dH ₂ O	0.5	0.25	0.125	0.0625	0.03125	0.015625	dH ₂ O	Negative control	Negative control	Negative control	dH ₂ O	G
H	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	dH ₂ O	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Values displayed are the final drug concentrations (µg/ml) after 1:2 dilution with bacterial inoculum.

Negative control: 200 µl sterile Middlebrook 7H9 broth.

Positive control: 100 µl bacterial inoculum with 100 µl sterile Middlebrook 7H9 broth.

(Test plate for DNM-2 was prepared in a similar fashion, but with a highest concentration of 12 µg/ml and a lowest concentration of 0.00586 µg/ml.)

3.1.6.2 Preparation of inoculum and inoculation of test plate

Performed according to section 3.1.4.2

3.1.6.3 Culture, incubation and reading of results

Performed according to section 3.1.4.3

3.2 DNA isolation

3.2.1 Bacterial cultures

M. tuberculosis working cultures were prepared as described in section 3.1.3. One hundred microliters of each culture was inoculated and spread out on Middlebrook 7H11 plates. Three separate agar plates were inoculated for each isolate. The plates were heat-sealed in gas-permeable plastic bags and incubated at 37 °C in 5% CO₂ for one week after which they were transferred to the hot room for an additional 4-6 weeks until a lawn of growth was observed on each plate.

3.2.2 DNA isolation with the cetyltrimethylammoniumbromide method

The cetyltrimethylammoniumbromide (CTAB) method was used as previously described by Van Soolingen *et al*, with minor modifications^{174,191–193}. Preparation of all reagents and solutions are described in Appendix A. Colony growth were

harvested from the surface of Middlebrook 7H11 agar media with a sterile plastic loop and suspended in a sterile, round-bottomed 2 ml Eppendorf tube prefilled with 500 μ l of sterilized distilled water. The tubes were then closed and placed for 30 minutes in a heating-block set at 80 °C to heat-kill the live bacteria. Next the tubes were transferred to an Eppendorf thermomixer set at 60 °C and 70 μ l 10% SDS and 50 μ l proteinase K (10 μ g/ml) were added to it. The Eppendorf thermomixer was set at low shaking mode i.e. 10 second periods of mixing alternating with 10 second periods of no mixing. The Proteinase K is used to both digest protein to release DNA from cells and to inactivate DNases that may degrade DNA. The action of Proteinase K is potentiated by SDS. After one hour 100 μ l 5M NaCl and then 100 μ l 10% CTAB, both preheated to 60 °C, were added to each tube and thoroughly mixed by inverting the tube by hand. The tubes were then subjected to another 15 minutes in the Eppendorf thermomixer set at 60 °C together with low-shaking mode. CTAB is a surfactant that, together with NaCl binds to and remove polysaccharides from lysed bacterial suspensions¹⁹². To purify the DNA further, 700 μ l of a chloroform:isoamyl alcohol (24:1) mixture was added to all the tubes and mixed by inverting 20 to 25 times by hand. This led to the formation of a homogenous white “milky” solution. The tubes were then centrifuged for 10 minutes at 15625.503 x g and the upper aqueous phase (\pm 700 μ l) transferred to sterile microcentrifuge tubes containing 700 μ l cold isopropanol (Sigma-Aldrich, SA). It was then carefully mixed by inverting the tubes several times so that the precipitated DNA could be seen as a thin thread. All the tubes were then kept overnight at 4 °C and then centrifuged at 9245.86 x g for 10 minutes at 4 °C. Thereafter the isopropanol was decanted and each pellet washed with 50 μ l 80% cold ethanol (Merck, SA) followed by centrifugation for a

further 5 to 10 minutes at 13314.038 x g. This washing step was repeated once and the pellet left to dry by evaporation for 30 minutes at room temperature, by placing each opened tube upside-down on a clean paper towel. The heat-killed, isolated and cleaned DNA was then kept at 4 °C in 55 µl of 1x TE buffer until use.

3.2.3 Estimation of DNA purity, concentration and quality

DNA purity was estimated with the A_{260}/A_{230} and A_{260}/A_{280} absorbance ratios using the NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and DNA quality was visualized by electrophoresing 1 µl extracted DNA in a 1% agarose gel (Appendix A.20)^{174,194}.

Nucleotides, DNA and RNA all absorb at a wavelength of 260 nm and most other expected substances in molecular samples at either higher or lower wavelengths. By also measuring absorbance at 230 nm and 280 nm, contaminants can be excluded by looking at A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios. The A_{260}/A_{280} and A_{260}/A_{230} absorbance ratios were therefore used to evaluate the purity of DNA. With the A_{260}/A_{280} ratio, “pure” DNA is expected to have a A_{260}/A_{280} absorbance ratio of ± 1.8 and a A_{260}/A_{230} absorbance ratio of $\pm 2.0-2.2$. If the ratios are considerably lower than these values, then it may indicate the presence of substantial amounts of impurities that absorb light at either 280 nm or 230 nm. Two microliters of each DNA sample were therefore loaded onto the absorbance platform in order for the instrument to measure absorbance¹⁹⁴.

Gel electrophoresis was used to assess DNA quality. First, a 1% agarose gel was prepared. A casting tray and 20-well plastic comb were cleaned with 70% alcohol followed by the proper positioning of the plastic comb inside the casting tray. The open ends of the tray were then secured with masking tape to prevent the gel from leaking out of the tray. Next 1X Tris-borate-EDTA (TBE) buffer was freshly prepared by adding 100 ml of 10X TBE to 900 ml distilled water (Appendix A.21). The final 1% agarose solution was prepared by adding 1.4 grams of agarose (Seakem LE Agarose, Whitehead Scientific, SA) to 140 ml of the 1X TBE buffer. The agarose was then dissolved in the 1X TBE buffer by heating it in a microwave oven and the full amount was then poured into the casting tray once it cooled down to approximately 40 to 45 °C. It was then left for 30 to 40 minutes to solidify at room temperature (25 °C) after which the comb and masking tape were removed. The gel was then carefully placed in an electrophoresis tank and 1 X TBE buffer was added to the tank so that its surface was covered with approximately 1-2 mm of fluid.

For each DNA sample, 5 µl of gel loading dye (Appendix A.22) was first dispensed onto a sheet of parafilm. Next the DNA was mixed inside its tube by tapping it lightly with a finger a couple of times and then 1 µl of the DNA was transferred directly into the drop of gel loading dye. The two were then mixed by pipetting up and down 3 to 4 times. The resultant 6 µl drop was immediately transferred to its appointed well in the gel, before continuing with the next sample. The last well was loaded with 5 µl gel loading dye together with 1 µl of a DNA molecular weight marker (DNA Molecular Weight Marker II, Roche, SA). The ladder served as a

molecular weight marker, so that the size of DNA fragments in the gel could be determined.

The gel was then covered with a lid so that the negative (black) cathode was positioned closest to the DNA samples in their wells and the positive (red) anode positioned furthest away from the DNA samples. DNA samples then migrated toward the positively charged electrode. The electrical power source was set at 100 Volts and allowed to run for one hour. The current remained between 35 and 50% of the Volts for the duration of the experiment. Next the gel was removed from its tank and placed inside the Syngene G:Box gel imaging system for image capturing and printing with the GeneSnap software package (Syngene, Maryland, USA). The size and brightness of bands were compared with that of the molecular weight marker as a rough estimate of the quality and quantity of the DNA.

3.3 Genotyping using IS 6110 restriction fragment length polymorphism fingerprinting

The IS 6110 restriction fragment length polymorphism (RFLP) fingerprinting method was used to genotype *M. tuberculosis* isolates, as previously described by Van Soolingen *et al.* with a few modifications^{191,195,196}. The main six steps in this method included (1.) restriction and (2.) gel electrophoresis on the first day followed by (3.) Southern blotting and (4.) hybridization on the second day; and (5.) washing of hybridized membrane and (6.) band detection in the darkroom on the third day. Preparation of all reagents and solutions are described in Appendix A.

3.3.1 Restriction

PvuII restriction endonuclease (Roche, SA) was used to digest *M. tuberculosis* DNA by cleaving at IS 6110. The appropriate volumes of DNA and sterile distilled water used were determined by evaluating the gel electrophoresis pictures and NanoDrop results to make a visual estimation of the DNA concentration. The DNA and sterile distilled water were then mixed together with 2.5 µl of buffer (Roche Diagnostics, Mannheim, Germany) and 1.5 µl *PvuII* restriction enzyme (Roche Diagnostics, Mannheim, Germany) in a sterile 1.5 ml micro-centrifuge tube to prepare a final volume of 22 µl. Each sample required 2 to 15 µl DNA and 7 to 20 µl sterile distilled water to give good quality IS 6110 RFLP bands. All samples were mixed using a pipette and then placed in a floater in a water bath and incubated at 37 °C for four hours.

3.3.2 Gel electrophoresis

Gel electrophoresis was performed to separate the DNA fragments. A 1% agarose gel was prepared as described earlier. After the restriction process has been completed the tubes were removed from the water bath, and 5 µl of RFLP loading dye added to each (Appendix A.23). The gel was then loaded with DNA samples, but the first and last lanes were reserved for the Jacks Standard molecular weight marker (0.7 – 15 kilo base pairs) that served as an external reference. The electrical power source was set at 100 Volts and switched on until all the samples have moved out of their wells. The voltage was subsequently reduced to 35 Volt and the process allowed to continue overnight for approximately 18 to 20 hours.

3.3.3 Southern blotting

Southern blotting employs electrophoresis to transfer DNA fragments from an agarose gel to a membrane. For this study, we used the Hybond-N+ membrane (Amersham-GE Healthcare Life Sciences, UK) which is a positively charged nylon membrane. It was first submerged in distilled water for a few seconds before it was soaked in 10X SSC for five minutes. The nylon membrane was then positioned on the VacuGene XL vacuum blotting unit (Amersham-GE Healthcare Life Sciences, UK) with the gel on top of it. After ensuring that the edges have formed a good seal, the VacuGene XL Blotting Pump was switched on and set to deliver 55 millibars suctioning power. The gel was then flooded with a 1:100 dilution of HCl for 20 minutes, followed by flooding with Soak 1 solution for 20 minutes, Soak 2 solution for a further 20 minutes and 10XSSC solution for a final 90 minutes. With the 10XSSC solution, the suctioning power was increased to 60 millibars. Care was taken to ensure that each solution completely covered the surface of the gel and that it was first removed with the vacuum pump aspirator before the next solution was added. Thereafter the gel was discarded and the membrane carefully lifted and removed from the vacuum blotter and placed on paper towel in order to air dry for five minutes. The membrane was then briefly irradiated under ultraviolet light in the UVP-CL1000 cross-linker (Ultra-Violet Products, Cambridge, UK) in order to strengthen cross-links between the DNA fragments and the nylon membrane.

3.3.4 Hybridization

The blotted nylon membrane was rolled up and placed into a hybridization cylinder. As a pre-hybridization step, 20 ml of hybridization buffer (Amersham-GE Healthcare Life Sciences, UK) was pre-warmed to 37 °C and poured into the cylinder. The cylinder was then incubated for 30 minutes at 41.5 °C in the rotating hybridization oven. While pre-hybridization was in progress, the hybridization probe solution was prepared. Hybridization probe, a 245 base pair (bp) PCR fragment of IS 6110, was removed from the freezer and thawed on ice. Based on the intensity of subsequent band formation, certain components of the hybridization probe solution had to be optimized. The amount of hybridization probe varied between 5 and 7.5 µl; and the volume of distilled water varied between 12.5 and 15 µl. The calculated amounts of hybridization probe and distilled water were then mixed together in a micro-centrifuge tube followed by boiling for five minutes before it was returned to the ice for roughly 10 minutes to cool down. Next 20 µl DNA labeling reagent (Amersham-GE Healthcare Life Sciences, UK) and 20 µl glutaraldehyde (Amersham-GE Healthcare Life Sciences, UK) were added to this micro-centrifuge tube and incubated for ten minutes in a water bath at 37 °C. This provided a final hybridization probe solution of 60 µl. The pre-hybridization buffer was decanted into a sterile glass bottle and the full volume of hybridization probe solution was then added to it. The resultant hybridization buffer was added to the hybridization cylinder and the membrane was left to hybridize overnight in the rotating hybridization oven at 6 revolutions per minute and 42 °C.

3.3.5 Washing of membrane

The hybridization buffer that was used overnight was discarded and the nylon membrane rinsed with 30 ml primary wash buffer, which was immediately discarded as well. Another 30 ml of primary wash buffer was added to the hybridization cylinder and incubated for 30 minutes in the rotating hybridization oven at 42 °C. The primary wash buffer was discarded and the nylon membrane rinsed twice with secondary wash buffer (2XSSC) for 5 to 10 minutes at room temperature on a Stuart Orbital SSL1 shaker (Cole-Parmer, Illinois, USA). The solution was discarded and the nylon membrane placed into a plastic bucket.

3.3.6 Detection of banding patterns

Exposure of the film and the development process were performed in a dark room. Eight milliliters of detecting reagent (Luminata Forte Western HRP Substrate, Merck, SA) was added to the plastic bucket containing the hybridized membrane. The membrane was carefully moved around in the bucket with a tweezer for two minutes to ensure good coverage of the whole surface. The detecting agent was then drained and covered in cling wrap taking care to avoid and wipe out all air bubbles that may enter in-between the layers. Next, the membrane was placed into a cassette with Amersham Hyperfilm ECL (Amersham-GE Healthcare Life Sciences, UK) and the film exposed to the membrane for two minutes. The film was removed and placed in image developer until bands could be observed. It was then immediately placed in fixer solution until the background appeared clear. The film was thoroughly rinsed before it was hanged to air-dry.

3.3.7 Reading and interpretation of results

The BioNumerics version 6.0 software package (Applied Maths, Sint-Maartens-Latem, Belgium) was used to visually analyze IS 6110 RFLP banding patterns^{197,198}. This software was also used to construct a dendrogram that approximates the degree of similarity between banding patterns^{197,198}. It specifically uses the unweighted pair group clustering method of averages (UPGMA) and the Dice coefficient to show how well patterns cluster together^{197,199}. IS 6110 RFLP genotype patterns were compared to stored profiles at the University of KwaZulu-Natal and Stellenbosch University for IS 6110 RFLP family designations^{199,200}. Isolates in a IS 6110 RFLP family usually share at least two-thirds of their banding patterns and a similarity index of at least 70% was therefore used to signify a IS 6110 RFLP family^{199,201}. A 5% tolerance level was allowed for band matching within IS 6110 RFLP families. Each IS 6110 RFLP family was also interrogated for the presence of clusters i.e. at least two isolates with at least five bands and identical banding patterns; and for non-clustering isolates i.e. isolates that do not have at least one other isolate with an identical banding pattern, also called “unique” isolates^{199,200}.

3.4 *gyrA* investigations

The same 32 *M. tuberculosis* isolates used for drug susceptibility investigations in section 3.1.1 were included in this experiment. A 762 base pair region of the *gyrA* gene, that includes the QRDR (codons 74 to 113), were investigated for mutations that may confer fluoroquinolone resistance. This experiment incorporated primers

and amplification conditions previously described by Dookie *et al*¹⁷⁴. DNA isolation, quality determination and quantity estimation have been described in section 3.2.2 and section 3.2.3.

3.4.1 Polymerase chain reaction

Dookie *et al* previously designed the forward and reverse primers used in this study with Primer-3 open source software^{174,202,203}. The designed primers were manufactured by *metabion*, Munich Germany^{174,204}. See table 3.9 for primer characteristics. Polymerase chain reaction (PCR) was performed using the Expand High Fidelity PCR System dNTPack kit (Roche, Mannheim, Germany) that includes all reagents required for the PCR reaction, except DNA and primers. The different components of the master mix were first aliquoted together with the primers into PCR tubes (on ice), followed by the addition of extracted DNA. The final reaction mixture for each isolate was 50 µl and is presented in table 3.10. After the master mix preparation and the addition of DNA, the final reaction mixture was loaded into the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, San Diego, USA) and the DNA amplified according to the cycling conditions described in table 3.11. Gel electrophoresis was used as described before to evaluate whether the amplification process was successful. The amplicons were stored at 4 °C until further processing.

Table 3.9 Primers used to PCR amplify a 762 base pair region of the *gyrA* gene¹⁷⁴.

Primer	Nucleotide sequence	Molecular weight	Lyophilized concentration	Amount of primer used per reaction	Melting temperature
Forward	5'-CGA TTG CAA ACG AGG AAT AG-3'	6199	81.8 nmol	10 pmol (1 μ l)	56 °C
Reverse	5'-GGC CAG TTT TGT AGG CAT CA-3'	6148	90.6 nmol	10 pmol (1 μ l)	58 °C

Table 3.10 Master mix components for each 50 μ l reaction.

Master Mix component	Concentration	Volume (μ l)
PCR Nucleotide Mix (dNTP)	neat	1
PCR Buffer (Expand High Fidelity Buffer)	10X	5
MgCl ₂	25 mM	3.3
Forward primer	10 pmol (stock solutions were 100 pmol)	1
Reverse primer	10 pmol (stock solutions were 100 pmol)	1
DNA polymerase* (Expand High Fidelity Enzyme Mix)	3.5 U/ μ l	0.75
PCR grade water	neat	33.95
DNA template (added last)	1:100 to 1:1000 dilution of original concentration**	4

*The DNA polymerase enzyme (Expand High Fidelity Enzyme Mix) in this kit consists of Taq DNA polymerase as well as a second DNA polymerase with proofreading activity.

**Concentration depended on estimated DNA concentration.

Table 3.11 PCR cycling conditions.

	Initial denaturation	Denaturation	Annealing	Extension	Final extension
Temperature	94 °C	94 °C	53 °C	72 °C	72 °C
Duration	2 minutes	45 seconds	45 seconds	45 seconds	7 minutes

Number of PCR cycles: 40 (denaturation, annealing and extension).

3.4.2 Gel electrophoresis of PCR product

Gel electrophoresis and the Nanodrop instrument were used to estimate the purity, concentration and quality of each amplicon, as described in section 3.2.3. Amplicons were diluted with 10X TE buffer to an estimated 200 ng / 2 µl concentration. Thirty microliters of this DNA concentration were used for *gyrA* sequencing for each *M. tuberculosis* isolate.

