

The effect of potential host-directed therapies on the control of mycobacterial infection

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Declaration

I, Azure-Dee Natasha Pillay, confirm that this master's dissertation, titled "The Effect of Potential Host-Directed Therapies on the Control of Mycobacterial Infection," is my original and independent work. It is submitted in fulfilment of the requirements for the Degree of Master of Medicine in Science at the University of the Witwatersrand, Johannesburg. This work has not been submitted previously for any degree or examination at any other institution.

Signature of candidate:

Date: 28 March 2025

Declaration

To gain a comprehensive understanding of the Mycobacterial Growth Inhibition Assay (MGIA), the wet laboratory activities were performed by Dr. Koari Levy Fonseca, a postdoctoral researcher at the Institute for Health Science Research Germans Trias i Pujol (IGTP), Barcelona, Spain.

Table of Contents

Declaration.....	I
Declaration.....	II
Dedication.....	VI
Acknowledgments.....	VII
Abbreviations.....	IX
List of Figures.....	XI
List of Tables.....	XIV
Abstract.....	XV
Introduction.....	1
1.1 Background.....	1
1.2 Global impact of TB.....	1
1.3 The spectrum of TB infection to disease.....	2
1.4 Strain diversity in TB infection.....	6
1.5 Current treatment.....	7
1.5.1 Isoniazid.....	9
1.5.2 Rifampicin.....	9
1.5.3 Pyrazinamide.....	10
1.5.4 Ethambutol.....	10
1.5.5 Second-line drugs.....	12
1.6 Host-directed therapies (HDTs).....	13
1.7 NSAIDs.....	14
1.7.1 Aspirin.....	15
1.7.2 Ibuprofen.....	16
1.8 TB and HDTs in mouse models.....	17
1.9 Human cell lines (HCLs) as a model to study HDTs.....	19
1.9.1 THP-1 cells.....	20
1.10 The mycobacterial growth inhibition assay (MGIA).....	20
2. Rationale of the study.....	22
2.1 Hypothesis.....	22
2.2 Aim.....	22
2.3 Objective 1.....	22
2.4 Objective 2.....	23
3. Materials and methods.....	25
3.1 Materials.....	25

3.2	Culturing of <i>Mycobacterium tuberculosis</i> (<i>M.tb</i>)	25
3.3	Broth microdilution assay	25
3.4	Chequerboard assay	27
3.5	THP-1 culturing, maintenance, and differentiation	28
3.6	Mycoplasma detection for cell contaminants	29
3.7	THP-1 cell culturing	30
3.8	MTT assay	31
3.9	Extraction of genomic DNA	32
3.10	Quantification and visualization of gDNA	33
3.10.2	Spoligotyping	34
3.10.3	Polymerase Chain Reaction (PCR)	34
3.10.4	Hybridisation and detection	35
3.11	Infection of THP-1 cells.....	36
3.12	Mycobacterial Growth Inhibition Assay (MGIA)	38
3.13	Statistical analysis	38
4.	Results.....	39
4.1	Broth microdilution assays	39
4.2	Chequerboard assay	44
4.3	Mycoplasma detection of THP-1 cells for viability assay	49
4.4	Viability assay using MTT.....	50
4.5	Spoligotyping of strains	58
4.6	THP-1 infections	59
4.7	Mycobacterial Growth Inhibition Assay (MGIA)	66
5.	Discussion	75
5.1	Broth microdilution assays	75
5.1.1	Determination of MICs for the NSAIDs and FLD drugs	75
5.2	Chequerboard assays.....	76
5.2.1	Optimization and setting up of a chequerboard assay using the FLDs.....	76
5.3	Mycoplasma contamination	77
5.4	Viability assay using MTT assay	77
5.5	Quantification and visualization of genomic DNA and Spoligotyping	77
5.6	THP-1 infections	78
5.7	MGIA assay	79
5.8	A retrospective data analysis on the XDR-TB pilot study.....	79
5.8.1	Body mass index (BMI).....	79

5.8.2 Gender	80
5.8.3 Age	80
5.8.4 Hepatitis (hep C)	81
5.8.5 Smoking and alcohol misuse	81
5.8.6 TB drug sensitivity	82
6. Limitations	84
7. Conclusion	85
8. References	86
9. Appendix.....	97
9.1 Appendix A	97
9.2 Appendix B	100
9.3 Turnitin report	101

Dedication

This dissertation is lovingly dedicated to the two most amazing people in my life: my Mom and my daughter.

Mommy, your memory continues to inspire me. You were an amazing woman who kept me grounded through the highs and lows of life. I know you are immensely proud watching over us. Thank you for your guidance.

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Abbreviations

μl	microlitre
μg/ml	micrograms per millimeter
2D	two dimensional
AFRI	African
ASA	aspirin
ATP	adenosine triphosphate
BCG	Baccille Calmatte- Guérin
BDQ	bedaquiline
Bj	Beijing
BPaLM	bedaquiline, pretomanid, linezolid, and moxifloxacin
CAS	Central Asian strain
CD4	cluster of differentiation
CFU	colony forming units
CoA	coenzyme A
COVID-19	coronavirus disease
COX	cyclooxygenase
DCL	diclofenac
DNA	deoxyribonucleic acid
DR	drug resistance
DS	drug-sensitive
EAI	East Asian Indian
EMB	ethambutol
FICI	fractional inhibitory concentration index
FLDs	first-line drugs
HCL	human cell line
HDT	host-directed therapy
Hepatitis C	hep C
HREC	Human Research Ethics Committee
IBU	ibuprofen
IFN- γ	interferon-gamma
IL	interleukin
INH	isoniazid
kg	kilogram
LAM	Latin American Mediterranean
LOX	lipooxygenase
LPS	lipopolysaccharide
LTBI	latent TB infection
LXA4	lipoxin A4
LXB4	leukotriene B4
<i>M.tb</i>	<i>Mycobacterium tuberculosis</i>
MDR-TB	multi-drug-resistant TB
MGIA	mycobacterial growth inhibition assay

MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MIRU-VNTR	mycobacterial interspersed repetitive units-variable number tandem repeat
ml	millilitre
MTBC	<i>Mycobacterium tuberculosis</i> complex
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
MXF	moxifloxacin
NK	natural killer cells
NSAIDs	non-steroidal anti-inflammatory drugs
PG	prostaglandin
PGD ₂	prostaglandin D ₂
PGE ₂	prostaglandin E ₂
PGI ₂	prostacyclin
pH	potential of hydrogen
POA	pyrazinoic acid
PZA	pyrazinamide
RIF	rifampicin
RNA	ribonucleic acid
RPT	rifapentine
RR	rifampicin-resistant
SLD	second-line drugs
STM	streptomycin
TB	tuberculosis
TbD1	tuberculosis deletion region 1
THP-1	Tohoku Hospital Pediatrics-1 human leukemia monocytic cell line
TNF- α	tumor necrosis factor-alpha
TTP	time to positivity
TXA	thromboxane
WHO	World Health Organization
XD-TB	extensive-drug-resistant TB

List of Figures

Figure 1. Distribution of new TB cases globally.....	2
Figure 2. The transmission and pathogenesis of <i>M.tb</i>	3
Figure 3. The continuum from TB infection to active disease progression	5
Figure 4. Illustrates The evolution of <i>M.tb</i> and the geographical classification of lineages and sub- lineages.....	7
Figure 5. A diagram that illustrates the modes of action of FLDs.....	11
Figure 6. The therapeutic effect of HDTs may shift the disease outcome from active to latent TB by modulating the host immune response	14
Figure 7. The mechanism of action that NSAIDs have in the COX and 5-LOX pathway.....	17
Figure 8. The workflow for the MGIA assay, including PBMC isolation and ex vivo stimulation.....	21
Figure 9. A flowchart outlining the investigation of the potential effects of HDTs on the control of <i>M.tb</i> infection	24
Figure 10. The 96-well plate set up for the broth microdilution assay	26
Figure 11. A flow diagram of the 3D chequerboard assay for RIF, INH, and EMB	28
Figure 12. The step-by-step procedure of mycoplasma detection using the MycoStrip™ detection kit.....	30
Figure 13. A schematic representation of the MTT assay workflow for evaluating the cytotoxic effects of NSAIDs or FLDs.....	32
Figure 14. Flow diagram of gDNA extraction using CTAB, quantification of gDNA using the NanoDrop, and Spoligotyping	36
Figure 15. THP-1 cell infection and CFU determination with H37Rv, Bj, and LAM strains of <i>Mycobacterium tuberculosis</i>	37
Figure 16. The broth microdilution assay for ASA and IBU against the H37Rv strain of <i>Mycobacterium tuberculosis</i> using the standard 2-fold broth microdilution method.....	39
Figure 17. The broth microdilution assay for IBU against the H37Rv strain of <i>Mycobacterium tuberculosis</i> using the standard 2-fold broth microdilution method.....	40

Figure 18. The broth microdilution assay for FLDs against <i>Mycobacterium tuberculosis</i> , H37Rv strain using 2-fold dilutions in the broth microdilution method.....	40
Figure 19. The broth microdilution assay for FLDs, and NSAIDs against <i>Mycobacterium tuberculosis</i> , Bj strain using <i>the standard</i> 2-fold broth microdilution method.....	41
Figure 20. The broth microdilution assay for FLDs, and NSAIDs against <i>Mycobacterium tuberculosis</i> , LAM strain using <i>the standard</i> 2-fold broth microdilution method	42
Figure 21. An optimization and setting up of a chequerboard assay using the FLDs against the H37Rv strain of <i>Mycobacterium tuberculosis</i>	44
Figure 22. An optimization and setting up of a chequerboard assay using the FLDs with lowered starting concentrations of RIF and INH.....	45
Figure 23. An optimization and setting up of a chequerboard assay using the FLDs.....	46
Figure 24. MIC experiments set up to assess the efficacy of RIF against three strains of <i>Mycobacterium tuberculosis</i>	47
Figure 25. An optimization and setting up of a chequerboard assay using lowered starting concentrations for RIF and INH.....	47
Figure 26. An optimization and setting up of a chequerboard assay.....	48
Figure 27. A MycoStrip™ detection assay to test for common cell contaminants like Mycoplasma in the THP-1 cell line culture.....	49
Figure 28. A viability assay using MTT at 1 x MIC 4 and 24 hours.....	50
Figure 29. A viability assay using MTT at 1 x MIC 48 and 72 hours.....	51
Figure 30. A viability assay using MTT at 5 x MIC for 4 and 24 hours.....	52
Figure 31. A viability assay using MTT at 5 x MIC for 48 and 72 hours.....	53
Figure 32. A viability assay using MTT at 10 x MIC for 4 and 24 hours.....	54
Figure 33. A viability assay using MTT at 10 x MIC for 48 and 72 hours.	55
Figure 34. Illustrating the quantification of gDNA on an agarose gel and the NanoDrop for spoligotyping	56
Figure 35. THP-1 infected with <i>Mycobacterium tuberculosis</i> H37Rv.....	57
Figure 36. THP-1 infected with <i>Mycobacterium tuberculosis</i> Bj	58
Figure 37. THP-1 infected with <i>Mycobacterium tuberculosis</i> LAM	59

Figure 38. An optimization of FLDs at 5 x, and 10 x MIC in the THP-1 cell line.....	60
Figure 39. THP-1 infected with <i>Mycobacterium tuberculosis</i> H37Rv.....	61
Figure 40. THP-1 infected with <i>Mycobacterium tuberculosis</i> Bj	62
Figure 41. THP-1 infected with <i>Mycobacterium tuberculosis</i> LAM	63
Figure 42. A schematic representation of the sample cohort and groups that were used for MGIA assay.. ..	64
Figure 43 A standard curve was generated, TPP was plotted against CFU for each input volume and a regression analysis was done to obtain the equation of the curve.....	65
Figure 44. Healthy volunteer PBMCs were used to optimize the MGIA assay.....	66
Figure 45. An analysis of clinical PBMC samples from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates MGIA results.....	67
Figure 46 An analysis of clinical PBMC samples from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis ‘ illustrates gender and age.....	69
Figure 47. An analysis of clinical PBMC samples from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates hep C and alcohol intake patterns.....	70
Figure 48. An analysis of clinical PBMC samples from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates drug sensitivity and microbial cure.....	71
Figure 49. A flowchart summarizing the study findings on the potential effects of HDTs in controlling <i>Mycobacterium tuberculosis</i> infection.....	72

List of Tables

Table 1. List of NSAID or FLDs and solvents prepared at stock concentrations and desired working concentrations.....	25
Table 2. List of drugs for MTT viability assay.....	31
Table 3. PCR primers used to amplify the DR sequences for genotyping.....	34
Table 4: MIC data for the different <i>Mycobacterium tuberculosis</i> strains.....	42
Table 5. Demographics of clinical characteristics of participants in the XDR-TB study.....	68
Table A1. PBMC cell count for HV samples.....	84
Table A2. PBMC cell count for clinical samples.....	84
Table A3. Pooled PBMC samples for experiment 1.....	85
Table A4. Pooled PBMC samples for experiment 2.....	86

Abstract

Before the COVID-19 pandemic, tuberculosis (TB) remained the leading cause of death among bacterial infectious diseases, particularly in developing regions such as Southern Africa with high human immunodeficiency virus (HIV) co-infection rates.. The emergence of drug-resistant TB strains further exacerbates the global burden. Key challenges in TB treatment include prolonged therapy duration and extensive lung damage. Host-directed therapies (HDTs) have been proposed as adjuncts to standard TB regimens to mitigate inflammation, reduce lung injury, and potentially shorten treatment duration. Despite promising results in animal models, clinical trials for HDTs are still in the early stages. This study evaluated two candidate HDTs—aspirin (ASA) and ibuprofen (IBU)—for their potential synergy with anti-TB drugs. Using a THP-1 human macrophage in vitro model, ASA and IBU were tested alongside standard TB drugs.

Additionally, a retrospective analysis of clinical samples from a prospective cohort study assessed the effect of adjunctive IBU in TB treatment. The mycobacterial growth inhibition assay (MGIA) was used to measure the ability of peripheral blood mononuclear cells (PBMCs) from IBU-treated individuals to control *Mycobacterium tuberculosis* (*M.tb*) infection. Results from an XDR-TB pilot study showed no significant improvement in bacterial clearance with IBU. Further investigation revealed no significant impact of ASA or IBU on mycobacterial control in THP-1 macrophages or MGIA. Additionally, different *M.tb* strains exhibited no differential responses to these treatments. These findings suggest ASA and IBU do not influence TB infection outcomes, highlighting the need for alternative therapeutic strategies.

Introduction

1.1 Background

Tuberculosis (TB) is a communicable disease that mostly affects the lungs (pulmonary TB) and can cause disease in other organs (extrapulmonary TB) (1). TB is triggered by intracellular, facultative aerobic, rod-shaped bacteria, *Mycobacterium tuberculosis* (*M.tb*). The *M.tb* complex encompasses species that are phylogenetically related (*Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium canetti* and *Mycobacterium mungi*) (2). Mycobacteria are non-spore-forming bacteria, ranging between 2-4 µm in size, and exhibit a distinctive lipid-rich cell wall that gives them their characteristic acid-fast properties (3). Robert Koch first identified these organisms in 1882 (4). Over the past two centuries, tuberculosis has caused more fatalities compared to other infectious diseases (5).

The Bacille Calmette-Guérin (BCG) vaccine is currently the only available TB vaccine and is routinely given to infants (6). In adults, BCG protection against pulmonary disease varies, with some preliminary evidence of benefit in adolescents whose childhood dose was augmented by a second booster dose of BCG (7). Therefore, new, more effective vaccines are urgently needed. However, the inadequacy of clear immune response correlates of protection against TB is a key factor hampering the advancement of novel TB vaccines (7). The management of TB through chemotherapy has been effective in controlling the disease and attempting its elimination. However, the long treatment regimen often leads to non-adherence and the emergence of drug resistance, which significantly affects TB control initiatives worldwide.

1.2 Global impact of TB

TB is a worldwide public health challenge. In 2019, it was the leading infectious pathogen killer (8) , but it was surpassed in 2020 when the coronavirus killed more people (9). A comparison of TB cases in 2019 (7.1 million cases) and 2020 (5.8 million cases) showed a notable decrease in newly confirmed TB cases globally (10). In 2022, the World Health Organization (WHO) reported that the South-East Asian Region was burdened with 46% of the global TB patient burden, followed by the WHO African Region (23%) and then the WHO Western Pacific (18%) (11). Smaller proportions were attributed to the Eastern Mediterranean (8.1%), the Americas (3.1%), and Europe (2.2%). TB can affect anyone and any organ, but

most individuals are adults. In 2021, 55% of TB cases were reported in men and 33% in women; 88% were older than 15 years, while 12% were children aged 14 years and younger. (12). Globally, two-thirds of new TB cases were reported in eight nations, each with a minimum of 100,000 incident cases. India has the highest burden, contributing to 2% of the global incidence, followed by Indonesia at 9.2% and China at 7.4%, ranking third. The Philippines accounts for 7.0%, and Pakistan contributes 5.8%. The countries with the lowest incidence among this group include Nigeria at 4.4%, Bangladesh at 3.6%, and the Democratic Republic of Congo, which has the lowest rate at 2.9%, (12) as mapped in Figure 1. It is, therefore, critical to understand the pathogenesis and the dynamics that trigger immune responses throughout TB infection and subsequent disease.

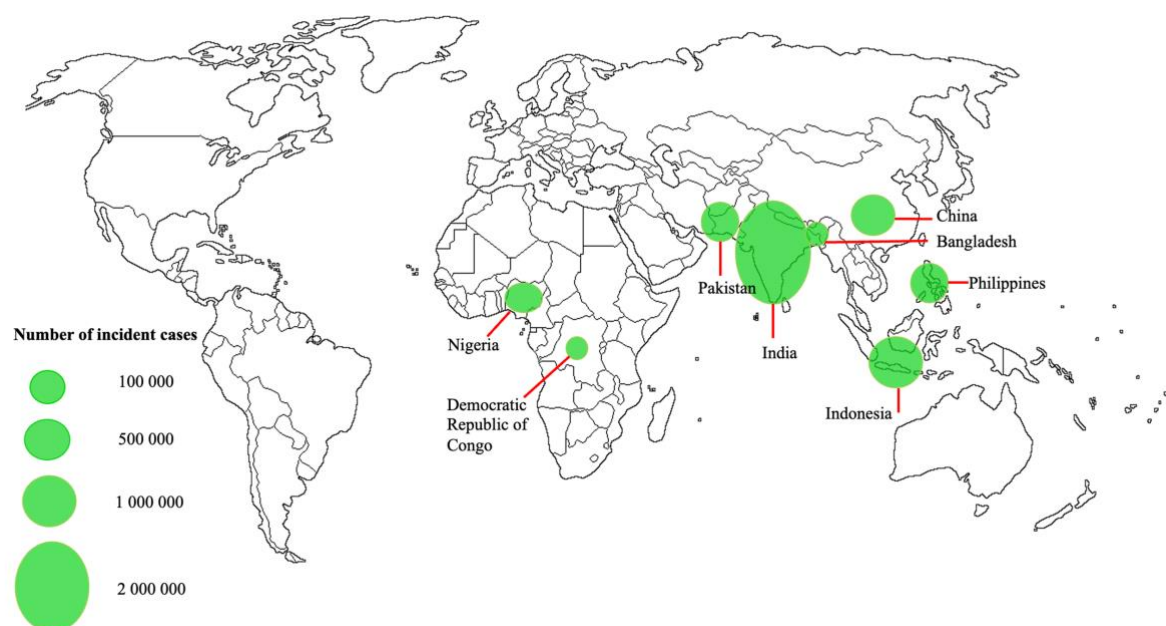


Figure 1. Distribution of new TB cases globally.

