Antimicrobial, synergistic and autophagic effects of Medicines for Malaria Venture Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae*

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DECLARATION

I, **Eric Mensah**, hereby declare that the work contained in this thesis is my own original work and neither whole nor part of it has been previously submitted for a degree at this or any other University or tertiary institution.

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Signature of Candidate

15 June 2023

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Date

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I would like to thank my heavenly Father, Almighty God for giving me strength, wisdom and guiding me throughout this PhD journey. All the glory and praise belong to You!

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Antimicrobial, synergistic and autophagic effects of Medicines for Malaria Venture Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae*

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SUMMARY

Antimicrobial resistance in *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* is emerging globally. Due to the limited treatment options, the World Health Organization has designated *M. tuberculosis* and *N. gonorrhoeae* as two critical and high-priority pathogens for research and development of novel antibiotic drugs. Particularly for *M. tuberculosis*, new agents that can induce autophagy processes directed at clearing intracellular *M. tuberculosis* from host cells is highly needed.

To speed up the discovery and development of novel agents, the Medicines for Malaria Venture (MMV) group developed the Pathogen Box, containing a collection of 400 novel drug compounds. The Pathogen Box was originally assessed primarily for anti-malarial properties but, in the initial screen, has been shown to contain compounds potentially also effective against several other microorganisms, including *M. tuberculosis*. The aim of this study was to explore the antibiotic potential, including synergistic and autophagic effects, of this diverse compound library of the MMV Pathogen Box.

As a first step, the identities and resistance profiles of clinical strains of *M. tuberculosis* and *N. gonorrhoeae* selected for use in this study were confirmed, using GeneXpert MTB/RIF and MTBDR*plus* assays, followed by whole genome sequencing (WGS). Broth microdilution assay was used to determine the pathogen-specific minimum inhibitory and minimum bactericidal concentrations (MICs/MBCs) of the Pathogen Box compounds (PBCs) against reference strains of *M. tuberculosis* and *N. gonorrhoeae*. Finally, a checkerboard assay approach was

used to determine synergy between the active compounds if used in combination with reference drugs. Time-kill kinetics was performed to determine bactericidal or bacteriostatic activity. Selecting priority compounds for further investigation was based on the following criteria: (1) MIC and MBC for *N. gonorrhoeae* ≤ 10 µM; and (2) MIC and MBC for *M. tuberculosis* ≤ 0.625 µM.

Five PBCs, MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413, showed potent activity against both susceptible and multidrug-resistant *M. tuberculosis* strains at MIC and MBC below 0.625 µM. Except for MMV687696, the remaining four PBCs were clearly bactericidal. Combining the PBCs with isoniazid or rifampicin demonstrated either synergistic or additive activity, with fractional inhibitory concentration indexes ranging between 0.18 and 2.60. The five selected anti-TB PBCs recorded low cytotoxicity in murine-derived macrophages and effectively suppressed the growth of intracellular *M. tuberculosis*. Western blotting analysis was used to assess the potential of the five selected PBCs to induce autophagy against intracellular *M. tuberculosis* in host cells. All compounds induced some level of LC3 lipidation and LC3II/LC3I, although this was not statistically significant compared to controls. Notably, inhibition of the autophagic flux reversed the anti-mycobacterial activity of MMV676603, MMV687146, and MMV687180.

Eight PBCs, MMV676501, MMV002817, MMV688327, MMV688508, MMV024937, MMV687798 (Levofloxacin), MMV021013, and MMV688978 (Auranofin), demonstrated potent activity against resistant strains of *N. gonorrhoeae* at a MIC and MBC of ≤ 10 µM. All the compounds showed potent bactericidal activity between 4 and 24 hrs, with time-kill kinetics similar to that of ceftriaxone. The *N. gonorroheae* active PBCs in combination with ceftriaxone showed either synergistic or additive activity with fractional inhibitory concentration indexes ranging between 0.40 to 1.8.

Conclusion. This study has identified novel compounds with potent activity against both resistant and susceptible strains of *N. gonorrhoeae* and *M. tuberculosis*. The study has also identified compounds that can suppress the growth of intracellular *M. tuberculosis* with the potential to induce autophagy at high concentrations. Overall, the study results point to promising anti-gonococcal and anti-TB drug leads worthy of further exploration.

LIST OF ABBREVIATIONS

ADD	Additive
AMR	Antimicrobial resistance
APC	Antigen-presenting cell
ATCC	American Type Culture Collection
ATG	Autophagy-related proteins
AST	Antimicrobial sensitivity testing
AZM	Azithromycin
BCG	Bacille Calmette-Guérin
BDQ	Bedaquiline
BMDM	Bone marrow-derived macrophages
CDC	Centers for Disease Control and Prevention
CEACAM	Carcinoembryonic antigen cell adhesion molecule
CFU	Colony-forming unit
CFX	Cefixime
CIP	Ciprofloxacin
CO2	Carbon dioxide
CMP-NANA	Cytidine 5'- monophosphate N-acetylneuraminic acid
CRO	Ceftriaxone
DALYs	Disability-adjusted life-years
DGI	Disseminated gonococcal infection
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DR-TB	Drug-resistant tuberculosis
DST	Drug susceptibility testing
ECOFF	Epidemiological cut-off
EIS	Enhanced intracellular survival
ESC	Extended-spectrum cephalosporin
ESAT-6	Early secretory antigenic target 6
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EBSS	Earle's Balanced Salt Solution
FBS	Fetal bovine serum

FICI	Fractional inhibitory concentration index
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HBHA	Heparin-binding haemagglutinin
HIV	Human Immunodeficiency virus
IND	Indifference
IFN	Interferon
INH	Isoniazid
IGRA	Interferon-gamma release assay
LC3	Light chain 3 beta
LJ	Löwenstein–Jensen
LOS	Lipooligosaccharide
LPA	Line probe assay
LPS	Lipopolysaccharides
LTBI	Latent tuberculosis infection
MBC	Minimum bactericidal concentration
MDC	Monodansylcadaverine
MDR	Multidrug-resistant
MHC	Major histocompatibility complex
MGIT	Mycobacteria growth indicator tube
MIC	Minimum inhibitory concentration
MBC	Minimum bactericidal concentration
MMV	Medicines for malaria ventures
PID	Pelvic inflammatory disease
MRSA	Methicillin-resistant *Staphylococcus aureus*
Mtb	*Mycobacterium tuberculosis*
MTBc	*Mycobacterium tuberculosis* complex
NOS	Nitric oxide
NTM	Nontuberculous mycobacteria
Opa	Opacity-associated protein
Por	Porin proteins
PAS	Phagophore assembly site
PBCs	Pathogen Box Compounds

PBS	Phosphate-Buffered Saline
PRRs	Pattern recognition receptors
PTLD	Post-TB lung disease
PZA	Pyrazinamide
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene fluoride membrane
R&D	Research and development
ROS	Reactive Oxygen Species
RFP	Red fluorescent protein
SPC	Spectinomycin
SOD	Superoxide dismutase
Tfp	Type IV pilus
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TST	Tuberculin skin test
SYN	Synergy
RR-TB	Rifampicin-Resistant Tuberculosis
TB	Tuberculosis
TFEB	Transcriptional Factor ED
UN	United Nations
WHO	World Health Organization
XDR	Extensively drug-resistant

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LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

PUBLICATIONS

Mensah E, Peters RPH, Fourie PB. Antimicrobial and synergistic effects of MMV Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis.* Abstract OA03-219-08, Abstract Book. 53rd Union World Conference on Lung Health, Virtual, 2022. https://theunion.org/sites/default/files/2022-11/Abstract_Book_2022-compressed.pdf.

PAPERS IN PREPARATION

Mensah E, Peters RPH, Fourie PB (2023) Antimicrobial effects of Medicines for Malaria Venture Pathogen Box compounds on strains of *Neisseria gonorrhoeae.* Revision submitted for publication in *Antimicrobial Agents and Chemotherapy*.

Mensah E, Peters RPH, Fourie PB (2023) Antimicrobial and synergistic effects of Medicines for Malaria Venture Pathogen Box compounds on drug-resistant strains of *Mycobacterium tuberculosis.* To be submitted to *Journal of Antimicrobial Chemotherapy.*

Mensah E, van den Bout I, Peters RPH, Fourie PB (2023) Autophagic effects of Pathogen Box compound and intracellular control of *Mycobacterium tuberculosis*. To be submitted to *Cell Host & Microbe.*

CONFERENCE PRESENTATIONS

Mensah E, Peters RPH, Fourie PB. Antimicrobial and synergistic effects of MMV Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis*. Presented at the 53rd Union World Conference on Lung Health, virtual, from 8-11 November 2022.

Mensah E, Peters RPH, Fourie PB. Antimicrobial effects of MMV Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis*. Poster presentation at Faculty of Health Science Research Day from 23-24 August 2022.

CHAPTER 1: Introduction

1.1 Study rationale

Treatment options for My*cobacterium tuberculosis* and *Neisseria gonorrhoeae* are limited due to emergence of global resistance (WHO, 2020b; Mallick *et al*., 2022; Cámara *et al*., 2012; Pleininger *et al.,* 2022). Due to this, the WHO has designated *M. tuberculosis* and *N. gonorrhoeae* as critical and high priority pathogen to advance the search and development of novel drugs (WHO, 2017; Tacconelli *et al*., 2018).

1.1.1 WHO priority pathogens

The extensive use of antibiotics is driving global antimicrobial resistance leading to the rapid evolution of resistant pathogens, resulting in more extended hospital stay, higher cost of treatment, and high morbidity and mortality rates (Cassini *et al*., 2019; Murray *et al*., 2022). The ever-increasing rate of drug-resistant bacterial infections, coupled with the limited antibiotic arsenals available to tackle infectious diseases, has invigorated the search for novel and effective drugs (WHO, 2017; Tacconelli *et al*., 2018; Pleininger *et al*., 2022), including drugs that can induce cellular processes such as autophagy (Campbell and Spector, 2012; Kim *et al.*, 2012; Kimmey and Stallings, 2016; Giraud-Gatineau *et al.*, 2020). Due to the limited treatment options, the WHO has developed a priority list of antibiotic-resistant bacteria to advance research and development (R&D) of effective drugs for such pathogens (WHO, 2017; Tacconelli *et al*., 2018). Fluoroquinolone-resistant *Mycobacterium tuberculosis,* and 3rd generation cephalosporin-resistant *Neisseria gonorrhoeae* are classified as critical and high-priority pathogens in need of new therapeutic interventions (WHO, 2017; Tacconelli *et al*., 2018).

The WHO priority pathogens are defined as the most resistant bacterial pathogens at a global level that have limited treatment options and are responsible for high morbidity burden and all-

cause mortality. The potential for rapid spread and difficulty confronted when treating these resistant organisms make it critically important that appropriate interventions and public health policies that are grounded in scientific evidence, are developed as a matter of urgency. These consequences pose a significant public health concern and affect service delivery. Research and development of novel compounds that show potent activity against these pathogens, or agents that potentiate the actions of existing drugs are urgently needed to combat the rise of antibiotic resistance in pathogens currently posing a threat to public health (WHO, 2017; Tacconelli *et al*., 2018; WHO, 2020b).

Mycobacterium tuberculosis is a major intracellular pathogen of humans that remains the leading cause of death globally from a single infectious agent (WHO, 2020b). In 2021, 10.6 million incident tuberculosis (TB) cases (WHO, 2022) and 1.6 million deaths were reported (WHO, 2022). Approximately one-quarter of the world's population is latently infected with *M. tuberculosis*, of which about 5% to 10% of people latently infected develop tuberculosis throughout their lives (WHO, 2020a). Drug-resistant TB (DR-TB) is a risk factor fuelling the ongoing TB epidemic and increasing the morbidity and mortality of TB, as well as jeopardizing the TB control program (WHO, 2020b; Mallick *et al*., 2022; Omar *et al*., 2022). Whereas TB is treatable and curable, current anti-TB regimens are losing their effectiveness due to the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (Seddon *et al*., 2021; Mallick *et al*., 2022; Omar *et al*., 2022). It is estimated that approximately 5% of global TB cases are caused by MDR *M. tuberculosis* strains, accounting for roughly 500 000 cases of newly diagnosed MDR infections annually (WHO, 2020b). Only 52% of all MDR-TB patients are effectively treated globally, and at best just one out of every three patients with XDR-TB responds to treatment (WHO, 2017; WHO, 2022). The total number of patients treated for MDR TB increased from 122 726 in 2015 to 156 205 in 2018 and 177 099 in 2019 (WHO, 2020b). The increasing number of patients treated for MDR TB, with associated morbidity, permanent disability and mortality, coupled with the limited therapeutic options for DR-TB highlight the need for the search and development of novel and effective anti-TB drugs (Dheda *et al*., 2019).

Gonorrhoea is a common sexually transmitted disease worldwide with a high morbidity burden (Maduna *et al*., 2020; Suay-García and Pérez-Gracia, 2020). *Neisseria gonorrhoeae* caused 82 million new cases of gonococcal infections in 2020 (WHO, 2020). Increasing evidence shows that the actual number of these infections is under-reported because of inadequate clinical or

diagnostic infrastructure, poor reporting systems, and high rates of asymptomatic infections (Tapsall, 2009; Walker and Sweet, 2012; Rice *et al*., 2017). Severe complications such as increased risk of tubal factor infertility, pelvic inflammatory disease, ectopic pregnancy, and adverse pregnancy outcomes can occur without effective treatment (Little, 2006; WHO, 2011, Burnett *et al*., 2012). The evolution of *N. gonorrhoeae* strains resistant to all drugs recommended for treatment is a major public health concern (Unemo *et al*., 2012; WHO, 2012). The emergence of multidrug-resistant and extensively drug-resistant strains has worsened the situation, making *N. gonorrhoeae* a superbug, requiring urgent development of new drugs and therapeutic options (Cámara *et al*., 2012; Pleininger *et al.,* 2022). The development of high-level resistance to last resort ceftriaxone, leading to untreatable gonorrhoeae, coupled with the limited pipeline of new anti-gonococcal drugs highlights the urgent need to discover or develop new treatment agents, even for susceptible *N. gonorrhoeae*, since ceftriaxone is the only treatment option currently (Pleininger *et al*., 2022; Unemo and Nicholas, 2012).

1.1.2 The MMV Pathogen Box compounds (PBCs)

To accelerate the discovery of novel drug compounds, the Medicines for Malaria Venture (MMV) group has developed the Pathogen Box, an open-access library consisting of a collection of 400 novel and reference drugs (https://www.mmv.org). These compounds were originally identified from a screen of ~4 million chemicals on basis of their low toxicity to mammalian cells (http://www.pathogenbox.org/). The novel compounds have diverse chemical scaffolds distinct from presently available antibiotics (Duffy *et al.*, 2017). Each of the compounds has been tested for cytotoxicity and has shown values acceptable for the initial drug discovery program. The Pathogen Box compounds (PBCs) have demonstrated biological activity against specific pathogenic microorganisms in a screen that was initially mostly directed at protozoal parasites responsible for tropical diseases, in particular malaria. However, it also included compounds with activity against *M. tuberculosis* and to a lesser extent, compounds with activity against other microorganisms including kinetoplastids, helminths, cryptosporidiosis, toxoplasmosis, and dengue (Ballell *et al*., 2013; Duffy *et al*., 2017; Veale, 2019). The activity of the PBCs against resistant forms of several key pathogens, including *M. tuberculosis* and *N. gonorrhoeae,* has not been sufficiently explored and provides scientists with the opportunity to screen and identify new drug agents against pathogens with limited treatment options.

Among the Gram-negative bacteria, the activity of the PBCs has been reported in few studies.

The compound MMV675968 demonstrated antimicrobial activity against different strains of *Acinetobacter baumannii* and *Escherichia coli* by targeting dihydrofolate reductase (Songsungthong *et al*., 2019, Sharma *et al*., 2022). The dual combination of MMV675968 with last resort antibiotics colistin and meropenem exhibited a synergistic effect against strains of *E. coli*, including MDR strains (Sharma *et al*., 2022). In a screen of the PBCs against *Vibrio cholerae*, two compounds MMV687807 and MMV675968 were found that effectively inhibited growth (Kim *et al*., 2021). RNA sequencing analysis revealed that these compounds affect multiple cellular functions including iron homeostasis, carbon metabolism, and biofilm formation (Kim *et al*., 2021).

1.1.2 Autophagy-inducing agents and control of intracellular *M. tuberculosis*

An emerging field in tuberculosis pathogenesis research is autophagy and its role in controlling intracellular parasites such as *M. tuberculosis* (Klionsky and Emr, 2000; Yoshimori and Noda, 2008; Deretic and Levine, 2009). Autophagy is a key factor in antimicrobial defense mechanisms (Claude-Taupin *et al.*, 2017). During this process, non-functional or redundant intracellular components such as long-lived proteins and damaged organelles are sequestered into autophagosomes which then fuse with lysosomes for degradation (Deretic, 2015; Claude-Taupin *et al.*, 2017). Autophagy facilitates the host response against the destruction of intracellular pathogens and connects the innate and adaptive immune function (Deretic, 2010; Jo, 2010). In bacterial infection, targeting of the substrate involves ubiquitination of the bacteria, and recognition of the ubiquitin by autophagy receptors (Kimmey and Stallings, 2016). The autophagy receptors interact with LC3 to recruit bacteria to autophagosomes (Kimmey and Stallings, 2016). Autophagy is central to the destruction of intracellular pathogens such as *M. tuberculosis, Salmonella* spp., *Coxiella* spp., and *Legionella* spp. in an autophagy process called xenophagy facilitated by specific receptors (Espert *et al*., 2015; Paulus and Xavier, 2015; Lam *et al.*, 2017). However, these pathogens can prevent autophagy through different mechanisms. These include interference with autophagy machinery, masking themselves with host proteins to avoid recognition and inhibition of fusion of autophagosomes with lysosomes; making them proliferate inside the macrophages and initiate cellular necrosis while inhibiting apoptosis (Huang and Brumell, 2014; Kimmey and Stallings, 2016; Siqueira *et al*., 2018). Increasing studies are confirming the ability of *M. tuberculosis* to inhibit the cellular autophagy machinery, using proteins such as the heparin-binding haemagglutinin (HBHA), Early secretory antigenic target-6 (ESAT-6), and enhanced intracellular survival (EIS) (Paulus and

Xavier, 2015; Lam *et al.*, 2017; Zheng *et al.*, 2017).

The essential roles of antibiotics and specific compounds in inducing/enhancing autophagy is increasingly reported. Isoniazid and pyrazinamide (PZA), as well as reactive oxygen species (ROS), which are also produced by antibiotics' interactions with the host, was found to induce autophagy in *M. tuberculosis*-infected macrophages (Kim *et al.*, 2012). It was observed that without autophagy, isoniazid and pyrazinamide were unable to clear *M. tuberculosis* from macrophages (Kim *et al.*, 2012). *Mycobacterium marinum*-infected *Drosophila* flies lacking autophagy gene 7 (*atg7)* had a low survival rate, which could not be rescued with antibiotics (Kim *et al.*, 2012). Similarly, in a more recent study (2020) by Giraud-Gatineau and colleagues, bedaquiline (BDQ), a recently developed antibiotic against multidrug-resistant *M. tuberculosis*, was found to increase macrophage lysosomal activity and induced autophagy activation. Bedaquiline was able to reprogram macrophages into potent bactericidal phagocytes to enhance intracellular killing of *M. tuberculosis*, *Staphylococcus aureus* and *Salmonella typhimurium* that were naturally insensitive to BDQ (Giraud-Gatineau *et al.*, 2020). Thus, it is certain that not only do antibiotics directly kill pathogens, but they also induce autophagy to enable the immune system to eliminate intracellular bacteria directly. Since current anti-TB chemotherapy for treating MDR-TB and XDR-TB have low efficiency, drugs that can induce autophagy has the potential to modulate host cell response towards enhanced immunity, and to aid in the elimination of intracellular pathogens (Kim *et al.*, 2012; Kimmey and Stallings, 2016; Giraud-Gatineau *et al.*, 2020). Induction and regulation of autophagy is promising because autophagy works at the host cellular level to enhance intracellular killing of both replicating and non-replicating pathogens, including MDR and XDR strains, where pathogen resistance is unlikely to evolve (Campbell and Spector, 2012). In the face of increasing drug resistance, not only is it crucial to find antibiotics or antibiotic combinations that are effective against drug-resistant pathogens, but it is also vital to have an agent that can overcome pathogen-initiated autophagy inhibition and facilitate autophagosome-lysosome fusion within macrophages (Kim *et al.*, 2012; Paulus and Xavier, 2015; Lam *et al.*, 2017).

1.2 Aim

This study aimed to identify the antimicrobial, synergistic, and autophagic effects of the MMV PBCs against resistant strains of two bacterial pathogens of global public health importance, *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae.*

1.3 Objectives

1. To confirm the identity, antimicrobial susceptibility testing and resistance mechanisms of *M. tuberculosis* strains used in these experiments using GeneXpert/MTBDRplus assays, and *N. gonorrhoeae* strains using API NH system, E-test, and whole genome sequencing.[1]
2. To determine the pathogen-specific MICs and MBCs of selected drug molecules in the Pathogen Box for resistant strains of *M. tuberculosis* and *N. gonorrhoeae* by using a broth dilution method.
3. To identify lead compounds from the Pathogen Box with antibiotic activity against *M. tuberculosis* and *N. gonorrhoeae* based on low MIC and MBC and to describe mutations that might govern resistance to the selected compounds using RNA transcriptomics and whole genome sequencing.[2]
4. To compare the pathogen-specific *in vitro* kill and host cell toxicity kinetics of selected PBCs with that of reference medications commonly used against *M. tuberculosis* or *N. gonorrhoeae*.
5. To investigate the potential of selected Pathogen Box lead compounds against intracellular *M. tuberculosis* for inducing/enhancing autophagy processes or potentiating existing drug actions directed at clearing host cells of the infecting organism using MDC staining, LC3 puncta detection by immune fluorescence laser confocal microscopy, and Western Blot analysis.[3]

1.4 Hypothesis

Specific objective 1

H0: The identity, antimicrobial susceptibility testing and resistance mechanisms of *M. tuberculosis* and *N. gonorrhoeae* strains used in these experiments confirm suitability for use in the proposed experiment.

Specific objectives 2 and 3

H0: The Pathogen Box contains compounds with potential therapeutic effects based on MICs

[1] Whole genome sequencing of the *Neisseria gonorrhoeae* strain was not performed. Note the text on page 150.
[2] RNA transcriptomics and whole genome sequencing were not performed. Compounds eventually selected had known mechanisms of action.
[3] Immune fluorescence laser confocal microscopy was not performed. Note the text on page 127.

and MBCs in resistant strains of the bacterial pathogens *M. tuberculosis* and *N. gonorrhoeae.*

Specific objective 4

H0: One or more of the prioritised PBCs show adequate bacterial kill activity to *M. tuberculosis* and *N. gonorrhoeae* to be advanced as therapeutic lead agents for further characterisation.

Specific objective 5

H0: One or more of the Pathogen Box lead compounds induce/enhance autophagy processes in clearing *M. tuberculosis* from host cells.

1.5 Thesis structure and overview of chapters

This study investigated the antimicrobial activity of PBCs against *M. tuberculosis* and *N. gonorrhoeae*. In this thesis, the literature and experimental result chapters of *M. tuberculosis* and *N. gonorrhoeae* are presented in different parts or chapters. This thesis is presented in a publication format and consists of six chapters:

(i) **Chapter 1** (general introduction);

(ii) **Chapter 2** (literature review, consisting of three parts: part 1, part 2 and part 3);

(iii) **Chapter 3** (first research manuscript);

(iv) **Chapter 4** (second research manuscript);

(v) **Chapter 5** (third research manuscript); and

(vi) **Chapter 6** (general discussion and conclusion, consisting of two parts: part 1 and part 2).

The results chapters in the thesis are presented as three research manuscripts, each prepared in the style as prescribed by the guidelines of the journals where the manuscripts are targeted for publication.

Figure 1.1 depicts the thesis structure and chapter overview.

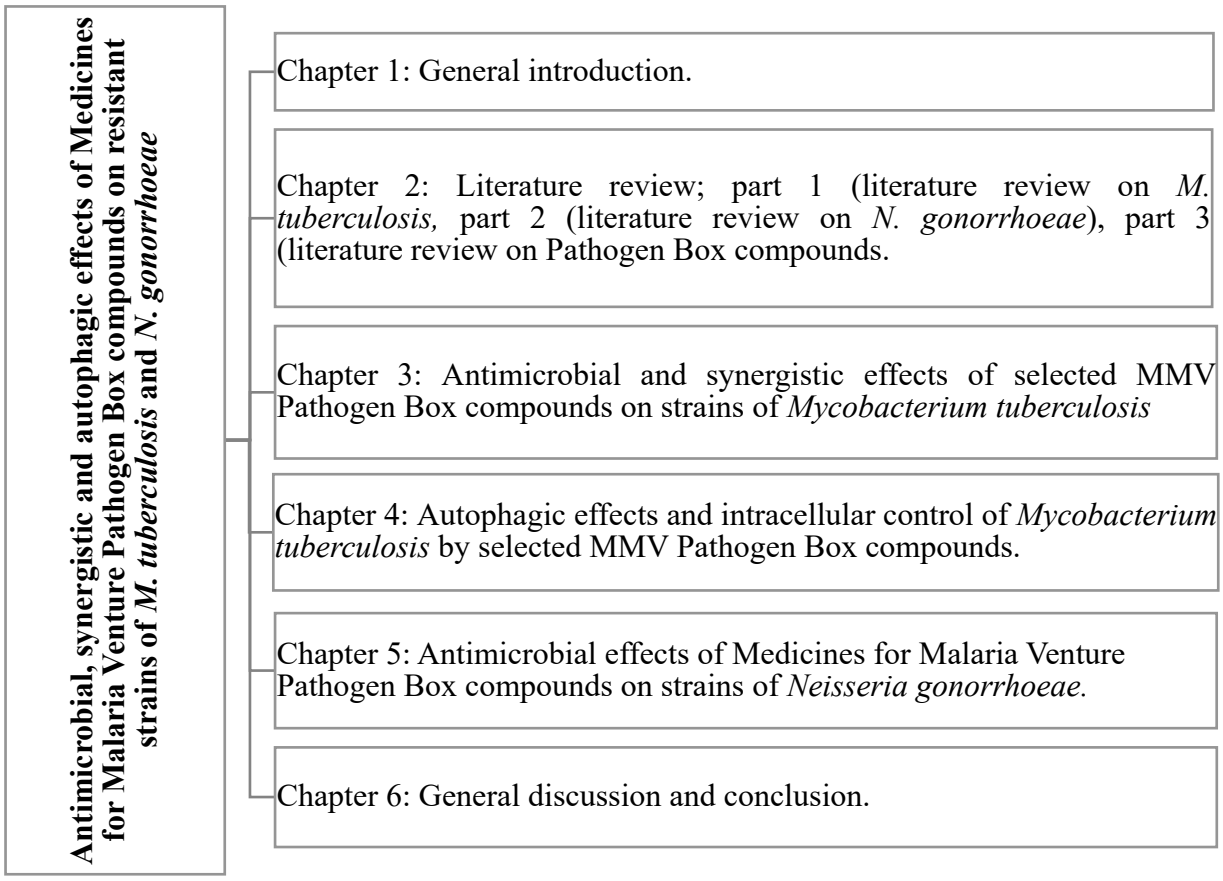

Figure 1. 1: Thesis structure and chapter overview

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CHAPTER 2: Literature Review

The emergence of antimicrobial resistance in *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* is a major public health concern. The need for new agents to treat bacterial infections with limited treatment option is a critical global priority. Due to this, the World Health Organization (WHO) developed a list of antibiotic-resistant 'priority' pathogens designated as critical, high, and medium to advance the search and development of antibiotics for such pathogens (WHO, 2017; Tacconelli *et al*., 2018). The Medicines for Malaria Ventures group developed the Pathogen Box to speed up the discovery and development of novel compounds (Ballell *et al*., 2013; Duffy *et al*., 2017).

This chapter is divided into three parts. Parts 1, 2 and 3 provide a literature review of *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, and the Medicines for Malaria Venture Pathogen Box, respectively.

2.1 Part 1: *Mycobacterium tuberculosis*

2.1.1 Epidemiology of *M. tuberculosis*

Tuberculosis (TB) is a major cause of death from a single infection, ranking higher than HIV/AIDS (Gorlenko *et al*., 2020; WHO, 2020a). TB is spread by aerosol when sick TB patients expel the bacteria in the air (Getahun *et al*., 2015; Cerceo *et al*., 2016). TB normally affects the lungs (pulmonary) but can also spread to other sites (extrapulmonary) (WHO, 2020b). *M. tuberculosis* survives in macrophages after a primary infection stage in most patients, allowing it to escape the host immune system, resulting in latent tuberculosis infection (LTBI) (Mack *et al*., 2009). According to the WHO, approximately one-quarter of the world's population is latently infected with *M. tuberculosis* (WHO, 2020a). The risk of TB disease after infection depends on several factors, the most important being weakened immunological status (Getahun *et al*., 2015; Cerceo *et al*., 2016). Most of the infected individuals show no symptoms of TB and are also not infectious but have an increased risk of progression to active TB disease and become infectious. On average, 5% to 10% of people latently infected will develop tuberculosis disease throughout their lives, with the majority developing the disease during the first five years of infection (WHO, 2020a). Approximately, 75% of people who develop the active disease after coming into contact with TB do so within a year after the index patient's TB diagnosis, and 97% develop TB within two years (Behr *et al*., 2018).

Since antiquity, TB has been a leading cause of illness and death in human society. In 2021, 10.6 million individuals worldwide developed tuberculosis (WHO, 2022c). In the same year, approximately 1.6 million TB deaths were recorded in HIV-negative people and 187,000 deaths among HIV-positive people (WHO, 2022c). Men and women respectively accounted for 56.5% and 32.5% of the people who developed TB in 2021, and 11% were children (aged <15 years) (WHO, 2022c). Among the total number of infected individuals, only 6.7% were individuals living with HIV (WHO, 2022c). Thirty high TB burden countries account for almost 90% of cases of TB per year. Geographically, the majority of patients who were developed TB in 2019 originated from South-East Asia (45%), Africa (23%), and the Western Pacific (18%) (WHO, 2022c). Few cases were reported in the Eastern Mediterranean (8.1%), the Americas (2.9%), and Europe (2.2%). Eight countries including India (28%), Indonesia (9.2%), China (7.4%), the Philippines (7.0%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%), and the Democratic Republic of the Congo (2.9%). (3.6%) accounted for two-thirds of the global total (WHO, 2022c). In 2021, South Africa reported 3.3% of the global total TB cases and is counted as one of the highest-burden TB countries in the world (WHO, 2021b).

2.1.2 Classification of Mycobacteria

Mycobacterium is the only genus in the *Mycobacteriaceae* family (Eisenstadt and Hall, 1995). *Mycobacterium* spp. are non-spore-forming, non-motile bacilli, that are thin, slightly curved to straight (Tortoli, 2006). There are currently more than 170 species in the genus *Mycobacterium* (Tortoli, 2006; Tortoli, 2014). Most of these species are environmental microorganisms found in soil, food, and vegetation that have never been linked to human illness, while others are known for causing human diseases (Esteban and Muñoz-Egea, 2017).

Medically important mycobacterial species are grouped into *M. tuberculosis* complex (MTBc) and nontuberculous mycobacteria (NTM) (Jhun *et al*., 2017). MTBc are causative agents of human and animal tuberculosis, which includes the species *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. microti,* and *M. canettii* (Jhun *et al*., 2017; Tan *et al*., 2018). NTM species are associated with a pulmonary disease resembling tuberculosis, lymphadenitis, localized skin, and subcutaneous tissues infections (Hautmann,1997; Jhun *et al*., 2017). The species include *M. avium*, *M. intracellulare*, *M. haemophilum*, *M. marinum*; *M. fortuitum*, *M. chelonae*, *M. abscessus*, and *M. massiliense (*Falkinham, 2013; Tan *et al*., 2018).

2.1.3 General characteristics of Mycobacteria

The genus *Mycobacterium* comprises more than 100 species, all of which are closely related based on their 16S rRNA sequences (Tortoli, 2006). Mycobacteria are non-sporulated rods that are aerobic, non-motile, and do not possess capsules or produce toxins (Rastogi *et al*., 2001). *Mycobacterium* species are characterized by the possession of thick, waxy, lipid-rich hydrophobic cell walls that is high in mycolic acids, which make them unique from other bacteria (Collins *et al*., 1984). Due to the high lipid content, these bacteria resist de-staining using weak mineral acids or acid-alcohol solutions that are commonly employed during the Gram stain (Talbot and Raffa, 2015). As a result, mycobacteria and members of the related genus, *Nocardia* spp. are categorized as acid-fast organisms (Talbot and Raffa, 2015). Acid-fast bacteria, therefore, retain dyes when heated and treated with acidified organic compounds (Talbot and Raffa, 2015).

Mycobacteria are ubiquitous bacteria that inhabit different environmental niches such as surface or tap water (including chlorinated water) and soil sources (Kazda *et al*., 2009). Mycobacteria, such as *M. tuberculosis* and *M. leprae*, are obligatory pathogens, while *M. avium* is an opportunistic pathogen, while other species are saprophytes (De Chastellier, 2009). *M. tuberculosis* can also infect various animal species, even though humans are the primary host. *M. tuberculosis* is an aerobic bacterium that thrives in tissues with a high oxygen concentration, such as the lungs (Ramakrishnan *et al*., 2020).

The colony morphology of mycobacteria differs among some species, ranging from rough or smooth colonies, and from pigmented to non-pigmented (Talbot and Raffa, 2015). The color of the colonies varies from white to orange or pink (Talbot and Raffa, 2015). The culture of mycobacteria is performed by a combination of solid and liquid media (Adler *et al*., 2005). Middlebrook medium (agar-based or liquid-based) and Löwenstein–Jensen (LJ) (agar-based) medium are the two media used to culture *M. tuberculosis*. These media contain inhibitors such as malachite-green to prevent contaminants from over-growing *M. tuberculosis* (Adler *et al*., 2005). *Mycobacterium tuberculosis* colonies are small and buff-colored when cultured on either Middlebrook 7H11 or LJ medium. *M. tuberculosis* is a slow-growing bacteria. Most other bacteria, such as *Escherichia coli*, have an *in vitro* generation time of about 20 minutes, however, *M. tuberculosis* has a generation time of 18 hours to 24 hours (Talbot and Raffa, 2015). As a result, it takes about 4 to 6 weeks for a visible colony to appear on either type of media (Ojha *et al*., 2008). This delayed growth makes it difficult for timely diagnosis of *M.*

tuberculosis. The microorganism grows in parallel groups, to produce serpentine cording and can form pellicle biofilm on the air-media interface in a surfactant-free liquid medium culture (Ojha *et al*., 2008).

2.1.4 Virulence factors of *M. tuberculosis*

The survival of *M. tuberculosis* within a host requires the expression of different genetic determinants involved in the host-pathogen interaction, to allow the bacteria to overcome both physiological and environmental stress (Meena, 2010). Over the long course of evolution, *M tuberculosis* has become adaptable inside host cells, with the capability of evading and modifying their response to infection (Forrellad *et al*., 2013). A variety of *M. tuberculosis* virulent genes encode enzymes responsible for the biosynthesis of cell surface proteins, diverse lipids, regulators, and proteins of signal transduction (Axelrod *et al*., 2008; Stanley and Cox, 2013). Compared to other bacterial pathogens, which produce specific virulence factors such as toxins, fimbriae, and capsules, *M. tuberculosis* induces pathology via a complex combination of virulence genes and host responses (Meena, 2010).

Virulence in *M. tuberculosis* is coordinated by a set of genes and there are over 300 virulence genes in *M. tuberculosis* (Forrellad *et al*, 2013). These virulence genes encode proteins responsible for cell envelop (cell wall proteins, lipoproteins, and secretion system), lipid biosynthesis, and proteins that evade the human immune system and inhibit the phagocytic activity of macrophages (Forrellad *et al*, 2013). The important virulence genes are involved in phagosome arrest and inhibition of apoptosis, oxidative and nitrosative stresses, enzymatic reaction (protein kinases, proteases, metalloproteases), sigma factor, gene expression regulators, and transcriptional regulators (Forrellad *et al*, 2013; Mikheecheva *et al*, 2017). The products of the virulence genes are vital to the various stages of *M. tuberculosis*: adhesion, colonization of mucosal membranes, survival under stress conditions, invasion, and evasion of the human host immune response (Mikheecheva *et al*, 2017).

Several structural and physiological properties that contribute to mycobacterial virulence include: (i) *M. tuberculosis* can modulate the bactericidal mechanism of the macrophages by inhibiting phagosome-lysosome fusion to continue long-term survival in macrophages and cause necrosis (ii) The slow generation time of *M. tuberculosis* makes it difficult for the immune system to recognize and trigger a sufficient immune response to eliminate the bacteria (iii) *M. tuberculosis* counter the toxic effects of reactive oxygen intermediates produced during

phagocytosis (iv) Antigen 85 complex help walling off the bacilli from the immune system and enable tubercle formation (v) The high cell wall lipid content makes the bacterial impermeable and accounts for resistance to antimicrobial agents and (vi) The cord factor blocks macrophage activation by IFN-γ, elicits production of TNF-α and causes *M. tuberculosis* to form cords (Gupta *et al*., 2012; Huang and Brumell, 2014; Kimmey and Stallings, 2016).

2.1.5 Pathogenesis of *M. tuberculosis*

Mycobacterium tuberculosis is transmitted by inhaling small droplet nuclei (1-5 μm in diameter) containing bacilli from infectious patients (Lee, 2016). The tracheal and bronchial epithelium is the first line of defense to ward off the pathogen (Tellier *et al*., 2019). Bacteria enter the lungs where they survive the first line of defense (Middleton *et al*., 2002; Tellier *et al*., 2019). In the lung, alveolar macrophages phagocytose the bacteria and entrap them in the phagosome (de Martino, 2014). The innate immune response is activated when pattern recognition receptors (PRRs), primarily Toll-like receptors (TLRs) on alveolar and interstitial macrophages as well as dendritic cells recognize *M. tuberculosis* in the alveolar space and engulf it (Jo *et al*., 2007). These antigen-presenting cells (APCs) present *M. tuberculosis* antigens along with major histocompatibility complex (MHC) class II molecules to CD4 T-helper (Th) lymphocytes, as well as in association with MHC-1 and CD1 to CD8 T-cells in a lesser extent (Russell *et al*., 2009). Additionally, dendritic cells are also responsible for recruiting other cells of the immune system, T cell activation, and disseminating the infection (Lin *et al*., 2014). The phagosome containing *M. tuberculosis* can fuse with the lysosome to form a phagolysosome to kill the bacteria (Rohde *et al*., 2007). *M. tuberculosis* however has several mechanisms to evade and survive in the human host. These include interference with autophagy machinery, masking themselves with host proteins to avoid recognition and inhibition of fusion of autophagosomes with lysosomes; making them proliferate inside the macrophages and initiate cellular necrosis while inhibiting apoptosis (Huang and Brumell, 2014; Kimmey and Stallings, 2016; Siqueira *et al*., 2018).

Primed T-cells can also recognize and activate macrophages to launch anti-mycobacterial killing via secretion of interferon (IFN) and tumor necrosis factor (TNF) to control bacterial replication (Pandey and Sassetti, 2008). The host immune response of the majority (90%) of immunocompetent individuals usually limits infection such that the patients do not develop TB (Russell, 2007). *M. tuberculosis* multiplies inside macrophages during active TB and is released when macrophages die (Mishra and Surolia, 2018). When the bacilli survive the host

defense mechanism, the bacteria gain access to the lung parenchyma and stimulate a cell-mediated pro-inflammatory response leading to the formation of a protective barrier shell called a granuloma (Pagán and Ramakrishnan, 2018). *M. tuberculosis* bacilli remain viable but dormant in the granuloma for many years after infection (Ramakrishnan, 2012). Stable granuloma normally results in latent infection, which can be diagnosed by a positive tuberculin skin test (TST) or interferon-gamma release assay (IGRA) (Ramakrishnan, 2012). Latent TB can however be reactivated into clinical disease (Verma *et al*., 2021). Factors such as HIV co-infection, diabetes mellitus, smoking, renal failure, and malnutrition can activate the progression of latent TB to active disease (Ai *et al*., 2016; Sharan *et al*., 2020). Reactivation or primary disease is considered as a failure of the CD4 T-cell immunity (Dheda *et al*., 2005). *M. tuberculosis* in the blood can be disseminated to different organs such as the peripheral lymph nodes, central nervous system, and kidney (Moule and Cirillo, 2020). Disseminated TB causes severe TB disease and usually occurs in patients co-infected with HIV and children and is known as miliary TB (Moule and Cirillo, 2020).

2.1.6 Antibiotic resistance and global prevalence of drug-resistant *M. tuberculosis*

Mycobacterium tuberculosis is one of the most successful pathogens causing infectious diseases. Besides its innate ability to modulate the human host defense mechanisms to survive, *M. tuberculosis* is intrinsically resistant to many antibiotics (Nguyen, 2016). As a result, there are limited chemotherapeutic options for tuberculosis treatment (Nguyen, 2016). Prolonged regimens with the same few drugs have resulted in the evolution of strains with increasing resistance to anti-TB drugs (WHO, 2022b). The extensive use of antibiotics has created a selective pressure leading to the rapid evolution of resistance in *M. tuberculosis*, from mono-drug resistant to multidrug-resistant, extensively drug-resistant, and totally drug-resistant strains (Mallick *et al*., 2022; Omar *et al*., 2022). Although TB is treatable and curable, current anti-TB regimens are losing their effectiveness due to the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (Mallick *et al*., 2022; Omar *et al*., 2022). Drug-resistant TB (DR-TB) is a risk factor fuelling the ongoing TB epidemic and increasing the morbidity and mortality of TB, as well as jeopardizing the TB control program (Velayati *et al*., 2009; Klopper *et al*., 2013; Chakaya *et al*., 2021).

Multidrug-resistant *M. tuberculosis* is defined as resistance to at least isoniazid and rifampicin, two of the four commonly used first-line medicines used in the treatment of TB. Rifampicin resistance is an indicator of MDR-TB since more than 90% of rifampicin-resistant isolates are

also resistant to other drugs (Rahman *et al*., 2016). Pre-XDR TB is defined as *M. tuberculosis* strains that are rifampicin resistant (RR) or MDR but are also resistant to fluoroquinolones. Extensively drug resistant (XDR) TB is defined as TB caused by *M. tuberculosis* strains that are MDR-/RR-TB, resistant to fluoroquinolones, and to either bedaquiline and/or linezolid. (Viney *et al*., 2021). Totally drug resistance TB (TDR-TB) is defined as *M. tuberculosis* strains resistant to all TB drugs, leaving no options for effective treatment to the infected patient (Khawbung *et al*., 2021).

It is estimated that 5% of global TB cases are caused by MDR *M. tuberculosis* strains, accounting for roughly 500,000 cases of newly diagnosed MDR infections annually (WHO, 2021c). In 2019, MDR TB was diagnosed in 18% of previously treated patients and 3.3% of new TB cases. A total of 465 000 (range, 400 000 – 535 000) incident cases of rifampicin-resistant TB (RR-TB) was diagnosed, out of which 78% had MDR-TB (WHO, 2021c). Patients infected with MDR TB require second-line drugs for at least nine months and up to 20 months (WHO, 2021c). In the same year, 12 350 extensively drug-resistant (XDR) TB cases were diagnosed worldwide. Globally, only 57% of all DR-TB cases are successfully treated (WHO, 2021c).

2.1.7 Mechanisms of drug resistance in *M. tuberculosis*

Mycobacterium tuberculosis is noted for its intrinsic resistance to a wide array of antimicrobial agents such as β-lactams and macrolides (Gygli *et al*., 2017). The cell wall of *M. tuberculosis* is composed of different lipids that include mycolic acids that is covalently linked to the peptidoglycan layer via arabinogalactan, has low numbers of porins, and is much thicker and more hydrophobic (Gygli *et al*., 2017; Batt *et al*, 2020). This peculiar structure and composition of the cell wall envelope hinder the diffusion of hydrophobic molecules, including several antibiotics (Batt *et al*, 2020). Antibiotics may be enzymatically cleaved after penetrating the cell wall to render them ineffective (Kumar *et al*., 2022). A typical example is the enzymatic degradation of β-lactam antibiotics by β-lactamases, which hydrolyze the β-lactam ring of the antibiotics (Kumar *et al*., 2022). The enzymatic drug target modification and drug efflux generate drug resistance in *M. tuberculosis* (Gygli *et al*., 2017). Apart from the intrinsic resistance mechanisms, chromosomal mutations confer clinically relevant drug resistance in majority of *M. tuberculosis* (Gygli *et al*., 2017). Different mechanisms and levels of resistance are conferred by chromosomal mutation. Depending on the antibiotic used, there may be different mechanisms of resistance (Gygli *et al*., 2017; Batt *et al*., 2020) (Table 2.1).

Chromosomal mutation in the drug-target genes induces acquired resistance (Nguyen, 2016).

Rifampicin resistance (RR) is associated with mutations in the 81 bp rifampicin resistance determining region (RRDR). Mutations in RRDR of the *rpoB* gene such as S531L, D516V, H526D, and H526Y are associated with phenotypic rifampicin resistance (Gupta *et al*., 2020). Mutation in the catalase-peroxidase gene (*katG*) (such as S315T1*)*, enoyl-ACP reductase gene (*inhA*-15C > T), and its promoter, the alkyl hydroperoxide reductase gene (*ahpC*) (AhpC_9, AhpC_12) is associated with resistance to isoniazid (Guo *et al*., 2022; Isakova *et al*., 2018). Efflux pump genes, such as *drrA, drrB, efpA, Rv2459, Rv1634, and Rv1250*, were also shown to be associated with MDR tuberculosis (AlMatar *et al*., 2020). **Table 2.1** shows the different target genes and the mechanisms of resistance to some common antibiotics used to treat tuberculosis (Gygli *et al*., 2017; Batt *et al*., 2020).

Table 2. 1: Common targets of chromosomal mutations conferring drug resistance in *M. tuberculosis*

Antibiotic	Target gene	Mechanism of resistance
Rifampicin	*rpoB*	Drug target alteration
Isoniazid	*katG*	Abrogated prodrug activation
	inhA	Drug target alteration
	inhA promotor	Drug target overexpression
Linezolid	*rplC* , *rrl*	Drug target alteration
Pyrazinamide	*pncA*	Abrogated prodrug activation
Bedaquiline	*atpE*	Drug target alteration
	Promotor/*mmpR*	Overexpression of efflux pump Mmpl5
Delamanid/pretomanid	*fbiA/B/C* , *fgd1* , *ddn*	Abrogation of prodrug activation
Fluoroquinolones	*gyrA/B*	Drug target alteration

Table adapted from Batt *et al*. (2020) and Gygli *et al.* (2017)

2.1.8 Methods used to detect drug-resistant *M. tuberculosis*

Molecular diagnostic test including Xpert® MTB/RIF, Xpert® MTB/RIF Ultra (Cepheid), Xpert® MTB/XDR (Cepheid), line probe assay (LPAs) and whole genome sequencing (WGS) are used to diagnose drug-resistant tuberculosis (WHO, 2020d; Olawoye *et al*., 2021). LPA is a DNA strip-based test that detects the DNA of *M. tuberculosis* complex (MTBC) and its drug resistance profile, through binding of amplicons to probes that target specific components of the MTBC genome and common resistance-associated mutations (WHO, 2020d). Xpert MTB/RIF assay is a cartridge-based fully automated nucleic acid amplification test used as an

initial diagnostic test for TB and rifampicin-resistance detection within 2 hours (WHO, 2020d). The sensitivity of Xpert MTB/RIF is suboptimal, especially in smear-negative and HIV-associated TB patients, so MTB/RIF Ultra (Cepheid, Sunnyvale, USA) was developed to over this limitation. Xpert Ultra incorporates two different multicopy amplification targets (IS6110 and IS1081) to improve sensitivity. Xpert Ultra has a limit of detection of 16 bacterial colony forming units (cfu) per millilitre compared with 114 cfu/mL for Xpert MTB/RIF (WHO, 2020d). Xpert MTB/XDR (Cepheid, Sunnyvale, CA, USA) is a new molecular assay that detect *M. tuberculosis* with mutations to isoniazid (INH), ethionamide (ETH), fluoroquinolone (FQ), and second-line injectable drugs (SLIDs) (Penn-Nicholson *et al.,* 2022). WGS can accurately detect MDR- and XDR-TB (Olawoye *et al*., 2021).

2.1.9 Treatment of tuberculosis

A combination of multiple drugs with different mode of action is required for treatment of tuberculosis as a disease. The goal of treatment is to reduce or clear bacillary load and reduce the risk of TB transmission (Gygli *et al*., 2017). Drug-resistant TB strains are difficult to treat than drug susceptible strains. The management of drug resistant TB has been lately revolutionized by shorter all oral anti -TB regimen that is more effective, less toxic and better tolerated (WHO, 2022b).

2.1.9.1 Treatment of disease caused by drug susceptible strains of *M. tuberculosis*

Patients infected with drug-susceptible *M. tuberculosis* are commonly treated with a 6-month regimen comprising of a two-month intensive phase consisting of isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by a continuation phase of 4 months of treatment with isoniazid and rifampicin (WHO, 2022a). Patients aged 12 years or older, a four-month regimen consisting of isoniazid, rifapentine, and pyrazinamide (2HPMZ/2HPM) is recommended (WHO, 2022a). Additionally, a 4-month regimen consisting of rifampicin, isoniazid, and pyrazinamide with or without ethambutol (2HRZ(E)/2HR) is recommended for treating children and adolescents between 3 months and 16 years (WHO,2022a). Tuberculosis patients co-infected with HIV are recommended to receive the same treatment duration just as in HIV-negative TB patients (WHO, 2022a). Also, antiretroviral therapy should be initiated within two weeks after the start of TB treatment, irrespective of the patients' CD4 cell count (WHO, 2022a). Treatment success of 85% has been recorded in patients treated for susceptible TB (DS-TB) (WHO, 2020a).

2.1.9.2 Treatment of disease caused by drug-resistant strains of *M. tuberculosis*

According to recent evidence from the TB-PRACTECAL and ZeNix studies, a novel 6-month treatment regimen with a combination of bedaquiline, pretomanid, linezolid, and moxifloxacin (BPaLM) is suggested for the treatment of patients with MDR/RR-TB who have not been previously exposed to these drugs instead of 9 months regimen or the longer (≥18 months) regimen (**Table 2.2**) (Mirzayev *et al*., 2021). This regimen showed favourable efficacy and safety (WHO, 2022b; WHO, 2022d). In case of documented resistance to fluoroquinolone, bedaquiline, pretomanid, and linezolid (BPaL) combination is recommended (WHO, 2022b). Because of the slow response to BPaL, a three-month extension may be possible, bringing the total treatment duration to 9 months (WHO, 2022b, WHO, 2022d). The use of BPaLM and BPaL in countries such as Belarus, South Africa and Uzbekistan recorded a high treatment success of over 90%, suggesting their use in eligible patients with MDR/RR-TB and pre-XDR irrespective of patients' HIV status (Conradie *et al*., 2020, WHO, 2022b, WHO, 2022d). The evidence leading to the use of BPaLM or BPaL was limited to patients aged above 14 years (WHO, 2022b; WHO, 2022d). Also, no data is available for the use of these novel combinations in patients with severe forms of extrapulmonary TB and in pregnant women (WHO, 2022b). The treatment success rate for MDR/RR-TB has steadily improve. Between 2012 and 2018, the success rate increased from 50% to 59% globally (WHO, 2021b).

Table 2. 2: WHO grouping of medicines recommended for treatment of multidrug-resistant tuberculosis

Group	Step	Medicines
Group A	Include all three medicines	Levofloxacin OR moxifloxacin, Bedaquiline, Linezolid
Group B	Add one or both medicines	Clofazimine, Cycloserine OR terizidone
Group C	Add to complete the regimen and when medicines from groups A and B cannot be used	Ethambutol, Delamanid Pyrazinamide, Imipenem–cilastatin OR meropenem Amikacin (OR streptomycin) Ethionamide OR prothionamide p-Aminosalicylic acid+

From: WHO (2022b, WHO, 2022d).

2.1.10 Pipeline of new antibiotic agents for treatment of MDR-TB

The recent emergence of bedaquiline resistance threatens the effectiveness of novel 6-month treatment regimens for MDR-TB (Chesov *et al*., 2022; Kaniga *et al*., 2022). A recent study reported 15% prevalence of bedaquiline resistance in a MDR-TB cohort in Moldova (Chesov

et al., 2021). New drugs in clinical development (**Table 2.3**) give hope but given the long development period and overlapping mechanism of action, there is limited certainty of success. This emphasizes the need to use the available anti-TB drugs appropriately to benefit future generation.