3.4.3 Gene sequencing, reading and interpretation of results

PCR product purification and *gyrA* sequencing were performed in collaboration with Inqaba Biotec, Pretoria, South Africa. Briefly, PCR products were first cleaned using *ExoSAP-IT* (Affymetrix, Thermo Fisher Scientific, Massachusetts, USA) according to the package insert. One-directional sequencing was performed using the same primers used for *gyrA* PCR, with the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions (Table 3.9). Labeled products

were cleaned with the ZR DNA Sequencing Clean-Up Kit (Zymo Research, California, USA). The cleaned products were loaded into the ABI 3500XL analyzer using POP-7 Polymer (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). The final DNA sequences were trimmed, aligned and analyzed with BioEdit version 7.1 software package (Ibis Therapeutics, California, USA)^{205,206}. All sequences were compared to that of *M. tuberculosis* H37Rv.

3.5 Computational investigations

This work was performed in collaboration with the Molecular Bio-Computation and Drug Design Laboratory, School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa.

3.5.1 Molecular docking investigations

The previously published X-ray crystal structure of *M. tuberculosis* gyrase (PDB ID: 5BS8) complexed together with DNA, moxifloxacin and a magnesium ion were downloaded from the online Research Collaboration for Structural Bioinformatics Protein Data Bank (RCSB PDB)^{175,177,207}. Structural analysis was performed using the Discovery Studio 4.0 software package (Accelrys, San Diego, USA)^{208,209}. The complex was validated within its binding landscape to assure the effectiveness of the molecular docking algorithm before further evaluation of binding energies with other ligands. ChemBioDraw (CambridgeSoft, Massachusetts, USA) was used to prepare and energetically minimize nybomycin, DNM-2 and ciprofloxacin and ligands²¹⁰. The known moxifloxacin ligand binding domain was used to correctly

orientate the constructed ligands between the DNA residues. The LibDock module of the Discovery Studio software package was used for simulating molecular docking^{208,209}.

3.5.2 Molecular dynamics simulations

Molecular docking scores are known to be sometimes inaccurate in determining ligand binding affinity^{211,212}. Further computational investigations were therefore embarked upon that included molecular dynamics simulations together with free binding energy determination²¹³. The wild type form of the *M. tuberculosis* gyrase A subunit was changed using *Chimera* software (University of California, San Francisco, USA) to produce Ala90Val, Asp94Gly, Asp94His and Ala90Ser protein configurations²¹⁴. This same software was also used to add missing hydrogen molecules and to remove water molecules and non-standard amino acid residues²¹⁴. Molecular dynamics simulations for docking complexes of nybomycin, DNM-2 and ciprofloxacin with wild type and mutant *M. tuberculosis* gyrase were performed using the SANDER program within the AMBER 14.0 software package (University of California, San Francisco, USA)^{215,216}. Proteins and ligands were parameterized with the AMBER99SB force field as well as the Generalized Amber Force Field (GAFF)²¹⁷. Thereafter each complex was solvated with TIP3P water molecules and neutralized with the addition of sodium ions²¹⁸. Whilst maintaining periodic boundary conditions, the long-range electrostatics were treated using the particle-mesh Ewald method²¹⁹. Hereafter, partial minimization (1000 steps of steepest descent followed by 500 steps of conjugate gradient) was followed by full minimization (50 steps of steepest descent followed by 150 steps of conjugate

gradient). On completion of minimization, heat was added from 0 to 300° K for 5 picoseconds using Langevin Dynamics and then equilibrated at 300° K. A Berendsen barostat was used to maintain a 1 bar atmospheric pressure²²⁰. The temperature and pressure were kept constant for a period of 500 picoseconds. The SHAKE algorithm was used to constrain bonds that include hydrogen atoms^{221,222}. The final molecular dynamics simulation was conducted for 2 nanoseconds at the stabilized temperature of 300° K and pressure of 1 bar, without any additional constraints. A time step of 2 femtoseconds was employed with a distance cutoff of 12.0 Å for all the non-bonded interactions. Simulation trajectories were saved at every 1 picosecond time-point. Post-dynamics analysis employed the Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) method to calculate binding free energies for the full 2 nanosecond simulation period^{223,224}. Briefly, this method is explained by the following equations:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}} \dots \dots \dots (1)$$

$$\Delta G_{\text{bind}} = E_{\text{gas}} + G_{\text{sol}} - T\Delta S \dots \dots \dots (2)$$

$$E_{\text{gas}} = E_{\text{int}} + E_{\text{vdw}} + E_{\text{ele}} \dots \dots \dots (3)$$

$$G_{\text{sol}} = G_{\text{GB}} + G_{\text{SA}} \dots \dots \dots (4)$$

$$G_{\text{SA}} = \text{YSASA} \dots \dots \dots (5)$$

ΔG_{bind} : change of Gibbs (free) energy between ligand and protein

E_{gas} : gas-phase energy

G_{sol} : solvation free energy

E_{int} : internal energy

E_{vdw} : van der Waals energy

E_{ele} : Coulomb energy (electrostatic)

T: temperature in Kelvin

S: solute entropy

G_{GB} : polar solvation contribution

G_{SA} : nonpolar solvation contribution

YSASA: solvent accessible surface area

Bhakat *et al* published a detailed explanation of each parameter²²⁵. A free energy decomposition analysis was conducted with the SANDER program in AMBER 14.0 to determine the relative contribution of residue 90 and residue 94 of the gyrase enzyme to overall binding strength between each ligand and gyrase^{215,216}.

3.6 Selection and whole genome sequencing of *M. tuberculosis* mutants with increased nybomycin MICs

3.6.1 Selection of *M. tuberculosis* mutants with increased nybomycin MICs

M. tuberculosis V9124 is a fully drug-susceptible isolate belonging to the F15/LAM4/KZN IS 6110 RFLP family and has been previously obtained from a patient in Tugela Ferry, KwaZulu-Natal, South Africa. It forms part of the culture collection of the Department of Infection Prevention and Control at the University of KwaZulu-Natal. This clinical isolate has been well-characterized and its whole genome sequence was deposited in 2016 in the Sequence Read Archive (SRA) of the NCBI under the accession number SRP067784^{226,227}. This fast-growing *M. tuberculosis* isolate was chosen for the mutant selection experiment.

The classic Luria-Delbrück fluctuation analysis method was modified for mutant selection in this study^{125,228–230}.

3.6.1.1 Preparation of antibiotic dilutions and culture media

M. tuberculosis V9124 displayed a nybomycin MIC of 0.5 µg/ml and mutant selection was subsequently done by exposing the isolate to nybomycin concentrations of 0.125, 0.25, 0.5, 1, 2 and 4 µg/ml.

The equation below was used to calculate the amount of nybomycin required to conduct a mutant selection experiment four times with each of the five nybomycin concentrations:

$$\begin{aligned}\text{Drug required (gram)} &= \text{HDC} \times \text{AV} \times \text{DDF} \times \text{CF} \times 100/\text{P} \times \text{R} \\ &= \text{HDC} \times 20 \times 2 \times 10^{-6} \times 100/\text{P} \times 4 \\ &= \text{HDC}/\text{P} \times 0.016\end{aligned}$$

- HDC = Highest drug concentration to be tested (µg/ml)
- AV = Agar volume in each plate
- DDF = Double dilution factor. This was 2 for all drugs, because a double amount of drug was required in the first plate from where 1:2 dilutions were made to the subsequent plates
- CF = Conversion factor i.e. 10^{-6} to convert microgram to gram
- P = % purity of antimicrobial powder, as indicated by the manufacturer
- R = number of times experiment needs to be repeated

The calculated amount of nybomycin powder was dissolved with 1.6 ml DMSO in a sterile 30 ml universal container and further diluted with 14.4 ml sterile PBS to give a final volume of 16 ml.

For agar plate pouring, four rows of sterile 30 ml universal containers were positioned on a tube rack, with six containers in each row. The first five containers in each row represented the five nybomycin concentrations that were used and the sixth container the drug-free positive-growth control. Two milliliters PBS were added to all the containers, except the first four containers representing the highest nybomycin concentration. Four milliliters antibiotic stock solution were added to these four containers. Two-fold serial dilutions were performed by removing 2 ml from the first container and adding it to the second container. It was then thoroughly mixed by pipetting up-and-down five times and thereafter 2 ml were transferred to the third container. This procedure was repeated up to the fifth container after which 2 ml antibiotic containing solution was discarded so that 2 ml fluid remained behind in all six containers of each row.

Eighteen milliliters of freshly prepared Middlebrook 7H10 agar, maintained in its liquid state in a water bath set at 45 °C, was next added to each 2 ml of antibiotic working solution in the 30 ml container. Immediately after 18 ml of media was added to any particular container, its screw-cap was tightly closed and the container carefully tilted three times to mix its contents. The cap was then removed and the full 20 ml antibiotic-containing media promptly poured into an empty 90 mm Petri dish before the agar had time to solidify. The agar plates were closed and left on the benchtop until the agar solidified, after which the plates were

sealed in plastic bags and stored at 4-8 °C until use. Plates were used within one week of preparation.

3.6.1.2 Preparation of *M. tuberculosis* inoculum and inoculation of agar plates

One hundred microliters of *M. tuberculosis* V9124 stock culture was inoculated into 4 ml Middlebrook 7H9 broth in a 30 ml square media bottle (Nalgene, Thermo Fisher Scientific, Massachusetts, USA), and incubated in a shaking incubator at 37 °C until it reached a turbidity with an optical density at 600 nm (OD_{600nm}) of 1.0. This corresponds to approximately 1×10^8 CFU/ml. Next a 1:100 dilution was made by mixing 0.1 ml of this bacterial suspension with 9.9 ml sterile Middlebrook 7H9 broth, followed by a further 1:200 dilution through mixing 0.05 ml of the 1:100 dilution with 99.95 ml Middlebrook 7H9 broth. The final 1:20,000 dilution therefore contained approximately 1.5×10^3 CFU/ml.

Four milliliters of this low-density bacterial suspension was then dispensed into each of 25 separate 30 ml square media bottles. Only 20 square media bottles were to be used for the mutant selection, the extra five were used for positive growth controls and for determining the OD_{600nm} . The cultures were incubated at 37 °C in a shaking incubator until an OD_{600nm} of 0.9 to 1 was reached, representing the end of the logarithmic growth phase. The full contents of each bottle was then decanted directly into a separate 50 ml PPT. Each culture was mixed for 60 seconds with the vortex mixer and then sonicated twice (Misonix Sonicator s-4000, QSonica, Connecticut, USA) for ten seconds at an amplitude of 10%, reaching a power level of 10 Watt. A ten second pause was taken between

the two 10 second sonication periods to tilt the PPT three times to mix its contents. Each culture was then centrifuged at 3434.496 x g for 10 minutes at room temperature and its supernatant was decanted. After resuspending the sediment in 200 µl PBS with 1% Tween-80, each PPT was again mixed thoroughly by pipetting up-and-down 5 to 10 times to break down the clumps. The entire 200 µl volume of each of the 20 cultures were plated and spread out onto a separate antibiotic-containing plate. Similarly, the entire 200 µl volume of each of the five positive control cultures were plated and spread out onto separate antibiotic-free plates.

3.6.1.3 Culture, incubation and reading of results

Inoculated agar plates were kept in the biosafety cabinet for up to an hour until the surface was dry. The plates were then heat-sealed in gas-permeable plastic bags and incubated at 37 °C in 5% CO₂. After one week of incubation, the cultures were transferred to a hot room with a temperature of 37 °C and ambient air for an additional 2 to 4 weeks of incubation. Plates were read after 3 to 4 weeks' incubation, or later depending on when the positive controls indicated growth. Any growth on the plates containing 2 and 4 µg/ml nybomycin was regarded as potential drug-resistant mutants. Potential drug-resistant mutants were picked off and their level of resistance to nybomycin confirmed by agar dilution MIC determination with the multipoint inoculator as described in section 3.1.5.

3.6.2 Whole genome sequencing of *M. tuberculosis* mutants with increased nybomycin MICs

Whole genome sequencing (WGS) was performed in collaboration with the Kwazulu-Natal Research Innovation and Sequencing Platform in Durban, South Africa.

Two copies of the wild type *M. tuberculosis* V9124 isolate, five mutants displaying a nybomycin MIC of 2 µg/ml and five mutants displaying a nybomycin MIC of 4 µg/ml were selected for further investigation by WGS and comparative genomic analysis. DNA extraction was performed using the CTAB method and the quality of the extracted DNA confirmed with gel electrophoresis and the Nanodrop instrument, as described in sections 3.2.2 and 3.2.3. The Illumina MiSeq platform (Illumina, San Diego, USA) was employed and the following three basic steps were followed: (1.) library preparation, (2.) cluster generation and (3.) sequencing.

3.6.2.1 Library preparation

Transposomes are enzymes with open DNA ends that can insert themselves randomly into strands of DNA. The Nextera XT DNA Library Preparation Kit (Illumina, San Diego, USA) used in this study, employs a transposome designed to first fragment input DNA, leaving a staggered cut at the site where it inserted itself, and then to ligate partial adapter sequences to these single-stranded DNA overlays. This modified transposition reaction is called tagmentation and enables dual-index sequencing of pooled libraries. Three additional motifs are

subsequently inserted during a reduced cycle amplification process by using the partial adaptor sequences. This includes both sequencing primer binding sites for forward and reverse primers and index sequences that allows for pooling of libraries by enabling *in silico* sample identification and sorting. It further includes adapter regions that are either the same (i5) or complementary (i7) to the oligonucleotides fixed to the surface of the flow cell slide used downstream during cluster formation.

Sample preparation. Input DNA first had to be quantified and its quality confirmed as the Nextera XT kit is optimized to start with 1 ng DNA. The Qubit dsDNA HS Assay system (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA) is a fluorometric-based method and was used to quantify each DNA sample. Hereafter individual DNA samples were diluted in molecular-grade water to reach the appropriate starting concentration of 0.2 ng/μl.

Tagmentation of gDNA. Five microliters of normalized gDNA were added and mixed with 10 μl Tagment DNA Buffer[®] into each well of a MicroAmp 96-well PCR plate (Thermo Fisher Scientific, Massachusetts, USA). Next 5 μl of Amplicon Tagment Mix[®] was added and the plate centrifuged at 280 x g and 20 °C for 60 seconds. Thereafter the plate was placed on a preheated Veriti thermocycler (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) and its contents incubated at 55 °C for 5 minutes whereafter the temperature was decreased to at 10 °C (holding temperature). Five microliters Neutralizing Tagment Buffer[®] was subsequently mixed into each sample and the plate again centrifuged at 280 x g at 20 °C for 60 seconds. Tagmentation was completed with a final

incubation step at room temperature for 5 minutes. The result was 25 μ l neutralized and tagmented genomic DNA per sample.

Amplification of tagmented genomic DNA libraries. Five microliters each of Index 1 (i7) adapter and Index 2 (i5) adapter were added to each tagmented genomic DNA sample followed by 15 μ l Nextera PCR Master Mix. The final volume in each well was therefore 50 μ l. The MicroAmp 96-well PCR plate was centrifuged at 280 x g at 20 °C for 60 seconds and PCR amplification performed with a Veriti thermocycler under the conditions stipulated in table 3.12

Table 3.12 PCR conditions for amplification of tagmented DNA.

	Temperature	Duration
Initial denaturation	72 °C	3 minutes
	95 °C	30 seconds
*Denaturation	95 °C	10 seconds
*Annealing	55 °C	30 seconds
*Extension	72 °C	30 seconds
Final extension	72 °C	5 minutes
Holding temperature	10 °C	-

*12 cycles

Library cleanup with AMPure XP beads. The 96-well PCR plate was first centrifuged at 280 x g at 20 °C for 60 seconds where after the full contents of each well was transferred to a 0.8 ml well of a MicroAmp 96-well PCR plate. Next 30 μ l AMPure XP beads (Agencourt AMPure XP, Beckman Coulter Genomics, Minnesota, USA) were added to each well and the reagents mixed on a plate shaker at 695.485 x g for 2 minutes. After incubation at room temperature for

5 minutes, the PCR plate was positioned on a magnetic stand to allow the liquid to settle and become clear (approximately 2 minutes). The supernatant was aspirated and discarded from each well and the remaining contents were washed twice by adding 200 μ l 80% ethanol to each well and letting it stand for 30 seconds on the magnetic stand, followed by aspiration and discarding of the supernatant. Care was taken to remove all the residual ethanol from each well by carefully aspirating through a 20 μ l pipette tip. The plate was then allowed to air-dry on the magnetic stand for 15 minutes after which 52.5 μ l Resuspension Buffer was added to each well and the contents mixed by shaking the plate at 1800 revolutions per minute for 2 minutes. The midi plate was next incubated at room temperature for 2 minutes and placed back on the magnetic stand until the liquid was clear again (approximately 2 minutes). Finally, 50 μ l supernatant was transferred from each well to a new MicroAmp 96-well PCR plate.

Check library size distributions. To assess the library size distributions of each cleaned library, 1 μ l of neat library DNA was analyzed with the Labchip GX touch (PerkinElmer, Massachusetts, USA). Each sample was quantified using the Qubit instrument as described earlier.

Normalization of libraries by concentration. The quantity of each of the 12 libraries was normalized to 4 nM in an attempt to ensure that the pooled library will have an equal representation.

Pooling of libraries. The PCR plate was first centrifuged at 1000 x g at 20 °C for 60 seconds after which 5 μ l of each 4 nM library was transferred to a single clean

Eppendorf tube. The pooled libraries were then diluted to a final concentration of 12 pM according to the loading concentration required for the Illumina MiSeq sequencing platform. PhiX Control v3 (Illumina, San Diego, USA) was included as an internal sequencing control. This adapter-ligated library was derived from the small, well-characterized PhiX genome. The PhiX library provides a quality control for cluster generation, sequencing, and alignment. To accommodate for the low diversity of the *M. tuberculosis* genome, PhiX was added to the pooled library to a final concentration of 10%.

3.6.2.2 Cluster generation

Clustering is the process whereby clonal groups of template DNA are produced and attached to the surface of a flow cell (Illumina, San Diego, USA)²³¹. This on-board process ensures that multiple copies of each DNA fragment are available for sequencing²³¹. The flow cell is a glass slide with several physically separated lanes, each coated with two types of oligonucleotides: one type (P5) is complementary to the oligonucleotide sequence on one end of the DNA fragment (i5) and the other type (P7) is complementary to the oligonucleotide sequence on the other end of the DNA fragment (i7)²³¹.