The eight nations shown on the map account for two-thirds of all cases worldwide. (Adapted from WHO, Global Tuberculosis Report 2022).

1.3 The spectrum of TB infection to disease

M.tb is primarily transmitted via inhalation of aerosolized droplets; when someone with pulmonary TB coughs, sneezes, or even passively exhales (4) and expels viable bacilli. These are inhaled as aerosol droplets by an uninfected host and when tubercle bacilli reach the alveoli in the lung, an infection may result (4). The first line of the innate immune defence during natural infection involves phagocytosis of bacteria by macrophages, wherein the bacilli survive and replicate. Macrophages signal T and B immune cells to form a shell-like barrier called a

granuloma, which includes a diverse range of cell type, including macrophages, neutrophils, dendritic cells, and lymphocytes (12), thereby ensuring an immunological and physical barrier to contain bacilli (13). Some bacilli can enter the bloodstream and lodge in other organ systems (14). However, if the immune system cannot control bacilli replication, the granuloma increases in size and ruptures (13). If it ruptures into an airway, that person will become infectious as depicted in Figure 2. The innate immune system is rapidly triggered, with the adaptive immune system in tandem to manage the infection (15). Cytokines such as interleukin (IL)-2 and IL-23, are secreted by macrophages and dendritic cells, which promote the synthesis of interferon-gamma (IFN- γ) by T cells and natural killer (NK) cells. IFN- γ is a cytokine generated by T-cells, playing a significant role in detecting and removing pathogens (16). It plays an integral role in facilitating macrophage activation in the host by upregulating the expression of antigen-presenting molecules, thereby enhancing immune defence mechanisms. Furthermore, dendritic cells, which are abundant in the airways, migrate to the draining lymph nodes along with the bacteria, facilitating the presentation of antigens to immature T cells. This stimulates CD4⁺ T-cells, which then migrate to the lungs to suppress *M.tb* growth (17).

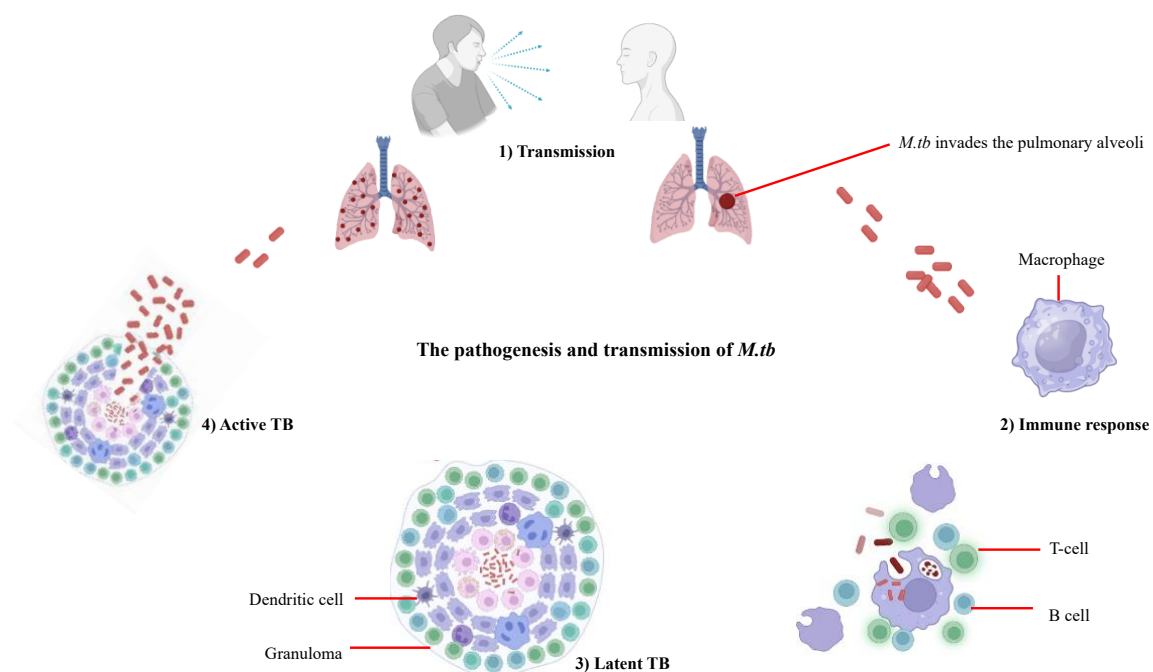


Figure 2. The transmission and pathogenesis of *M.tb*

1) An infected individual with active TB coughs or sneezes and expels bacilli to an uninfected person. **2)** Once *M.tb* is inhaled it invades the alveoli and is then engulfed by macrophages. It is internalized, the cell's internal machinery is taken over and replication begins. **3)** T-cells activate macrophages to kill *M.tb*, and in some people, the granuloma can control the infection. **4)** However, the infection can overcome the immune system and spread throughout the body. The granuloma can rupture and spread locally within the lung. It can be released into the airways, allowing the bacilli to be expelled through coughing, sneezing, or speaking. It can also enter the bloodstream and spread to other organs, leading to extrapulmonary TB (Adapted from Paulson, 2013).

A successful transmission is influenced by the bacterial load in aerosolized droplets, the closeness to an infected individual, and the length of exposure to the infectious person (18). Although *M.tb* primarily affects the lungs, it is capable of causing disease in nearly any area of the body (7). In most instances, TB predominantly affects the lungs, known as pulmonary TB, which is contagious (19), whereas TB in other organs is classified as extrapulmonary (20). Different manifestations of the disease have distinct symptoms linked to different host responses to the bacterium. There are four possible outcomes when a TB bacterium invades the lung of a new host: Latent TB occurs when the infection is contained without clinical sickness, although bacilli remain harboured (21). In contrast, the infection may advance to active TB, manifesting as a clinically symptomatic disease (22). Bacilli that enter a non-replicative or slow-growing state, enabling it to remain inside the host for prolonged periods without eliciting symptoms, are known as dormant bacteria (23). If the host's immune system becomes weakened or compromised, these dormant bacteria can transition into the active, transmissible form. For many years, researchers have been able to distinguish two infected states: (I) active TB, where mycobacteria can be cultured and can be transmitted as in the cases of pulmonary TB (7), and (II) latent tuberculosis infection (LTBI), where the individual remains asymptomatic and non-infectious, with no viable *M.tb*. detectable. However, Achkar and Jenny-Avital, 2011 have identified additional intermediate stages of infection that exist between active tuberculosis disease and latent infection. More recently, a five-state framework as a pathophysiological spectrum to understand TB has been proposed and is illustrated in Figure 3, with some definitions below (24).

1.3.1 Eliminated TB infection refers to individuals who was previously exposed to *M.tb* but have eradicated infection, may be controlled through innate and or adaptive immune responses or eliminated through TB treatment.

1.3.2 Latent TB infection, one in which viable *M.tb* bacteria are present but disease progression is unlikely, with no symptoms developing.

1.3.3 Incipient TB infection occurs with viable *M.tb* bacteria that is detected early but remains contained, likely to progress to active TB disease in the absence of intervention but has not induced clinical symptoms, microbiological evidence, or radiographic abnormalities that are consistent with active TB disease. Gordhan et al., 2019 described how metabolomics and transcriptomics identify biomarkers to help distinguish disease states and monitor treatment responses. Metabolic processes produce small biochemical molecules in response to genetic

and physiological alterations. Mycobacteria undergo metabolic shifts during disease progression, leading to distinct metabolite profiles. Several metabolite biomarkers have been identified in different human samples, as specific metabolic biomarkers are upregulated in TB patients. Measuring metabolites before and after TB treatment can help assess drug efficacy, and notably, a personalized treatment plan based on an individual's metabolic profile can be developed (25).

1.3.4 Subclinical TB disease where viable *M.tb* is detected without causing clinical TB symptoms, but it may lead to other abnormalities that can be identified through microbiological tests and radiology.

1.3.5 Active TB disease occurs when viable *M.tb* bacteria induce clinical symptoms that are detected by microbiological tests and radiology and are consistent, as per the WHO definition (26).

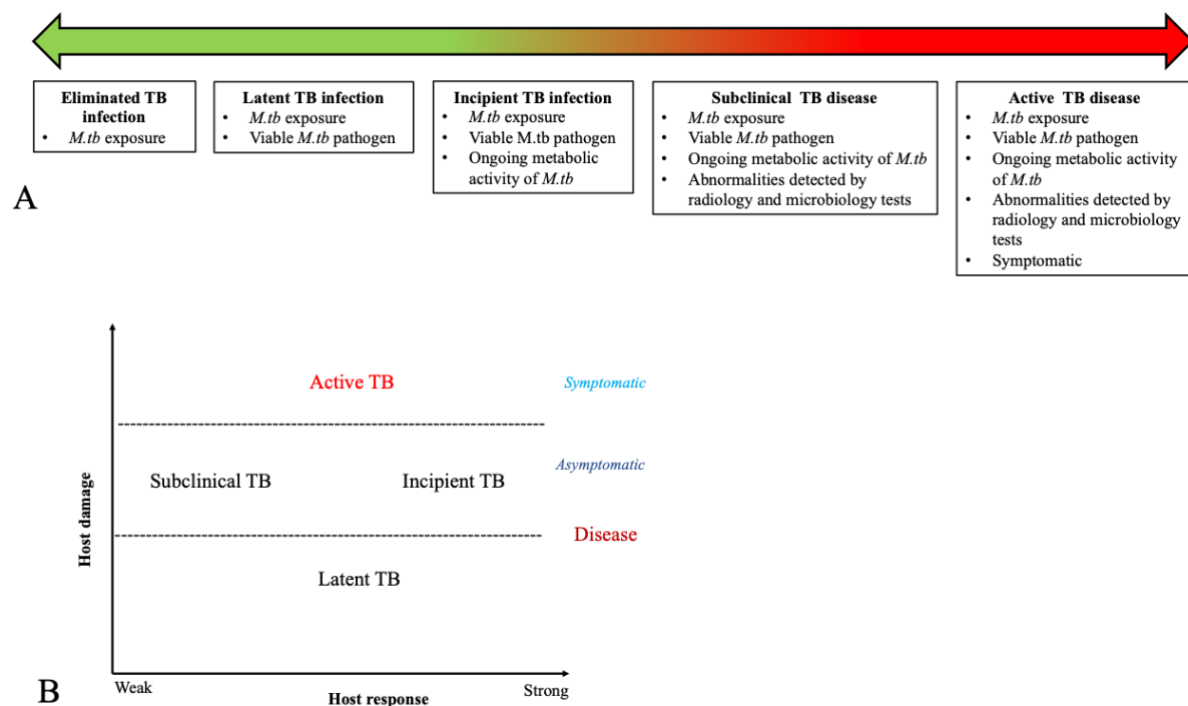


Figure 3. The continuum from TB infection to active disease progression.

A) Illustrates the five stages of TB disease, from TB infection to active disease. **B)** The host's damage-response framework with TB infection. These stages cause different amounts of damage to the cells which in turn elicits a host response. The disease is evident when the damage threshold is exceeded to a level where homeostasis is impaired. The extent of host damage varies significantly between latent TB infection and active TB. In LTBI, the host's immune response contains the infection, causing insufficient damage to elicit clinical symptoms. In contrast, active TB results in more extensive damage and symptomatic disease. (Adapted from Drain et al., 2018 and Achkar and Jenny-Avital, 2011).

The progression and outcomes of TB are significantly influenced by the genetic and phenotypic diversity of *M.tb*, as well as host-specific factors (24, 27) . This underscores the importance of understanding the genetic heterogeneity among *M.tb* strains and its implications for disease dynamics.

1.4 Strain diversity in TB infection

The *Mycobacterium tuberculosis* complex (MTBC) comprises seven unique phylogenetic lineages (28), each correlated with geographically distinct populations (29) as illustrated in Figure 4. Whole-genome sequencing has assisted in categorizing the lineages into modern and ancient, based on the *M.tb* deletion region 1 (TbD1). Ancient strains that contain the TbD1 region include the Indo-oceanic, West Africa 1, and West Africa 2 lineages. In contrast, the East Asian, Euro-American, and East-African-India strains are identified as modern strains, which do not belong to a single phylogenetic group. Lineage 1 is predominantly found in the Indo-Oceanic region and is associated with disseminated TB and meningitis (30). Lineage 5 is localized to Western Africa (30). Prevalent strains in South Africa include Latin American Mediterranean (LAM) and Beijing (Bj). Before 1965, the Bj strain was not present in Cape Town, South Africa but by 2011, 20% of TB cases were subsequently due to the Bj strain (31). Bj strains constitute the majority of all strains in East Asia and account for a minimum 13% of all strains globally (32). Bj strains of *M.tb* are regarded as more virulent and are linked to drug-resistant TB (32) . Faye et al., 2023 reported that 18.8% of LAM strain infections of TB are predominantly found in Gauteng, Northern Cape, and Kwa-Zulu Natal (33). The LAM strain is reported to cause cavitory disease and is also associated with drug resistance (34). Apart from this, there is limited understanding of how these strains respond to treatment or how their immune responses to these strains vary. Further research into these two strains will allow a better understanding of the importance of how they respond when exposed to the combination of TB drugs and host-directed therapies (HDTs).

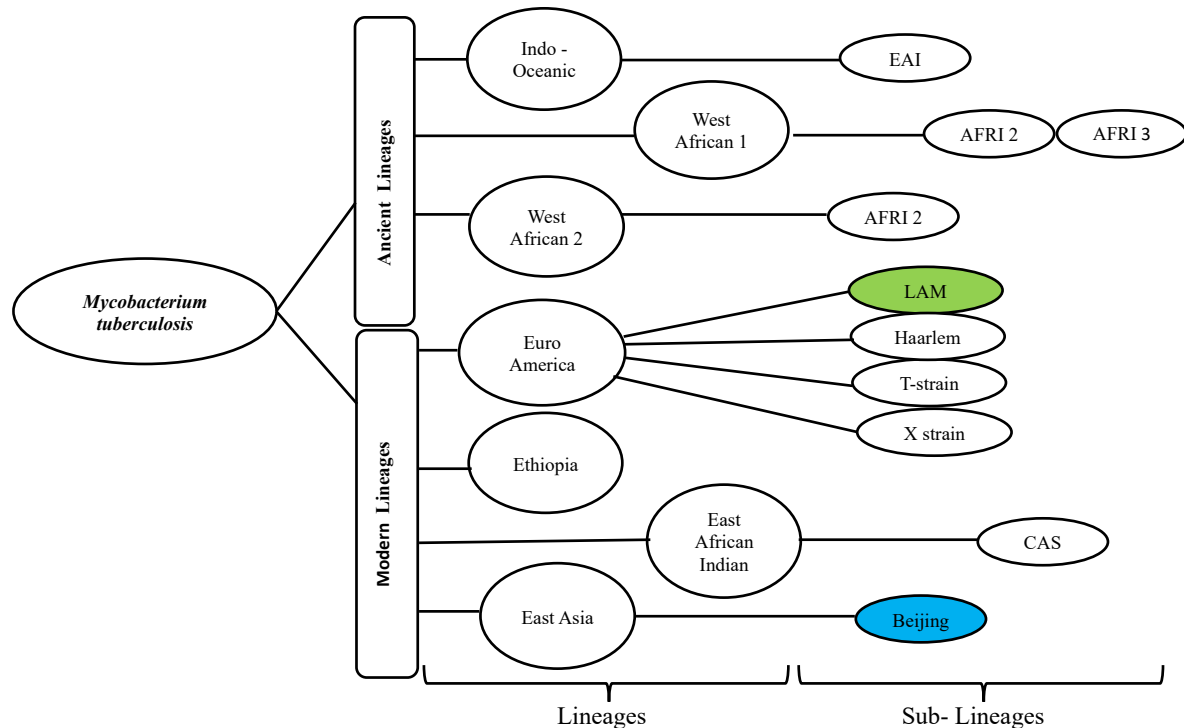


Figure 4. Illustration of the evolution of *M.tb* and the geographical classification of lineages and sub-lineages. The MTBC is made up of seven phylogenetic lineages. Lineages 1 and 3 are confined to the geographical regions of East Africa, Central, South, and East Asia, while Lineages 2 and 4 have the broadest distribution. The highlighted sub-lineages are those adapted from Coscolla and Gagneux, 2014. Lineages 2, and 4 (green, and blue, respectively) represent the strains used as they are most prevalent in South Africa. Abbreviations: EAI, East Asian Indian, CAS, Central Asian strain; AFRI, African; LAM, Latin American-Mediterranean.

1.5 Current treatment

Effective TB treatment for drug-sensitive (DS) TB includes four first-line drugs (FLDs): isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) (35). This treatment regimen involves two phases: i) the primary treatment phase, lasting two months, aims to target the rapidly growing bacilli that are killed in most people, which leads to the resolution of clinical symptoms. ii) The continuation phase which lasts four months, with two drug therapies (RIF and INH) to eliminate any persisting bacteria (19). Occasionally, FLD therapy fails to cure TB, resulting in treatment failure and the emergence of drug resistance (DR). According to the WHO (2023) classification, drug-resistant TB can be grouped into five categories. Isoniazid-resistant TB refers to resistance to isoniazid, with or without resistance to

other first-line TB drugs, while still remaining susceptible to RIF. Rifampicin-resistant TB (RR-TB) is defined as resistance to rifampicin, with or without resistance to other first-line drugs. Multidrug-resistant TB (MDR-TB) occurs when the strain is resistant to at least both INH and RIF. Pre-extensively drug-resistant TB (pre-XDR-TB) is MDR-TB that also shows resistance to any fluoroquinolone and either bedaquiline (BDQ) or linezolid (LZD). Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB that is also resistant to any fluoroquinolone and any three injectable second-line (SLD) (36). The WHO, 2022 endorsed bedaquiline, pretomanid, and linezolid (BPaL) and bedaquiline, pretomanid, linezolid, and moxifloxacin (BPaLM), involving a recommended daily treatment of 600 mg of linezolid for 6 months to treat RR-TB, marking a shift from the previous treatment regime of 9-18 months. These recommendations are based on two clinical trials that showed that the daily dose of 600 mg of linezolid delivers a significant therapeutic effect while minimizing adverse side effects compared to higher dosages. Most treatment regimens are for six months for DS-TB and extended for up to 18 months in the case of MDR-TB cases (37). In the present study, alongside the THP-1 cell line, a retrospective data analysis was done on a cohort of samples collected in a study carried out in Tbilisi, Georgia, on individuals with pre-XDR and XDR-TB. This study investigated the efficacy and safety of IBU as an adjunctive treatment. This analysis is relevant for evaluating treatment outcomes, characterizing drug resistance patterns, and identifying clinical factors that may influence prognosis in a high-burden setting. This will assist in highlighting potential gaps in treatment strategies and contributing to the broader understanding of managing highly drug-resistant TB in similar epidemiological contexts.