Novel drug compounds to complement the available TB-regimen are needed to improve tuberculosis (TB) control. Although some of the compounds in clinical trial belongs to classes that is already approved for treatment of TB, the bulk belong to novel classes (WHO, 2021b).

Table 2. 3: TB drug development pipeline by mechanism of action and study phase (WHO, 2021c)

Drug	Class of compound	Mechanism of action	Phase
Sutezolid (PNU-100480)	Oxazolidinone	Inhibits protein synthesis (23S ribosome)	IIb
Delpazolid (LCB01-0371)	Oxazolidinone	Inhibits (23S ribosome)	IIb
TBI-223	Oxazolidinone	Inhibits (23S ribosome)	IIb
SPR 720	Benzimidazole	GyrB bacterial DNA synthesis inhibitor	Ia/Ib
GSK2556286 (GSK-286)		Cholesterol catabolism inhibitor	Ia/Ib
Bedaquiline	Diarylquinoline	Inhibits ATP synthase and bacterial respiration	III
GSK3036656 (Gsk 656)	Oxaborole	LeuRsS inhibitor (protein synthesis)	IIa
Delamanid	Nitroimidazole	Cell wall synthesis inhibitor, inhibits cell respiration	IV
Pretomanid	Nitroimidazole	Cell wall synthesis inhibitor, inhibits cell respiration	
SQ109	1,2 ethylene diamine	MmpL3 inhibitor (Cell eall synthesis)	IIb
Pyrifazimine (TB1-166)	Riminophenazine	Inhibits ion transport and bacterial respiration	IIa
Telacebec (Q203)	Imidazopyridine	QcrB (ATP synthesis) and cell respiration inhibitor	IIa
TBA-7371	Azaindole	Inhibits cell wall synthesis (DprE1)	IIa
BTZ-043	Benzothiazone	Inhibits cell wall synthesis (DprE1)	Ib/IIa
Macrozinone (PBTZ169)	Benzothiazone	Inhibits cell wall synthesis (DprE1)	Ib/IIa
OPC-167832	Carbostyril	Inhibits cell wall synthesis (DprE1)	Ib/IIa
TBAJ-587	Diarylquinoline	Inhibits ATP synthase	Ia/Ib
TBAJ-876	Diarylquinoline	Inhibits ATP synthase	Ia/Ib
BVL-GSK098	Amido piperidine	Ethionamide (cell wall synthesis inhibitor) booster	Ia/Ib

2.1.11 Prevention and control of tuberculosis

The prevention of new infections and development of active disease are crucial for deceasing

morbidity and mortality. The main purpose of TB prevention is to reduce TB transmission, prevent progression of latent TB into active disease and reduce the risk of transmission of TB to persons in close contact with individuals with TB disease (WHO, 2020a). It was found that among individuals who develop TB within 15 years of exposure, the probabilities of developing active disease within one, two and five years are respectively 5%, 62% and 83% (WHO, 2022a). The high-risk TB group includes patients living with human immunodeficiency virus (PLHIV), individuals with immunodeficiency conditions and people in contact with TB patients (WHO, 2020a). It is recommended that individuals in this priority group receive TB preventive therapy (TPT). Vaccination with Bacillus Calmette-Guérin (BCG) in children is a major strategy for TB prevention and control (Voss *et al*., 2018). Despite the low efficacy of BCG, it has been reported that BCG vaccination prevents about 120 000 childhood death in a year (Voss *et al*., 2018).

2.1.12 Autophagy

After a primary infection, *M. tuberculosis* survive in macrophages in most subjects, allowing the bacteria to evade the immune system and cause latent tuberculosis (Mack *et al*., 2009). During LTBI, *M. tuberculosis* resists the fusion of autophagosome and lysosome to establish residency in the macrophage cytoplasm (Mack *et al*., 2009). The progression to active tuberculosis is determined by intracellular survival and mycobacterial multiplication (Deretic, 2021). Among the innate host defence mechanisms, autophagy has been shown to induce *M. tuberculosis* clearance by targeting the cytosolic bacteria to lysosomes for degradation (Chai *et al*., 2019; Deretic *and* Levine, 2009; Deretic, 2021).

Autophagy is an evolutionarily conserved homeostatic process in eukaryotic cells that involves the sequestration of components of the cytosol including long-lived proteins and damaged organelles within double-membrane-bound autophagosomes which then fuses with lysosomes for degradation (Songane *et al*., 2012; Huang and Brumell, 2014; Wu *et al*., 2022). This maintains cellular viability and balance, keeps cells healthy, and restores normal cell function in a eukaryotic cell (Deretic and Levine 2009; Deretic, 2021). Autophagy has been found to play a role in many critical biological processes, including cellular response to starvation, clearance of inclusion bodies in neurodegenerative diseases, cell survival and death, cancer, and host defence (Deretic, 2010). Autophagy has been found to facilitates the host response against the destruction of intracellular pathogens and connect the innate and the adaptive immune function (Espert *et al*., 2015). Autophagy is therefore central to the clearance of intracellular pathogens

such as *M. tuberculosis, Salmonella* spp., *Coxiella* spp., and *Legionella* spp. in an autophagy process called xenophagy facilitated by specific receptors (Espert *et al*., 2015; Paulus and Xavier, 2015).

2.1.12.1 Mechanism of autophagy induction

Autophagy is a highly conserved homeostatic cytoplasmic degradation process that is activated by starvation, endoplasmic reticulum (ER) stress, and autophagy modulators, among others (Klionsky and Emr, 2000; Yorimitsu *et al*., 2006; Deretic, 2012). This catabolic process maintains cellular homeostasis. It requires the orchestration of several molecules and a dynamic membrane rearrangement to achieve a complete flux. The canonical pathway of starvation-induced autophagy starts with the formation of the sequestering compartment. This process involves the formation of a phagophore by inducing an omega-shaped subdomain of the endoplasmic reticulum (ER) membrane (Klionsky and Emr, 2000; Yorimitsu *et al*., 2006). The autophagosome is formed when the double membrane phagophore expands until it closes in on itself by engulfing around recyclable cellular/ cytoplasmic materials (Deretic, 2012). This is followed by the fusion of the autophagosome with a lysosome to form an autolysosome (Kim *et al*., 2012; Klionsky *et al*., 2014), inside which the cargo is enzymatically degraded under acidic conditions (Galluzzi *et al*., 2017).

Autophagy can be divided into three steps, namely: initiation (**Figure 2.1**), execution, and maturation (Liang *et al*., 2007; Shang *et al*., 2011). Each stage of the process is regulated by the sequential recruitment and action of autophagy related (ATG) proteins (Liang *et al*., 2007; Shang *et al*., 2011).

The three protein complexes that regulate autophagy include UNC51-like kinase 1 (ULK1), comprising of ULK1, ATG13, RB1CC1/FIP200; and ATG101 class III PtdIns3K, comprising of ATG14, BECN1, PIK3R4/VPS15; and PIK3C3/VPS34 and ATG16L1, comprising of ATG16L1, ATG5, and ATG12. Nutrition starvation is the most studied initiator of cellular autophagy (Klionsky *et al*., 2021). Autophagy is promoted by the energy sensor, adenosine monophosphate (AMP)-activated protein kinase (AMPK). AMPK regulates cellular metabolism to maintain energy homeostasis (Liang *et al*., 2007; Shang *et al*., 2011).

Conversely, autophagy is inhibited by the mammalian target of rapamycin (mTOR), a central regulator of cell growth that combines growth factor and nutrient signals (Lee *et al*., 2010; Kim *et al*., 2011). AMPK promotes autophagy by directly activating Ulk1 through phosphorylation

of Serine 317 (Ser 317) and Serine 777 (Ser 777) during glucose starvation (Kim *et al*., 2011; Galluzzi *et al*., 2017).

On the other hand, in the presence of sufficient nutrients, high mTOR activity prevents Ulk1 activation by phosphorylating Ulk1 Ser 757 and breaking the interaction between AMPK and Ulk1(Lee *et al*., 2010; Kim *et al.,* 2011). When mTOR is inactivated, ULK1 complex forms that lead to activation of the PtdIns3K, which results in the creation of PtdIns3P-rich regions on the omegasome's surface (He and Klionsky, 2009; Bello-Perez *et al*., 2020). These domains are recognized by human WD-repeat protein interacting with phosphoinositides proteins (WIPI), which leads to the recruitment of the ATG16L1 complex. This facilitates the lipidation of LC3-I to form LC3-II (Polson *et al*., 2010; Dooley *et al*., 2015). Receptors such as SQSTM1/p62 bind to ubiquitinated cargo and LC3-II to facilitate selective autophagy (Seto *et al*., 2013; Sargazi *et al*., 2021).

In bacterial infection, targeting of the substrate involves ubiquitination of the bacteria, and recognition of the ubiquitin by autophagy receptors. The autophagy receptors interact with LC3 to recruit bacteria to autophagosome (Kimmey and Stallings, 2016).

The cytoplasmic cargo includes worn-out organelles, proteins, and intracellular bacteria (Deretic and Levine, 2009). Expansion of the phagophore through membrane addition leads to the sequestering of the cargo into autophagosome (Deretic and Levine, 2009). These autophagosomes are decorated with RAB7 (Gutierrez et al., 2004; Jager et al., 2004), causing them to fuse with lysosomes and create autolysosomes, where the cargo is destroyed (**Figure 2.1**) (Klionsky *et al*., 2021).

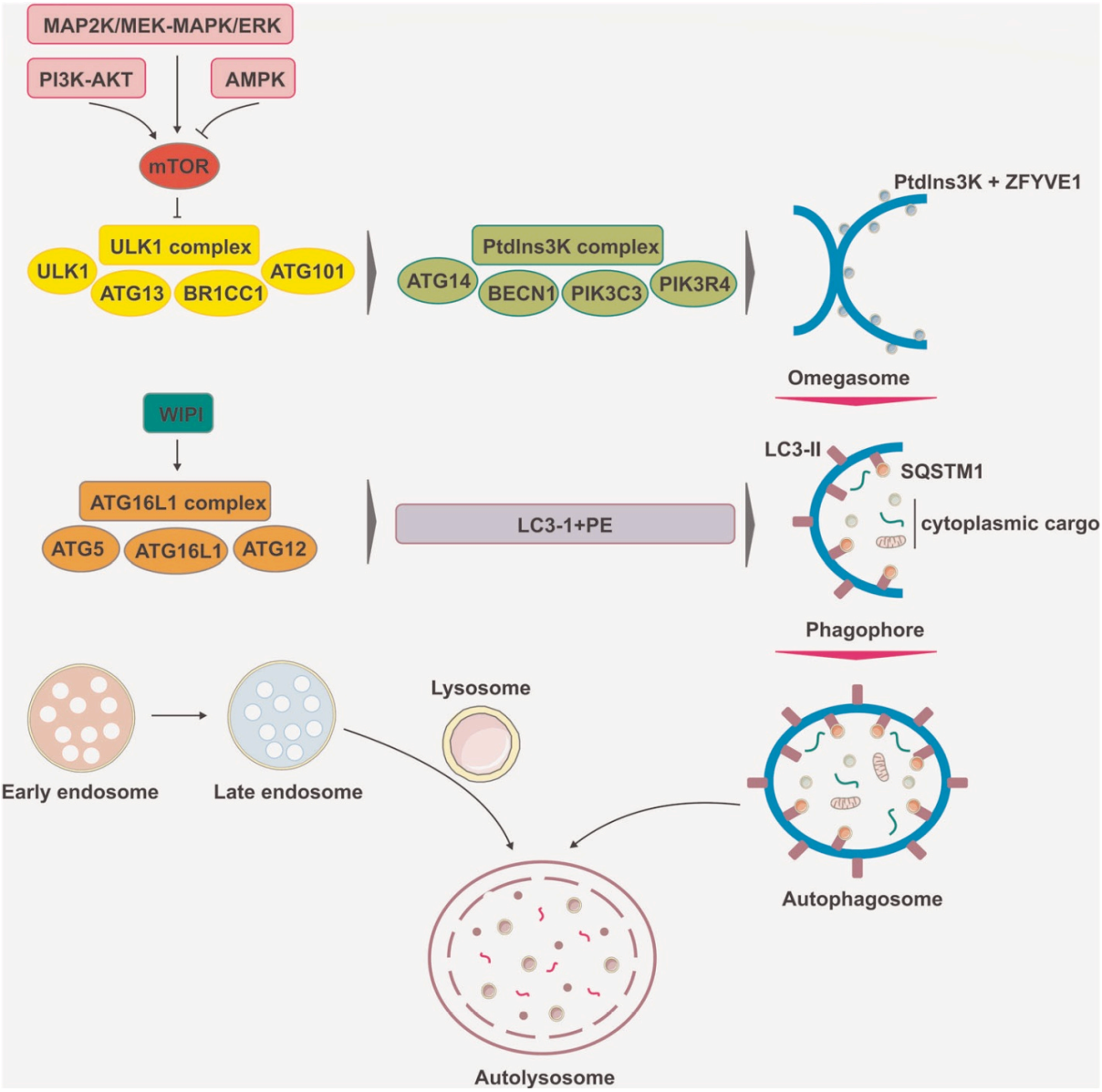

Figure 2. 1: Genes involved in autophagy induction

The figure highlights the three-protein complex included in Autophagy induction: ULK1 complex, including ULK1, RB1CC1, ATG13, andATG101; PtdIns3K complex, including the ATG14, BECN1, PIK3R4/VPS15, PIK3C3/VPS34, and ATG16L1 complex, including ATG5, ATG12, and ATG16L1 (Sargazi et al., 2021)

2.1.12.2 Autophagy-inducing agents and control of intracellular *M. tuberculosis*

Antibiotics are routinely used in the treatment of bacterial infections, by targeting cellular targets to either inhibit or kill bacteria. However, anti-TB chemotherapy is currently facing several hurdles, including HIV coinfection, adherence issues, and the rise of multidrug-resistant *M. tuberculosis* strains (Heidary *et al*., 2022; Reta *et al*., 2022). As a result, finding

treatments via drug discovery, identifying targets, and developing fast-acting medications to treat human tuberculosis have become top priorities (WHO, 2017). Most of target-based discovery programs are not likely to be effective. Understanding the host response and micro-environment of *M. tuberculosis*-infected macrophages could lead to the development of potent treatments against *M. tuberculosis* infection, as well as provide strategies for chemotherapeutic intervention. Increasing evidence suggests that some antibiotics and molecules can have an impact also on the human immune system by modulating the functions of immune cells (Wallis and Hafner, 2015). Identification of new agents that can boost the innate defenses by reprogramming macrophages to become more phagocytic, killing intracellular *M. tuberculosis,* has the potential to improve treatment outcome, reduce relapse, and combat tuberculosis (Wallis and Hafner, 2015; Kim *et al*., 2012)**.** The essential role of autophagy in controlling intracellular parasites such as *M. tuberculosis* is an emerging field in tuberculosis pathogenesis research (Kim *et al*., 2012).

Besides starvation, the essential roles of antibiotics and specific compounds in inducing/enhancing autophagy are increasingly reported (Kim *et al.*, 2012). Isoniazid and pyrazinamide, as well as reactive oxygen species (ROS), which are also produced by antibiotics' interactions with the host, were found to induce autophagy in *M. tuberculosis*-infected macrophages (Kim *et al.*, 2012). It was observed that without autophagy, isoniazid and pyrazinamide were unable to clear *M. tuberculosis* from macrophages (Kim *et al*., 2012). *M. marinum*-infected Drosophila flies lacking the *atg7* autophagy gene, had a low survival rate, which could not be rescued with antibiotics (Kim *et al.*, 2012). Similarly, in a more recent study (2020) by Giraud-Gatineau and colleagues, bedaquiline, a recently developed antibiotic against multidrug-resistant *M. tuberculosis*, was found to increase macrophage lysosomal activity and induced autophagy activation. Bedaquiline was able to reprogram macrophages into potent bactericidal phagocytes to enhance the intracellular killing of *M. tuberculosis*, *S. aureus,* and *Salmonella typhimurium* that was naturally insensitive to bedaquiline (Giraud-Gatineau *et al.*, 2020). Thus, it is certain that not only do antibiotics directly kill pathogens, but they also induce autophagy to enable the immune system to eliminate intracellular bacteria directly.

Bedaquiline and linezolid were found to induce autophagy and autolysosome formation, and significantly reduced intracellular mycobacterial survival (Giraud-Gatineau *et al.*, 2020). Bedaquiline also potentiates the antimycobacterial activity of pyrazinamide (Giraud-Gatineau *et al.*, 2020).The combination of the two drugs was highly bactericidal on *M. tuberculosis*,

which resulted in an 83% decrease in colony-forming units (Giraud-Gatineau *et al.*, 2020). Bedaquiline, however, did not potentiate the activity of isoniazid, rifampicin, and ethambutol (Giraud-Gatineau *et al.*, 2020). The potentiation of the activity of pyrazinamide by bedaquiline was likely because of the effect of bedaquiline in increasing lysosomal acidification in host cell (Giraud-Gatineau *et al.*, 2020). The lysosomal pathway was not activated by any of the standard anti-TB drugs, including rifampicin, isoniazid, pyrazinamide and ethambutol (Giraud-Gatineau *et al.*, 2020).

Ambroxol, NSC 18725 (a pyrazole derivative), and aminopyrimidine compound (name as compound 2062) induced autophagy processes directed against *M. tuberculosis* (Choi *et al.*, 2018; Arora *et al.*, 2020; Bryk *et al*., 2020). At a dose of 1 to 10 µM, Ambroxol was capable of inducing autophagy both *in vitro* and *in vivo* and enhanced the mycobacterial killing of *M. tuberculosis* in macrophages (Choi *et al.*, 2018). Ambroxol was also able to potentiate the antimycobacterial activity of rifampicin in a murine tuberculosis model (Choi *et al.*, 2018). NSC 18725, a pyrazole derivative that was potent against fast and slow-growing Mycobacteria, was synergistic with INH, induce autophagy and inhibited the survival of *M. tuberculosis* in human macrophages (Arora *et al.*, 2020). In the screening of more than 2000 compounds, aminopyrimidine compound (name as compound 2062) was found to enhance the production of nitric oxide (NO) and TNFα in macrophages (Bryk *et al*., 2020). Although it had no direct effect on *M. tuberculosis* in Bone marrow- derived macrophage (BMDM) when tested alone, its combination with sub-optimal concentration of rifampicin (0.5 µM) reduced the burden of *M. tuberculosis* in BMDM (Bryk *et al*., 2020). The beneficial effect of compound 2062 was associated with activation of host transcriptional factor ED (TFED) (Bryk *et al*., 2020). TFED is a master regulator of lysosomal biogenesis and lysosomal activation (Bala and Szabo, 2018). This effect enhanced autophagy, lysosomal acidification, and lysosomal degradation (Bryk *et al*., 2020). Since current anti-TB chemotherapy for treating MDR-TB and XDR-TB have low efficiency, host-directed therapy has the potential to modulate host cell response to enhance immunity against pathogen eradication (Giraud-Gatineau *et al.*, 2020).

2.1.12.3 Methods for detecting or monitoring autophagy

An experimental approach to determine if a compound or a new drug modulate autophagy for therapeutic purposes is critical (Klionsky *et al*., 2021). It is important to use essential criteria and acceptable standards to determine if the compound truly affects/induce autophagy (Klionsky *et al*., 2021). Laser confocal microscopy and Western blot assay are mostly used for

detecting or monitoring autophagy (Kim *et al*., 2012; Giraud-Gatineau *et al*., 2020; Klionsky *et al*., 2021). Microtubule-associated protein 1 light chain 3-II (LC3B-II) is the form of LC3 recruited to the autophagosomal membrane, which is a widely used specific marker of autophagy (Lucocq and Hacker, 2013; Klionsky *et al*., 2021). Beclin-1, ATG-5, and ATG-7 proteins are autophagy regulators used to detect autophagy in a western blot assay besides LC3B-II (Yuk *et al*., 2009; Kim *et al*., 2012; Giraud-Gatineau *et al*., 2020). The autophagic flux is a key step for confirming if a compound induces autophagy or not. The pH sensitivity differences exhibited by tandem green fluorescent protein (GFP), which labeles neutral autophagosome, and red fluorescent protein (RFP), which labels acidic autolysosome is used to monitor the progression from autophagosome to autolysosome (Klionsky *et al*., 2021).

An accumulation of LC3 might reflect a reduction in autophagosome turnover (Kovács *et al*., 1987; Kovács *et al*., 1988), or the inability of turnover to keep up with increased autophagosome formation (Chu, 2006). Inefficient fusion with lysosomes inhibits autophagosome maturation to autolysosomes (Fass *et al*., 2006), while decreased flux might be due to inefficient degradation of the cargo once fusion has occurred (Kovács *et al*.,1982). To enable a correct interpretation of the result, autophagy markers such as LC3B-II is complemented by assays to measure overall autophagic flux (Giraud-Gatineau *et al*., 2020). That is, autophagy activity does not include only the increased synthesis in LC3B, or increase in autophagosome, but very importantly flux through the entire system, which includes lysosomes and the subsequent degradation of the end products (Klionsky *et al*., 2021). Therefore, autophagic substrate is monitored dynamically over time to confirm colocalization with lysosome and whether the substrate are broken down. The flux is accurately measured by using differences in the amount of LC3B-II between samples in the absence and presence of lysosomal protease inhibitors such as bafilomycin and chloroquine (Mizushima and Yoshimori, 2007, Yuk *et al*., 2009; Kim *et al*., 2012).

Measuring the degradation of p62 (SQSTM1/sequestosome 1) is another method for identifying the autophagic flux (Klionsky *et al*., 2021). Cargo protein 62 **(**p62) binds directly to LC3 and GABARAP family proteins in humans. p62 is degraded by autophagy and serve to link ubiquitinated proteins to the autophagic machinery to promote their breakdown in the lysosome (Klionsky *et al*., 2021). Since p62 accumulates when autophagy is inhibited, and decreased when autophagy is induced, p62 is used as a marker to study autophagic flux (Ichimura and Komatsu, 2010; Klionsky *et al*., 2021).

2.1.13 Conclusion

Mycobacterium tuberculosis is responsible for causing more than 10.6 million incident tuberculosis cases and 1.6 million deaths in 2021 (WHO, 2022c). *M. tuberculosis* remains the leading cause of death globally from a single infectious agent (WHO, 2022c). Approximately one-quarter of the world's population is latently infected with *M. tuberculosis*, of which about 5% to 10% of people latently infected develop tuberculosis throughout their lives (WHO, 2020a). Over the long course of evolution, *M tuberculosis* has become adaptable inside host cells, with the capability of evading and modifying their response to infection to survive in the host (Forrellad *et al*., 2013). These include interference with autophagy machinery, masking themselves with host proteins to avoid recognition and inhibition of fusion of autophagosomes with lysosomes; making them proliferate inside the macrophages and initiate cellular necrosis while inhibiting apoptosis (Huang and Brumell, 2014; Kimmey and Stallings, 2016; Siqueira *et al*., 2018).

Besides its innate ability to modulate the human host defense mechanisms to survive, *M. tuberculosis* is intrinsically resistant to many antibiotics (Nguyen, 2016). The evolution of resistance in *M. tuberculosis*, from mono-drug resistant to multidrug-resistant, extensively drug-resistant, and totally drug-resistant strains is a public health concern (Mallick *et al*., 2022; Omar *et al*., 2022). Although TB is treatable and curable, current anti-TB regimens are losing their effectiveness due to the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains (Mallick *et al*., 2022; Omar *et al*., 2022). Due to this, the WHO has designated *M. tuberculosis* as a critical priority pathogen to advance the search for novel drugs (WHO, 2017). The identification of new agents to complement current anti-TB regimens and agents that can boost the innate defenses by reprogramming the host immune cells to be more phagocytic, such as induction of autophagy, to kill intracellular infections, including MDR and XDR tuberculosis, where drug resistance is unlikely is a high research priority (Kim *et al*., 2012; Wallis and Hafner, 2015).

2.2 Part 2: *Neisseria gonorrhoeae*

2.2.1 Epidemiology

Gonorrhoea is a sexually transmitted infection (STI) that continues to remain a major public health concern globally. Over the past decade, the incidence of gonorrhea has increased sharply in many countries (Lin *et al*., 2021). The bacterium infects the human mucosal surfaces of both

men and women, leading to a localized genital tract infection. *Neisseria gonorrhoeae* can cause symptomatic or asymptomatic infections at the urogenital sites such as the cervix, urethra, rectum, and oro-/nasopharynx (Unemo *et al*., 2019). In 2020, WHO estimated 82.4 million (48 million to 130 million) new gonococcal infections among adults and adolescents between 15 to 49 years, with a global incident rate of 23 per 1000 in men and 19 per 1000 in women and with the highest overall incidence observed in sub-Saharan Africa (Rowley *et al*., 2019; WHO, 2021c). Most of the cases were reported in the WHO South-East Asia region, Western Pacific region, and Africa region (WHO, 2021c). Increasing reports suggest that the actual number of these infections is under-reported because of inadequate clinical or diagnostic infrastructure, poor reporting systems, and high rates of asymptomatic infections (Edwards and Apicella, 2004; Walker and Sweet, 2011).

Gonococcal infections result in limited immunity; hence individuals can become recurrently infected (Quillin and Seifert, 2018). These infections, if left untreated, can lead to serious sequelae such as pelvic inflammatory disease (PID), an increased risk of tubal factor infertility, ectopic pregnancy, adverse pregnancy outcomes, disseminated gonococcal infection (DGI), and facilitate the transmission of the human immunodeficiency virus (Little, 2006; Burnett *et.al*, 2012). *N. gonorrhoeae* attach to sperms (James-Holmquest *et al*., 1974; Harvey *et al*., 2000) and are transmitted from men to their partners via ejaculates, which contain a high number of gonococci (Cohen *et al*., 1994). However, it is not fully clear how women maintain efficient transmission to their partners. The surface of *N. gonorrhoeae* successfully bind and enter urethral epithelial cells of men. It is believed that the cervicovaginal microbiota secretes bacterial sialidases that first desialylate *N gonorrhoeae* lipooligosaccharide (LOS) to efficiently transmit from women to men (Ketterer *et al*., 2016). Mother-to-child transmission during birth can also lead to neonatal blindness (Unemo and Shafer, 2014). Infections with *N. gonorrhoeae* are associated with the acquisition and spread of additional sexually transmitted infections such as *Chlamydia trachomatis*, in addition to HIV (Unemo and Shafer, 2014). Most of these gonococcal infections are in populations that are at high risk for STIs, such as men who have sex with men and commercial sex workers (Maduna *et al*., 2020).

2.2.2 Classification of *Neisseria*

The genus *Neisseria* consists of at least 21 members including *N. gonorrhoeae* and *N. meningitidis* (Hoffman and Weber, 2009). The latter is the leading cause of human bacterial meningitis (Hoffman and Weber, 2009). The other members are non-pathogenic, although

some cause opportunistic infections in humans (e.g. *N. lactamica*) (Marri *et al*., 2010). At least eight non-pathogenic commensal *Neisseria* spp. make up a substantial proportion of the human nasal and oropharyngeal flora (Marri *et al*., 2010). The other non-pathogenic *Neisseria* spp. are capable of colonizing hosts such as herbivorous mammals, dolphins, non-human primates, cats, dogs, insects, and birds (Liu *et al*., 2015). Phylogenetic analysis reveals that *N. meningitidis* and *N. gonorrhoeae* share a common ancestor but have separate lineages and occupy different niches: the nasopharyngeal mucosa and genital mucosa, respectively (Maiden, 2008; Maiden and Harrison, 2016).

2.2.3 General characteristics

Neisseria gonorrhoeae is a Gram-negative diplococci bacterium that was first described by Albert Neisser in 1879, and in 1882 Leistokow reported the cultivation of the gonococcus (Simmons, 1985). It is an oxidase-positive (possesses cytochrome c oxidase) and catalase-positive (can convert hydrogen peroxide to oxygen), an aerobe, and utilizes glucose but not maltose and sucrose (Ng and Martin, 2005). *N. gonorrhoeae* is facultatively intracellular and under the microscope, the bacteria appear in pairs (diplococci) (Ng and Martin, 2005). Gonococci are found inside polymorphonuclear leukocytes in clinical specimens (PMNs) and are unviable if dehydrated or exposed to non-physiological temperatures (Ng and Martin, 2005; Spence *et al*., 2008). *N. gonorrhoeae* move using twitching motility and is non-spore-forming (Ng and Martin, 2005).

Strict growth requirement includes 5% atmospheric CO_2 and a temperature of between 35 to 37^{O} C in humid conditions *(Spence et al., 2008).* It is a fastidious microorganism that requires special nutrients to grow *in vitro* (Kellog *et al*., 1963; Wesley Catlin, 1973). It requires iron to grow and typically extracts it from the human host, hence the medium must be supplemented with iron (Kellog *et al*., 1963). Additionally, glucose, lactate, or pyruvate must be added to the media to serve as the pathogen's carbon source (Wesley Catlin, 1973). Kellogg's supplements or gonococcal additives are mostly added to the agar or broth to meet the nutritional requirements (Kellogg *et al.,* 1963). *N. gonorrhoeae* grows on enriched media such as chocolate agar, Thayer–Martin agar, and New York City agar plates. *N. gonorrhoeae* can also grow in developed fastidious broth (Takei *et al.,* 2005). Colonies appear within 12 to 48 hours on agar plates; however, viability declines after 48 hours (Spence *et al*., 2008). On agar plates, four distinct *N. gonorrhoeae* colonial morphologies are noticeable due to the phase-variable expression of type IV pilus (Tfp) and opacity protein (Opa) (Kellog *et al*, 1963; Walstad *et al*.,

1977). At some times, colonies may be piliated and appear opaque in color (Opa+). Colony types 1 and 2 are small, shiny, and convex due to the expression of the pilus (Kellog *et al*, 1963; Walstad *et al.,* 1977). Piliated colonies may suddenly lose the pili and look dull and flat as in colony types 3 or 4. Opa- colonies are translucent (Kellog *et al*, 1963; Walstad *et al.,* 1977).

2.2.4 Major virulence factors in *N. gonorrhoeae*

The cell wall of *N. gonorrhoeae* is made up of an inner and outer membrane, separated by a thin layer of peptidoglycan (Ward *et al*., 1978). The outer membrane is made up of lipooligosaccharides, Opacity-associated proteins, pili, porin, and peptidoglycan which are crucial to the biology of the organism (Hill *et al*., 2016). *N. gonorrhoeae* possesses several virulence factors including opacity-associated protein expression, lipooligosaccharide expression (LOS), elaboration of pili, Por protein expression, and IgA1 protease production that facilitates host adaptation (Hill *et al*., 2016).

2.2.4.1 Opacity-associated protein (Opa)

Opacity-associated proteins (Opa) are highly variable outer membrane proteins that cause colonies to appear opaque due to inter-gonococcal aggregation (Connelly *et al*., 1990). Opa proteins are members of a multigene family (Connelly *et al*., 1990). Each of the genes consists of conserved, semi-variable, and two hypervariable regions, with the hypervariable section of the proteins positioned on the outside of the outer membrane (Bhat *et al*., 1991).

Opacity-associated proteins expression varies as infection proceeds, though it is not required for the initial attachment of gonococci to the host (Swanson *et al*., 1992). Gonococci Opa-expressing bacteria can be found in epithelial cells and neutrophils (Swanson *et al*., 1988; Jerse *et al*., 1994). The differential expression of Opa determines the invasive capacity of *N. gonorrhoeae* (Makino *et al*., 1991). The Opa proteins act as adhesins that bind to several receptors found on different cells and tissues to facilitate more intimate attachment and initiation of microcolony development (Dehio *et al*., 1998).

2.2.4.2 Pili

Type IV pili (Tfp) are retractable appendages found to protrude from the outer membrane and are known to be involved in mediating cell mobility, surface attachment, and subsequent clustering (Quillin and Seifert, 2018; Unemo *et al*., 2019). Tfp are the primary mode of

movement for gonococcus (Quillin and Seifert, 2018; Unemo *et al*., 2019). The development of pili is a crucial prerequisite for infection as this structure is essential for attaching to human mucosal epithelial cells (Swanson *et al*., 1987), vaginal epithelial cells (Tramont *et al*., 1980), human polymorphonuclear leukocytes (PMNs; neutrophils) (Virji and Heckels, 1986), and fallopian tube mucosa (Draper *et al*., 1980). Pili was first considered to be a prime target for vaccine candidate due to their prominent surface position and the presence of pilus-specific antibodies in genital secretions (Tramont *et al*., 1980). However, two well-known vaccine trials were unsuccessful due to indications that the pilus protein(s) underwent antigenic variation (Boslego *et al*., 1991).

Besides promoting adhesion to host cells, Type IV pili are involved in bacterial twitching motility, DNA transformation, and biofilm development (Heckels, 1989). *Neisseria gonorrhoeae* is inherently competent for transformation (Goodman and Scocca, 1988). It can take up exogenously produced *Neisseria*-specific DNA with a 10-bp uptake sequence (Goodman and Scocca, 1988).

2.2.4.3 Porin protein

Porin proteins (Por) are hydrophilic transmembrane channel proteins (Britigan *et al*., 1985). It is the most abundant outer membrane protein in the gonococcus accounting for almost 60% of the entire protein content (Britigan *et al*., 1985*).* Por's molecular size varies between strains, but it only exists as a single protein species within each strain (Johnston *et al.,* 1976). Due to this, Por is used for the serological classification (Johnston *et al*., 1976)*,* with nine different serovars of gonococcus being identified (Sandstrom *et al.,* 1982). Porins allow the transport of ions and other small molecules such as nutrients across the outer membrane and contribute to the survival of the gonococcus (Judd, 1989). *Neisseria gonorrhoeae* porin increases attachment and impairs the ability of phagocytes to kill the bacteria. Other functions include invading host cells, resting the action of complement factors, and contributing to antimicrobial resistance (Massari *et al*., 2003; Olesky *et al*, 2006; Madico *et al*., 2007; Deo *et al*, 2018).

After the bacteria is attached, the gonococcal Por protein translocates from the outer membrane into synthetic black lipid membranes and epithelial cell membranes (Weel *et al*., 1991). Porin proteins can also enter infected cells' mitochondria, where it forms porin channels in the inner membrane of the mitochondria and increases permeability (Müller *et al*., 2002). As a result, cytochrome c and other proteins are released, causing infected cells to undergo apoptosis

(Müller *et al*., 1999). Por protein can also regulate phagosome maturation by altering the phagosomal protein composition via an increase in early endocytic markers and a decrease in late endocytic markers, which ultimately causes phagosome maturation delay (Mosleh *et al*., 1998).

2.2.4.4 Lipooligosaccharide

Neisseria gonorrhoeae possesses lipopolysaccharide (LPS), an important component of the outer membrane that contributes to the virulence of the gonococcus (Britigan *et al*., 1985) (**Figure 2.2**). These glycolipids operate as virulence factors in addition to maintaining the outer membrane's structure (Britigan *et al*., 1985; Banerjee *et al.,* 1998). LPS is made up of lipid A, a core oligosaccharide, and a polymer of repeating polysaccharide (Britigan *et al*., 1985). The lipid A moiety acts as an anchor to the membrane. In contrast, gonococcal LPS consists of lipid A and core polysaccharide and truncated non-repeating oligosaccharide instead of the repeating polysaccharide, hence gonococcal LPS has been designated as lipooligosaccharide (LOS) (Britigan *et al*., 1985; Banerjee *et al*., 1998). As a result of its surface exposure, gonococcal LOS is one of the principal immune targets besides the outer membrane protein Por (Hook *et al*., 1984; Apicella *et al*., 1986). LOS in gonococcus is harmful to fallopian tube mucosa, causing the ciliary cells to shed off (Gregg *et al.,* 1981). **Figure 2.2** depicts the cell structure of *N. gonorrhoeae*.

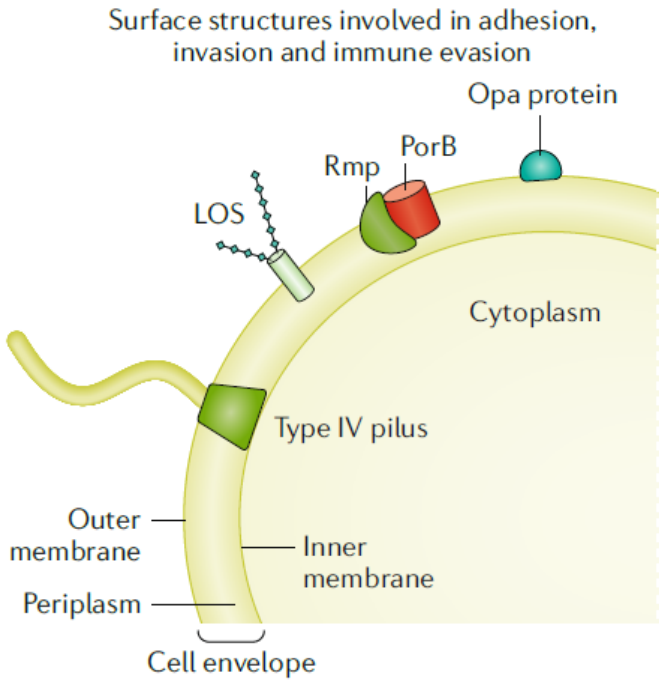

Figure 2. 2: Cell structure of *Neisseria gonorrhoeae*

Neisseria gonorrhoeae are diplococci (individual cell walls are ~0.6–1 µm in diameter). The cell wall consists of a cytoplasmic membrane, a periplasmic space containing peptidoglycan and an outer membrane containing lipooligosaccharide (LOS). It also has a long and thin type IV pilus, Opa proteins and localized porin (PorB) (Unemo *et al*., 2019).

The variable oligosaccharide section of LOS has the potential to mimic host glycosphingolipids to facilitate bacterial entry (van Putten and Robertson, 1995b). Additionally, the sialylation of gonococcal LOS makes the bacteria resistant to serum killing (Parsons *et al*., 1989; de la Paz *et al*., 1995). Thus, gonococcal LOS increases gonococcal pathogenicity by enabling bacterial translocation over the mucosal barrier and confers resistance to normal human serum (Elkins *et al*., 1992; van Putten, 1993).

2.2.4.5 IgA1 protease

The immunoglobin A (IgA) protease is another virulence component of *N. gonorrhoeae* (Halter *et al*., 1989). After release from the cell, the protein goes through several endo-proteolytic cleavages, which causes the IgA protease to mature (Pohlner *et al*., 1987). During infection, the mature IgA1 protease specifically targets and cleaves IgA1 at the proline-rich hinge region of the IgA1 heavy chain (Halter *et al*., 1984). Since the human IgA2 subclass lacks a susceptible

duplicated octameric amino acid sequence, gonococcal IgA protease is unable to cleave it. Lysosome-associated membrane protein 1 (LAMP1) is also cleaved by *Neisseria* IgA protease, which results in lysosome modification and subsequently bacterial survival (Hooper *et al*., 2000; Ayala *et al*., 2002).

2.2.5 Pathogenesis

Following transmission, *N. gonorrhoeae* colonises the mucosal epithelium of the urogenital tract to replicate and be transmitted to new individuals (Quillin and Seifert, 2018). The majority of the gonococcus' genes are adapted to colonization and survival because it cannot survive outside of a human host (Quillin and Seifert, 2018). *N. gonorrhoeae* mainly colonizes mucosal surfaces by attaching to different epithelial surfaces (**Figure 2.3**) (Quillin and Seifert, 2018). Bacterial adhesion to the mucosal epithelium is the primary factor for establishing infection and the first stage of pathogenesis (Quillin and Seifert, 2018). This is mediated by many bacterial surface structures, such as type IV pili, opacity (Opa) proteins, LOS, and the outer membrane protein porin (also known as PorB) (Hill *et al*., 2016). Pili are implicated as adhesions in early pathogenesis. The pilus is responsible for initial attachment to host epithelial cells and the establishment of sustained infections (Higashi *et al*., 2007). During initial infection, micro-colony formation on columnar epithelial cells commences approximately 1 to 2 hours post-infection (van Putten and Robertson, 1995; Braun and Stein, 2004). After the micro-colonies attain a cell density of 100+ diplococci, cytoskeletal rearrangement, and host protein aggregation emerge, which result in pilus-mediated attachment of the gonococcus to the CD46 host cell-surface receptor (Kallstrom *et al*., 1997; Nassif *et al*., 1999). Once bound, the pilus structures on certain microorganisms retract through PilE depolymerization (Wolfgang *et al*., 1998), promoting tighter interaction with the host cells by causing Opa to bind to the CEACAM receptors (Virji *et al*.,1996; Chen *et al*., 1997). After CEACAM binds, the host cell undergoes actin polymerization and rearrangement, which causes the bacteria to be engulfed, undergo transcellular transcytosis, and release the bacteria into the subepithelial layer (Wang *et al*., 1998; Billker *et al*., 2002). Pili and Opa coordinated expression vary greatly *in vivo* (James and Swanson, 1978). Gonococcus isolated from the urethra of males usually co-express pili and one of the multiple Opa proteins (Swanson *et al*., 1992). Opa expression in women, however, differs according to whether the patient is on oral contraceptives and the stage of her menstrual cycle (James and Swanson, 1978). Bacteria isolated from the cervix at mid-cycle express Opa, whereas those isolated during menstruation

are usually Opa-negative (Draper *et al*., 1980). Universally, gonococcus isolated from infected fallopian tubes is mostly Opa-negative, although bacteria expressing Opa can be recovered from the same patient's cervix (Draper *et al*., 1980). More proteolytic enzymes in cervical secretion during menses than during the follicular phase may explain the above observation. Subsequently, due to Opa proteins' high sensitivity to trypsin-like enzymes, non-Opa expressing cells may be selected (Draper *et al*., 1980).

In contrast, it has been observed that fallopian epithelial tube cell cultures do not express CECAM receptors in light of recent studies showing Opa interactions with these receptors (Swanson *et al.,* 2001). However, it was found that gonococci continued to adhere and invade in the absence of these receptors. Subsequently, CECAM expression or its absence may facilitate *in vivo* phenotypic selection of distinct gonococcal populations on different tissues (Sintsova *et al.,* 2015). Overall, Opa expression improves the fitness of gonococci in the female vaginal tract. Most re-isolates from female disseminated infections generally lack Opa expression (Cole *et al*., 2010).

2.2.5.1 Induction of inflammatory response

The inflammatory response to gonococcal infections occurs as a result of host cells' interaction with bacterial virulence factors (Virji and Heckels, 1986; Higashi *et al*., 2007). The massive influx of neutrophils to the site of infection, resulting in the production of a pustular discharge, is the hallmark symptom of a non-complicated gonorrhea infection (Virji and Heckels, 1986; Higashi *et al*., 2007).

Opacity-associated proteins (Opa) expression was initially believed to be fully involved in polymorphonuclear leucocytes (PMN) (Rest *et al*., 1982; Rest *et al.,* 1985; Ramsey *et al*., 1995). It was however observed that following the attachment of gonococci to the mucosa, pro-inflammatory cytokines IL-6 and TNF-α, as well as chemokine IL-8, were produced leading to the recruitment of neutrophils at the site of infection (Ramsey *et al*., 1995). Additionally, gonococci release LOS and lipoproteins upon arrival at the sub-epithelial layer, which further stimulates cytokine production because the Toll-like receptors (TLRs) on immune cells detect these outer membrane components (Makepeace *et al*., 2001; Fisette *et al*., 2003).

Host cells also use cytoplasmic NOD-like receptors (NLRs) to respond to bacterial peptidoglycan fragments which also contribute to the secretion of more pro-inflammatory cytokines (Kaparakis *et al*., 2010). Despite the active influx of PMNs to the site of infection,

gonococci have evolved mechanisms to survive both the oxidative and nonoxidative defense mechanisms (Kaparakis *et al.,* 2010).

Survival appears to be correlated with gonococci modulating IL-17 expression to selectively activate Th17-dependent host defensive systems (Ovcinnikov and Delektorskij, 1971). Gonococci also combat substantial oxidative stress by producing a variety of enzymes during the inflammation response to detoxify hydrogen peroxide (H_2O_2), superoxide anions ($O_2•-$), and hydroxyl radicals (Seib *et al*., 2005; Criss and Seifert, 2012). Gonococci must eliminate H_2O_2 since in the presence of ferrous ions, the Fenton reaction (Fe2+ + H2O2 → Fe3+ + OH. + OH−) can generate additional hydroxyl radicals (Seib *et al*., 2006).

Catalase, in conjunction with a periplasmic cytochrome-c peroxidase (Ccp), is used by the gonococcus to remove H_2O_2 which significantly increases the organism's ability to resist in vitro neutrophil killing (Stohl *et al*., 2005; Seib *et al*., 2006). Superoxide dismutase enzymes (SOD) normally remove superoxide ions by converting superoxide to H_2O_2 and water (Seib *et al*., 2005). However, most *N. gonorrhoeae* strains lack detectable SOD activity, this suggests that oxidants may be eliminated by a different mechanism (Hill, 2011). Manganese ions (Mn^{2+}) are used by *N. gonorrhoeae* to prevent the buildup of reactive oxygen species (Seib *et al*., 2005).

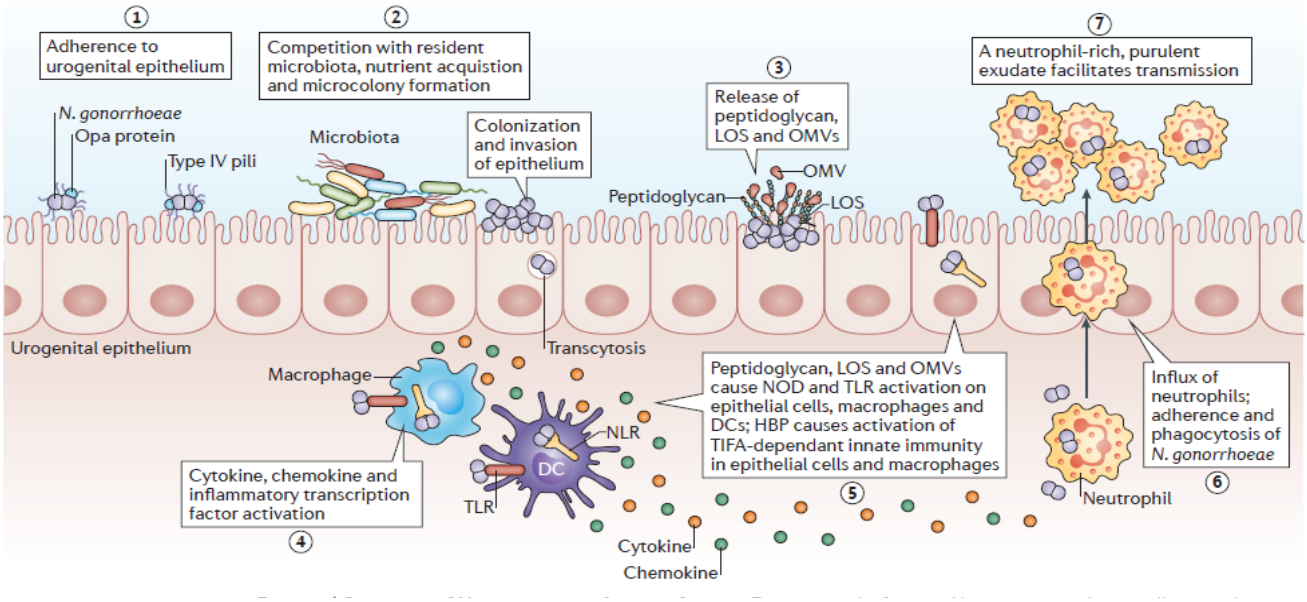

Figure 2. 3: Overview of the pathways of *Neisseria gonorrhoeae* infection in the human host

(Quillin and Seifert, 2018)

2.2.5.2 The need for iron from the host

Iron is a vital nutrient required by gonococci (Kellog *et al*., 1963). The pathogen, therefore, expends substantial resources on scavenging the element from the host. It is even more challenging during an infection because the human host decreases the amount of free iron in the bloodstream in response to inflammation by limiting iron availability (Seib *et al*., 2006). Although humans store their iron sequestered in iron-proteins complexes such as hemoglobin, ferritin, lactoferrin, and transferrin, *Neisseria* can scavenge iron from transferrin and hemoglobin by expressing receptors for both lactoferrin and transferrin which give them a selective advantage within the host (Jordan and Saunders, 2009; Skaar, 2010). *Neisseria* directly extracts iron from transferrin since they do not form siderophores. The iron transport system of *Neisseria* consists of two large surface proteins, namely transferrin-binding protein A (TbpA) and transferrin-binding protein B (TbpB) (Noinaj *et al.,* 2012). These surface proteins are present in all clinical Neisseria isolates (Noinaj *et al.,* 2012). TbpA is an important outer membrane transporter that is crucial for iron uptake and can bind both to apo- and iron-containing transferrin, whereas TbpB, a surface-exposed lipoprotein, only binds to iron-bound transferrin (Noto and Cornelissen, 2008). Due to a similar affinity between the bacterial receptor and transferrin for iron, the gonococcus can compete with the host for this essential nutrient (Cornelissen *et al*., 1992). The expression of the transferrin receptor was shown to be required for gonococcal infectivity (Cornelissen *et al.*, 1998).

2.2.5.3 Serum resistance

Neisseria gonorrhoeae can evade the host immune system responses. The endless ability of the gonococci to modify surface structures coupled with antigenic and phase variation enables the bacteria to escape recognition by the host (Quillin and Seifert, 2018). Antibody-mediated killing varies between patients presenting with genital infections (Kasper *et al*., 1977). Gonococcal surface components are the primary targets of antibody-dependent complement killing; LPS-specific antibodies are the most effective at eliciting bactericidal responses (Ward *et al*., 1978). Stable and unstable serum resistance are the first two types of serum resistance described in *N. gonorrhoeae* (Schoolnik *et al*., 1976; Winstanley *et al.,* 1984).

Unstable serum resistance mostly occurs in human serum, various mucosal secretions, and within professional phagocytes. Unstable serum resistance results from the modification of gonococcal LOS by the addition of sialic acid molecules to terminal galactose residues using

cytidine 5'- monophosphate N-acetylneuraminic acid (CMP-NANA) (Mandrell *et al*., 1993). In addition to altering bacterial resistance to complement-mediated bacterial killing, sialylation of LOS mediates gonococci entry into host mucosal cells (van Putten, 1993). It has been demonstrated that gonococcal cells harboring lightly sialylated LOS molecules are more efficient at invading host epithelial cells than heavily sialylated-LOS variants but are more susceptible to complement-mediated killing (Parsons *et al*., 1989; de la Paz *et al*., 1995). Heavy sialylation of LOS renders gonococci resistant to the human serum by masking the target sites for bactericidal antibodies (Parsons *et al*., 1989; de la Paz *et al*., 1995), which prevents the activation of the complement cascade (Elkins *et al*., 1992).

On the contrary, stable serum resistance is caused by the faulty insertion of the C5b-C9 membrane attack complex in serum-resistant strains (Joiner *et al*., 1983; Joiner *et al*., 1985a; Lewis *et al*., 2013). In addition to this deposition defect, blocking antibodies is also believed to cause the C3 complement component to be directed onto a different site on the outer membrane which also hinders bacterial killing (Joiner *et al*., 1985b). Complement resistance is undoubtedly crucial for microorganisms causing disseminated infection, but its importance for microorganisms that cause mucosal infections is not clear. However, the presence of complement activation inhibitors in the seminal plasma indicates some level of complement activity in the mucosa (Brooks *et al*., 1981).

Gonococci that express Por1A bind more effectively to complement factor H (Ram *et al.,* 1998). As factor H down-regulate alternative complement activation, this binding contributes to serum resistance in disseminated strains (Ram *et al.,* 1998). Opacity-associated proteins also induces the activation of the classical complement pathway by binding to the C4b-binding protein, which once again down-regulates complement activation (Ngampasutadol *et al*., 2005). Since factor H and C4b-binding sites on the Por proteins prevent functional complement deposition, these sites may need modification in vaccine development to help resolve challenges associated with serum resistance (Blom and Ram, 2018).