The pooled single-stranded DNA library was loaded into a flow cell of the onboard cluster module within the MiSeq instrument. Single DNA fragments were then captured by the surface-bound oligonucleotides (P5) on the flow cell followed by the formation of complementary strands by polymerase enzyme. The resultant double-stranded DNA was denatured and the original template washed away. The

non-binding end of the DNA fragment then bent over to hybridize to the second type of oligonucleotide (P7) on the flow cell surface. Polymerase enzyme once again produced a complementary strand, thereby forming a double-stranded bridge. Subsequent denaturation resulted in two separate DNA molecules attached to the flow cell surface. This isothermal process is called “bridge” amplification and through repeated cycles of denaturation and extension results in the amplification of single DNA strands into millions of separate clonal clusters across the flow cell surface, each cluster containing approximately 1000 copies. Bridge amplification and cluster formation were completed after washing away of all the reverse strands, leaving only forward strands for sequencing.

3.6.2.3 Gene sequencing

The Illumina MiSeq sequencing platform is a next generation sequencing (NGS) technology that uses a method similar to capillary electrophoresis sequencing^{126,232}. The major advancement with NGS is that millions of DNA fragments are sequenced simultaneously instead of just one fragment at a time¹²⁶. For this study, 2 x 250 bp paired-end sequencing was performed using the MiSeq v2 (500 cycle) reagent kit (Illumina, San Diego, USA). This kit provides up to 15 million reads per run and a maximum output of 8.5 giga base pairs¹²⁶.

DNA polymerase enzyme catalyzes the addition of four fluorescently labeled dNTPs into growing nucleic acid chains during cycles of DNA synthesis¹²⁶. Laser excitation and imaging are used to identify each fluorescently labeled dNTP as soon as it is incorporated into the growing nucleic acid chain¹²⁶. The intensity of

the signal thus produced is measured and used by the MiSeq software for creating chromatogram peaks and thereby for assigning nucleobases¹²⁶. After each read, the nucleotide label is enzymatically cleaved to permit the next dNTP to be incorporated during the following cycle¹²⁶.

3.6.3 Bioinformatics analysis of whole genome sequencing results

Bioinformatics analysis of gene sequences was performed in collaboration with the KwaZulu-Natal Research Innovation and Sequencing Platform in Durban, South Africa.

Raw sequencing reads were obtained in FASTQ format from the Illumina MiSeq instrument. Quality filtering and trimming were performed using the Trimmomatic software tool (Rheinisch-Westfälische Technische Hochschule, Aachen, Germany)^{233,234}. This process involved both the removal of adaptor sequences used for sequencing and the deletion of regions with low base call quality, as determined by Phred+33 scoring²³⁴. Sequences were therefore trimmed from their ends to the loci where low-quality base calling appears^{233,234}. Hereafter, trimmed genome sequence reads from the 12 genomic DNA samples were each mapped against the *M. tuberculosis* H37Rv reference genome (Ensembl number: ASM19595v2) using the Burrows-Wheeler Aligner software package²³⁵⁻²³⁸. Single nucleotide polymorphism (SNP) calling was conducted for each of the samples using Samtools software^{239,240}. To correct for “strand bias” characteristic to Illumina sequencing data, all SNPs were further filtered using BCFtools^{239,241}. The resultant SNP data was analyzed with the TBProfiler tool to identify the presence

of known drug resistance mutations, as previously described^{142,242}. The TBProfiler tool is able to identify most of the common SNPs that confer resistance to isoniazid, rifampicin, pyrazinamide, ethambutol, fluoroquinolones, aminoglycosides, capreomycin, and ethionamide^{142,242}. SNPs were also annotated and analyzed with the snpEff software and Variant Effect Predictor (VEP) online tools to help identify and describe their basic effects^{243,244}.

All SNPs potentially resulting in amino acid changes were tabulated using Excel and those common to all 10 mutant sequences, as well as the 2 wild type control sequences, were removed. SNPs present in PE and PPE genes were also filtered out. PE and PPE are two large mycobacteria-specific families of genes that contain proline-glutamic acid (PE) or proline-proline-glutamic acid (PPE) motifs. Many of the PE and PPE genes appear to play a role in antigenic variation and immune modulation, but they are remarkably polymorphic and therefore difficult to study²⁴⁵. SNPs present in the mutant sequences, but not in the wild type sequences, were considered as potentially associated with *M. tuberculosis* mutants with increased nybomycin MICs.

CHAPTER 4 – RESULTS

4.1 Introduction

Following previous work published by Hiramatsu *et al* and Arai *et al*, the *in vitro* inhibitory effect of nybomycin was explored in three *Mycobacterium tuberculosis* clinical isolates^{143,165,166}. The results thereof prompted the screening of susceptibility of a diverse collection of bacterial isolates to various quinoline compounds. *M. tuberculosis* was subsequently further investigated with IS 6110 RFLP fingerprinting, confirmatory nybomycin susceptibility testing and the testing of DNM-2, a compound closely related to nybomycin. Mycobacterial DNA gyrase enzyme was investigated as the potential site where nybomycin binds to and exert its effect. This was attempted through *gyrA* quinolone resistance-determining region (QRDR) sequencing and *in silico* investigations. Nybomycin resistant mutants were also obtained and their genomes sequenced for comparison with the wild type organism to elucidate the possible mechanism of resistance of *M. tuberculosis* to nybomycin.

4.2 *In vitro* inhibitory effect of quinolones on various bacterial species

After encouraging *in vitro* susceptibility results were obtained with nybomycin and *M. tuberculosis*, the study was expanded with testing of a larger and more diverse collection of *M. tuberculosis* isolates as well as various other bacterial species. The goal was to determine whether (1.) nybomycin exhibits an inhibitory effect on any of the bacterial species and (2.) whether nybomycin acts as a reverse

antibiotic in these same isolates. In a similar fashion, we also included and investigated four quinoline containing drugs that are currently registered for human use: quinine, chloroquine, mefloquine and primaquine.

4.2.1 *M. tuberculosis*

Three *M. tuberculosis* isolates were screened for susceptibility ($\mu\text{g/ml}$) to nybomycin using Middlebrook 7H9 broth with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt as growth detecting reagent. This was done in triplicate and the results are displayed in table 4.1.

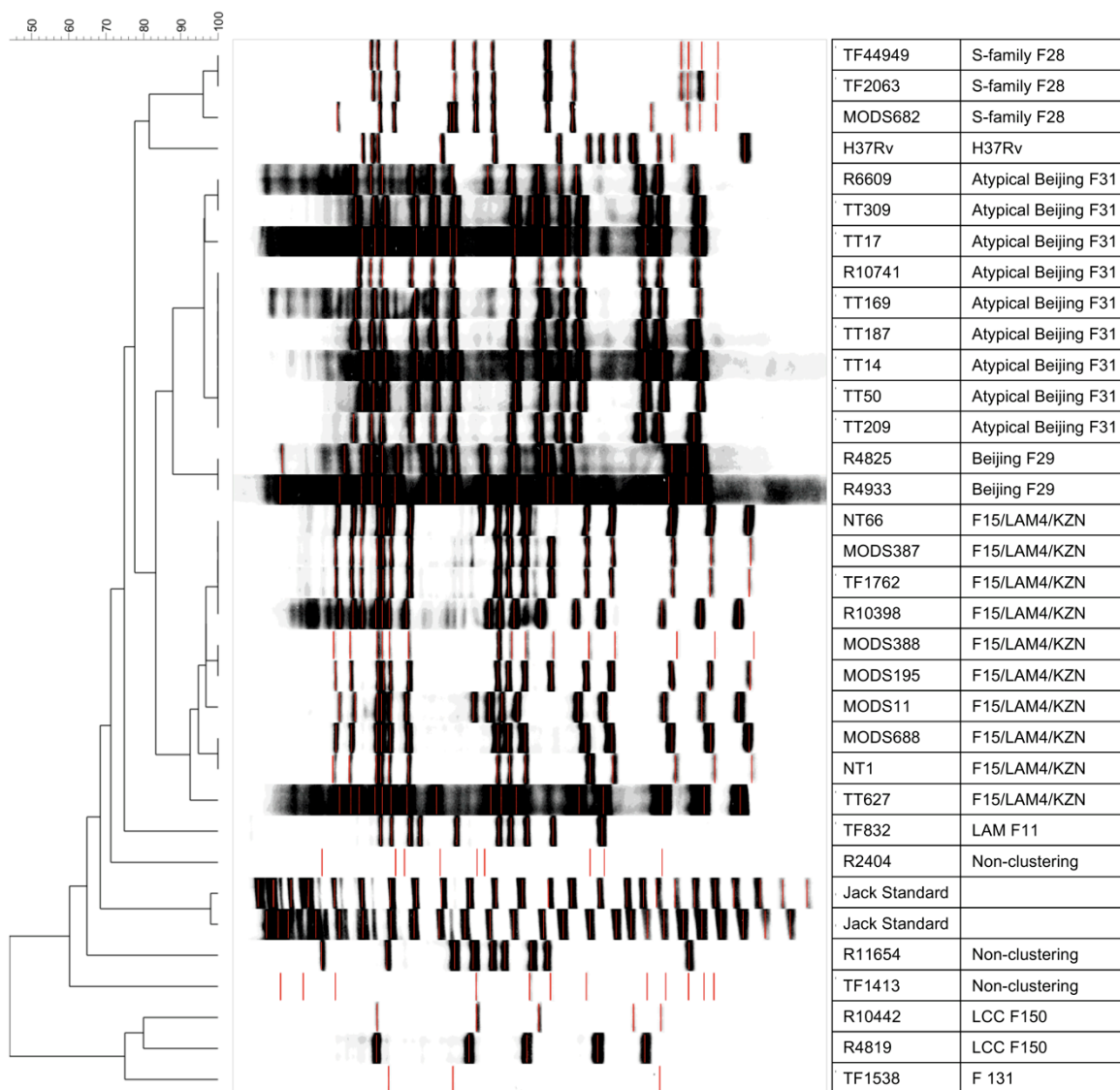
Table 4.1 Nybomycin minimum inhibitory concentrations ($\mu\text{g/ml}$) of fluoroquinolone-susceptible and fluoroquinolone-resistant *M. tuberculosis* isolates.

<i>M. tuberculosis</i> Isolate number	Phenotypic classification	A	B	C	Final MIC
TF 1538	Susceptible	6.25	6.25	3.125	6.25
MODS 11	MDR	12.5	12.5	25	12.5
MODS 387	XDR	1.5625	1.5625	0.78125	1.5625

Nybomycin minimum inhibitory concentration (MIC) values for the fluoroquinolone-susceptible isolates TF 1538 and MODS 11 were two to three double dilutions higher than that of MODS 387, the fluoroquinolone-resistant isolate. The low nybomycin MIC ($\mu\text{g/ml}$) seen with the XDR isolate, as well as the inverse relationship between fluoroquinolone susceptibility and nybomycin MIC ($\mu\text{g/ml}$) values, provided an impetus for further investigation of nybomycin activity against *M. tuberculosis*.

In order to compare the effect of nybomycin on fluoroquinolone-susceptible and fluoroquinolone-resistant *M. tuberculosis* isolates more thoroughly, and to ensure global appeal and applicability of the results of this study, a collection of 32 *M. tuberculosis* isolates was assembled consisting of a wide variety of IS 6110 RFLP genotypes and drug susceptibility profiles. The most common IS 6110 RFLP banding pattern was F15/LAM4/KZN (n=10) followed by Atypical Beijing F31 (n=9), S-family F28 (n=3), non-clustering banding patterns (n=3), Beijing F29 (n=2), LCC F150 (n=2) and one isolate each of F131 and LAM F11. The IS 6110 RFLP banding patterns, IS 6110 RFLP family name designations and dendrogram results are displayed in figure 4.1.

Figure 4.1 IS 6110 RFLP banding patterns, IS 6110 RFLP family name designations and dendrogram results.



Drug susceptibility testing results obtained with Middlebrook 7H10 agar dilution and multipoint inoculation^{183,184,189} were used to categorize the *M. tuberculosis* isolates into DS (n=3), MDR (n=7), pre-XDR (n=6) and XDR (n=15) phenotypes, as displayed in table 4.2. *M. tuberculosis* susceptibility to nybomycin was investigated in the same manner and MIC ($\mu\text{g/ml}$) results are also displayed in

table 4.2. All MIC ($\mu\text{g/ml}$) investigations were done in triplicate with *M. tuberculosis* H37Rv as susceptible control. Raw data for MIC investigations are displayed in Appendix B.

Table 4.2 *M. tuberculosis* drug susceptibility results obtained with the multipoint inoculation method.

Isolate Number	IS 6110 RFLP Genotype	Agar dilution MIC ($\mu\text{g/ml}$)					Drug Resistance Phenotype
		Isoniazid	Rifampicin	Amikacin	Ofloxacin	Nybomycin	
H37Rv	H37Rv	0.125	0.25	1	0.5	1	Susceptible
TF 1538	F131	0.0625	0.125	1	0.5	1	Susceptible
TF 1413	Non-clustering	0.0625	0.25	1	0.5	1	Susceptible
TF 832	LAM F11	0.125	0.25	1	0.5	1	Susceptible
MODS 11	F15/LAM4/KZN	64	2	2	0.5	1	MDR
TT 14	Atypical Beijing F31	16	> 64	1	0.5	1	MDR
TT 17	Atypical Beijing F31	8	> 64	1	0.5	1	MDR
TF 44949	S-family F28	16	> 64	1	0.5	0.25	MDR
MODS 688	F15/LAM4/KZN	> 64	64	2	0.5	1	MDR
TF 2063	S-family F28	16	64	0.25	0.5	0.25	MDR
MODS 682	S-family F28	16	> 64	1	0.5	1	MDR
TT 50	Atypical Beijing F31	32	> 64	1	4	1	Pre-XDR
TT 309	Atypical Beijing F31	32	64	>64	0.5	1	Pre-XDR
TT 627	F15/LAM4/KZN	> 64	> 64	1	4	1	Pre-XDR
NT 1	F15/LAM4/KZN	32	2	1	16	1	Pre-XDR
NT 66	F15/LAM4/KZN	32	> 64	1	8	1	Pre-XDR
R 11 654	Non-clustering	32	64	1	8	0.5	Pre-XDR
TT 169	Atypical Beijing F31	32	64	> 64	16	1	XDR
R 2 404	Non-clustering	2	16	> 64	8	0.25	XDR
R 4 825	Beijing F29	32	> 64	> 64	8	1	XDR
TT 187	Atypical Beijing F31	32	64	> 64	16	1	XDR
TT 209	Atypical Beijing F31	32	32	> 64	16	1	XDR
R 6 609	Atypical Beijing F31	32	16	> 64	16	0.5	XDR
R 10 741	Atypical Beijing F31	> 64	> 64	> 64	16	1	XDR
R 10 398	F15/LAM4/KZN	32	> 64	> 64	4	1	XDR
R 4 819	LCC F150	8	64	4	4	1	XDR
R 10 442	LCC F150	16	> 64	> 64	2	1	XDR
MODS 388	F15/LAM4/KZN	32	> 64	> 64	4	0.5	XDR
MODS 387	F15/LAM4/KZN	64	> 64	> 64	4	0.5	XDR
TF 1762	F15/LAM4/KZN	64	> 64	> 64	4	0.5	XDR
MODS 195	F15/LAM4/KZN	32	> 64	> 64	4	0.5	XDR
R 4 933	Beijing F29	16	> 64	> 64	8	0.5	XDR

The collection of 32 *M. tuberculosis* isolates comprised of 12 fluoroquinolone-susceptible and 20 fluoroquinolone-resistant phenotypes. Nybomycin MIC values of *M. tuberculosis* isolates ranged between 0.25 and 1.0 µg/ml and did not show much variation across the different resistance phenotypes. These results differ markedly from the initial screening results obtained with the MTT assay. The MTT assay was therefore employed a second time to verify the results obtained with the agar dilution with multipoint inoculation by investigating 23 of the 32 *M. tuberculosis* isolates. At this time, a deoxynybomycin derivative (DNM-2) donated by Paul Hergenrother (Department of Chemistry, University of Illinois, USA)¹⁷⁹, was also tested using the same 23 isolates and methodology. All MIC (µg/ml) investigations were performed in triplicate, with *M. tuberculosis* H37Rv as control. The final nybomycin and DNM-2 MIC results are shown in table 4.3 and raw data for MIC investigations are displayed in Appendix B.

Table 4.3 MIC ($\mu\text{g/ml}$) values of *M. tuberculosis* isolates for nybomycin and a deoxynybomycin derivative (DNM-2) obtained with the MTT assay

Isolate Number	IS 6110 RFLP Genotype	Drug Resistance Phenotype	Multipoint inoculator method	MTT assay	
			Nybomycin ($\mu\text{g/ml}$)	Nybomycin ($\mu\text{g/ml}$)	DNM-2 ($\mu\text{g/ml}$)
H37Rv	H37Rv	Susceptible	1	2	1.5
TF 1538	F131	Susceptible	1	2	3
TF 1413	Non-clustering	Susceptible	1	2	3
TF 832	LAM F11	Susceptible	1	2	3
MODS 11	F15/LAM4/KZN	MDR	1	2	3
TT 17	Atypical Beijing F31	MDR	1	1	3
TF 44949	S-family F28	MDR	0.25	2	3
MODS 688	F15/LAM4/KZN	MDR	1	2	3
TF 2063	S-family F28	MDR	0.25	2	3
TT 50	Atypical Beijing F31	Pre-XDR	1	1	3
TT 309	Atypical Beijing F31	Pre-XDR	1	1	3
TT 627	F15/LAM4/KZN	Pre-XDR	1	1	1.5
NT 1	F15/LAM4/KZN	Pre-XDR	1	2	3
NT 66	F15/LAM4/KZN	Pre-XDR	1	1	1.5
TT 169	Atypical Beijing F31	XDR	1	1	1.5
R 2 404	Non-clustering	XDR	0.25	2	1.5
R 4 825	Beijing F29	XDR	1	1	1.5
R 6 609	Atypical Beijing F31	XDR	0.25	1	1.5
R 10 741	Atypical Beijing F31	XDR	1	1	3
R 4 819	LCC F150	XDR	1	2	1.5
R 10 442	LCC F150	XDR	1	2	3
MODS 388	F15/LAM4/KZN	XDR	0.5	1	1.5
MODS 387	F15/LAM4/KZN	XDR	0.5	2	1.5

M. tuberculosis isolates displayed nybomycin MIC values that ranged between 0.25 and 1.0 $\mu\text{g/ml}$ with the agar dilution method and between 1.0 and 2.0 $\mu\text{g/ml}$ with the MTT assay. For DNM-2, MIC values ranged between 1.5 and 3.0 $\mu\text{g/ml}$. From these results, there appears to be no direct or inverse relationship between MIC ($\mu\text{g/ml}$) values obtained for ofloxacin, nybomycin and DNM-2 with any of the tested *M. tuberculosis* isolates.