The previous definition described pre-XDR TB as TB caused by *M.tb* strains resistant to RIF and INH, accompanied by resistance to either a fluoroquinolone or a second-line injectable, but not to both. In 2021, the WHO recommended new definitions for pre-XDR and XDR-TB patients which states that pre-XDR-TB strains are now resistant to RIF, INH, and fluoroquinolone. Currently, XDR TB is defined as resistance to RIF, INH, fluoroquinolone, and at least one drug from Group A (bedaquiline or linezolid), which replaced the second-line

injectables used in the previous definitions. In Eastern Europe, XDR-TB's current definition has much poorer treatment outcomes than the former definition (38). Treatment success for XDR TB under the current definition was alarmingly low at 31%, compared to 54% under the former definition. Part of the research covered in this work, a retrospective data analysis will be conducted on XDR-TB patients from Georgia, Tbilisi, collected between 2017 and 2019. It would be interesting to compare the WHO 2021 recommendations with this cohort, as covered in Objective 2.

1.5.1 Isoniazid

Isoniazid (INH) is a potent and targeted drug that has been a key part of treatment for TB since 1952 (39). *M.tb* is highly susceptible to INH. It specifically targets the long- α -chain-branched, β -hydroxylated fatty acids known as mycolic acids, which make up the key elements in the cell wall of *M.tb* and it enters the cell via passive diffusion (40). After administration, the pro-drug, gets converted into an active drug by the bifunctional heme enzyme, catalase-peroxidase encoded by *katG*. Once INH is converted to the active form, isonicotinoyl-NAD, it binds and inhibits InhA, which is involved in mycolic acid synthesis as indicated in Figure 5 (41). The catalase enzyme eliminates reactive intermediates which have various cellular targets and promotes the persistence and virulence of *M.tb*. within macrophages (41). The laboratory strain of *M.tb* has a minimum inhibitory concentration (MIC) of 0.025 (42). INH targets actively replicating tubercle bacilli but does not affect non-replicating bacilli or anaerobic bacteria (43). It is associated with neurotoxicity and hepatotoxicity adverse reactions (39).

1.5.2 Rifampicin

Rifampicin (RIF) was first used clinically in 1972 and has excellent sterilizing activity against *M.tb*. It suppresses bacterial transcription, by targeting the bacterial DNA-directed RNA polymerase thereby affecting the transcriptional process, which is important for gene expression as seen in Figure 5 (43). The multi-subunit core is structurally conserved and consists of α I, α II homodimers, and β , β' and ω subunits, which are encoded by the genes *rpoA*, *rpoB*, *rpoC*, and *rpoZ* respectively (44). The β subunit encoded by the *rpoB* gene, serves as the target for RIF, which binds and prevents elongation of transcription, ultimately leading to the killing of the bacilli (43). It works best against rapidly proliferating bacilli and has a MIC of 0.003 – 0,006 μ g/ml in the laboratory strain (42). It is associated with gastrointestinal upset and hepatotoxicity (39).

1.5.3 Pyrazinamide

Pyrazinamide (PZA) is used to shorten TB treatment. It is distinguished from traditional antibiotics by targeting multiple pathways, including those involved in ATP synthesis, and the pantothenate/coenzyme A essential for the survival of persister cells. In *M.tb*, the *pncA* gene encodes for the pyrazinamidase enzyme, which hydrolyzes PZA to produce pyrazinoic acid (POA) which is subsequently exported to the extracellular environment (45). When exposed to an acidic environment, POA undergoes protonation to form its protonated derivative, HPOA, which subsequently traverses through the bacterial membrane and builds up inside *M.tb* (46). This potential of hydrogen (pH)-dependent model, in which HPOA can break the membrane and lower intra-bacterial pH, continues to be a prevalent mechanism of PZA build-up (47). However, pH-independent activity of POA has been reported where PZA binds to and inhibits aspartate decarboxylase PanD. In turn, it converts L-aspartate to beta-alanine, which delays the biosynthesis of coenzyme A (CoA), which has also been reported, and is illustrated in Figure 5 (48). CoA plays a crucial role in lipid production, including essential components of the cell envelope and virulence factors, as well as in lipid breakdown. This serves as the main source of energy for the bacillus during TB infection (48). PZA prevents the growth of semi-dormant bacilli in acidic environments and remains active against *M.tb* at a pH of 5.5. However, at neutral pH, it has poor activity and an MIC of 6.25-50 µg/ml in the laboratory strain (49). It causes hypersensitivity and gastrointestinal upset (39).

1.5.4 Ethambutol

Ethambutol (EMB) augments the efficacy of adjunctive drugs such as quinolones, rifamycins, and aminoglycosides. It inhibits arabinosyl transferases (*embCAB* operon), blocking arabinose polymerization into arabinan, a key component of arabinogalactan and lipoarabinomannan in the mycobacterial cell wall Figure 5 (49). This will lead to mycolic acid accumulating within the cell wall, causing cell death. Dizziness, blurred vision, and nausea are side effects that are associated with EMB. It has a MIC of 0.5-2 µg/ml (50) and is active against proliferating bacilli (39). When FLDs fail to effectively treat TB disease, relapse, and disease transmission continue and contribute to promoting the emergence of drug resistance in individuals. Therefore, treatment with second-line drugs (SLDs) is needed. Drug-resistant TB (DR-TB) is correlated with increased death rates in the global TB epidemic (51). DR-TB can either be contracted, which is when an individual is directly infected with a DR-TB strain, but it can also develop when drug resistance mutations develop during TB treatment. In 2022, South Africa experienced a particularly high prevalence of drug-resistant tuberculosis (DR-TB), accounting

for 35% of all diagnosed multidrug-resistant TB (MDR-TB) cases in the African region (36). Most DR-TB patients in South Africa are infected with multiple TB strains; both contracted and evolved DR-TB strains (51).

The MIC of an antimicrobial drug is the lowest concentration that inhibits the visible growth of a microorganism. A lower MIC indicates that less drug is required for bacterial growth to be inhibited. This information can be utilised to determine the optimal drug dosage required to achieve successful treatment and cure. Tang et al., 2002 report how the MICs of some TB drugs can serve as a means for improved treatment outcomes for MDR/RR-TB (52). The interactive effects of these TB drugs are an important aspect to understand, as drug-drug interactions when used in combination can exhibit synergy or antagonism. A chequerboard assay can be used to assess the drugs in a 2D matrix where the dose of each drug is linearly increasing on one axis. The fractional inhibitory concentration index (FICI) denotes the sum of the FICs of each drug (53). It is calculated by dividing the MIC of the combination drug by the MIC of the single drug. Eliopoulous and Moellering, 1996 define synergy as where when the FICI is less than 0.5, the drugs work better together than individually, additive or indifferent with FICI between 0.5-4, where the drugs work well together as they do alone, and antagonistic with a FICI more than four, where the drugs interfere with each other, reducing effectiveness (54).

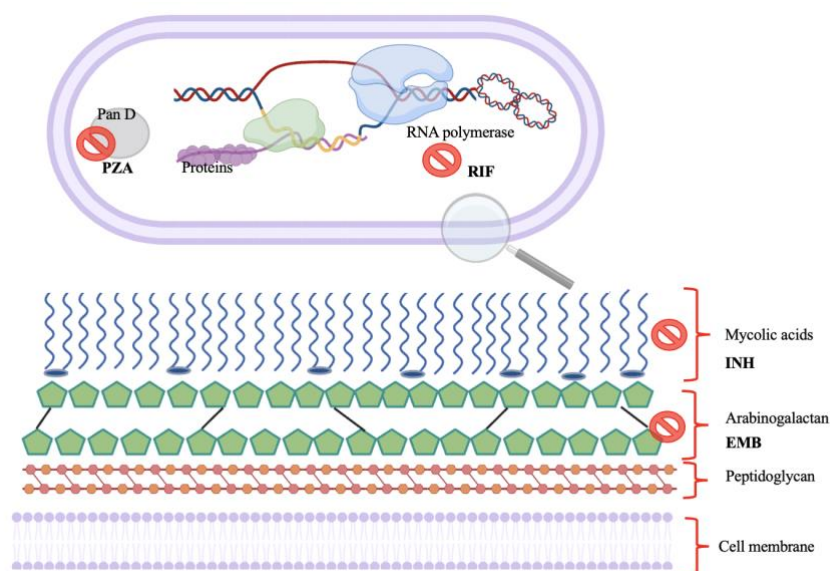


Figure 5. A diagram that illustrates the modes of action of FLDs.

The rod-shaped bacilli are encompassed by a remarkable cell wall structure, which is a distinguishing feature of *M.tb*. It consists of a phospholipid bilayer, followed by the complex macromolecule peptidoglycan found outside the plasma membrane. Arabinogalactan is a polysaccharide covalently bonded to peptidoglycan, and EMB targets the biosynthesis of this structure. INH targets the biosynthesis of mycolic acids. RIF targets RNA polymerase, which is responsible for gene expression. PanD is targeted by PZA, which plays an essential role in energy synthesis that affects coenzyme A, subsequently impacting lipid biosynthesis. As indicated in the text, PZA also has other mechanisms of action. Adapted from Drain et al. 2018 and Achkar and Jenny-Avital, 2011.

1.5.5 Second-line drugs

In 2022, treatment for DR-TB was updated into three categories. First, a six-month oral regimen for people with MDR/RR-TB (and is extendable by three months if necessary), consisting of BDQ, pretomanid, linezolid, and moxifloxacin (MXF) collectively referred to as BPaLM. The second oral regime entailed 9 months for people with MDR/RR-TB and can be extended by two months. Thirdly, treatment involves an extended regimen of 18–24 months that includes the injectable drug amikacin. (36). RR-TB causes the largest amount of TB deaths worldwide (55). Many trials using BPaL with 1200 mg of linezolid have shown 90% efficacy but significant toxicity and adverse events. Haley et al., 2023 investigated the use of BPaL in the United States of America for RR-TB, which required less than half the time that was endorsed by the US guidelines in 2019 (56). BDQ is a first-class diarylquinoline and kills dormant and replicating bacilli by its novel mechanism of action, which differs from other drugs (57), as discussed above. It targets the c-ring of adenosine triphosphate (ATP) synthase, which is critical for the proliferation and persistence of *M.tb*. Furthermore, it causes a buildup of pressure within the electron transport chain and depletes bacterial ATP levels, resulting in cell death (58). Studies evaluating BDQ in a mouse model with various drug combinations, including BDQ + INH, RIF, PZA, or MXF, under a high initial bacillary load of 7.2 log₁₀ CFU, showed that after eight weeks of treatment, regimens containing BDQ caused lung homogenates to be culture-negative in 70–100% of cases of mice (59). In the group of mice treated with two or three combinations of INH, PZA, RIF, and MXF and no BDQ, the lung homogenates remained culture-positive. The BDQ-INH-RIF and BDQ-MXF-RIF combinations exhibited lower activity compared to BDQ-PZA, thus indicating a synergistic effect between PZA and BDQ. Similar findings were recorded (60) in two long-term experiments set up to study the effect of BDQ alone or concurrently with the FLDs; RIF, INH, and PZA in aerosol-infected mice (60). In the first experiment drug combinations containing BDQ, PZA together or alone plus a third drug, outperformed the FLD like RIF, INH, and PZA. At month 1 CFU evaluation, clofazimine + BDQ + PZA combination was the third best combination pair rated in bacterial killing, and pretomanid was antagonistic. These drugs were administered for 3 months and displayed greater potency than the 4-month FLD regimen. In experiment two; three-drug combinations involving BDQ+ PZA and either rifapentine (RPT) or MXF were investigated over two months, and it yielded curing 100% and 67%, respectively. When compared to the four months of FLD treatment which had no curing effect, the combination of BDQ, pretomanid, and MXF yielded 50% of mice to be cured. The bacterial-killing activity is similar to that observed by Ibrahim and colleagues, 2007, where BDQ

combinations were more effective and also demonstrated the capacity to prevent recurrence following treatment discontinuation, a finding not previously reported (59). These results illustrate the killing effectivity of BDQ-containing shortened treatment protocols for DS and DR-TB when administered together with pretomanid.

SLDs possess a wide range of anti-microbial activity and are commonly used in the treatment of different bacterial infections. Adverse reactions that are associated with SLD are rashes and headaches (39). MDR patients require SLDs which are expensive and more toxic than FLDs. Therefore, TB treatment adherence is often poor, with alternative and shortened treatment regimens urgently needed. Even though TB mortality rates are slowly decreasing globally, TB prevalence has risen, along with the swift development of resistance to TB medications, leading to increased TB-related deaths (6) (37). This long duration of medication leads to low adherence, side effects, and give rise to drug resistance therefore making alternative treatments a necessity (61). These limitations therefore, require the exploration of HDTs as a possible solution.

1.6 Host-directed therapies (HDTs)

Over thousands of years, the co-evolution of hosts and pathogenic bacteria has led to intricate processes that influence infection outcomes, including illness progression, treatment efficacy, and mortality rates. The use of HDTs as immunological adjuvants has recently gained traction (62). This approach may interfere with pathogen infection and colonization, boost host immune responses, reduce inflammation, and promote treatment efficacy. HDTs are a treatment option that uses small biological compounds that have antibacterial or beneficial effects targeting the host (63). HDTs are a new approach to exploit and augment host factors linked to pathogen control by modulating immune mechanisms to either make the environment less favourable for the pathogen to exist (61). They can also dampen adverse immune responses that are responsible for tissue damage (14). HDTs have the potential to augment treatment for both DS and DR TB since the pathogen is not subjected to direct selective pressure, combined with the fact that host-targeting compounds minimize the development of drug resistance (61). Moreover, for latent TB infection (LTBI), the clearance of non-replicating bacilli may be facilitated by HDTs (61) (Figure 5). Additionally, HDTs may allow shortening the time of TB treatment, consequently increasing patient adherence (Figure 6). Finally, HDT could allow dose lowering of the FLDs and thereby reduce the probability of drug-induced toxicity. Many

repurposed drugs, target the inflammatory response which could potentially improve patient outcomes. Some examples include: corticosteroids which are used as an adjunct treatment for TB meningitis (64). Another example, metformin demonstrated to enhance immune responses against *M.tb* by stimulating autophagy and mitigating inflammation (64). Since hyperinflammation is associated with severe outcomes and organ damage, repurposing existing drugs with anti-inflammatory properties may offer a faster route to therapeutic intervention. By reducing excessive inflammation, these drugs could help limit tissue damage, improve recovery, and minimize the likelihood of enduring complications or permanent sequelae that arise from conditions such as cytokine storms or other inflammatory-driven diseases. Therefore, by investigating the effects of non-steroidal anti-inflammatory drugs (NSAIDs) as host-directed therapy in a human cell line (HCL) model, we aim to understand their potential impact on immune modulation and disease outcomes. This study will investigate the potential specific HDTs may augment TB therapy.

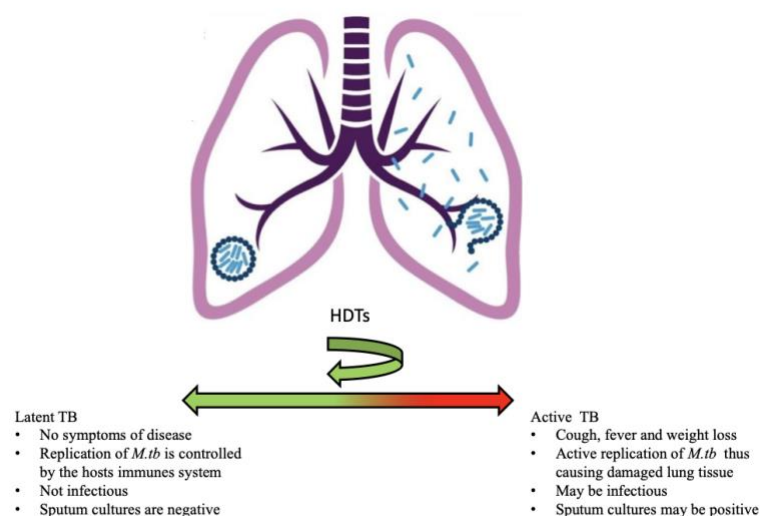


Figure 6. The therapeutic effect of HDTs may shift the disease outcome from active (red) to latent TB (green) by altering the host immune response. HDTs have the potential to enhance bacterial clearance, reduce inflammation, and improve treatment outcomes when used alongside conventional anti-TB therapy. The pathogenesis and transmission of *M.tb* occurs when an infected individual with active TB coughs or sneezes and expels bacilli to an uninfected person. Adapted from Palucci and Delogu, 2018.

1.7 NSAIDs

NSAIDs like aspirin (ASA) and ibuprofen (IBU) are already widely used in combination with antibiotics (65). They are inexpensive, easily available over the counter, and relatively safe to use. NSAIDs modulate specific host immune pathways while decreasing immunopathology

and potentially improving clinical outcomes (66). Figure 7 indicates the two main pathways; cyclooxygenase and lipoxygenase (COX and 5-LOX) where NSAIDs have an effect. NSAIDs inhibit the formation of prostaglandins that suppress inflammation (67). Prostaglandins are effective intercellular messengers and mediators of the inflammatory response (68). They block cyclooxygenase enzymes (COX-1 and COX-2) which are involved in prostaglandin production (67). During mycobacterial infections, the prostaglandin E₂ (PGE₂) protects the cell against necrotic death (69).

1.7.1 Aspirin

Aspirin (ASA) is a salicylate anti-inflammatory drug commonly used for pain and inflammation management (70). Nonspecific esterases primarily hydrolyze aspirin into salicylic acid in the liver, with a smaller extent of hydrolysis occurring in the stomach (71). This minimizes the quantity of ASA that enters the systemic circulation, with approximately 68% of the dose absorbed as aspirin (72). Its serum half-life is approximately 20 minutes (73). The decrease in ASA concentration is accompanied by a rapid increase in salicylic acid concentration, which is eliminated by the kidneys (72). ASA suppresses prostaglandin (PG) and thromboxane (TXA) production to decrease induced inflammation. As seen in Figure 7, four bioactive PGs are produced ubiquitously; prostaglandin (PGE₂), prostacyclin (PGI₂), prostaglandin D₂ (PGD₂), and prostaglandin PGF_{2α} (69). In a patient with an acute TB infection, the PG increases immediately, then an inflammatory response is elicited by immune cells releasing tissue necrosis factor alpha (TNF-α), which depends on concentrations of both lipoxin A₄ (LXA₄) and leukotriene B₄ (LXB₄). The balance between these two axes is important as an imbalance may paradoxically cause immunosuppression with a resultant increase in *M.tb* bacillary load (37). ASA also inhibits cyclooxygenase-2 (COX-2) activity which adds to LXA₄ synthesis causing a decrease in TNF-α level and neutralizes the inflammation in patients with pulmonary TB (72). Consequently, the patient were able to manage the TB-related inflammation better. Long-term adverse effects of ASA are associated with irreversible liver or kidney damage (74). Clinical evidence suggests that the concomitant use of ASA with anti-TB drugs correlates with improved clinical outcomes of patients (66). However, in the mouse model, ASA appeared to antagonize INH's bactericidal effects in the treatment of murine pulmonary TB (70). Two clinical trials in TB meningitis patients have been conducted with ASA. Shoeman and colleagues reported no significant outcomes within the three randomized arms; placebo, low-dose ASA, and a high-dose group in children with

TB meningitis (74). Whilst in another study in India investigating the efficacy of ASA in 118 TB meningitis participants showed that ASA was well-tolerated and it decreased mortality and stroke incidents (75). Given these two limited studies, additional research in humans is required to confirm the clinical effectiveness of ASA over the long term.