2.2.6 Active immunity

It is well known that gonorrhoea neither elicit a protective immune response nor impart immune memory (Liu *et al*., 2011). This causes repeated infections. Even though specific antibodies that inhibit adherence are produced within the genital tract, these antibodies are short-lived (Tramont, 1977; Tramont *et al*., 1980). The ability of the bacteria to manipulate the

host cell response account for the unresponsiveness to gonococcal infection (Boulton and Gray-Owen, 2002). The transient reduction in the T-cell population appears to correlate with Opa protein interactions with CD4+ T-cells, which suppress T-cell activation (Boulton and Gray-Owen, 2002). Additionally, Opa-carcinoembryonic antigen cellular adhesion molecules (CEACAM1) T-cell interactions do not cause internalization of gonococci into the T-cells, in contrast to Opa-mediated interactions with CEACAM antigens on other cell types (Lee *et al.,* 2008). Opa-CEACAM1 interactions on B lymphocytes prevent antibody formation (Lee *et al*., 2008). Opa-CEACAM1 interactions do not stimulate the internalization of dendritic cells, rather, engulfment is mediated by the interaction of lipooligosaccharide (LOS) with DC-SIGN antigens (Zhang *et al*., 2006).

The variation of LOS composition further gives gonococcus an opportunity for immune evasion (Zhang *et al.,* 2006). Lipooligosaccharide molecules mostly interact with Toll-like receptors to activate immune cells (Lu *et al*., 2005). However, deacylation of LOS following interaction with its cognate Toll-like receptors can also moderate immune response by downregulation of antibody production (Lu *et al*., 2005). Additional modulation of the human response is observed with gonococcal activation of IgM-specific memory B-cells in a T-independent manner (So *et al*., 2012). This elicits a non-specific polyclonal immunoglobulin immune response without forming specific immunologic memory cells to gonococcal infection (So *et al*., 2012).

2.2.7 Antibiotic resistance in *N. gonorrhoeae*

Without any effective vaccine, antibiotics are used to effectively control gonorrhea. However, the emergence of resistance to all major classes of antibiotics makes it more difficult to treat gonococcal infections (**Figure 2.4**) (Unemo and Nicholas, 2012).

Sulfonamides were introduced in 1930 and were used to successfully treat gonococcal infections (Kampmeier, 1983). However, resistance emerged by mid-1940, making the drug ineffective against gonococcal infections (Dunlop, 1949). In 1949, sulfonamides were no longer used to treat gonococcal infections (Sköld, 2000). Penicillin was introduced in the mid-1940s as a drug of choice (Willcox, 1970). During this time, the gonococci were extremely sensitive and reported a MIC of 0.004-0.01 µg/mL for penicillin (Willcox, 1970). In 1958, decreased susceptibility to both penicillin and streptomycin was reported in stains of *N. gonorrhoeae* (Cradock-Watson *et al*., 1958). MICs gradually increase over time, such that the MICs for some strains were greater than 2 µg/mL between the late 1970s and early 1980s,

indicating resistance (CDC, 2007).

In 1987, penicillin was no longer used since treatment failure was widespread (Faruki, *et al.,* 1985). Moreover, in the 1980s, the Centers for Disease Control and Prevention (CDC) withdrew tetracycline as a treatment option due to increasing resistance to tetracycline (CDC, 1985). Fluoroquinolones were introduced in 1996 as a treatment option for gonococcal infections, but resistance to this antibiotic class soon emerged and was widespread, and by 2007, the CDC removed fluoroquinolones as a treatment for gonorrhoeae because of treatment failure (CDC, 2007). In the same year, the CDC added *N. gonorrhoeae* to the list of "superbugs", highlighting the difficulties in treating gonococcal infections due to increasing resistance to azithromycin, beta-lactams, spectinomycin, tetracycline, and fluoroquinolones (CDC, 2007).

Expanded-spectrum cephalosporins (i.e., ceftriaxone, and cefixime) are effective for treating gonorrhoea (Unemo and Shafer, 2014). In 2009, reports of treatment failure with ceftriaxone emerged in the treatment of pharyngeal gonorrhea (Tapsall *et al*., 2009; Tapsall, 2009a; Tapsall, 2009b). In 2010 and 2011, *N. gonorrhoeae* strains with resistance to ceftriaxone and cefixime were isolated in France and Japan (Ohnishi *et al*., 2012; Unemo *et al*., 2012).

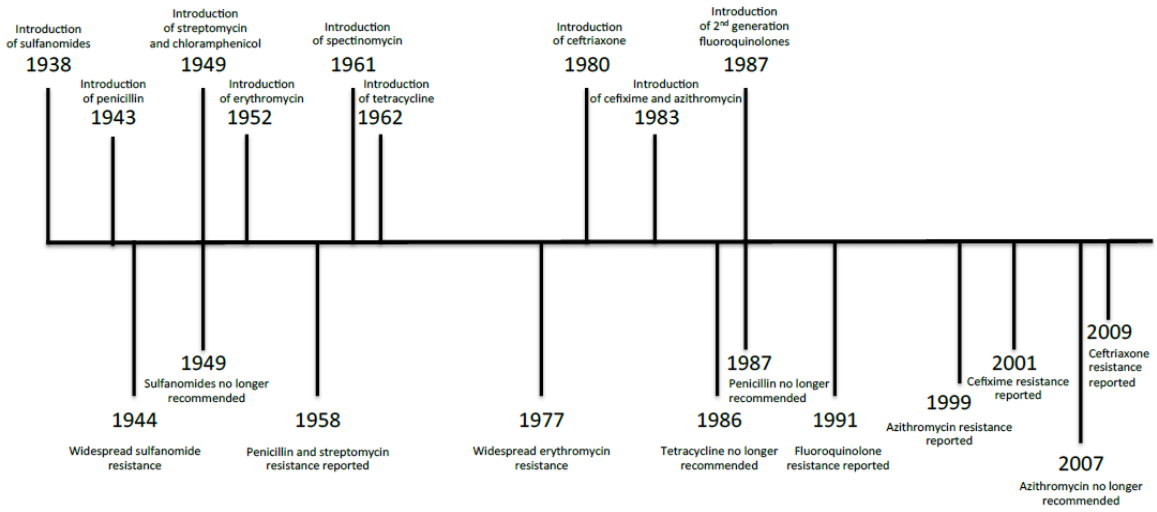

Figure 2. 4: Timeline of the development of antibiotic-resistant *N. gonorrhoeae*

Since the introduction of the first antibiotics, sulfonamides, for the treatment of gonococcal infections in 1938, N. gonorrhoeae has evolved resistance to all the classes of drugs used to treat gonorrhoea (Unemo and Shafer, 2011).

2.2.7.1 Global prevalence of drug-resistant *N. gonorrhoeae*

The extraordinary capacity of *N. gonorrhoeae* to develop and spread resistance to new antibiotic classes poses a significant public health threat globally (Unemo and Nicholas, 2012). *N. gonorrhoeae* has developed resistance to sulfonamides, macrolides, penicillins, tetracyclines, and fluoroquinolones in most parts of the world (Unemo and Shafer, 2014). Sporadic resistance and decreased susceptibility to third generation cephalosporins, and spectinomycin are increasingly reported (Unemo and Nicholas, 2012). Moreover, multi-drug-resistant and extensively drug-*resistant N. gonorrhoeae* strains are commonly isolated in many countries (Tapsall, 2009b; Unemo and Nicholas, 2012).

The data from the WHO's global antimicrobial resistance surveillance for *N. gonorrhoeae* indicates that the countries reporting gonococcal antimicrobial resistance (AMR) and resistant isolates have increased compared to previous reports of gonococcal AMR (Unemo *et al*., 2021). In 2017 to 2018, AMR data on at least one or more drugs were reported by 73 countries. Resistance or decreased susceptibility to ceftriaxone was reported by 31% (21/68) of reporting countries and to cefixime by 47% (21/51) of reporting countries (Unemo *et al*., 2021). Azithromycin resistance was reported by 84% (51/61) of reporting countries and ciprofloxacin resistance by 100% (70/70) of reporting countries (Unemo *et al*., 2021). Since the isolation of the H041 strain in Japan, other XDR strains have been detected in Catalonia, Spain (Camara *et al*., 2012), and in Quimper, France (F89 strain) (Unemo *et al*., 2011). The isolation of these XDR strains in sex tourists, sex workers, long-distance truck drivers, homosexual men, and people undergoing forced migration suggests the potential for global transmission of these strains (Unemo and Nicholas, 2012; Hill *et al.,* 2016). Without new effective treatment options, gonococcal infections will soon become difficult to treat and cure (WHO, 2011).

The yearly proportion of resistance across countries was 0 to 100% to ciprofloxacin, 0 to 60% to azithromycin, 0 to 21% to ceftriaxone, and 0 to 22% to cefixime. Surveillance was scarce in eastern Europe, central America, and the Caribbean, and in the Eastern Mediterranean, WHO African, and South-East Asian regions (Unemo *et al*., 2021). Antimicrobial resistance in *N. gonorrhoeae* is increasing globally; strains with resistance to both azithromycin and ceftriaxone have been reported in many countries (WHO, 2011).

2.2.8 Mode of action and mechanisms of antibiotic resistance

Based on the modes of action, commonly used antibiotics to treat infections are generally

classified into three main classes (Lewis, 2010). Antibiotics that act on protein synthesis included tetracyclines (e.g. tetracycline and doxycycline), macrolides (e.g. erythromycin and azithromycin), aminoglycosides (e.g. gentamicin, kanamycin, streptomycin, and oxazolidinones), and spectinomycin (Lewis, 2010). Antibiotics that act on the cell wall include glycopeptides (e.g., vancomycin, teicoplanin, telavancin, bleomycin and ramoplanin), and β-lactams (e.g., penicillins, ceftriaxone, and cefixime) (Lewis, 2010). Drugs that act to inhibit nucleic acid consist of fluoroquinolones (i.e ciprofloxacin and ofloxacin). Other antibiotic classes are drugs that inhibit folate synthesis (e.g., sulfonamides) and drugs active against membranes (i.e. polymyxins) (Lewis, 2010). The high mutational rate in many genes encoding resistance determinants and the natural ability to acquire resistant genes from other bacteria has led to the increasing global antimicrobial resistance in *N. gonorrhoeae* (Młynarczyk-Bonikowska *et al*., 2020). The resistance is either intrinsic or acquired (Młynarczyk-Bonikowska *et al*., 2020) (**Table 2.4**). Antibiotic resistance can be chromosomally, or plasmid-mediated (Młynarczyk-Bonikowska *et al*., 2020). Plasmid-mediated resistance is observed for tetracycline via the production of TetM protein which prevents tetracycline action, and penicillin via the expression of β-lactamase (Młynarczyk-Bonikowska *et al*., 2020). In chromosomally mediated resistance, mechanisms of **resistance** include modification of the drug, alterations of the drug target, efflux of the drug out of the cell, and changes in the permeability of the cell (Młynarczyk-Bonikowska *et al*., 2020).

Table 2. 4: Mechanisms of antibiotic resistance in *N. gonorrhoeae*

Mechanism	Examples	Intrinsic or Acquired
Drug Modification	*N. gonorrhoeae* strains harbor plasmids that code for β-lactamases which hydrolyze the β-lactam ring of β-lactam antibiotics (Maduna *et al*., 2020).	Acquired
A decreased influx of antibiotics into the cell	*penB*-mutation in porB1b: Increases resistance to third-generation cephalosporins, penicillin (Zhao *et al*., 2005). and tetracycline *penC*-mutation in pilQ reduces the entry of tetracycline and penicillin (Lee *et al*., 2010; Unemo *et al*., 2011).	Both
Alteration of antibiotic targets	*penA*-mutations in PBP2 led to a decreased rate of acylation by penicillin (Unemo M, Shafer, 2011). *rpsJ*-Mutation in the ribosomal protein S10 results in a high level of resistance to tetracycline (Maduna *et al*., 2020). *gyrA* and *parC*-mutations in DNA gyrase and topoisomerase IV cause resistance to fluoroquinolones (Maduna *et al*., 2020)	Acquired
Increased efflux out of the cell via multi-drug efflux pumps	The pump MtrC–MtrD–MtrE and its repressor MtrR contribute to *N. gonorrhoeae* resistance through antimicrobial efflux resulting in resistance to different antimicrobials. These mutations cause overexpression of the efflux pump (Hagman *et al*., 1995)	Both

2.2.9 Diagnostic methods used to detect drug-resistant *N. gonorrhoeae*

Antibiotic susceptibility testing of *Neisseria gonorrhoeae* colonies is performed by Etest. Susceptibility to azithromycin, ceftriaxone, cefixime, ciprofloxacin, penicillin G, and tetracycline are commonly checked (Maduna *et al*., 2020). Multidrug-resistant (MDR) *N. gonorrhoeae* is defined as resistance to either cephalosporin or azithromycin plus resistance to at least two other antibiotics (ciprofloxacin, erythromycin, penicillin, and tetracycline) (Martin *et al*., 2019). Extensively drug-resistant (XDR) *N. gonorrhoeae* is defined as resistant to cephalosporin and azithromycin, plus resistance to at least two other antibiotics (ciprofloxacin, erythromycin, penicillin, and tetracycline) (Martin *et al*., 2019). Whole genome sequencing is used to identify the various resistant genes or mechanisms as applied to the reference agent (Martin *et al*., 2019; Maduna *et al*., 2020).

2.2.10 Treatment of gonorrhoea

In clinical practice, gonococcal infection is typically treated empirically during the initial clinical visit; thus, testing for antibiotic susceptibility is rarely done prior to antibiotic prescription (CDC, 2015). Due to the emergence of high-level resistance to all extended-spectrum cephalosporins (ESCs), including the most potent ceftriaxone and cefixime, dual antibiotic therapy consisting of ceftriaxone and azithromycin was introduced as the first-line empirical therapy for uncomplicated anogenital and pharyngeal gonorrhea in Australia (Bignell and Unemo, 2013), Canada (Public Health Agency of Canada, 2013), Europe (Bignell and Unemo, 2013), USA (CDC, 2015) and WHO (WHO, 2016) for the past decade. This treatment generally consisted of a single 250–500 mg dose of ceftriaxone along with 1–2 g of azithromycin.

Because of the increasing resistance to azithromycin and the potent anti-commensal activity of the dual therapy, some countries including the USA and UK have removed azithromycin from the treatment regimen; ceftriaxone monotherapy is now recommended for treating *N. gonorrhoeae* infections (Ohnishi *et al*., 2011; Cyr *et al*., 2020). The emergence of gonococcal infections with resistance to ceftriaxone and cefixime is reported in different countries (Unemo *et al*., 2011; Unemo and Nicholas, 2012), making *N. gonorrhoeae* a superbug, requiring urgent development of new treatment options. **Table 2.5** shows the treatment guidelines suggested by the USA, the European Union, Australia, Canada, Brazil, Southern Africa, and the WHO.

Table 2. 5: Antimicrobial therapy recommended for the treatment of gonococcal infection

Country/region/ organization	Therapy	Special situations	Year guideline published	Reference
WHO	CRO 250 mg IM + AZM 1 g PO or CFX 400 mg PO + AZM 1 g PO	If local recent data confirm susceptibility, one antimicrobial in a single dose is a possibility: CRO 250 mg IM or CFX 400 mg PO or SPC 2 g IM	2016	WHO, 2016
Europe	CRO 500 mg IM + AZM 2 g PO	If CRO is unavailable, or antimicrobial injection is impossible, or patient refuses to take the medication: CFX 400 mg PO + AZM 2 g PO If AZM is unavailable, or patient cannot take oral medication: CRO 500 mg IM In case of ESC resistance, or if patient is allergic to penicillin or cephalosporin: SPC 2 g IM + AZM 2 g PO	2013	(Bignell and Unemo, 2012)
USA	CRO 250 mg IM + AZM 1 g PO	If CRO is unavailable: CFX 400 mg PO + AZM 1 g PO	2015	CDC, 2015
Canada	CRO 250 mg IM + AZM 1 g PO or CFX 800 mg PO	If CRO is unavailable: SPC 2 g IM + AZM 1 g PO If a patient is allergic to cephalosporin: AZM 2 g PO	2013	Public Health Agency of Canada, 2013
Australia	CRO 500 mg IM + AZM 1 g PO	None	2021	(Lahra *et al.,* 2021)
Brazil	CIP 500 mg PO + AZM 500 mg PO or CRO 500 mg IM + AZM 500 mg PO	If a patient is allergic to cephalosporin: AZM 2 g PO If patient is < 18-year-old or pregnant: CRO 500 mg IM + AZM 500 mg PO	2015	Notícias *et al*., 2015
Southern African	CRO 500mg IM or CFX 800mg PO	In case of confined oropharyngeal infection, increase the dose of 1g IM	2022	Peters *et al*., 2022

AZM, azithromycin; CFX, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; SPC, spectinomycin; ESC, extended-spectrum cephalosporin.

2.2.11 Pipeline of new antibiotics for treatment of gonorrhoea

Neisseria gonorrhoeae has rapidly emerged from exquisitely susceptible into a superbug, with the ability to demonstrate an XDR phenotype (Pleininger *et al*., 2022). The absence of an effective vaccine, coupled with the limited pipeline and availability of new drugs has raised global fear about the emergence of untreatable gonorrhoea (Lin *et al*., 2021). Due to this, the WHO has designated fluoroquinolone and third-generation cephalosporin-resistant *N. gonorrhoeae* as a high-priority pathogen for research and development of new antimicrobial agents (Tacconelli *et al*., 2018).

Clinical trials involving delafloxacin (Hook *et al*., 2019) and solithromycin (Hook *et al*., 2015; Oldach, 2017) produced unsatisfactory outcomes as both agents failed to demonstrate non-inferiority to ceftriaxone-based regimes. At present, gepotidacin and zoliflodacin are the most promising antibiotics in clinical development (Taylor *et al*, 2018a; Taylor *et al*., 2018b). In Phase 2 clinical trial, both drugs demonstrated potent activity in eradicating urogenital gonorrhoea, however, treatment failure occurred at the oropharyngeal site (Taylor *et al*, 2018a; Taylor *et al*., 2018b). Since the oropharyngeal site is a crucial site of infection, it is unlikely for any of these agents to be promoted as monotherapy for gonorrhoea. The pre-clinical pipeline is empty of any new agent that is likely to advance to clinical development to treat gonococcal infections (WHO, 2021a). This underscores the need to increase research and investment in the discovery of new anti-gonococcal agents.

2.2.12 Prevention and control

Public health measures play a key role in the prevention and treatment of AMR gonococcal infections, and several interventions have been outlined in the WHO's Global Action Plan against *N. gonorrhoeae* (WHO, 2012). A robust surveillance system that can quickly detect antibiotic-resistant *N. gonorrhoeae* infections can facilitate focused efforts to outbreaks within the population. Improving diagnostics to detect AMR in *N. gonorrhoeae* would be useful to identify individuals at risk of treatment failure and will also benefit the treatment of resistant infections. Furthermore, increasing knowledge, advocacy, and awareness of drug-resistant *N. gonorroheae* among health officials, clinicians, and the public may play vital in the prevention and treatment of drug-resistant gonococcal infections (WHO, 2012).

2.2.13 Need for an effective vaccine against gonorrhoea

The need for an effective vaccine has become crucial since *N. gonorrhoeae* continues to develop resistance to all antibiotics recommended for treatment (Jerse *et al*., 2014). It is well established that individuals treated for gonorrhea can be repeatedly infected without immunological memory formation (Quillin and Seifert, 2018). In a study using an experimental gonococcal infection model in men, it was discovered that within 21 days of the initial infection, there was no protection against repeated infections with the same strain (Schmidt *et al*., 2001). *N. gonorrhoeae* uses several mechanisms to evade the adaptative human immune system to establish an infection (Cahoon and Seifert, 2011). *N. gonorrhoeae* escape the host immunity by undergoing antigenic and phase variation of the surface-exposed type IV pili, LOS, and Opa proteins (Cahoon and Seifert, 2011). The carbohydrate structures of LOS mimic host molecules to evade the adaptive immune system (Mandrell *et al*., 1988; Mandrell, 1992; Gulati *et al*., 1996). Majority of *N. gonorrhoeae* LOSs exhibit cross-reactivity with antibodies that target human glycosphingolipid surface antigens, notably on human erythrocytes; this mimics the human surface antigens and makes it more difficult to develop an effective vaccine (Mandrell *et al*., 1988; Mandrell, 1992; Gulati *et al*., 1996). Additionally, *N.* gonorrhoeae actively suppress the immune system by regulating the production of IL-10 from mouse iliac lymph node cells, CD4+ T cells, and genital tract explants by regulating transforming growth factor-β (TGFβ) cytokine production in the vaginal cells of BALB/c mouse and the type 1 regulatory T cell activity of CD4+ T cells (Liu *et al*., 2012; Liu *et al*, 2014). This prevents the development of T helper 1 and T helper 2 cells (Liu *et al*., 2012; Liu *et al*, 2014). Also, dendritic cells exposed to gonococcus were no longer able to stimulate the proliferation of CD4+ T cells (Zhu *et al*., 2012). Although *N. gonorrhoeae* induces a large innate immune response from the human host and suppresses the adaptive immune response, these interactions on both arms of the human host immune system can prolong infection and cause repeated infections in high-risk groups of the population (Liu *et al*., 2011).

Since the 1970s, several attempts to produce an effective vaccine against *N. gonorrhoeae* have been unsuccessful (Lin *et al*., 2021). Generally, the obstacle to vaccine development is attributed to the lack of naturally acquired immunity to *N. gonorrhoeae* because of antigenic variation, coupled with different immune evasion mechanisms and its exclusive restriction to humans (Quillin and Seifert, 2018). Petousis-Harris and colleagues first reported a potential protective immunity against gonorrhea using MeNZB vaccine against *N. meningitidis*.

MenNZB vaccination resulted in lower infection rates with an estimated vaccine effectiveness of 31% (Petousis-Harris *et al*., 2017). A follow-up study reported a 24% effectiveness of MeNZB vaccine against hospitalizations due to gonococcal infections, supporting the cross-protectivity of the vaccination (Paynter *et al*., 2019). However, effectiveness was low and decreased to 9% in 5 years (Kenyon, 2019). Other studies have backed the meningococcal serogroup B vaccines' potential gonorrhea protection (Azze, 2019; Whelan *et al*., 2016). Studies on the rates of gonorrhea in Norway and Cuba both revealed a decrease in incidence after vaccination with MenB (Azze, 2019; Whelan *et al*., 2016).

Although MeNZB vaccine is not available, a newer serogroup B vaccine, 4CMenB known as Bexsero is available (Semchenko *et al*., 2019; Semchenko *et al*., 2020). This vaccine has the same outer membrane vesicle (OMV) components as in MeNZB, in addition to three recombinant proteins of Neisserial heparin binding antigen (NHBA) which is conserved and expressed on the surface of *N. gonorrhoeae* (Semchenko *et al*., 2019; Semchenko *et al*., 2020). This target was found to be crucial for gonococcal survival and colonization (Semchenko *et al*., 2019; Semchenko *et al*., 2020). Bexsero successfully generated anti-gonococcal NHBA and OMVs antibodies, offering a source of protection against *N. gonorrhoeae* (Semchenko *et al*., 2019). These findings were validated in mouse studies, where rapid clearance and decreased burden of *N. gonorrhoeae* was reported with several antibodies targeting *N. gonorrhoeae* surface proteins, including NHBA (Leduc *et al*., 2020). Clinical trials for Bexsero are currently in Phase II (NCT04350138) and is estimated to be completed in August 2023 (Looker *et al*., 2023). Besides OMVs and NHBA, there are several potential vaccine targets. In general, an ideal vaccine target should be highly conserved among *N. gonorrhoeae* strains (Lin *et al*., 2021).

2.2.14 Conclusion

Neisseria gonorrhoeae is an established high-priority pathogen that continues to remain a major public health concern globally. Over the past decade, the incidence of gonococcal infections has increased sharply in many countries (Tacconelli *et al*., 2018). *N. gonorrhoeae* uses several mechanisms including antigenic and phase variation of the surface-exposed type IV pili, LOS, and Opa proteins to manipulate and evade the human immune system to establish an infection (Cahoon and Seifert, 2011). Without effective treatment, these infections can result in serious sequelae such as ectopic pregnancy, and adverse pregnancy outcomes, pelvic inflammatory disease, increased risk of tubal factor infertility, disseminated gonococcal

infection (DGI), and facilitate the transmission of HIV (Burnett *et al*., 2012; WHO, 2011). Repeated gonococcal infections occur even after treatment because *N. gonorrhoeae* infection neither elicits a protective immune response nor imparts immune memory (Quillin and Seifert, 2018). The development of *N. gonorrhoeae* strains resistant to all antibiotics recommended for treatment, including third-generation cephalosporins, fluoroquinolones, azithromycin, β-lactams, and tetracycline poses a major public health challenge (Tapsall, 2009a; Tapsall, 2009b; Unemo and Nicholas, 2012). The lack of effective vaccines to prevent infections, coupled with the increase in antibiotic resistance and the limited pipeline highlights the crucial need to invest in research and development of new drugs.

2.3 Part 3: The Medicines for Malaria Venture Pathogen Box

2.3.1 MMV Pathogen Box

In order to accelerate the discovery of novel drug compounds, the Medicines for Malaria Venture (MMV) group developed the Pathogen Box (**Figure 2.5**), a collection of 400 novel drugs and reference compounds in a set of five 96-well micro-titre plates labelled A- E (Duffy *et al.*, 2017). Within each well is 10 mM of drug compound in a 10 µL of dimethyl sulphoxide (DMSO) solution. These 400 compounds are mostly novel synthetic chemicals that were initially selected from a screen of over 4 million chemicals due to their low toxicity to mammalian cells. Each of the compounds has been tested for cytotoxicity and has shown values within levels considered acceptable for an initial drug discovery programme (**Figure 2.5**) (http://www.pathogenbox.org/).

Figure 2. 5: The MMV Pathogen Box

2.3.2 Antibiotic activity of the Pathogen Box compounds (PBCs)

The PBCs have demonstrated biological activity against specific pathogenic microorganisms in a screen that was initially mostly directed at protozoal parasites responsible for tropical diseases, in particular malaria (Ballell *et al*., 2013; Veale, 2019). However, it also includes compounds with activity against *M. tuberculosis* and to a lesser extent, compounds with activity against other microorganisms. The compounds have diverse chemical scaffolds distinct from presently available antibiotics. The mechanisms of action of the compounds are mostly unknown (Bleicher *et al*., 2003; Congreve *et al*., 2005).

Initial screens showed that the majority of the compounds have specific activity against malaria, tuberculosis and kinetoplastids (**Figure 2.6**). A total of 315 copies of the Pathogen Box have been distributed to scientists in 44 countries since January 2016, including to the Department of Medical Microbiology of the University of Pretoria.

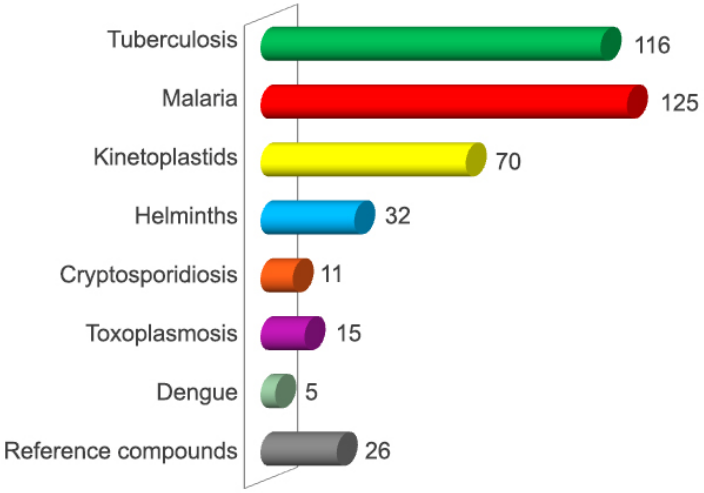

Figure 2. 6: Composition of the MMV Pathogen Box

The plate mapping of the 400 compounds included in the PBox, including the biological data and DMPK data with chemical structures (as SMILES or with illustrations of the structures), can be found via the following link, covering the essential information on the compounds: https://www.mmv.org/mmv-open/pathogen-box/about-pathogen-box.

Appendix E shows extracts from the anti-*M. tuberculosis* activity data of the PBCs (the full data are available at the website link shown above with Fig. 2.6).

The average MIC90 of the compounds against both replicating and non-replicating forms of *M. tuberculosis* range from < 0.01 to >100 µM. Some of the compounds in the PBC have also

shown activity against other pathogens: helminths (n=32 compounds), toxoplasmosis (n=15 compounds), dengue (n=15 compounds), and cryptosporidium (n=11 compounds) (Duffy *et al.*, 2017). However, activity against resistant forms of certain key pathogens, such as *M. tuberculosis* and *N. gonorrhoeae,* has not been sufficiently explored. Screening, and identifying new drug agents against pathogens with limited treatment options, other than the malaria parasite, have since been undertaken.

Following the discovery of the 116 compounds with activity in a primary screen, few studies have explored the activity of individual compounds against *M. tuberculosis* strains. Based on **Appendix E**, showing the cellular targets of MMV PBCs against *M. tuberculosis*, the following five compounds have been chosen as candidates for further exploration in this study: MMV153413, MMV676603, MMV687146, MMV687696 and MMV687180.

Inhibitors of novel cellular target of *M. tuberculosis* including proteasome (Tyagi *et al*., 2020), ketol-acid reductoisomerase (Bayaraa *et al*., 2020), decaprenyl-phosphoryl-β-D-ribofuranose oxidoreductase DprE1 (Sukheja *et al*., 2017), cytochrome *bc1* complex (Pethe et al., 2013, Kang *et al*., 2014), demethylmenaquinone methyltransferase menG (Sukheja *et al*., 2017) and siderophore biosynthesis inhibitor (Gupte *et al.,* 2008) have been reported. These compounds were highly effective against both susceptible and multidrug-resistant *M. tuberculosis* (**Appendix E**). Compound MMV687812 was active against both replicating and non-replicating *M. tuberculosis* by targeting specific GyrB ATPase (Shirude *et a*l., 2013). MMV090930 was also effective against susceptible, resistant, and persistent *M. tuberculosis* by targeting demethylmenaquinone methyltransferase menG required for respiration (Wang *et al*., 2013). There was a synergy between MMV687145 and isoniazid against *M. tuberculosis* (Abrahams *et al*., 2016). MMV687146 was superior to isoniazid in *in vitro* assay, coupled with low cytotoxicity in Vero cells (Pieroni *et al.,*2011). MMV687180 decreased colony forming units in a manner similar to moxifloxacin (Remuiñán *et al*.,2013). Both MMV687696 and MMV676603 are in clinical trials.

It is clear that other compounds which showed activity in primary screen against susceptible *M. tuberculosis* are likely to be active also in resistant strains because these drug molecules target novel cellular targets and resistance is often associated with mutations in target encoding genes (Gygli et al., 2017). Based on this, the five best compounds with low MIC from MMV primary screen against susceptible *M. bovis* BCG and *M. tuberculosis* H37Rv strains (Ballell et al., 2013) were selected for synergy and autophagy studies for *M. tuberculosis*.

The activity of the PBCs against other Gram-negative bacteria have been reported in few studies. MMV675968 (a diaminoquinazoline analog) was found to inhibit four *A. baumannii* strains at MIC ranging between 1.6 µM to 10 µM by targeting dihydrofolate reductase (Songsungthong *et al*., 2019). Similarly, MMV675968 demonstrated antimicrobial activity against *E. coli* strains by targeting dihydrofolate reductase (Sharma *et al*., 2022). The dual combination of MMV675968 with last resort antibiotics colistin and Meropenem exhibited a synergistic effect against strains of *E. coli*, including MDR strains (Sharma *et al*., 2022). In a screen of the PBCs against *V. cholerae*, two compounds MMV687807 and MMV675968 were found that effectively inhibit growth (Kim *et al*., 2021). RNA sequencing analysis revealed that these compounds affect multiple cellular functions including iron homeostasis, carbon metabolism, and biofilm formation (Kim *et al*., 2021).

During *in vitro* screening of the 400 PBCs to identify novel anti-staphylococcal compounds using both methicillin-resistant (ATCC 700699) and methicillin-sensitive (ATCC 292130) *S. aureus* strains, 13 compounds were found to show highly potent activity against both biofilm and planktonic state (Bhandari *et al.*, 2018). The MIC of these compounds ranged between 0.5 µM to 45.41 µM. Nine and 4 of the 13 compounds were already known to show activity against tuberculosis and kinetoplastids, respectively (Bhandari *et al.*, 2018).

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CHAPTER 3: Antimicrobial and synergistic effects of selected MMV Pathogen Box compounds on strains of *Mycobacterium tuberculosis*

Chapter 3 is formatted according to the editorial style of the Journal of Antimicrobial Chemotherapy.

3.1 Introductory note

Objectives addressed	To confirm the identity, antimicrobial susceptibility testing and resistance mechanisms of *Mycobacterium tuberculosis* using GeneXpert/MTBDR*plus assays.* To determine the pathogen-specific MICs and MBCs of selected drug molecules in the Pathogen Box for resistant strains of *Mycobacterium tuberculosis* by using a broth dilution method. To define lead compounds from the Pathogen Box with antibiotic activity against *Mycobacterium tuberculosis* based on low MIC and MBC. To profile pathogen-specific *in vitro* kill kinetics of selected PBCs against *Mycobacterium tuberculosis.*
Publication status	Ready for submission
Authors	Eric Mensah, Remco P. H. Peters & P. Bernard Fourie
Journal	Journal of Antimicrobial Chemotherapy
Publisher	Oxford University Press

3.2 Abstract

Background: Identification of new agents and therapeutic options to treat patients with *Mycobacterium tuberculosis* is a globally recognised high priority.

Objective: We explored the antibiotic potential, including synergistic effects, of Medicines for Malaria Ventures (MMV) Pathogen Box compounds (PBCs) against strains of *M. tuberculosis* using a standard *in vitro* approach.

***Methods**:* GeneXpert MTB/RIF and MTBDR*plus* assays were used to confirm the identity and wild-type or resistance profiles of the reference strain (MTB H37Rv 25618), one fully susceptible clinical isolate, and one MDR-TB isolate of *M. tuberculosis*. Five MMV PBCs, namely MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 were selected from the supplied set of 116 compounds, based on low minimum inhibitory concentration (MIC) and cellular target. Broth microdilution assay was used to determine the pathogen-specific minimum MIC and minimum bactericidal concentration (MBC) of the PBCs against strains of *M. tuberculosis*. A checkerboard assay was used to determine synergistic inhibitory effects of the five compounds, or of individual compounds used in combination with isoniazid and rifampicin. Time-kill kinetics was performed to determine bactericidal or bacteriostatic activity.

***Results**:* Compounds MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 showed activity against *M. tuberculosis* strains, with MIC and MBC values well below 0.625 µM. All the PBCs were bactericidal, except MMV687696. Synergistic or additive activity in combination with isoniazid and rifampicin was shown, with fractional inhibitory concentration indexes ranging between 0.2 and 2.6.

***Conclusions**:* Five selected MMV PBCs individually showed promising bactericidal activity against *M. tuberculosis* strains, as well as synergistic and additive antimicrobial properties in combination with rifampicin and isoniazid.

Keywords**:** *Mycobacterium tuberculosis*, MMV Pathogen Box, *in vitro* bactericidal activity, drug development.

3.3 Background

Despite notable advances in diagnostic and chemotherapy options to detect and treat tuberculosis, *Mycobacterium tuberculosis* (Mtb) continues to infect and kill millions of people globally.[1,2]. Furthermore, drug-resistant TB (DR-TB) jeopardises TB control programme efforts.[3,4]. Often the result of ineffective drug regimens or inappropriate treatment practices, multidrug-resistant TB (MDR-TB) adds to the mortality burden through increased risk of incurable disease, permanent disability or death.[1,2,5]. In this context, the World Health Organization (WHO) has designated *M. tuberculosis* as a critical priority pathogen to advance the search for and development of new drugs.[6,7].

To support discovery and development of novel drugs, the Medicines for Malaria Venture

(MMV) group developed the Pathogen Box, a collection of 400 drug compounds that demonstrated biological activity in a screen that was initially directed at protozoal parasites responsible for tropical diseases, especially malaria.[8,9] Most compounds are novel synthetic chemicals that were initially selected from a screen of ~4 million chemicals on basis of their low toxicity to mammalian cells. These compounds have diverse chemical scaffolds distinct from presently available antibiotics. Each of the compounds has been evaluated for cytotoxicity and has shown values within levels considered acceptable for the initial drug discovery program. Besides malaria, these compounds have also demonstrated biological activity against other pathogenic organisms (http://www.pathogenbox.org/). Some of the compounds showed activity in a primary screen against susceptible *M. bovis* BCG and *M. tuberculosis* H37Rv strains using whole-cell phenotypic screening.[8,9,10] However, activity against resistant forms of *M. tuberculosis* has not been explored, providing an opportunity to screen and identify new drug molecules with activity against this pathogen.

Five Pathogen Box compounds (PBCs) were selected for this study, based on their low MIC values shown in the primary screens (https://www.mmv.org/mmv-open/pathogen-box/about-pathogen-box), and with novel cellular targets of interest.[8,9]

(1) Compound MMV676603 is a sulfur-containing compound belonging to the nitrobenzothiazinone class, that has demonstrated strong potency and specificity for mycobacteria by inhibiting the synthesis of *M. tuberculosis* arabinogalactan biosynthetic enzyme DprE1 (decaprenylphosphoryl-beta-D-ribose 2-epimerase).[11] DprE1 is an important enzyme of the cell wall assembly.[12,13]

(2) Compound MMV687696 belongs to a new class of compound, imidazopyridine amide, also known as telacebec (Q203), that has demonstrated antituberculosis activity by blocking the respiratory cytochrome *bc*1 complex, resulting in decreased intracellular ATP, the energy source of most cellular enzymes.[14]

(3) Compound MMV687146 is an indolecarboxamide derivative that has shown anti-TB activity by actively interacting with the active domain of *M. tuberculosis* proteasome.[15]

(4) Compound MMV153413 belongs to a new class of compound called thiophene. Thiophenes exert anti-TB activity by selectively inhibiting polyketide synthase 13 (Pks13), which subsequently inhibits mycolic acid biosynthesis leading to rapid mycobacterial cell death.[16]

(5) Even though the cellular target of compound MMV687180 is unknown, it inhibits *M.*

tuberculosis at a MIC of 0.2 µM (Table 3.2). These compounds were included for exploration of antimicrobial potential against wild-type or resistant strains of *M. tuberculosi*s, and for synergistic effects in combination with each other or with the first-line anti-TB drugs rifampicin and isoniazid.

3.4 Material and Methods

3.4.1 Chemicals, reagents, and preparation of Middlebrook 7H11 agar and 7H9 broth

Middlebrook 7H11 Agar Base and Middlebrook 7H9 Broth Base (BD Diagnostics, USA) were used to prepare the agar and broth, respectively. The Middle-brook agar and broth were supplemented with OADC (BD Diagnostics, USA) and glycerol (Merck KGaA, Darmstadt, Germany) according to the manufacturer's instructions. Isoniazid (Merck KGaA, Darmstadt, Germany) and rifampicin (Merck KGaA, Darmstadt, Germany) were used to determine the resistant profile of *M. tuberculosis*. The compounds and drugs were dissolved in 90% DMSO (Merck KGaA, Darmstadt, Germany) to prepare different concentrations. Rifampicin and isoniazid were dissolved in 90% DMSO and filtered sterilized with 0.22 µM GVS sterile membrane filter (Merck KGaA, Darmstadt, Germany) and stored at –80°C. PrestoBlue™ High Sensitivity (HS) Cell Viability Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as a growth indicator.

3.4.2 *M. tuberculosis* strains, antimicrobial susceptibility profile, and resistance mechanisms

Mycobacterium tuberculosis H37Rv ATCC was used as a fully susceptible reference strain. Two clinical strains of *M. tuberculosis* isolated from patients with active tuberculosis were collected. GeneXpert and MTBDRplus (Hain Lifescience, Germany) confirmed the identity and resistance profile of *M. tuberculosis* strains. The molecular line probe assay (LPA) (Hain Lifescience, Germany) was used to confirm mutations associated with resistance to isoniazid, rifampicin, fluoroquinolones, and second-line injectable antibiotics in an already known profile of *M. tuberculosis* strains.[17] The PCR primers shown in Sup table 3.S4 were used for the amplification of the MTB gene targets.

GeneXpert MTB/RIF and MTBDRplus assays confirmed that *M. tuberculosis* isolates were at least resistant to rifampicin and isoniazid, harboring *rpo*BMUT1(D516V) and *Kat*GMUT1(S315T1) mutations. Mutations associated with resistance to fluoroquinolones, kanamycin, capreomycin, and amikacin was not detected in any of the strains. Using a broth

dilution assay, a MIC of 30 µM for rifampicin and 100 µM for isoniazid were recorded for the above MDR *M. tuberculosis* isolate. **Table 3.1** shows the resistance profile of the *M. tuberculosis* isolates.

Table 3. 1: Resistance profile and mechanism of resistance of *M. tuberculosis* isolates

Isolate ID		Resistance profile	Mechanism of resistance
Ro	MTB H37Rv ATCC 25618	Susceptible	-
Clinical strain 1 (R1)	MDR-TB	Rifampicin resistant	rpoBWT3/ rpoBWT4 (absent), *rpo*BMUT1(D516V) present
		Isoniazid resistant	*kat*GWT (absent), *kat*GMUT1(S315T1) present
	Pre-XDR TB	Fluoroquinolones(susceptible) Kanamycin/Capreomycin/ amikacin (susceptible)	gyrAWT 1/2/3 present. gyrB WT present rrsWT1/2 present eis WT1/2/3 present
	XDR TB	Susceptible	-
Clinical strain 2 (R2)	Susceptible	Susceptible	-

Antimicrobial susceptibility profile of *M. tuberculosis* H37Rv ATCC 25618 and clinical strains isolated from patients with active tuberculosis. MTB, *M. tuberculosis*; MDR, multidrug-resistant; XDR, extensively drug-resistant. There was no indication of MDR, pre-XDR or XDR in susceptible strains.

3.4.3 Selection and preparation of PBCs

Five PBCs with low MIC and novel cellular targets from MMV primary screen against susceptible *M. bovis* BCG and *M. tuberculosis* H37Rv strain were selected. These are: MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413. The biological data and DMPK data with chemical structures can be found via the following link, covering the essential information on the compounds: https://www.mmv.org/mmv-open/pathogen-box/about-pathogen-box.

A 10 mM stock solution for each of the selected PBCs provided by Medicines for Malaria Venture was dissolved in DMSO (Merck KGaA, Darmstadt, Germany) and deionized distilled water to prepare stock solutions and stored at –80°C. The final concentration of DMSO (Merck KGaA, Darmstadt, Germany) in each assay well was always lower than 1%. The tolerance of mycobacteria at 1% DMSO (Merck KGaA, Darmstadt, Germany) was assessed, and this concentration of solvent did not affect the growth of bacteria.

3.4.4 Determination of MIC and MBC of PBCs by the broth dilution method

The MIC of the PBCs against strains of *M. tuberculosis* was determined using the microdilution

method, with modification by using PrestoBlue™ HS Cell Viability Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA). A two-fold serial dilution of the PBCs with concentration ranging from 0.00975 to 10 µM in a 96-well flat-bottom microtitre plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was used to investigate the pathogen-specific MIC of the five selected PBCs against strains of *M. tuberculosis*. The plates were sealed with paraffin (Sigma-Aldrich, Germany), placed in a biohazard bag (Lasec, South Africa), and incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 37°C.

After seven days of incubation, PrestoBlue™ HS Cell Viability Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used as a growth indicator. A change in colour from blue to red was an indication of bacterial growth. The lowest concentration with no colour change was recorded as the MIC. Subsequently, an aliquot was taken from MIC assays where there is no visible growth and plated on Middlebrook 7H11 agar (BD Diagnostics, USA) supplemented with OADC (BD Diagnostics, USA). The MBC was determined as the lowest concentration that produce a 99.9% (3-log) decrease in visible bacterial growth. The experiment was repeated three times for each of the five selected compounds.

3.4.5 Checkerboard-based determination of PBC synergies

The ability of the compounds to work in combination, and with two of the frontline anti-TB drugs, isoniazid, and rifampicin at lower concentrations was assessed as previously described.[18,19,20] In brief, a positive flag *M. tuberculosis* in MGIT was centrifuged (Labnet International Inc, USA) at 3 500 × *g* at 4°C and the supernatants were discarded. The pellets were washed twice and resuspended in Phosphate buffered saline (PBS) (Sigma-Aldrich, USA).

Bacterial inoculum (0.6 McFarland standard; ~1 x 10^7–10^8 CFU/mL) was prepared in PBS using DensiCHEK™ Plus instrument (bioMérieux SA, Marcy l'Etoile, France). Subsequently, the compounds and convectional drugs, rifampicin (Merck KGaA, Darmstadt, Germany), and isoniazid (Merck KGaA, Darmstadt, Germany) were added at different concentrations. A two-fold serial dilution of each compound or antibiotic (rifampicin or isoniazid) to at least double the MIC was distributed into each well of a 96-well microtitre plate to obtain varying concentrations.

One compound or antibiotic (rifampicin or isoniazid) of the combination was distributed in rows in ascending concentrations. The other compound or antibiotic (rifampicin or isoniazid)

was similarly distributed among the columns. Each of the wells contained unique combinations of concentrations. The bacterial inoculum was diluted in Middlebrook 7H9 broth until an inoculum of 5×10^5 CFU/mL was achieved.The 96-well flat-bottom microtitre plates were incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 37°C for 7 days. The fractional inhibitory concentration index (FICI) was calculated for each compound used at the given concentration. The FICI was calculated using the formula, FICI= (MIC of agent A in combination)/ (MIC of agent A alone) + (MIC of agent B in combination)/ (MIC of agent B alone). A FICI value of ≤ 0.5 was considered synergistic, between > 0.5 but ≤ 1.25 as an additive, ≤ 4 as indifference, and > 4.0 as antagonistic.[20] The experiment was repeated three times for each combination.

3.4.6 Time-kill kinetics

To determine if the five compounds that show activity against MDR *M. tuberculosis* are bactericidal or bacteriostatic antibacterial *in vitro*, a time-kill assay was performed as previously described.[21] *M. tuberculosis* culture solution (adjusted to ~5×10^6 CFU/mL) was inoculated in a Middlebrook 7H9 broth (BD Diagnostics, USA) containing 20 x MIC concentration of the compounds in 48-well flat-bottom microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and incubated at 37°C for a maximum of 14 days. The 20 x MIC is chosen to stimulate the exposure of the compounds to the *M. tuberculosis* that has a slow growth rate and exhibits a high degree of natural resistance.[22]

At time intervals of 3, 6, 10, and 14 days, colony counting was conducted by plating serial 10-fold dilutions (10^{-1}, 10^{-2}, 10^{-3},10^{-4}, and 10^{-5}) of the culture directly on Middle-brook 7H11 agar supplemented with OADC (BD Diagnostics, USA) and incubated at 37° C for least 21 days. Colonies were counted after incubation of the plates at 37°C for 3 to 4 weeks and viability was expressed in log CFU/mL. A colony counter (Lasec, South Africa) was used to determine the CFU/mL. A ≥ 3 log10 reduction within 14 days was designated as bactericidal and < 2 log reduction as bacteriostatic. The assay was repeated thrice. The number of colonies was counted, recorded in CFU/mL, and was used to generate a graph of log CFU/mL against time (days).

3.5 Results

3.5.1 Antimicrobial activity of PBCs against strains of *M. tuberculosis*

Table 3.2 shows the compound class/common name, molecular formula/ structure from MMV

as well as the mode of action of the selected MMV PBCs. These PBCs demonstrated potent activity against strains of *M. tuberculosis* at a low MIC and MBC ranging between 0.0095 to 0.625 µM. MMV676603, MMV687146, and MMV687696 recorded a MIC of < 0.1 µM. MMV687180 recorded a MIC of between 0.156 to 0.3125 µM, and MMV153413 recorded the highest MIC of 6.25 µM. The antimicrobial activity of these MMV PBCs was based on the inhibition of *M. tuberculosis* cytochrome bc_1 complex, proteasome, and cell wall biosynthesis as previously reported.[11,14,15,16] The MIC of MMV676603, MMV687146, and MMV687696 was however lower in susceptible *M. tuberculosis* H37Rv and clinical *M. tuberculosis* when compared to the MDR *M. tuberculosis* strain. The MIC of MMV687180 and MMV153413 was similar in susceptible and MDR *M. tuberculosis* strains.

3.5.2 Combination testing of PBCs with rifampicin and isoniazid against MDR *M. tuberculosis*

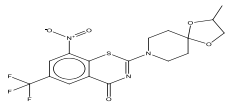
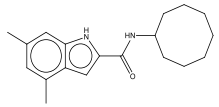
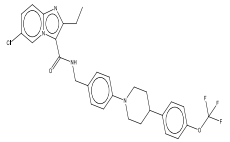
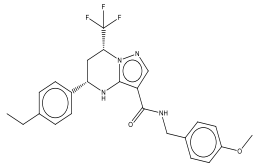
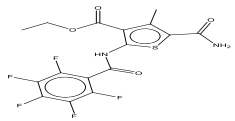
Among the five PBCs, there was either synergistic or additive activity against MDR *M. tuberculosis* with a fractional inhibitory concentration index ranging between 0.23 to 2.4 (**Table 3.3; Supplementary Table 3.S.1-3.S.3**).

The dual combination of rifampicin or isoniazid with each of the five selected PBCs resulted in a potent activity and produced a FICI between 0.2 to 2.6 against MDR *M. tuberculosis* (**Table 3.4; Table 3.5**). A combination of isoniazid with MMV676603 and isoniazid with MMV687696 reduced the MIC of both drug/compound by at least four-fold and produced a FICI of 0.41. The combination of isoniazid with MMV687146 reduced the MIC of both drug/compound by ~2 and produced a FICI of 1.14 (**Table 3.5**).

The most pronounced effect of the combination was demonstrated by the combination of rifampicin with MMV676603, and rifampicin with MMV687696, which reduced the MIC of rifampicin by ~ five-fold and ~ 10-fold, respectively, and produced a FICI of < 0.3. In these combinations, the MIC of both MMV676603 and MMV687696 were reduced by ~13-fold.

The combination of rifampicin with MMV687696 reduced the MIC of each drug/compound by ~2.5-fold and produced additive activity with a FICI of 0.74 (**Table 3.4**).

Table 3. 2: Pathogen-specific MIC and MBC of five selected PBCs against strains of *M. tuberculosis*

MMV ID	Compound class (Common name)	Molecular formula/ structure *	Mode of action	MTB H37Rv 25618 strain		Susceptible MTB clinical strain		MDR MTB clinical strain	
				MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)	MIC (µM)	MBC (µM)
MMV676603	Benzothiazinone	$C_{17}H_{16}N_3O_5F_3S$ 	Inhibit mycobacterial enzyme DprE1 (decaprenylphosphoryl-beta-D-ribose 2-epimerase), which subsequently inhibits cell wall arabinans	0.00976	0.00976	0.00976	0.00976	0.078125	0.15625
MMV687146	Indole-2-carboxamide	$C_{19}H_{26}N_2O$ 	Inhibit MTB proteasome.	0.0195	0.0195	0.0195	0.0195	0.0391	0.078125
MMV687696	Imidazopyridine amide (Telacebec) (Q203)	$C_{29}H_{28}ClF_3N_4O_2$ 	Inhibit MTB cytochrome bc_1 complex.	0.0391	0.3125	0.0195	0.3125	0.078125	0.3125
MMV687180		$C_{24}H_{25}N_4O_2F_3$ 	Unknown	0.3125	0.3125	0.15625	0.3125	0.15625	0.15625
MMV153413	Thiophene	$C_{16}H_{11}N_2O_4F_5S$ 	Polyketide synthase 13 (Pks13) inhibition, which subsequently inhibits MTB mycolic acid biosynthesis	0.3125	0.3125	0.625	0.625	0.625	0.625

Note: Profile of five MMV PBCs with potent activity against strains of *M. tuberculosis*. MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MTB, *M. tuberculosis*; *Information provided by Medicines for Malaria Venture.

Table 3. 3: Effects of combining MMV676603 with other drug compounds against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV676603	0.078125	0.03125	1.2	ADD		
MMV687146	0.039	0.03125				
MMV676603	0.078125	0.015625	0.4	SYN		
MMV687696	0.078125	0.015625				
MMV676603	0.078125	0.03125	0.625	ADD		
MMV687180	0.156	0.03125				
MMV676603	0.078125	0.015625	0.225	SYN		
MMV153413	0.625	0.015625				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - 4 as indifference. MMV676603 was combined with MMV687146, MMV687696, MMV687180, and MMV153413 at a concentration lower than their respective MIC.

Table 3. 4: Effect of combination of Rifampicin with the five PBCs against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
Rifampicin	30	6.25	0.29	SYN		
MMV676603	0.078125	0.00625				
Rifampicin	30	12.5	0.74	ADD		
MMV687146	0.0391	0.0125				
Rifampicin	30	3.125	0.2	SYN		
MMV687696	0.078125	0.00625				
Rifampicin	30	25	1.0	ADD		
MMV687180	0.156	0.025				
Rifampicin	30	30	1.8	IND		
MMV153413	0.625	0.50				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. Rifampicin was combined with MMV676603, MMV687146, MMV687996, MMV687180, and MMV153413 at a concentration lower than their respective MIC.