**4.2.2 *Neisseria gonorrhoeae*, *Escherichia coli*, *Klebsiella pneumoniae*,
Enterobacter cloacae, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*,
Enterococcus faecalis and *Staphylococcus aureus***

A selection of Gram-positive and Gram-negative bacterial isolates exhibiting a wide range of susceptibility to fluoroquinolones was included for investigation. Drug susceptibility testing was performed in triplicate using agar dilution methodology with multipoint inoculation. *N. gonorrhoeae* ATCC 49226 and *E. coli* ATCC 25722 served as controls. MIC ($\mu\text{g/ml}$) results for the different bacterial isolates are presented in table 4.4 and table 4.5.

Table 4.4 MIC ($\mu\text{g/ml}$) values of *N. gonorrhoeae* isolates for ciprofloxacin, nalidixic acid, nybomycin, quinine, chloroquine, mefloquine and primaquine with the multipoint inoculation method

<i>N. gonorrhoeae</i> Isolate number	Ciprofloxacin	Nalidixic acid	Nybomycin	Quinine	Chloroquine	Mefloquine	Primaquine
ATCC 49226	< 0.0625	2	4	128	> 128	16	128
526	< 0.0625	2	1	128	> 128	16	128
840	< 0.0625	2	1	128	> 128	4	64
924	< 0.0625	2	2	128	> 128	4	64
556	< 0.0625	16	2	128	> 128	8	128
373	< 0.0625	8	8	128	> 128	16	> 128
310	< 0.0625	2	0.5	128	> 128	4	64
360	0.25	8	4	128	> 128	16	> 128
20	0.25	8	4	128	> 128	4	64
172	0.25	16	2	128	> 128	4	128
391	0.25	8	1	128	> 128	4	64
277	0.5	8	4	128	> 128	16	128
342	0.5	8	2	128	> 128	16	128
524	0.5	16	2	128	> 128	16	64
938	0.5	8	0.25	128	> 128	2	32
336	1	8	2	128	> 128	16	128
345	1	16	2	128	> 128	16	128
462	1	8	0.5	> 128	> 128	4	64
219	2	8	4	128	> 128	16	64
74	2	8	4	> 128	> 128	16	128
227	2	16	4	> 128	> 128	16	> 128
108	4	16	4	> 128	> 128	16	> 128
119	4	16	2	> 128	> 128	16	128
236	4	8	2	128	> 128	16	> 128
251	4	16	4	128	> 128	16	> 128
267	4	16	4	128	> 128	16	> 128
296	4	8	8	128	> 128	16	128
688	4	8	4	128	> 128	16	> 128
819	4	8	4	> 128	> 128	16	128
766	4	8	0.5	> 128	> 128	0.5	16

For *N. gonorrhoeae* isolates, the ciprofloxacin MIC values ranged between < 0.0625 and 4 $\mu\text{g/ml}$; and the nalidixic acid MIC values between 2 and 16 $\mu\text{g/ml}$. MIC values with nybomycin ranged between 0.25 and 8.0 $\mu\text{g/ml}$ and showed no direct or inverse relationship with MIC values from the quinolones. One isolate had

a mefloquine MIC of 0.5 µg/ml and another isolate had a mefloquine MIC of 2 µg/ml. Mefloquine MIC values for the other 28 isolates ranged between 4 and 16 µg/ml. All *N. gonorrhoeae* isolates displayed quinine MIC values \geq 128 µg/ml and all chloroquine MIC values were $>$ 128 µg/ml. Twenty-eight *N. gonorrhoeae* isolates exhibited primaquine MIC values between 64 and \geq 128 µg/ml, while one isolate had a primaquine MIC of 32 µg/ml and another a primaquine MIC of 16 µg/ml. The mostly high MIC values obtained with quinine, chloroquine, mefloquine and primaquine provided insufficient incentive for further investigation of these drugs against *N. gonorrhoeae*. Nybomycin MIC values are moderately lower and may warrant further scientific exploration.

Table 4.5 MIC ($\mu\text{g/ml}$) values of *E. coli*, *K. pneumoniae*, *E. cloacae*, *P. aeruginosa*, *A. baumannii*, *S. aureus* and *E. faecalis* isolates for ciprofloxacin, nalidixic acid, nybomycin, quinine, chloroquine, mefloquine and primaquine with the multipoint inoculation method.

Test organism	Ciprofloxacin	Nalidixic acid	Nybomycin	Quinine	Chloroquine	Mefloquine	Primaquine
<i>E. coli</i> ATCC 25722	< 0.125	8	> 16	128	> 128	64	> 128
<i>E. coli</i> no. 1	< 0.125	16	> 16	128	> 128	> 64	> 128
<i>E. coli</i> no. 2	2	16	> 16	128	> 128	> 64	> 128
<i>E. coli</i> no. 3	2	16	> 16	128	> 128	> 64	> 128
<i>E. coli</i> no. 4	32	16	> 16	128	> 128	> 64	> 128
<i>E. coli</i> no. 5	32	16	> 16	128	> 128	> 64	> 128
<i>K. pneumoniae</i> no. 1	< 0.125	16	> 16	128	> 128	> 64	> 128
<i>K. pneumoniae</i> no. 2	0.25	16	> 16	128	> 128	> 64	> 128
<i>K. pneumoniae</i> no. 3	2	16	> 16	128	> 128	> 64	> 128
<i>K. pneumoniae</i> no. 4	16	16	> 16	128	> 128	> 64	> 128
<i>E. cloacae</i> no. 1	< 0.125	8	> 16	128	> 128	> 64	> 128
<i>E. cloacae</i> no. 2	8	8	> 16	128	> 128	> 64	> 128
<i>E. cloacae</i> no. 3	32	8	> 16	128	> 128	> 64	> 128
<i>E. cloacae</i> no. 4	32	16	> 16	128	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 1	0.25	8	> 16	128	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 2	0.5	8	> 16	64	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 3	8	8	> 16	64	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 4	16	8	> 16	64	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 5	16	8	> 16	64	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 6	16	8	> 16	64	> 128	> 64	> 128
<i>P. aeruginosa</i> no. 7	32	8	> 16	128	> 128	> 64	> 128
<i>A. baumannii</i> no. 1	0.25	8	> 16	128	> 128	64	> 128
<i>A. baumannii</i> no. 2	2	8	> 16	128	> 128	64	> 128
<i>A. baumannii</i> no. 3	4	8	> 16	128	> 128	> 128	> 128
<i>A. baumannii</i> no. 4	16	8	> 16	128	> 128	64	> 128
<i>A. baumannii</i> no. 5	> 32	16	8	> 128	> 128	32	> 128
<i>S. aureus</i> no. 1	0.5	32	4	> 128	> 128	32	> 128
<i>S. aureus</i> no. 2	0.5	32	8	> 128	> 128	32	> 128
<i>S. aureus</i> no. 3	2	32	4	> 128	> 128	32	> 128
<i>S. aureus</i> no. 4	> 32	32	4	> 128	> 128	32	> 128
<i>E. faecalis</i> no. 1	1	32	16	> 128	> 128	32	> 128
<i>E. faecalis</i> no. 2	2	32	16	> 128	> 128	32	> 128
<i>E. faecalis</i> no. 3	> 32	32	8	> 128	> 128	32	> 128
<i>E. faecalis</i> no. 4	> 32	32	8	> 128	> 128	32	> 128

Table 4.5 displays MIC ($\mu\text{g/ml}$) values obtained with five *E. coli*, four *K. pneumoniae*, four *E. cloacae*, seven *P. aeruginosa*, five *A. baumannii* four *S.*

aureus and four *E. faecalis* isolates. Each genus tested included fluoroquinolone-susceptible and fluoroquinolone-resistant isolates. All Gram-negative isolates displayed nybomycin MIC values $> 16 \mu\text{g/ml}$, except for one *A. baumannii* isolate with a nybomycin MIC of $8 \mu\text{g/ml}$. Nybomycin MIC values for the *E. faecalis* isolates ranged between 8 and $16 \mu\text{g/ml}$, and for *S. aureus* between 4 and $8 \mu\text{g/ml}$. No direct or inverse relationship was observed between MIC values of quinolones and those of nybomycin. All Gram-negative isolates had mefloquine MIC values of $\geq 64 \mu\text{g/ml}$, except for one *A. baumannii* isolate with a mefloquine MIC of $32 \mu\text{g/ml}$. All Gram-positive isolates displayed a mefloquine MIC of $32 \mu\text{g/ml}$. All Gram-negative isolates had MIC values for quinine of $128 \mu\text{g/ml}$, except for all seven *P. aeruginosa* isolates that exhibited a quinine MIC of $64 \mu\text{g/ml}$. All Gram-positive isolates displayed quinine MIC values of $> 128 \mu\text{g/ml}$. All Gram-positive and Gram-negative isolates displayed chloroquine and primaquine MIC values of $> 128 \mu\text{g/ml}$.

4.3 *In silico* effect of nybomycin and DNM-2 on *M. tuberculosis* gyrase enzyme

4.3.1 *gyrA* QRDR sequencing

The most common *gyrA* QRDR mutations in the collection of 32 *M. tuberculosis* isolates were identified to inform the subsequent *in silico* investigations. This was done in order to ensure that insights obtained through computational methods can be linked to the previous *in vitro* susceptibility test results. DNA sequencing results are presented in table 4.6.

Table 4.6 DNA sequencing results of the *gyrA* QRDR from 32 *M. tuberculosis* isolates.

Isolate Number	IS 6110 RFLP Genotype	Drug Resistance Phenotype	Mutations			
			Codon	Codon Sequence Change	Amino Acid Change	Codon Change
H37Rv	H37Rv	Susceptible	----	----	----	----
TF 1538	F131	Susceptible	----	----	----	----
TF 1413	Non-clustering	Susceptible	----	----	----	----
TF 832	LAM F11	Susceptible	----	----	----	----
MODS 11	F15/LAM4/KZN	MDR	----	----	----	----
TT 14	Atypical Beijing F31	MDR	----	----	----	----
TT 17	Atypical Beijing F31	MDR	----	----	----	----
TF 44949	S-family F28	MDR	----	----	----	----
MODS 688	F15/LAM4/KZN	MDR	----	----	----	----
TF 2063	S-family F28	MDR	----	----	----	----
MODS 682	S-family F28	MDR	----	----	----	----
TT 50	Atypical Beijing F31	Pre-XDR	----	----	----	----
TT 309	Atypical Beijing F31	Pre-XDR	----	----	----	----
TT 627	F15/LAM4/KZN	Pre-XDR	91	TCG → CCG	Ala → Pro	A91P
NT 1	F15/LAM4/KZN	Pre-XDR	94	GAC → GGC	Asp → Gly	D94G
NT 66	F15/LAM4/KZN	Pre-XDR	90	GCG → GTG	Ala → Val	A90V
R 11 654	Non-clustering	Pre-XDR	94	GAC → TAC	Asp → Tyr	D94Y
TT 169	Atypical Beijing F31	XDR	94	GAC → CAC	Asp → His	D94H
R 2 404	Non-clustering	XDR	94	GAC → GGC	Asp → Gly	D94G
R 4 825	Beijing F29	XDR	94	GAC → GGC	Asp → Gly	D94G
TT 187	Atypical Beijing F31	XDR	94	GAC → GGC	Asp → Gly	D94G
TT 209	Atypical Beijing F31	XDR	94	GAC → AAC	Asp → Asn	D94N
R 6 609	Atypical Beijing F31	XDR	88	GGC → TGC	Gly → Cys	G88C
R 10 741	Atypical Beijing F31	XDR	94	GAC → GGC	Asp → Gly	D94G
R 10 398	F15/LAM4/KZN	XDR	90	GCG → GTG	Ala → Val	A90V
R 4 819	LCC F150	XDR	----	----	----	----
R 10 442	LCC F150	XDR	----	----	----	----
MODS 388	F15/LAM4/KZN	XDR	90	GCG → GTG	Ala → Val	A90V
MODS 387	F15/LAM4/KZN	XDR	90	GCG → GTG	Ala → Val	A90V
TF 1762	F15/LAM4/KZN	XDR	90	GCG → GTG	Ala → Val	A90V
MODS 195	F15/LAM4/KZN	XDR	90	GCG → GTG	Ala → Val	A90V
R 4 933	Beijing F29	XDR	94	GAC → GGC	Asp → Gly	D94G

As expected, only the fluoroquinolone-resistant isolates contained mutations in the QRDR of *gyrA*. However, both of the XDR isolates from the LCC F150 RFLP family had no mutations in the QRDR of *gyrA*. None of the ofloxacin resistant

isolates had more than one mutation. A90V and D94G were the most common codon changes and were present in six isolates each. There were also one each of D94H, D94Y, D94N, A91P and G88C codon changes.

4.3.2 Molecular docking investigations

The previously published X-ray crystal structure of *M. tuberculosis* gyrase (PDB ID: 5BS8) complexed together with DNA, moxifloxacin and a magnesium ion was reproduced and validated within its binding landscape, to assure the effectiveness of the molecular docking algorithm for further evaluation of binding energies with other ligands^{177,208,209}. Nybomycin, DNM-2 and ciprofloxacin ligands were subsequently constructed with the help of the ChemBioDraw software tool (CambridgeSoft, Massachusetts, USA) and oriented between DNA residues, similar to moxifloxacin²¹⁰. LibDock scores for nybomycin, DNM-2 and ciprofloxacin were compared to the results obtained with the validated moxifloxacin complex and all three ligands displayed scores higher than that obtained with moxifloxacin. At 152.12 kcal/mol, nybomycin displayed the highest docking score of all ligands evaluated in this study. The molecular docking scores are presented in table 4.7.

Table 4.7 Molecular docking scores and molecular weight of nybomycin, DNM-2, ciprofloxacin and moxifloxacin against wild type *M. tuberculosis* gyrase

Ligands	Molecular Weight	LibDock score (kcal/mol)
Nybomycin	298.30	152.12
DNM-2	296.33	148.69
Ciprofloxacin	331.35	148.24
Moxifloxacin	401.44	116.33

4.3.3 Molecular dynamics simulations

The binding free energies (kcal/mol) of nybomycin and DNM-2 were assessed by comparing them with that of ciprofloxacin. Both nybomycin and DNM-2 exhibited strong binding affinities to both wild type gyrase and mutants, with values ranging between -39.21 and -45.46 kcal/mol for nybomycin and between -28.50 and -44.87 kcal/mol for DNM-2. Binding free energies results are presented in table 4.8. The specific contribution of the amino acid residues coded by codons 90 and 94 of *M. tuberculosis* GyrA to overall ligand binding strength was evaluated by analyzing the per-residue interaction energy decomposition results, as presented in table 4.9. Residue 90 was found to play a comparatively bigger role than residue 94 in the overall binding strength of gyrase to all three ligands.

Table 4.8 Binding free energies of nybomycin, DNM-2 and ciprofloxacin against wild type and mutated *M. tuberculosis* gyrase.

Ligands	Binding free energies (kcal/mol) toward <i>M. tuberculosis</i> gyrase				
	Wild type	Mutants			
	(Ala90 and Asp94)	Ala90Val	Asp94Gly	Asp94His	Ala90Ser
Nybomycin	-40.74	-45.46	-39.21	-44.02	-39.97
DNM-2	-33.80	-44.87	-39.42	-28.50	-42.39
Ciprofloxacin	-38.72	-41.1	-22.36	-30.14	-43.61

Table 4.9 Per-residue interaction energy decomposition of *M. tuberculosis* gyrase in ligand binding for residue 90 and 94.

Ligands	Anchoring residues of <i>M. tuberculosis</i> GyrA	Per-residue interaction energy decomposition (kcal/mol) towards <i>M. tuberculosis</i> gyrase				
		Wild type (Ala90, Asp94)	Mutants			
			Ala 90 Val	Asp 94 Gly	Asp 94 His	Ala 90 Ser
Nybomycin	Residue no. 90	-0.152 +/- 0.094	-0.105 +/- 0.056	-0.023 +/- 0.031	-0.031 +/- 0.022	-0.125 +/- 0.061
	Residue no. 94	-0.003 +/- 0.009	-0.009 +/- 0.011	0.005 +/- 0.002	0.002 +/- 0.003	0.006 +/- 0.005
DNM-2	Residue no. 90	-0.099 +/- 0.080	-0.230 +/- 0.139	-0.050 +/- 0.059	-0.491 +/- 0.126	-0.030 +/- 0.044
	Residue no. 94	0.005 +/- 0.003	-0.004 +/- 0.006	0.005 +/- 0.003	-0.001 +/- 0.007	0.007 +/- 0.006
Ciprofloxacin	Residue no. 90	-0.368 +/- 0.163	-0.128 +/- 0.135	0.107 +/- 0.064	-0.181 +/- 0.380	-0.148 +/- 0.089
	Residue no. 94	0.014 +/- 0.007	-0.013 +/- 0.009	0.008 +/- 0.004	0.001 +/- 0.005	0.003 +/- 0.010

Finally, the residues involved in stabilizing ligands inside the binding pocket of *M. tuberculosis* gyrase containing a Ala90Val mutation were identified. Eleven residues were found to be key for all three of the ligands and included Arg114, Gly800, Val802, Ser803, Arg1259, Gly1260, Glu1278, DC1464, DA1465, DT3-1480 and DG5-1481. In addition to this, Gly1237 and Asp1238 were key residues for nybomycin and DNM-2, but not for ciprofloxacin. Lys1261 was determined as a key residue only with DNM-2 and ciprofloxacin. Residues considered to be key in only one of the ligands were DA1482 with nybomycin, Asp801 with DNM-2 and Glu1236 and Mg1496 with ciprofloxacin.

4.4 Investigation of *M. tuberculosis* mutants with increased nybomycin MICs using whole genome sequencing

The Nextera XT DNA library preparation kit (Illumina, San Diego, USA) was used for DNA library preparation and quality indicators indicated that clustering was achieved with a raw density of 1017 K/mm² and a passing filter of 92.4%.

The Illumina MiSeq sequencer together with the MiSeq v2 (500 cycle) reagent kit were used for whole genome sequencing (WGS). A 2 x 250 paired-end configuration was used and yielded a total of 9233 mega base pairs i.e. approximately 769 mega base pairs per sample. Full genomic coverage was achieved for each of the isolates. The quality of the sequencing process was confirmed with the PhiX Control v3.

Using the Burrows-Wheeler Aligner (BWA) tool, an average of 95% (and minimum of 94.9%) of each of the trimmed gene sequences were paired with the *M. tuberculosis* H37Rv reference genome^{235–237}. Cleaned and aligned sequences were submitted to the TB Profiler tool for identification of single nucleotide polymorphisms currently known to be associated with resistance to clinically used drug¹⁴². None of the resistance-conferring mutations in the software's database were identified in any of the 12 samples. This supports the notion that nybomycin may have a novel mechanism of action.