1.7.2 Ibuprofen

Ibuprofen (IBU) is a non-salicylate anti-inflammatory drug and when combined with TB medications, is thought to modulate immune responses that impacts TB progression (76). It is commonly used to reduce pain, inflammation, and fever. Ibuprofen's FDA-approved daily dose is 3200 mg, and repeated treatment leads to a plasma maximum concentration of 90.4 g/ml (77). The pharmacokinetic profile of IBU, demonstrates a short half-life of three hours and a good safety profile when compared to other NSAIDs. It functions by inhibiting the activity of cyclooxygenases, COX-1, and COX-2 enzymes, which are involved in prostaglandin synthesis, signalling molecules crucial in inflammation, pain, and fever reduction. COX-1 is important in sustaining normal physiological functions, like preserving the stomach lining, whereas COX-2 is predominantly linked with inflammation and the synthesis of prostaglandins due to injury or disease. In prior work, an animal model with active TB was used to demonstrate that once-daily administration of IBU improved the ability to cope with infection (78). This lowered both the lesion count and size, along with the bacterial burden in the lungs of IBU-treated animals, relative to the control group (78). IBU inhibits COX-1 and COX-2, thereby suppressing the production of prostaglandin H₂ and thromboxane. In the advanced phase of TB infection, the suppression of prostaglandin E₂ (PGE₂) suppresses phagocytosis, impairs bacterial clearance, and suppresses the production of T-helper cytokines (37). Tumor necrosis factor (TNF) is a cytokine essential for controlling (79) TB. It has many important functions, including activating macrophages, facilitating immune cell migration to infection sites, and forming granulomas. Neutralizing TNF affects infection control, causing granuloma disintegration and increased susceptibility to TB and other diseases. Humans treated with TNF-neutralizing medications appear to be more susceptible to TB. Animal models are being utilized to investigate how TNF-neutralizing drugs cause TB reactivation (79). Furthermore, IBU has been shown to have strong effects in lowering bacterial population in the lung tissue of *M.tb.* infected mice. Furthermore, in murine infection models, IBU was found to improve treatment efficacy and to have synergistic effects with pyrazinamide (70). As a result, it may be a viable choice or adjuvant in future anti-TB regimens. IBU is well-tolerated with few side effects and mostly affects the gastrointestinal tract. Employing a high-capacity spot culture growth

suppression assay to screen the antimicrobial potency of drugs against *M.tb* (80) demonstrated inhibition of *M.tb* by IBU in vitro; however, it was unable to reduce bacterial count in the lungs and spleen of mice when administered at doses of 10, 20, or 40 mg/kg (80).

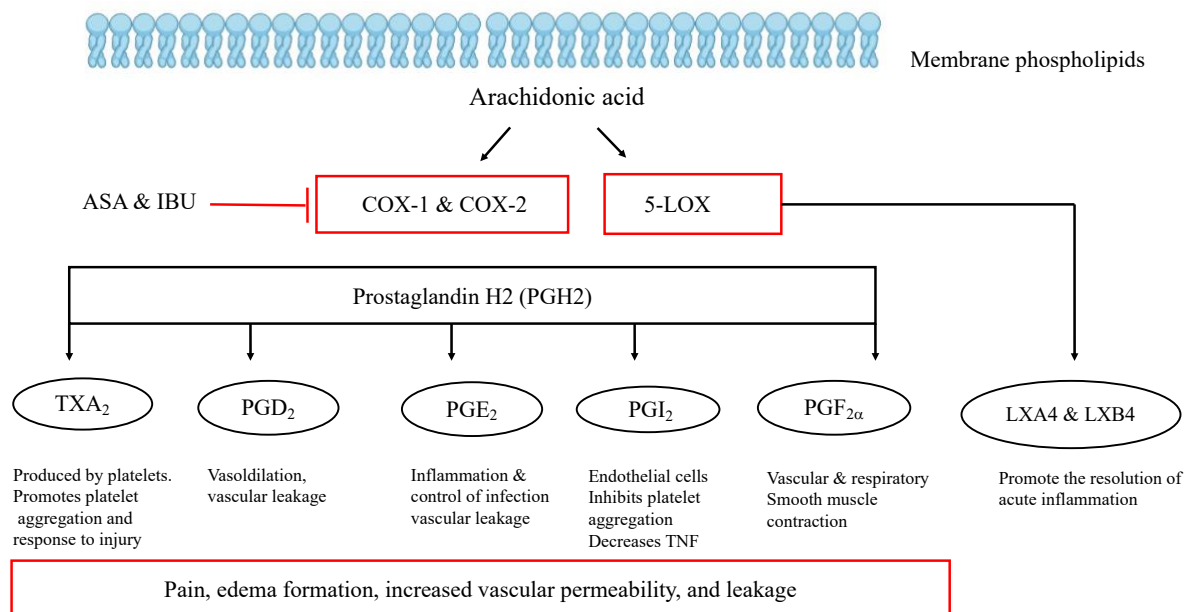


Figure 7. The mechanism of action of the NSAIDs in the COX and 5-LOX pathway. The COX and LOX-5 pathways involve NSAIDs blocking the COX-1, COX-2, and LOX enzymes. By blocking the COX enzymes, NSAIDs reduce prostaglandin synthesis. Additionally, they enhance the conversion of arachidonic acid to other products through 5-LOX. Abbreviations: COX (cyclooxygenase), TXA (thromboxane), PG (prostaglandin), PGD₂ (prostaglandin D₂), PGE₂ (prostaglandin E₂), PGI₂ (prostacyclin I₂), PGF_{2α} (prostaglandin F_{2α}), LOX (lipoxygenase), LXA₄ (lipoxin A₄), LXB₄ (leukotriene B₄). (Adapted from Kroesen et al; 2017, Nunes-Alves et al., 2014 & N.K. Panchal and E. Prince Sabina 2023).

1.8 TB and HDTs in mouse models

Infectious disease research primarily relies on mice as the most used model. Following *M.tb* infection, mice and humans show similar immune responses however, the disease pathology is different in that mice do not develop well-formed granulomas (81). During the 19th century, significant contributions were explored to the causes of infectious diseases in animal models by Louis Pasteur and Robert Koch (82). Their findings provided a deeper understanding of the disease's pathology, transmission, and potential treatments (83). Variables that impact the susceptibility of murine *M.tb* infection are; the age of the mouse, generally mice younger than 3 weeks are not used in infectious experiments as they have underdeveloped immune systems (83). The WHO reports that TB affects more males than females, and similarly, male mice are more susceptible compared to females (36) (84). Aerosol infection mimics the transmission route in humans and is more virulent as the immune response is delayed when compared to

intravenous infection. When intravenously infected, there is rapid TB development and higher doses can be administered (83). The most significant determination is the lineage of the mouse being used as the major histocompatibility complex (MHC) or H2-complex in mice, plays a significant role in immune response and experimental outcomes (85). CH3eB/ FeJ mice develop active TB (86) , whereas BALB/c mice are resistant (87).

In a mouse model, IBU reduces inflammation caused by *M.tb* thereby serving as a potential adjunctive treatment to reduce lung injury (88). Kroesen and colleagues used a murine model to investigate the preventive and therapeutic effects of low-dose ASA alone, individually or together with the four TB treatment drugs, in addition to the influence of the BCG vaccine on TB disease advancement (88). Mice received an intravenous infection with a single administration of the *M.tb* H37Rv laboratory strain. The bacillary load and lung pathology were measured by immunohistochemistry and cytokine levels. Additionally, inflammatory markers in both serum and lung tissue were examined. The group who received low-dose ASA had increased survival and anti-inflammatory effects. Furthermore, mice that received a combination of low-dose ASA and TB drugs exhibited smaller lung lesions and lower bacillary loads.

In another study, the potential of ASA and IBU to synergize with INH, a frontline TB drug, was investigated (70). The mice were exposed to the H37Rv strain through aerosol infection and received treatment five times a week with similar doses of ASA and IBU. Isoniazid was administered individually and in combination with ASA or IBU. The results showed that isoniazid as a short-course treatment cleared the aerosol infection in mice while ASA or IBU alone yielded no effect. The combination of IBU and isoniazid decreased CFU counts while ASA with isoniazid yielded an increased CFU count when compared to isoniazid alone.

The antimycobacterial activity of diclofenac (DCL), another known NSAID, was studied *in vitro* and in a mouse model to ascertain its efficacy when used in combination with streptomycin (STM) (89). Dutta et al. demonstrated that administration of the combination therapy comprising DCL and STM resulted in 100% survival of mice over a 28-day observation period, with no detectable signs of disease. In contrast, monotherapy with DCL or STM yielded survival rates of 60% and 70%, respectively.

The studies mentioned above have conflicting data, suggesting that additional research is required to clarify how the activity of TB drugs may be enhanced by HDTs. *In vivo* studies using mice have enhanced our understanding of the effect of adjunctive HDTs, and the model, holds both advantages and disadvantages. Mouse models are advantageous as reagents for recombinant cytokine analysis and antibodies are readily available (21). As the most frequently utilized model for drug development in TB, it is practical, economical, and requires limited quantities of test compounds (90). Research laboratories often test novel compounds in mouse models before pre-clinical evaluation is done (90). The disadvantage of mouse models is they do not faithfully mimic the pathogenesis of TB in humans (91). Hence, the requirement for further clinical trials in humans to confirm murine results. Moreover, there is a need for alternative models based on human specimens to rapidly and better understand the effects of adjunct therapy for TB disease. Human cell lines (HCLs) function as a potentially effective model for investigating the effects of HDTs on TB treatment.

This dissertation will investigate HDT activity in human cell lines using the endogenous mechanisms that play a role in targeting *M.tb*. In infected macrophages, autophagy is activated by HDT which upregulates the synthesis of antimicrobial peptides. Autophagy is an evolutionary homeostatic housekeeping cellular process that is triggered by various stress signals like microbial infections. Some HDT drugs can remodel the granuloma structure, making the drug more accessible to the infected cell. They can also suppress the pro-inflammatory responses and increase the anti-inflammatory responses, thereby reducing inflammation and tissue damage associated with TB (92).

1.9 Human cell lines (HCLs) as a model to study HDTs

HCLs are often used to understand the biochemical pathway of experimental drug compounds against TB (93, 94). Experiments with HCLs offer advantages, as many cells with identical genetic material can be grown and studied, in their infected and uninfected stages (95) HCLs can be divided into i) primary cells directly derived from the original tissue of interest and humans and ii) cultured cell lines, which are produced from a primary culture. Using Tohoku Hospital Pediatrics-1 (THP-1) cells in the proposed research will help to clarify the recapitulation of various immune effects.

1.9.1 THP-1 cells

In 1980, THP-1 cells, which are monocytic-like cells, were derived from a boy diagnosed with acute monocytic leukemia (M5 subtype) (96-98). It serves as an important resource for studying a disease like TB (96). Macrophages are phagocytic cells, among the most significant immune cells in TB that take part in the destruction of infected mycobacteria. When circulating monocytes encounter lipopolysaccharide (LPS), a macromolecular component of gram-positive bacterial cell walls, they initiate the onset of the adaptive immune response. Monocytes move towards lymph nodes, to transform into dendritic cells and display antigens to CD4⁺ and CD8⁺ T cells. These circulating monocytes also differentiate into macrophages and phagocytose the invading pathogen (96). Chemical mediators, chemokines, and cytokines are secreted, as inflammatory responses to the infection site (99). Within the macrophage, nutrient deprivation, expression of antimicrobial peptides, as well as the synthesis of reactive nitrogen and oxygen radicals occur to control the infection (100). However, sometimes cells are not able to completely clear an infection, which allows the mycobacteria to replicate inside the cell (100). In this study, this cell line was used to understand the relationship between the host and pathogen during infection with *M.tb* and if bacterial replication was affected after the administration of TB- drugs with NSAIDs as an adjunctive treatment.

When using THP-1 cells, it is important to have a clear insight into how the cytotoxicity of experimental drugs could affect the cell's functionality, therefore this was measured by assessing their ability to damage or kill cells (101). Several methods are commonly used in laboratory settings to evaluate drug-induced cytotoxicity. A colorimetric assay, 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT), quantifies cell viability based on the transformation of a yellow substrate (MTT) into purple formazan by mitochondrial enzymes in viable cells, indicating mitochondrial activity 37°C (102). The amount of formazan is directly dependent on the viable cell count, and a decrease in viability reflects cytotoxicity. The viability is quantified at 540 nm using a spectroscopic multi-plate reader. This study will provide a clearer insight into drug interactions and the function of HDTs in TB treatment.

1.10 The mycobacterial growth inhibition assay (MGIA)

MGIA is an assay that evaluates the capacity of host cells to suppress mycobacterial growth when induced by an effective specific immune response (103). It assesses the complex interplay between the humoral and cellular components of the host, both of which serve crucial

functions in the immunological response to mycobacterial infection. The principle of MGIA is based on the concept that when an effective, targeted immune response is triggered, host cells will control mycobacterial growth in *ex vivo* cell culture. Indirect markers for MGIA is TNF and IFN- γ cytokine, which is synthesized by the immune cells in response to the infection. Evaluating markers CD69 and CD25 provide insight into the activation status of immune cells, a crucial factor in the regulation of infection (104). Peripheral blood mononuclear cells (PBMCs) or whole blood are used to assess the capacity to suppress mycobacterial growth after *in vitro* infection, with cells cultured in the automated BACTEC MGIT 960 system (Becton Dickinson, BD) to quantify bacterial populations. Each MGIT tube has silica gel at the bottom, which remains non-fluorescent in an oxygen-rich mycobacterial growth medium but becomes fluorescent once bacterial growth, which consumes oxygen, reaches a detectable fluorescence threshold. The time to positivity (TTP) is recorded and varies based on the initial bacterial load used to inoculate the MGIT tube. By creating a standard curve, it will be possible to determine the correlation between the initial bacteria number and the TTP. The standard curve can be utilized to convert the TTP into a bacterial count, expressed in colony-forming units (CFUs) (105). Figure 8 below illustrates the MGIA assay.

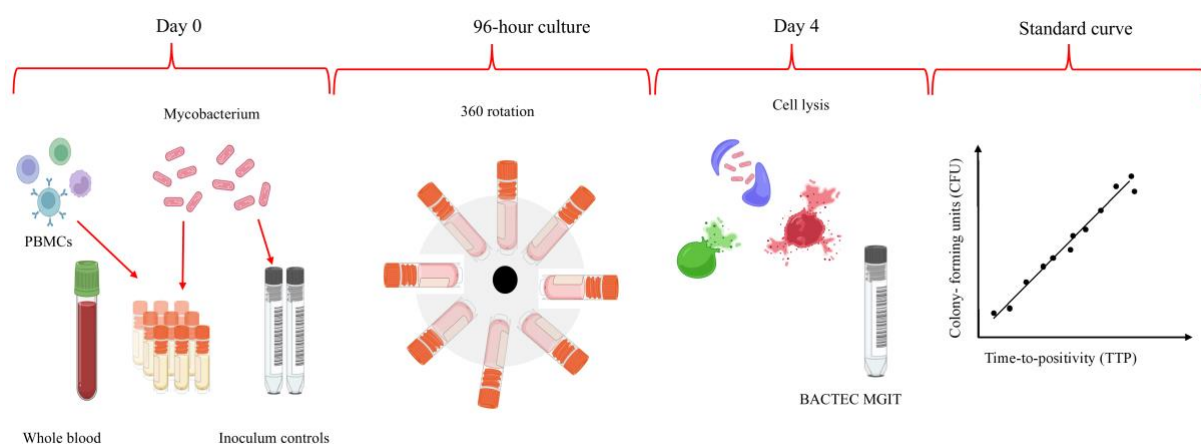


Figure 8. The workflow for the MGIA assay including PBMC isolation and *ex vivo* stimulation. Day 0; PBMCs are infected with BCG and controls are set up as well. It is allowed to incubate for 96-hours and on Day 4 cells are lysed and the cell suspension was introduced to MGIT tubes and placed on the BACTEC instrument till TTP is reached. The TTP is plotted on a standard curve against the CFU (Adapted from Tanner et al., 2017).

Moreover, this assay also provides an opportunity to mimic the host response and could assist in selecting novel vaccine candidates (103, 106, 107). Several studies have demonstrated that by using the whole blood BACTEC method, early clinical evaluation of new anti-TB drugs is

possible (108). The MGIA model was used to evaluate the effectiveness of a vaccine that is shown to reduce the bacillary load in an ex vivo model (108). Therefore, the MGIA has the potential to minimize both the time and resources needed for the preclinical assessment of drug interactions.

2. Rationale of the study

2.1 Hypothesis

We hypothesized that ASA and IBU will enhance the effectiveness in killing *M.tb* in cell-culture-based systems. We also hypothesized that distinct lineages of *M.tb* will behave differently in these models.

2.2 Aim

To investigate the effect of potential HDTs on the treatment of mycobacterial infection in HCLs (using the THP-1 cell line) and in a PBMC - MGIA model by investigating the effect in clinical specimens from patients with XDR-TB.

2.3 Objective 1

To assess if *M.tb* strains respond differently to ASA or IBU in conjunction with first-line TB drugs (RIF, INH, and EMB) in a THP-1 cell line. Clinical strains were used in the THP-1 cell line experiment and the data analysis comprised: (I) a comparison of drug response with and without ASA/IBU within individual strains and (II) a comparison of drug responses between strains, using the laboratory strain, H37Rv. To achieve this, the following were carried out.

1. The MICs for the NSAIDs and FLDs will be determined in vitro.
2. The MICs of combination drugs and synergy with the FLDs drugs will be determined by using an in vitro chequerboard assay.
3. THP-1 cell culture contaminants will be assessed using the Mycoplasma detection kit.
4. The MTT assay will be utilized to evaluate the effect of NSAIDs and FLDs drugs on THP-1 cell viability.
5. The genetic profile of the clinical mycobacterial strains will be verified using Spoligotyping.
6. The effect of treatment of THP-1 infection with different strains of *M.tb* when treated with FLDs with or without NSAIDs will be evaluated by assessing colony-forming units (CFU).

2.4 Objective 2

A retrospective data analysis from a previous: “Pilot Study to Estimate the Potential Efficacy and Safety of Using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis”, was done. It was a prospective, randomized, controlled clinical trial aimed at assessing the role of IBU in enhancing TB treatment which was conducted at the National Center for Tuberculosis and Lung Diseases in Georgia Tblisis. The trial focused on patients with pulmonary pre-XDR and XDR-TB. The primary aim was to evaluate the potential efficacy and safety of IBU in improving TB treatment outcomes, including microbiological and clinical responses, combined with FLDs. The study design trial involved 24 participants randomized into two groups: one treated with FLD treatment alone and the other treated with FLD plus a daily dose of 400 mg ibuprofen for two months. The main objective was to assess the proportion of patients achieving microbiological or clinical benefits, such as negative sputum cultures and improved radiological outcomes. The rationale was based on preclinical evidence that IBU, a repurposed NSAID with anti-inflammatory properties, may reduce inflammation-induced lung damage and improve TB treatment outcomes.

An overview of the methods that was used to ascertain the aims and objectives is illustrated in a flow diagram in Figure 9. A structured approach were used to determine if ASA and IBU enhanced any bacterial clearance *in vitro* and *ex vivo* models. The *in vitro* model explored the MICs of single drugs and then the combination of FLDs in the chequerboard assay. Then cytotoxicity assays were carried out, and Spoligotyping of *M.tb* strain conformations were done before infection of the THP-1 cell line. The *ex vivo* exploration were done as a retrospective data analysis to explore any additive effect IBU might have had on pre-XDR and XDR-TB patients from Tbilisi, Georgia.