Table 3. 5: Effect of combination of Isoniazid with the five Pathogen Box compounds against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
Isoniazid	100	25	0.41	SYN		
MMV676603	0.078125	0.0125				
Isoniazid	100	50	1.14	ADD		
MMV687146	0.0391	0.025				
Isoniazid	100	25	0.41	SYN		
MMV687696	0.078125	0.0125				
Isoniazid	100	100	1.32	IND		
MMV687180	0.156	0.05				
Isoniazid	100	100	2.6	IND		
MMV153413	0.625	1				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. Isoniazid was combined with MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 at a concentration lower than their respective MIC.

3.5.3 Time-kill kinetics of Pathogen Box compounds against MDR *M. tuberculosis*

After confirming the activity of the five selected compounds in *M. tuberculosis* strains, the bactericidal activity of the compounds to kill MDR *M.* tuberculosis was evaluated using 20 x MIC. Bactericidal activity against MDR *M. tuberculosis* was defined as a 99.9 % reduction of the initial inoculum after 14 days of incubation with the selected compounds. As shown in **Figure 3.1**, all the compounds demonstrated bactericidal activity against clinical MDR *M. tuberculosis* except MMV687696 after duplicate measurements. The variance was limited in the repeated measurements and within the acceptable range. After 10 days, MMV676603, MMV687146, and MMV687180 showed rapid bactericidal activity. MMV153413 also demonstrated full bactericidal activity after 14 days.

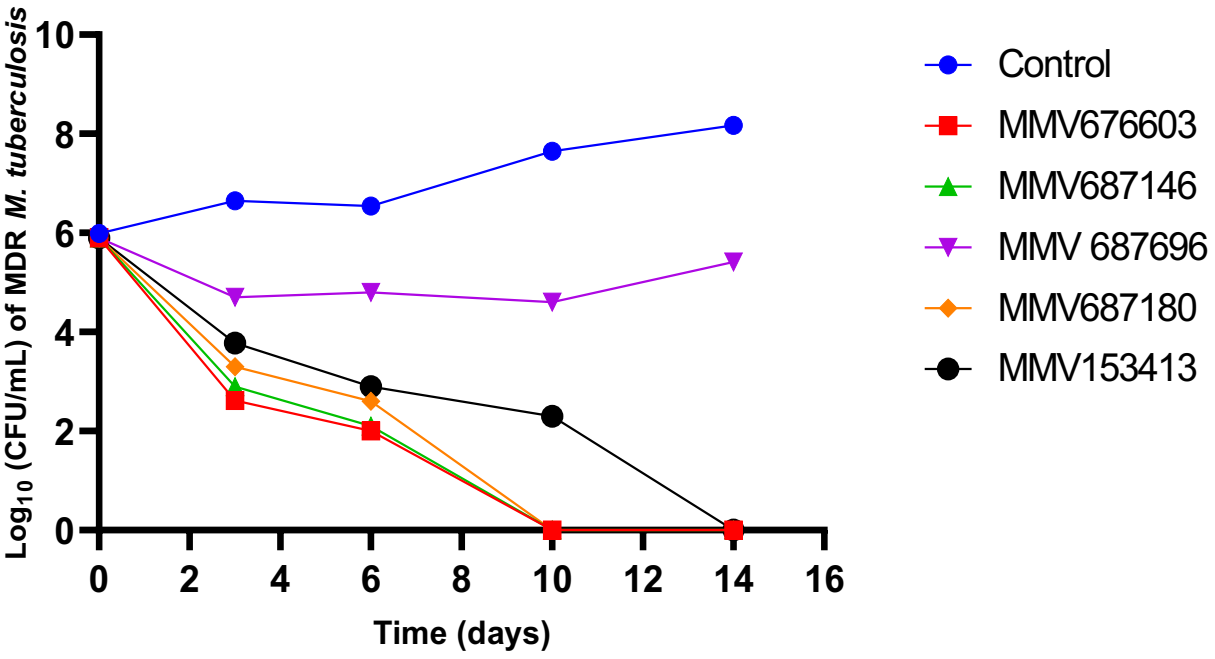


Figure 3. 1: Time-kill kinetics

(five selected PBCs at 20 x MIC against MDR M. tuberculosis over a 14-days incubation period at 37° C. DMSO was used as a negative control)

3.6 Discussion and conclusions

Mycobacterium tuberculosis is an established priority pathogen that is increasingly becoming difficult to treat due to the emergence of multi-/extensively drug-resistant strains.[2,5,23] *M. tuberculosis* is intrinsically resistant to different classes of antibiotics and can also evolve acquired resistance mutations to anti-TB drugs, making it difficult to treat TB infections.[4,24] Drug-resistant *M. tuberculosis* is a public health concern as it is difficult to cure, requiring lengthy treatment with potent drug combinations. Even though MDR *M. tuberculosis* strains have increased in recent decades, the rate at which new drugs are developed has decreased. The emergence of bedaquiline resistance threatens the effectiveness of novel treatment regimens for MDR-TB.[4,25,26] In the face of limited effective chemotherapy options against drug-resistant TB infections, the discovery, and development of highly effective new anti-TB drugs and combinations with novel cellular targets are desperately needed.

Compounds MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 showed potent activity against susceptible and MDR *M. tuberculosis* strains at a MIC and MBC

of between 0.00976 to 0.625 µM. The elevated MIC of these compounds in MDR *M. tuberculosis* stains compared to susceptible strains may be due to resistance mechanisms such as increased efflux of compounds out of the cell or reduced influx of compounds into the cell.[27] (Laws *et al*., 2022). MMV676603, MMV687146, and MMV687180 exhibited excellent *in vitro* sterilizing activity after 10 days and MMV153413 after 14 days against MDR *M. tuberculosis*, with no resistant mutants isolated at 20 x MIC. The bactericidal activity of these compounds is consistent with that reported in the previous studies.[11,16] All the PBCs were bactericidal except MMV687696 and were either synergistic or additive with fractional inhibitory concentration index ranging between 0.2 to 2.60. The synergistic and additive activity of the compounds with rifampicin and isoniazid suggest their potential inclusion in combination therapy for different forms of TB, including drug-resistant forms.

Inhibition of multiple components of the *M. tuberculosis* cell wall is an efficacious target for drug development. MMV676603 and MMV153413 inhibit the biosynthesis of *M. tuberculosis* cell wall arabinans and Pks13 which subsequently inhibits mycolic acid biosynthesis, respectively.[11,16] Arabinans and mycolic acid are important components of the *M. tuberculosis* cell wall.[24] Inhibition of these targets led to cell lysis and bacterial death, which account for the bactericidal and sterilizing ability of these compounds.[11,16] The use of a genome-scale CRISPRi chemical-genetic revealed that mutations in rv0678 conferred a low level of resistance to Benzothiazinone DprE1 inhibitors (MMV676603) in *M. tuberculosis.*[28] An F79S mutation in Pks13 conferred resistance in *M. tuberculosis*.[28] In resistant *M. tuberculosis*, MMV153413 inhibited mycolic acid biosynthesis in a much lesser extent than in wild-type *M. tuberculosis.*[16] Results from this current study confirmed the activity of these two compounds against *M. tuberculosis*. Additionally, the combination of MMV676603 with isoniazid and rifampicin in the present study resulted in a synergistic activity against MDR *M. tuberculosis*. MMV676603 was also found to kill *M. tuberculosis* in macrophages and the lung and spleen of BALB/c mice. Macrophages treated with MMV676603 were protected and even at the highest dose, there were no adverse anatomical, physiological, or behavioural effects in mice after a month.[11] This shows that these compounds have the potential to potentiate anti-TB drugs to treat MDR tuberculosis.

Compound MMV687146 was recently identified as a potential proteasome inhibitor against *M. tuberculosis.*[15] This compound inhibited *M. tuberculosis* by actively interacting with the active domain of the *M. tuberculosis* proteasome.[15] The activity of MMV687146 against *M.*

tuberculosis in our study is consistent with previous report.[15] MMV687146 demonstrated bactericidal activity after ten days and either additive or synergistic activity with the compounds tested, including isoniazid and rifampicin against MDR *M. tuberculosis*. Proteasomes are multi-subunit proteolytic complexes that play a vital role in various cellular functions.[29] Proteasomes function to degrade proteins in a regulated manner and are required to cause disease.[30] *M. tuberculosis* lacking the proteasome could not maintain infection in the TB mouse model.[31] This indicates that proteosomes are essential for the survival of *M. tuberculosis* in the host and are involved in the host immune system's resistance to nitric oxide stress.[32,33] Since the proteasome is vital to the survival and pathogenesis of *M. tuberculosis*, it is an attractive target for the development of agents that may inhibit *M. tuberculosis.*[34,35] Due to increasing resistance, drugs that can inhibit bacterial defenses against the host antimicrobial effectors such as nitric oxide could aid to win the battle against tuberculosis.

Compound MMV687696 (Q203) was potent against MDR *M. tuberculosis* and demonstrated synergistic activity with both isoniazid and rifampicin. This combination reduced the MIC of isoniazid and rifampicin by 4-fold and ~10-fold, respectively. MMV687696 was found to block the bacteria growth by targeting the respiratory cytochrome bc_1 complex.[14] From the time-kill curve, MMV687696 was slow acting, resulting in bacteriostatic activity against MDR *M. tuberculosis* after three weeks. Lu and colleagues (2018) reported similar time-kill kinetics at 30 x MIC. Inhibition of respiratory chain activity by MMV687696 (Q203) was found to be incomplete.[36] Genetic knock-out or inhibition of cytochrome *Bd* (a branch of the mycobacterial respiratory chain) with aurachin D invoked bactericidal activity of MMV687696 (Q203).[36] However, in a mouse model, MMV687696 reduced the bacterial burden of *M. tuberculosis* H37Rv by 90%, 99%, and 99.9% after treatment with 0.4, 2, and 10 mg respectively after four weeks.[14,37] Sequence analysis demonstrated that T313A and T313I mutations in *qcrB* were associated with resistance to MMV687696 (Q203).[35] The combination of Q203 with the efflux inhibitor, verapamil, increased the potency of Q203 against *M. tuberculosis* both *in vitro* and *ex vivo*, suggesting that efflux pumps are linked to the activity of MMV687696.[38] Further research on the antimicrobial activity of this compound against *M. tuberculosis* is imperative.

Although compound MMV687180 showed bactericidal activity against MDR *M. tuberculosis*, the mode of action is unknown. Two or more susceptible drugs, with potent bactericidal and sterilizing activity in combination therapy, are known to be capable of treating most cases of tuberculosis.[39,40]

Limitations: Only five compounds were selected from the supplied set of 116 compounds, based on low MIC and novel cellular target against *M. tuberculosis* H37Rv strain. The small amount of active ingredient available restricted the assessment of MIC, MBC, and synergies in triplicate and time-kill kinetics assessment in duplicate measurements. The variance was limited, however, in the repeated measurements and within the acceptable range. For the same reason, the potential emergence of resistance of the selected compounds against *M. tuberculosis* in the time-kill assay was not determined.

Conclusions: The study demonstrated that the five PBCs included in the study have potent activity against strains of *M. tuberculosis* at a low MIC and MBC. These compounds were bactericidal, with the potential to be synergistic or additive to current anti-TB regimens for tuberculosis.

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We declare no conflicts of interest.

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The authors have no conflict of interest to declare.

Transparency declarations

None to declare.

Ethics approval

Ethical approval was obtained from the Research Ethics Committee, Faculty of Health Sciences, University of Pretoria (Ethics Reference No.: 170/2021).

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Supplementary tables

Table 3.S. 1: Effect of combination of MMV687146 and other lead drugs against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV687146	0.039	0.015625	0.6	SYN		
MMV687696	0.078125	0.015625				
MMV687146	0.039	0.0625	2.4	IND		
MMV687180	0.078125	0.0625				
MMV687146	0.039	0.0625	1.7	IND		
MMV153413	0.625	0.0625				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - 4 as indifference. MMV687146 was combined with MMV687696, MMV687180, and MMV153413 at a concentration lower than their respective MIC.

Table 3.S. 2: Effect of combination of MMV687696 and other lead drugs against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV687696	0.078125	0.03125	0.8	ADD		
MMV687180	0.078125	0.03125				
MMV687696	0.078125	0.0625	0.9	ADD		
MMV153413	0.625	0.0625				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - 4 as indifference. MMV687696 was combined with MMV687180 and MMV153413 at a concentration lower than their respective MIC.

Table 3.S. 3: Effect of combination of MMV687180 and other lead drugs against MDR *M. tuberculosis*

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV687180	0.078125	0.0625	0.9	ADD		
MMV153413	0.625	0.0625				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - 4 as indifference. MMV687180 was combined with MMV153413 at a concentration lower than their respective MIC.

CHAPTER 4: Autophagic effects and intracellular control of *Mycobacterium tuberculosis* by selected MMV Pathogen Box compounds

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Chapter 4 is formatted according to the editorial style of Cell Host & Microbe.

4.1 Introductory note

Objectives addressed	To determine the host cell toxicity of selected Pathogen Box compounds (PBCs). To investigate the potential of selected Pathogen Box lead compounds against intracellular *Mycobacterium tuberculosis* for inducing/enhancing autophagy processes or potentiating existing drug actions directed at clearing host cells of the infecting organism using Western Blot analysis.
Publication status	In preparation
Authors	Eric Mensah, Iman Van Den Bout, Remco P. H. Peters & P. Bernard Fourie
Journal	Cell Host & Microbe
Publisher	Cell Press

4.2 Abstract

Background: Identification of novel agents that can induce autophagy in host cells directed against *Mycobacterium tuberculosis* (*Mtb*) is urgently needed to treat both susceptible and resistant forms of tuberculosis. This study explored the autophagic effect of five selected Pathogen Box lead compounds against *Mtb.*

Methods: PrestoBlue™ High Sensitivity Cell Viability Reagent was used to assess the viability of murine monocyte-derived macrophages (MDMs) after treatment with PBCs MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 for 48 hrs. Microplate reader was used to measure the fluorescence or absorbance. Western blotting assay was used to investigate the autophagic activity of these compounds and the effect on clearing *M.*

tuberculosis from host cells using the colony-forming unit count.

Results: Compared to control group, acceptable MDMs viability of 99.9%, 88.6%, 91.9% and 87.7% were respectively recorded after treatment with MMV676603 (1 µM), MMV687146(1 µM), MMV687696 (1 µM) and MMV687180 (2 µM). A reduced viability of MMV153413 was recorded after treatment with 5 µM. MMV676603, MMV687146, MMV687696 and MMV687180 induced LC3-II lipidation after treatment. A significant LC3II/LC3I ratio was recorded (p value = 0.02) after treatment with MMV687146, when compared to the control. *Mtb* infection had an effect on β-actin and suppressed its expression. Treatment with the PBCs further reduced the level of β-actin. There was significant intracellular suppression of *Mtb* in infected macrophages measured by colony- forming units count after treatment with MMV686603 (p = 0.0002), MMV687146 (p = 0.0015) and MMV687180 (p = 0.0011) when compared to the control group. Inhibition of autophagy flux with bafilomycin A1 significantly reversed the anti-mycobacterial activity of MMV676603 (p = 0.0019) and MMV687146 (p = 0.0235) but not MMV687180 (p = 0.19), MMV687696 (p = 0.91) and MMV153413 (p = 0.73).

Conclusions: The results demonstrated that the five selected PBCs have low cytotoxicity in murine-derived macrophages and effectively suppressed the growth of intracellular *M. tuberculosis*. The compounds induced LC3B lipidation and increased LC3II/LC3I ratio. Blocking of the autophagic flux significantly reversed the anti-mycobacterial activity of MMV676603 and MMV677146, suggesting autophagy as a possible mechanism in inhibiting intracellular growth of *M. tuberculosis*.

Keywords: Autophagy, autophagic flux, MMV Pathogen Box, *Mycobacterium tuberculosis*, intracellular killing, cell viability.

4.3 Background

Mycobacterium tuberculosis is a major intracellular pathogen of humans that kills millions of patients globally.[1,2,3]. Despite the multitude of effective host defense mechanisms deploy against *Mtb*, the bacterium manages to persist and actively replicates in phagocytic cells via a variety of escape pathways including prevention of phagosomal acidification,[4,5] inhibition of phagolysosome fusion,[6,7] and phagosomal rupture.[8,9] Due to this, agents that can boost our innate defenses or reprogram the immune cells, particularly macrophages to overcome the *Mtb* escape mechanisms are highly needed to improve the cure rate, reduce relapse, and combat

tuberculosis.[10,11,12]

Autophagy, an essential homeostatic intracellular control process that delivers damaged cytosolic components for lysosomal degradation is a key factor in antimicrobial defense mechanisms.[13,14,15] During this process, double membrane autophagosomes engulf cytosolic substrates such as *Mtb* and deliver them along microtubules to fuse with acidic lysosomes, resulting in the digestion of the enclosed content.[13] Autophagy facilitates the host response against the destruction of intracellular pathogens and connects the innate and adaptive immune function.[16,17] The role of some antibiotics in modulating the functions of immune cells via inducing or enhancing autophagy processes directed against intracellular pathogens such as *Mtb* is recognized.[18,19,20] Due to the rise in antibiotic resistance in *Mtb* to almost all drugs introduced for treatment, anti-Tb drugs become less effective, and TB is becoming difficult to treat.[3,21,22] Therefore, not only is it crucial to find new agents or antibiotic combinations with a novel mode of action against *Mtb*, but it is also vital to have an agent that can overcome pathogen-initiated autophagy inhibition and facilitate autophagosome-lysosome fusion within macrophages.[10,11,19,20] The induction of autophagy is promising since autophagy works at the host cellular level to enhance intracellular killing of both replicating and non-replicating pathogens, including MDR and XDR strains, where pathogen resistance is unlikely to evolve.[23]

Besides demonstrating activity against malaria parasites, the PBCs provided by MMV have also shown activity against other microorganisms, including *Mtb.* These compounds have cytotoxicity values within levels considered acceptable for the initial drug discovery program in human liver cells.[24,25,26] In our recent study, five MMV PBCs namely: MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 demonstrated potent activity against strains of *Mtb* at a low MIC and MBC of between 0.01 to 0.625 µM.[27] It was hypothesised that one or more of the lead PBCs may induce/enhance autophagy processes in clearing *M. tuberculosis* from host cells because of the promising *in vitro* activity of the selected compounds (refer Chapter 3). This study explored the autophagic effects of five selected MMV PBCs against *Mtb.*

4.4 Materials and Methods

4.4.1 Cell culture, reagents, and antibodies

Murine monocyte-derived macrophages (MDMs) was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Merck KGaA, Darmstadt, Germany)

supplemented with 10% fetal bovine serum (FBS) (Merck KGaA, Darmstadt, Germany) in a 5% CO_2 incubator (Stuart incubator Si500, Bibby Scientific Group, UK) at 37^{O}C. Bafilomycin A1(Merck KGaA, Darmstadt, Germany) and rapamycin (Merck KGaA, Darmstadt, Germany) were used to inhibit and induce autophagy, respectively. Anti-LC3B antibody (AB192890) (Biocom Diagnostic, South Africa) and anti-SQSTM1/ p62 antibody (P0067) (Biocom Diagnostic, South Africa) were used to monitor autophagy and autophagic flux. β-actin (Merck KGaA, Darmstadt, Germany) was used as a loading control.

4.4.2 Bacterial strain, and growth conditions

Mycobacterium tuberculosis H37Rv 25618 strain was used in this study. A positive flag for *Mtb* in MGIT was centrifuged (Labnet International Inc, USA) at 3500 × *g* for 15 min at 4°C and the supernatants discarded. The pellets were washed twice and resuspended in phosphate buffered saline (PBS) (Sigma-Aldrich, USA). Bacterial inoculum (0.6 McFarland standard; ~1 x 10^7–10^8 CFU/mL) was prepared in PBS using DensiCHEK™ Plus instrument (bioMérieux SA, Marcy l'Etoile, France). Subsequently, the inoculum was adjusted to ~5 × 10^6 CFU/mL in six-well plates containing MDMs. The plates were incubated in a 5% CO2 incubator (Stuart incubator Si500, Bibby Scientific Group, UK) at 37^{O}C.

4.4.3 Preparation of PBCs

MMV (https://www.mmv.org) provided the PBCs in 96-well plates, containing 10 µL of a 10 mM dimethyl sulfoxide (DMSO) solution of each compound. Five PBCs with novel cellular targets and low MIC from MMV primary screen against susceptible *M. bovis* BCG and *M. tuberculosis* H37Rv strain were selectd.[24,25,26] These were: MMV676603 (Pbc 1), MMV687146 (Pbc 2), MMV687696 (Pbc 3), MMV687180 (Pbc 4), and MMV153413 (Pbc 5). The biological data and DMPK data with chemical structures can be found via the following link, covering the essential information on the compounds: https://www.mmv.org/mmv-open/pathogen- box/about-pathogen-box. The compounds were diluted in 90% DMSO (Sigma Aldrich, USA) and deionized distilled water to create a stock solution of 1 mM and stored at -80°C. The final concentration of DMSO (Sigma Aldrich,USA) in all the assay wells was less than 0.5%.

4.4.4 Assessment of cell viability

Cell viability of MDMs after exposure to the selected PBCs was determined using

PrestoBlue^{TM} HS cell viability reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) as previously described.[28] Briefly, a 96-well microtitre plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was seeded with 1.0 X 10^4 MDMs and then treated with 1 µM of MMV676603 (Pbc 1), MMV687146 (Pbc 2), and MMV687696 (Pbc 3), 2 µM of MMV687180 (Pbc 4), and 5 µM of MMV153413 (Pbc 5) for 48 hours. At the end of the treatment, cell viability was assessed by adding PrestoBlue^{TM} HS cell viability reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) and examined using a microplate reader (Agilent BioTek Epoch, Santa Clara, CA, United States) with the excitation/emission wavelengths set at 560/590 nm. The percentage cell viability was determined from the following Equation:

$$\% \text{ cell viability} = \left(\frac{\text{Average OD of treated cells}}{\text{Average OD of control cells}}\right) x\ 100$$

4.4.5 Infection of macrophages, intracellular killing, and colony-forming unit assays

MDMs were infected with *Mtb* H37Rv at a multiplicity of infection (moi) at 10 for 2 hrs to allow phagocytosis. Extracellular or unbound bacteria were removed by washing with PBS (Merck KGaA, Darmstadt, Germany) twice and overlaid with DMEM/F-12 (Merck KGaA, Darmstadt, Germany) supplemented with 10% FBS (Merck KGaA, Darmstadt, Germany) containing the PBCs and incubated in a 5% CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 37^{O}C. Infected cells were treated for 24 and 72 hrs for autophagy induction and intracellular killing assay, respectively. To quantify the intracellular growth of *Mtb* H37Rv, the supernatant was aspirated, and the infected cells were treated with 1 X PBS containing 0.1% Triton X-100 (Merck KGaA, Darmstadt, Germany) to induce lysis of macrophages. Next, 10-fold serial dilutions of cell lysates were prepared and plated in duplicates on Middlebrook 7H11 agar (BD Diagnostics, USA) supplemented with 10% OADC (BD Diagnostics, USA). Plates were incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 37°C and colonies were counted after 3 weeks of incubation.

4.4.6 Western Blot analysis

The expression of key autophagy markers such as LC3B and p62 were quantified in the presence and absence of the PBCs according to the manufacturer's instruction. MDMs (1 × 10^5 cells) were cultured in 6-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany) and infected with *Mtb* at moi of 10 for 2 hrs as previously described,[18] following treatment with PBCs for 24 hrs. MDMs were either treated with 1 µM rapamycin (Merck KGaA, Darmstadt,

Germany) or starved, as a positive control for autophagy induction experiments. For starvation, MDMs were deprived of FBS (Merck KGaA, Darmstadt, Germany) for 24 hours. Bafilomycin (Merck KGaA, Darmstadt, Germany), a lysosomal protease inhibitor, was added in the presence and absence of the compounds to monitor autophagic flux. MDMs were harvested, and the protein extracted in 1 x NuPAGE™ LDS Sample buffer (Merck KGaA, Darmstadt, Germany), supplemented with β-mercaptoethanol (Merck KGaA, Darmstadt, Germany). The protein was resolved by SDS-PAGE (Thermo Fisher Scientific Inc., Waltham, MA, USA) alongside a protein standard marker (Bio-Rad). Resolved proteins were blotted onto PVDF membranes (Thermo Fisher Scientific Inc., Waltham, MA, USA) followed by blocking with PBS-T (Merck KGaA, Darmstadt, Germany) containing 2% bovine serum albumin (Merck KGaA, Darmstadt, Germany) for 1 h at 25°C. Next, the PVDF membrane (Thermo Fisher Scientific Inc., Waltham, MA, USA) was probed for LC3B (1: 1000 dilution) and p62/SQSTM1 (1:1000 dilution) for 1 hr at 25°C. The membrane was washed three times in 5 mL PBS-T and incubated with anti-rabbit IgG HRP-linked secondary antibody (1:5000) for 1 hr at 25°C. Protein bands were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) and exposure to CL Xposure film (Thermo Fisher Scientific Inc., Waltham, MA, USA). The band intensities were quantified using Azure Imager C400 (Azure Biosystems, Dublin, CA) and ImageJ software.

4.4.7 Statistical analysis

GraphPad Prism 9 software (GraphPad Software, Inc.) was used for statistical analysis. A two-tailed sample t-Test on mean values was used to compare the band intensity of LC3-II and p62, and the number of viable *Mtb* in the macrophages treated with the PBCs. Differences between groups were considered statistically significant when $P < 0.05$.

A two-sample t-Test on mean values was used to compare the band intensity of LC3-II and p62, and the number of viable *Mtb* in the macrophages treated with the PBCs.

4.5 Results

4.5.1 MDMs viability after exposure to PBCs

The cytotoxicity of the five PBCs in MDM'S was assessed after 48 hrs using a PrestoBlue^{TM} HS cell viability reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) as a growth indicator in three replicates. Compounds MMV676603 (Pbc 1), MMV687146 (Pbc 2), and

MMV687696 (Pbc 3) recorded low toxicity in MDM, with viability of 99.9%, 88.6%, and 91.9% respectively after treatment with 1 µM of each of the compounds when compared to untreated cells. At 2 µM concentration, MMV687180 (Pbc 4) recorded a viability of 87.68% after treatment; MMV153413 (Pbc 5) recorded the highest cytotoxicity, with a viability of 68.60% after treatment with 5µM (**Figure 4.S2**) when compared to untreated cells or control.

4.5.2 Effect of PBCs on autophagy-related proteins in MDMs

To determine whether the five selected PBCs induce or regulate autophagy, murine-derived macrophages were infected with *Mtb* and treated with and without each of the five selected PBCs and analysed for the expression levels of autophagy-associated proteins: LC3 and p62.

Compared to the *Mtb* control group, treatment with MMV6603 (Pbc 1), MMV687146 (Pbc 2), MMV687696 9 (Pbc 3) and MMV687180 (Pbc 4) induced lipidation of endogenous LC3-II (**Figures 4.1, 4.2, and 4.S1**). A significant LC3II/LC3I ratio was recorded (p = 0.02) after treatment with MMV687146, when compared to *Mtb* control (**Figure 4.3**).

Mycobacterium tuberculosis infection induced p62 formation. Treatment with MMV153413 (Pbc 5) further increased the level of p62. The addition of bafilomycin A1(Merck KGaA, Darmstadt, Germany) to treated cells increased the level of LC3-II, total LC3, and LC3-II/LC3-I (**Figures 4.1, 4.2, 4.3, 4.4 and 4.S.1**).

Protein p62 remained relatively the same after treatment with bafilomycin A1 (Merck KGaA, Darmstadt, Germany) (**Figure 4.5**).

Mycobacterium tuberculosis infection suppressed the expression level of β-actin. Treatment of *Mtb*-infected cells further suppressed β-actin formation (**Figure 4.6**).

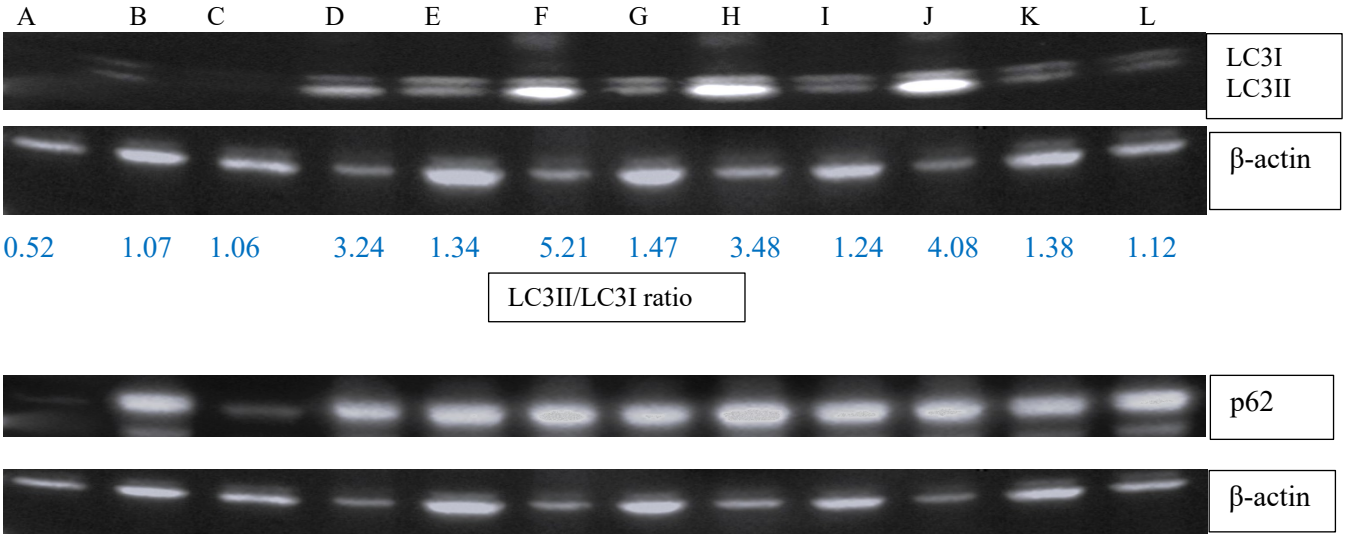

Figure 4. 1: Effects of PBCs on autophagy-related factors

Blot A. MDMs were infected with M. tuberculosis (moi = 10) for 2 hr. and incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM),) and Pbc 3 (1 µM) 2 µM of Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM) or rapamycin (1 µM) or starved. Cells were harvested and subjected to western blot analysis for LC3II, LC3I/II, and p62, and for β-actin as a loading control. The alphabets represent different treatment conditions. A-macrophages only, B-macrophages + Mtb, C-starved macrophages, D-macrophages + Mtb + Baf, E-macrophages + Mtb + Pbc 1, F-macrophages + Mtb + Pbc 1 + Baf, G-macrophages + Mtb + Pbc 2, H-macrophages + Mtb + Pbc 2 + Baf, I-macrophages + Mtb + Pbc 3, J-macrophages + Mtb + Pbc 3+ Baf, K-macrophages + Mtb + Pbc4, L-macrophages + Mtb + Pbc 5. Baf, bafilomycin A1; Mtb, M. tuberculosis

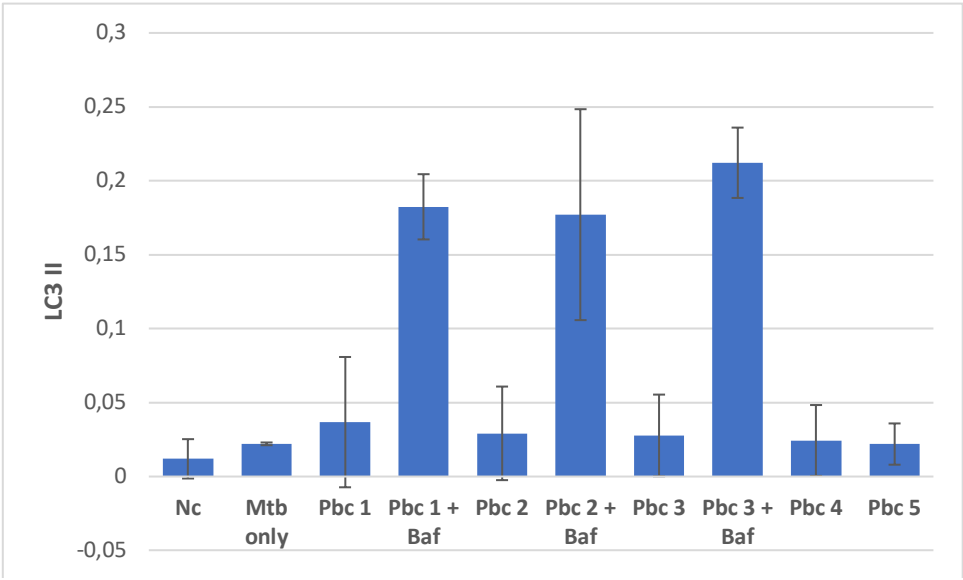

Figure 4. 2: Effect of PBCs on LC3II

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM) and Pbc 3 (1 µM), Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis for LC3II and for β-actin as a loading control. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis; Baf, bafilomycin

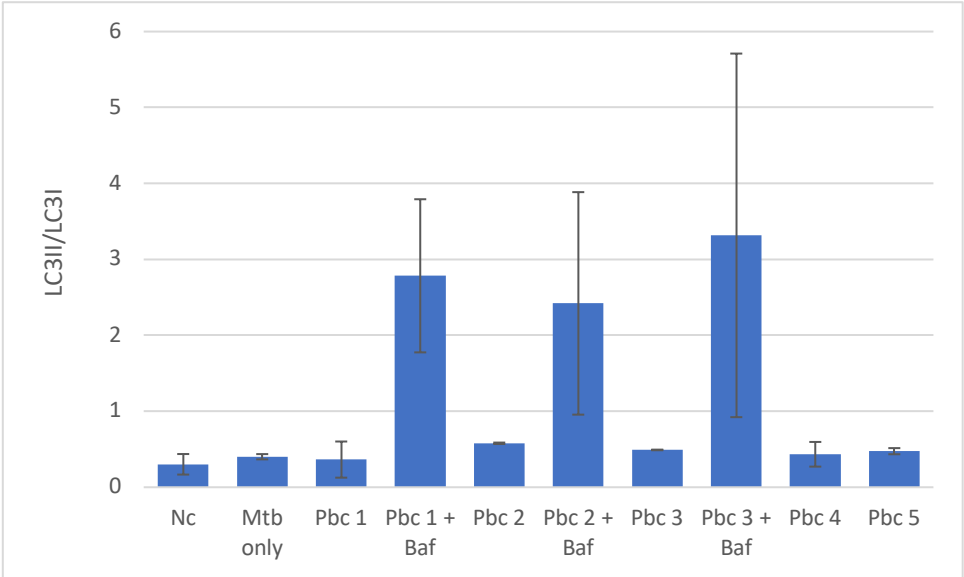

Figure 4. 3: Effect of PBCs on LC3II/LC3I

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM) and Pbc 3 (1 µM), Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis for LC3 II/I and for β-actin as a loading control. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis; Baf, bafilomycin.

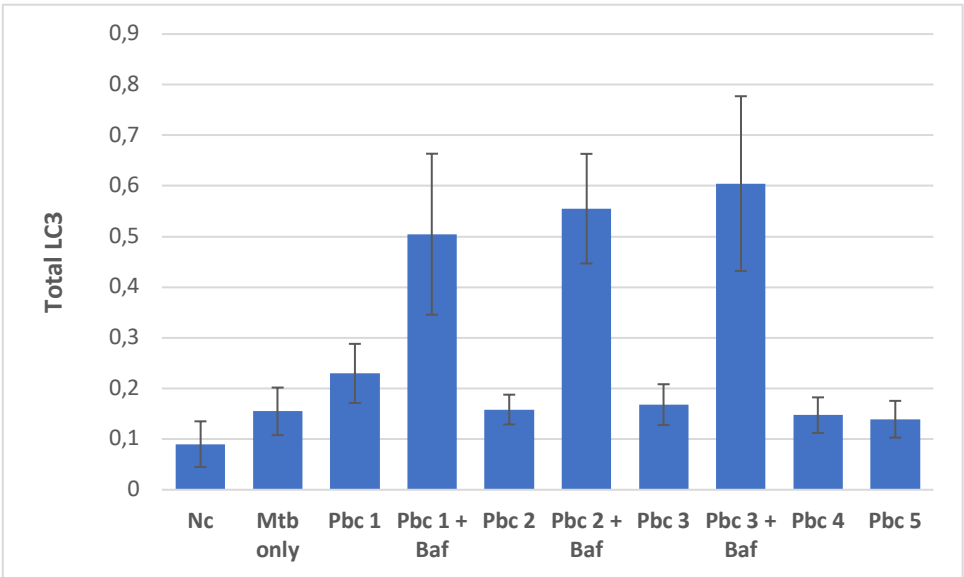

Figure 4. 4: Effect of PBCs on total LC3

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM) and Pbc 3 (1 µM) 2 µM of Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis for total LC3 and for β-actin as a loading control. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis; Baf, bafilomycin.

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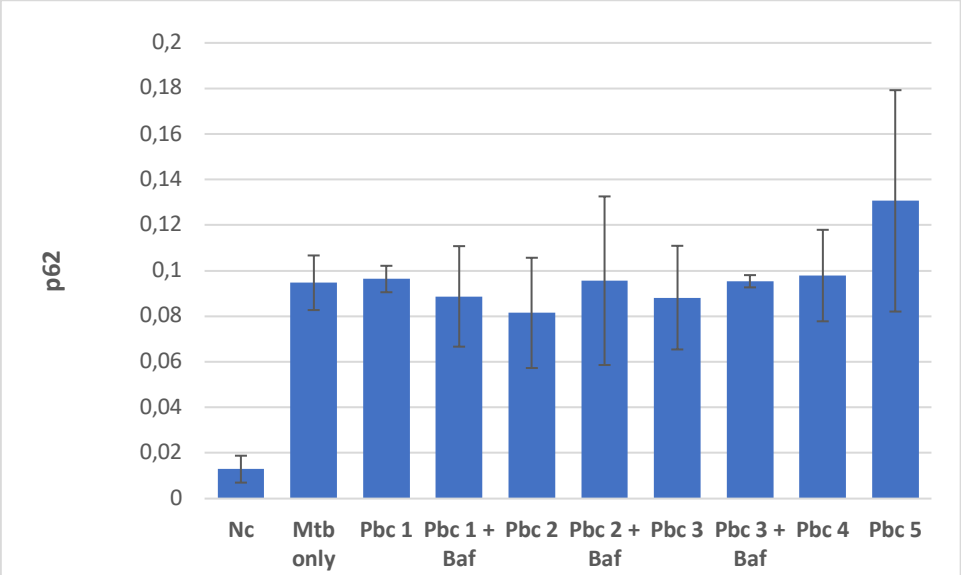

Figure 4. 5: Effect of PBCs on p62

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM),) and Pbc 3 (1 µM), Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis for p62 and for β-actin as a loading control. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Baf, bafilomycin; Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis.

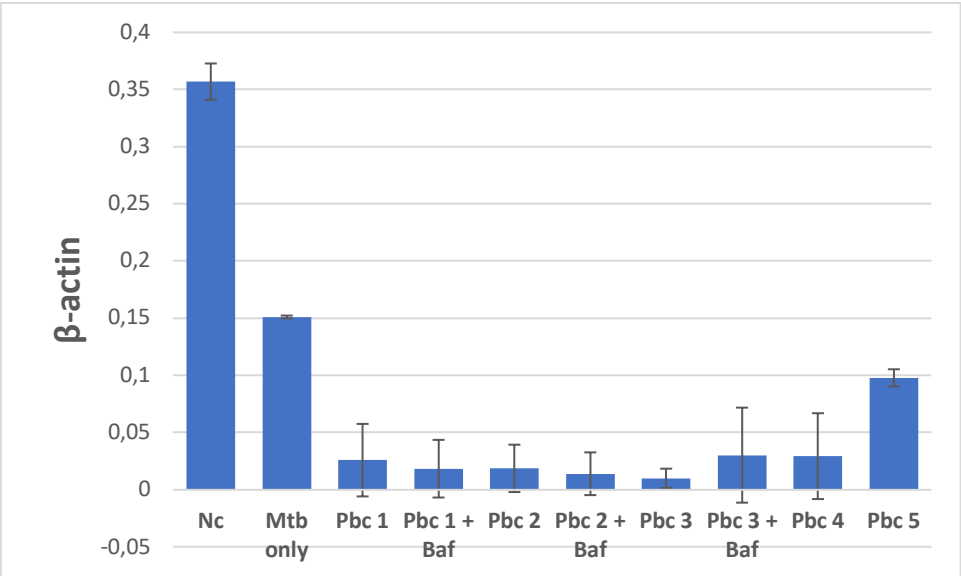

Figure 4. 6: Effect of five selected PBCs on β-actin

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 µM), Pbc 2 (1 µM), Pbc 3 (1 µM), Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Baf, bafilomycin; Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis.

4.5.3 Pathogen Box compounds suppress intracellular growth of *M. tuberculosis*

All five of the selected PBCs inhibited the growth of intracellular *Mtb*. There was a fast clearance of intracellular *M. tuberculosis* by MMV676603 and MMV687146, when compared to other treatment groups. A significant intracellular suppression of *M. tuberculosis* in infected macrophages after treatment with MMV686603 (p value = 0.0002), MMV687146 (P value = 0.0015) and MMV687180 (p value = 0.0011) was observed when compared to untreated control group (**Figure 4.7**). Inhibition of autophagy flux with bafilomycin A1 (Merck KGaA, Darmstadt, Germany) significantly reversed the anti-mycobacterial activity of MMV676603 (p = 0.002) and MMV687146 (p = 0.0235) but not MMV687180 (p = 0.19), MMV687696 (p = 0.91) and MMV153413 (p = 0.73).

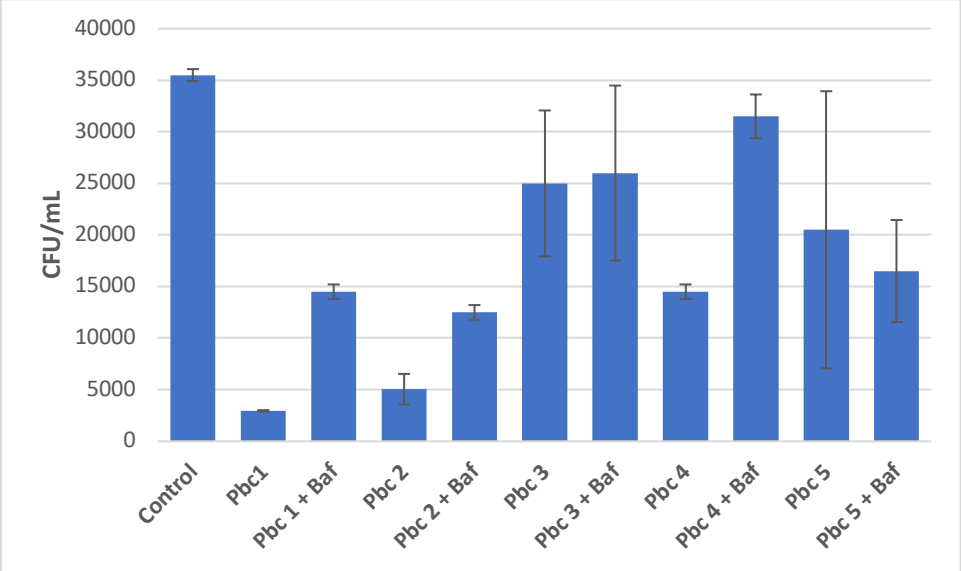

Figure 4. 7: PBCs suppress intracellular growth of *M. tuberculosis*

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with MMV676603 (1 µM), MMV687146 (1 µM), MMV687696 (1 µM), MMV687180 (2 µM) and MMV153413 (5 µM) for 72 hr. The cells were also treated with Baf-A (100 nM). After treatment, cells were lysed in 1 X PBS containing 0.1% Triton X-100 to induce lysis of macrophages. 10-fold serial dilutions of cell lysates were prepared and plated in duplicates on Middlebrook 7H11 agar supplemented with 10% OADC and incubated at 37 °C and colonies were counted after 3 weeks. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Baf, bafilomycin; Pbc, Pathogen Box compound; Control (untreated); Mtb, M. tuberculosis; Pbc 1, MMV676603; Pbc 2, MMV687146; Pbc 3, MMV687696; Pbc 4, MMV687180; Pbc 5, MMV153413.

4.6 Discussion

Mycobacterium tuberculosis has several mechanisms to escape the host immune antibacterial defense to survive and multiply within host cells.[5,7,8] The increasing emergence of Mtb resistant to existing anti-TB drugs highlights the need to develop new agents that have an impact on cellular functions, besides their bactericidal activity.[3,21,22] There is an urgent need for new treatment agents and therapeutic options for TB, including host-directed therapy.[10,11] There is increasing demand for agents that can induce autophagy to reprogram immune cells to become more phagocytic against intracellular *Mtb*, where pathogen resistance is unlikely to develop.[23] The autophagic effect of five selected PBCs against *Mtb* was explored to identify novel therapeutics for TB. Compared to the control, the PBCs induced LC3-II lipidation and increased LC3II/LC3I ratio, resulting in the suppression of intracellular *Mtb*. Inhibition of autophagic flux with bafilomycin A1 reversed the anti-mycobacterial activity of the compounds.

The PBCs demonstrated low cytotoxity in murine-derived macrophages. MMV676603 (1 µM), MMV687146 (1 µM), MMV687696 (1 µM), and MMV687180 (2 µM) recorded low toxicity in MDMs, with viability of 99.86%, 88.59%, 91.89% and 87.68% respectively following treatment. MMV153413 (5 µM) recorded the least viability of 68.60% after treatment. Compounds with high viability and low cytotoxicity effects are acceptable and useful as promising compound for development as anti-TB drug. However, compounds with high cytotoxic effect such as MMV153413 (Pbc 5) might be lethal and not useful in human. MMV recorded $Hep2_{CC20}$ of > 80 µM for MMV676603 (Pbc 1) and MMV687146 (Pbc 2). However, $Hep2CC_{20}$ ranging between 1.69 to 14.1 was reported for MMV687696 (Pbc 3), MMV687180 (Pbc 4) and MMV153413. The low cytotoxicity of MMV676603 (Pbc 1) and MMV687146 (Pbc 2), and the high cytotoxicity of MMV153413 was consistent with our study. Compounds with high antimycobacterial activity and low cytotoxicity with high protection index are of great interest as a hit compound for drug development as a new antitubercular agents.[29,30,31,32] Cytotoxic effects of anti-TB drugs are lethal in humans and causes adverse events such as bone marrow suppression and peripheral neuropathy and are often associated with poor adherence leading to the emergence of drug-resistant TB.[33,34]

Our results demonstrated endogenous LC3-II lipidation by MMV6603 (Pbc 1), MMV687146 (Pbc 2), MMV687696 9 (Pbc 3) and MMV687180 (Pbc 4); and a significant LC3II/LC3I ratio (p value = 0.02) after treatment with MMV687146 (Pbc 2), when compared to *Mtb* control

which may contribute to the anti-mycobacterial activity of the compound in macrophages. In this study, MDMs were infected with *Mtb* for two hours and treated with PBCs for 24 hrs. The results showed that incubation with *Mtb* increases p62 expression. Treatment with Pbc 5 further increased p62 expression. It was observed that *Mtb* infection had an effect on β-actin and suppressed its expression. Treatment with the PBCs further reduced the level of β-actin. The addition of bafilomycin to treated cells increased the level of LC3-II, total LC3, and LC3-II/LC3-I. The amount of LC3-II directly *correlated* with the number of autophagosomes.[35,36] The high LC3-II and significant LC3II/LC3I ratio after treatment with MMV676603 and MMV687146 ($p = 0.020$) shows that during autophagy, cytoplasmic components such as *Mtb* are sequestered into autophagosomes which then fuse with lysosomes for degradation.[37] Autophagy-inducing drugs or compounds significantly induce LC3-II lipidation at concentrations ranging between 3.6 -10 μM.[18,19,38,39,40] The low concentration of Pathogen compounds used in this study may account for the insignificant lipidation of LC3II. Ibrutinib, bedaquiline, and vitamin D3 are known autophagy-inducing agents. Treatment of THP-1 cells with vitamin D3 significantly resulted in upregulation of p62.[39] However, ibrutinib and bedaquiline treatment decreased the level of p62.[19,40]

To accurately assess the relationship between the effect of PBCs on the growth of intracellular *M. tuberculosis*, and its potential role in inducing autophagy, we used bafilomycin A1, an autophagy inhibitor that prevents the fusion of autophagosomes and lysosomes to block autophagic flux induced by PBCs.[41] This resulted in an increased accumulation of LC3B-II, suggesting that the increased amount of LC3B induced by PBCs was not due to the inhibition of autophagic flow. Treatment with MMV676603 (Pbc 1) (p value =0.0002), MMV687146 (Pbc 2) (p =0.0015) and MMV687180 (Pbc4) (p =0.0011) significantly suppressed intracellular growth of *M. tuberculosis*. The addition of bafilomycin A1 reversed the ant-mycobacterial activity of MMV676603 (Pbc 1) and MMV687146 (Pbc 2), indicating that the effect on intracellular *M. tuberculosis* was dependent on its role in inducing autophagy. Drugs or compounds involved in the modulation of autophagy, particularly those involved in effective control of intracellular *M. tuberculosis* growth, have shown to be promising against the intracellular killing of both replicating and non-replicating pathogens, including MDR and XDR strains, where pathogen resistance is unlikely to emerge.[23]

It was observed that *Mtb* infection suppressed β-actin. Treatment with PBCs further decreased the level of β-actin. The actin cytoskeleton is an important protein framework that is involved

in cellular processes such as signaling, trafficking and motility; and is underlying the plasma membrane.[42,43] Destabilization of the actin cytoskeleton of macrophages with cytochalasin D resulted in the reduction of entry of mycobacteria. Dutta and colleagues (2022) observed a strong correlation between actin content and entry of mycobacteria into macrophages, suggesting the involvement of actin cytoskeleton in mycobacterial infection. Actin-mediated mycobacterial entry has been suggested as a potential target for future anti-TB therapeutics.[44] Further research on the effect of *Mtb* and PBCs on actin suppression is imperative.

Limitations: The following limitations potentially impacting the study findings are acknowledged. Measurements of autophagy related proteins (LC3-II, LC3II/LC3I and p62) and intracellular killing of *Mtb* by PBCs were limited to two replicates instead of three, because of the restricted amount of PBCs received from MMV. A third repeat would have strengthened the statistical power of the study. Similarly, not enough active ingredient was available to fully explore the potential of the five selected compounds for inducing LC3 puncta formation in treated macrophages using immune fluorescence laser confocal microscopy. Descriptions of the potential of these compounds to induce lysosomal biosynthesis and colocalization of autophagosomes (containing *Mtb*) and lysosomes were, therefore, of necessity limited to the investigations as described above for this study.

Conclusion: This study showed that the selected PBCs have low cytotoxicity in macrophages and effectively suppressed the growth of intracellular *Mtb.* Inhibition of the autophagic flux reversed the anti-mycobacterial activity of the compounds, suggesting its role in inducing autophagy against intracellular *Mtb* and warrant further research to confirm the autophagic activity of the MMV compounds explored in this study.