After gene annotation and sequence variant analysis, the results were exported to Microsoft Excel and the following gene variants were manually removed:

(1.) variants present in both the wild type and mutant sequences, (2.) variants from PE and PPE coding regions (3.) synonymous variants and (4.) variants located in intergenic regions. Gene variants unique to each of the ten mutant isolates were also filtered out, except where they occur in the same gene.

The final list of 22 genes with one or more gene variants potentially linked to phenotypic resistance to nybomycin are presented in table 4.10, on pages 105 through 108. Gene variants are described by highlighting the (1.) gene name, (2.) *M. tuberculosis* H37Rv locus, (3.) change type, (4.) reference nucleotide sequence, (5.) mutated nucleotide sequence, (6.) reference amino acid sequence (7.) amino acid and (8.) whether the changes were found in a mutant with a nybomycin MIC of 2 µg/ml or 4 µg/ml.

Table 4.10 Gene variants potentially responsible for phenotypic resistance to nybomycin (*continued on next page*)

Gene				Affected isolates		Change type	Reference nucleotide sequence	Allele	Reference amino acid/s	Amino acid change
No	Name	H37Rv Locus	Position	MIC 2 $\mu\text{g/ml}$	MIC 4 $\mu\text{g/ml}$					
1.	<i>psfP</i>	Rv0018c	21795	D, F	H, I, L	missense variant	G	A	P	S
			22412		I	disruptive inframe deletion	TCGCCTGAG ACCGCCCC GGCCAGAAT CGGTTGGGT CTGGCCGTA GTCGTAGTC GACGACGTC GGCGACGA CGACAGTGA CGTTGTCGG GGCCGC	T	G226_G256 del	-
2.		Rv0338c	403406	F	H	missense variant	A	C	I	M
3.	mkl	Rv0655	752148	D, F, G		missense variant	C	T	A	V
			752297		H	missense variant	C	G	Q	E
4.		Rv0823c	917610		J, L	start-lost mutation and conservative inframe deletion	ACGCCGGG CTGGGTTGG ATTGCCCGC CTCCTGCTC ATCGCGCTG CGCGCTCTG CATCGT	A	M1_A12del	-
5.		Rv1046c	1168716		L	frameshift variant	T	TC	A	fs
			1168717	C	I	frameshift variant	T	TG	E	fs

Table 4.10 Gene variants potentially responsible for phenotypic resistance to nybomycin (*continued from previous page*)

Gene			Position	Affected isolates		Change type	Reference nucleotide sequence	Allele	Reference amino acid/s	Amino acid change
No	Name	H37Rv Locus		MIC 2 $\mu\text{g/ml}$	MIC 4 $\mu\text{g/ml}$					
6.		Rv1435c	1612595	D		missense variant	C	G	G	A
			1612606	F		disruptive inframe insertion	T	TGGTAACGGTGCGCCCGGGATC	-	IPGAPLP
			1612625	E		conservative inframe insertion	A	ATCGGGACCGGGGCGCCAGGGG	-	TPGAPVP
					I	conservative inframe insertion	A	ATCGGTACCGGTGCGCCAGGGG	-	TPGAPVP
			1612630	F		disruptive inframe insertion	T	TACCGGGGCGCCAGGGATCGGG	-	PIPGAPV
				G		disruptive inframe insertion	T	TACCGGTGCGCCAGGGGTCGGG	-	PTPGAPV
					J	disruptive inframe insertion	T	TACCGGTGCGCCCGGGGACGGG	-	PSPGAPV
			1612631	F		conservative inframe insertion	A	ACCGGTGCGCCAGGGATCGGTC	-	GPIPGAP
			1612642	C		disruptive inframe insertion	A	AGGGGTGCGGACCGGTGCGCCC	-	GAPVPTP
					I	disruptive inframe insertion	A	AGGGATCGGTACCGGTGCGCCC	-	GAPVIP
			1612646	E		conservative inframe insertion	A	ATCGGGACCGGTGCGCCCGGGG	-	TPGAPVP
					H	conservative inframe insertion	A	ATCGGGACCGGGGCGCCAGGGG	-	TPGAPVP
					I	conservative inframe insertion	A	ATCGGTACCGGTGCGCCAGGGG	-	TPGAPVP
					L	conservative inframe insertion	A	ATCGGGACCGGTGCGCCAGGGG	-	TPGAPVP
1612647		H	conservative inframe insertion	T	TCGGTACCGGTGCGACAGGGAG	-	LPVAPVP			
1612657		K	disruptive inframe insertion	T	TGCGCCAGGGATGGGTACCGGG	-	PVPIPGA			
7.		Rv1588c	1789650	D		missense variant	C	T	A	T
			1789678	D, G	I	frameshift variant	C	CG	H	fs
8.	<i>pks7</i>	Rv1661	1879841		H, L	missense variant	T	G	V	G

Table 4.10 Gene variants potentially responsible for phenotypic resistance to nybomycin (*continued from previous page*)

Gene				Affected isolates		Change type	Reference nucleotide sequence	Allele	Reference amino acid/s	Amino acid change
No.	Name	H37Rv Locus	Position	MIC 2 µg/ml	MIC 4 µg/ml					
9.	cut1	Rv1758	1989054	F		frameshift variant	TTCAGAGAG GACTTCATC GATGC	T	F	fs
			1989055		H, J	frameshift variant	TCAGAGAGG ACTTCATCG ATGCG	T	R	fs
10.	gnd1	Rv1844c	2094909	C, F		conservative inframe insertion	TGACAGC	T	A92_V93del	-
11.		Rv1883c	2133467	C, E	H, J	conservative inframe insertion	G	GGTCGCATGCCGTCAC	-	VTACD
			2133469	C		conservative inframe insertion	T	TCGCATGCCGTCACCG	-	AVTAC
					J	conservative inframe insertion	T	TCCCATGCCGGCACC	-	GVPAW
			2133471		L	disruptive inframe insertion	G	GCATGCCGTCACCTCA	-	EVTAC
12.		Rv2262c	2534561		H, J	frameshift variant	CGG	C	R	fs
13.		Rv2415c	2713078	G	H, J, K, L	conservative inframe insertion	A	ACCC	-	G
14.		Rv2492	2807873	E, F	K	frameshift variant	A	AT	E	fs
15.		Rv2522c	2839459	C	K	frameshift variant	ACGCGCTCA ACCAGCTCG CGAGCAGA CGCAGAGTC GCATGAAAA TCCGGATTT CGATGCGAT TCTGCGTCT GCT	A	R	fs

Table 4.10 Gene variants potentially responsible for phenotypic resistance to nybomycin (*continued from previous page*)

Gene				Affected isolates		Change type	Reference nucleotide sequence	Allele	Reference amino acid/s	Amino acid change
No	Name	H37Rv Locus	Position	MIC 2 $\mu\text{g/ml}$	MIC 4 $\mu\text{g/ml}$					
16.	<i>affC</i>	Rv2673	2990584	D	J	frameshift variant and stop-lost and splice region variant	CAGCGGTG ATCGCAAGC GCGGCGAG CCGGGCGC AGCGGGTC ACCGCCATC GGGACT	C	Q	fs
17.		Rv3023c	3381641	F	L	missense variant	G	T	Q	K
18.		Rv3645	4083347	G	I	missense variant	A	C	T	P
19.	<i>dppD</i>	Rv3663c	4102108		H, J	frameshift variant	C	CG	R	fs
20.	<i>glpK</i>	Rv3696c	4138437		K	missense variant	G	C	A	G
			4139181		I	conservative inframe insertion	A	ACCC	G191dup	-
			4139183	C, E	I, K, L	disruptive inframe insertion	A	ACCC	p.G191dup	-
			4139190	C		conservative inframe insertion	C	CCCA	-	LG
21.		Rv3728	4176301		K	missense variant	A	G	I	V
			4176351	F		missense variant	A	C	E	D
22.	<i>sigM</i>	Rv3911	4400659		H, I	frameshift variant	CA	C	T	fs

CHAPTER 5 – DISCUSSION AND CONCLUSION

5.1 *In vitro* inhibitory effect of quinolines on various bacterial species

5.1.1 *Mycobacterium tuberculosis*

This is the first study to investigate the *in vitro* effect of nybomycin and DNM-2 on fluoroquinolone-resistant *M. tuberculosis* isolates. During the initial investigations, *in vitro* exposure of three *M. tuberculosis* isolates to nybomycin using the MTT assay suggested that nybomycin may act as a reverse antibiotic for this bacterial species. This prompted the testing of the susceptibility of various bacterial species to nybomycin and other quinoline-containing compounds.

A collection of 32 *M. tuberculosis* isolates with diverse IS 6110 RFLP banding patterns and fluoroquinolone susceptibility profiles were assembled. The three *M. tuberculosis* isolates investigated during the initial susceptibility testing were included in this collection. Nybomycin minimum inhibitory concentrations (MIC) reported with the multipoint inoculator method were relatively low and ranged from 0.25 to 1.0 µg/ml. No “reverse antibiotic effect” could be demonstrated, even when the original three isolates were retested. Repeat testing of a subset of 23 isolates, again using the MTT assay and including the three isolates investigated at the outset, also failed to reproduce the “reverse antibiotic effect”.

These discrepant results were further investigated. The nybomycin powder used during the initial investigation was procured from SantaCruz Biotechnology

(Dallas, Texas, USA) and the nybomycin powder for all subsequent investigations from BioAustralis (Smithfield, New South Wales, Australia). However, it was established that SantaCruz, obtained their nybomycin powder from BioAustralis. Further inquiry into the matter prompted BioAustralis to perform HPLC analysis on their different stocks. This revealed that the nybomycin batch procured through SantaCruz consisted of approximately 4.5% deoxynybomycin, whereas no deoxynybomycin could be detected in all subsequent batches procured through BioAustralis. Slight differences between batches of culture-produced antimicrobials are inevitable and highlight the importance of the development of a chemical synthesis pathway. All further interrogation of the susceptibility testing methods was unsuccessful in determining any additional factor that could have accounted for this discrepancy. The exact origin of this discrepancy could therefore not be satisfactorily explained. However, MIC results obtained with the multipoint inoculator method and subsequent MTT assay were in agreement with each other and were performed using larger numbers of *M. tuberculosis* isolates.

The relatively low nybomycin MIC results observed for all *M. tuberculosis* isolates indicate that nybomycin possess a mechanism of action unrelated to fluoroquinolone resistance. However, these results do not completely exclude the possibility of nybomycin acting as a reverse antibiotic, as it may be that this effect is masked due to the presence of at least one additional antibacterial mechanism. Such a mechanism would make it possible for susceptibility to occur in the presence of fluoroquinolone resistance. Computational methods were therefore employed in an attempt to clarify this issue.

To describe the cytotoxic and apoptotic potential of nybomycin, *in vitro* investigations using Vero, HeLa and A549 cell lines were prepared. Unfortunately, upon adding nybomycin stock solution to EMEM, the nybomycin crystalized and lysed all the mammalian cells within a few hours. *In vivo* toxicity investigations were therefore not further pursued.

The cytotoxic potential of nybomycin and DNM-2 have been investigated in the past. *In vivo* toxicity studies showed that 250 mg/kg nybomycin was well tolerated by mice when the compound was suspended in peanut oil and administered intraperitoneally¹⁴⁵. However, these preliminary toxicity studies were reported in 1955 and there have since been no further reports published specifically on nybomycin toxicity. Egawa *et al* reported DNM to be non-toxic to non-cancerous cell lines in 2000^{179,246}. More recently toxicity investigations were performed for DNM and several DNM derivatives, including DNM-2¹⁷⁹. None of the tested compounds caused significant hemolysis of red blood cells; and they produced no appreciable intercalation of DNA with the DNA intercalation assay¹⁷⁹. *In vivo* evaluation of DNM-2 also did not produce any observable toxicity when 50 mg/kg doses were administered to mice through oral lavage¹⁷⁹.

The *in vitro* susceptibility of 23 *M. tuberculosis* isolates to DNM-2 were investigated using the MTT assay and MIC values were in a similar range as for nybomycin. Like nybomycin, DNM-2 also failed to exhibit a “reverse antibiotic effect”. The range of unconventional drug concentrations tested deserves further explanation. Paul Hergenrother (Department of Chemistry, University of Illinois, USA) donated 5.76 mg DNM-2 antibiotic powder for this project¹⁷⁹. As this was a

very small amount of DNM-2 to work with and because DNM-2 is very poorly soluble in DMSO and water, it was decided to meticulously follow the donors' instructions regarding antibiotic stock solution preparation. This resulted in the unusual final drug concentrations reported in this study whereby 1:2 dilutions were prepared to start from 12 µg/ml.

Parkinson *et al* reported a peak serum DNM-2 concentration of 12.8 µg/ml in mice, after an oral dose of 50 mg/kg. This is 12.6 times higher than the highest nybomycin MIC and 4.2 times higher than the highest DNM-2 MIC observed for *M. tuberculosis* in this study¹⁷⁹. It is therefore conceivable that DNM-2 and nybomycin, or related compounds, may reach sufficient levels in human blood to make it useful for clinical use against infection with *M. tuberculosis*.

5.1.2 *Neisseria gonorrhoeae*

A collection of 32 *N. gonorrhoeae* isolates displaying a wide range of susceptibilities to fluoroquinolones were assembled for *in vitro* investigation. This study was the first to investigate the *in vitro* susceptibility of *N. gonorrhoeae* isolates to nybomycin. Nybomycin MICs (µg/ml) obtained with the multipoint inoculator method ranged from 0.25 to 8.0 µg/ml, but showed no association with fluoroquinolone MIC results. Based on these nybomycin susceptibility results, infections due to both ciprofloxacin susceptible and ciprofloxacin resistant *N. gonorrhoeae* may respond to treatment with nybomycin. In view of the looming crises of untreatable infections due to *N. gonorrhoeae*, these results deserve

further exploration²⁴⁷. Nybomycin may conceivably be able to act as a microbicide in *N. gonorrhoeae* infection.

The same collection of 30 *N. gonorrhoeae* isolates were also exposed to anti-malarial drugs: quinine, chloroquine, primaquine, mefloquine²⁴⁸. MICs obtained for quinine, chloroquine and primaquine were high and the results are in agreement with the work done by Mehaffey *et al* who concluded that they are inactive against *N. gonorrhoeae*. This present study was the first to investigate the *in vitro* antibacterial effect of these compounds against fluoroquinolone-resistant isolates. Mehaffey *et al* also reported that mefloquine displayed marginal *in vitro* effectivity against less than 10 percent of their collection of 105 *N. gonorrhoeae* isolates²⁴⁸. In this present study, mefloquine MICs for *N. gonorrhoeae* ranged from 4 to 16 µg/ml for 28 of the 30 isolates. The peak serum concentration of mefloquine after an oral dose of 250 mg drug has previously been reported as 0.656 to 1.018 µg/ml²⁴⁹. Mefloquine is therefore unlikely to achieve adequate serum levels to be useful against infections due to *N. gonorrhoeae*²⁴⁹⁻²⁵¹.

5.1.3 *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus* and *Enterococcus faecalis*

A collection of various commonly encountered human bacterial pathogens, including *E. coli* (n=6), *K. pneumoniae* (n=4), *E. cloacae* (n=4), *P. aeruginosa* (n=7), *A. baumannii* (n=5), *S. aureus* (n=4) and *E. faecalis* (n=4), with different levels of susceptibilities to fluoroquinolones, was exposed to nybomycin as well as

four anti-malarial drugs. What made this study different from others is the fact that anti-malarial drugs were investigated against both fluoroquinolone-susceptible and fluoroquinolone-resistant isolates. This was done in order to identify any “reverse antibiotic effect” with regards to fluoroquinolone susceptibility.

All Gram-negative isolates exhibited nybomycin MICs of > 16 µg/ml, except for one *A. baumannii* isolate with a nybomycin MIC of 8 µg/ml. The results agree with most previous studies that reported nybomycin to be inactive against Gram-negative bacteria^{143,148}. The only exception was Strelitz *et al* who reported in 1955 that nybomycin exhibits activity against two out of the seven investigated *E. coli* isolates and against the single investigated *K. pneumoniae* isolate¹⁴⁵. Their results were never replicated and stand in contrast to all subsequent reports^{143,148}. Morimoto *et al* identified efflux pumps to be the cause of resistance to nybomycin in *E. coli* isolates and postulated that this may be the mechanism of resistance (MOR) to nybomycin in all Gram-negative bacterial species¹⁴⁸.

Nybomycin MICs for Gram-positive bacteria ranged from 4 to 8 µg/ml for *S. aureus* (n=4) and 8 to 16 µg/ml for *E. faecalis* (n=4). This antibacterial activity is moderate compared to that reported by other investigators and no “reverse antibiotic effect” could be observed^{143,145,166}. This disagreement between the previously reported and the current results may be explained by the difference in methodology used. Previous reporters employed micro broth dilution assays and paper disk methods whereas this present study used a solid agar dilution methodology^{143,145}. The small sample size in this study unfortunately prevents firm conclusions to be drawn.

MIC results obtained with quinine, chloroquine, mefloquine and primaquine were less encouraging. All Gram-positive and Gram-negative isolates displayed MICs > 128 µg/ml for both chloroquine and primaquine. MIC results with quinine and mefloquine were ≥ 64 µg/ml for all isolates, except for the Gram-positive isolates that exhibited mefloquine MICs of 32 µg/ml. These results are in keeping with published results from other investigators^{250–255}. None of these anti-malarial drugs can reach adequate concentrations in human serum to be considered feasible treatment options of bacterial infections caused by bacterial species investigated in this study^{250,256–259}.

5.2 *In silico* effect of nybomycin and DNM-2 on *M. tuberculosis* gyrase enzyme

In silico methodologies were employed in an attempt to explain the *in vitro* inhibitory effects of nybomycin and DNM-2 on *M. tuberculosis* isolates. Ligand-based investigation methods such as pharmacophore modeling and quantitative structure-activity relationship methods are often employed for this purpose. However, based on the known association between nybomycin and fluoroquinolone-resistant *S. aureus*, a structure-based approach was followed as it was anticipated that nybomycin and DNM-2 act by inhibiting *M. tuberculosis* gyrase directly^{143,166,179}.