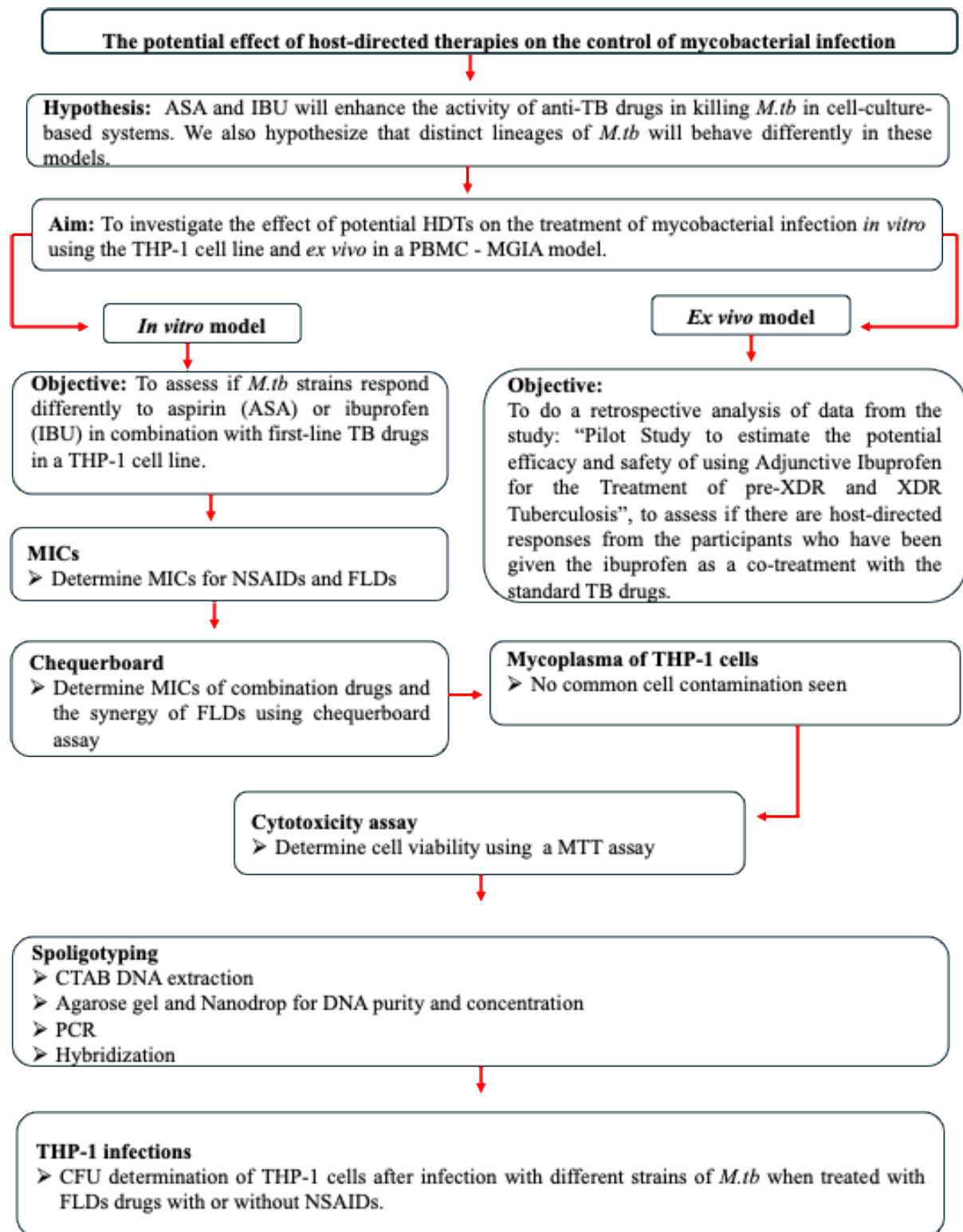


Figure 9. A flowchart outlining the investigation of the potential effects of HDTs on the control of *Mycobacterium tuberculosis* infection. *In vitro* and *ex vivo* models were explored to ascertain if ASA and IBU will enhance the activity of TB drugs. Mycoplasma contamination testing was done to ensure contamination-free THP-1 cell lines were used. The other lab experiments that were conducted were CTAB DNA extraction, PCR, Spoligotyping, and determination of CFU infection.

3. Materials and methods

3.1 Materials

Appendix A contains preparation instructions as well as a list of all the items used in this study

3.2 Culturing of *Mycobacterium tuberculosis* (*M.tb*)

A 1 ml aliquot of frozen *M.tb* was thawed at room temperature and inoculated into 5 ml of 7H9 (Difco™, Becton, Dickinson and Company USA) Middlebrook broth, supplemented with 0.1% oleic acid-albumin-dextrose-catalase (OADC) (Difco™, Becton, Dickinson and Company USA), 0.5% glycerol (Sigma-Aldrich, USA), and 0.05% Tween 80 (Sigma-Aldrich, USA), (referred to as 7H9), and incubated until the optical density (OD_{600nm}) reached 0.5–0.8, indicating exponential growth. For solid media, 7H11 agar quarter plates were prepared with OADC (Difco™, Becton, Dickinson and Company, USA) and 0.5% glycerol (Sigma-Aldrich, USA), (referred to as 7H11). Once the culture reached the desired OD, it was either used for experiments or 1 ml aliquots were transferred to 2 ml O-ring tubes (Eppendorf, Germany) and stored at -80°C.

3.3 Broth microdilution assay

The non-steroidal anti-inflammatory drugs (NSAIDs) and first-line drugs (FLDs) were obtained in powder form from Sigma-Aldrich, USA and stored at room temperature and 2°C - 8°C, respectively. The drugs were reconstituted to the desired stock concentrations, as detailed in Table 1, using 99.8% dimethylsulfoxide (DMSO) (Sigma-Aldrich, USA) or distilled water (dH₂O) (Gibco™, Thermo Fisher Scientific, USA). For experiments, the required concentrations were further diluted in Middlebrook 7H9 liquid media.

Table 1. List of NSAID or FLDs and solvents used to prepare stock concentrations and working concentrations.

NSAID or FLDs Drug	Solvent	Stock Concentration (mg/ml)	Working concentration (µg/ml)
ASA	DMSO	40	720.64
IBU	DMSO	40	720.64
INH	dH ₂ O	0.1	6.4
RIF	DMSO	0.1	0.768
EMB	dH ₂ O	10	200

In a 96-well round bottom plate (Thermo Fisher Scientific, USA), 50 µl of 7H9 media was added using a multichannel pipette to all rows (2-12) except row 1. To the first well in row 1, 100 µl of media was added, and an equivalent amount of diluent (DMSO) was added to the second well. Then in duplicate wells, 100 µl of the working concentration of drugs was added to the remaining wells. The positive drug control was RIF. By using a multi-channel pipette, 50 µl of the drug was transferred from row 1 to row 2, mixed up and down four times, and then 50 µl was transferred from row 2 to row 3, and then row 3 to row 4, and so on until row 12. In row 12, 50 µl was removed and discarded, see Figure 10. Next, 50 µl of a 1:500 diluted culture ($OD_{600nm} = 0.5-0.8$), which brought the bacterial concentration to 10^3-10^5 colony-forming units (CFU) /ml, was added to all wells except row 1. This diluted the drug concentration to half. The plates were closed with lids and placed in zip-lock bags and incubated at 37°C in a 5% CO₂ incubator. On days seven and fourteen, the plates were examined for growth by using an inverted mirror, and scoring was done as growth (+++), no growth (-), or partial growth (+/-) if approximately 50% of the area shows bacterial growth on a record sheet. The last row that does not show bacterial growth represents the MIC of the compound. In addition, 10 µl of AlamarBlue® (Thermo Fisher Scientific, USA) was added to all the wells, and the plates were scored for colour change after 20 hours of incubation. AlamarBlue® contains resazurin, a blue and fluorescent dye. In metabolically active cells, AlamarBlue® is reduced to resorufin, which is pink and highly fluorescent. The reduction occurs due to the activity of intracellular enzymes, reflecting cellular respiration and metabolic activity. The blue indicates low metabolic activity or non-viable cells, and the pink indicates high metabolic activity and viable cells.

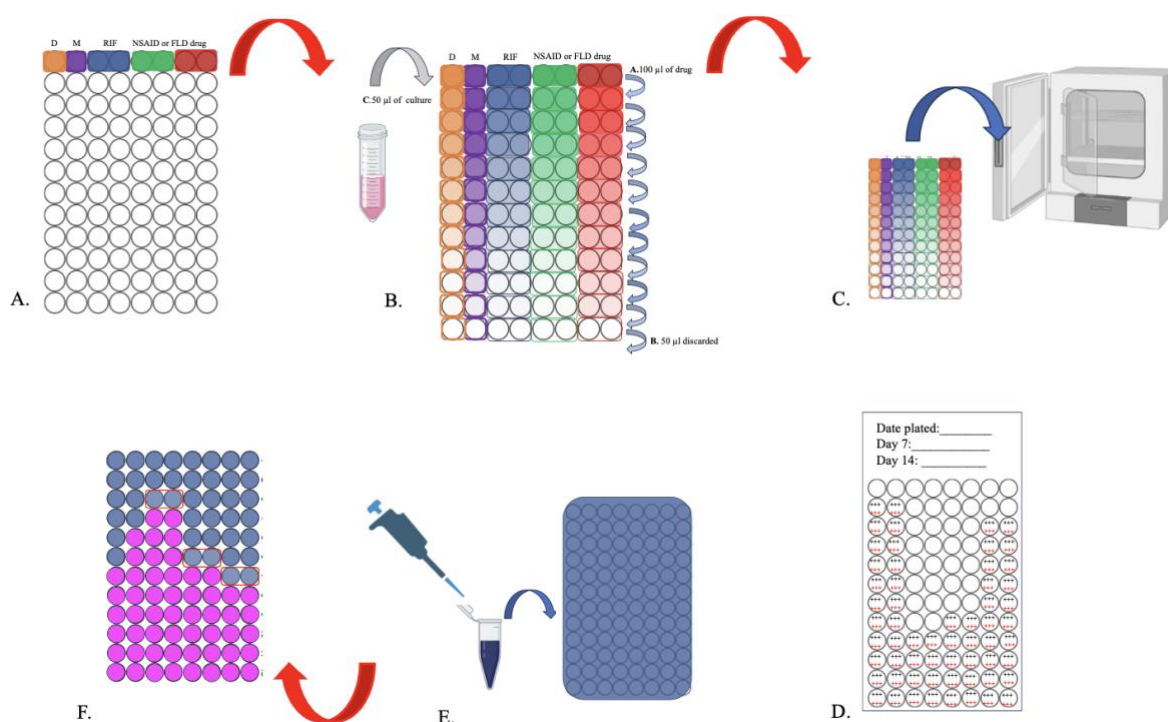


Figure 10. The 96-well plate set up for the broth microdilution assay.

(A) In a 96-well plate, media was added to the wells (grey) in all the rows except for the first row (blue). In the first row 1, NSAIDs or FLDs were added in duplicate (dark blue). (B) The dilution series was performed (shown by the curved arrows) and then 50 µl of diluted bacteria were added to the wells except for row 1 (C) The plates were incubated at 37°C, 5% CO₂ for 7 and 14 days whereafter growth was scored (D) Scoring of bacterial growth at day 7 and 14 were recorded. (E) 10 µl of AlamarBlue® was added and incubated for 20 hours in all the wells. (F) The plates were visually read, pink = bacterial growth while blue = no growth. The plates were photographed and the MICs were determined.

(42). As shown in the plate setup in Figure 11, 50 µl of the highest concentration of rifampicin (RIF) was added to well A1 of a 96-well round-bottom plate (Thermo Fisher Scientific, USA), with the concentration decreasing across columns A1 to A9. Then, 50 µl of the most concentrated dilution of INH was added to column H9 to H2, with no drug in well H1. Subsequently, 50 µl of EMB was added to all wells, excluding H1. For synergy 1, the ethambutol (EMB) concentration was 2 µg/ml, for synergy 2; the EMB was 1 µg/ml, and for synergy 3 the EMB was 0.5 µg/ml. All plate layouts were standardized as the plate layout in Figure 11. Then, 50 µl of the bacterial culture was added throughout the plate. The negative control was set up in 4 wells in column 11 with 100 µl of 7H9, and the positive control (no drug) was set up in the remaining 4 wells in column 11 with 50 µl of 7H9 and 50 µl of bacteria. The empty wells were filled with 100 µl of water. The plates were sealed in zip-lock bags and incubated at 37°C in a 5% CO₂ incubator. On days seven and fourteen, plates were examined for growth using an inverted mirror, and scoring was done as growth (+++), no growth (-), or partial growth (+/-) if 50% on a record sheet. The last row that does not demonstrate growth

represents the MIC of the compound. On day fourteen, 10 µl of AlamarBlue® was added, and the plates were scored for colour change after 20 hours of incubation. Pink denoted growth, while blue represented no growth. The last row in the dilution series that did not demonstrate growth represented the MIC of the drug. This methodology was adapted from Charles R. Bonapace, 2002, and Bhusal et al., 2005 (108, 109). The plates were photographed and the MICs were determined.

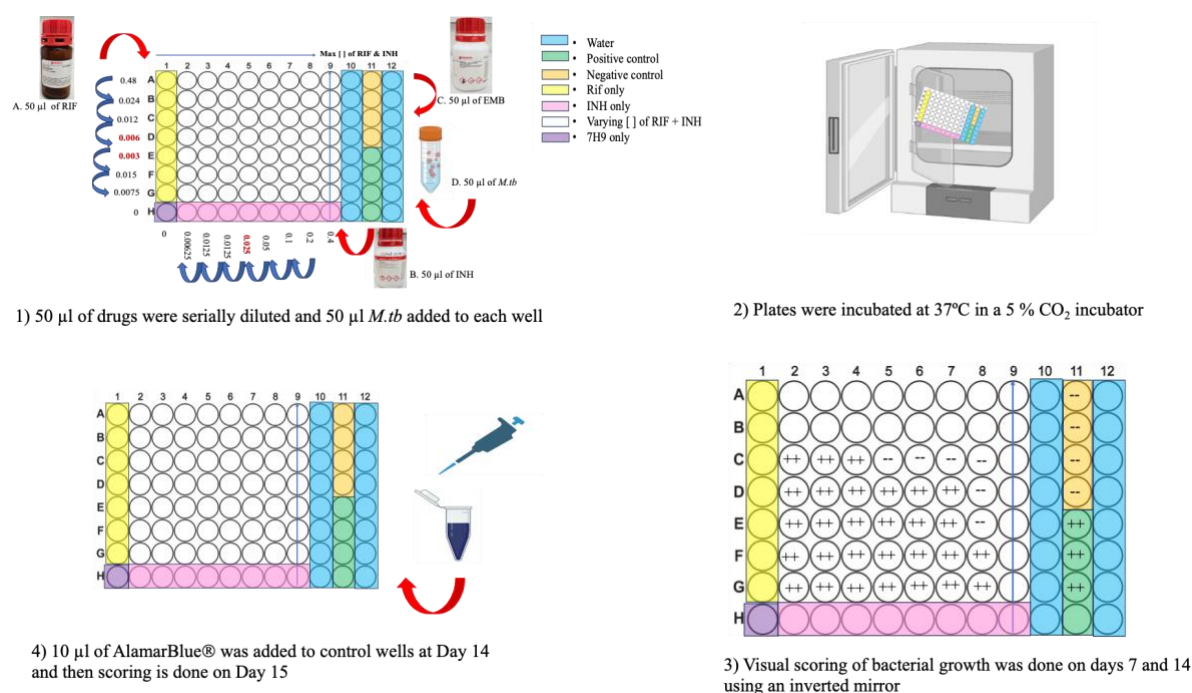


Figure 11. A flow diagram of the 3D chequerboard assay for RIF, INH, and EMB.

A 96-well plate was set up with drugs at varying concentrations from the highest to the least concentrated dilutions of RIF; 1. (B) varying concentrations from the highest to the least concentrated dilutions of INH were added to the 96-well plate. 1. (C) EMB was added to all wells, then 1. (D) The bacteria were added to each well, and growth was noted on days 7 and 14; 2) The plates were incubated at 37°C in a humidified incubator with 5% CO₂, a positive control (green) and a negative control was set up in each plate, water (blue) was added to the empty wells. (3) On days 7 and 14, visual scoring of bacterial (orange) growth was done using an inverted mirror. Plates were incubated at 37°C in a humidified incubator with 5% CO₂. (4) 10 µl of AlamarBlue® was added to each well and scoring was done on day 15.

3.5 THP-1 culturing, maintenance, and differentiation

Complete Roswell Park Memorial Institute (cRPMI) (Sigma-Aldrich, USA) media was pre-warmed in a 37°C water bath. One vial of THP-1 cells was removed from the liquid nitrogen and placed on ice. The vial was gently agitated in the water bath and the cap was kept dry to minimize the risk of contamination. Thawing was done rapidly (approximately 2 minutes) by removing the vial from the water bath immediately when thawing was observed, it was disinfected by spraying with 70% ethanol. Using a Pasteur pipette (Sigma-Aldrich, USA), 1 ml

of warm cRPMI was added into the vial, mixed by gentle pipetting, and the thawed cells and crpmI were transferred to a 15 ml Falcon tube (Greiner Bio-One, Germany) containing 10 ml of cRPMI. The 15 ml Falcon tube was centrifuged at 300 rpms for 5 minutes, the supernatant was discarded, and the pellet was resuspended in 5 ml of warm cRPMI. The 5 ml cell suspension was seeded into a small flask (T25) (Greiner Bio-One, Germany). Cells were checked under the microscope and labelled as passage 1 (P1) on the side of the flask. This was then incubated at 37°C in a 5% CO₂ incubator. Cells were maintained and expanded by checking the flask regularly every 2-3 days. Once the cells reached confluence, they were gently resuspended by pipetting with a serological pipette, transferred to a 15 ml Falcon tube, and centrifuged at 300 g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of warm cRPMI. The cell count was performed by using a sterile 1.5 ml Eppendorf tube (Sigma-Aldrich, USA), with 10 µl of cell suspension mixed with 10 µl of trypan blue (Sigma-Aldrich, USA), and 10 µl of the mixture was then loaded onto a Neubauer hemocytometer slide (Sigma-Aldrich, USA). Manual cell counting was performed in two diagonally opposite quadrants and the viable cell count was recorded and calculated using the following equation:

$$\text{Cell count} = \frac{\text{count in quadrant A/B} + \text{count in quadrant C/D}}{2} \times \text{dilution factor} \times 10^4$$

3.6 Mycoplasma detection for cell contaminants

A MycoStrip™ detection kit (InvivoGen, USA) was used to test for cell culture contaminants in the THP-1 cell cultures, as illustrated in Figure 12. One ml of cell culture supernatant was transferred to a 1.5 ml Eppendorf tube and it was centrifuged at 16 000 rpm for 5 minutes. Following centrifugation, the supernatant was discarded, and 500 µl of phosphate-buffered saline (PBS) was added, then mixed by pipetting up and down. The reaction buffer from the kit was vortexed and 5 µl was transferred to a sterile Eppendorf. Then 5 µl of the sample was added. Then it was incubated at 65°C for 40 minutes. Next, 200 µl of migration buffer (supplied in the kit) was added to the processed sample tube and mixed well. Lastly, 100 µl of the processed sample was added to the cassette (supplied in the kit) well. The results were read after 5 minutes. A positive control, which contains mycoplasma DNA, was supplied by the kit and was set up in the same manner as the samples.