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Supplementary Material

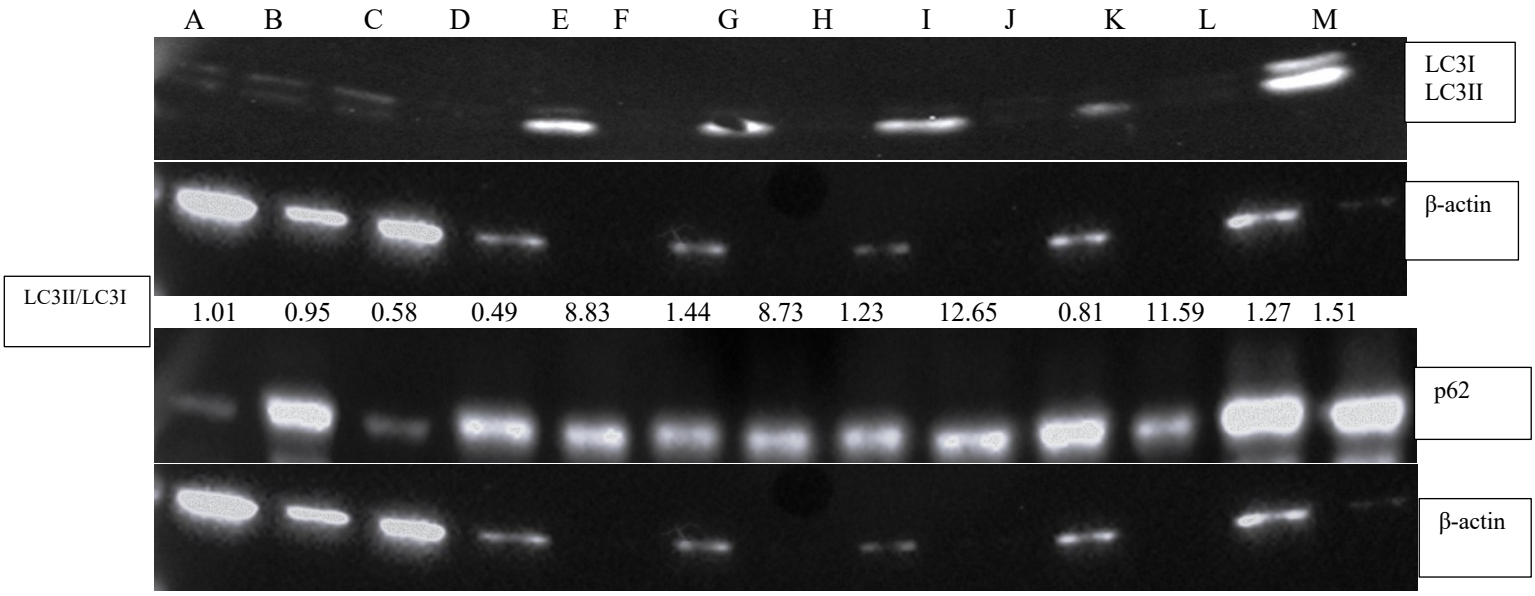


Figure 4.S. 1: ***Effect of PBCs on autophagy-related factors***

Blot B. MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with or without each of Pbc 1 (1 μM), Pbc 2 (1 μM), Pbc 3 (1 μM) 2 μM of Pbc 4 (2 μM) and Pbc 5 (5 μM) for 24 hr. The cells were also treated with Baf-A (100 nM) or rapamycin (1 μM) or starved. Cells were harvested and subjected to western blot analysis for LC3II, LC3I/II, and p62, and for β-actin as a loading control. The alphabets represent different treatment conditions. A-macrophages only, B-macrophages + Mtb, C-macrophages + rapamycin, D-macrophages + Mtb + Pbc 1, E-macrophages + Mtb + Pbc 1 + Baf, F-macrophages + Mtb + Pbc 2, G-macrophages + Mtb + Pbc 2 + Baf, H-macrophages + Mtb + Pbc 3, I-macrophages + Mtb + Pbc 3+ bafilomycin, J-macrophages + Mtb + Pbc4, K-macrophages + Mtb + Pbc 4 + Baf, L-macrophages + Mtb + Pbc5, M-macrophages + Mtb + Pbc5 + Baf. Baf, bafilomycin A1; Mtb, M. tuberculosis.

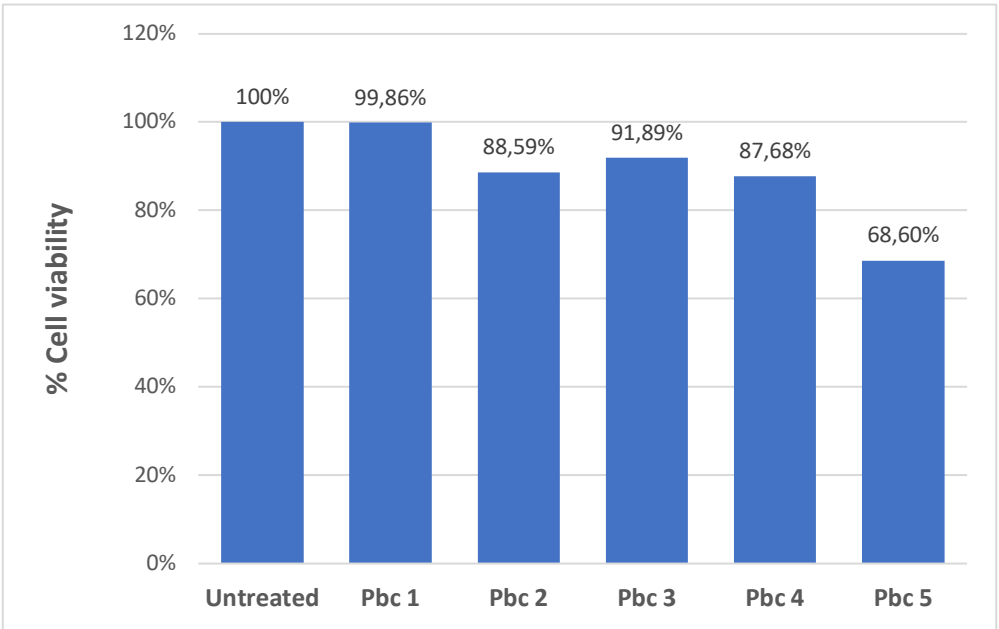
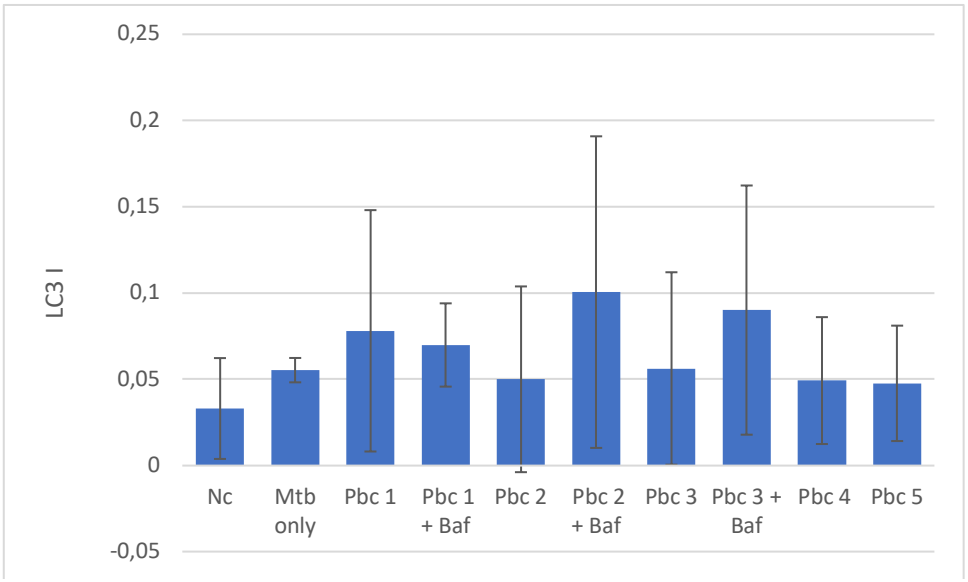
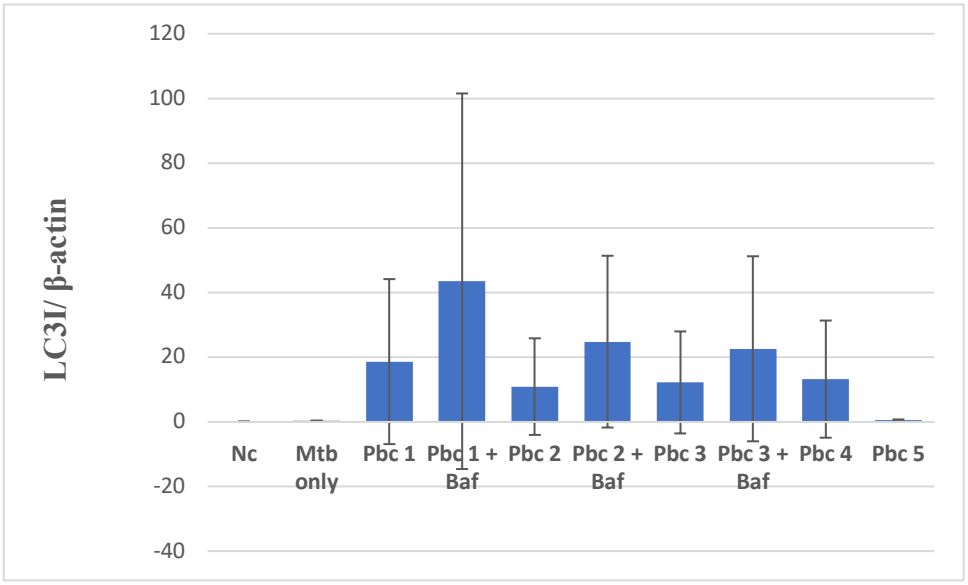


Figure 4.S. 2: ***Viability of five selected Box compounds in Murine derived macrophages***

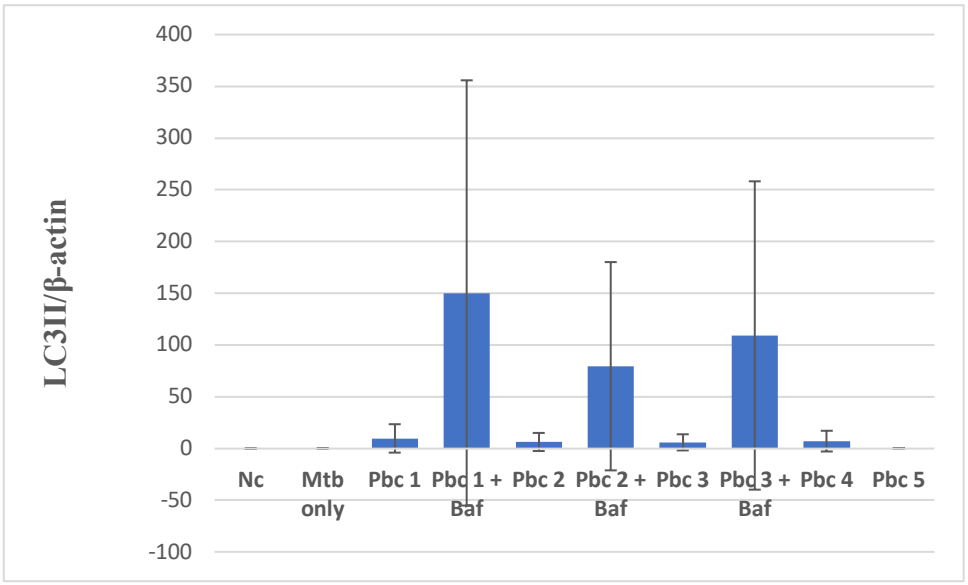
Treated with Pbc 1 (1 μM), Pbc 2 (1 μM), Pbc 3 (1 μM), Pbc 4 (2 μM) and Pbc 5 (5 μM) for 48 hr. The viability of macrophages post-treatment wasdetected using PrestoBlue HS cell viability reagent and examined using a microplate reader with the excitation/emission wavelengths set at 560/590 nm. Pbc 1, MMV676603; Pbc 2, MMV687146; Pbc 3, MMV687696; Pbc 4, MMV687180; Pbc 5, MMV153413.



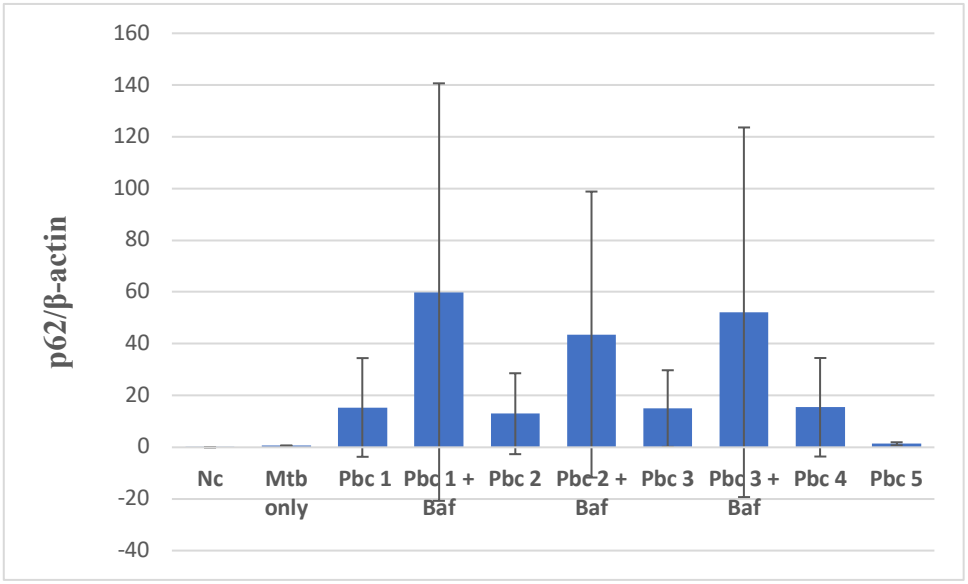
(A)



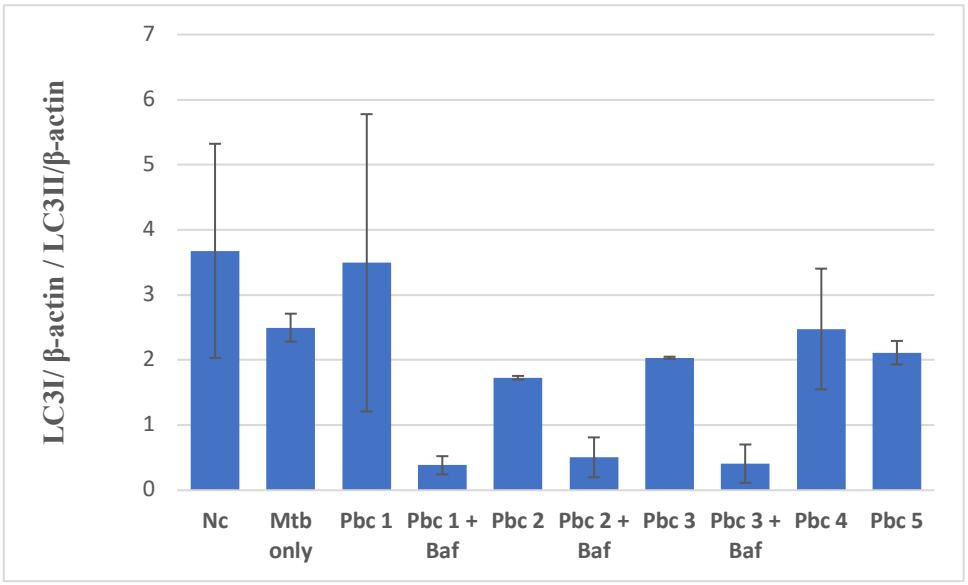
(B)



(C)



(D)



(E)

Figure 4.S. 3: ***Effect of PBCs on LC31(A), LC3I/β-actin (B), LC3II/ β-actin (C); p62/ β-actin (D), and LC3I/ β-actin / LC3II/β-actin***

MDMs were infected with M. tuberculosis (moi = 10) for 2 hr, incubated with Pbc 1 (1 µM), Pbc 2 (1 µM), Pbc 3 (1 µM), Pbc 4 (2 µM) and Pbc 5 (5 µM) for 24 hr. The cells were also treated with Baf-A (100 nM). Cells were harvested and subjected to western blot analysis. Differences were compared by Student's t -test. Data are expressed as the means ±standard error of the mean. Baf, bafilomycin; Pbc, Pathogen Box compound; Nc, negative control (untreated); Mtb, M. tuberculosis.

CHAPTER 5: Antimicrobial effects of Medicines for Malaria Venture Pathogen Box compounds on strains of *Neisseria gonorrhoeae*

Chapter 4 is formatted in the editorial style of Antimicrobial Agents and Chemotherapy.

5.1 Introductory note

Objectives addressed	To confirm the identity, antimicrobial susceptibility testing and resistance mechanisms of *N. gonorrhoeae* using API NH system, E-test, and whole genome sequencing. To determine the pathogen-specific MICs and MBCs of selected drug molecules in the Pathogen Box for resistant strains *Neisseria gonorrhoeae* by using a broth dilution method. To define lead compounds from the Pathogen Box with antibiotic activity against *N. gonorrhoeae* based on low MIC and MBC. To profile pathogen-specific in vitro kill kinetics of selected Pathogen Box compounds against *N. gonorrhoeae*.
Publication status	Revision submitted
Authors	Eric Mensah, P. Bernard Fourie & Remco P. H. Peters
Journal	Antimicrobial Agents and Chemotherapy
Publisher	Oxford Academic

.

5.2 Abstract

Background: Therapeutic options for *Neisseria gonorrhoeae* are limited due to emerging global resistance. New agents and treatment options to treat patients with susceptible and multi-extensively drug-resistant *N. gonorrhoeae* is a high priority.

Objective: This study used a *in vitro* approach to explore the antimicrobial potential, as well as synergistic effects of MMV PBCs against ATCC and clinical *N. gonorrhoeae* strains.

Methods: Micro broth dilution assay was used to determine pathogen-specific MIC and MBC of the PBCs against susceptible and resistant *N. gonorrhoeae* strains, with modification, by adding PrestoBlueTM HS Cell Viability Reagent. A checkerboard assay was used to determine synergy between the active compounds and in conjunction with ceftriaxone. Time-kill kinetics was performed to determine if the compounds were either bactericidal or bacteriostatic.

Results: The PBCs: MMV676501, MMV002817, MMV688327, MMV688508, MMV024937, MMV687798 (Levofloxacin), MMV021013, and MMV688978 (Auranofin) showed potent activity against resistant strains of *N. gonorrhoeae* at a MIC and MBC of ≤ 10 µM. Besides the eight compounds, MMV676388 and MMV272144 were active against susceptible *N. gonorrhoeae* strains, also at MIC and MBC of ≤ 10 µM. All the compounds were bactericidal and were either synergistic or additive with fractional inhibitory concentration index ranging between 0.40 to 1.8.

Conclusions: The study identified eight novel PBCs with potent activity against clinically susceptible and resistant strains of *N. gonorrhoeae,* having the potential to serve as primary or adjunctive therapy to treat gonococcal infections.

Keywords: *Neisseria gonorrhoeae*, novel compound, drug discovery, MMV Pathogen Box, *in vitro* bactericidal activity.

5.3 Background

Gonococcal infections caused by *Neisseria gonorrhoeae* are important sexually transmitted infections globally (1, 2). According to the World Health Organization (WHO), approximately 106 million new cases of gonococcal infections occur annually worldwide (3). Increasing evidence suggests that the actual number of these infections is under-reported because of inadequate clinical or diagnostic infrastructure, poor reporting systems, and high rates of asymptomatic infections (4-7). Without effective treatment, these infections can result in severe complications such as pelvic inflammatory disease, increased risk of tubal factor infertility, ectopic pregnancy and adverse pregnancy outcomes, and facilitate the transmission of the human immunodeficiency virus (HIV) (8-10).

The emergence of *N. gonorrhoeae* strains resistant to all drugs recommended for treatment,

including third-generation cephalosporins, azithromycin, fluoroquinolones, tetracyclines, and β-lactams, is a major public health concern (3,11,12,13). Fluoroquinolone and third-generation cephalosporin-resistant *N. gonorrhoeae is* designated as a high-priority pathogen (14). Dual therapy with ceftriaxone and azithromycin has been the mainstay for the treatment of gonococcal infections for the past decade (15-18). However, because of the potent anti-commensal activity of the dual therapy and increasing resistance to azithromycin, the USA and UK have removed azithromycin from the treatment regimen; ceftriaxone monotherapy is now recommended for treating *N. gonorrhoeae* infections (19). Emergence of infections with resistance to ceftriaxone is reported in different countries, making *N. gonorrhoeae* a superbug, requiring urgent development of new drugs and therapeutic options (10, 20-26). The continuous increase in antibiotic resistance, coupled with the limited pipeline and availability of new drugs has raised global concern about the emergence of untreatable gonorrhoea. No currently available vaccine to prevent infections highlights the critical need to develop new treatment agents, even for susceptible *N. gonorrhoeae*, since ceftriaxone is the only treatment option currently.

To accelerate the discovery of novel drug compounds, the Medicines for Malaria Venture (MMV) group has developed the Pathogen Box, a collection of 400 drugs that have demonstrated biological activity against specific pathogenic organisms in a screen that was initially mostly directed at protozoal parasites responsible for tropical diseases, in particular malaria (27). These 400 compounds are mostly novel synthetic chemicals that were initially selected from a screen of over 4 million chemicals due to their low toxicity to mammalian cells. Each of the compounds has been tested for cytotoxicity and has shown values within levels considered acceptable for an initial drug discovery program (28). The activity of the PBCs against some bacteria including *A. baumannii* (29) and *S. aureus* (30) has been reported. However, activity against resistant forms of several key pathogens, including *N. gonorrhoeae*, has not been explored. In this study, we conducted *in vitro* testing of the antibiotic potential, including synergistic effects of MMV PBCs against *N. gonorrhoeae* strains.

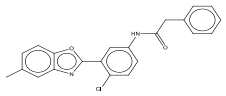
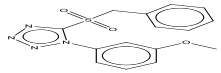
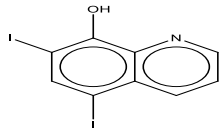
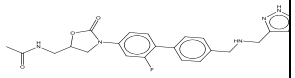
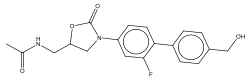
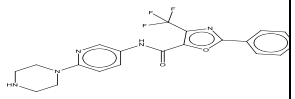
5.4 Results

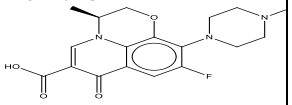
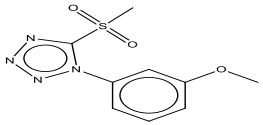
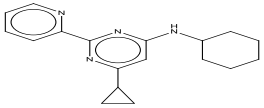
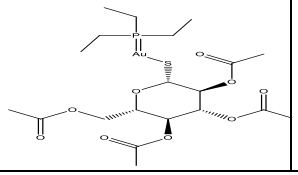
5.4.1 Testing for antimicrobial activity of PBCs against *N. gonorrhoeae* strains

Table 5.1 shows the pathogen-specific MIC and MBC of PBCs against *N. gonorrhoeae* strains. All the 400 drug molecules, including reference drugs, were screened against *N. gonorrhoeae*

isolates at 10 µM. After the primary screen, 25 compounds showed antibacterial activity to a different degree against *N. gonorrhoeae* isolates at 10 µM (**Figure 5.S.1**). Since the MIC of the most effective drug, ceftriaxone is ≤ 0.189 µM (≤ 0.125 µg/mL) (31), concentrations ranging between 0.156 to 10 µM was used to identify compounds with full antibacterial activity and determine the pathogen-specific MIC_{90} and MBC using a two-fold serial dilution broth microdilution (**Figure 5.S.2**). The activity of these compounds was repeated in triplicate. Eight compounds: MMV676501 (Pbc 1), MMV002817 (Pbc 3), MMV688327 (Pbc 4, Radezolid), MMV688508 (Pbc 5), MMV024937 (Pbc 6), MMV687798 (Pbc 7, Levofloxacin), MMV021013 (Pbc 9) and MMV688978 (Pbc 10, Auranofin) showed full antibacterial activity against the *N. gonorrhoeae* ATCC (Figure 5.S.3C) and clinical strains (**Figures 5.S.3A & 5.S.3B**). In addition, MMV676388 (Pbc 2) and MMV272144 (Pbc 8) showed full antibacterial activity against the *N. gonorrhoeae* ATCC 49266 strain but only partial antibacterial activity against the clinical strains. Among the ten drug compounds, two were reference drugs: MMV687798 (Pbc 7, Levofloxacin), and MMV688978 (Pbc 10, Auranofin). The remaining eight were novel compounds with potent antibacterial activity against *N. gonorrhoeae* strains. The MIC and MBC were much lower in the susceptible ATCC *N. gonorrhoeae* strains than in the clinical strains in most of the compounds (Table 5.2). The MIC_{90} of MMV676501 (Pbc 1) was 0.625 µM for all the isolates irrespective of the resistance profile. The MIC_{90} and MBC of MMV687798 (Pbc 7, Levofloxacin) were < 0.0195 µM in reference strain and 5 µM in clinical strain 1 (Ciprofloxacin MIC= 0.5 mg/liter) and 10 µM in clinical 2 (Ciprofloxacin MIC= 2 mg/liter). The MIC of MMV688978 (Pbc 10, Auranofin) (0.3125 µM), MMV002817 (Pbc 3) (2.5 µM), MMV688327 (Pbc 4, Radezolid) (2.5 µM), MMV024937 (Pbc 6) (10 µM), and MMV021013 (Pbc 9) (5 µM) were the same in both clinical strain 1 and clinical strain 2 (**Table 5.1**). The MBC was determined as the lowest concentration that produce a 99.9% (3Log) decrease in visible bacterial growth.

Table 5. 1: Pathogen-specific MIC and MBC of PBCs against susceptible and resistant *N. gonorrhoeae* strains

MMV ID	Compound class (Common name)	Molecular formula/structure *	Pathogen Box target*	Mode of action	HepG2 CC_{20} (µM) *	ATCC 49226		Clinical strain 1		Clinical strain 2	
						MIC_{90} (µM)	MBC (µM)	MIC_{90} (µM)	MBC (µM)	MIC_{90} (µM)	MBC (µM)
MMV676501 (Pbc 1)	-	$C_{11}H_5N_3O_2Cl_2S_2$ 	*M. tuberculosis*	unknown	2.64	0.625	0.625	0.625	1.25	0.625	1.25
MMV676388 (Pbc 2)	5-Sulfonyl tetrazole	$C_{15}H_{14}N_4O_3S$ 	*M. tuberculosis*	unknown	1.85	5	5	ǂ	ǂ	ǂ	ǂ
MMV002817 (Pbc 3)	Diiodohydroxyquinoline (Iodoquinol)	$C_9H_5NOI_2$ 	*Lymphatic filariasis-onchocerciasis*	Chelates ferrous ions required for amoebic metabolism	2.53	1.25	1. 25	2.5	2.5	2.5	2.5
MMV688327 (Pbc 4)	Oxazolidinone (Radezolid)	$C_{22}H_{23}N_6O_3F$ 	*M. tuberculosis*	Inhibit 50S ribosomal subunit during protein synthesis	8	0.3125	0.3125	2.5	2.5	2.5	2.5
MMV688508 (Pbc 5)	Oxazolidinone	$C_{19}H_{19}N_2O_4F$ 	*M. tuberculosis*	Inhibit 50S ribosomal subunit during protein synthesis	5.87	0.625	0.625	2.5	5	2.5	5
MMV024937 (Pbc 6)	-	$C_{20}H_{18}N_5O_2F_3$ 	*Plasmodium falciparium*	Unknown	13.4	5	5	10	10	10	10

MMV ID	Compound class (Common name)	Molecular formula/structure *	Pathogen Box target*	Mode of action	HepG2 CC_{20} (µM) *	ATCC 49226		Clinical strain 1		Clinical strain 2	
						MIC_{90} (µM)	MBC (µM)	MIC_{90} (µM)	MBC (µM)	MIC_{90} (µM)	MBC (µM)
MMV687798 (Pbc 7)	Quinolone (Levofloxacin)	$C_{18}H_{20}N_3O_4F$ 	Broad range spectrum	Inhibit bacterial DNA gyrase and topoisomerase IV.	>80	≤ 0.0195	≤ 0.0195	5	5	10	10
MMV272144 (Pbc 8)	Heteroaromatic Sulfones	$C_9H_{10}N_4O_3S$ 	*M. tuberculosis*	unknown	0.764	10	10	ǂ	ǂ	ǂ	ǂ
MMV021013 (Pbc 9)	2-Pyridyl-4-aminopyrimidine	$C_{18}H_{22}N_4$ 	*M. tuberculosis*	unknown	0.629	1.25	1.25	5	5	5	5
MMV688978 (Pbc 10)	(Auranofin)	$C_{20}H_{34}AuO_9PS$ 	Rheumatoid arthritis drug recently repurposed against Amebiasis	Inhibits bacterial thioredoxin reductase.	1.74	0.156	0.156	0.3125	0.3125	0.3125	0.3125

Profile of ten PBCs with potent activity against *N. gonorrhoeae* strains at ≤ 10 µM. ǂ: Antibacterial activity to different degree at 10 µM; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration. NA, not applicable; * Information provided by Medicines for Malaria Venture.

5.4.2 Time-kill kinetics of PBCs against *N. gonorrhoeae*

After confirming the antibacterial activity of the ten PBCs with best MIC_{90} or MBCs against two *N. gonorrhoeae* clinical strains (**Table 5.1**), we selected eight (excluding Pbc 2 and Pbc 8) and examined whether these molecules (all at 3 x MIC) exhibit bacteriostatic or bactericidal activity via standard time-kill kinetic assay over 24 hrs. As presented in **Figure 5.1**, all eight compounds showed bactericidal activity against clinical strain 1, which was chosen because the strain showed high resistance to azithromycin. After 4 hrs, auranofin exhibited rapid bactericidal activity. Additionally, the time-kill curve of the combination of the four best compounds, and in conjunction with ceftriaxone found to be either synergistic or additive was also plotted (**Table 5.3 and 5.4; Figure 5.2**). The dual combinations demonstrated bactericidal activity over 24 hours. The compounds showed similar time-kill activity to ceftriaxone in our experimental design. After 6 hours, the combination of Pbc 6 and Pbc 10 rendered the culture sterile (**Fig 5.2**).

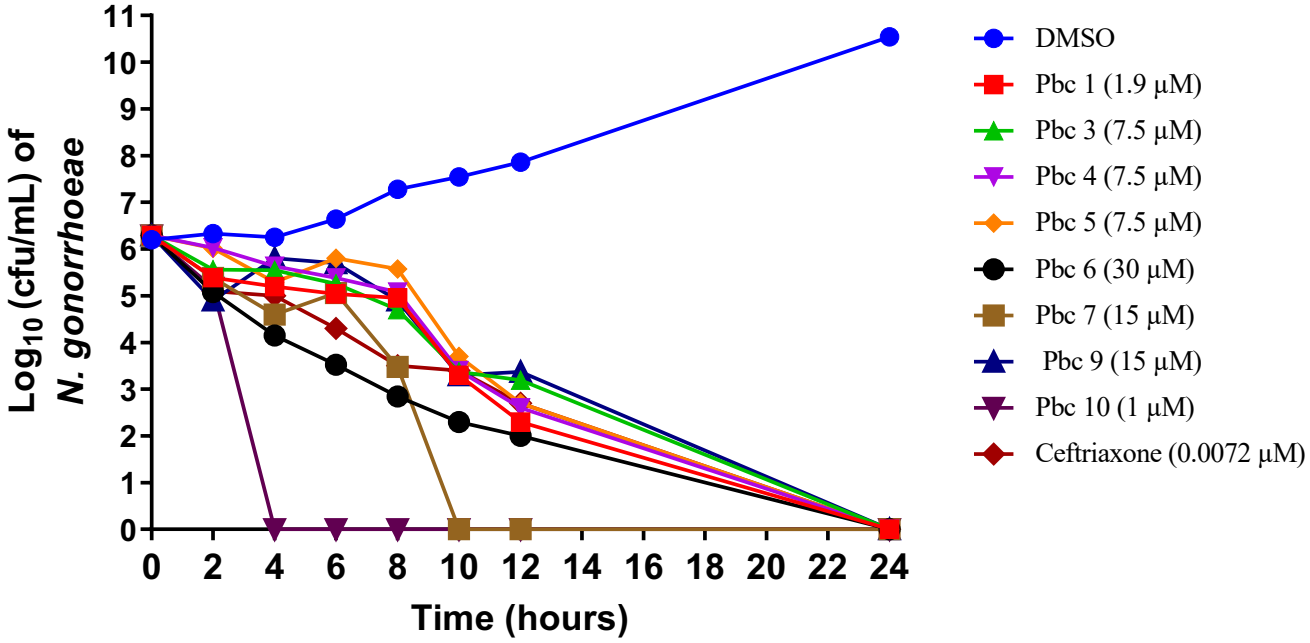


Figure 5. 1: Time-kill analysis of PBCs against *N. gonorrhoeae*

(at 3x MIC, over a 24-hour incubation period at 35° C. DMSO served as a negative control)

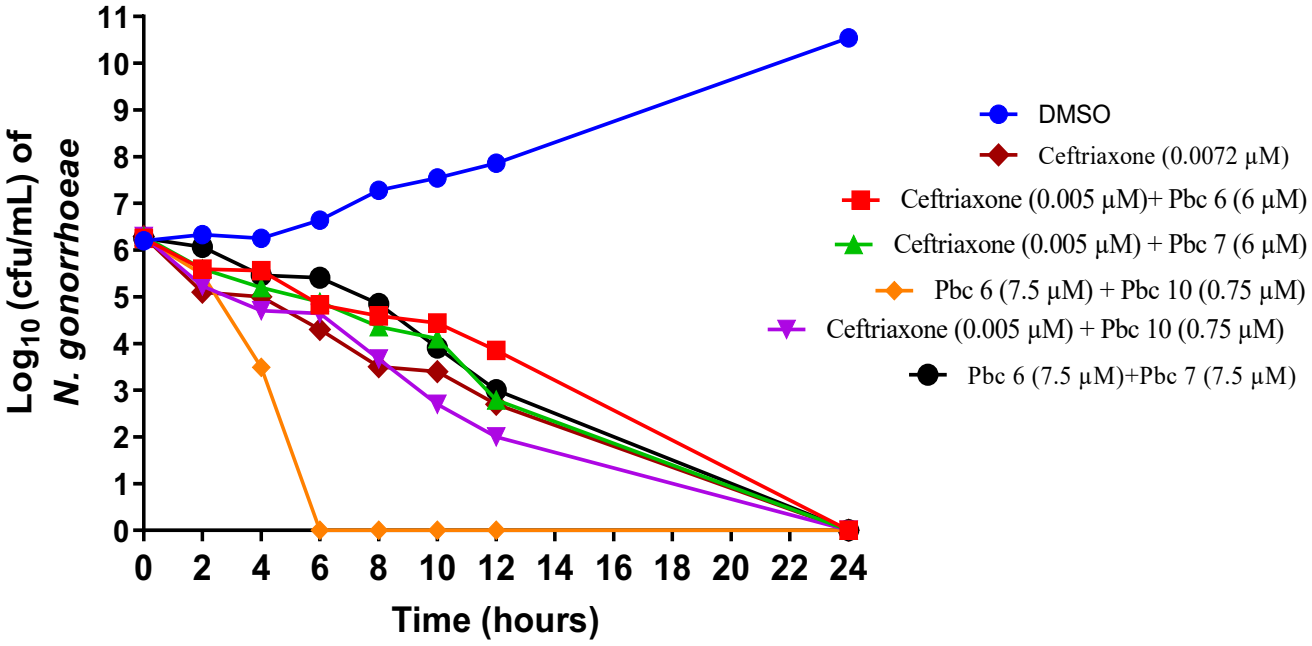


Figure 5. 2: Time-kill kinetics against *N. gonorrhoea* of the combination of the four best PBCs, individually and in combination with ceftriaxone

(at 3x MIC, over a 24-hour incubation period at 35°C. DMSO served as a negative control)

5.4.3 Combination testing of PBCs and ceftriaxone

The ability of the eight PBCs to work in combination individually and with ceftriaxone was investigated. Ceftriaxone was selected because it is the drug of choice for treating *N. gonorrhoea*. As presented in **Tables 5.2**, **5.3, and 5.S.1 to 5.S.7**, the compounds showed synergistic and additive activity against *N. gonorrhoeae* isolates. Fractional inhibitory concentration indices (FICIs) ranged from 0.45 to 1.8.

The four best PBCs: Pbc 1, Pbc 6, Pbc 7 (levofloxacin), and Pbc 10 (auranofin) were selected and each combined in pairs or with ceftriaxone (**Tables 5.2 and 5.3**).

There were additive effects between ceftriaxone and MMV024937 (Pbc 6), MMV687798 (Pbc 7 Levofloxacin) and MMV688978 (Pbc 10 Auranofin) (**Table 5.3**).

Table 5. 2: Effect of combining the four best PBCs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV676501 (Pbc 1)	0.625	0.5	1.3			IND
MMV024937 (Pbc 6)	10	5				
MMV676501 (Pbc 1)	0.625	0.5	1.8			IND
MMV687798 (Pbc 7, Levofloxacin)	5	5				
MMV676501 (Pbc 1)	0.625	0.5	1.6			IND
MMV688978 (Pbc 10, Auranofin)	0.3125	0.25				
MMV024937 (Pbc 6)	10	2.5	0.75		ADD	
MMV687798 (Pbc 7, Levofloxacin)	5	2.5				
MMV024937 (Pbc 6)	10	2.5	1.05		ADD	
MMV688978 (Pbc 10, Auranofin)	0.3125	0.25				
MMV687798 (Pbc 7,Levofloxacin)	5	2.5	1.3			IND
MMV688978 (Pbc 10, Auranofin)	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. The four best compounds: MMV676501, MMV024937, MMV687798, and MMV688978 were combined at a concentration lower than their respective MIC.

Table 5. 3: Effect of combining ceftriaxone with the four best PBCs individually

Test compounds	MIC in nM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
Ceftriaxone	2.44	0.156	1.44			
MMV676501 (Pbc 1)	625	500			IND	
Ceftriaxone	2.44	0.156	0.84			
MMV024937 (Pbc 6)	10 000	2 000			ADD	
Ceftriaxone	2.44	0.156	1.04			
MMV687798 (Pbc 7, Levofloxacin)	5000	2000			ADD	
Ceftriaxone	0.244	0.781	1.12			
MMV688978 (Pbc 10, Auranofin)	312.5	250			ADD	

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. Ceftriaxone was combined with MMV676501, MMV024937, MMV687798, and MMV688978 at a concentration lower than their respective MICs.

5.5 Discussion

Neisseria gonorrhoeae is one of the high-priority pathogens defined by the WHO, because of its exceptional capacity to acquire resistance to all antimicrobials used as first-line drugs for treatment (10, 14, 25). Even though *N. gonorrhoeae* continues to evolve resistance rapidly, the rate at which new antibiotics are discovered and developed has steadily decreased (32, 33). The emergence of multidrug-resistant and extensively drug-resistant strains has worsened the situation. The development of high-level resistance to last resort ceftriaxone, leading to untreatable gonorrhoea, coupled with the limited pipeline of new anti-gonococcal drugs highlights the urgent need to discover new antibacterial agents (6, 21, 33, 34). We screened the MMV PBCs that have previously demonstrated biological activity against specific pathogenic organisms to identify novel treatment options or drug molecules with activity against *N. gonorrhoeae* using an *in vitro* approach instead of *in silico* or mechanistic approach (35, 36, 37). We identified ten PBCs with potent activity against *N. gonorrhoeae* strains, with the potential for further research. Two of the compounds are reference drugs, auranofin, and

levofloxacin while the other eight are novel compounds.

Broth microdilution assay was used to explore the antimicrobial activity of MMV PBCs in two clinical strains, and a reference *N. gonorrhoeae* ATTC 49266 using an established broth. We used the micro broth dilution instead of agar dilution method as it is a convenient approach to MIC testing of *N. gonorrhoeae* (35, 38, 39, 40). Results of the microdilution method using broth have been reported as consistent with that of the agar dilution method (41). Eight compounds: MMV676501 (Pbc 1), MMV002817 (Pbc 3), MMV688327 (Pbc 4, Radezolid), MMV688508 (Pbc 5), MMV024937 (Pbc 6), MMV687798 (Pbc 7, Levofloxacin), MMV021013 (Pbc 9), and MMV688978 (Pbc 10, Auranofin) showed potent activity against two resistant strains of *N. gonorrhoeae* at MIC and MBC of ≤ 10 µM, and with similar time-kill activity as ceftriaxone. Besides these eight compounds, MMV676388 (Pbc 2) and MMV272144 (Pbc 8) showed sterilising activity against susceptible *N. gonorrhoeae* ATCC strains. The reference strains recorded lower MIC and MBC than the clinical strains. The resistant clinical strains have mutations such as GyrA, *tetM* and bla_{TEM} which might account for the high MIC of *N. gonorrhoeae* active MMV PBCs . All the eight compounds with activity against resistant *N. gonorrhoeae* strains were bactericidal with fractional inhibitory concentration index ranging between 0.40 to 1.8. Based on their MIC and time-kill kinetics, the four best compounds: MMV024937 (Pbc 6), MMV687798 (Pbc 7, Levofloxacin), and MMV688978 (Pbc 10, Auranofin), had an additive activity with ceftriaxone, suggesting their potential use as a combination therapy.

A limitation of this study was the restricted amount of compound supplied, preventing a higher number of replicate investigations. Assessment of MIC and MBC was done in triplicate while time kill kinetics were assessed in duplicate measurement (**Tables 5.S8 - 5.S12**). Variance in the repeated measurements was limited and within the acceptable range.

The finding of auranofin activity against *N. gonorrhoeae* in this study is consistent with the report of Elkashif and Seleem (2020), that found auranofin activity in MDR *N. gonorrhoeae* (35). After four hours, auranofin exhibited rapid killing and complete eradication of bacterial inoculum. There was a complete bactericidal activity and rapid eradication of a high bacterial inoculum after six hours with the combination of MMV688978 (Pbc 10, auranofin) and MMV024937 (Pbc 6). In a follow-up *in vivo* study by Elhassanny and colleagues (2022), auranofin was found to significantly reduce *N. gonorrhoeae* from the vagina of infected mice (42). Further research on auranofin as a potential drug, alone or in combination, against *N.*

gonorrhoeae is warranted.

Even though the two clinical strains were ciprofloxacin-resistant, levofloxacin (MMV687798, Pbc 7) demonstrated bactericidal activity against *N. gonorroheae* strains. Levofloxacin is more active than ciprofloxacin against several organisms including *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacteroides fragilis* and *Clostridium* species (43). Levofloxacin was highly active against several Gram-negative bacteria including *N. meningitidis, H. influenzae, Proteus* spp., *P. mirabilis, M. morganii and E. cloacae* (44). Consistent with the findings of the current study, *N. gonorrhoeae* was highly susceptible to levofloxacin at a concentration of less than or equal to 10µ M and showed additive effect with ceftriaxone and with other NG active PBCs.

This study reported the activity of eight novel compounds from the Pathogen Box with potent activity against *N. gonorrhoeae.* Six (MMV676501, MMV002817, MMV688327, MMV688508, MMV024937 and MMV021013) showed full activity in clinical strains. At 3x MIC, the drug molecules demonstrated bactericidal activity. MMV002817 (Pbc 3) and MMV024937 (Pbc 6) showed activity against Lymphatic filariasis-onchocerciasis and *Plasmodium falciparum*, respectively in primary screen (27, 28). The remaining compounds have shown activity against *M. tuberculosis*. The mechanism of action of MMV676501 (Pbc 1), MMV676388 (Pbc 2), MMV272144 (Pbc 8) and MMV021013 (Pbc 9) is unknown (27, 28, 45). It is possible that the compounds investigated in other organisms may result from differences in cellular targets that may be effective against resistant strains. Besides auranofin and levofloxacin, MMV024937 (Pbc 6) was one of the best compounds that rapidly eradicated a high *N. gonorrhoeae* bacterial inoculum (~1 x 10^6) even at 1 x MIC (**Figures 5.1 and 5.S.4**). After 6 hours, auranofin combined with MMV024937 (Pbc 6) completely eradicated the bacteria (**Figure 5.2**).

The activity of the *N. gonorrhoeae* active PBCs is unknown in other Gram-negative bacteria. Few of the other Pathogen Box compounds have demonstrated activity against Gram-negative bacteria. MMV675968 (a diaminoquinazoline analog) inhibited different strains of *A. baumannii*, *E. coli,* and *V. cholerae* (30, 46, 47). Also, MMV687807 effectively inhibited the growth of *V. cholerae* (47).

Based on the data provided by MMV, the cytotoxicity profile of the *N. gonorrhoeae* active Pathogen Box compounds in human liver cells is promising (**Table 5.1**). Drug metabolism and

pharmacokinetics (DMPK) of these compounds in mice have also been studied by MMV (https://www.mmv.org/mmv-open/pathogen-box/about-pathogen-box).

Since the pre-clinical pipeline remains largely empty of new agents that are likely to advance to development for gonorrhoea treatment (48), further studies on *N. gonorrhoeae* active compounds from MMV Pathogen Box is urgently needed. This study had a few limitations. First and foremost, an *in vitro* approach was used for initial testing instead of *in silico* or mechanistic approach to investigate the antimicrobial potential of 400 MMV Pathogen Box compounds against strains of *N. gonorrhoea* (36). Also, the potential emergence of resistance of the active PBCs against *N. gonorrhoeae* in the time-kill assay was not explored (37).

5.6 Conclusions

In conclusion, we reported ten PBCs including two reference drugs, auranofin, and levofloxacin, that have promising *in vitro* antibacterial activity against *N. gonorrhoeae* strains. The activity of MMV PBCs has not been explored for *N. gonorrhoeae* before. This study points to additional options for future anti-gonococcal drug research, in particular encouraging further investigations of the compounds for their therapeutic potential in primary or adjunctive treatment regimens for gonococcal infection.

5.7 Materials and Methods

5.7.1 *N. gonorrhoeae* strains, antimicrobial susceptibility testing, and resistance mechanisms

N. gonorrhoeae ATCC 49226 and ATCC 19424 reference strains (KWIK-STIK™ Plus, Microbiologics) were purchased and used for quality control. Stored clinical strain 1 (SRS5471848), and clinical strain 2 (SRS5471840), were used; these had been collected from symptomatic male patients in Johannesburg, South Africa between March 2018, and April 2019 (13). Urethral swabs (Copan Diagnostics, Italy) were collected and immediately inoculated on New York City (NYC) agar medium (Thermofisher Scientific, USA) and incubated at 35^0C in a 5% CO_2 incubator (Heracell™ VIOS 250i CO2 incubator, Thermofisher Scientific, USA) for 24 hours. Presumptive colonies were identified using Gram staining, a rapid oxidase test, and the API NH system (bioMérieux, France). This was followed by antibiotic susceptibility testing to azithromycin, ceftriaxone, cefixime, ciprofloxacin, penicillin G, and tetracycline using Etest (bioMérieux, France).

The MICs were interpreted using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints. In the case of azithromycin, the epidemiological cut-off (ECOFF) value of 1.0 mg/liter was used for interpretation (www.eucast.org). The whole genome sequencing (WGS) data for stored clinical strain 1 (SRS5471848), and clinical strain 2 (SRS5471840) have previously been published, providing information on the various resistant genes or mechanisms as applied to the reference agent (13). **Table 5.4** shows the antimicrobial profile and the mechanism of antibiotic resistance of the *N. gonorrhoeae* clinical isolates (13).

Table 5. 4: Antimicrobial susceptibility profiles and mechanisms of resistance of *N. gonorrhoeae* clinical isolates

Isolate (Accession number)	**MIC (mg/L) of antibiotics**						**Resistance associated mutations**
	Azithromycin (0.016 -256)‡	Ceftriaxone (0.016 -256) ‡	Cefixime (0.016 -256) ‡	Ciprofloxacin (0.002-32) ‡	Tetracycline (0.016 -256) ‡	Penicillin (0.016-256) ‡	
ATCC AT49266	0.032 (S)	<0.002 (S)	<0.016 (S)	<0.002 (S)	0.047(S)	<0.016 (S)	-
Clinical strain 1 (SRS5471848)	0.75 (S)	<0.002 (S)	<0.016 (S)	0.5 (R)	12 (R)	0.38 (R)	GyrA, *tetM*, *bla*TEM
Clinical strain 2 (SRS5471840)	0.38 (S)	0.002 (S)	<0.016 (S)	2 (R)	12 (R)	3 (R)	GyrA, *tetM*, *bla*TEM

Antimicrobial susceptibility profile of *N. gonorrhoeae* ATCC 49266 and clinical strains isolated from symptomatic male patients. S, susceptible; R, resistant. ‡ Range of antibiotics tested.

5.7.2 Chemicals and preparation of chocolate agar and liquid broth for *N. gonorrhoeae*

GC base medium (BD Difco, USA), BBL Haemoglobin Powder (Sigma-Aldrich, USA), and BBL IsovitaleX Enrichment (BD Difco, USA) was used to prepare chocolate agar. Brain Heart Infusion (Sigma-Aldrich, USA), BBL IsovitaleX Enrichment (BD Difco, USA), Agarose, and fetal bovine serum (Thermofisher Scientific, USA) were used to prepare the fastidious broth.

PrestoBlue™ High Sensitivity (HS) Cell Viability Reagent (Thermofisher Scientific, USA) was used as a growth indicator (49, 50). Brain heart infusion broth (37.0 g/L) (Merck, Germany) was supplemented with 5% fetal bovine serum (Merck, Germany), 1% IsovitaleX (gonococcal additive) (BD Difco, USA), and 1% agarose solution (0.75%) (Merck, Germany) to prepare the liquid broth for *N. gonorrhoeae*.

5.7.3 Establishment of liquid broth and cultivation of *N. gonorrhoeae*

The liquid broth was prepared and distributed into 96-well flat-bottom microtitre plates (Lasec, South Africa), followed by inoculation with *N. gonorrhoeae* isolates. After incubation (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 35°C under 5% CO2 for 24 h, *N. gonorrhoeae* multiplied and grew well in the established liquid broth (fastidious broth). PrestoBlue™ HS Cell Viability Reagent (Thermofisher Scientific, USA) indicated macroscopic growth, and colony count ranged from 1.0 x 10^4 to ~ 1.0x 10^7 and from 1.0 x 10^6 to ~ 1.0 x 10^{11} after 24 hrs.

5.7.4 Preparation of PBCs

MMV provided the Pathogen Box in 5 (A-E) 96-well plates, with each plate consisting of 80 10 µL of 10 mM compounds. The plate mapping of the 400 compounds included in the Pathogen Box, including the biological data and DMPK data with chemical structures (as SMILES or with illustrations of the structures), can be found via the following link, covering the essential information on the compounds: https://www.mmv.org/mmv-open/pathogen-box/about-pathogen-box. The compounds were prepared/diluted according to the manufacturer's instructions by dissolving the compounds in DMSO (Sigma Aldrich, USA) and deionized distilled water to create a stock solution of 1 mM and stored at -80°C. In assessing the activity of the compounds against *N. gonorrhoeae*, the final concentration of DMSO (Sigma Aldrich, USA) used in all the assay wells was below 1%. *N. gonorrhoeae* tolerance to 1% DMSO (Sigma Aldrich, USA) was examined, and this concentration of solvent did not affect the bacteria growth/viability.

5.7.5 Determination of MIC and MBC of PBCs by the broth dilution method

The MIC_{90} of each of the PBCs was determined using broth microdilution assay, as previously described (35, 38,39, 40, 41). All the 400 drug molecules including reference drugs were tested against Clinical strain 1 (SRS5471848) and Clinical strain 2 (SRS5471840) at 10 µM. After

the primary evaluation, compounds that showed partial to full inhibition were selected and repeated in triplicate in a 96-well flat-bottom microtitre plate (Lasec, South Africa) using a two-fold broth microdilution assay at a concentration ranging from 0.156 to10 µM to identify compounds with full activity and determine pathogen-specific MIC_{90} and MBC. These compounds were also tested against the susceptible *N. gonorrhoeae* ATCC 49266 strain. PrestoBlue™ HS Cell Viability Reagent (Thermofisher Scientific, USA) was used as a growth indicator as previously described (49, 50). A change in colour from blue to pink is an indication of bacterial growth. The lowest concentration with no colour change was recorded as the MIC_{90}. Subsequently, an aliquot was taken from MIC_{90} assays where there was no visible growth and plated on chocolate agar. The MBC was determined as the lowest concentration that produce a 99.9% (3Log) decrease in visible bacterial growth. The experiment was repeated three times for only compounds which show activity in the primary evaluation.

5.7.6 Checkerboard-based determination of PBC synergies

The ability of the compounds to work in combination, and in conjunction with conventional antibiotic ceftriaxone, used in the treatment of gonorrhoea at a reduced MIC was assessed as previously described (51, 52). An overnight *N. gonorrhoeae* inoculum (1.0 McFarland standard) was prepared in 0.85% (w/v) sodium chloride (NaCl) solution (Merck KGaA, Darmstadt, Germany) using DensiCHEK™ Plus instrument (bioMérieux SA, Marcy l'Etoile, France). Subsequently, the compounds and ceftriaxone (Merck KGaA, Darmstadt, Germany) were added at different concentrations. A two-fold serial dilution of each compound to at least double the MIC was distributed into each well of a 96-well microtitre plate to obtain varying concentrations. One compound of the combination was distributed in rows in ascending concentrations. The other compound was similarly distributed among the columns. Each of the wells contained unique combinations of concentrations of two compounds. The bacterial inoculum was diluted in the broth to an inoculum of 5×10^5 CFU/mL. The 96-well flat-bottom microtitre plates were incubated at 35°C for 24 hrs. in a 5% CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA). The fractional inhibitory concentration index (FICI) was calculated for each compound used at the given concentration. The FICI was calculated using the formula, FICI= (MIC of agent A in combination)/ (MIC of agent A alone) + (MIC of agent B in combination)/ (MIC of agent B alone). A FICI value of ≤ 0.5 was considered as synergistic activity, between > 0.5 but ≤ 1.25 as an additive activity, ≤ 4 as indifference, and > 4.0 as antagonistic activity (53, 54).