Molecular docking investigations were used to predict whether nybomycin and DNM-2 will bind to wild type *M. tuberculosis* gyrase with an affinity comparable to that seen with fluoroquinolones. The resultant LibDock scores (kcal/ml) of

nybomycin and DNM-2 indicate that these two compounds have at least similar, if not higher, affinity for wild type *M. tuberculosis* gyrase compared to ciprofloxacin and moxifloxacin. These results therefore support the notion that nybomycin and DNM-2 act on *M. tuberculosis* gyrase. The results are also in agreement with the *in vitro* results obtained with fluoroquinolone-susceptible *M. tuberculosis* isolates.

The value of the LibDock scoring system is restricted because it is calculated by taking only a limited number of factors into account including molecular shape, electrostatic interactions, van der Waals interactions, Coulombic interactions and hydrogen-bond formations^{40,211}. Molecular dynamics simulations were therefore employed to further refine the molecular docking investigations. Molecular dynamics simulations aim to mimic the natural motion of proteins by considering ligand position and orientation inside the binding pocket, as well as taking time, temperature and atmospheric pressure into consideration^{40,211}.

With the molecular dynamics studies, the investigation of the binding affinity between the ligands and the wild type *M. tuberculosis* gyrase was expanded by including the examination of *M. tuberculosis* gyrase coded by DNA containing common fluoroquinolone resistance-conferring mutations. Globally it has been reported that in the vast majority of fluoroquinolone-resistant *M. tuberculosis* isolates the resistance results from only a handful of mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene^{173,176,260,261}. Unlike other organisms such as *K. pneumoniae* and *S. aureus*, the *gyrB* gene is very seldom responsible for clinical resistance to fluoroquinolones in *M. tuberculosis*^{173,176,260,261}. Most fluoroquinolone resistance-conferring mutations

are located in either the 90th, 91st or 94th codon of *gyrA*^{174,175,260,261}. In light of the unexpected *in vitro* susceptibility results obtained with nybomycin and DNM-2 in the current study, it was decided prudent not to assume this to be the case with the selection of *M. tuberculosis* isolates used in this study. To this end, the QRDR of *gyrA* from all 32 *M. tuberculosis* isolates were sequenced and analyzed. With the exception of two isolates, all ofloxacin-resistant isolates harbored codon changes in *gyrA* known to confer clinical resistance to fluoroquinolones in *M. tuberculosis*^{174,175,260,261}. The two isolates without mutations in the investigated DNA segment of *gyrA* both belong to the LCC F150 IS 6110 RFLP family. Genetic analysis is warranted to identify the mutations that lead to fluoroquinolone resistance in these isolates, but falls outside the scope of the present study.

The accuracy and therefore the interpretation of molecular dynamics simulations are problematic²⁶²⁻²⁶⁴. Force fields are the most widely regarded source for potential error²⁶²⁻²⁶⁴. Force fields consist of equations that are used to govern the forces interacting on all the atoms in a given experiment^{263,264}. They are largely based on Newtonian physics and make poor use of quantum physics²⁶²⁻²⁶⁴. Several force fields have been developed to choose from but none are considered truly accurate^{263,265,266}. A second concern commonly encountered with regards to molecular dynamics simulations is the difficulties experienced in deciding on the length of sampling time^{262,263}. Both too short and too long sampling times may provide erroneous results and no definite guidelines exist with which to satisfactorily address this issue²⁶²⁻²⁶⁴. Due to the inherent inaccuracies observed with molecular dynamics simulations, care should also be taken not to make quantitative

comparisons, but to rather restrict analyses to qualitative conclusions^{263,264,267}. No direct comparisons of binding affinity were therefore made between the ligands.

The Molecular Bio-Computation and Drug Design Laboratory (School of Health Sciences, University of KwaZulu-Natal, Durban, South Africa) performed the computational experiments and included ciprofloxacin, nybomycin and DNM-2 in the molecular dynamics simulations.

Binding free energy results generated through molecular dynamics studies showed that ciprofloxacin binds stronger to gyrase with a Ala90Ser mutation than to wild type gyrase. This is in keeping with results from Blower and Aldred *et al* and supports the validity of this experiment^{175,177}. As expected, ciprofloxacin also binds stronger to wild type gyrase than to gyrase containing either Asp94Gly or Asp94His mutations. However, ciprofloxacin appears to have a slightly lower affinity for wild type gyrase compared to gyrase containing a Ala90Val mutation. This unexpected finding could not be explained upon further interrogation of the investigation methodology and results.

Nybomycin bound notably stronger to gyrase containing either Ala90Val or Asp94His mutations than to wild type gyrase, whereas bonds of comparable strength were observed when either Asp94Gly or Ala90Ser mutations were present. Nybomycin seems to form substantial bonds with *M. tuberculosis* gyrase regardless of whether any of the common fluoroquinolone resistance-conferring mutations are present in GyrA.

DNM-2 bound notably stronger to gyrase containing either one of Ala90Val, Asp94Gly or Ala90Ser mutations than to wild type gyrase, but weaker bonds formed when gyrase contained a Asp94His mutation. Similar to nybomycin, DNM-2 appears to bind strongly to *M. tuberculosis* gyrase regardless of whether any of the common fluoroquinolone resistance-conferring mutations are present in GyrA.

Further examination of the molecular dynamics simulation results included a per-residue interaction energy decomposition analysis of the bonds between *M. tuberculosis* gyrase and the three ligands. The specific contributions of residue 90 and 94 to the strength of binding of the ligand-gyrase complex as a whole were hereby approximated. For all three ligands, residue 90 appears to have played a predominant role in ligand binding compared to residue 94. However, the contribution of residue 94 cannot be disregarded on the basis of this analysis alone, because it does not take into account conformational changes that may be the result of different amino acid variations of codon 94. Any resultant conformational changes in the gyrase binding pocket may lead to other gyrase residues playing a more significant role in ligand binding.

Molecular dynamics simulations were also used to identify key residues involved in stabilizing the ligands inside the *M. tuberculosis* gyrase binding pocket harboring a Ala90Val mutation. The majority i.e. eleven residues were found to be key for all three of the ligands, illustrating potential similarity in mechanism of action between nybomycin, DNM-2 and ciprofloxacin with regards to *M. tuberculosis* gyrase.

In silico investigations identified a strong affinity between the two test compounds (nybomycin and DNM-2) and *M. tuberculosis* gyrase, irrespective of the presence of common fluoroquinolone resistance-conferring mutations in *gyrA*. A “reverse antibiotic effect” was not identified and the computational investigations therefore support the *in vitro* drug susceptibility results of this study. *In silico* investigations provided important information towards the unraveling of the mechanism of action of nybomycin and DNM-2 in *M. tuberculosis* isolates.

5.3 Investigation of *M. tuberculosis* mutants with increased nybomycin MICs using whole genome sequencing

Wild type *M. tuberculosis* V9124 was experimentally exposed to different concentrations of nybomycin. Mutants with increased nybomycin MICs were selected and WGS was used to compare the genomes of the mutants with that of the wild type. Analysis of the WGS results identified 22 genes that may possibly explain the nybomycin MIC difference between the wild type isolate and the mutants. Each gene is discussed separately in subsequent paragraphs.

Rv0018c is the locus of *pstP*, a 1545 base pair (bp) gene coding for a regulatory protein called phospho-serine/threonine phosphatase (PstP)^{268,269}. An identical missense variant was identified in this gene for five of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. It is the first gene in an operon consisting of five genes, all of which are considered important in the regulation of cell wall synthesis, cell division and cell shape^{270–273}. The first gene downstream to *pstP* is *rodA*, located at Rv0017c²⁶⁸. This gene codes for RodA, a non-classical

transglycosylase involved in the synthesis of septum-peptidoglycan during cell wall formation^{268,274}. RodA further assists in stabilizing the FTSZ ring, which is the bacterial homolog to tubulin, during cell division²⁶⁸. The following gene downstream is *pbpA*, located at Rv0015c²⁶⁸. Its product is a penicillin-binding protein called PbpA that is involved in the final stages of peptidoglycan synthesis through its transpeptidase function^{268,274}. PbpA is further said to play a role in determining the shape of bacterial cells^{268,274}. The last two genes that form part of this operon are *pknA* and *pknB* in positions Rv0015c and Rv0014c respectively²⁶⁸. They code for two crucial transmembrane serine/threonine protein kinases (STPKs) designated protein kinase A (PknA) and protein kinase B (PknB)²⁶⁸. Both are crucial in the regulation of certain morphological changes associated with cell division and cell differentiation^{268,270,273,275}. Bacteria employ surface-located sensor proteins to transfer information from the extra-cellular to the intra-cellular area, in order to effectively survive in and respond to their environment²⁷⁶. Regulation of these sensor proteins is complex and involves the balancing of a reversible phosphorylation process mediated by kinases and phosphatases²⁷⁶. STPKs undergo auto-phosphorylation of their serine/threonine residues and then participate in the functioning of other transmembrane signal processes through transferal of its phosphate moiety^{270,276}. PstP is the sole serine/threonine phosphatase in *M. tuberculosis* and regulates STPKs as well as other signal proteins by dephosphorylating their phosphorylated serine/threonine residues^{268,270,276}. It was previously shown that PstP is constantly required for pathogen survival and mutations in *pstP* are therefore likely to have a detrimental effect on cell growth and pathogenicity^{270,276}. PstP, PknA and PknB are independently indispensable for *in vitro* growth and the *in vivo* survival of

*M. tuberculosis*²⁷⁴. Not surprisingly, PstP has already been identified as an attractive target for drug discovery²⁷⁵. Interestingly, Arai *et al* previously noticed morphological changes in *Mycobacterium smegmatis* cells treated with nybomycin similar to that seen in *M. tuberculosis* with mutations in their *pknA* or *pknB* genes^{165,272}. However, the authors did not find any mutations in *pknA* or *pknB* and did not specifically comment on whether any mutations were observed in the upstream *pstP* gene.

Rv0338c is the locus of a currently unnamed 2649 bp long gene with an unclear function^{268,269}. The gene product is possibly an iron-sulfur-binding reductase involved in intermediary metabolism and respiration²⁶⁸. An identical missense variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. DeJesus *et al* published a groundbreaking study in 2017 where they used fully saturated Himar1 transposon libraries to investigate and describe different gene regions of the *M. tuberculosis* genome in terms of whether or not it is essential for cell viability²⁷⁷. Rv0338c was accordingly classified as essential for the viability of *M. tuberculosis* bacilli.

Rv0655 is the locus of *mkl*, a 1080 bp gene coding for a putative ribonucleotide-transport ATP-binding protein named Mkl^{268,269}. An identical missense variant was identified in this gene for three of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. Mkl is also called MceG because of its close relation to the functioning of the mce multiprotein complexes in *M. tuberculosis*^{278,279}. These multiprotein complexes are coded by one of four mce operons (1 to 4) and function similarly to ABC transporter permeases and substrate-binding proteins^{278,279}.

Mce-1 and Mce-4 are respectively involved with fatty acid and cholesterol uptake, both crucial for cell survival²⁷⁹. Cholesterol is important for maintaining a chronic infection and both Mce-1 and Mce-4 have been shown to be integral survival and virulence factors^{279,280}. MceG/Mkl is coded outside these operons, but are required for their functioning^{278,279}.

Rv0823c is the locus of a currently unnamed 1170 bp long gene with a poorly defined function^{268,269}. Predictions have this gene code for a transcriptional regulatory protein involved with fatty acids and other currently undefined metabolic processes^{268,269}. According to the Universal Protein Knowledgebase (UniProt) consortium, this gene should be named *dus* due to its homology to proteins found in other organisms²⁸¹. Its function is hereby more specifically predicted to be that of a tRNA-dihydrouridine synthase²⁸¹. This transcriptional regulatory protein is said to catalyze the synthesis of a dihydrouridine residue located in the D-loop of most tRNAs²⁸¹. The exact role of dihydrouridine in tRNA is not currently known. In this study, a start-lost mutation together with a conservative inframe deletion was detected in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. According to work done by DeJesus *et al*, this gene is not considered essential for cell viability²⁷⁷.

Rv1046c is the locus of a currently unnamed 525 bp long gene coding for an uncharacterized protein^{268,281}. It is predicted to be a functional partner of Rv1047, which is a potential transposase required for the transposition of IS 1081^{281,282}. In the current study, a frame-shift variant was detected in this gene for three of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. However, analysis of

saturated Himar1 transposon libraries by DeJesus *et al* classifies this gene as non-essential for the viability of *M. tuberculosis* bacilli²⁷⁷. Interestingly, another gene probably involved in the transposition of IS 1081 has been identified in this study as potentially related to the mechanism of action of nybomycin. This gene is located at Rv3023 and is discussed in a later paragraph. Only one of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin harbored a mutation in both Rv1046c and Rv3023.

Rv1435c is the locus of a currently unnamed 609 bp long gene coding for a protein that is secreted into the extra-cellular space^{268,281,283}. It is probably secreted via a classical twin-arginine translocation (Tat)-dependent pathway and is not considered essential for the virulence or survival of *M. tuberculosis*^{277,283}. This study identified several conservative as well as disruptive inframe insertions in this gene for a number of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. Interestingly, it was previously reported that this gene contains at least five imperfect repeats consisting of seven amino acids each²⁶⁸. The bioinformatics analysis software may therefore have incorrectly identified and annotated such variations in bp positions 1612606, 1612625, 1612630, 1612631, 1612642, 1612646 and 1612647. Nevertheless, disruptive inframe insertions were also found in three isolates at bp position 1612630 and in two further isolates at bp position 1612642.

Rv1588c is the locus of a currently unnamed 669 bp long gene coding for REP13E12, a partial repeat protein that is part of the REP13E12 family of proteins^{268,284}. The REP13E12 family consists of seven copies of a similar gene

sequence and is found throughout the *M. tuberculosis* genome^{284,285}. This protein has been linked to DNA regulation and the classical RecA/LexA SOS response to DNA damage^{268,284,285}. A frameshift variant was identified in this gene for three of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin. The presence of multiple copies of this gene in *M. tuberculosis* possibly testifies to its importance for DNA-repair and bacterial survival, but makes it a poor focus for drug development.

Rv1661 is the locus of *pks7*, a 6381 bp long gene coding for a probable polyketide synthase named Pks7^{268,269}. Apart from a predicted but poorly defined function in lipid metabolism, this protein is also potentially involved in certain intermediate steps relating to the synthesis of polyketides which may be involved in secondary metabolism processes^{268,269}. Results from the study by DeJesus *et al* do not classify this gene as essential for cell viability²⁷⁷. However, Rousseau *et al* pointed out that *pks7* has only been observed in pathogenic mycobacterial species and has an apparent role during infection²⁸⁶. It is thus considered a virulence factor for *M. tuberculosis* and a potential drug target^{280,286}. An identical missense variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv1758 is the locus of *cut1*, a 525 bp gene coding probably for a cutinase named Cut1^{268,269}. The primary substrate for cutinases is cutin, the waxy outer layer of plants²⁸⁷. However, cutin is not present in the natural life cycle of *M. tuberculosis*, so cutinases probably have other functions in this organism²⁸⁷. Work done by DeJesus *et al* classify this gene as non-essential for cell viability²⁷⁷. A frameshift

variant was identified in this gene for three of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv1844 is the locus of *gnd1*, a 1458 bp gene coding for a possible 6-phosphogluconate dehydrogenase named Gnd1^{268,269}. This enzyme is probably involved in the early stages of the pentose phosphate pathway (PPP) where it catalyzes the early oxidative decarboxylation of 6-phosphogluconate to produce ribulose 5-phosphate and carbon dioxide as well as reduce NADP to NADPH^{268,269}. This gene was not considered essential for cell viability by DeJesus *et al*²⁷⁷. A conservative inframe insertion was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv1833c is the locus of a currently unnamed 462 bp long gene coding for an uncharacterized protein^{268,281}. DeJesus *et al* do not classify this gene as essential for cell viability²⁷⁷. Conservative inframe insertions were identified in four of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv2262c is the locus of a currently unnamed 1083 bp long gene coding for an uncharacterized protein possibly involved in lipid metabolism^{268,281}. DeJesus *et al* classify this gene as non-essential for cell viability²⁷⁷. A frameshift mutation identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv2415c is the locus of a currently unnamed 894 bp long gene coding for an uncharacterized protein possibly involved in DNA binding and repair^{268,281}.

Results from the study by DeJesus *et al* do not classify this gene as essential for cell viability²⁷⁷. A conservative inframe insertion was identified in this gene for five of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv2492 is the locus of a currently unnamed 753 bp long gene coding for an uncharacterized protein with an undetermined function^{268,281}. DeJesus *et al* placed this gene within a region of the *M. tuberculosis* genome that they identified as non-essential for cell viability²⁷⁷. A frameshift variant was identified in this gene for three of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv2522c is the locus of a currently unnamed 1413 bp long gene coding for an uncharacterized protein with an undetermined function^{268,281}. DeJesus *et al* classifies this gene as non-essential for cell viability²⁷⁷. A frameshift variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv2673 is the locus of *aftC*, a 1302 bp gene coding for a possible arabinofuranosyl transferase named AftC^{268,269}. AftC is involved in the biosynthesis of the mycolylarabinogalactan-peptidoglycan (mAGP) complex that forms an essential part of the mycobacterial cell wall^{268,269,288}. It is considered essential for cell viability^{277,288}. A frameshift variant, stop-lost and splice region variant were identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3023c is the locus of a currently unnamed 1248 bp long gene coding for a protein that possibly functions as a transposase for IS *1081*^{268,281}. Although it is required for the transposition of IS *1081*, it is not considered essential for cell viability²⁷⁷. An identical missense variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3645 is the locus of a currently unnamed 1650 bp long gene coding for a protein that is predicted to be a conserved transmembrane protein with an unknown function^{268,281}. DeJesus *et al* classified this gene as essential for cell viability²⁷⁷. An identical missense variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3663c is the locus of *dppD*, a 1647 bp gene coding for a putative ATP-binding protein that forms part of an ABC transporter involved the uptake of small peptides^{268,269,289}. This transmembrane protein is known as DppABCD and although not considered essential for cell viability, it has been identified as a virulence factor^{277,280,289}. A frameshift variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3696c is the locus of *glpK*, a 1554 bp gene coding for a probable glycerol kinase named GlpK^{268,269}. GlpK is considered important for the regulation of glycerol uptake and metabolism^{268,269}. It is involved in the first step of the glycerol kinase pathway by catalyzing the phosphorylation of glycerol to produce sn-glycerol 3-phosphate^{268,269}. DeJesus *et al* did not classify this gene as essential

for cell viability²⁷⁷. A disruptive inframe insertion was identified in this gene for five of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3728 is the locus of a currently unnamed 3198 bp long gene coding for a conserved two-domain membrane protein that is probably involved in sugar or drug efflux^{268,281}. Gupta *et al* observed that this gene is upregulated when the bacterium is exposed to various anti-tuberculosis drugs and is therefore possibly important in the development of MDR-TB²⁹⁰. Several efflux-pumps have been identified in *M. tuberculosis* that have been linked to resistance to various anti-tuberculosis drugs²⁹¹. DeJesus *et al* categorized this gene as non-essential for cell viability²⁷⁷. Two missense variants were identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Rv3911 is the locus of *sigM*, a 669 bp gene coding for an initiation factor named SigM^{268,269}. This protein helps the attachment of RNA polymerase to certain initiation sites^{268,269}. SigM is one of 13 sigma factors coded by *M. tuberculosis* and is considered an alternative to the main sigma factor, SigA²⁸⁰. SigM is not considered essential for cell viability by DeJesus *et al*²⁷⁷. However, it controls the expression of four *esat-6* homologs and sigma factors have been identified as potential drug targets^{277,281}. A frameshift variant was identified in this gene for two of the ten *M. tuberculosis* V9124 isolates exposed to nybomycin.