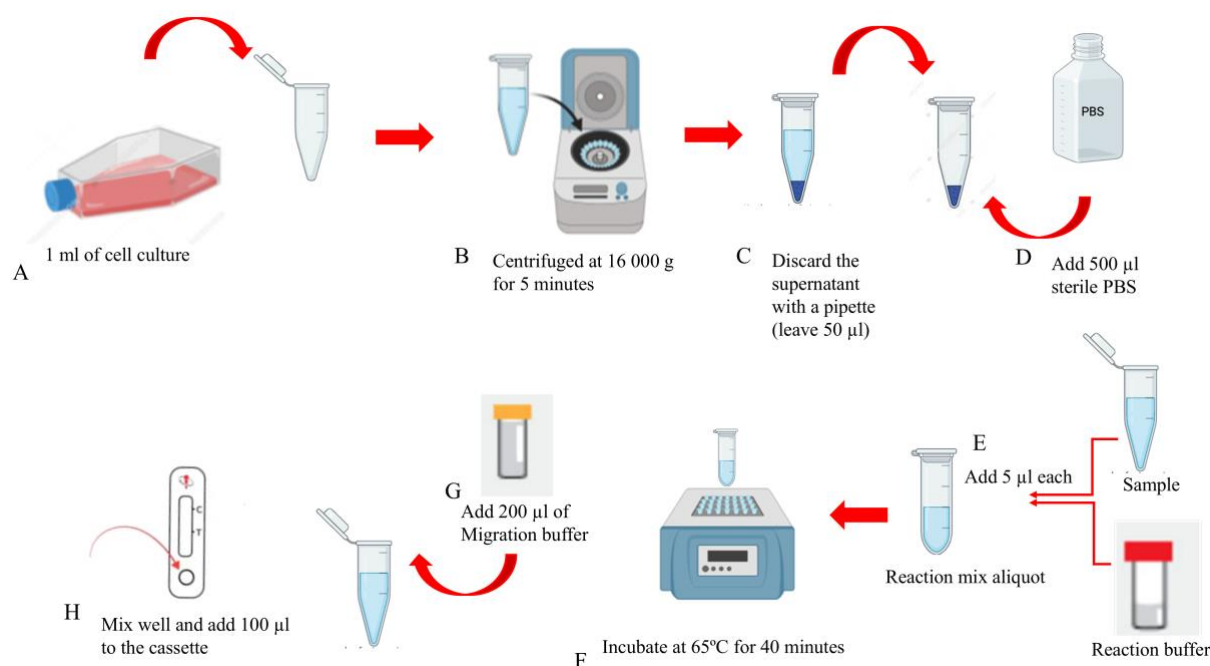


Figure 12. The step-by-step procedure of mycoplasma detection using the MycoStrip™ detection kit. **A)** 1 ml of cell culture was transferred to a microcentrifuge tube. **B)** The tube was centrifuged for 5 minutes at 16 000 g. **C)** The supernatant was discarded. **D)** Then, the pellet was resuspended with 500 µl of PBS. **E)** Equal volumes of the sample and the reaction buffer were added to a tube to form the reaction mix. **F)** The sample was incubated for 40 minutes at 65°C. **G)** 200 µl of migration buffer was added to the sample. **H)** Then 100 µl of the mixed sample was loaded into the well on the cassette, and the results were read after 5 minutes.

3.7 THP-1 cell culturing

The culturing of cells was done at $2-4 \times 10^5$ viable cells/ ml and sub-culturing was done when cells reached 8×10^5 cells/ ml. The cells were not allowed to exceed 1×10^6 cells/ ml as per American Type Culture Collection (ATCC) recommendations. Cells were resuspended by gently pipetting up and down using a serological pipette, transferred to a 15 ml Falcon tube, and centrifuged at 300 rpm for 5 minutes. The supernatant was discarded, and the pellet was gently resuspended in 1 ml of warm cRPMI. Cells were counted, using a 1.5 ml Eppendorf tube with 10 µl of cell suspension and 10 µl of trypan blue, after which 10 µl was then loaded onto a Neubauer hemacytometer slide. The concentration was adjusted where in a 24-well plate, 500 µl containing 2.5×10^5 cells were added in each well, using warm cRPMI. Phorbol 12-Myristate 13-Acetate (PMA) (Sigma-Aldrich, USA) was prepared to a concentration of 20 ng/ml, and 500 µl was added to each well. The plate was incubated at 37°C in a 5% CO₂ incubator for 48 hours. Thereafter, the PMA- media was removed by washing carefully with warm 1 x PBS, and 1 ml of warm cRPMI was added. This was followed by incubation for 24

hours at 37°C in a 5% CO₂ incubator. The cells were treated with different concentrations of NSAIDs and FLDs as indicated in Table 2. A 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to determine the viability of THP-1 cells.

Table 2. List of drugs for MTT viability assay

Drug	Concentration (µg/ml)	Volume added to each well
Media only	n/a	1 ml of media
DMSO only	n/a	1 ml of media and 18 µl of DMSO
IBU only	360.32	1 ml of IBU drug
ASA only	360.32	1 ml of ASA drug
IBU + FLDs	90.08 + 0.025 +0.006	500 µl of IBU + 500 µl of FLDs
ASA+ FLDs	180.16 + 0.025 +0.006	500 µl of ASA + 500 µl of FLDs

*FLDs refer to INH and RIF as EMB was eliminated from the chequerboard.

3.8 MTT assay

For the viability assay at the appropriate time point (4 hours, 24 hours, 48 hours, and 72 hours) the media from each well was removed, and it was replaced with 500 µl of fresh media. Then 50 µl of the 12-mM MTT (Sigma-Aldrich, USA) stock solution was added to each well and the media-only well. The plates were incubated at 37°C in 5% CO₂ for 3 hours. All the media except for 125 µl was removed from each well, and then 250 µl of DMSO (Sigma-Aldrich, USA) was added to each well, it was mixed by pipetting up and down and incubated for 10 minutes at 37°C in a 5% CO₂ incubator. Each well was mixed again, 100 µl of the suspension was transferred into duplicate wells to a 96-well plate, and the absorbance was read at an absorbance of 540 nm. The process is outlined in Figure 13.

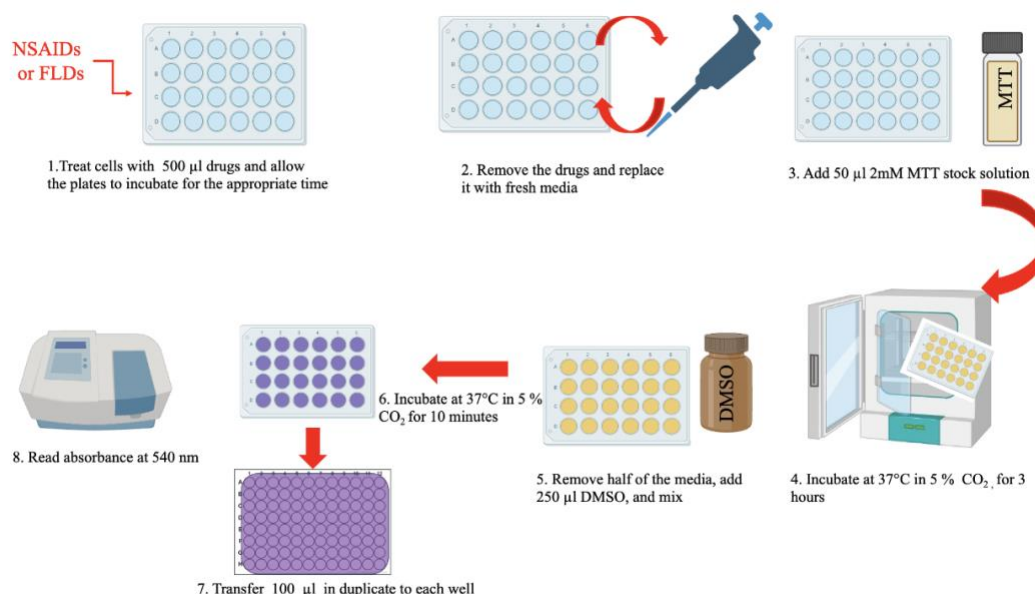


Figure 13. A schematic representation of the MTT assay workflow for evaluating the cytotoxic effects of NSAIDs or FLDs.

1) THP-1 cells were treated with drugs and plates incubated at 37°C in a 5% CO₂ for the appropriate amount of time. 2) At the time point, the drug was removed and replaced with fresh media. 3) Then 50 µl of MTT reagent was added to each well. 4) The plates were incubated at 37°C in 5% CO₂ for an additional 3 hours. 5) Half of the media was removed, and 250 µl of DMSO was added, followed by mixing using a pipette. 6) The plates were incubated at 37°C in 5% CO₂ for 10 minutes. 7) 100 µl of the suspension was transferred into duplicate wells to a 96-well plate and the absorbance was measured at 540 nm.

3.9 Extraction of genomic DNA

Genomic DNA (gDNA) was extracted from frozen cultures using the cetyltrimethylammonium bromide (CTAB/NaCl) method (110). The cultures were allowed to thaw, after which they were centrifuged at 13,000 revolutions per minute (rpms) for 10 minutes. The supernatant was removed, and the pellets were resuspended in 500 µl of 1x Tris-EDTA (TE) buffer to prevent nucleic acid degradation. The cells were incubated on a heating block at 80°C for 30 minutes. The tubes were removed from the heating block and were cooled before adding 50 µl of lysozyme (10 mg/ml) to each tube. The samples were gently inverted and then incubated at 37°C for one hour. This assisted the lysis of the bacterial cell wall. Subsequently, 70 µl of 10% SDS (pre-warmed at 37°C for one hour) and 50 µl of proteinase K (10 mg/ml) were added to each tube. The tubes were gently inverted to mix and then incubated at 60°C for two hours to facilitate protein degradation. Meanwhile, 5 M NaCl and CTAB were preheated to 60°C. After the 1-hour incubation, 100 µl of prewarmed NaCl and 100 µl of CTAB were added to each tube, followed by incubation at 60°C for 15 minutes to aid in the removal of polysaccharides that could interfere with gDNA extraction. Thereafter, an equivalent volume (700 µl) of

chloroform:isoamyl alcohol (24:1 v/v) was added, and the mixture was vigorously shaken to form a homogeneous white solution. The samples were subsequently centrifuged at 13,000 rpm for 10 minutes, allowing the cell debris to separate into the organic phase while the gDNA remained in the aqueous phase. The upper aqueous layer was carefully transferred into a sterile 2 ml O-ring screw cap tube. An equal volume of isopropanol (~600 µl) was then added to the tube to precipitate the DNA, followed by gentle inversion three times to facilitate precipitation. The tubes were centrifuged at 13,000 rpm for 10 minutes, and the resulting pellet was washed with 500 µl of 70% ethanol. Another centrifugation at 13,000 rpm for 10 minutes was performed to eliminate any residual salts from the precipitation step. The supernatant was discarded, and the pellet was dried at 45°C for 10 minutes using a SpeedVac (Thermo Fisher Scientific™). Finally, the pellet was resuspended in 60 µl of 1 x TE buffer, and the concentration, purity, and integrity of the extracted gDNA were then assessed.

3.10 Quantification and visualization of gDNA

The concentration and purity of the purified gDNA was quantified using the Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific™, USA). DNA concentration was determined by measuring absorbance at 260 nm, and purity was assessed using the A260/A280 ratio, which reflects the DNA-to-protein ratio. Samples with an A260/A280 ratio between 1.8 and 2.0 were deemed to be of acceptable purity.

3.10.1 Evaluation of gDNA Integrity Using Agarose Gel Electrophoresis

The integrity of the extracted gDNA was assessed using a 1% agarose gel. Firstly, 0.3 g of SEAKEM® agarose powder (Lonza, India) was melted in 30 ml of 1x Tris-Acetic Acid-EDTA (TAE) buffer in a microwave. To cool the solution, the bottom of the flask was placed under cold running water for 40–60 seconds. After cooling, 5 µl of SYBR™ Safe DNA gel stain (Invitrogen Thermo Fisher Scientific, USA) was added to the agarose solution and mixed by gentle swirling. The mixture was then poured into a gel casting tray and left to solidify at room temperature for 15–20 minutes. Once solidified, the gel was placed in an electrophoresis chamber filled with 1 x TAE buffer, ensuring it was completely submerged. A 5 µl volume of DNA sample was mixed with 1 µl of 6 x gel loading dye (Thermo Fisher Scientific, USA) (containing 0.25% bromophenol blue, 0.25% xylene cyanol, and 30% glycerol) on a piece of parafilm before being loaded into the gel wells. Electrophoresis was carried out at 90 V for 45 minutes. The DNA bands were visualized under UV light using the SynGene G:Box (SynGene, United Kingdom) and analyzed with SnapGene version 7.12 acquisition software.

3.10.2 Spoligotyping

The various *M.tb* genotype families and lineages were identified by spoligotyping, also known as spacer oligonucleotide typing, which is a genotyping method used to explore the genetic variation in the direct repeat (DR) region of the *M.tb* genome (111). The absence and presence of 43 spacer sequences are used to classify the different genotype families (111). Biotin-labeled PCR products were hybridized with complementary oligonucleotides immobilized on a membrane. These were subsequently detected through a chemical interaction involving streptavidin and electrochemiluminescence (ECL) reagents.

3.10.3 Polymerase Chain Reaction (PCR)

The DR regions in the extracted gDNA was amplified using PCR. The primers, BCG, and *M.tb* H37Rv controls were provided in a kit by Mapmygenome (Hyderabad, India). The primers (Table 3) were reconstituted by adding 1 ml of nuclease-free Hclone water (Cytiva, USA).

Each reaction was prepared in a final volume of 25 µl, consisting of 6.5 µl of Hyclone nuclease-free water, 12.5 µl of ReadyMix™ Taq PCR (Sigma-Aldrich, USA) reaction mix, 2 µl of DRa primer, 2 µl of DRb primer, and 2 µl of extracted gDNA. The PCR tubes were briefly centrifuged using a mini centrifuge (Bio-Rad Laboratories, USA) to ensure proper mixing before being placed in a thermal cycler (Bio-Rad Laboratories, USA) for amplification. The PCR cycling conditions were as follows: an initial denaturation at 95°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, and extension at 72°C for 30 seconds. A final extension was carried out at 72°C for 10 minutes. Upon completion, the biotinylated PCR products were stored at 4°C for a maximum of 7 days before hybridization, as per Vitol et al., 2006 (112).

Table 3: PCR primers used to amplify the DR sequences for genotyping (Adapted from(113))

Primer	5' to 3' sequence	Annealing temperature	Storage
DRa (biotin)	GGTTTTGGGTCTGACGAC	55 °C	4 °C
DRb	CCGAGAGGGGACGGAAAC	55 °C	-20 °C

3.10.4 Hybridisation and detection

A 25 µl PCR tube was prepared, and 150 µl of 2X SSPE/0.1% sodium dodecyl sulphate (2X SSPE/0.1% SDS) was added to each tube. The tubes were then placed in a Bio-Rad thermocycler at 99°C for 10 minutes to denature the PCR product. Immediately after, the tubes were transferred to ice to prevent DNA re-annealing until they were ready for membrane loading. The spoligotyping membrane provided in the kit was pre-washed twice with warmed 2X SSPE/0.1% SDS and incubated in a hybridization oven at 60°C for 10 minutes. Next, the membrane was placed in the mini blotter on a foam cushion, ensuring that the loading wells aligned parallel to the membrane's lines and that the snipped edge was positioned in the bottom left to indicate orientation. Excess fluid was removed with a paper towel, and plastic screws were used to secure the blotter. Any remaining buffer was aspirated with a pipette before 170 µl of the PCR product was loaded into each well. Additionally, 2X SSPE/0.1% SDS was added to the first and last wells of the 45-well membrane. The kit-supplied controls, H37Rv, BCG, and a non-template control (NTC), were included, followed by duplicate loading of the unknown strain samples in the remaining wells. The mini blotter was placed on a horizontal surface inside the hybridization oven and incubated for one hour at 60°C. After incubation, any residual sample was aspirated before removing the membrane from the blotter. The membrane was then washed twice with 2X SSPE/0.5% SDS for five minutes at 60°C. For chemiluminescent detection, hybridized biotin-labeled PCR products were bound to spacer-oligos on the membrane. The membrane was transferred to a roller bottle containing 40 ml of 2X SSPE/0.5% SDS and 10 µl of Streptavidin-peroxidase conjugate (500U/ml), then incubated at 42°C for one hour. The membrane was subsequently washed twice with 2X SSPE/ 0.5% SDS for five minutes at 42°C. The membrane was placed in a glass bowl, and 10 ml of detection solution (prepared by mixing solutions 1 and 2 from the ECL detection kit, GE Healthcare) was added. The solution was gently swirled for 90 seconds before being discarded. The membrane was enclosed in a clear plastic sleeve and exposed to the three X-ray films improves confidence in the spacer pattern results for 20 minutes. The X-ray films were developed manually in a darkroom using the following sequence: developing solution, water, fixer solution, and a final water rinse, with each step lasting 30 seconds. The films were then hung to air dry. Once dried, the spoligotyping patterns were analyzed by identifying the presence or absence of small black squares at each DR locus on the X-ray film. These unique patterns corresponded to the genotypes of the strains. The SPOTCLUST spoligotyping website (<https://tbinsight.cs.rpi.edu/runspotclust.html>) was used to determine the strain type. Each

black square was assigned a binary value of 1, indicating the presence of a spacer, while blank spaces were assigned a value of 0. This process is illustrated in Figure 14.

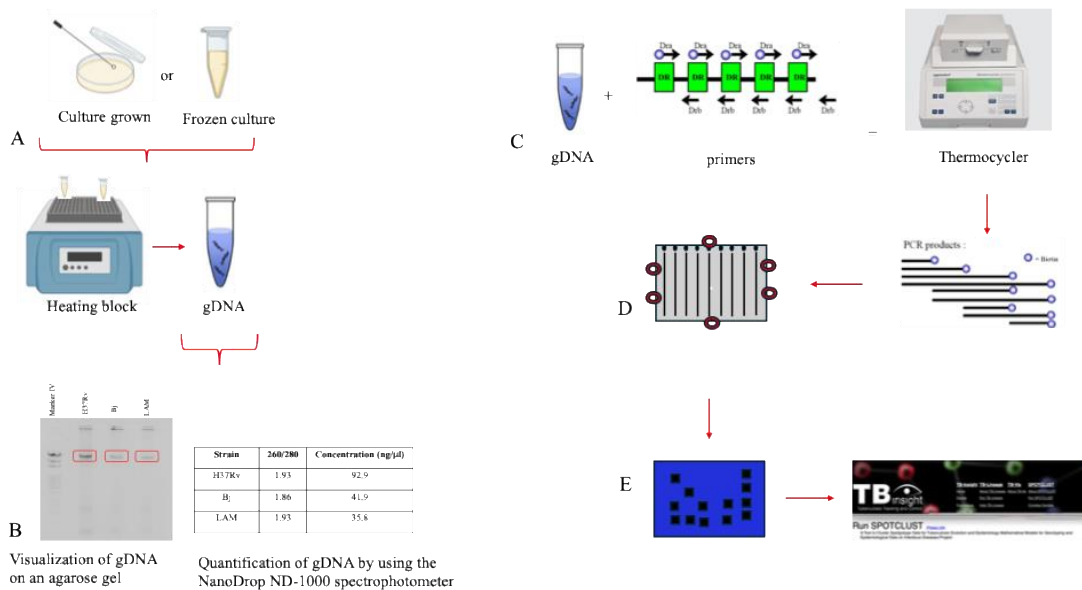


Figure 14. A flow diagram of gDNA extraction using CTAB, quantification of gDNA using the NanoDrop, and Spoligotyping.