5.7.7 Time-kill kinetics 0.078125

To determine if the compounds that show activity against resistant *N. gonorrhoeae* strains are either bacteriostatic or bactericidal antibacterial *in vitro*, a time-kill assay was performed (55). Briefly, an overnight *N. gonorrhoeae* inoculum (1.0 McFarland standard) was prepared in 0.85% (w/v) sodium chloride (NaCl) solution (Merck KGaA, Darmstadt, Germany) using DensiCHEK™ Plus instrument (bioMérieux SA, Marcy l'Etoile, France) and diluted in the established fastidious broth containing 3 x MIC of the compound to an inoculum of ~5 × 10^6 CFU/mL. Azithromycin (0.2 µM) was used as a positive control and DMSO (0.5%) served as a negative control. At 0, 2, 4, 6, 8, 10, 12, and 24 hrs, an aliquot from each sample was serially diluted (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) and plated onto chocolate agar plates. Plates were incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) in 5% CO_2 for 24 h at 35°C to determine the CFU/mL. If the initial bacterial CFU/mL was reduced by at least 3 $\log_{10}$ over 24 hours, the test compound was considered bactericidal, and < 2log reduction bacteriostatic.

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We declare no conflicts of interest.

Transparency declarations

No declarations to record.

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Supplementary material

Figure 5.S. 1: Primary screen of PBCs against *N. gonorrhoeae* strains

(Plates A to E)

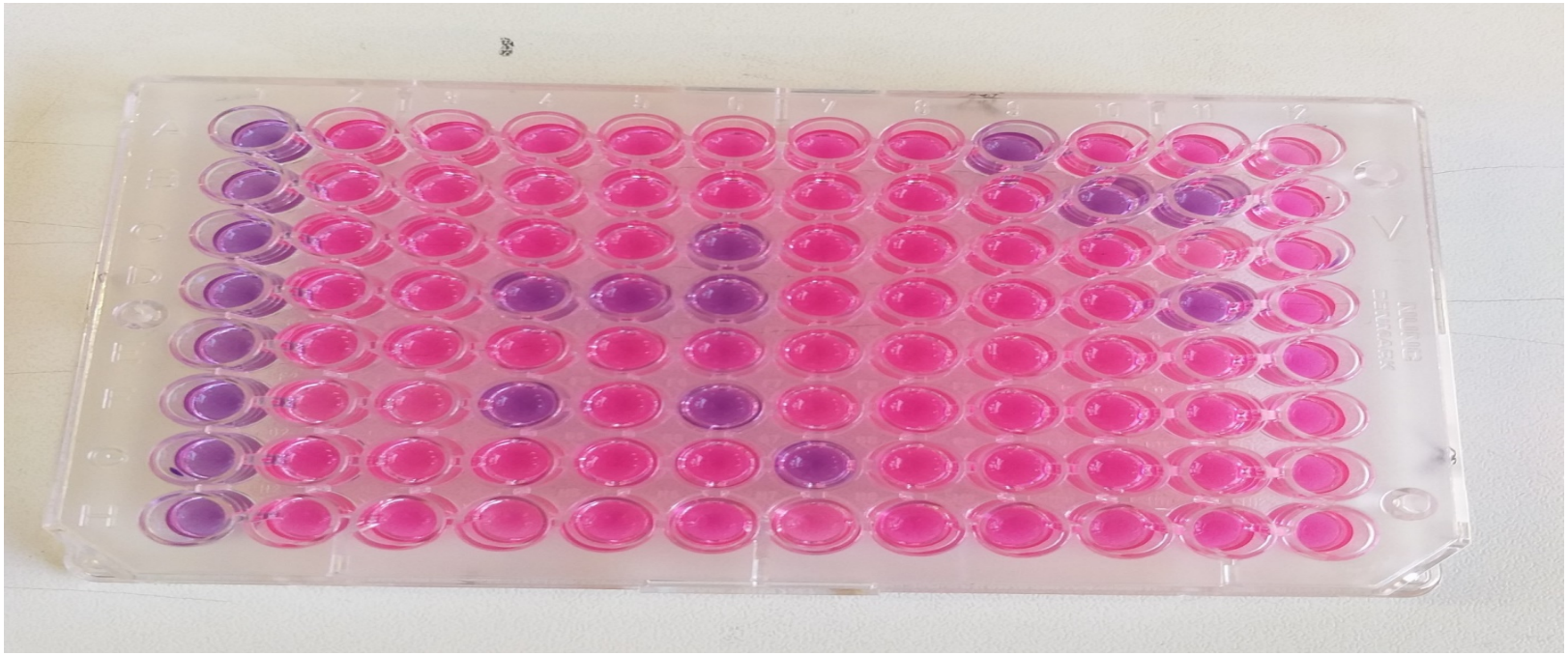


Plate A: Well 1 was used as a negative control and well 12 as a positive control. Each of the wells from well 2 to 11 contains a single Pathogen Box compound at 10 µM. MMV676501, MMV102872, MMV676477, MMV688943, MMV661713, MMV688793, MMV688942, MMV676409, MMV676388, MMV688936, and MMV676558 showed partial to full inhibitory activity against *N. gonorrhoeae.*

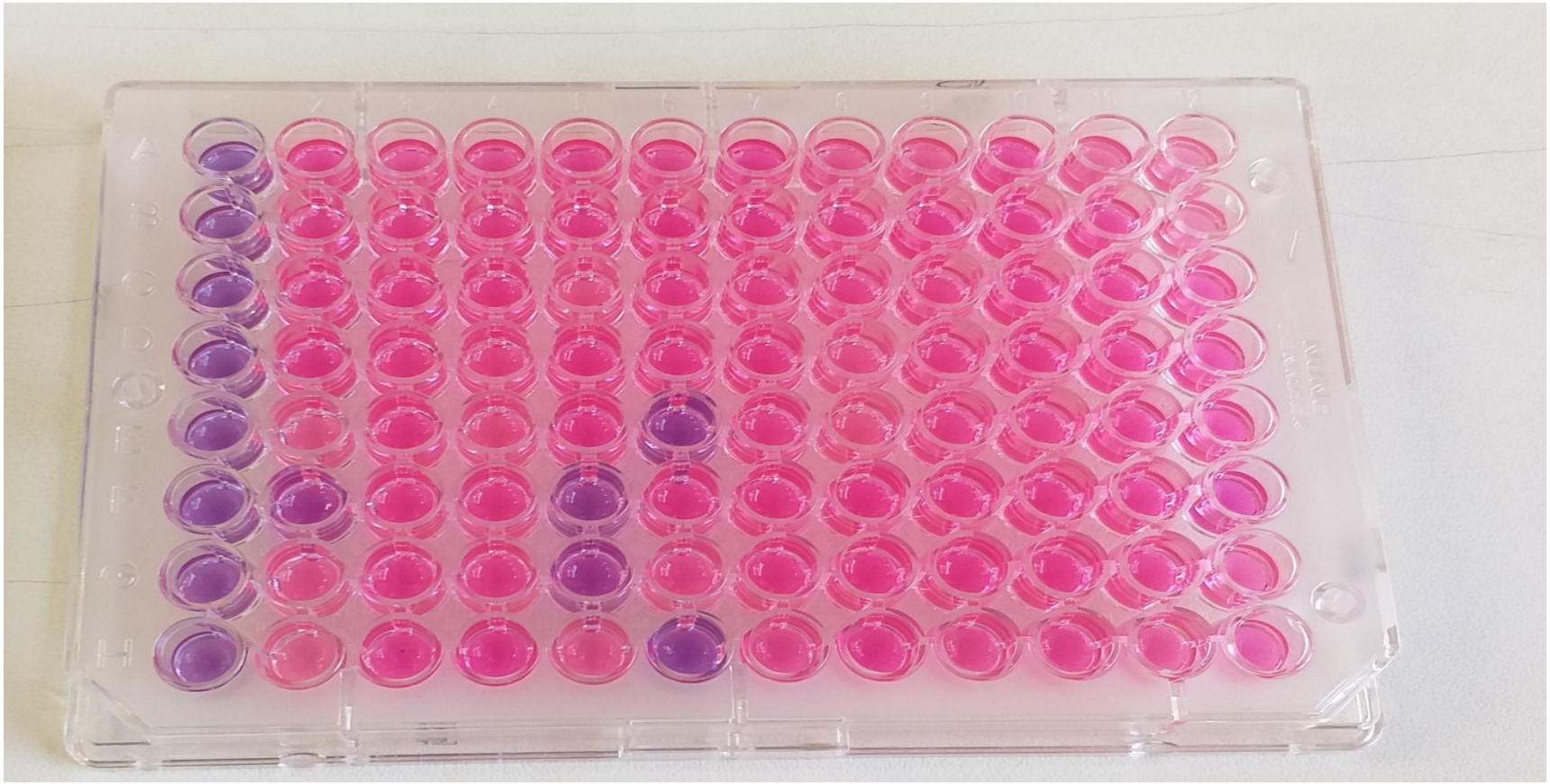


Plate B: Well 1 was used as a negative control and well 12 as a positive control. Each of the wells from well 2 to 11 contains a single Pathogen Box compound at 10 µM. MMV002817, MMV637229, MMV019189, MMV689480, and MMV019742 showed partial to full inhibitory activity against *N. gonorrhoeae.*

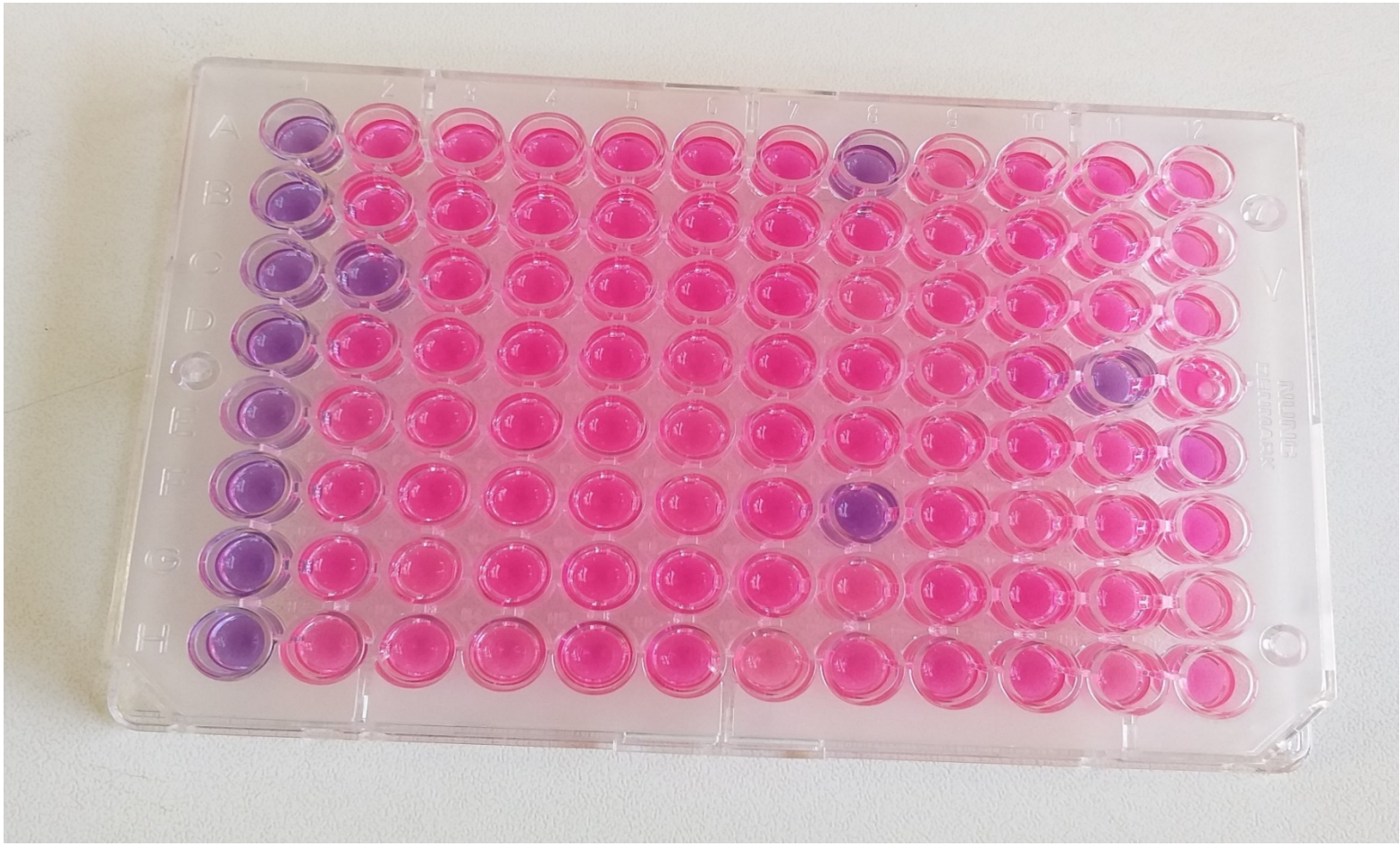


Plate C: Well 1 was used as a negative control and well 12 as a positive control. Each of the wells from well 2 to 11 contains a single Pathogen Box compound at 10 µM.MMV688327, MMV688508, MMV1030799, and MMV687807 showed partial to full inhibitory activity against *N. gonorrhoeae*

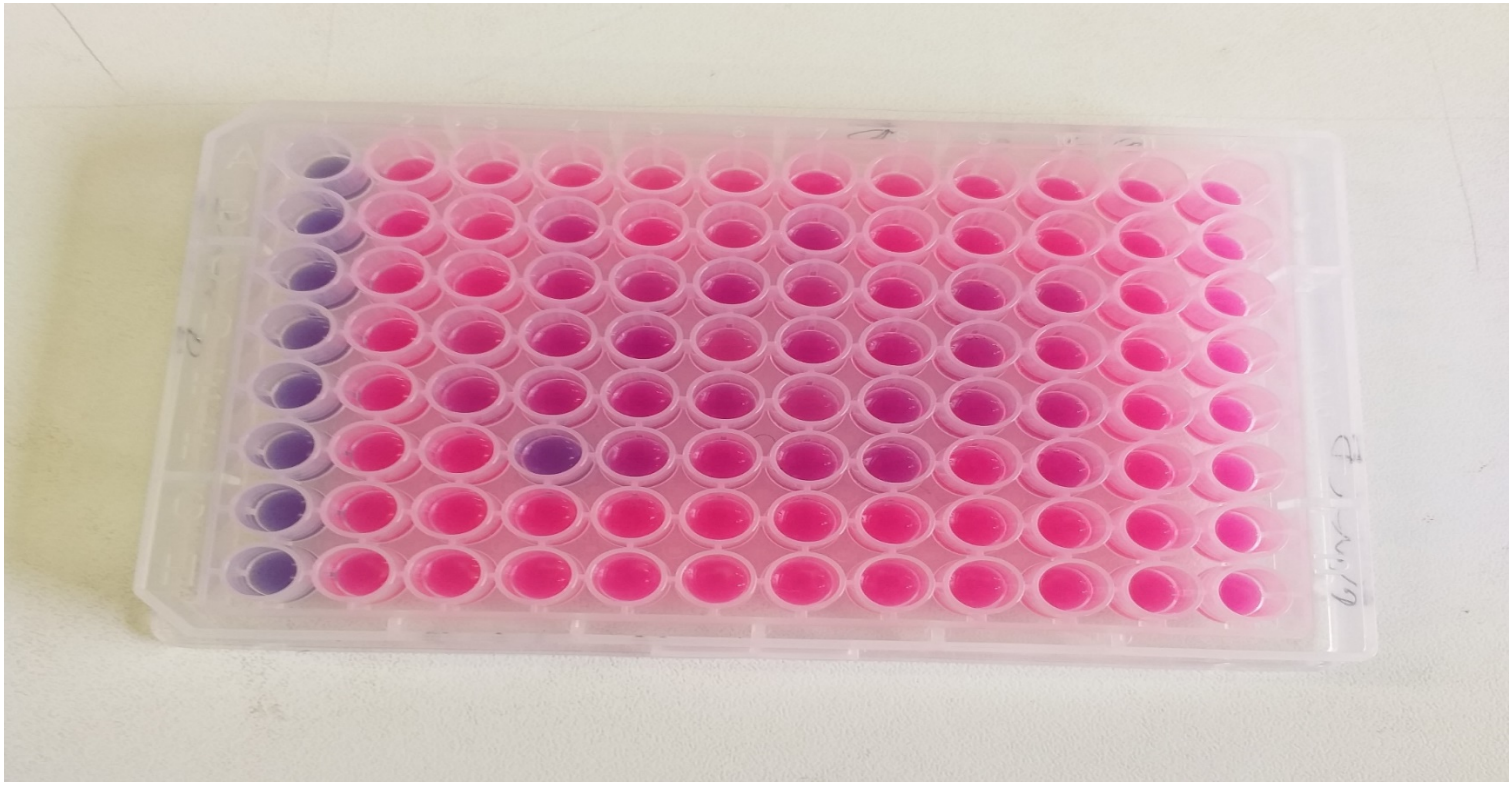


Plate D: Well 1 was used as a negative control and well 12 as a positive control. Each of the wells from well 2 to 11 contains a single Pathogen Box compound at 10 µM. Only MMV024937 showed full inhibitory activity against *N. gonorrhoeae.*

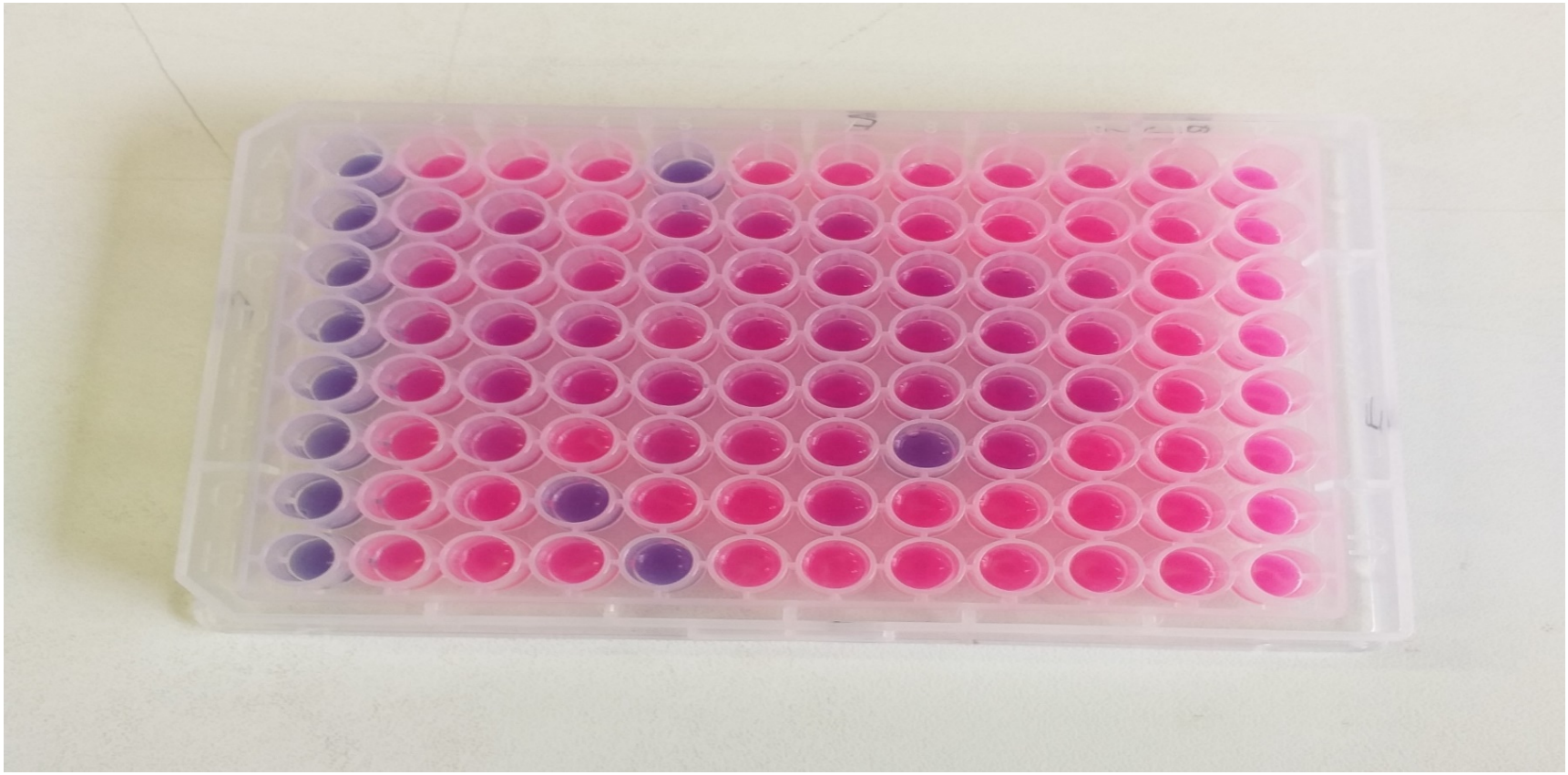
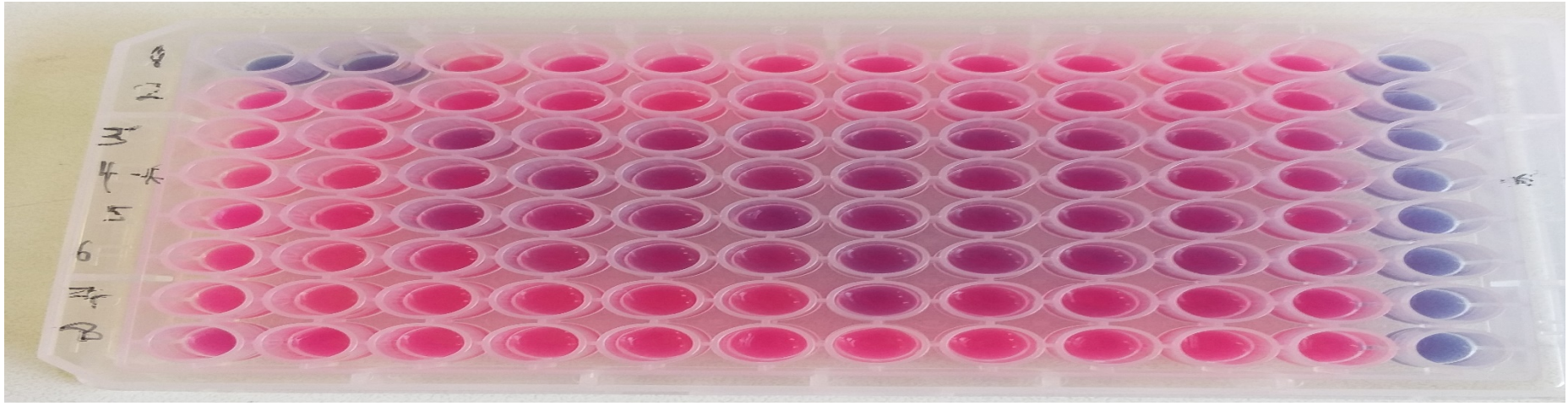
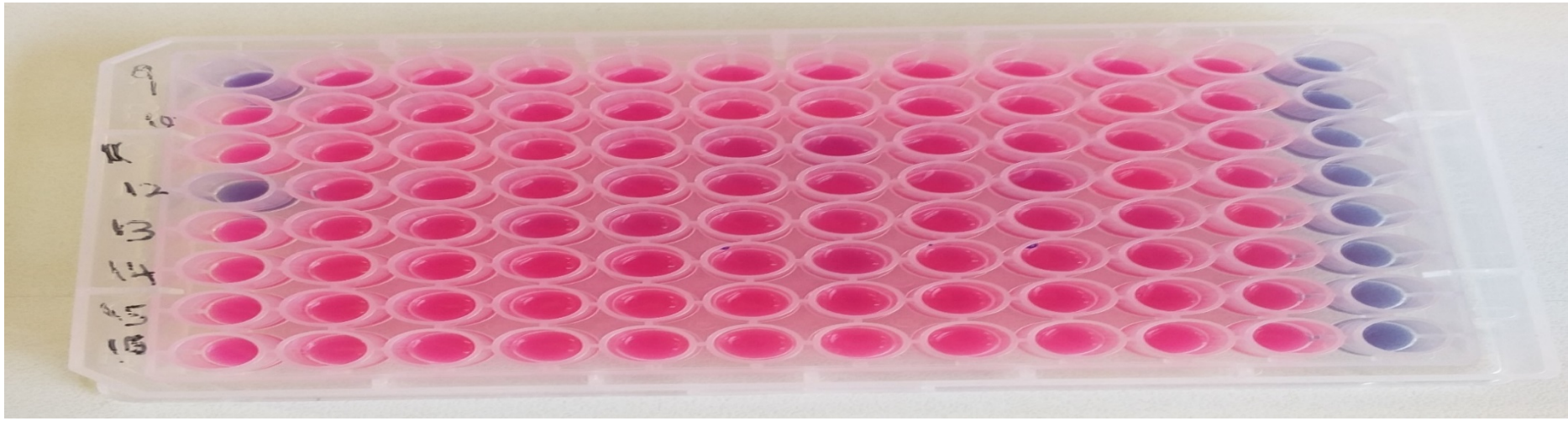


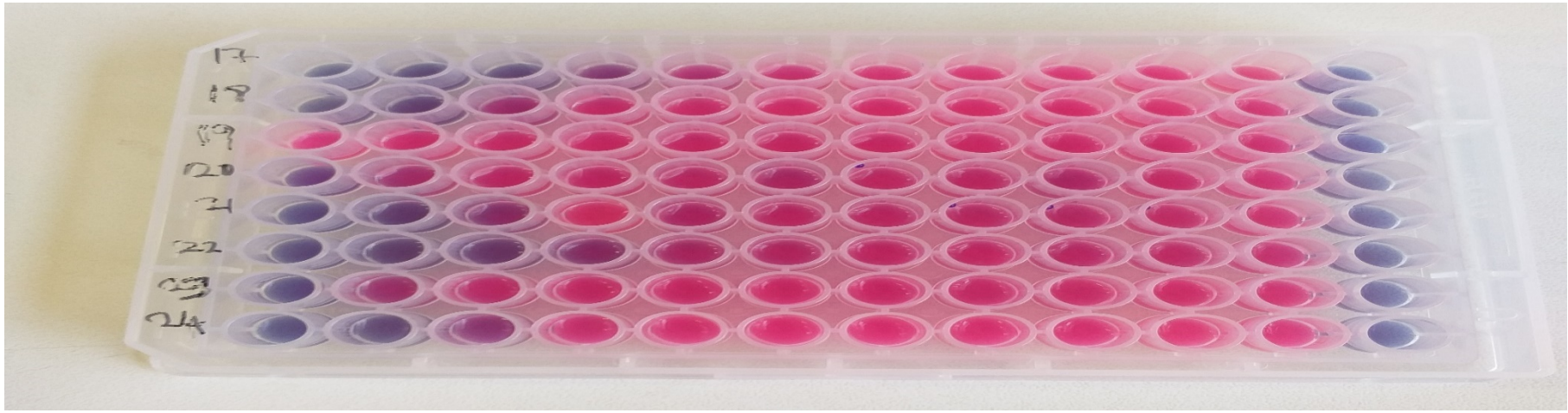
Plate E: Well 1 was used as a negative control and well 12 as a positive control. Each of the wells from well 2 to 11 contains a single Pathogen Box compound at 10 µM. MMV687798, MMV272144, MMV021013, and MMV688978 showed partial to full inhibitory activity against *N. gonorrhoeae.*

Figure 5.S. 2: Selection of PBCs showing full activity in primary screening against *N. gonorrhoeae*

Pathogen Box compounds showing partial to full inhibition were selected and screened in duplicate to select compounds with full activity. Well, 11 and 12 were used as positive and negative control respectively. Ten Pathogen Box compounds MMV676501, MMV676388, MMV002817, MMV688327, MMV688508, MMV024937, MMV687798, MMV272144, MMV021013 and MMV688978 showed full inhibition against *N. gonorrhoeae.*







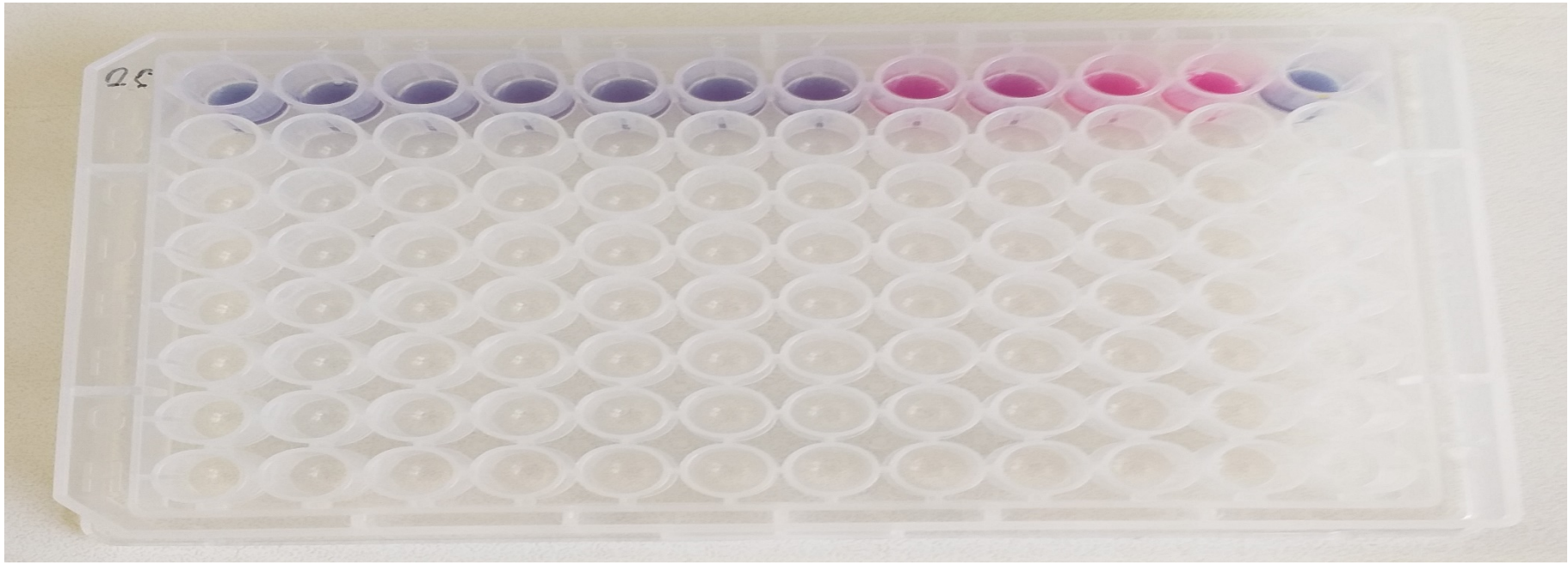


Figure 5.S. 3: Determination of PBC-specific MIC against strains of *N. gonorrhoeae* using a two-fold micro-broth dilution assay

The concentrations ranged from 0.0195 – 10 µM. The concentration decreases from well 1 to well 10. Well 11 served as a positive control and well 12 as a negative control. The experiment was repeated in triplicate against *N. gonorrhoeae* clinical strain 1 (Sup Fig 3B), clinical strain 2 (Sup Fig 3A), and reference strain ATCC 49266 (Sup Fig 3C). The median value was used as the MIC. MIC: minimum inhibitory concentration. 1, MMV676501; 2, MMV676388; 3, MMV002817; 4, MMV688327; 5, MMV688508; 6, MMV024937; 7, MMV687798 (Levofloxacin); 8, MMV272144; 9, MMV021013; 10, MMV688978 (Auranofin).

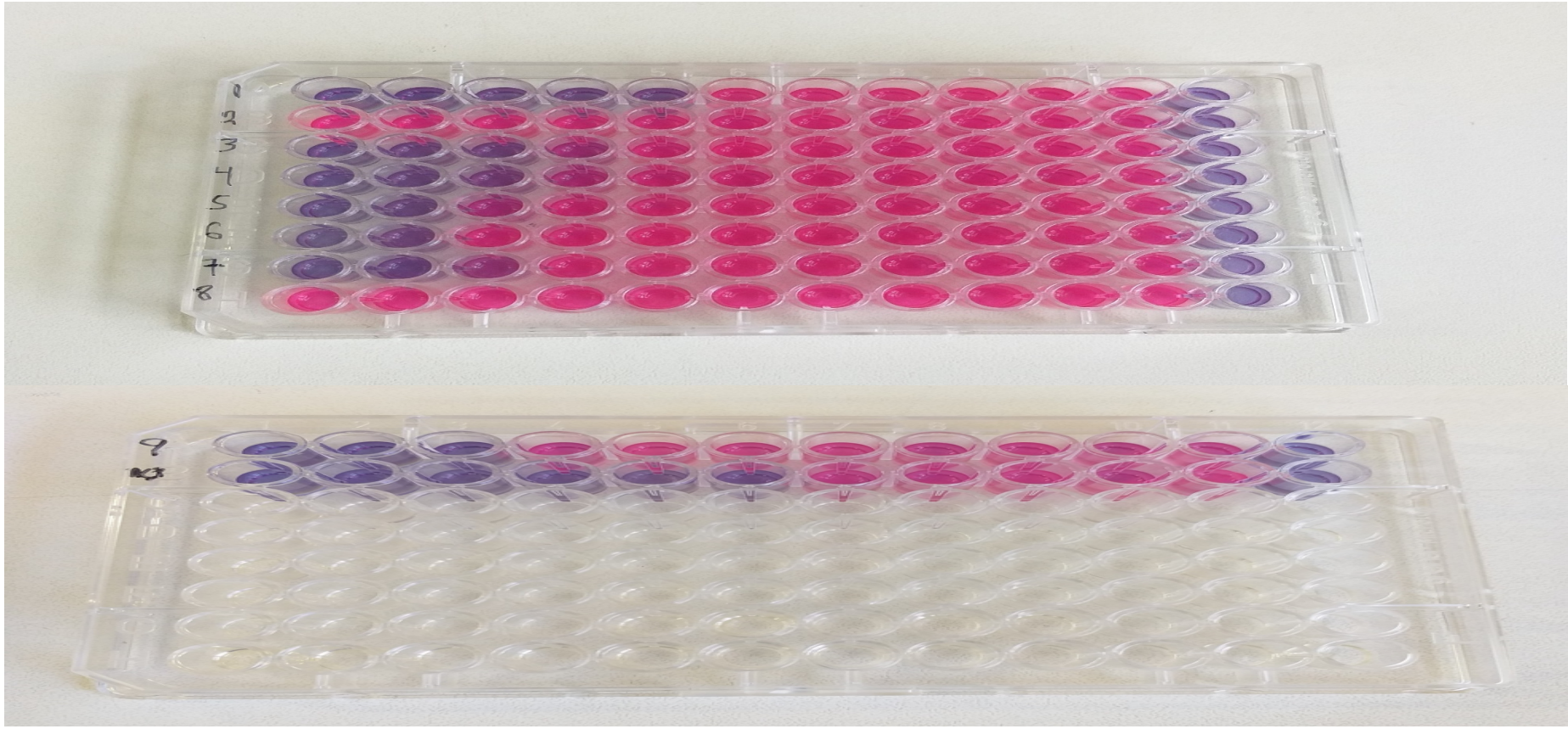


Figure 5.S.3.A

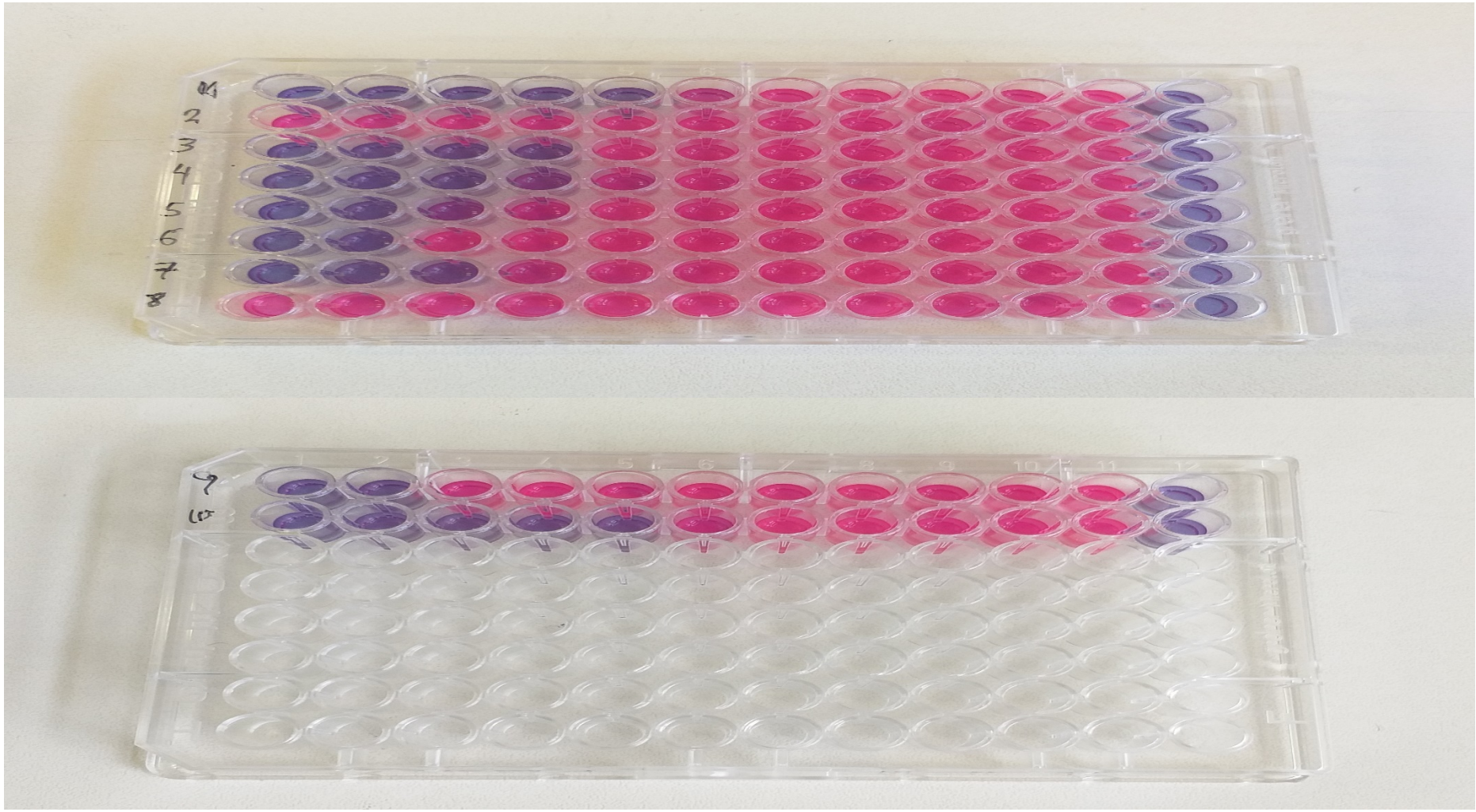


Figure 5.S.3.B

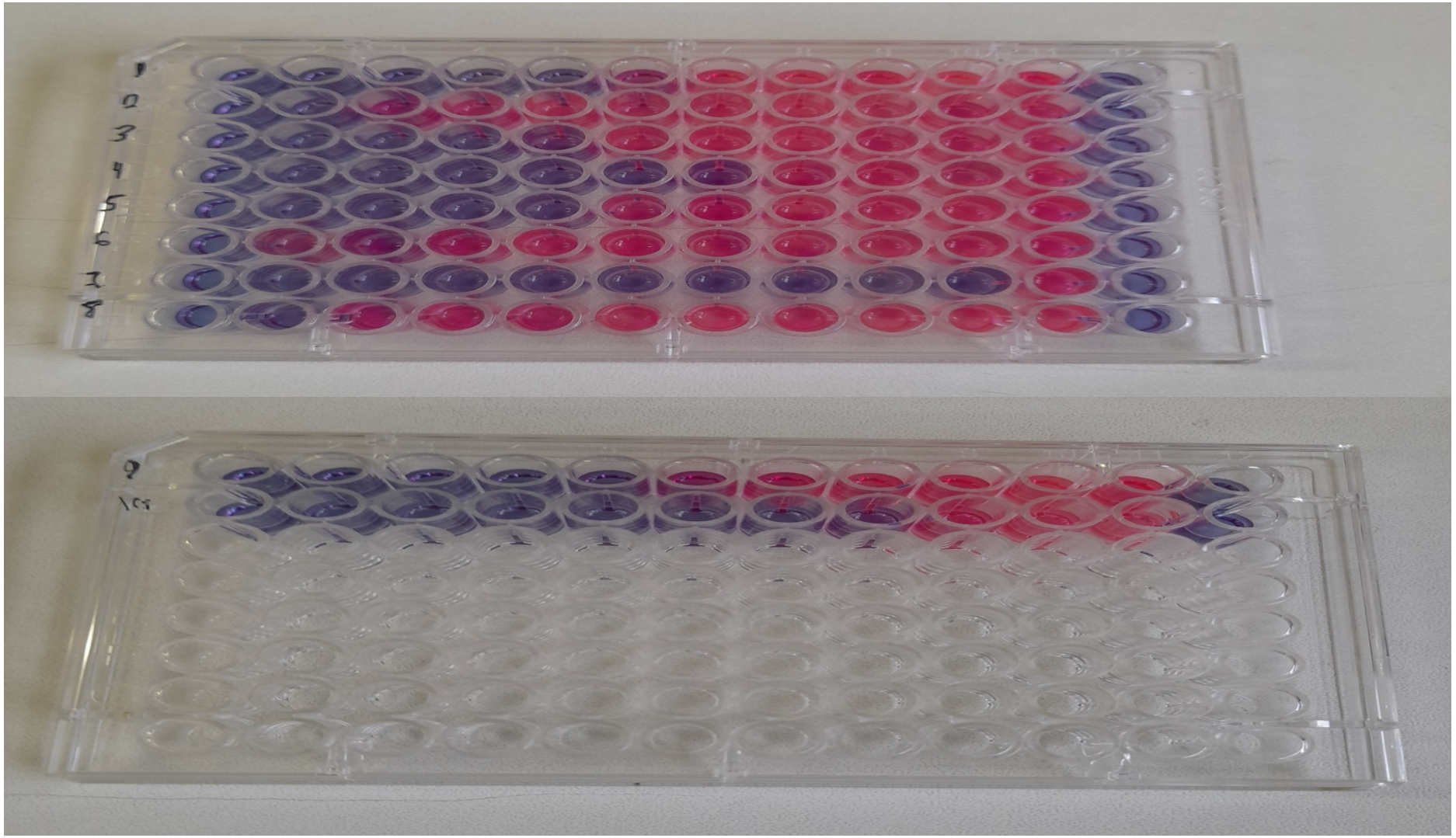


Figure 5.S.3.C

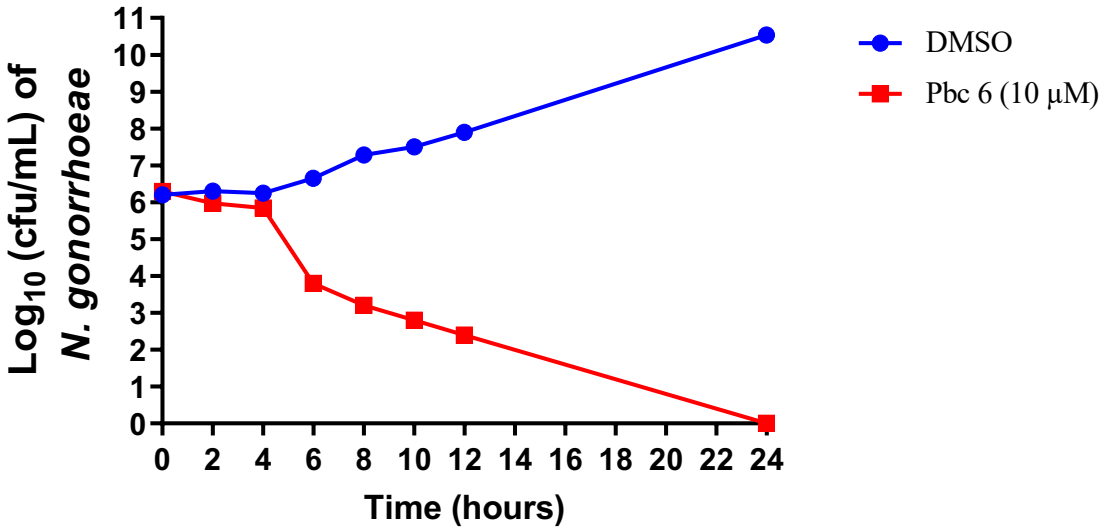


Figure 5.S. 4: Time-kill analysis of MMV024937 (Pbc 6) (at 1 x MIC) against *N. gonorrhoeae*

(over a 24-hour incubation period at 35°C. DMSO served as a negative control)

Table 5.S. 1: Effect of combination of compound MMV676501 with other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV676501	0.625	0.5	1.0	ADD		
MMV002817	2.5	0.5				
MMV676501	0.625	0.5	1.0	ADD		
MMV688327	2.5	0.5				
MMV676501	0.625	0.5	1.0	ADD		
MMV688508	5	0.5				
MMV676501	0.625	0.5	1.3	IND		
MMV024937	10	5				
MMV676501	0.625	0.5	1.8	IND		
MMV687798	5	5				
MMV676501	0.625	0.25	0.9	ADD		
MMV021013	5	2.5				
MMV676501	0.625	0.5	1.6	IND		
MMV688978	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV676501 was combined with MMV002817, MMV688327, MMV688508, MMV024937, MMV687798, MMV021013, and MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 2: Effect of combination of compound MMV002817 and other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV002817	2.5	1	0.8	ADD		
MMV688327	2.5	1				
MMV002817	2.5	1	0.6	ADD		
MMV688508	5	1				
MMV002817	2.5	0.5	0.7	ADD		
MMV024937	10	5				
MMV002817	2.5	0.5	1.2	ADD		
MMV687798	5	5				
MMV002817	2.5	0.5	1.2	ADD		
MMV021013	5	5				
MMV002817	2.5	0.25	0.9	ADD		
MMV688978	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV002817 was combined with MMV688327, MMV688508, MMV024937, MMV687798, MMV021013, and MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 3: Effect of combination of compound MMV688327 and other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV688327	2.5	0.5	0.4	SYN		
MMV688508	2.5	0.5				
MMV688327	2.5	0.5	0.7	ADD		
MMV024937	10	5				
MMV688327	2.5	0.5	1.2	ADD		
MMV687798	5	5				
MMV688327	2.5	0.5	1.2	ADD		
MMV021013	5	5				
MMV688327	2.5	0.5	1.8	IND		
MMV688978	0.3125	0.5				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV688327 was combined with MMV688508, MMV024937, MMV687798, MMV021013, and MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 4: Effect of combination of compound MMV688508 and other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV688508	2.5	0.5	0.7	ADD		
MMV024937	10	5				
MMV688508	2.5	0.5	1.2	ADD		
MMV687798	5	5				
MMV688508	2.5	0.5	1.2	ADD		
MMV021013	5	5				
MMV688508	2.5	0.25	0.9	ADD		
MMV688978	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV688508 was combined with MMV024937, MMV687798, MMV021013, and MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 5: Effect of combination of compound MMV024937 and other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV024937	10	2.5	0.75	ADD		
MMV687798	5	2.5				
MMV024937	10	2.5	0.75	ADD		
MMV021013	5	2.5				
MMV024937	10	2.5	1.05	ADD		
MMV688978	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV024937 was combined with MMV687798, MMV021013and with MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 6: Effect of combination of compound MMV687798 and other lead drugs

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV687798	5	2.5	1	ADD		
MMV021013	5	2.5				
MMV687798	5	2.5	1.3	IND		
MMV688978	0.3125	0.25				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV687798 was combined with MMV021013 and with MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 7: Effect of combination of compounds MMV021013 and MMV688978

Test compounds	MIC in µM		FICI	Interpretation		
	MIC alone	MIC in combination		SYN	ADD	IND
MMV021013	5	1.25	0.625	ADD		
MMV688978	0.3125	0.125				

FICI, fractional inhibitory concentration index; Add, additive; Syn, synergistic; IND, indifference; A FICI value of ≤ 0.5 was considered a synergistic activity; between > 0.5 but ≤ 1.25 as an additive activity; > 1.25 - ≤ 4 as indifference. MMV021013 was combined with MMV688978 at a concentration lower than their respective MIC.

Table 5.S. 8: Pathogen-specific MIC and MBC of Pathogen Box compounds against Clinical strain 1 (SRS5471848)

	MIC replicate 1 (µM)	MIC replicate 2 (µM)	MIC replicate 3 (µM)	Median MIC (µM)	Mean ± sd MIC (µM)	MBC replicate 1 (µM)	MBC replicate 2 (µM)	MBC replicate 3 (µM)	Median MBC (µM)	Mean ± sd MBC (µM)
MMV676501 (Pbc 1)	0.625	0.625	0.625	0.625	0.625 ± 0	0.625	0.625	1.25	0.625	0.833 ± 0.36
MMV002817 (PBC 3)	2.5	1.25	2.5	2.5	2.08 ± 0.72	2.5	2.5	2.5	2.5	2.5 ± 0
MMV688327 (Pbc4, Radezolid)	2.5	2.5	1.25	2.5	2.08 ± 0.72	2.5	2.5	2.5	2.5	2.5 ± 0
MMV688508 (Pbc 5)	2.5	5	2.5	2.5	3.33 ± 1.44	5	5	5	5	5 ± 0
MMV024937 (Pbc 6)	5	10	10	10	8.33 ± 2.89	10	5	10	10	8.33 ± 2.89
MMV687798 (Pbc 7, Levofloxacin))	5	2.5	5	5	4.17 ± 1.44	5	5	5	5	5 ± 0
MMV021013 (Pbc 9)	5	10	5	5	6.67 ± 2.89	5	5	5	5	5 ± 0
MMV688978 (Pbc 10, Auranofin)	0.3125	0.3125	0.156	0.3125	0.26 ± 0.9	0.3125	0.3125	0.3125	0.3125	0.3125 ± 0

The MIC and MBC of each Pathogen Box compound against *N. gonorrhoeae* was repeated in triplicate. MIC, minimum inhibitory concentration; ; sd, standard deviation; MBC, minimum bactericidal concentration; Pbc, pathogen box compound.

Table 5.S. 9: Pathogen-specific MIC and MBC of Pathogen Box compounds against Clinical strain 2 (SRS5471840)

	MIC replicate 1 (µM)	MIC replicate 2 (µM)	MIC replicate 3 (µM)	Median MIC (µM)	Mean ± sd MIC (µM)	MBC replicate 1 (µM)	MBC replicate 2 (µM)	MBC replicate 3 (µM)	Median MBC (µM)	Mean ± sd MBC (µM)
MMV676501 (Pbc 1)	0.625	0.625	0.625	0.625	0.625 ± 0	0.625	0.625	0.625	0.625	0.625 ± 0
MMV002817 (PbC 3)	2.5	2.5	2.5	2.5	2.5 ± 0	2.5	2.5	2.5	2.5	2.5 ± 0
MMV688327 (Pbc 4, Radezolid)	2.5	2.5	2.5	2.5	2.5 ± 0	2.5	2.5	2.5	2.5	2.5 ± 0
MMV688508 (Pbc 5)	5	2.5	2.5	2.5	3.33 ± 1.44	2.5	5	5	5	4.17 ± 1.44
MMV024937 (Pbc 6)	10	10	10	10	10 ± 0	10	10	10	10	10 ± 0
MMV687798 (Pbc 7, Levofloxacin)	10	10	10	10	10 ± 0	10	10	10	10	10 ± 0
MMV021013 (Pbc 9)	5	2.5	5	5	4.17 ± 1.44	5	5	5	5	5 ± 0
MMV688978 (Pbc 10, Auranofin)	0.3125	0.3125	0.3125	0.3125	0.3125 ± 0	0.3125	0.3125	0.3125	0.3125	0.3125 ± 0

The MIC and MBC of each Pathogen Box compound against *N. gonorrhoeae* was repeated in triplicate. MIC, minimum inhibitory concentration; ; sd, standard deviation; MBC, minimum bactericidal concentration; Pbc, pathogen box compound.