Arai *et al* previously exposed *M. smegmatis* with a nybomycin MIC of 1 µg/ml to increased concentrations of nybomycin¹⁶⁵. *M. smegmatis* colonies with a nybomycin MIC of 10 µg/ml were selected and investigated using WGS¹⁶⁵.

According to a preliminary analysis of their WGS results, the authors hypothesized that nybomycin targets the mycobacterial genome and not specific proteins¹⁶⁵. They proceeded to test this hypothesis by investigating the binding affinity of nybomycin to plasmid DNA using a competitive inhibitor experiment using nybomycin, a nybomycin probe and a dummy probe¹⁶⁵. The investigators concluded that nybomycin has general DNA binding activity and that its binding strength varies depending on the exact DNA sequence involved¹⁶⁵. However, these results are not in agreement with earlier nybomycin research conducted by Hiramatsu *et al* with *S. aureus* that implicated gyrase to be the site of action¹⁴³. Differences between *S. aureus*, *M. smegmatis* and *M. tuberculosis*, as well as between plasmid DNA and genomic DNA, may have been responsible for the different conclusions that were reached with the different studies. The present study was not designed in a way that could support or disprove the prediction of Arai *et al* that nybomycin directly binds to and inhibit various areas of the *M. tuberculosis* genome¹⁶⁵.

For this study, WGS was conducted with its primary aim to identify a potential mechanism whereby *M. tuberculosis* resists the action of nybomycin. With this approach, the mechanism whereby nybomycin acts on *M. tuberculosis* can potentially be deduced much easier, but only after its MOR has been confirmed.

None of the ten *M. tuberculosis* isolates with increased MICs for nybomycin harbored any significant mutations in *gyrA* or *gyrB*. Also, none of the 22 genes identified as potentially responsible for resistance of *M. tuberculosis* to nybomycin can be confidently associated with a MOR that may involve fluoroquinolones, such

as efflux pumps or inactivating enzymes. The only potential exception is Rv3728 that codes for a poorly described transmembrane pump, known to be upregulated in the presence of certain anti-tuberculosis drugs. More broadly speaking, WGS did not identify any known drug resistance determinants responsible for resistance to the currently used anti-tuberculosis drugs. This indicates that *M. tuberculosis* probably employs a novel MOR against nybomycin. It further suggests that nybomycin may use a novel mechanism of action against *M. tuberculosis*.

Although it is possible that any of these 22 genes may be associated with the mechanism whereby *M. tuberculosis* resist nybomycin, the establishment of such a link was not pursued in this study. Further investigations are required and may include comparing nybomycin MICs of wild type *M. tuberculosis* with that of *M. tuberculosis* mutants created through gene knock-out experiments, where each one of the 22 genes identified in this study have been knocked out to create a separate mutant.

5.4 Conclusion

This was the first study to investigate the *in vitro* effect of nybomycin against various commonly encountered drug-resistant human bacterial pathogens. *In vitro* drug susceptibility testing with DNM-2, a chemically altered compound closely related to nybomycin, was also conducted with the *M. tuberculosis* isolates. Fluoroquinolone-susceptible and fluoroquinolone-resistant isolates were compared in order to identify whether a “reverse antibiotic effect” could be elicited. With the *in vitro* investigations, no “reverse antibiotic effect” could be identified in any of the

bacterial species, but *M. tuberculosis* and *N. gonorrhoeae* isolates displayed relatively low MIC values for nybomycin. This effect was irrespective of the presence of fluoroquinolone resistance. Nybomycin also displayed some inhibitory activity against *S. aureus* and *E. faecalis* isolates, albeit to a much lesser extent than that seen with *M. tuberculosis*. Exposure of *M. tuberculosis* isolates to DNM-2 produced results similar to that observed with nybomycin.

Gyrase enzyme was predicted to be a target site for the binding of nybomycin and DNM-2 whereby they exert an inhibitory effect on *M. tuberculosis*. This hypothesis was supported by molecular docking investigations. Molecular dynamics simulations further confirmed the importance of residue 90 of GyrA for the binding affinity of the test ligands to gyrase enzyme. Residue 94 was also identified as important for ligand binding affinity, but this was probably an indirect effect through conformational changes of the gyrase binding pocket and thus implies the involvement of other gyrase residues.

The *in vitro* nybomycin and DNM-2 susceptibility testing results together with the molecular docking and molecular dynamics simulation results suggest the presence of an additional mechanism of action, apart from the mechanism involving gyrase enzyme. This is supported by WGS results that also indicate that resistance to nybomycin is unlikely to be directly related to quinolone resistance, as no mutations were observed in the *gyrA* or *gyrB* genes.

WGS results identified 22 genes that are possibly responsible for a mechanism whereby *M. tuberculosis* resist the inhibitory action of nybomycin. This should be

the starting point for further investigations and could include gene knock-out experiments as well as gene expression profiling. Further investigation of these potential resistance determinants, may very well lead to the discovery of a novel mechanism whereby nybomycin and DNM-2 act against *M. tuberculosis*. There are however, several hurdles facing *in vivo* investigations with nybomycin. Most important is the issue of poor solubility in all solvents routinely used. For *in vivo* investigations, it may be prudent to rather focus on derivatives that have been designed to have better solubility, such as DNM-2. Other concerns with nybomycin is its high cost and batch-to-batch variability that are inherent to culture-derived antimicrobials. DNM-2 is manufactured through chemical synthesis and therefore has much less batch-to-variability and may be easier to produce in larger quantities. Nybomycin and DNM-2 are considered serious drug leads for the fight against *M. tuberculosis* and deserves further exploration. This is especially urgent in light of the escalating crises of drug-resistant *M. tuberculosis* infection.

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APPENDIX A – MEDIA AND REAGENTS

A.1 Middlebrook 7H9 broth

1. Add 4.7 gram Difco Middlebrook 7H9 Broth base (Becton Dickinson, USA), 2 ml glycerol (Rochelle Chemicals & Lab Equipment, SA) and 0.5 ml Tween-80 (Merck, SA) to 900 ml distilled water in a 3 liter Erlenmeyer flask. For diluting drugs, the Tween-80 was omitted.
2. Gently swirl flask to dissolve powder completely
3. Sterilize by autoclaving at 121 °C for 15 minutes
4. Cool down to room temperature
5. Add 100 ml OADC enrichment (BBL Middlebrook OADC Enrichment, Becton Dickinson, USA) containing oxidase, albumin, dextrose and catalase
6. Dispense 3 to 5 ml volumes into 30 ml universal containers (Sterilin Polypropylene 30 ml Universal Container, Thermo Fisher Scientific, Massachusetts, USA)
7. If mixing with vortex mixer to reduce clumping is anticipated, then aseptically add 3 to 5 sterile glass beads (diameter of 5 mm) per container
8. Store at 4 to 8 °C until use

A.2 Chocolate agar plates

1. Prepare a double strength base by adding 36 gram GC Agar Base (Oxoid, England) to 500 ml distilled water in a 3 liter Erlenmeyer flask

2. Slowly bring to boil and swirl flask and to dissolve powder completely
3. In a separate Erlenmeyer flask, dissolve 20 gram Bacto Hemoglobin powder (Oxoid, England) in 500 ml distilled water
4. Sterilize both solutions separately by autoclaving at 121 °C for 15 minutes
5. Cool down both solutions in a water bath to 50 °C
6. Reconstitute 10 ml of Vitox growth supplement (Oxoid, England) and add to the GC base medium dissolved in 500 ml
7. Aseptically add the 500 ml hemoglobin solution to the GC base medium
8. Mix the two solutions by gently swirling the flask
9. Dispense 20 ml media into each petri dish with a diameter of 90 mm
10. Leave at room temperature until have agar fully solidified
11. Pack in plastic packets and store at 4 to 8 °C until use

A.3 Brain heart infusion broth

1. Add 37 gram Brain Heart Infusion base (Oxoid, England) to 1000 ml distilled water in a 3 liter Erlenmeyer flask
2. Slowly bring to boil and swirl flask and to dissolve powder completely
3. Sterilize by autoclaving at 121 °C for 15 minutes
4. Cool down at room temperature
5. Dispense into the required volumes
6. Store at 4 to 8 °C until use

A.4 Middlebrook 7H11 agar plates

1. Add 19 gram BBL Seven H11 Agar Base (Becton Dickinson, USA) and 5 ml glycerol (Rochelle Chemicals & Lab Equipment, SA) to 900 ml distilled water in a 3 liter Erlenmeyer flask
2. Gently swirl flask to dissolve powder completely
3. Sterilize by autoclaving at 121 °C for 10 minutes
4. Cool down in a water bath to 50 to 55 °C
5. Aseptically add 100 ml OADC enrichment (BBL Middlebrook OADC Enrichment, Becton Dickinson, USA) containing oxidase, albumin, dextrose and catalase
6. Gently swirl flask to mix contents
7. Dispense 20 ml media into each petri dish with a diameter of 90 mm
8. Leave at room temperature until have agar fully solidified
9. Pack in plastic packets and store at 4 to 8 °C until use

A.5 McFarland turbidity standards 0.5 and 1.0

1. Add 0.5 ml of concentrated H₂SO₄ (Merck, SA) to 49.5 ml of sterilized distilled water to make a 1% H₂SO₄ solution
2. Add 0.175 gram of BaCl₂·2H₂O (Sigma-Aldrich, SA) to 10 ml of sterilized distilled water to make a 1% BaCl₂·2H₂O solution
 - For a McFarland standard of 0.5, mix 0.05 ml of the 1% BaCl₂·2H₂O solution with 9.95 ml of the 1% H₂SO₄ solution

- For a McFarland standard of 1.0, mix 0.1 ml of the 1% BaCl₂·2H₂O solution with 9.9 ml of the 1% H₂SO₄ solution
3. Cover tube with tinfoil and store away from direct sunlight

A.6 Phosphate buffered saline (PBS)

1. Add 10 PBS tablets (Oxoid, England) to 1000 ml distilled water in a 3 liter Erlenmeyer flask
2. If it is required that bacterial clumping be minimized, add (1%) 10 ml Tween-80 (Merck, SA)
3. Slowly bring to boil and swirl flask and to mix contents
4. Sterilize by autoclaving at 121 °C for 15 minutes
5. Cool down at room temperature
6. Dispense into the required volumes
7. Store at 4 to 8 °C until use

A.7 Storage media for bacterial isolates (excluding *M. tuberculosis*)

1. Add 3.7 gram Brain Heart Infusion base (Oxoid, England) and 20 ml glycerol (Rochelle Chemicals & Lab Equipment, SA) to 80 ml distilled water. This provide brain heart infusion broth with 20% glycerol medium
2. Slowly bring to boil and swirl flask and to dissolve powder and glycerol
3. Sterilize by autoclaving at 121 °C for 15 minutes
4. Cool down to 4 °C and dispense 1 ml amounts together with sterilized glass beads into cryovials.

A.8 MTT Solution

- Dissolve 25 mg MTT powder (Sigma-Aldrich, SA) in 5 ml PBS

A.9 50% DMF solution

- Mix 10 ml DMF with 10 ml sterile distilled water

A.10 SDS solutions

- SDS (Sigma-Aldrich, SA) is toxic and SDS particles easily disperse through the air, so therefore work inside a fume hood and wear an appropriate mask
 - To prepare a 10% SDS solution, dissolve 4 gram SDS in 36 ml sterile distilled water in a 50 ml polypropylene tube
 - To prepare a 20% SDS solution, dissolve 8 gram SDS in 40 ml sterile distilled water in a 50 ml polypropylene tube
1. Place tube in a water bath at 30 to 35 °C to help dissolve the powder
 2. Leave the suspension standing overnight on the bench top to allow the foam to settle
 3. Filter sterilize with a 0.22 micron Millipore filter (Merck, SA) and leave the suspension on the benchtop again, to let the foam settle
 4. To avoid precipitation, store at room temperature rather than in a fridge

A.11 1:1 SDS(20%)-DMF(50%) solution

1. Add 20 ml 20% SDS to 20 ml 50% DMF in a 50 ml polypropylene tube
2. Cover polypropylene tube with tinfoil and store away from direct light

A.12 Middlebrook 7H10 agar

1. Add 19 gram Difco Middlebrook 7H10 Agar base (Becton Dickinson, USA) and 5 ml glycerol (Rochelle Chemicals & Lab Equipment, SA) to 900 ml distilled water in a 3 liter Erlenmeyer flask
2. For susceptibility testing purposes, add an extra 10% of the Difco Middlebrook 7H10 Agar powder i.e. 1.9 gram to the flask. This is to account for the 10% agar loss when making 1:10 dilutions with antibiotic working solutions.
3. Gently swirl flask to dissolve powder completely
4. Sterilize by autoclaving at 121 °C for 10 minutes
5. Cool down in water bath to 50 to 55 °C
6. Aseptically add 100 ml OADC enrichment (BBL Middlebrook OADC Enrichment, Becton Dickinson, USA) containing oxidase, albumin, dextrose and catalase
7. Gently swirl flask to mix contents
8. Dispense into the required volumes
9. Leave at room temperature until agar have fully solidified
10. Pack in plastic packets and store at 4 to 8 °C until use

A.13 GC agar for *N. gonorrhoeae* susceptibility testing

1. Add 36 gram GC Agar Base (Oxoid, England) to 1000 ml distilled water in a 3 liter Erlenmeyer flask. Add an extra 10% of the Mueller Hinton agar base i.e. 3.6 gram to the flask. This is to account for the 10% agar loss when making 1:10 dilutions with antibiotic working solutions.
2. Slowly bring to boil and swirl flask and to dissolve powder completely
3. Sterilize by autoclaving at 121 °C for 15 minutes
4. Cool down in a water bath to 50 to 55 °C
5. Reconstitute two 10 ml amounts of Vitox growth supplement (Oxoid, England) and add to the GC base medium dissolved in 1000 ml
6. Gently swirl flask to mix contents
7. Dispense into the required volumes
8. Leave at room temperature until agar have fully solidified
9. Pack in plastic packets and store at 4 to 8 °C until use

A.14 Mueller Hinton agar

1. Add 38 gram Mueller Hinton agar base (Oxoid, England) to 1000 ml distilled water in a 3 liter Erlenmeyer flask
2. For susceptibility testing purposes, add an extra 10% of the Mueller Hinton agar base i.e. 3.8 gram to the flask. This is to account for the 10% agar loss when making 1:10 dilutions with antibiotic working solutions.
3. Slowly bring to boil and swirl flask and to dissolve powder completely
4. Sterilize by autoclaving at 121 °C for 15 minutes

5. Cool down in a water bath to 50 to 55 °C
6. Dispense into the required volumes
7. Leave at room temperature until agar have fully solidified
8. Pack in plastic packets and store at 4 to 8 °C until use

A.15 10 mg/ml Proteinase K

1. Mix 1 ml of 20 mg/ml Proteinase K stock (Qiagen, Whitehead Scientific, SA) with 1 ml of autoclaved distilled water
2. Store at 4 °C until use

A.16 5M NaCl solution

1. Dissolve 14.6 gram NaCl (Sigma-Aldrich, SA) in 50 ml distilled water
2. Sterilize by autoclaving at 121 °C for 15 minutes
3. Store at 4 to 8 °C until use

A.17 10% CTAB solution

1. Add 10 gram CTAB (Sigma-Aldrich, SA) to 100 ml sterilized distilled water
2. Heat to 65 °C until powder has completely dissolved

A.18 Chloroform:Isoamyl alcohol (24:1)

- Mix 1 ml of isoamyl alcohol (Sigma-Aldrich, SA) into 24 ml chloroform (Sigma-Aldrich, SA)

A.19 TE Buffer

1. Add 1.21 gram Trizma base (Sigma-Aldrich, SA) to 80 ml distilled water
2. Adjust pH to 8.0 using HCl (Merck, SA)
3. Add 0.37 gram EDTA (Sigma-Aldrich, SA)
4. Check the final pH and adjust to a final volume of 100 ml
5. Sterilize by autoclave at 121 °C for 15 minutes
6. This produces a 10x TE Buffer solution
7. To prepare a 1x TE Buffer solution, add 100 ml of the 10x TE buffer to 900 ml sterilized distilled water
8. Store at room temperature

A.20 1% Agarose gel

1. Add 1.4 gram agarose powder (Seakem LE Agarose, Whitehead Scientific, SA) to 140 ml 1x TBE buffer
2. Heat in microwave to dissolve the powder
3. After cooling at room temperature to approximately 45 °C, pour gel into the casting gel tray

A.21 TBE Buffer

1. Dissolve 108 gram Trizma base (Sigma-Aldrich, SA), 55 gram Boric acid (Sigma-Aldrich, SA) and 9.3 gram EDTA (Sigma-Aldrich, SA) in 900 ml sterilized distilled water
2. Adjust the final volume to 1000 ml
3. To prepare a 1x TBE Buffer solution, add 100 ml of the 10x TE buffer to 900 ml sterilized distilled water
4. Store at room temperature

A.22 Gel loading dye for gel electrophoresis

1. Measure out and mix 50 gram glycerol (Merck, SA), 5 ml 1M Tris/HCl (pH 7.5), 5 ml 100mM EDTA (Sigma-Aldrich, SA), 0.05 gram Bromophenol blue (Sigma-Aldrich, SA) and 300 μ l 10 mg/ml RNase (Qiagen, Sigma-Aldrich, SA)
2. Add distilled water to a final volume of 100 ml
3. Heat in a water bath to 100 °C for 15 minutes, until reagents have completely dissolved
4. Store at 4 to 8 °C for a maximum of one year
5. Before use, dispense 980 μ l into a cryovial and add 20 μ l GelRed Nucleic Acid Stain (Biotium, Sigma-Aldrich, SA) to complete the gel loading dye preparation