A) CTAB was performed on frozen or grown culture to yield genomic DNA. **B)** The quality of the extracted gDNA was evaluated using agarose gel electrophoresis and by using the NanoDrop. **C)** The gDNA was annealed with primers, and a biotin-labeled product was formed. **D)** Hybridized of PCR-biotin labeled product on a membrane was developed onto an x-ray film. **E)** The x-ray was analyzed on SPOTCLUST.

3.11 Infection of THP-1 cells

Cultures of H37Rv, Bj, and LAM were grown to an exponential phase with an OD_{600nm} of 0.5. THP-1 cell culturing and maintenance followed the steps outlined in Section 3.5. When the cell density reached 2.5×10^5 cells /1.5 ml of media, 1.5 ml of the cell suspension was seeded into each well of a 24-well plate. THP-1 monocytes were differentiated into adherent, activated macrophages, by adding 100 nM phorbol myristate acetate (PMA) (Sigma-Aldrich, USA) together with 150 U of recombinant human gamma interferon (IFN- γ). This was followed by incubation for three days at 37°C with 5% CO₂. After differentiation, the medium was replaced with 1 ml of mycobacterial inoculum (at a ratio of 10 bacteria per THP-1 cell) and incubated for four hours at 37°C with 5% CO₂ to allow phagocytosis. To quantify extracellular bacteria after four hours, the supernatant was aspirated into a sterile 2 ml O-ring tube. A 1:10 dilution series was performed by transferring 100 μ l of undiluted supernatant into 900 μ l of 7H9 medium in a labeled tube (10^{-1}), followed by thorough vortexing. This process was repeated for subsequent dilutions (10^{-2} , 10^{-3} , and 10^{-4}). From each dilution, 100 μ l was plated in

duplicate on quarter 7H11 agar plates, spread evenly, and incubated at 37°C for four weeks. For the remaining wells (24, 48, and 72 hours), the medium was carefully removed, and the wells were washed with warm RPMI + 10% FBS before it was discarded. Each well was then replenished with 1 ml of the appropriate treatment condition (ASA, ASA + FLDs, IBU, IBU + FLDs, and FLDs). The plates were incubated at 37°C with 5% CO₂ for the duration of the experiment. At each designated time point (24, 48, and 72 hours), the wells were washed with RPMI + 10% FBS, and the medium was replaced. The macrophages were lysed, and the intracellular bacterial quantification was performed as described previously with the extracellular bacterial quantification. Figure 15 provides an overview of the experimental steps.

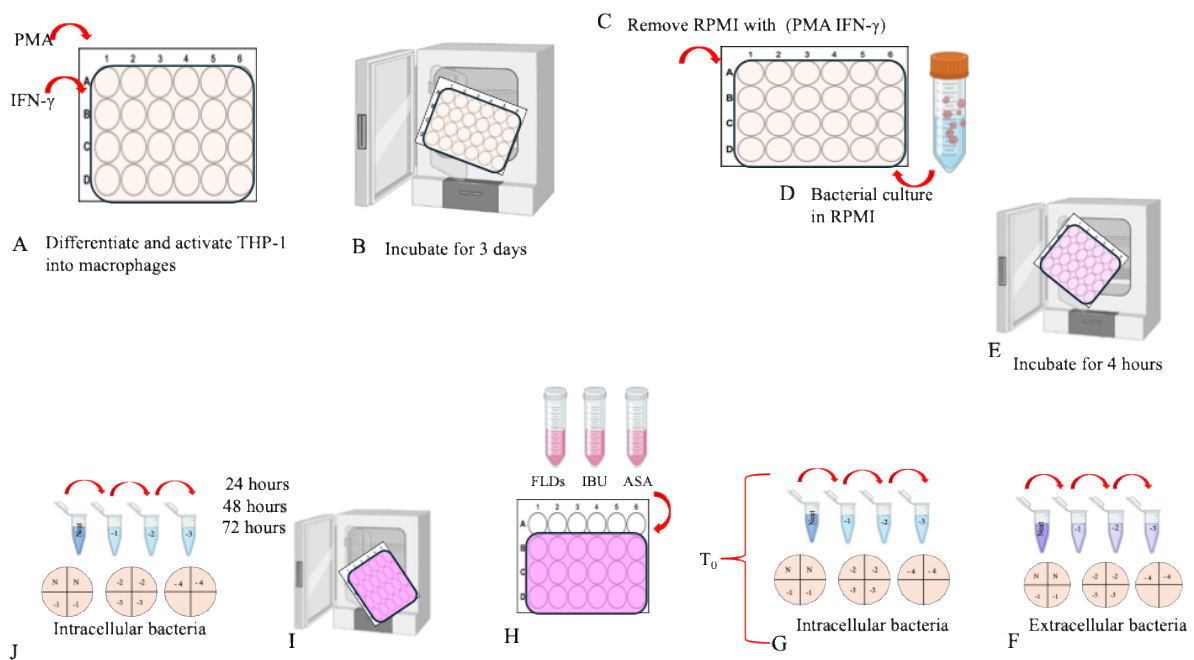


Figure 15. THP-1 cell infection and CFU determination with H37Rv, Bj, and LAM strains of *Mycobacterium tuberculosis*.

A) THP-1 cells were plated in 24 well plates, then differentiated and activated by adding PMA and IFN-γ. **B)** The plates were incubated at 37°C, with 5% CO₂ for 3 days. **C)** The supernatant was removed. **D)** The cells were infected with *M.tb*. **E)** Infection time was 4 hours. **F)** The extracellular supernatant was removed from each well and serially diluted, and then 100 μl plated in duplicate on 7H11 plates. **G)** Then, the intracellular bacteria were lysed by adding 10 ml of 10% SDS and serially diluted and plated (this was T₀). **H)** For the remainder of the time points the media containing bacteria was removed, and then the appropriate drugs were added. **I)** The plates were allowed to incubate at 37°C, 5% CO₂ for 24, 48, and 72 hours, respectively. **J)** Then, the intracellular bacteria were lysed by adding 10 ml of 10% SDS, and serially diluted, plated, and incubated for 4-6 weeks and CFUs were determined.

3.12 Mycobacterial Growth Inhibition Assay (MGIA)

The Mycobacterial Growth Inhibition Assay (MGIA) is a functional immunological assay used to evaluate the host's antimicrobial activity against mycobacteria. To gain an understanding of the MGIA assay, I observed a laboratory scientist performing the wet lab activities. PBMCs were thawed and co-cultured with BCG. Then it was incubated for 96- hours, after which the bacterial growth was measured. The assay measures the net growth or inhibition of *M.tb*, providing clarity into the immune system's functional capacity to limit bacterial replication. After the incubation, the mixture was transferred into a liquid-based culture system, such as the Mycobacteria Growth Indicator Tube (MGIT) system. The MGIT system detects bacterial growth by monitoring oxygen depletion or fluorescence changes, which indicate microbial metabolism. TTP (time-to-positivity) was recorded as the time (in hours or days) it takes for *M.tb* to reach a detectable growth level in the culture. A shorter TTP indicates higher bacterial growth (weaker immune control). A longer TTP indicates lower bacterial growth (stronger immune control). A retrospective data analysis on the raw data was done using GraphPad Prism 10, raw data can be found in Appendix A Table 1- 4. The clinical data analysis was done using Microsoft Excel. Ethical approval for this study was granted by the Human Research Ethics Committee (HREC) under certificate number M240993. The corresponding documentation is available in Appendix Section B. Ethical clearance for the use of *M.tb* strains in this study was covered under approvals obtained for the parent studies from which the strains were derived, namely the BMG (protocol number: M140265) and MGIT-Plus (protocol number: M110833) studies. Both studies were approved by the HREC of the University of the Witwatersrand. All experimental procedures adhered to the ethical guidelines stipulated in these protocols.

3.13 Statistical analysis

For the MIC experiments, visual reading of broth microdilution plates was used to determine the MICs of NSAIDs and FLDs. In the case of RIF, a range of MIC values was observed across certain strains (H37Rv and LAM). Drug-to-drug interactions were assessed using the chequerboard assay, and the FICI was calculated to categorise the nature of drug interactions (synergy, additivity, indifference, or antagonism). For the MTT assay, raw absorbance values were corrected by subtracting background readings from blank wells. These corrected values were then normalised to media-only controls (untreated cells = 100% viability). Technical replicates within each plate were averaged to generate a single value per condition per biological replicate. Data were analysed in GraphPad Prism 10 using descriptive statistics

(mean $\pm$ standard error of the mean, SEM). One-way analysis of variance (ANOVA) was applied to compare conditions, with statistical significance set at $p < 0.05$. For spoligotyping, binary patterns were recorded, and matches or mismatches were used to assign the corresponding *M. tb* strain lineage. For the MGIA, data were expressed relative to control wells, and the degree of mycobacterial growth inhibition was determined after plotting bacterial growth curves. For the THP-1 infection assay, CFU counts were converted to Log₁₀ CFU/ml and plotted over time. Treatment conditions were compared to the infected, no-drug control using a two-way ANOVA with Tukey's multiple comparison test. Statistical significance was defined as $p < 0.05$.

4. Results

4.1 Broth microdilution assays

Since the THP-1 cell line was going to be infected with different strains of *M.tb* it was important to establish the MICs of each strain so that the correct drug concentration was used.

The broth microdilution assay was optimized and performed using the H37Rv lab strain of *Mycobacterium tuberculosis* (*M.tb*), by setting up three biological repeats (BR) as described in the methods Section 3.3. The plates were scored visually using an inverted mirror at days 7 and 14, after which AlamarBlue® was added to the plates and allowed to incubate for another 24 hours. On day 15, the plates were visually assessed, with pink indicating bacterial growth and blue indicating no growth. The plates were then photographed, and the lowest minimum inhibitory concentration (MIC) that inhibited the growth of *M.tb* was recorded.. The starting concentrations for the NSAIDs were 720.64 μ g/ml and that for RIF was 0.768 μ g/ml. The MIC results for the ASA were consistent at 360.12 μ g/ml, however, the MIC of IBU was variable in each of the three experiments carried out. The MIC results were 90.08, 22.52, and 45.04 μ g/ml as indicated in Figure 16. Hence, the MIC determination of IBU was repeated to establish a more reproducible result.

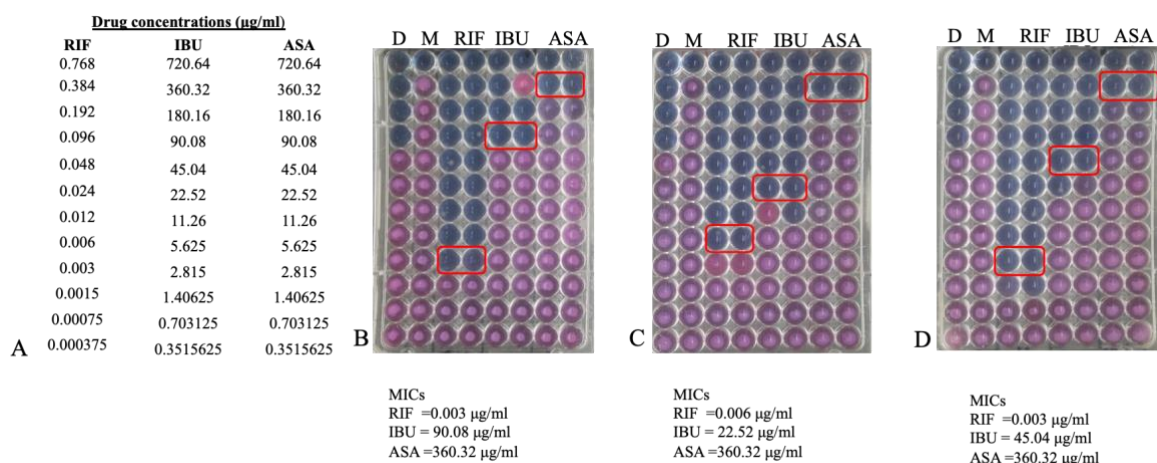


Figure 16. The broth microdilution assay for ASA and IBU against the H37Rv strain of *Mycobacterium tuberculosis* using the standard 2-fold broth method.

After 14 days of growth at 37°C, AlamarBlue® was added to each well to assess the presence of viable cells. **A)** A table that illustrates the drug concentrations for RIF, IBU, and ASA in the dilution series. **B)** Biological Replicate - BR 1, the 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and diluent (D) controls were also serially diluted in the first and second columns and the RIF, IBU, and ASA were serially diluted in the remaining columns. **C)** BR 2 with the same layout as in B. **D)** BR 3 was carried out with the same layout as B. The pink represents bacterial growth, and the blue indicates no growth. The lowest drug concentration that inhibits the growth of bacteria is indicated by the red boxes.

with a starting concentration of 720.64 µg/ml for IBU, the range was narrowed to 90.08 µg/ml by the repetition of replicates as indicated in Figure 17, however, the third repeat for IBU was indeterminate, and this might be due to a pipetting error. By visual reading of the MICs on the 96-well plate, it was therefore concluded that the MIC for IBU was 90.08 µg/ml.

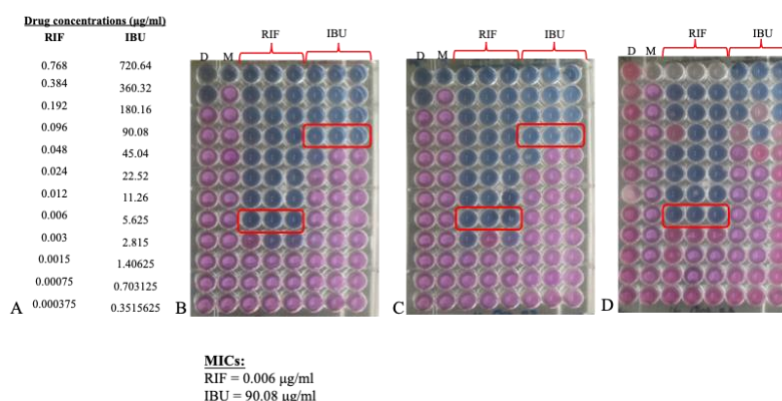


Figure 17. The broth microdilution assay for IBU against the H37Rv strain of *Mycobacterium tuberculosis* using the standard 2-fold broth microdilution method.

A) A table that illustrates the drug concentrations for the dilutions of RIF and IBU. **B)** Media (M), and Diluent (D) controls were placed in the first and second columns, followed by RIF and IBU, respectively, in 3 columns each. The pink represents bacterial growth, and the blue indicates no growth. The lowest drug concentration that inhibited the growth of bacteria was the MIC of that compound as indicated by the red boxes. **C)** Media (M), and Diluent (D) controls were placed in the first and second columns, followed by RIF and IBU, respectively, in 3 columns each. The pink represents bacterial growth, and the blue indicates no growth. The lowest drug concentration that inhibited the growth of bacteria was the MIC of that compound as indicated by the red boxes. **D)**

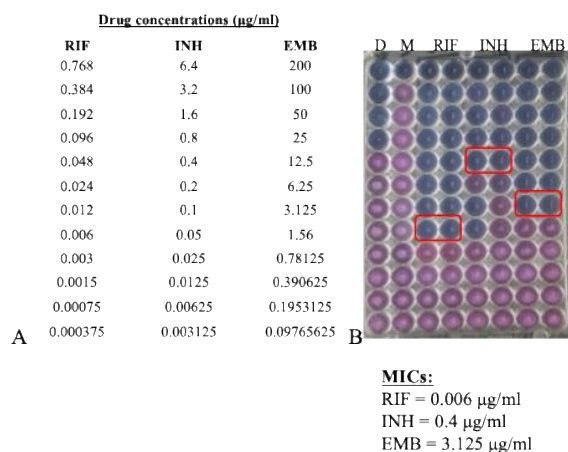


Figure 18. The broth microdilution assay for FLDs against *Mycobacterium tuberculosis*, H37Rv strain using the standard 2-fold broth microdilution method.

After 14 days of growth at 37°C, AlamarBlue® was added to each well to assess the presence of viable cells. **A)** A table that illustrates the drug concentrations for RIF, INH, and EMB in the dilution series. **B)** The 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and Diluent (D) controls in the first and second columns followed by RIF, INH, and EMB in duplicate in the consecutive columns were serially diluted. The pink represents bacterial growth and the blue indicates no growth. The lowest drug concentration that inhibits the growth of the bacteria was the MIC as indicated by the red boxes. The experiment was conducted in triplicate.

6.4 µg/ml, and 200 µg/ml, respectively. The MICs for RIF varied between 0.003, 0.006, and a range was therefore established. The MIC for INH was 0.4 µg/ml, and EMB 3.125 µg/ml, which correlated to previously published data (42), as seen in Figure 18.

Different lineages of *M.tb* can exhibit varying responses to antimicrobial agents due to their genetic diversity and specific resistance mechanisms. These differences may impact the MIC values and how effectively drugs inhibit bacterial growth. Therefore, it was important to establish the MICs for Bj and LAM, which is seen in Figures 19 and 20. As shown in Figure 19, the starting concentrations for RIF, INH, and EMB were 0.768 µg/ml, 6.4 µg/ml, and 200 µg/ml, respectively, and resulted in MICs of 0.024 µg/ml, 0.2 µg/ml, and 1.56 µg/ml with the Bj strain, which is similar to that observed for H37Rv, except MIC for RIF was four-fold higher. The starting concentrations for the NSAIDs were 720.64 µg/ml, and the MICs were established at 360.12 µg/ml for ASA and 180.16 µg/ml for IBU. The Bj strain has a higher

	RIF	INH	EMB			Drug concentrations (µg/ml)							
				D	M	RIF	INH	EMB	RIF	IBU	ASA		
	0.768	6.4	200						0.768	720.64	720.64		
	0.384	3.2	100						0.384	360.32	360.32		
	0.192	1.6	50						0.192	180.16	180.16		
	0.096	0.8	25						0.096	90.08	90.08		
	0.048	0.4	12.5						0.048	45.04	45.04		
	0.024	0.2	6.25						0.024	22.52	22.52		
	0.012	0.1	3.125						0.012	11.26	11.26		
	0.006	0.05	1.56						0.006	5.625	5.625		
	0.003	0.025	0.78125						0.003	2.815	2.815		
	0.0015	0.0125	0.390625						0.0015	1.40625	1.40625		
	0.00075	0.00625	0.1953125	0.00075	0.703125	0.703125							
A.	0.000375	0.003125	0.09765625	B.					C.	0.000375	0.3515625	0.3515625	D.
					MICs RIF = 0.024 µg/ml INH = 0.2 µg/ml EMB = 1.56 µg/ml								
								MICs RIF = 0.024 µg/ml ASA = 360.32 µg/ml IBU = 180.16 µg/ml					

After 14 days of growth at 37°C, AlamarBlue® was added to each well to assess the presence of viable cells. **A)** A table that illustrates the drug concentrations for RIF, INH, and EMB in the dilution series. **B)** The 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and Diluent (D) controls in the first and second columns followed by RIF, INH, and EMB in duplicate in the consecutive columns were serially diluted. The pink represents bacterial growth and the blue indicates no growth. The lowest drug concentration that inhibits the growth of the bacteria was indicated by the red boxes. **C)** A table that illustrates the drug concentrations for RIF, ASA, and IBU. **D)** The 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and Diluent (D) controls were also serially diluted in the first and second columns and then RIF, ASA, and IBU were serially diluted in the remaining columns. The experiment was conducted in triplicate.

were 0.768 µg/ml, 6.4 µg/ml, and 200 µg/ml respectively, and yielded the MICs for LAM with RIF, INH, and EMB were 0.006 -0.012 µg/ml, 0.2 µg/ml, and 1.56 µg/ml, which is similar to that of H37Rv except for RIF. The starting concentrations for the NSAIDs were 720.64 µg/ml, and the MICs were established at 360.12 µg/ml for ASA and 180.16 µg/ml for IBU.