Table 5.S. 10: Pathogen-specific MIC and MBC of Pathogen Box compounds against *N. gonorrhoeae* ATCC AT49266 strain

	MIC replicate 1 (µM)	MIC replicate 2 (µM)	MIC replicate 3 (µM)	Median MIC (µM)	Mean ± sd MIC (µM)	MBC replicate 1 (µM)	MBC replicate 2 (µM)	MBC replicate 3 (µM)	Median MBC (µM)	Mean ± sd MBC (µM)
MMV676501 (Pbc 1)	0.625	0.625	0.625	0.625	0.625 ± 0	0.625	0.625	0.625	0.625	0.625 ± 0
MMV676388 (Pbc 2)	5	5	5	5	5 ± 0	5	5	5	5	5 ± 0
MMV002817 (PBC 3)	1.25	1.25	1.25	1.25	1.25 ± 0	1.25	1.25	1.25	1.25	1.25 ± 0
MMV688327 (Pbc4, Radezolid)	0.3125	0.3125	0.3125	0.3125	0.3125 ± 0	0.3125	0.3125	0.3125	0.3125	0.3125 ± 0
MMV688508 (Pbc 5)	0.625	0.625	0.625	0.625	0.625 ± 0	0.625	0.625	0.625	0.625	0.625 ± 0
MMV024937 (Pbc 6)	5	5	5	5	5 ± 0	5	5	5	5	5 ± 0
MMV687798 (Pbc 7, Levofloxacin))	<0.0195	<0.0195	<0.0195	<0.0195	<0.0195 ± 0	<0.0195	<0.0195	<0.0195	<0.0195	<0.0195 ± 0
MMV272144 (Pbc 8)	10	10	10	10	10 ± 0	10	10	10	10	10 ± 0
MMV021013 (Pbc 9)	1.25	1.25	1.25	1.25	1.25 ± 0	1.25	1.25	1.25	1.25	1.25 ± 0
MMV688978 (Pbc 10, Auranofin)	0.15625	0.15625	0.15625	0.15625	0.15625 ± 0	0.15625	0.15625	0.15625	0.15625	0.15625 ± 0

The MIC and MBC of each Pathogen Box compound against *N. gonorrhoeae* was repeated in triplicate. MIC, minimum inhibitory concentration; ; sd, standard deviation; MBC, minimum bactericidal concentration; Pbc, pathogen box compound.

Table 5.S. 11: Time kill kinetics of dual combination of Pathogen Box compounds against *N. gonorrhoeae*

	Time (hrs)							
	0	2	4	6	8	10	12	24
DMSO (cfu/mL)	**Mean:** 1.02×10^6 **R1:** 1.01×10^6 **R2:** 1.03×10^6	**Mean:** 2.27×10^6 **R1:** 2.1×10^6 **R2:** 2.4×10^6	**Mean:** 1.74×10^6 **R1:** 1.68×10^6 **R2:** 1.8×10^6	**Mean:** 4.4×10^6 **R1:** 4.3×10^6 **R2:** 4.5×10^6	**Mean:** 1.9×10^7 **R1:** 1.8×10^7 **R2:** 2.0×10^7	**Mean:** 3.55×10^7 **R1:** 3.3×10^7 **R2**: 3.8×10^7	**Mean:** 6.8×10^7 **R1:** 6.0×10^7 **R2:** 7.6×10^7	**Mean:** 3.45×10^{10} **R1:** 3.3×10^{10} **R2:** 3.6×10^{10}
Ceftriaxone + MMV688978 (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.26×10^6 **R1:** 1.2×10^6 **R2:** 1.32×10^6	**Mean:** 1.7×10^5 **R1:** 1.6×10^5 **R2:** 1.8×10^5	**Mean:** 5.0×10^4 **R1:** 4.9×10^4 **R2:** 5.1×10^4	**Mean:** 4.4×10^4 **R1:** 4.0×10^4 **R2:** 4.8×10^4	**Mean:** 4.7×10^3 **R1:** 4.6×10^3 **R2:** 4.8×10^3	**Mean:** 5.0×10^2 **R1:** 4.0×10^2 **R2:** 6×10^2	**Mean:** 1.0×10^2 **R1:** 1.0×10^2 **R2:** 1.0×10^2	0
Ceftriaxone + MMV6887798 (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.26×10^6 **R1:** 1.2×10^6 **R2:** 1.32×10^6	**Mean:** 4.0×10^5 **R1:** 3.8×10^5 **R2:** 4.2×10^5	**Mean:** 1.81×10^5 **R1:** 1.7×10^5 **R2:** 1.92×10^5	**Mean:** 7.9×10^4 **R1:** 7.0×10^4 **R2:** 8.8×10^4	**Mean:** 2.3×10^4 **R1:** 2.1×10^4 **R2:** 2.5×10^4	**Mean:** 1.25×10^4 **R1:** 1.2×10^4 **R2:** 1.3×10^4	**Mean:** 6.0×10^2 **R1:** 5.0×10^2 **R2:** 7.0×10^2	0
Ceftriaxone + MMV024937 (Pbc 6) (cfu/mL)	**Mean:** 1.1×10^6 **R1:** 1.01×10^6 **R2:** 1.2×10^6	**Mean:** 3.9×10^5 **R1:** 3.8×10^5 **R2:** 4.0×10^5	**Mean:** 3.6×10^5 **R1:** 3.5×10^5 **R2:** 3.7×10^5	**Mean:** 6.8×10^4 **R1:** 6.5×10^4 **R2:** 7.1×10^4	**Mean:** 3.9×10^4 **R1:** 3.8×10^4 **R2:** 4.0×10^4	**Mean:** 2.8×10^4 **R1:** 2.5×10^4 **R2:** 3.1×10^4	**Mean:** 7.0×10^2 **R1:** 6.5×10^2 **R2:** 7.5×10^2	0
MMV024937 (Pbc 6)+ MMV688978 (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.15×10^6 **R1:** 1.1×10^6 **R2:** 1.2×10^6	**Mean:** 3.0×10^5 **R1:** 2.5×10^5 **R2:** 3.5×10^5	**Mean:** 3.1×10^3 **R1:** 3.0×10^3 **R2:** 3.2×10^3	0	0	0	0	0
MMV024937 (Pbc 6) + MMV6887798 (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.15×10^6 **R1:** 1.1×10^6 **R2:** 1.2×10^6	**Mean:** 4.4×10^5 **R1:** 4.0×10^5 **R2:** 4.8×10^5	**Mean:** 2.9×10^5 **R1:** 2.7×10^5 **R2:** 3.1×10^5	**Mean:** 2.6×10^5 **R1:** 2.5×10^5 **R2:** 2.7×10^5	**Mean:** 7.0×10^4 **R1:** 6.4×10^4 **R2:** 7.6×10^4	**Mean:** 8.0×10^3 **R1:** 7.0×10^3 **R2:** 9.0×10^3	**Mean:** 1.0×10^3 **R1:** 9.0×10^2 **R2:** 1.1×10^3	0

The time-kill kinetics of dual combination of Pathogen Box compounds. 3 x MIC of each compound in combination found to be additive was used against ~ 1.0×10^6 *N. gonorrhoeae*. R1, repeat 1; R2, repeat 2.

Table 5.S. 12: Time kill kinetics of Pathogen Box compounds against *N. gonorrhoeae*

	Time (hrs)							
	0	2	4	6	8	10	12	24
DMSO (cfu/mL	**Mean:** 1.02 x 10^6 **R1:** 1.01 x 10^6 **R2:** 1.03 x 10^6	**Mean:** 2.27 x 10^6 **R1:** 2.13 x 10^6 **R2:** 2.4 x 10^6	**Mean:** 1.74 x 10^6 **R1:** 1.68 x 10^6 **R2:** 1.8 x 10^6	**Mean:** 4.4 x 10^6 **R1:** 4.3 x 10^6 **R2:** 4.5 x 10^6	**Mean:** 1.9 x 10^7 **R1:** 1.8 x 10^7 **R2:** 2.0 x 10^7	**Mean:** 3.55 x 10^7 **R1:** 3.3 x 10^7 **R2:** 3.8 x 10^7	**Mean:** 6.8 x 10^7 **R1:** 6.0 x 10^7 **R2:** 7.6 x 10^7	**Mean:** 3.45 x 10^{10} **R1:** 3.3 x 10^{10} **R2:** 3.6 x 10^{10}
MMV676501 (Pbc 1) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.55 x 10^5 **R1:** 2.8 x 10^5 **R2:** 2.3 x 10^5	**Mean:** 1.7 x 10^5 **R1:** 1.62 x 10^5 **R2:** 1.78 x 10^5	**Mean:** 1.1 x 10^5 **R:1** 1.0 x 10^5 **R2:** 1.2 x 10^5	**Mean:** 8.9 x 10^4 **R1:** 8.5 x 10^4 **R2:** 9.3 x 10^4	**Mean:** 1.96 x 10^3 **R1:** 1.92 x 10^3 **R2:** 2.0 x 10^3	**Mean:** 2.0 x 10^2 **R1:** 1.6 x 10^2 **R2:** 2.4 x 10^2	0
MMV002817 (Pbc 3) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 3.6 x 10^5 **R1:** 3.5 x 10^5 **R2:** 3.7 x 10^5	**Mean:** 3.5 x 10^5 **R1:** 3.4 x 10^5 **R2:** 3.6 x 10^5	**Mean:** 1.81 x 10^5 **R1:** 1.8 x 10^5 **R2:** 1.82 x 10^5	**Mean:** 5.0 x 10^4 **R1**: 4.5 x 10^4 **R2:** 5.5 x 10^6	**Mean:** 2.35 x 10^3 **R1:** 2. x 10^3 **R2:** 2.4 x 10^3	**Mean:** 1.5 x 10^3 **R1:** 1.4 x 10^3 **R2:** 1.6 x 10^3	0
MMV688327 (Pbc 4, Radezolid) (cfu/mL)	**Mean:** 1.04 x 10^6 **R1:** 1.02 x 10^6 **R2:** 1.06 x 10^6	**Mean:** 9.95 x 10^5 **R1:** 9.9 x 10^5 **R2:** 1.0 x 10^6	**Mean:** 4.4 x 10^5 **R1:** 4.2 x 10^5 **R2:** 4.6 x 10^5	**Mean:** 2.4 x 10^5 **R1:** 2.3 x 10^5 **R2:** 2.5 x 10^5	**Mean:** 1.2 x 10^5 **R1:** 1.1 x 10^5 **R2:** 1.3 x 10^5	**Mean:** 3.0 x 10^4 **R1:** 2.0 x 10^4 **R2:** 4.0 x 10^4	**Mean:** 4.0 x 10^3 **R1:** 3.0 x 10^3 **R2**: 5.0 x 10^3	0
MMV688508 (Pbc 5) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.02 x 10^6 **R2:** 1.03 x 10^6	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2**: 1.0 2 x 10^6	**Mean:** 2.05 x 10^5 **R1:** 2.0 x 10^5 **R2:** 2.1 x 10^5	**Mean:** 6.8 x 10^5 **R1:** 6.5 x 10^5 **R2:** 7.1 x 10^5	**Mean:** 3.7 x 10^5 **R1:** 3.5 x 10^5 **R2:** 3.9 x 10^5	**Mean:** 5.0 x 10^3 **R1:** 4.5 x 10^3 **R2:** 5.0 x 10^3	**Mean:** 4.5 x 10^3 **R1:** 4.0 x 10^3 **R2:** 5.0 x 10^3	0
MMV024937 (Pbc 6) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 1.23 x 10^5 **R1:** 1.15 x 10^5 **R2:** 1.3 x 10^5	**Mean:** 1.41 x 10^5 **R1:** 1.4 x 10^5 **R2:** 1.42 x 10^5	**Mean:** 3.25 x 10^4 **R1:** 3.0 x 10^4 **R2:** 3.5 x 10^4	**Mean:** 6.75 x 10^2 **R1:** 6.0 x 10^2 **R2:** 7.5 x 10^2	**Mean:** 1.4 x 10^2 **R1:** 1.3 x 10^2 **R2**: 1.5 x 10^2	**Mean:** 1.0 x 10^2 **R1:** 1.0 x 10^2 **R2:** 1.0 x 10^2	0
MMV687798 (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.4 x 10^4 **R1:** 2.3 x 10^4 **R2:** 2.5 x 10^4	**Mean:** 4.25 x 10^4 **R1**: 4.0 x 10^4 **R2:** 4.5 x 10^4	**Mean:** 1.17 x 10^4 **R1:** 1.14 x 10^4 - **R2:** 1.2 x 10^4	**Mean:** 3.05 x 10^3 **R1:** 3.0 x 10^3 **R2:** 3.1 x 10^3	0	0	0
MMV021013 (Pbc 9) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 8. x 10^4 **R1:** 7.0 x 10^4 **R2:** 9.0 x 10^4	**Mean:** 6.3 x 10^5 **R1:** 6.2 x 10^5 **R2:** 6.4 x 10^5	**Mean:** 5.6 x 10^5 **R1:** 5.5 x 10^5 **R2:** 5.7 x 10^5	**Mean:** 8.0 x 10^4 **R1:** 7.0 x 10^4 **R2:** 9.0 x 10^4	**Mean:** 2.2 x 10^3 **R1:** 2.0 x 10^3 **R2:** 2.4 x 10^3	**Mean:** 2 .35 x 10^3 **R1:** 2.3 x 10^3 **R2:** 2.4 x 10^3	0
MMV688978 (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1**: 1.0 x 10^6 **R2**: 1.02 x 10^6	**Mean:** 1.4 x 10^5 **R1:** 1.36 x 10^5 **R2:** 1.44 x 10^5	0	0	0	0	0	0
Ceftriaxone (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.7 x 10^5 **R1:** 1.25 x 10^5 **R2:** 1.45 x 10^5	**Mean:** 9.7 x 10^4 **R1:** 9.6 x 10^4 **R2:** 9.8 x 10^4	**Mean:** 1.95 x 10^4 **R1:** 1.9 x 10^4 **R2:** 2.0 x 10^4	**Mean**: 3.5 x 10^3 **R1:** 3.0 x 10^3 **R2:** 4.0x 10^3	**Mean:** 2.9 x 10^3 **R1:** 2.5 x 10^3 **R2:** 3.3 x 10^3	**Mean:** 5.5 x 10^2 **R1:** 5.0 x 10^2 **R2:** 6.0 x 10^2	0

CHAPTER 6: General discussion and concluding remarks

6.1 Global challenge of antimicrobial resistance and priority pathogens

The emergence of antimicrobial resistance and the spread of antibiotic-resistant bacteria pose major threats to public health, jeopardising the treatment of potentially fatal infections and increasing morbidity and mortality. Due to limited treatment options, the WHO has developed a list of priority pathogens and classified *M. tuberculosis*, and fluoroquinolone and 3rd generation cephalosporin-resistant *N. gonorrhoeae* as critical and high-priority pathogens (WHO, 2017; Tacconelli *et al*., 2018). These pathogens spread rapidly, are very difficult to treat and affect service delivery. Research and development of novel compounds that show potent activity against these pathogens, or agents that potentiate the actions of existing drugs are urgently needed to combat the rise of antibiotic resistance in pathogens currently posing a threat to public health (WHO, 2017; Tacconelli *et al*., 2018; WHO, 2020). The Medicines for Malaria Venture group compiled the Pathogen Box, an open access diverse compound library primarily used to screen for anti-malarial properties but subsequently offered to other interested parties for exploration of wider screening as antibiotic agents targeting other pathogens. This study explored the antibiotic potential, including synergistic and autophagic effects of the MMV Pathogen Box compounds against two of the WHO priority pathogens: *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae* to identify lead compounds with activity against these pathogens.

6.2 *Mycobacterium tuberculosis*

6.2.1 General discussion

Tuberculosis remains a major public concern globally, causing about 10.6 million incident TB cases and over 1.6 million deaths in 2021 (WHO, 2022). Although TB is preventable, treatable, and curable, current anti-TB regimens are losing their effectiveness due to the emergence of drug-resistant strains (WHO, 2020; Mallick *et al*., 2022; Omar *et al*., 2022). Drug-resistant tuberculosis is a health risk, contributing to the ongoing TB epidemic and increasing the morbidity and mortality of TB (Chakaya *et al*., 2021; WHO, 2021). Often, insufficient, and incomplete treatment regimens lead to antimicrobial resistance. Of 157 903 patients diagnosed with drug-resistant tuberculosis in 2020, 132,222 were rifampicin-resistant or MDR-TB, and 25, 681 were XDR-TB (WHO, 2022). Increasing evidence suggests that MDR-TB is a key

contributor of Post-TB Lung Disease (PTLD), a disability and suffering that requires rehabilitation (Akkerman *et al*., 2020; Hsu *et al*., 2020; Allwood *et al*., 2021; Migliori *et al*., 2021). Also, half of the lifetime disability-adjusted life-years (DALYs) caused by TB is caused by PTLD (Duarte *et al*., 2021; Mpagama *et al*., 2021).

Inside the host cells, *M. tuberculosis* has become adaptable, with the capability of evading and modifying its response to survive (Flannagan *et al*., 2009; Forrellad *et al*., 2013; Chai *et al*., 2020; Chandra *et al*., 2022). Some of the mechanisms used by *M. tuberculosis* include interfering with the autophagy machinery, masking themselves with most cells to escape detection, inhibiting the fusion of autophagosomes with lysosomes to proliferate in macrophages, and causing cellular necrosis while blocking apoptosis (Flannagan *et al*., 2009; Huang and Brumell, 2014; Kimmey and Stallings, 2016; Siqueira *et al*., 2018; Chai *et al*., 2020; Chandra *et al*., 2022). The role of several antibiotics and compounds that induce or enhance autophagy and cellular processes directed at clearing intracellular pathogens such as *M. tuberculosis,* is gradually emerging (Kim *et al*., 2012; Giraud-Gatineau *et al*., 2020). To effectively combat *M. tuberculosis*, besides new agents or antibiotic combinations with novel cellular targets, agents or combinations that can induce autophagy processes and facilitate autophagosome-lysosome fusion within macrophages are highly needed to successfully treat patients with different forms of TB (Klionsky and Emr, 2000; Kim *et al*., 2012; Giraud-Gatineau *et al*., 2020).

In this thesis, **the first four objectives explored the antimicrobial and synergistic effects of MMV Pathogen Box compounds on strains of *M. tuberculosis***. These objectives were covered in Chapter 3, in which the antibiotic potential, including synergistic effects of MMV676603, MMV687146, MMV687696, MMV687180, and MMV153413 were explored against wild-type or resistant strains of *M. tuberculosi*s using a standard *in vitro* approach. These compounds showed potent activity against *M. tuberculosis* strains, with MIC and MBC values ranging between 0.01 to 0.625 µM. Additionally, all the Pathogen Box compounds demonstrated bactericidal activity with no resistant mutants isolated at 20 x MIC after 10 to 14 days, except MMV687696. The combination of the five Pathogen Box compounds with each other, and with isoniazid or rifampicin produced a fractional inhibitory concentration index ranging between 0.2 to 2.6, indicating either a synergistic or additive activity against MDR *M. tuberculosis*. The *in vitro* bactericidal activity of these compounds is consistent with the previous studies (Makarov *et al*., 2013; Wilson *et al*., 2013). The synergistic or additive activity

of the selected PBCs with each other and with rifampicin or isoniazid suggest their potential inclusion in combination therapy for treating susceptible and resistant forms of tuberculosis. These compounds with novel cellular targets have the potential to potentiate existing TB drugs. Overall, the MMV Pathogen Box contains compounds with promising activity against *M. tuberculosis.* Further exploration of their potential in the development of additional treatment options for tuberculosis is appropriate.

Objective five explored the autophagic effects of five selected Pathogen Box compounds against *M. tuberculosis* using murine-derived macrophages. The cytotoxicity studies revealed that compounds MMV676603, MMV687146, MMV687696 and MMV687180 have acceptable toxicity levels in murine-derived macrophages when compared to untreated cells. We observed LC3-II lipidation after treatment with MMV6603, MMV687146, MMV687696 and MMV687180, and a significant LC3II/LC3I ratio (p value = 0.02) after treatment with MMV687146, when compared to control. MMV676603 ($p < 0.001$), MMV687146 ($p = 0.002$), and MMV687180 ($p = 0.001$) significantly suppressed the growth of intracellular *M. tuberculosis* after three days of treatment. However, blocking the autophagy flux with bafilomycin A1 significantly reversed the anti-mycobacterial activity of MMV676603 ($p = 0.002$) and MMV687146 ($p = 0.024$), suggesting that the effect on intracellular *M. tuberculosis* was dependent on autophagy induction. Autophagy is a lysosomal degradative process, a major mechanism for killing intracellular drug-susceptible, drug-resistant, and dormant *M. tuberculosis* (Klionsky and Emr, 2000; Campbell and Spector, 2012; Kim *et al*., 2012; Arora *et al*., 2020; Giraud-Gatineau *et al*., 2020).

This study also showed that compounds such as MMV676603 and MMV687146 that induce endogenous lipidation of LC3-II and increased LC3II/LC3I ratio resulted in faster clearance of intracellular *M. tuberculosis*. Patients diagnosed with multidrug-resistant tuberculosis (MDR-TB) are often treated with a complex and lengthy multidrug treatment regimen that are expensive and highly toxic for at least nine months and up to 20 months. Cytotoxicity effects of anti-TB drugs are lethal in humans and causes adverse events such as bone marrow suppression and peripheral neuropathy and are often associated with poor adherence leading to the emergence of drug-resistant TB (Conradie *et al.,* 2020; Wasserman *et al*., 2022). Due to this, compounds with strong antimycobacterial activity with low cytotoxicity and high protection index are of great interest as a hit compound for drug development as a new anti-Tb agent (Biava *et al*., 2006; Niles *et al*., 2008; Patel *et al.,* 2019; Bigelow *et al.,* 2022). The

potential to develop a safe combination therapy with these compounds is promising.

The use of bafilomycin A1 to block the fusion between autophagosomes and lysosomes reversed the anti-mycobacterial activity of MMV676603 and MMV687146. This shows that fusion of the autophagosomes containing *M. tuberculosis* with lysosomes is key in control of intracellular *M. tuberculosis* infection. In the absence of effective vaccines to halt tuberculosis transmission, new agents or therapeutic combinations that can induce autophagy or overcome pathogen-initiated autophagy inhibition in macrophages is needed to improve the cure rate, reduce relapse, and combat tuberculosis (Chandra *et al..,* 2022; Gafa *et al*., 2022; Jeong *et al*., 2022; Mehta *et al*., 2022). Further research on the combination of these Pathogen Box compounds with other conventional anti-TB drugs against intracellular *M. tuberculosis* is urgently needed. Bedaquiline increased macrophage lysosomal activity and induced autophagy activation, leading to reprogramming of macrophages into potent bactericidal phagocytes to enhance the intracellular killing of *M. tuberculosis (*Giraud-Gatineau *et al*., 2020). In a clinical trial using a combination of bedaquiline, pretomanid, and linezolid, a cure rate of 90% was reported among patients diagnosed with multidrug-resistant and extensively drug-resistant *M. tuberculosis* (Conradie *et al*., 2020). Further research on these compounds may provide additional effective combination therapies for tuberculosis.

Limitations: Due to the limited amount of Pathogen Box compounds raw material available, investigations using laser confocal microscopy of LC3B puncta formation, co-localization of the autophagosome (containing *Mtb*) with the lysosome, and whether these compounds increase macrophage lysosomal activity, could not be performed. Future research on the autophagic activity of these compounds by confocal microscopy is indicated. For the same reason, only a single concentration of the PBCs could be investigated for the Western blot and colony-forming unit assays. Further studies using different concentrations to establish the optimal concentration at which these compounds induce autophagy, are indicated.

6.2.2 Conclusions and future research

In conclusion, this study demonstrated that five Pathogen Box compounds have potent anti-mycobacterial activity against susceptible and resistant strains of *M. tuberculosis* at low MIC and MBC values. These compounds were bactericidal and showed synergistic and additive activity with each other and with either rifampicin or isoniazid. The synergistic or additive activity of the Pathogen Box compounds with each other and with rifampicin or isoniazid

suggest their potential inclusion in combination therapy for treating tuberculosis. Additionally, the intracellular suppression of *M. tuberculosis* by these compounds with low cytotoxic effect is promising in developing a safe novel combination regimen for tuberculosis. MMV676603 and MMV687146 induced endogenous LC3-II lipidation and increased LC3II/LC3I ratio, which resulted in significant clearance of intracellular *M. tuberculosis* after three days. These compounds have the potential to augment the host defense mechanisms to overcome pathogen-initiated autophagy inhibition and facilitate autophagosome-lysosome fusion within macrophages to enhance intracellular killing of *M. tuberculosis*. The compounds have the potential to reduce treatment duration of tuberculosis.

Moving forward, future research on the assessment of these compounds in animal models such as a guinea pig model, mouse model, rat model and non-human primate models are needed to evaluate the clinical usefulness before a potential phase 1 clinical trial in humans. Evaluations of the safety, tolerability, pharmacokinetics and pharmacodynamics as well as steady-state dosing of these compounds is needed in pigs as a basis for the development of the compounds. It is important to identify organs for compound toxicity and assess the severity of adverse events. Also, the minimum compound exposure required to achieve antimycobacterial effectiveness and time to positivity or clearance needs to be investigated after treatment. Pig models offer advantages for preclinical assessment because they have similar anatomical and physiological features to that of humans and can predict drug response accurately before advancing to human clinical trials.

6.3 *Neisseria gonorrhoeae*

6.3.1 General discussion and concluding remarks

Neisseria gonorrhoeae is a high-priority pathogen defined by WHO due to the constant rise in the number of gonococcal infections and an increasing prevalence of resistance to all currently available first-line drugs approved for treatment (Tacconelli *et al*., 2018; WHO, 2020; Shaskolskiy *et al*., 2022). An estimated 106 million new gonococcal infections are documented annually globally (WHO, 2012), and many more infections go unreported (Walker and Sweet, 2012; Rice *et al*., 2017). If left untreated, gonococcal infections can lead to severe secondary sequelae such as pelvic inflammatory disease, infertility and an increased risk of ectopic pregnancy, and facilitate the transmission of the human immunodeficiency virus (HIV) (Little, 2006; WHO, 2011; Burnett *et al*., 2012). Ceftriaxone monotherapy is the only recommended

drug for treating both susceptible and resistant *N. gonorrhoeae* infections in most countries (Cyr *et al*., 2020). However, the evolution of infections with high-level resistance to ceftriaxone is increasingly reported, prompting the fear of untreatable gonorrhoea (Cámara *et al*., 2012; Deguchi *et al*., 2016; Lefebvre *et al*., 2017; Lahra *et al*., 2018; Pleininger *et al*., 2022). Zoliflodacin and gepotidacin are the most promising antimicrobial drugs currently in phase 3 clinical trial (Lewis, 2019). Both drugs showed strong efficacy in treating urogenital gonorrhoea in Phase 2 clinical trial; however, treatment failure occurred at the oropharyngeal site (Taylor *et al*., 2018a; Taylor *et al*., 2018b). Since the oropharyngeal site is an important site of infection, it is unlikely for any of these agents to be promoted as monotherapy for gonorrhoea. The absence of effective vaccines to prevent infections, coupled with the limited pipeline of new anti-gonococcal drugs highlights the critical need for the discovery and development of new anti-gonococcal agents.

This study identified ten Pathogen Box compounds with potent activity against *N. gonorrhoeae* strains at MIC and MBC of ≤ 10 µM; two are reference drugs, auranofin, and levofloxacin, and the remaining eight are novel compounds. MMV676501, MMV002817, MMV688327, MMV688508, MMV024937, MMV687798 (Levofloxacin), MMV021013 and MMV688978 (Auranofin) demonstrated full activity against two resistant strains of *N. gonorrhoeae* and were all bactericidal with time-kill kinetics similar to that of ceftriaxone. The bactericidal activity of auranofin and the rapid killing of bacterial inoculum after four hours is consistent with previous report (Elkashif and Saleem, 2020). Also, after 6 hours, the combination of auranofin and MMV024937 resulted in the complete eradication of high bacterial inoculum. The dual combination of the Pathogen Box compounds with each other and with ceftriaxone exhibited a rapid bactericidal activity and a fractional inhibitory concentration index ranging between 0.40 to 1.8, indicating a synergistic or additive activity. This finding suggests their potential use as a combination therapy. Combination therapy targeting different pathways is an effective strategy to combat the emergence of antimicrobial resistance (Park *et al*., 2016; Coates *et al*., 2020). Currently, in most countries, ceftriaxone monotherapy is used to treat gonococcal infections instead of the combination of azithromycin and ceftriaxone because of the increasing resistance and potent anti-commensal activity of the dual therapy (Cyr *et al*., 2020). The dual combination of *N. gonorrhoeae* active Pathogen Box compounds with ceftriaxone is promising since combination therapy maximises bactericidal killing and reduces or delays the emergence of resistance compared to monotherapy (Zusman, 2013). The *N. gonorrhoeae* active Pathogen Box compounds demonstrated activity against *M. tuberculosis*, *Plasmodium falciparum* and

Lymphatic filariasis-onchocerciasi in primary screen with different modes of action (Ballell *et al*., 2013; Duffy *et al*., 2017; Veale, 2019). It is possible these compounds may have different cellular targets in *N. gonorrhoeae* and therefore warrant further research.

For the first time, we have discovered compounds in the MMV Pathogen Box with potent activity against *N. gonorrhoeae* strains. This provides additional opportunity for future anti-gonococcal drug research. It is likely for *N. gonorrhoeae* to develop resistance to zoliflodacin and gepotidacin because of its exceptional capacity to acquire resistance. The bactericidal and additive or synergistic activity of *N. gonorrhoeae* active Pathogen compounds against clinically resistant *N. gonorrhoeae* strains, coupled with their low toxicity shows their promising potential to treat gonococcal infections. There is urgent need for effective vaccines to prevent infections and reduce the fear of untreatable gonorrhoeae because of the ability of *N. gonorrhoeae* to develop resistance to all antibiotics introduced as first line drugs (Terkelsen *et al*., 2017; Tacconelli *et al*., 2018; Masone, 2022; Pleininger *et al*., 2022).
This study identified novel compounds with potent activity against strains of *N. gonorrhoeae*. The following study limitations are acknowledged. First, we used a standard *in vitro* approach for initial screening instead of in silico or mechanistic approach to identify *N. gonorrhoeae* active compounds (Nishida *et al*., 2020). This approach was chosen because of the convenience to screen all 400 drug molecules against *N. gonorrhoeae* strains. Second, we did not explore the potential evolution of resistance of the active Pathogen Box compounds against *N. gonorrhoeae* in the time-kill kinetic assay (Allan-Blitz *et al*., 2022).

6.3.2 Conclusions and future research

This study has identified novel Pathogen Box compounds with promising *in vitro* antibacterial activity against susceptible and resistant *N. gonorrhoeae* strains with a time kill-kinetics similar to that of ceftriaxone. This is the first study to explore the activity of the Pathogen Box compounds against *N. gonorrhoeae* stains and provided additional resources for future anti-gonococcal drug research. The rapid killing and the synergistic or additive activity of these compounds suggest their potential for further research as a novel option for primary or adjunctive therapy for the treatment of gonococcal infection.

The identification of two reference drugs (levofloxacin and auranofin) with activity in *N. gonorrhoeae* showed that this approach for screening for compounds can be used for drug discovery and development programs. The agar dilution method is used for antibiotic

susceptibility testing for *N. gonorrhoeae* (CLSI, 2021). However, the micro broth dilution method is a rapid and convenient method for MIC testing. Using a fastidious broth, the results of the microdilution method was consistent with that of the agar dilution method (Wu *et al*., 2018). The huge costs, time-consuming research, and approval process associated with the development of entirely new drugs make drug repurposing to be more advantageous (Brown, 2015). This approach can be used to screen drugs currently undergoing clinical trials or approved drugs used for the treatment of other diseases. This may reveal anti-gonococcal agents leading to the discovery of new anti-gonococcal therapy. The rapid emergence of antimicrobial resistance in *N. gonorrhoeae* makes it highly crucial to have a library of compounds or drugs with potent anti-gonococcal activity that can be assessed and developed.

Next, an investigation of the safety and *in vivo* activity of the *N. gonorrhoeae* active Pathogen Box compounds in murine model to assess clinical usefulness is urgently needed. Information on safety, tolerability, pharmacokinetics, pharmacodynamics, and efficacy or microbiological cure is needed for these compounds to be considered for clinical trials in humans. The ability of any of these compounds to treat both urogenital and oropharyngeal gonorrhoea will be considered a hit compound for drug development as a new anti-gonococcal agent. The effects of these compounds on commensal species such as lactobacilli are important to be investigated since lactobacilli species hinder *Neisseria* colonization (Breshears *et al*., 2015).

Priority pathogens pose a significant threat to global public health due to limited treatment options to treat drug-resistant bacterial infections. Infections caused by priority pathogens are very difficult to treat, spread rapidly, affect service delivery, and are associated with high morbidity and mortality rate (WHO, 2017; Tacconelli *et al*., 2018). As a result, drug discovery for priority pathogens is global research priority to expand treatment options, combat drug resistance, improve treatment outcome and prevent outbreaks (WHO, 2017; Tacconelli *et al*., 2018; WHO, 2020b). Developing entirely new drugs are costly, time consuming and face challenges with approval (Årdal *et al*., 2020). To speed up the discovery and development of new drugs, the Medicines for Malaria Ventures group has developed already available open access diverse compound library such as the Pathogen Box, Pandemic Response Box, Global health priority Box, MMVSola and Malaria libre (http://www.mmv.org/mmv-open).The diverse mechanisms of actions of these compounds present opportunity for research and development of new anti-microbial drugs and a starting point for structure-based drug design for infections with limited treatment pipelines. Through this open-source drug discovery

initiative, several new anti-viral, antibacterial and antiparasitic drug compounds have been discovered for development as new agents (http://www.mmv.org/mmv-open). Phenotypic high-throughput screening of MMV Pathogen Box in Vero cells identified new drug compounds with antiviral activity against SARS-COV-2 (Coimbra *et al*., 2022). This study screened the Pathogen Box compounds against strains of *M. tuberculosis* and *N. gonorrhoeae*, and identified new anti-gonococcal and antimycobacterial agents, as well as autophagy inducing agents that suppressed the growth of intracellular *M. tuberculosis*. This has contributed to the global search of novel compounds against priority pathogens with limited pipelines for research and development.

Following the successes of the first Pathogen Box, MMV has discontinued promoting new Pathogen Box compounds. However, it has provided chemical structures or similes for each of the 400 drug compounds in the Pathogen Box for the synthesis of lead compounds by different groups for further research. We would be interested in synthesizing lead antimycobacterial and anti-gonococcal compounds from the Pathogen Box for future projects.

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APPENDIX SECTION

APPENDIX A: Reagents, media, buffers, antibiotics, and biomarker agents used in the experimental procedures

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1. Middlebrook 7H9 Broth Base (g/L)

- Ammonium sulfate 0.5 g
- Disodium phosphate 2.5 g
- Monopotassium phosphate 1.0 g
- Sodium citrate 0.1 g
- Magnesium sulfate 0.05 g
- Calcium chloride 0.000
- Zinc sulfate 0.001 g
- Copper sulfate 0.001 g
- Ferric ammonium citrate 0.04 g
- L-Glutamic acid 0.5 g
- Pyridoxine 0.001 g
- Biotin 0.0005 g

Suspend 2.35 g of Middlebrook 7H9 broth base powder (BD Diagnostics, USA) in 450 mL of distilled water and mix to completely dissolve the powder. Add 2 mL of glycerol and heat to dissolve. Autoclave the bottle containing the Middlebrook 7H9 broth at 121°C for 15 min. Aseptically add 50 ml of OADC growth supplement.

2. Middlebrook 7H11 Agar Base (g/L)

- Casein enzymatic hydrolysate 1.0 g
- Ammonium sulfate 0.5 g
- Monopotassium phosphate 1.5 g
- Disodium phosphate 1.5 g
- Sodium citrate 0.4 g
- Magnesium sulfate 0.05 g
- L-Glutamic acid 0.5 g
- Ferric ammonium citrate 0.04 g

- Pyridoxine 0.001 g
- Biotin 0.0005
- Malachite green 0.001 g
- Agar 15.0 g

Suspend 10.25 g of Middlebrook 7H11 agar base powder (BD Diagnostics, USA) in 450 mL of distilled water and mix well to completely dissolve the powder. Add 2.5 mL of glycerol and heat to dissolve. Autoclave the bottle containing the Middlebrook 7H11 at 121°C for 15 min. Aseptically add 50 ml of OADC growth supplement.

3. **Middlebrook OADC Growth Supplement (per vial)**
   - Bovine Albumin Fraction V 2.5 g
   - Dextrose 1.0 g
   - Catalase 0.002 g
   - Oleic acid 0.025 g
   - Sodium chloride 0.425 g
   - Distilled water 50 mL
4. **Ziehl-Neelsen (ZN) staining reagents**
   - Carbol fuchsin
   - 20% acid alcohol
   - Methylene blue dye (Counterstain) or Malachite green
5. **Reagents and buffers for DNA extraction, PrimeXtract™ KIT (Longhorn Vaccines and Diagnostics, Bethesda, USA)**
   - **200 µL 100%** ethanol
   - **200 µL** Lysis buffer
   - **500 µL** Wash buffer-1
   - **500 µL** Wash buffer -2
   - **50 µL** Elution solution
6. **Reagents and buffers for MTBDR*plus* v2 assay and MTBDR*sl* v2 assay (Hain Lifescience, Germany)**

   ❖ **Master Mix, PCR**
   - Master Mix-A (mix AM-A)
   - Master Mix-B (mix AM-B)

❖ **Strips & Buffers**

- DNA Strips
- Denaturation solution (DEN)
- Hybridization buffer (HYB)
- Stringent Wash solution (STR)
- Rinse Solution (RIN)
- Conjugate (CON)
- Substrate (SUB)

❖ **Control strain**

- *M. tuberculosis* H37Rv 25618

7. The Qubit™ Working Solution

- Qubit™ Reagent
- Qubit™ Buffer

Add 19.9 mL of Qubit Reagent to 100 µL of Qubit buffer, and mix using a vortex mixer.

8. Brain Heart Infusion Broth (g/ L)

• Beef heart	5 g
• Calf brains	12.5 g
• Disodium hydrogen phosphate	2.5 g
• D(+)-glucose	2 g
• Peptone	10 g
• Sodium chloride	5 g

Suspend 37 g of BHI broth base powder (Merck, Germany) in 1 L of distilled water and mix well to completely dissolve the powder. Autoclave the bottle containing BHI broth at 121°C for 15 min.

9. Hemoglobin

10. Isovitalex

• Vitamin B_{12}	0.01 g
• Thiamine Pyrophosphate	0.1 g
• L-Glutamine	10.0 g
• Ferric Nitrate	0.02 g

- Adenine 1.0 g
- Thiamine Hydrochloride 0.003 g
- Guanine Hydrochloride 0.03 g
- L-Cysteine Hydrochloride 25.9 g
- p-Aminobenzoic Acid 0.013 g
- L-Cystine 1.1 g
- Nicotinamide Adenine Dinucleotide 0.25 g
- Dextrose 100.0 g

Add the diluent to the lyophilized vial and shake to assure a complete solution.

11. Chocolate agar

- Proteose Peptone 15.0 g
- Corn Starch 1.0 g
- Dipotassium Phosphate 4.0 g
- Monopotassium Phosphate 1.0 g
- Sodium Chloride 5.0 g
- Agar 10.0 g

Add 7.2 g of GC agar base powder (BD Difco, USA) in 100ml of distilled water and mix well to completely dissolve the powder. In a separate autoclave bottle, add 2.0 g of haemoglobin powder to 100 ml of distilled water to prepare 2% solution. Autoclave the GC base medium and haemoglobin solution for 15 min at 121°C. Cool the solution to approximately 50° C, add 100 ml of haemoglobin solution and 2ml of reconstituted Isovitalex enrichment to 100 ml of GC base medium. Mix gently and distribute into sterile petri dishes.

12. Fetal bovine serum

13. Glycerol

14. 0.85% (w/v) sodium chloride (NaCl) solution (100 mL)

Dissolve 0.85g of NaCl powder in 100 ml of distilled water and mix. Autoclave at 121°C for 15 min.

15. PrestoBlue^{TM} HS cell viability reagent

16. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)

- Glucose
- Sodium pyruvate
- Stable glutamine
- $NaHCO_3$
- Phenol red
- HEPES

Add 450 mL of Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Merck KGaA, Darmstadt, Germany) to 50 mL of Fetal bovine serum.

17. Trypsin

18. PBS

- Phosphate buffer 0.1 M
- Potassium chloride 0.0027 M
- Sodium chloride 0.137 M

Add two tablets to 400 mL of distilled water and autoclave at 121°C for 15 min.

19. Antibiotics

- Rapamycin (Autophagy inducing agents)
- Bafilomycin A1

20. Biomarkers used to study autophagy

- Anti-LC3B antibody (AB192890)
- Anti-SQSTM1/ p62 antibody (P0067)
- HRP-conjugated anti-rabbit IgG antibody (AP307P)

21. Precast NUPAGE Novex 4-12% Bis-Tris gels

22. MOP running buffer

MOPS (50mM),

Tris base (50mM)

0.1% SDS (0.1%)

EDTA (1mM)

23. NuPAGE 4 x LDS buffer supplemented with 2.5% β-mercaptoethanol.

24. PVDF (0.2 um)

25. Transfer buffer

Tris

Glycine (39 mM)

Methanol

SDS (0.0375%)

26. PBS-T

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10m mM
KH_2PO_4	1.8 mM
Tween-20	0.2%

Add 10 ml of 20% Tween-20 (Merck KGaA, Darmstadt, Germany) to 1000 ml of PBS(Merck KGaA, Darmstadt, Germany). Gently shake to dissolve and autoclave at 121°C for 15 min.

27. Pierce ECL Western blotting reagent

28. List of Suppliers

- Becton Dickinson, New Jersey, United States of America
- Biocom Africa (PTY) LTD
- Longhorn Vaccine and diagnostic, Bethesda, United States of America
- Hain Lifescience, Germany
- Merck KGaA, Darmstadt, Germany
- Sigma-Aldrich GmbH, Munich, Germany
- Thermo Fisher Scientific, Massachusetts, United States of America

APPENDIX B: Experimental procedures for *N. gonorrhoeae*

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1. **Revival of *N. gonorrhoeae* from a frozen glycerol stock**
   i. Remove *N. gonorrhoeae* glycerol (Merck KGaA, Darmstadt, Germany) stock from the -80 freezer and allow it to thaw.
   ii. Aseptically pipette 100 µL of the glycerol (Merck KGaA, Darmstadt, Germany) stock into 5 mL BHI broth (Merck KGaA, Darmstadt, Germany) supplemented with Isovitalex (gonococcal supplement) (BD Difco, USA) and incubate overnight in a 5% CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) overnight at 37^{O}C.
   iii. Next, inoculate 20 µL of the overnight broth on New York City Agar plates (Thermofisher Scientific, USA) and streak using a sterile loop.
   iv. Incubate the plates at 37^{O}C for 24 hrs in a 5% CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA).
2. **Gram-staining procedures**
   i. Prepare bacterial smear by mixing pure colony with a drop of sterile saline (0.85%) on a microscopic slide.
   ii. Fix the smear by passing it through a Bunsen burner for 5 s and air dry.
   iii. Flood the air-dried smear with crystal violet for 1 min and rinsed off with tap water for 10s.
   iv. Flood slide with mordant: Gram's iodine for 1 min, and rinsed off with tap water for 10 s.
   i. Flood slide with a decolourising agent (acetone-alcohol decolouriser) for 30 s and wash off with tap water for 10s.
   ii. Flood with a counterstain, safranin for 1 min, and wash off with tap water for 10 s.
   iii. Dry Gram stain slide with a blotting paper and examine microscopically using high-power oil immersion objective lens (Primo Star microscope, Carl Zeiss Microscopy GmbH, Jena, Germany).
3. **Oxidase test**
   i. Place a sterile filter paper on a sterile petri plate.

ii. Soak the filter paper with an oxidase reagent and allow it to dry.

iii. Pick up a colony of the bacteria from overnight culture using a sterile inoculating loop and make a smear on the reagent-soaked filter paper.

iv. Observe the colour change after 1 min.

4. Preparation of frozen *N. gonorrhoeae* glycerol stocks

i. Pick a pure colony of *N. gonorrhoeae* from an overnight culture and inoculate in a BHI broth supplemented with Isovitalex.

ii. Incubate the inoculated broth in a CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 35^{O}C for 18-24 hours.

iii. Add 750 µL of the overnight broth to a sterile cryotube containing 750 µL of 40% glycerol.

iv. Store *N. gonorrhoeae* glycerol stock at -20^{O}C for long-term storage

5. Procedure for Disc diffusion method for antibiotic susceptibility testing (Wiegand et al., 2008)

i. Streak the *N. gonorrhoeae* isolates, including the reference strains onto New York City Agar plates (Thermofisher Scientific, USA) using a sterile inoculating loop.

ii. Incubate plates for 18-24 h in a CO2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 35-37^{O}C.

iii. Suspended pure culture in a sterile 0.85% (w/v) NaCl solution (Merck KGaA, Darmstadt, Germany) and adjust the suspension's turbidity to a McFarland 0.5 by adding sterile saline, if the turbidity is high or by adding bacterial if it is low using DensiCHEK (bioMérieux SA, Marcy l'Etoile, France).

iv. Dip a sterile cotton swab into the suspension (pressing against the inside of the tube to remove excess fluid) and inoculate and swab the entire surface of the New York City agar plates (Thermofisher Scientific, USA) in three directions. After turbidity adjustment, the bacterial suspension should be used within 15 minutes.

v. Remove the antibiotic disk and place it in the centre of the inoculated agar using sterile forceps.

vi. Incubate the plates within 15 min of disk application in a 5% CO_2 incubator (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 35-37^{O}C.

vii. Read MIC at the point where the ellipse intersects the scale.

6. Preparation of Fastidious broth for culturing *N. gonorrhoeae*

i. Weigh 18.5 g of BHI powder (Merck KGaA, Darmstadt, Germany) and dissolve it in 500 mL of distilled water and gently heat to dissolve completely.

ii. Weigh 0.75 g of agarose into 100 mL of distilled water and gently heat it to dissolve completely.

iii. Mix 97 mL of BHI (Merck KGaA, Darmstadt, Germany) (37 g/mL) + 5 ml of fetal bovine serum (Merck KGaA, Darmstadt, Germany) + 1 mL of 0.75% agarose (Merck KGaA, Darmstadt, Germany) to prepare the broth.

iv. Sterilize by autoclaving at 121°C for 15 minutes.

v. Cool the broth to approximately 50^{O}C and add 1 mL of IsovitaX (BD Difco, USA) to prepare the fastidious broth.

7. Preparation of ceftriaxone, azithromycin, auranofin, and levofloxacin stock solution

i. Dissolve the powder of the antibiotic in 90% DMSO(Merck KGaA, Darmstadt, Germany) to prepare a stock of 10mM for each of the antibiotics. After determining the potency of the antibiotic, the amount of powder to used was determined using the formula:

Mass = Concentration x Volume x Molar mass

ii. Thus, weigh 78.502 mg of azithromycin (Merck KGaA, Darmstadt, Germany) , 66.16 mg of ceftriaxone (Merck KGaA, Darmstadt, Germany) , and 36.137 mg of levofloxacin(Merck KGaA, Darmstadt, Germany) and add to 10 ml of 90% DMSO to completely dissolve.

iii. Filtered sterilized with 0.22 µM GVS sterile membrane filter (Merck KGaA, Darmstadt, Germany) .

iv. The final concentration of DMSO (Merck KGaA, Darmstadt, Germany) in all the assay wells was less than 1%.

v. Aliquot the prepared stock solution and store in a clean freezer at -80°C for up to six months.

8. Dilution of Pathogen Box compound

i. Add 90 µL of 90% DMSO (Merck KGaA, Darmstadt, Germany) to 10 µL of 10 mM (initial concentration) to prepare 1 mM stock concentration.

ii. Pipette 10 uL of 1 ml stock concentration to a new plate and add 90 uL of deionised distilled water to prepare a stock concentration of 100µm.

9. Broth microdilution (BMD) method for susceptibility testing of Pathogen Box compounds against *N. gonorrhoeae* strains

A. Preparation of microtitre plates for MIC and MBC determination.

i. A concentration ranging from 0.156 to 10 µM was used to determine the pathogen-specific MIC and MBC of the Pathogen Box compounds.

ii. Add 100 µL of fastidious broth to each of the wells of the 96-well microtitre plate (Lasec, South Africa).

iii. Next, add 100 µL of the compound at a working concentration of 20 µM to the 1st well of each row in the 96-well microtitre plate (Lasec, South Africa). This will result in a 1:2 dilution of the working solution and will be the starting (highest) concentration to be tested. (If a 20 µM working solution is used, the first well will have a concentration of 10 µM after the 1:2 dilution).

iv. Using a multichannel pipette, set to a volume of 100 µL, the compound-broth mixture is mixed in well 1.

v. Then 100 µL is transferred from well 1 to well 2 and mixed. This process is repeated until well 10. Once well 10 is mixed, the 100 uL in the pipette tip is discarded. Each well now contains a 100 µL mixture of compound and growth medium (broth) with progressive two-fold serial dilutions.

vi. Wells 11 and 12 will only contain the fastidious broth.

vii. Well 11 was used as a growth control (broth plus bacterial inoculum, no compound) and well 12 was used as a sterility control (broth only).

viii. The prepared microtitre plates may be used immediately or stacked, sealed, and stored in a clean freezer at -80°C for up to 6 months.

B. Inoculum preparation, and inoculation of the BMD plate

i. Prepare a 0.5 McFarland standard culture suspension from an overnight bacterial culture. The 0.5 McFarland standard bacterial suspension will contain approximately 1-2 x 10^8 CFU/mL.

ii. Next, 0.5 mL of the 0.5 McFarland turbidity standard bacterial suspension was added to 4.5 mL of sterile saline within 15 minutes of preparation. This resulted in a 1:10 dilution and yield a concentration of 10^7 CFU/mL.

iii. Subsequently, 5 µL of this 10^7 CFU/mL bacterial suspension was inoculated into a well containing 100 µL volume (broth + compound), this resulted in a final solution in each well of $5x10^5$ CFU/mL.

C. MIC Determination

i. Within 15 minutes of inoculum preparation, inoculate wells 1 to 11 of the 96-well microtitre plate with 5 µL of the inoculum. Well 11 serves as a growth control (contains broth and inoculum) and well 12 serves as the sterility control (broth only).

ii. The plates will be incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 35°C in 5% CO2 for 24 hours.

iii. After incubation, 10 µl of PrestoBlue™ HS Cell Viability Reagent (Thermofisher Scientific, USA) will be added to each well and incubated for 30 minutes.

iv. A change in colour from blue to pink/red is an indication of bacterial growth. The lowest concentration with no colour change will be recorded as the MIC.

D. MBC determination

i. Subsequently, an aliquot will be taken from MIC assays where there is no visible growth and then plated on New York City (NYC) agar plates (Thermofisher Scientific, USA). The plates will be incubated overnights.

ii. The MBC was determined as the lowest concentration that is able to produce a 99.9% decrease in visible bacterial growth.

iii. The experiment was repeated three times for only compounds which show activity in the first screen.

10. Procedure for time-kill kinetics

i. An overnight culture of *N. gonorrhoeae* was diluted in fresh fastidious broth and incubated until the inoculum was ~5×10^6 CFU/mL was achieved.

ii. Next, the bacterial solution was exposed to 3 × MIC of the tested compounds or drugs.

iii. DMSO (Merck, Germany) was used as a negative control and azithromycin as a positive control.

iv. After 0, 2, 4, 6, 10 and 12 hrs, an aliquot was collected, diluted (10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}), and plated onto GC II agar supplemented with hemoglobin (Sigma-Aldrich, USA) and 1% IsoVitaleX (BD Difco, USA).

v. Plates were incubated (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) at 37°C with 5% CO_2 for 24 hours.

vi. Colonies between $30 \leq x \leq 300$ will be counted.

vii. The number of colony forming units per ml will be determined from the following equation:

$$\text{CFU/ml} = \left(\frac{\text{number of colonies}}{\text{Amount plated x dilution}}\right)$$

11. Checkerboard assay for combination testing.

i. A 1.0 McFarland standard suspension from overnight *N. gonorrhoeae* isolates was prepared and diluted in a fresh broth to achieve a bacterial inoculum of 5×10^5 CFU/mL.

ii. The compounds and convectional drug ceftriaxone (Merck, Germany) were added at different concentrations along with bacteria-containing media.

iii. Increasing concentrations of one compound were added to half the MIC of another compound to determine the lowest and best concentrations with synergy.

iv. The plates were incubated for 24 hours at 37°C in the presence of 5% CO_2.

v. After incubation, PrestoBlue™ HS Cell Viability Reagent (Thermofisher Scientific, USA) will be added to each well and incubated for 30 minutes.

vi. A change in colour from blue to pink/red is an indication of bacterial growth. The lowest dual concentration with no colour change will be recorded as the MIC of those compounds/drugs.