A.23 Gel loading dye for IS 6110 RFLP

1. First prepare a 1% Double dye stock solution by dissolving 1 gram Bromophenol Blue (Sigma-Aldrich, SA) and 1 gram Xylene Cyanole (Sigma-Aldrich, SA) in 100 ml sterilized distilled water
2. Mix 5 ml of this 1% double dye stock solution with 5 ml 10x TBE buffer, 25 ml glycerol (Merck, SA) and 15 ml distilled water to prepare the sample loading dye
3. Store at 4 to 8 °C until use

A.24 SSC solutions

1. Mix 175 gram 3M NaCl (Sigma-Aldrich, SA) and 88 gram 0.3M Trisodium citrate dihydrate (Merck, SA) in 800 ml sterilized distilled water
2. Use HCl (Merck, SA) and sterilized distilled water to adjust the final pH to 7.0 and the final volume to 1000 ml
3. This provides a 20x SSC solution
 - To prepare a 10x SCC solution, add 500 ml of 20x SCC to 500 ml sterilized distilled water
 - To prepare a 2x SCC solution (Secondary Wash Buffer), add 100 ml of 20x to 900 ml sterilized distilled water

A.25 Soak I Solution

- Dissolve 20 gram 0.5M NaOH pellets (Sigma-Aldrich, SA) and 87.66 gram 1.5M NaCl (Sigma-Aldrich, SA) in 1000 ml sterilized distilled water

A.26 Soak II Solution

1. Dissolve 62.6 gram 0.5M Trizma base (Sigma-Aldrich, SA) and 87.67 gram 1.5M NaCl (Sigma-Aldrich, SA) in 800 ml sterilized distilled water
2. Use HCl (Merck, SA) and sterilized distilled water to adjust the final pH to 7.2 and the final volume to 1000 ml

A.27 Primary Wash Buffer

1. Dissolve 360 gram 6M Urea (Sigma-Aldrich, SA), 4 gram SDS (Sigma-Aldrich, SA) and 25 ml 20x SSC in 800 ml sterilized distilled water
2. Use sterilized distilled water to make up a final volume of 1000 ml

A.28 Hybridization buffer

1. Add 30 ml 5M NaCl to 270 ml Hybridisation buffer (Amersham, UK)
2. Use a magnetic stirrer with stirrer bar to make sure the NaCl fully dissolves
3. While on the magnetic stirrer, heat the solution 60 °C and slowly add 15 gram Blocking agent (Amersham, UK) to it
4. Store at -20 °C

APPENDIX B – RAW DATA (MINIMUM INHIBITORY CONCENTRATIONS)

B.1 MIC ($\mu\text{g/ml}$) results for *Mycobacterium tuberculosis* with multipoint inoculator

<i>M. tuberculosis</i> Isolate number	Isoniazid				Rifampicin				Amikacin				Ofloxacin				Nybomycin			
	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC
H37Rv	0.125	0.125	0.125	0.125	0.25	0.25	0.0625	0.25	1	1	4	1	1	0.5	0.5	0.5	1	1	1	1
TF 1538	0.0625	<0.03125	0.0625	0.0625	0.125	0.125	0.125	0.125	1	1	4	1	0.5	0.5	0.5	0.5	1	1	1	1
TF 1413	0.0625	<0.03125	0.0625	0.0625	0.125	0.25	0.25	0.25	1	1	4	1	0.5	0.5	0.5	0.5	1	1	1	1
TF 832	0.0625	0.125	0.125	0.125	0.25	0.25	0.25	0.25	1	1	4	1	0.5	0.5	0.5	0.5	1	1	1	1
MODS 11	64	64	32	64	2	2	2	2	2	2	4	2	0.5	0.5	0.5	0.5	1	1	1	1
TT 14	16	16	16	16	64	> 64	> 64	> 64	1	1	4	1	0.5	0.5	0.5	0.5	1	1	0.5	1
TT 17	8	4	8	8	64	> 64	> 64	> 64	1	1	4	1	0.5	0.5	0.5	0.5	1	1	0.5	1
TF 44949	16	16	16	16	8	> 64	> 64	> 64	0.25	0.5	2	1	0.5	0.5	0.25	0.5	0.5	0.25	0.25	0.25
MODS 688	> 64	> 64	> 64	> 64	64	> 64	64	64	2	2	4	2	0.5	0.5	0.5	0.5	1	1	1	1
TF 2063	16	16	64	16	8	> 64	64	64	0.25	0.25	0.5	0.25	0.5	0.5	0.5	0.5	0.5	0.25	0.125	0.25
MODS 682	16	16	16	16	> 64	> 64	> 64	> 64	1	1	4	1	0.5	0.5	0.5	0.5	1	1	1	1
TT 50	32	32	64	32	64	> 64	> 64	> 64	1	1	4	1	2	4	4	4	1	1	1	1
TT 309	32	64	32	32	64	64	32	64	> 64	> 64	>64	> 64	0.5	0.5	0.5	0.5	1	1	1	1
TT 627	> 64	> 64	> 64	> 64	64	> 64	> 64	> 64	1	1	4	1	4	4	4	4	1	1	0.5	1
NT 1	32	64	32	32	2	4	2	2	1	1	4	1	16	16	16	16	1	1	1	1
NT 66	32	32	32	32	64	> 64	> 64	> 64	1	1	4	1	8	8	8	8	1	1	0.5	1
R11 654	32	32	32	32	64	> 64	64	64	1	0.5	4	1	8	4	8	8	1	0.5	0.5	0.5
TT 169	32	32	64	32	>64	64	64	64	> 64	> 64	>64	> 64	16	16	16	16	1	1	1	1
R2 404	16	32	32	32	16	16	16	16	> 64	> 64	>64	> 64	8	8	8	8	NG	0.125	0.25	0.25
R4 825	8	16	16	16	> 64	> 64	> 64	> 64	> 64	> 64	>64	> 64	8	8	8	8	1	1	1	1
TT 187	32	64	32	32	64	64	64	64	> 64	> 64	>64	> 64	16	16	16	16	1	1	1	1
TT 209	32	64	32	32	32	64	32	32	> 64	> 64	>64	> 64	16	16	16	16	1	1	1	1
R6 609	32	32	32	32	16	16	8	16	> 64	> 64	>64	> 64	16	16	16	16	0.5	0.5	0.5	0.5

R10 741	> 64	> 64	32	> 64	64	>64	> 64	> 64	> 64	> 64	>64	> 64	16	16	16	16	1	1	1	1
R10 398	64	32	32	32	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	4	4	4	4	1	0.5	1	1
R4 819	8	8	NG	8	> 64	64	64	64	8	4	4	4	4	4	4	4	1	1	0.5	1
R10 442	16	16	4	16	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	4	2	2	2	1	0.5	1	1
MODS 388	32	64	32	32	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	8	4	4	4	0.5	0.5	0.5	0.5
MODS 387	64	64	> 64	64	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	4	4	4	4	0.5	0.5	0.5	0.5
TF 1762	32	64	64	64	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	4	4	4	4	0.5	0.5	0.5	0.5
MODS 195	32	64	16	32	> 64	> 64	> 64	> 64	> 64	> 64	>64	> 64	4	4	4	4	0.5	0.5	0.5	0.5
R4 933	16	16	16	16	64	> 64	> 64	> 64	> 64	> 64	>64	> 64	8	8	4	8	0.5	0.5	0.5	0.5

B.2 MIC ($\mu\text{g/ml}$) results for *Neisseria gonorrhoeae* with multipoint inoculator

<i>N. gonorrhoeae</i> Isolate number	Ciprofloxacin				Nalidixic Acid				Nybomycin			
	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC
ATCC 49226	0.0625	< 0.0625	< 0.0625	< 0.0625	1	2	2	2	4	2	4	4
526	< 0.0625	< 0.0625	< 0.0625	< 0.0625	2	2	2	2	1	2	1	1
840	< 0.0625	< 0.0625	< 0.0625	< 0.0625	2	2	2	2	1	1	1	1
924	< 0.0625	< 0.0625	< 0.0625	< 0.0625	2	2	2	2	2	2	2	2
556	< 0.0625	< 0.0625	< 0.0625	< 0.0625	16	16	16	16	2	2	2	2
373	< 0.0625	< 0.0625	< 0.0625	< 0.0625	8	8	8	8	4	8	8	8
310	0.0625	< 0.0625	< 0.0625	< 0.0625	1	2	2	2	0.5	1	0.5	0.5
360	0.25	0.5	0.25	0.25	8	8	8	8	4	4	4	4
20	0.25	0.5	0.25	0.25	8	4	8	8	4	4	4	4
172	0.25	0.25	0.25	0.25	16	16	16	16	2	2	2	2
391	1	0.25	0.25	0.25	8	4	8	8	1	1	1	1
277	0.5	0.5	0.5	0.5	8	8	8	8	2	4	4	4
342	0.5	0.5	0.5	0.5	8	8	8	8	2	2	2	2
524	0.25	0.5	0.5	0.5	8	16	16	16	2	2	4	2
938	0.5	0.5	0.5	0.5	4	8	8	8	0.25	0.25	0.25	0.25
336	1	1	2	1	8	8	8	8	2	2	2	2

345	1	1	1	1	16	8	16	16	2	2	2	2
462	1	1	1	1	8	8	8	8	1	0.5	0.5	0.5
219	1	2	2	2	8	8	8	8	4	4	4	4
74	1	2	2	2	8	8	4	8	2	4	4	4
227	2	2	2	2	16	16	16	16	4	4	4	4
108	4	2	4	4	16	16	16	16	4	4	4	4
119	4	4	4	4	4	16	16	16	2	2	2	2
236	4	2	4	4	8	8	8	8	2	4	2	2
251	4	4	4	4	16	16	16	16	4	4	4	4
267	2	4	4	4	16	16	16	16	4	4	4	4
296	2	4	4	4	8	8	8	8	8	8	8	8
688	4	4	4	4	8	4	8	8	4	2	4	4
819	4	4	4	4	8	8	8	8	4	4	4	4
766	4	4	4	4	8	8	8	8	0.5	1	0.5	0.5

N. <i>gonorrhoeae</i> Isolate number	Quinine				Chloroquine				Mefloquine				Primaquine			
	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC
ATCC 49226	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
526	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
840	128	128	128	128	> 128	> 128	> 128	> 128	4	8	4	4	64	64	64	64
924	128	128	> 128	128	> 128	> 128	> 128	> 128	4	4	4	4	64	64	64	64
556	128	128	> 128	128	> 128	> 128	> 128	> 128	8	8	8	8	128	128	128	128
373	128	> 128	128	128	> 128	> 128	> 128	> 128	16	8	16	16	> 128	> 128	> 128	> 128
310	128	128	128	128	> 128	> 128	> 128	> 128	4	4	4	4	128	64	64	64
360	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	> 128	> 128	> 128
20	128	128	128	128	> 128	> 128	> 128	> 128	4	4	4	4	64	64	64	64
172	128	> 128	128	128	> 128	> 128	> 128	> 128	4	4	4	4	128	128	128	128

391	128	128	128	128	> 128	> 128	> 128	> 128	4	4	4	4	64	64	64	64
277	128	128	128	128	> 128	> 128	> 128	> 128	8	16	16	16	128	128	128	128
342	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
524	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	32	64	64	64
938	128	128	> 128	128	> 128	> 128	> 128	> 128	2	2	2	2	32	32	64	32
336	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
345	128	128	128	128	> 128	> 128	> 128	> 128	8	16	16	16	128	128	128	128
462	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	4	4	2	4	64	128	64	64
219	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	64	64	64	64
74	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
227	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	> 128	> 128	> 128
108	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	128	> 128	> 128
119	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
236	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	> 128	> 128	> 128
251	128	> 128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	> 128	> 128	> 128
267	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	> 128	> 128	> 128
296	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
688	128	128	128	128	> 128	> 128	> 128	> 128	16	16	16	16	> 128	> 128	128	> 128
819	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	16	16	16	16	128	128	128	128
766	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	1	0.5	0.5	0.5	16	16	16	16

B.3 MIC ($\mu\text{g/ml}$) results for *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Staphylococcus aureus* with multipoint inoculator.

Bacterial isolate	Ciprofloxacin				Nalidixic Acid				Nybomycin			
	A	B	C	Final MIC	A	B	C	Final MIC	A	B	C	Final MIC
<i>E. coli</i> ATCC 25722	< 0.125	< 0.125	< 0.125	< 0.125	8	8	8	8	> 16	> 16	> 16	> 16
<i>E. coli</i> no. 1	< 0.125	< 0.125	< 0.125	< 0.125	16	16	16	16	> 16	> 16	> 16	> 16
<i>E. coli</i> no. 2	2	2	1	2	16	16	16	16	> 16	> 16	> 16	> 16

<i>E. coli</i> no. 3	2	4	2	2	16	16	16	16	> 16	> 16	> 16	> 16
<i>E. coli</i> no. 4	32	16	32	32	16	16	16	16	> 16	> 16	> 16	> 16
<i>E. coli</i> no. 5	32	32	32	32	16	16	16	16	> 16	> 16	> 16	> 16
<i>K. pneumoniae</i> no. 1	< 0.125	< 0.125	< 0.125	< 0.125	16	16	16	16	> 16	> 16	> 16	> 16
<i>K. pneumoniae</i> no. 2	0.5	0.25	0.25	0.25	16	16	16	16	> 16	> 16	> 16	> 16
<i>K. pneumoniae</i> no. 3	1	2	2	2	16	16	16	16	> 16	> 16	> 16	> 16
<i>K. pneumoniae</i> no. 4	16	16	16	16	16	16	16	16	> 16	> 16	> 16	> 16
<i>E. cloacae</i> no. 1	< 0.125	< 0.125	< 0.125	< 0.125	8	8	8	8	> 16	> 16	> 16	> 16
<i>E. cloacae</i> no. 2	8	8	8	8	8	8	8	8	> 16	> 16	> 16	> 16
<i>E. cloacae</i> no. 3	32	16	32	32	8	8	8	8	> 16	> 16	> 16	> 16
<i>E. cloacae</i> no. 4	32	32	32	32	16	16	16	16	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 1	0.25	0.25	0.25	0.25	8	8	8	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 2	0.5	1	0.5	0.5	8	8	4	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 3	8	8	8	8	8	8	8	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 4	8	16	16	16	8	8	8	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 5	16	16	16	16	8	8	4	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 6	16	16	16	16	8	8	8	8	> 16	> 16	> 16	> 16
<i>P. aeruginosa</i> no. 7	32	16	32	32	8	8	8	8	> 16	> 16	> 16	> 16
<i>A. baumannii</i> no. 1	0.25	0.25	0.25	0.25	8	16	8	8	> 16	> 16	> 16	> 16
<i>A. baumannii</i> no. 2	2	2	2	2	8	8	16	8	> 16	> 16	> 16	> 16
<i>A. baumannii</i> no. 3	2	4	4	4	8	8	8	8	> 16	> 16	> 16	> 16
<i>A. baumannii</i> no. 4	8	16	16	16	8	8	8	8	> 16	> 16	> 16	> 16
<i>A. baumannii</i> no. 5	> 32	> 32	> 32	> 32	8	16	16	16	8	16	8	8
<i>E. faecalis</i> no. 1	1	1	0.25	1	32	32	32	32	8	16	16	16
<i>E. faecalis</i> no. 2	2	2	2	2	32	32	32	32	8	16	16	16
<i>E. faecalis</i> no. 3	> 32	> 32	> 32	> 32	32	32	32	32	8	4	8	8
<i>E. faecalis</i> no. 4	> 32	> 32	> 32	> 32	32	32	32	32	4	8	8	8
<i>S. aureus</i> no. 1	0.5	1	0.5	0.5	32	32	32	32	4	4	4	4
<i>S. aureus</i> no. 2	0.5	0.5	0.5	0.5	32	32	32	32	2	8	8	8
<i>S. aureus</i> no. 3	4	2	2	2	32	32	32	32	4	8	4	4
<i>S. aureus</i> no. 4	> 32	> 32	> 32	> 32	32	32	32	32	4	4	4	4

<i>E. faecalis</i> no. 3	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	32	32	32	> 128	> 128	> 128	> 128
<i>E. faecalis</i> no. 4	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	32	32	32	> 128	> 128	> 128	> 128
<i>S. aureus</i> no. 1	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	8	32	32	> 128	> 128	> 128	> 128
<i>S. aureus</i> no. 2	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	16	32	32	> 128	> 128	> 128	> 128
<i>S. aureus</i> no. 3	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	32	32	32	> 128	> 128	> 128	> 128
<i>S. aureus</i> no. 4	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	> 128	32	32	32	32	> 128	> 128	> 128	> 128

B.4 MIC ($\mu\text{g/ml}$) results for *Mycobacterium tuberculosis* with MTT assay.

<i>M. tuberculosis</i> Isolate number	Nybomycin				DNM-2			
	A	B	C	Final MIC	A	B	C	Final MIC
H37Rv	2	2	2	2	1.5	1.5	1.5	1.5
TF 1538	2	2	2	2	3	3	3	3
TF 1413	2	2	2	2	3	3	3	3
TF 832	2	2	2	2	3	3	3	3
MODS 11	2	2	2	2	3	3	3	3
TT 17	1	1	1	1	3	1.5	3	3
TF 44949	2	1	2	2	3	3	3	3
MODS 688	2	2	2	2	3	3	3	3
TF 2063	2	1	2	2	3	1.5	3	3
TT 50	0.5	1	1	1	3	3	3	3
TT 309	1	1	1	1	1.5	3	3	3
TT 627	1	1	1	1	1.5	1.5	1.5	1.5
NT 1	2	1	2	2	3	3	3	3
NT 66	1	1	1	1	1.5	1.5	1.5	1.5
TT 169	1	1	1	1	1.5	1.5	1.5	1.5
R2 404	2	2	2	2	1.5	1.5	1.5	1.5
R4 825	1	1	1	1	1.5	1.5	1.5	1.5
R6 609	1	1	2	1	1.5	1.5	1.5	1.5
R10 741	1	1	1	1	3	1.5	3	3

R4 819	2	2	1	2	1.5	1.5	1.5	1.5
R10 442	2	2	2	2	3	3	3	3
MODS 388	1	1	1	1	3	1.5	1.5	1.5
MODS 387	2	2	2	2	1.5	1.5	1.5	1.5