APPENDIX C: *M. tuberculosis* - Experimental procedures

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1. Inoculation and incubation of *M. tuberculosis*

i. Label MGIT(BD Diagnostics, USA) with the specific label that contains identifying information.
ii. Reconstitute the MGIT (BD Diagnostics, USA) supplement and add 800 µL to MGIT (BD Diagnostics, USA).
iii. Add 200 µL of stored Mtb suspension to supplemented MGIT tube.
iv. Placed the inoculated MGIT in the BD BACTEC ™ MGIT ™ 960 instrument.
v. Incubate until the culture tubes flag positive.

2. Genomic Mtb. DNA extraction procedures using the PrimeXtractTM kit

i. Warm the elution solution in a heating block to 60 - 75ºC.
ii. In a clean 1.5 ml microcentrifuge tube, prepare the sample by combining 200 µL of 100% ethanol, 200 µL of Lysis Buffer, and 200 µL of the sample. Vortex for 5 seconds and speed down to remove liquid from the cap.
iii. Incubate at room temperature for 5 minutes.
iv. Transfer the entire contents of the prepared sample in step (ii) into the extraction column. Close the extraction column and centrifuge at 13 x 1000 g for 1 minute.
v. Remove the extraction column from the collection tube and discard the flow (elute) into a waste container. Place the column back into a collection tube.
vi. Add 500 µl of Wash Buffer 1 to the extraction column and centrifuge at 13 x 1000 g for 60 seconds.
vii. Remove tubes from centrifuge and discard flow through (eluate) into waste container. Place column back into collection tube.
viii. Add 500 µl of Wash Buffer 2 to the extraction column. Centrifuge for 60 seconds at 13 x 1000 g. Discard the flow-through from collection tube. Place column back into collection tube.
ix. Repeat step viii to wash the filter twice with Wash Buffer 2.
x. Centrifuge extraction column for an additional 1 minute at 13 x 1000 g to remove trace amounts of Wash Buffers from filter.

xi. Discard collection tube and transfer extraction column (with bound nucleic acid) into a clean 1.5 mL microcentrifuge tube

xii. Pipette 50 µl of pre-heated Elution Solution directly onto the silica filter in each column without touching the filter and incubate for 60 seconds.

xiii. Centrifuge the columns for 60 seconds at 13 x 1000 g. Remove and discard the column from the microcentrifuge tube.

xiv. Store nucleic acid at -20 to 4ºC until ready for use. For long-term storage, nucleic acid should be stored at -80ºC.

3. MTBDRplus VER.2 and MTBDRsl VER. 2.0 assay.

We followed the manufacturer's instructions (Hain Lifescience, Germany for the PCR amplification, preparation, and hybridization procedure (Lifescience H, 2015).

3.1 Preparation of PCR amplification Mix (50 µL total volume)

i. 35 µL primer nucleotide mix (amplification mix-B; AM-B).

ii. 10 µL of polymerase mix (amplification mix-A; AM-A)

iii. Add 5 µL of Mtb DNA

Table 1: PCR amplification cycling condition

15 min 95^{O}C	1 cycle
30 sec 95^{O}C	10 cycles
2 min 65 ^{O}C	
25 sec 65 ^{O}C	20 cycles
40 sec 50 ^{O}C	
40 sec 70 ^{O}C	
8 min 70 ^{O}C	**1 cycle**

Store amplification products at -20 ^{O}C until ready to be used.

3.2 Hybridization using GT-Blot

3.2.1 Preparation

i. Thoroughly clean the workbench with 1% bleach (sodium hypochlorite).

ii. Run both the A and B wash cycles in the GT-blot.

iii. Prepare reagents per the table calculations, following clean-up.

iv. Transfer the prepared reagents into the reagents bottles supplied with the GT-Blot

v. Return the bottles containing the GT-Blot reagent back to the machine.

vi. Next, screw a tubed lid on the bottles.

vii. Put strips on a clean paper surface using clean forceps and label them in accordance with the worksheet.

viii. Use a new or cleaned tray for each test, and check for any wear and tear.

Step-1:

a. Add 20 µL denaturation solution (DEN, blue) into the corner of each well.

Step 2:

b. Pipette 20 µL amplicon to DEN and pipette up and down to mix thoroughly.

c. Incubate it for 5 minutes before proceeding.

d. Change the tips or use a new tip for every sample loading.

e. Place the tray in GT-Blot with caution.

f. Close the lid of GT-Blot and press start.

g. Choose an appropriate number of wells (always in even number) and press start.

NB: Change the tips between each loading of the sample.

Step 3:

h. The machine automatically dispenses the hybridization solution to each of the wells that was selected.

i. "Add amplicon" message appeared or came up.

Step 4:

j. Using clean forceps, properly put the labelled strips in each well with the face-up.

k. Clean the lid of the GT-Blot

l. 'Press start'

NB: A fully automated GT-Blot machine performed all subsequent steps automatically.

4. Preparation of Middlebrook 7H9 broth (500 mL)

i. Suspend 2.35 g of Middlebrook 7H9 Broth Base into an autoclave bottle and add 450 mL containing distilled water.

ii. Add 2 mL of glycerol and heat to dissolve.

iii. Autoclave at 121^{O}C for 40 minutes.

iv. Cool the broth to about 50^{O}C and add 50 mL of OADC growth supplement.

v. Mix well before dispensing.

5. Preparation of Middlebrook 7H9 agar (500 mL)

i. Suspend 10.25 g of Middlebrook 7H11 Agar Base (BD Diagnostics, USA) into an autoclave bottle and add 450 mL containing distilled water.

ii. Add 2.5 ml of glycerol and boil to dissolve completely.

iii. Sterilize by autoclaving at 121^{O}C for 40 minutes.

iv. Cool to about 50^{O}C and add aseptically add 50 ml of OADC growth Supplement (BD Diagnostics, USA).

v. Mix thoroughly and dispense into a sterile petri dish (Lasec, South Africa).

6. Preparation of microtitre plates for MIC and MBC determination of Pathogen Box compounds against M. tuberculosis strains.

i. A concentration ranging from 0.156 to 10 µM is used to determine the pathogen-specific MIC and MBC of the Pathogen Box compounds.

ii. Add 100 µL of Middlebrook 7H9 broth (BD Diagnostics, USA) to each of the wells of the 96-well microtitre plate (Lasec, South Africa).

iii. Next, add 100 µL of the compound at a working concentration of 20 µM to the 1st well of each row in the 96-well microtitre plate (Lasec, South Africa). This will result in a 1:2 dilution of the working solution and will be the starting (highest) concentration to be tested. (If a 20 µM working solution is used, the first well will have a concentration of 10 µM after the 1:2 dilution).

iv. Using a multichannel pipette, set to a volume of 100 µL, the compound-broth mixture is mixed in well 1.

v. Then 100 µL is transferred from well 1 to well 2 and mixed. This process is repeated until well 10. Once well 10 is mixed, the 100 uL in the pipette tip is discarded. Each well now contains a 100 µL mixture of compound and Middlebrook 7H9 broth (BD Diagnostics, USA) with progressive two-fold serial dilutions.

vi. Well 11 was used as a growth control (broth plus bacterial inoculum, no compound) and well 12 was used as a sterility control (broth only).

vii. The prepared microtitre plates may be used immediately or stacked, sealed, and stored in a clean freezer at -80°C for up to 6 months.

6.1 Inoculum preparation and inoculation of the BMD plate

i. A positive flag culture MGIT (BD Diagnostics, USA) was centrifuged at 3500 × *g* for 10 min at room temperature and the supernatants were discarded.

ii. The pellets were washed twice with PBS (Merck, Germany) and re-suspended in Middlebrook 7H9 broth supplemented with OADC (BD Diagnostics, USA).

iii. The optical density was adjusted to 0.6, yielding ca. 10^7–10^8 CFU/mL.

iv. Next, 0.5 mL of the 0.5 McFarland turbidity standard bacterial suspension was added to 4.5 mL of sterile saline within 15 minutes of preparation. This resulted in a 1:10 dilution and yield a concentration of 10^7 CFU/mL.

6.2 MIC Determination

i. Within 15 minutes of inoculum preparation, inoculate wells 1 to 11 of the 96-well microtitre plate with 5 µL of the inoculum. Well 11 serves as a growth control (contains broth and inoculum) and well 12 serves as the sterility control (broth only).

ii. The plates were sealed with paraffin (Sigma-Aldrich, USA), placed in a biohazard bag, and incubated at room temperature for 7 days.

iii. After incubation, 10 µl of PrestoBlue™ HS Cell Viability Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) will be added to each well and incubated for 30 minutes.

iv. A change in colour from blue to pink/red is an indication of bacterial growth. The lowest concentration with no colour change will be recorded as the MIC.

6.3 MBC determination

i. Subsequently, an aliquot will be taken from MIC assays where there is no visible growth and then plated on Middlebrook 7H11 agar plates (BD Diagnostics, USA).

ii. The plates were sealed with paraffin (Sigma-Aldrich, USA), placed in a biohazard bag (Lasec, USA), and incubated at room temperature for at least 3 weeks.

7. Procedure for time-kill kinetics

i. A positive flag *M. tuberculosis* culture in MGIT (BD Diagnostics, USA) was adjusted to ~5 × 10^6 CFU/mL.

ii. Next, the bacterial solution was exposed to 20 × MIC of the tested compounds or drugs.

iii. DMSO (Merck, Germany) was used as a negative control.

iv. After 0, 3, 6, 10 and 14 days, an aliquot was collected, diluted (10^{-1},10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}), and plated onto Middlebrook 7H11 agar (BD Diagnostics, USA) supplemented with OADC (BD Diagnostics, USA).

v. Plates were incubated at 37°C for at least 21 days.

vi. A colony counter (Lasec) was used to determine the CFU/mL.

vii. Colonies between $30 \leq x \leq 300$ will be counted.

viii. The number of colony-forming units per ml will be determined from the following equation:

$$\text{CFU/ml} = \left(\frac{\text{number of colonies}}{\text{Amount plated x dilution}}\right)$$

8. Checkerboard assay for combination testing.

i. A positive flag *M. tuberculosis* culture in MGIT (BD Diagnostics, USA) was adjusted to ~5×10^6 CFU/mL.

ii. The compounds and convectional drug rifampicin and isoniazid were added at different concentrations along with bacteria-containing media.

iii. Increasing concentrations of one compound were added to half the MIC of another compound to determine the lowest and best concentrations with synergy.

iv. Plates were incubated at 37°C for at least 21 days.

v. After incubation, PrestoBlue™ HS Cell Viability Reagent was added to each well and incubated for 30 minutes.

vi. A change in colour from blue to pink/red is an indication of bacterial growth.

vii. The lowest dual concentration with no colour change will be recorded as the MIC of those compounds/drugs.

9. Culturing of murine-derived macrophages from frozen stock

i. Remove the cryovial containing the frozen cells from the liquid nitrogen storage. and immediately place it into a 37°C water bath.

ii. Thaw frozen cells rapidly (<1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.

iii. Move the vial to the laminar flow hood and wipe the outside with 70% ethanol before opening.

iv. Transfer the thawed cells dropwise into the centrifuge tube containing prewarmed DMEM F-12 (Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Merck KGaA, Darmstadt, Germany).

v. Centrifuge (Labnet International Inc, USA) the cell suspension at approximately 200 x g for 5–10 minutes.

vi. check the clarity of the supernatant and the visibility of a complete pellet.

vii. Aseptically decant the supernatant without disturbing the cell pellet.

viii. Resuspend the cells in complete DMEM F-12 (Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Merck KGaA, Darmstadt, Germany) and transfer them into 6-well tissue culture plates (Lasec, South Africa).

ix. Incubate cells at 37°C in a CO2 incubator (Stuart incubator Si500, Bibby Scientific Group, UK).

10. Procedure for passaging

i. Remove and discard spent DMEM F12 (Merck KGaA, Darmstadt, Germany) supplemented with FBS (Merck KGaA, Darmstadt, Germany) from the culture vessel.

ii. Gently add 1 X PBS to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth gently to wash the cells.

iii. Remove and discard 1 x PBS from the culture vessel.

iv. Gently add the prewarmed trypsin and rock the container to get complete coverage of the cell layer.

v. Incubate the culture vessel at room temperature for approximately 2 minutes.

vi. Use the microscope to observe cell detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 minutes. Also, tap the vessel to increase cell detachment.

vii. When ≥90% of the cells have detached, add twice the volume used for the dissociation reagent of prewarmed DMEM F-12 (Merck KGaA, Darmstadt, Germany) supplemented with 10% FBS (Merck KGaA, Darmstadt, Germany).

viii. Disperse the medium by pipetting over the cell layer surface several times.

ix. Transfer the cells to a 15 mL conical tube and centrifuge (Labnet International Inc, USA) at 200 x *g* for 5–10 minutes.

x. Resuspend the cell pellet in a minimal volume of prewarmed complete growth medium and remove a sample for counting.

xi. Determine the total number of cells and percent viability using a hemocytometer, cell counter, and trypan blue exclusion.

xii. Dilute the cell suspension by adding DMEM F12 (Merck KGaA, Darmstadt, Germany) supplemented with FBS (Merck KGaA, Darmstadt, Germany) and pipet the appropriate volume into new cell culture and return the cells to the 37^{O}C incubator (Stuart incubator Si500, Bibby Scientific Group, UK).

11. Cell viability of five selected Pathogen Box compounds

i. A 96-well microtitre plate was seeded with 1.0 x 10^4 macrophages.

ii. Treat cells with specific concentrations of the Pathogen Box compounds.

iii. Incubate the treated cells for 48 hours in a 5% CO_2 incubator (Stuart incubator Si500, Bibby Scientific Group, UK) at 37^{O}C.

iv. Add PrestoBlue™ HS Cell Viability Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA).

v. Incubate for 30 minutes.

vi. examined using a microplate reader (Agilent BioTek Epoch, Santa Clara, CA, United States) with the excitation/emission wavelengths set at 560/590 nm.

vii. The percentage of viable cells was calculated according to the following formula: % cell viability = $\left(\frac{\text{Average OD of treated cells}}{\text{Average OD of control cells}}\right) x\ 100$

12. Procedure for infection of murine-derived macrophages with Mtb and treatment of infected Macrophages with Pathogen Box control

i. A positive flag MGIT (BD Diagnostics, USA) was washed twice with PBS and the turbidity adjusted to 1.0 McFarland standard.

ii. Cells are seeded in 6 well microtitre plates (Lasec, South Africa)

iii. Infect macrophages with *M. tuberculosis* H37Rv at a multiplicity of infection (moi) at 10:1 for 2 hrs.

iv. Wash with 1 x PBS (Merck KGaA, Darmstadt, Germany) to remove any extracellular or unbound bacteria.

v. Add a complete growth medium (DMEM F-12 supplemented with 10% FBS) (Merck KGaA, Darmstadt, Germany) containing each of the Pathogen Box compounds (at specific concentrations) with or without bafilomycin (Merck KGaA, Darmstadt, Germany).

vi. Macrophage-infected Mtb was used as a s negative control and macrophage-infected Mtb treated with rapamycin was used a positive control for autophagy assay.

vii. Incubate the treated cells for 24 hours in a 5% CO_2 incubator (Stuart incubator Si500, Bibby Scientific Group, UK) at 37^{O}C.

13. Preparation of the western blot assay

13.1 Preparation of lysate

i. At termination, heat 150 – 200 µL of 1 x diluted LDS sample buffer (Merck, Germany) containing 2.5% β-mercaptoethanol in 2 ml Eppendorf tube (epp) on a hotplate to 80˚C.

ii. Take off medium and wash well once with PBS (Merck, Germany).

iii. Add hot loading buffer directly to the plate.

iv. Use a plunger of 1 ml syringe (Merck, Germany) to scrape cells together and collect lysate with a pipette and put in epp. Pipette up and down for a few times to enhance cell lysis.

v. Heat epp. to 80°C for 10 minutes.

vi. Centrifuge (Labnet International Inc, USA) at 14000 g for 20 minutes to remove nuclei and cell debris.

vii. Carefully transfer supernatant to clean epp.

13.2 Gel electrophoresis (loading protein sample and transfer of proteins from gel to membrane)

i. Load 20-30 µL of the sample onto gel placed in a gel running module containing MOPS running buffer (Merck KGaA, Darmstadt, Germany). Also, add 5 µL of protein ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

ii. Run gel at 100-120 V but stop before colour loading front reaches bottom of gel.

iii. Open the gel cassette and get gel into transfer buffer.

iv. Activate 0.2 µm PVDF (Thermo Fisher Scientific Inc., Waltham, MA, USA) in methanol.

v. Make stack using bottom-3 filter paper, gel membrane and 3 filter paper-top on the black side of transfer cassette.

vi. Transfer proteins in transfer buffer at 90V for 1.5 hours at 4°C or overnight at 60V.

vii. Stain membrane using Ponceau S solution (Thermo Fisher Scientific Inc., Waltham, MA, USA) to see if proteins transferred.

viii. Wash membrane in PBS-T (Merck KGaA, Darmstadt, Germany) once.

ix. Incubate membrane in 50 ml tube on rollerbank at room temperature for 1 h in 5 ml PBS-T (Merck KGaA, Darmstadt, Germany) containing 2% BSA (Merck KGaA, Darmstadt, Germany).

x. Remove the blocking solution and incubate membrane in 5 ml fresh blocking solution containing 1:1000 dilution of LC3 antibody (Biocom Diagnostic, South Africa), 1:5000 p62 antibody (Biocom Diagnostic, South Africa) and 1:5000 actin (Merck KGaA, Darmstadt, Germany) for 1 hour at RT on rollerbank.

xi. Wash 3 times in PBS-T (Merck KGaA, Darmstadt, Germany) each for 5 minutes.

xii. Incubate membrane in 5 ml blocking solution containing 1: 10 000 dilution secondary anti-mouse HRP antibody (Biocom Diagnostic, South Africa) for 1 hour at RT on rollerbank.

xiii. Wash membrane 3 times in PBS-T (Merck KGaA, Darmstadt, Germany) 5 minutes each.

13.3 Detection of autophagy-related factors

i. Make a 1:1 dilution of ECL substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) (600 µL of each) and pour it on the membrane.

ii. Take the ECL membrane (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a force for imaging using the gel doc system.

iii. LC3 II and p62 levels are compared with actin for each lane.

iv. Also controls such as starvation (6 hours in HBSS) and inhibitors such as bafilomycin A1 (Biocom Diagnostic, South Africa) were included to investigate effects on the total levels of autophagic flux.

14. Intracellular killing, and colony-forming unit assay

i. Take off medium and wash well once with PBS (Merck KGaA, Darmstadt, Germany).

ii. Add 1 X PBS (Merck KGaA, Darmstadt, Germany) containing 0.1% Triton X-100 (Merck KGaA, Darmstadt, Germany) to induce lysis of macrophages.

iii. Centrifuge (Labnet International Inc, USA) at 3500 × *g* for 10 min at room temperature and discard the supernatants.

iv. Prepare 10-fold serial dilutions ($10^{-1,}$ 10^{-2},10^{-3},10^{-4} and 10^{-5}) of cell lysates.

v. Plate on Middlebrook 7H11 agar (BD Diagnostics, USA) supplemented with 10% OADC (BD Diagnostics, USA).

vi. Incubate (Heracell™ VIOS 250i CO_2 incubator, Thermofisher Scientific, USA) plates at 37 °C and count colonies after 3 weeks of incubation.

References

Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature protocols. 2008 Feb;3(2):163-75.

Lifescience, H. (2015). GenoType MTBDRplus VER 2.0. Molecular Genetic Assay for Identification of the Mycobacterium tuberculosis Complex and its Resistance to Rifampicin and Isoniazid from Clinical Specimens and Cultivated Samples.

APPENDIX D: Laboratory data

Table 1: Time kill kinetics* of five selected Pathogen Box compounds against MDR *M. tuberculosis*

	Days				
	0	**3**	**6**	**10**	**14**
DMSO (cfu/mL)	**Mean:** 8.0×10^5 **R1:** 9.0×10^5 **R2:** 7.0×10^5	**Mean:** 4.5×10^6 **R1:** 4.9×10^6 **R2:** 4.1×10^6	**Mean:** 3.5×10^6 **R1:** 3.8×10^6 **R2:** 3.2×10^6	**Mean:** 4.5×10^7 **R1:** 5.5×10^7 **R2:** 3.5×10^7	**Mean:** 1.5×10^8 **R1:** 1.1×10^8 **R2:** 1.9×10^8
MMV676603 (cfu/mL)	**Mean:** 1.0×10^6 **R1:** 9.0×10^5 **R2:** 1.1×10^6	**Mean:** 4.2×10^2 **R1:** 5.0×10^2 **R2:** 3.4×10^2	**Mean:** 1.0×10^2 **R1:** 1.2×10^2 **R2:** 8.0×10^1	0	0
MMV687146 (cfu/mL)	**Mean:** 8.0×10^5 **R1:** 9.0×10^5 **R2:** 7.0×10^5	**Mean:** 8.0×10^2 **R1:** 1.0×10^3 **R2:** 6.0×10^2	**Mean:** 1.2×10^2 **R1:** 1.5×10^2 **R2:** 9.0×10^1	0	0
MMV687696 (cfu/mL)	**Mean:** 1.0×10^6 **R1:** 8.0×10^5 **R2:** 1.2×10^6	**Mean:** 5.0×10^4 **R1:** 6.6×10^4 **R2:** 3.4×10^4	**Mean:** 6.0×10^4 **R1:** 5.3×10^4 **R2:** 6.7×10^4	**Mean:** 4.0×10^4 **R1:** 3.4×10^4 **R2:** 4.6×10^4	**Mean:** 2.6×10^5 **R1:** 2.3×10^5 **R2:** 2.9×10^5
MMV687180 (cfu/mL)	**Mean:** 8.0×10^5 **R1:** 8.0×10^5 **R2:** 8.0×10^5	**Mean:** 2.0×10^3 **R1:** 1.0×10^3 **R2:** 3.0×10^3	**Mean:** 4.0×10^2 **R1:** 4.5×10^2 **R2:** 3.5×10^2	0	0
MMV153413 (cfu/mL)	**Mean:** 8.5×10^5 **R1:** 9.0×10^5 **R2:** 8.0×10^5	**Mean:** 6.0×10^3 **R1:** 5.0×10^3 **R2:** 7.0×10^3	**Mean:** 8.0×10^2 **R1:** 9.0×10^2 **R2:** 7.0×10^2	**Mean:** 2.0×10^2 **R1:** 3.0×10^2 **R2:** 1.0×10^2	0

*The time-kill kinetics of Pathogen Box compounds. 20 x MIC of each compound was used against ~ 1.0×10^6 MDR *M. tuberculosis*.
R1, repeat 1; R2, repeat 2

Table 2: Time kill kinetics of dual combination of Pathogen Box compounds against *N. gonorrhoeae*

	Time (hrs)							
	0	2	4	6	8	10	12	24
DMSO (cfu/mL)	**Mean:** 1.02 x 10^6 **R1:** 1.01 x 10^6 **R2:** 1.03 x 10^6	**Mean:** 2.27 x 10^6 **R1:** 2.1 x 10^6 **R2:** 2.4 x 10^6	**Mean:** 1.74 x 10^6 **R1:** 1.68 x 10^6 **R2:** 1.8 x 10^6	**Mean:** 4.4 x 10^6 **R1:** 4.3 x 10^6 **R2:** 4.5 x 10^6	**Mean:** 1.9 x 10^7 **R1:** 1.8 x 10^7 **R2:** 2.0 x 10^7	**Mean:** 3.55 x 10^7 **R1:** 3.3 x 10^7 **R2**: 3.8 x 10^7	**Mean:** 6.8 x 10^7 **R1:** 6.0 x 10^7 **R2:** 7.6 x 10^7	**Mean:** 3.45 x 10^{10} **R1:** 3.3 x 10^{10} **R2:** 3.6 x 10^{10}
Ceftriaxone + MMV688978 (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.26 x 10^6 **R1:** 1.2 x 10^6 **R2:** 1.32 x 10^6	**Mean:** 1.7 x 10^5 **R1:** 1.6 x 10^5 **R2:** 1.8 x 10^5	**Mean:** 5.0 x 10^4 **R1:** 4.9 x 10^4 **R2:** 5.1 x 10^4	**Mean:** 4.4 x 10^4 **R1:** 4.0 x 10^4 **R2:** 4.8 x 10^4	**Mean:** 4.7 x 10^3 **R1:** 4.6 x 10^3 **R2:** 4.8 x 10^3	**Mean:** 5.0 x 10^2 **R1:** 4.0 x 10^2 **R2:** 6 x 10^2	**Mean:** 1.0 x 10^2 **R1:** 1.0 x 10^2 **R2:** 1.0 x10^2	0
Ceftriaxone + MMV6887798 (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.26 x 10^6 **R1:** 1.2 x 10^6 **R2:** 1.32 x 10^6	**Mean:** 4.0 x 10^5 **R1:** 3.8 x 10^5 **R2:** 4.2 x 10^5	**Mean:** 1.81 x 10^5 **R1:** 1.7 x 10^5 **R2:** 1.92 x 10^5	**Mean:** 7.9 x 10^4 **R1:** 7.0 x 10^4 **R2:** 8.8 x 10^4	**Mean:** 2.3 x 10^4 **R1:** 2.1 x 10^4 **R2:** 2.5 x 10^4	**Mean:** 1.25 x 10^4 **R1:** 1.2 x 10^4 **R2:** 1.3 x 10^4	**Mean:** 6.0 x 10^2 **R1:** 5.0 x 10^2 **R2:** 7.0 x 10^2	0
Ceftriaxone + MMV024937 (Pbc 6) (cfu/mL)	**Mean:** 1.1 x 10^6 **R1:** 1.01 x 10^6 **R2:** 1.2 x 10^6	**Mean:** 3.9 x 10^5 **R1:** 3.8 x 10^5 **R2:** 4.0 x 10^5	**Mean:** 3.6 x 10^5 **R1:** 3.5 x 10^5 **R2:** 3.7 x 10^5	**Mean:** 6.8 x 10^4 **R1:** 6.5 x 10^4 **R2:** 7.1 x 10^4	**Mean:** 3.9 x 10^4 **R1:** 3.8 x 10^4 **R2:** 4.0 x 10^4	**Mean:** 2.8 x 10^4 **R1:** 2.5 x 10^4 **R2:** 3.1 x 10^4	**Mean:** 7.0 x 10^2 **R1:** 6.5 x 10^2 **R2:** 7.5 x 10^2	0
MMV024937 (Pbc 6)+ **MMV688978** (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.15 x 10^6 **R1:** 1.1 x 10^6 **R2:** 1.2 x 10^6	**Mean:** 3.0 x 10^5 **R1:** 2.5 x 10^5 **R2:** 3.5 x 10^5	**Mean:** 3.1 x 10^3 **R1:** 3.0 x 10^3 **R2:** 3.2 x 10^3	0	0	0	0	0
MMV024937 (Pbc 6) + **MMV6887798** (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.15 x 10^6 **R1:** 1.1 x 10^6 **R2:** 1.2 x 10^6	**Mean:** 4.4 x 10^5 **R1:** 4.0 x 10^5 **R2:** 4.8 x 10^5	**Mean:** 2.9 x 10^5 **R1:** 2.7 x 10^5 **R2:** 3.1 x 10^5	**Mean:** 2.6 x 10^5 **R1:** 2.5 x 10^5 **R2:** 2.7 x 10^5	**Mean:** 7.0 x 10^4 **R1:** 6.4 x 10^4 **R2:** 7.6 x 10^4	**Mean:** 8.0 x 10^3 **R1:** 7.0 x 10^3 **R2:** 9.0 x 10^3	**Mean:** 1.0 x 10^3 **R1:** 9.0 x 10^2 **R2:** 1.1 x 10^3	0

The time-kill kinetics of dual combination of Pathogen Box compounds. 3 x MIC of each compound in combination found to be additive was used against ~ 1.0 x 10^6 *N. gonorrhoeae*. R1, repeat 1; R2, repeat 2.

Table 3: Time kill kinetics of Pathogen Box compounds against *N. gonorrhoeae.*

	Time (hrs)							
	0	2	4	6	8	10	12	24
DMSO (cfu/mL	**Mean:** 1.02 x 10^6 **R1:** 1.01 x 10^6 **R2:** 1.03 x 10^6	**Mean:** 2.27 x 10^6 **R1:** 2.13 x 10^6 **R2:** 2.4 x 10^6	**Mean:** 1.74 x 10^6 **R1:** 1.68 x 10^6 **R2:** 1.8 x 10^6	**Mean:** 4.4 x 10^6 **R1:** 4.3 x 10^6 **R2:** 4.5 x 10^6	**Mean:** 1.9 x 10^7 **R1:** 1.8 x 10^7 **R2:** 2.0 x 10^7	**Mean:** 3.55 x 10^7 **R1:** 3.3 x 10^7 **R2:** 3.8 x 10^7	**Mean:** 6.8 x 10^7 **R1:** 6.0 x 10^7 **R2:** 7.6 x 10^7	**Mean:** 3.45 x 10^{10} **R1:** 3.3 x 10^{10} **R2:** 3.6 x 10^{10}
MMV676501 (Pbc 1) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.55 x 10^5 **R1:** 2.8 x 10^5 **R2:** 2.3 x 10^5	**Mean:** 1.7 x 10^5 **R1:** 1.62 x 10^5 **R2:** 1.78 x 10^5	**Mean:** 1.1 x 10^5 **R:1** 1.0 x 10^5 **R2:** 1.2 x 10^5	**Mean:** 8.9 x 10^4 **R1:** 8.5 x 10^4 **R2:** 9.3 x 10^4	**Mean:** 1.96 x 10^3 **R1:** 1.92 x 10^3 **R2:** 2.0 x 10^3	**Mean:** 2.0 x 10^2 **R1:** 1.6 x 10^2 **R2:** 2.4 x 10^2	0
MMV002817 (Pbc 3) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 3.6 x 10^5 **R1:** 3.5 x 10^5 **R2:** 3.7 x 10^5	**Mean:** 3.5 x 10^5 **R1:** 3.4 x 10^5 **R2:** 3.6 x 10^5	**Mean:** 1.81 x 10^5 **R1:** 1.8 x 10^5 **R2:** 1.82 x 10^5	**Mean:** 5.0 x 10^4 **R1**: 4.5 x 10^4 **R2:** 5.5 x 10^6	**Mean:** 2.35 x 10^3 **R1:** 2. x 10^3 **R2:** 2.4 x 10^3	**Mean:** 1.5 x 10^3 **R1:** 1.4 x 10^3 **R2:** 1.6 x 10^3	0
MMV688327 (Pbc 4, Radezolid) (cfu/mL)	**Mean:** 1.04 x 10^6 **R1:** 1.02 x 10^6 **R2:** 1.06 x 10^6	**Mean:** 9.95 x 10^5 **R1:** 9.9 x 10^5 **R2:** 1.0 x 10^6	**Mean:** 4.4 x 10^5 **R1:** 4.2 x 10^5 **R2:** 4.6 x 10^5	**Mean:** 2.4 x 10^5 **R1:** 2.3 x 10^5 **R2:** 2.5 x 10^5	**Mean:** 1.2 x 10^5 **R1:** 1.1 x 10^5 **R2:** 1.3 x 10^5	**Mean:** 3.0 x 10^4 **R1:** 2.0 x 10^4 **R2:** 4.0 x 10^4	**Mean:** 4.0 x 10^3 **R1:** 3.0 x 10^3 **R2**: 5.0 x 10^3	0
MMV688508 (Pbc 5) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.02 x 10^6 **R2:** 1.03 x 10^6	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2**: 1.0 2 x 10^6	**Mean:** 2.05 x 10^5 **R1:** 2.0 x 10^5 **R2:** 2.1 x 10^5	**Mean:** 6.8 x 10^5 **R1:** 6.5 x 10^5 **R2:** 7.1 x 10^5	**Mean:** 3.7 x 10^5 **R1:** 3.5 x 10^5 **R2:** 3.9 x 10^5	**Mean:** 5.0 x 10^3 **R1:** 4.5 x 10^3 **R2:** 5.0 x 10^3	**Mean:** 4.5 x 10^3 **R1:** 4.0 x 10^3 **R2:** 5.0 x 10^3	0
MMV024937 (Pbc 6) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 1.23 x 10^5 **R1:** 1.15 x 10^5 **R2:** 1.3 x 10^5	**Mean:** 1.41 x 10^5 **R1:** 1.4 x 10^5 **R2:** 1.42 x 10^5	**Mean:** 3.25 x 10^4 **R1:** 3.0 x 10^4 **R2:** 3.5 x 10^4	**Mean:** 6.75 x 10^2 **R1:** 6.0 x 10^2 **R2:** 7.5 x 10^2	**Mean:** 1.4 x 10^2 **R1:** 1.3 x 10^2 **R2**: 1.5 x 10^2	**Mean:** 1.0 x 10^2 **R1:** 1.0 x 10^2 **R2:** 1.0 x 10^2	0
MMV687798 (Pbc 7, Levofloxacin) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.4 x 10^4 **R1:** 2.3 x 10^4 **R2:** 2.5 x 10^4	**Mean:** 4.25 x 10^4 **R1**: 4.0 x 10^4 **R2:** 4.5 x 10^4	**Mean:** 1.17 x 10^4 **R1:** 1.14 x 10^4 - **R2:** 1.2 x 10^4	**Mean:** 3.05 x 10^3 **R1:** 3.0 x 10^3 **R2:** 3.1 x 10^3	0	0	0
MMV021013 (Pbc 9) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 8. x 10^4 **R1:** 7.0 x 10^4 **R2:** 9.0 x 10^4	**Mean:** 6.3 x 10^5 **R1:** 6.2 x 10^5 **R2:** 6.4 x 10^5	**Mean:** 5.6 x 10^5 **R1:** 5.5 x 10^5 **R2:** 5.7 x 10^5	**Mean:** 8.0 x 10^4 **R1:** 7.0 x 10^4 **R2:** 9.0 x 10^4	**Mean:** 2.2 x 10^3 **R1:** 2.0 x 10^3 **R2:** 2.4 x 10^3	**Mean:** 2 .35 x 10^3 **R1:** 2.3 x 10^3 **R2:** 2.4 x 10^3	0
MMV688978 (Pbc 10, Auranofin) (cfu/mL)	**Mean:** 1.01 x 10^6 **R1**: 1.0 x 10^6 **R2**: 1.02 x 10^6	**Mean:** 1.4 x 10^5 **R1:** 1.36 x 10^5 **R2:** 1.44 x 10^5	0	0	0	0	0	0
Ceftriaxone (cfu/mL)	**Mean:** 1.01 x 10^6 **R1:** 1.0 x 10^6 **R2:** 1.02 x 10^6	**Mean:** 2.7 x 10^5 **R1:** 1.25 x 10^5 **R2:** 1.45 x 10^5	**Mean:** 9.7 x 10^4 **R1:** 9.6 x 10^4 **R2:** 9.8 x 10^4	**Mean:** 1.95 x 10^4 **R1:** 1.9 x 10^4 **R2:** 2.0 x 10^4	**Mean**: 3.5 x 10^3 **R1:** 3.0 x 10^3 **R2:** 4.0x 10^3	**Mean:** 2.9 x 10^3 **R1:** 2.5 x 10^3 **R2:** 3.3 x 10^3	**Mean:** 5.5 x 10^2 **R1:** 5.0 x 10^2 **R2:** 6.0 x 10^2	0

The time-kill kinetics of dual combination of Pathogen Box compounds. 3 x MIC of each compound in combination found to be additive was used against ~ 1.0 x 10^6 *N. gonorrhoeae*. R1, repeat 1; R2, repeat

Table 4: Effect of Pathogen Box compounds on autophagy related proteins (experiment 1)

Treatment conditions	LC3I = top (1) LC3 II = bottom (2)		P62	β-actin
	Band No.	Adj. Volume (Int)		
Macrophages only	1	2852806	13409312	26685936
	2	2866446		
Macrophages + Mtb	1	3201432	80929240	10891620
	2	3051144		
Macrophages + Mtb + MMV676603 (Pbc 1)	1	1518070	72355488	3504420
	2	750448		
Macrophages + Mtb + MMV676603 (Pbc 1)+ Baf	1	2803826	57347584	45300
	2	24757344		
Macrophages + Mtb + MMV687146 (Pbc 2)	1	632028	50478568	2421000
	2	913012		
Macrophages + Mtb + MMV687146 (Pbc 2) + Baf	1	1944072	54450032	60900
	2	16968408		
Macrophages + Mtb + MMV687696 (Pbc 3)	1	893296	56542640	1160760
	2	1102174		
Macrophages + Mtb + MMV687696 (Pbc 3)+ Baf	1	2070150	73314995	66000
	2	26182800		
Macrophages + Mtb + MMV687180 (Pbc 4)	1	1233924	87897320	4058520
	2	994666		
Macrophages + Mtb + MMV687180 (Pbc 4)+ Baf	1	700600	40416152	53340
	2	8118280		
Macrophages + Mtb + MMV153413 (Pbc 5)	1	1273418	129407775	6716220
	2	1619254		
Macrophages + Mtb + MMV153413 (Pbc 5) + Baf	1	29158428	53025000	485940
	2	43938882		

Mtb, *Mycobacterium tuberculosis* H37Rv; Baf, bafilomycin; Pbc, Pathogen Box compound

Table 5: Effect of Pathogen Box compounds on autophagy related proteins (experiment 2)

Treatment condition	LC3I = top (1) LC3 II = bottom (2)		P62	β-actin
	Band No.	Adj. Volume (Int)		
Macrophages only	1	1588440	7405620	35964928
	2	825391		
Macrophages + Mtb	1	6447151	73052820	15813395
	2	6913984		
Macrophages + Mtb + Baf	1	9286701	67266120	5455190
	2	30096790		
Macrophages + Mtb + MMV676603 (Pbc 1)	1	16344340	85112400	361790
	2	21891802		
Macrophages + Mtb + MMV676603 (Pbc 1) + Baf	1	11142748	88385580	3775915
	2	58022163		
Macrophages + Mtb + MMV687146 (Pbc 2)	1	11284268	83531760	426595
	2	16619084		
Macrophages + Mtb + MMV687146 (Pbc 2) + Baf	1	21062181	103161480	2837120
	2	73310844		
Macrophages + Mtb + MMV687696 (Pbc 3)	1	12263989	88337940	426270
	2	15246889		
Macrophages + Mtb + MMV687696 (Pbc 3) + Baf	1	18088818	82417440	6210165
	2	73788726		
Macrophages + Mtb + MMV687180 (Pbc 4)	1	9644161	70879020	300755
	2	13319289		
Macrophages + Mtb + MMV153413 (Pbc 5)	1	9137190	81557160	10738130
	2	10255442		

Mtb, *Mycobacterium tuberculosis* H37Rv; Baf, bafilomycin; Pbc, Pathogen Box compound

Table 6: Intracellular suppression of Pathogen Box compounds against *M. tuberculosis* H37Rv strain

Treatment conditions	Replicate 1(cfu/mL)	Replicate 2 (cfu/mL)
Macrophages + *M. tuberculosis*	36000	35000
Macrophages + *M. tuberculosis* + MMV676603	2900	3000
Macrophages + *M. tuberculosis* + MMV676603 + Bafilomycin	14000	15000
Macrophages + *M. tuberculosis* + MMV687146	4000	6100
Macrophages + *M. tuberculosis* + MMV687146 + Bafilomycin	12000	13000
Macrophages + *M. tuberculosis* + MMV687696	20000	30000
Macrophages + *M. tuberculosis* + MMV687696 + Bafilomycin	32000	20000
Macrophages + *M. tuberculosis* + MMV687180	14000	15000
Macrophages + *M. tuberculosis* + MMV687180 + Bafilomycin	33000	30000
Macrophages + *M. tuberculosis* + MMV153413	11000	30000
Macrophages + *M. tuberculosis*+ MMV153413+ Bafilomycin	13000	20000

Pathogen Box compounds suppress intracellular growth of *M. tuberculosis.* MDMs were infected with *M. tuberculosis* (moi = 10) for 2 hr, incubated with MMV676603 (1 µM), MMV687146 (1 µM), MMV687696 (1 µM), MMV687180 (2 µM) and MMV153413 (5 µM) for 72 hr. The cells were also treated with Baf-A (100 nM). After treatment, cells were lysed in 1 X PBS containing 0.1% Triton X-100 to induce lysis of macrophages. 10-fold serial dilutions of cell lysates were prepared and plated in duplicates on Middlebrook 7H11 agar supplemented with 10% OADC and incubated at 37 °C and colonies were counted after 3 weeks.

APPENDIX E: MMV primary data for Pathogen Box compounds

Table 1: Primary assay data for antimicrobial activity of 116 Pathogen box compounds against *M. tuberculosis*

Rack	Position	MMV ID	Replicating form: Average MIC_{90} (µM)	Non-replicating form: Average MIC_{90} (µM)
Plate E	F10	MMV161996	14.4	13.4
Plate A	H07	MMV202458	14.6	12.2
Plate A	C10	MMV676395	8.4	7.8
Plate A	F09	MMV676406	0.6	1.3
Plate E	E11	MMV153413	< 0.25	0.2
Plate E	A03	MMV676468	17.4	6.0
Plate C	F09	MMV676478	0.9	0.8
Plate A	E07	MMV090930	0.7	0.3
Plate B	G02	MMV069458	6.4	7.8
Plate A	H06	MMV676539	7.7	4.8
Plate A	E09	MMV676571	0.4	1.6
Plate A	E11	MMV676589	5.4	13.7
Plate A	B08	MMV676603	< 0.01	< 0.01
Plate C	A07	MMV687145	0.7	1.4
Plate C	E05	MMV687146	0.01	0.01
Plate C	H04	MMV687172	7.4	7.6
Plate C	G07	MMV687180	0.2	1.1
Plate C	A04	MMV687239	4.5	3.6
Plate C	E06	MMV687696	0.01	0.01
Plate E	E10	MMV687813	3.5	2.9
Plate C	F06	MMV688262	0.08	0.2
Plate C	A08	MMV688327	0.5	0.3
Plate C	C02	MMV688508	0.5	0.3
Plate C	A05	MMV688122	2.5	0.09
Plate B	E02	MMV676386	22.6	10.4
Plate A	C07	MMV053220	23.2	13.7
Plate A	H09	MMV461553	0.9	3.8
Plate E	C11	MMV611037	68.4	4.5
Plate A	E10	MMV676445	12.7	7.0
Plate B	B02	MMV012074	> 100	2.0
Plate E	B09	MMV047015	> 100	16.1
Plate A	H08	MMV676474	> 100	16.4
Plate A	A09	MMV676501	35.2	2.8
Plate A	F11	MMV676509	3.6	> 100
Plate A	H10	MMV676520	> 100	16.7
Plate A	G06	MMV063404	> 100	6.0
Plate A	D08	MMV676555	> 100	4.6
Plate C	F05	MMV687138	> 100	4.0
Plate C	E07	MMV687170	> 100	8.4
Plate C	D07	MMV687188	> 50	1.6
Plate C	G06	MMV687273	1.2	5.3
Plate C	E04	MMV687699	> 100	2.9
Plate C	C05	MMV687730	0.3	97.3
Plate C	G04	MMV021660	1.2	2.8
Plate A	D07	MMV688554	> 100	19.8
Plate A	D09	MMV676383	3.1	1.4

Rack	Position	MMV ID	Replicating form: Average MIC_{90} (µM)	Non-replicating form: Average MIC_{90} (µM)
Plate A	F08	MMV676377	45.2	27.8
Plate A	C11	MMV676379	> 100	> 100
Plate E	D11	MMV676384	> 100	75.5
Plate A	B07	MMV676389	> 100	> 15.5
Plate A	D11	MMV676409	84.4	27.5
Plate D	G11	MMV676411	91.3	41.7
Plate A	A11	MMV676412	2.6	0.4
Plate A	E08	MMV676431	53.8	56.9
Plate A	C09	MMV676439	> 100	93.7
Plate A	D10	MMV676444	> 100	> 100
Plate A	F10	MMV676461	17.5	13.8
Plate E	G02	MMV146306	> 100	> 100
Plate E	B03	MMV676470	82.3	65.0
Plate E	D03	MMV200748	26.5	46.5
Plate E	C03	MMV676472	26.1	55.1
Plate A	F07	MMV676476	> 100	> 100
Plate A	B11	MMV676477	> 100	1.3
Plate A	B10	MMV102872	27.2	2.7
Plate A	A07	MMV676526	> 100	67.7
Plate E	H08	MMV393995	> 100	> 43.28
Plate A	G11	MMV676554	28.6	17.8
Plate A	G07	MMV676558	96.7	> 100
Plate A	C08	MMV676584	> 100	39.3
Plate A	G10	MMV676588	52.0	94.6
Plate A	G09	MMV676597	40.1	98.5
Plate C	F07	MMV687189	> 100	27.1
Plate C	C04	MMV687243	> 100	> 100
Plate C	D05	MMV687248	9.9	41.2
Plate C	C07	MMV687254	39.1	61.0
Plate E	D10	MMV687700	> 100	> 100
Plate C	D04	MMV687703	38.5	80.6
Plate C	H07	MMV024311	19.5	50.5
Plate E	E08	MMV687729	11.6	19.0
Plate C	A11	MMV687747	> 100	21.2
Plate C	F08	MMV687807	2.3	0.1
Plate D	F11	MMV687812	3.5	3.1
Plate A	A08	MMV688553	> 100	57.1
Plate A	G08	MMV688555	> 100	90.2
Plate E	G03	MMV688557	20.7	19.6
Plate C	C06	MMV687251	15.4	< 0.23
Plate A	A10	MMV676449	78.0	70.0
Plate A	B09	MMV676401	8.4	27.8
Plate E	C10	MMV676524	4.3	3.7
Plate C	D06	MMV688125	18.7	47.4
Plate C	B04	MMV688466	> 100	> 100
Plate C	B05	MMV687749	16.5	38.1
Plate C	F04	MMV023969	11.8	58.7
Plate E	C08	MMV687765	8.9	27.7
Plate C	D09	MMV688124	45.6	89.8
Plate A	H11	MMV676512	82.7	32.5
Plate E	F08	MMV272144	9.5	3.3
Plate E	H11	MMV495543	81.9	21.0
Plate E	G04	MMV021013		
Plate C	B07	MMV054312		
Plate E	F07	MMV228911		

Rack	Position	MMV ID	Replicating form: Average MIC90 (µM)	Non-replicating form: Average MIC90 (µM)
Plate A	E04	MMV553002		
Plate A	F04	MMV676388		
Plate E	F06	MMV688755		
Plate A	E06	MMV688756		
Plate C	H05	MMV688844		
Plate C	D10	MMV688845		
Plate C	B06	MMV688846		
Plate A	C04	MMV688888		
Plate A	B04	MMV688889		
Plate C	G09	MMV688891		
Plate A	F06	MMV688936		
Plate E	H04	MMV688939		
Plate D	B11	MMV688941		
Plate A	D04	MMV661713		
Plate E	B04	MMV688938		

Table 2: Cellular target of MMV Pathogen Box compounds against *Mycobacterium tuberculosis*

MMV ID	Structural class	Cellular target	Resistant profile	References
MMV153413 MMV461553	Pentafluobenzene containing thiophenes	Pks13 inhibition, which subsequently inhibit mycolic acid biosynthesis	Both susceptible and MDR strains	Wilson *et al*., 2013.
MMV019838 MMV687146		Inhibit MTB proteasome	Susceptible	Tyagi *et al*., 2020.
MMV687273	Adamantyl containing analogue	Trehalose inhibitor	Ethambutol resistant strains and susceptible strain.	Lee *et al*., 2003.
MMV687146	Indole-2-carboxamides	Target MmpL3, inhibit mycolic acid synthesis	Both susceptible and MDR strains	Pieroni *et al.,* 2011.
MMV687180		Target MmpL3, inhibit mycolic acid synthesis	Susceptible, MDR, XDR	Cooper, 2013
MMV676603	Benzothiazinone	Inhibit the biosynthesis of arabinans	Both susceptible and MDR strain	Makarov *et al*., 2009.
MMV090930		Target the enzymes decaprenyl-phosphoryl-β-D-ribofuranose oxidoreductase DprE1 and MoeW involved in cell wall and molybdenum cofactor biosynthesis	Susceptible, resistant, and persistent MTB	Wang et al., 2021.
MMV676409	4-(pyridine-2-yl) thiazole containing compound	Inhibit PyrG	Susceptible	Ballell *et al*., 2013. Mugumbate *et al.,* 2017.
MMV676411 MMV676383 MMV063404 MMV047015 MMV676384		Inhibit PyrG	Susceptibl, MDR, XDR strains	Ballell *et al*., 2013.
MMV687145 (Lethal synergy with IZH)	indazole sulfonamide	inhibits β-ketoacyl-ACP synthase (KasA)	Susceptible	Abrahams *et al*., 2016.
MMV687812	Amino pyrazinamide	Novel and Specific GyrB ATPase Inhibitor	Replicating and non-replicating MTB	Shirude *et a*l., 2013.
MMV202458	2-phenoxyacetamides	Mt-Guab2 Inhibitor	Susceptible	Usha *et al*., 2012.

MMV ID	Structural class	Cellular target	Resistant profile	References
MMV553002		a ketol-acid reductoisomerase inhibitor	Susceptible	Bayaraa *et al*., 2020.
MMV102872	Amide- containing	Inhibit transglycosylase	Susceptible MTB, MRSA strains	Cheng *et al*., 2010.
MMV687696	Imidazo[1,2-*a*] pyridine Amide	Inhibit cytochrome bc1 complex	Susceptible, MDR and XDR strains	Pethe *et al.,* 2013.
MMV676539		Anticholinesterase agent	Susceptible strain	Ballell *et al*., 2013; Ghanei-Nasab *et al*., 2016.
MMV676526	biphenyl amide	Inhibit demethylmenaquinone methyltransferase menG	Both susceptible and MDR	Sukheja *et al*., 2017.
MMV688756	Oxazolidinone	Protein biosynthesis inhibitor	Susceptible strain	Zhang *et al*., 2014.
MMV687700		Inhibit siderophore biosynthesis; Inhibit MbtA	Susceptible strain	Ferreras *et al*., 2005.
MMV688844		Predicted as inhibitor of ABC transport system	Susceptible strain	Braibant *et al*., 2000.
MMV688555 MMV688553		Disrupt DHSA hydrolase, which is involved in cholesterol metabolism.	Susceptible strain	Lack *et al*., 2010.
MMV688557		Predicted to be involve in amino and nucleotide sugar metabolism.	Susceptible and MDR strains	Rebollo-Lopez *et al*., 2015.

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APPENDIX F: PhD Committee and Research Ethics Committee approvals

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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

16 March 2021

Prof Bernard Fourie
Department of Medical Microbiology
Faculty of Health Sciences

Dear Prof Fourie

STUDENT: MENSAH E (PhD MEDICAL MICROBIOLOGY)

TITLE: Antimicrobial, synergistic and autophagic effects of Medicines for Malaria Venture Pathogen Box compounds on resistant strains of *Mycobacterium tuberculosis* and *Neisseria gonorrhoeae*

The above-mentioned student's protocol has been approved by the PhD committee.

We wish the student all the best with his studies.

Kind regards

PROF V STEENKAMP
CHAIR: PhD COMMITTEE

Deputy Dean: Teaching and Learning
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UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 18 March 2022 and Expires 18 March 2027.
- IORG #: IORG0001762 OMB No. 0990-0278 Approved for use through August 31, 2023.

Faculty of Health Sciences **Research Ethics Committee**

16 February 2023

Approval Certificate
Annual Renewal

Dear Mr E Mensah,

Ethics Reference No.: 170/2021 – Line 2
Title: Antimicrobial, synergistic and autophagic effects of Medicines for Malaria Venture Pathogen Box compounds on resistant strains of Mycobacterium tuberculosis and Neisseria gonorrhoeae

The **Annual Renewal** as supported by documents received between 2023-01-31 and 2023-02-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2023-02-15 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2024-02-16.
- Please remember to use your protocol number (170/2021) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 46 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2016 (Department of Health)

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