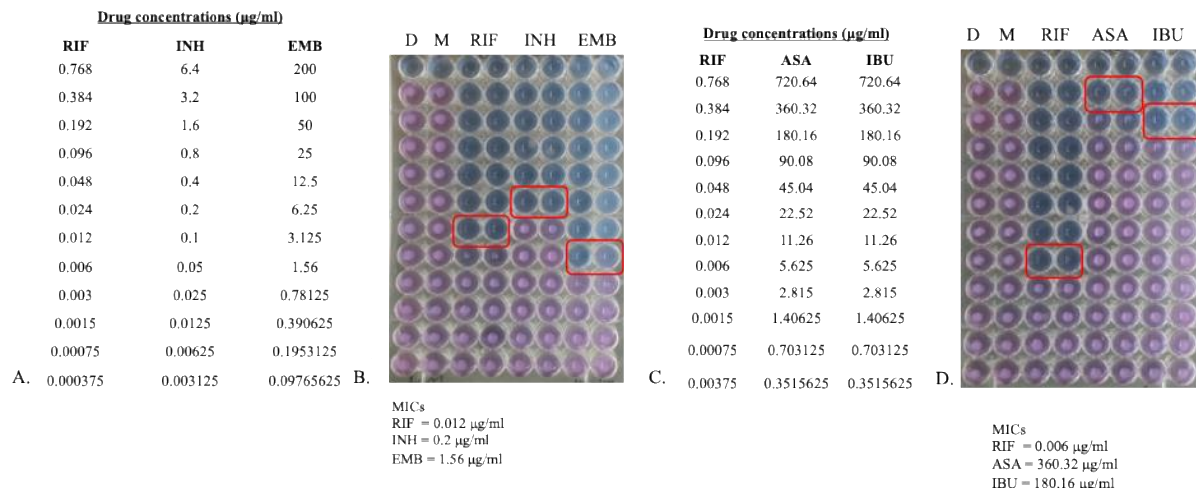


Figure 20. The broth microdilution assay for FLDs, and NSAIDs against *Mycobacterium tuberculosis*, LAM strain using the standard 2-fold broth microdilution method.

After 14 days of growth at 37°C, AlamarBlue® was added to each well to assess the presence of viable cells. **A)** A table that illustrates the drug concentrations for RIF, INH, and EMB in the dilution series. **B)** The 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and Diluent (D) controls were also serially diluted in the first and second columns, and then RIF, INH, and EMB in duplicate in the consecutive columns were serially diluted. The pink represents bacterial growth and the blue indicates no growth. The lowest drug concentration that inhibits the growth of the bacteria was indicated by the red boxes. **C)** A table that illustrates the drug concentrations for RIF, ASA, and IBU in the dilution series. **D)** The 96 well plate after 16 hours of incubation with AlamarBlue®. Media (M) and Diluent (D) controls were also serially diluted in the first and second columns and then RIF, ASA, and IBU were serially diluted in the remaining columns. This experiment was carried out in three times.

A summary of the MICs of the FLDs and NSAIDs against H37Rv, Bj, and the LAM strains is provided in Table 4

Table 4: MIC data for the different *Mycobacterium tuberculosis* strains

Drug	Minimum inhibitory Concentration (MIC) (µg/ml) for <i>M.tb</i> strains		
	H37Rv	Bj	LAM
ASA	360.32	360.32	360.32
IBU	90.08	180.16	180.16
INH	0.4	0.2	0.2
RIF	0.003-0.006	0.024	0.006 - 0.012
EMB	3.125	1.56	1.56

The MIC for ASA is consistent across all three strains at 360.32 µg/ml. This suggests that H37Rv is more susceptible to ASA, while Bj and LAM exhibit higher tolerance or potential resistance. For IBU, Bj and LAM have a MIC of 180.16 µg/ml, double that of H37Rv (90.08 µg/ml). Next, it was important to evaluate the interaction between two antimicrobial agents, and this was done by the chequerboard assay.

4.2 Chequerboard assay

The chequerboard assay was used to assess the potency of the FLD in combination. RIF and INH were diluted and the EMB was added at a fixed concentration of 0.5 µg/ml. As shown in Figure 21, bacterial growth was inhibited in the RIF-only wells and the combination wells, with bacterial growth (pink) observed only at lower drug levels of INH, up to 0.2 µg/ml alone, and in the positive control wells.

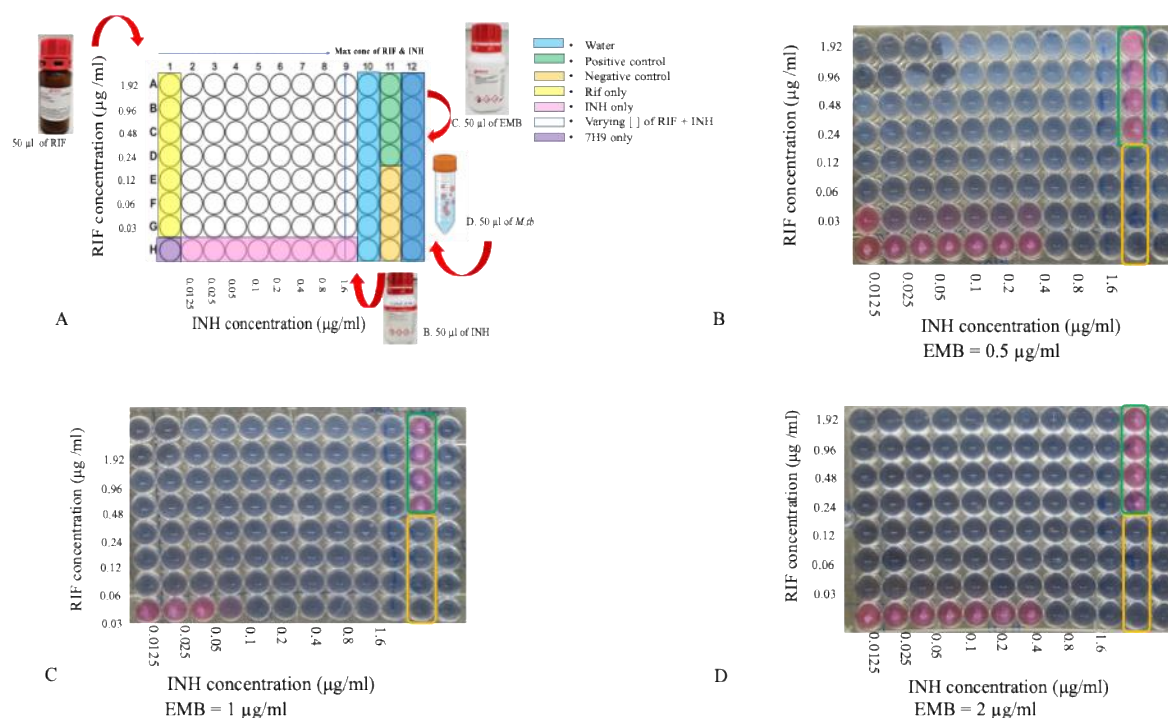


Figure 21. The optimization and setting up of a chequerboard assay using the FLDs against the H37Rv strain of *Mycobacterium tuberculosis*. A) Chequerboard assay plate layout setup with varying concentrations of RIF (in yellow wells), varying concentrations of INH (in pink wells), and a combination of RIF, INH, and EMB at a concentration of 2 µg/ml (in white wells). Empty wells in blue were filled with water, the positive control is represented in the green wells, and the negative control in the orange wells. B) **Synergy 1:** Chequerboard assay setup with varying concentrations of RIF, and INH at a concentration of 0.5 µg/ml of EMB. C) **Synergy 2:** Chequerboard assay setup with varying concentrations of RIF, and INH at a concentration of 1 µg/ml of EMB. D) **Synergy 3:** Chequerboard assay setup with varying concentrations of RIF, and INH at a concentration of 2 µg/ml of EMB. *This experiment was carried out in two independent experiments and yielded the same bacterial inhibition, as the MIC could not be read.

Since a combined MIC for the various drugs could not be established, the starting concentration of all drugs was revised and lowered from 1.92 µg/ml to 0.48 µg/ml for RIF, from 1.6 µg/ml to 0.4 µg/ml for INH whilst the concentration for EMB was kept constant at 0.5 µg/ml, 1 µg/ml and 2 µg/ml on each 96-well plate. The initial starting concentrations were determined by taking into consideration that each drug concentration is halved when another drug or bacterial

dose is added to a well, therefore increasing it three-fold. Despite using these lower concentrations, there was still complete inhibition of bacterial growth in the chequerboard assay, Figure 22.

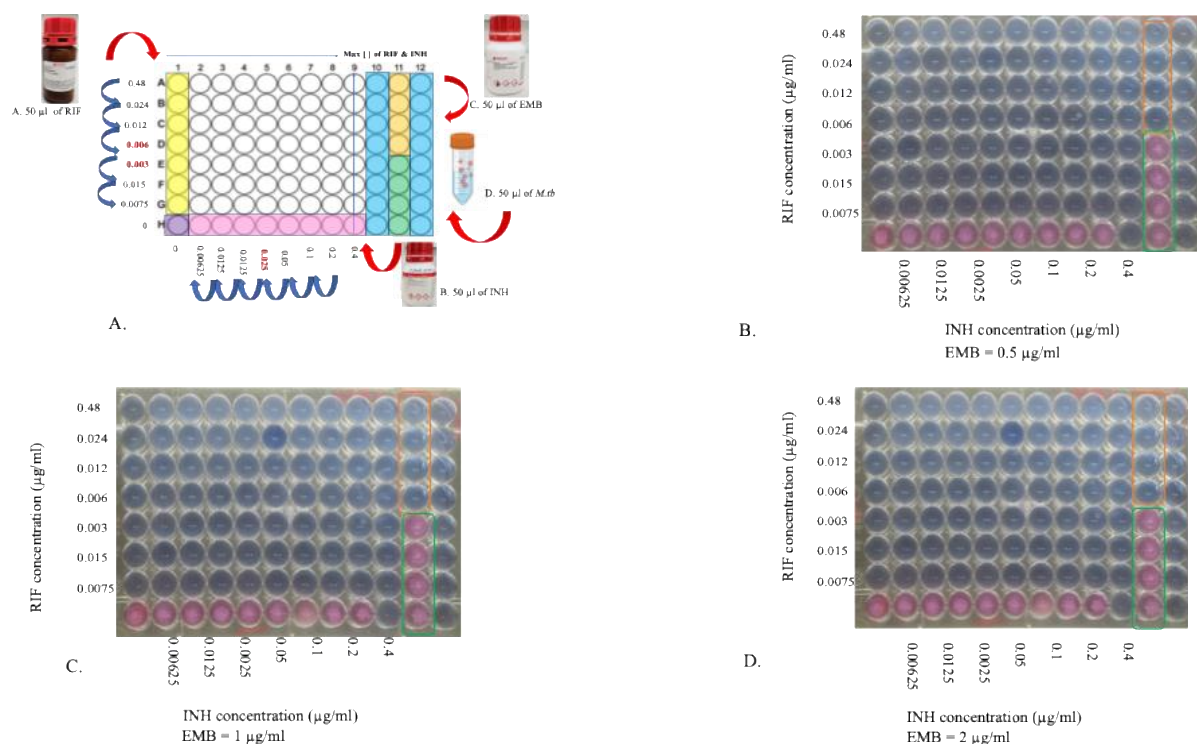


Figure 22. The optimization and setting up of a chequerboard assay using the FLDs with lowered starting concentrations of RIF and INH at 0.48 µg/ml and 0.4 µg/ml respectively.

A) Chequerboard assay plate layout setup with lowered starting concentrations of RIF (in yellow wells), lowered starting concentrations of INH (in pink wells), and a combination of RIF, INH, and EMB at a concentration of 2 µg/ml (in white wells). Empty wells in blue were filled with water, the positive control is represented in the green wells, and the negative control in the orange wells. **B) Synergy 1:** Chequerboard assay setup with varying concentrations of RIF, and INH with EMB at a concentration of 0.5 µg/ml. **C) Synergy 2:** Chequerboard assay setup with varying concentrations of RIF, INH and EMB at a concentration of 1 µg/ml. **D) Synergy 3:** Chequerboard assay setup with varying concentrations of RIF and INH with EMB at a concentration of 2 µg/ml. The experiment was carried out in triplicate and yielded the same bacterial inhibition as observed with the single drugs (Figure 18).

These results suggested that even at the lower than MIC concentrations, the three FLDs when combined, were still too potent. The synergy between the three FLDs was optimized by further lowering the starting drug concentrations from 0.48 µg/ml to 0.12 µg/ml for RIF and 0.4 µg/ml to 0.1 µg/ml for INH. Two plates were set up in this manner, and to one 0.5 µg/ml of EMB was added to plate one, but no EMB was added to the second plate since the EMB may also be responsible for killing the cells when combined with the RIF and INH. Once again, no bacterial

growth was observed in the RIF-only wells, Figure 23 B, suggesting that the strain of *M.tb* used may be hyper-sensitive to RIF.

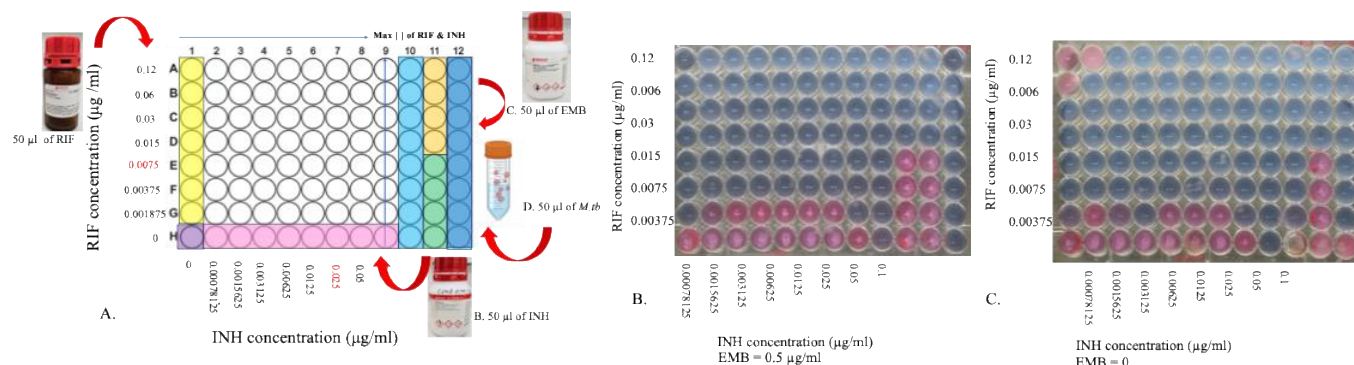
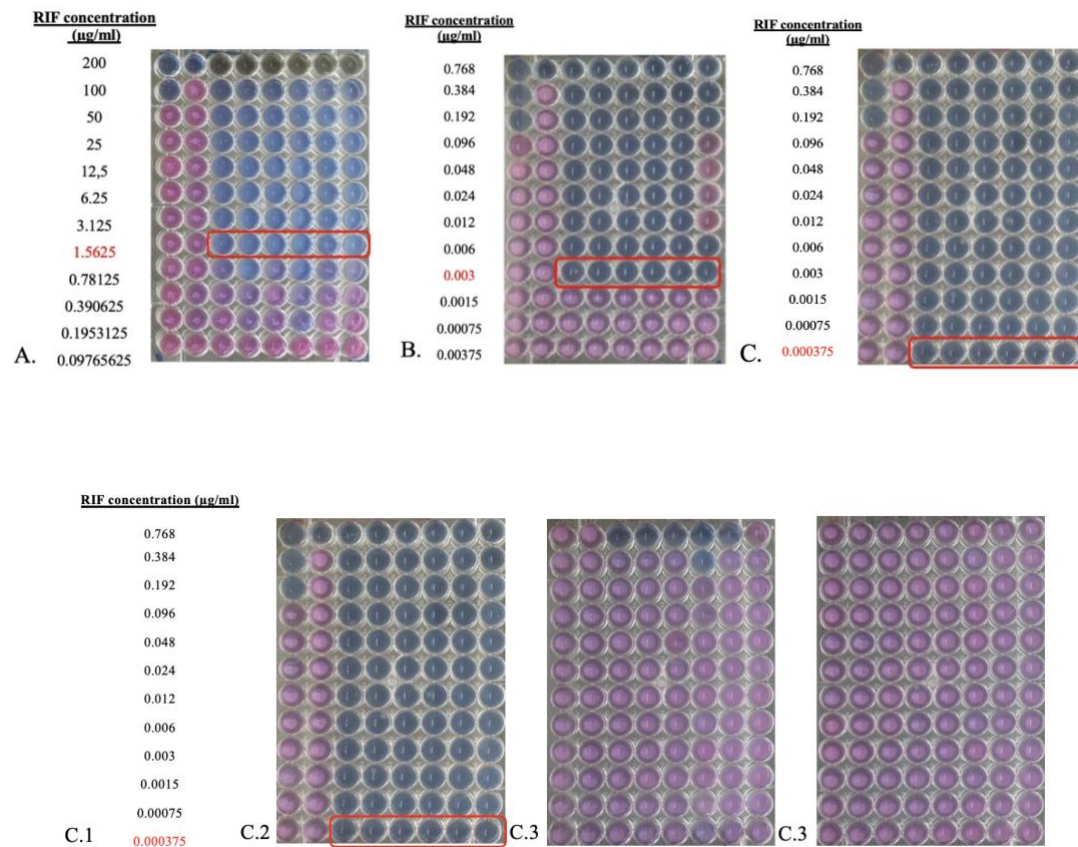


Figure 23. The optimization and setting up of a chequerboard assay using the FLDs.

The starting concentrations for RIF and INH at 0.12 µg/ml and 0.1 µg/ml, respectively, and only testing EMB at 0.5 µg/ml. **A)** Chequerboard assay plate layout setup with lowered starting concentrations of RIF (in yellow wells), lowered starting concentrations of INH (in pink wells), and a combination of RIF, INH, and EMB at a concentration of 0.5 µg/ml (in white wells). Empty wells in blue were filled with water, the positive control is represented in the green wells, and the negative control in the orange wells. In red note the expected MIC ranges previously reported. **B) Synergy 1:** Chequerboard assay setup with varying concentrations of RIF and INH with EMB at a concentration of 0.5 µg/ml. **C) Synergy 2:** Chequerboard assay setup with varying concentrations of RIF, INH, and 0 EMB. This experiment was carried out only once as no bacterial growth was observed.

In the absence of EMB, there was still no bacterial growth (Figure 23 C). To further understand the lack of synergy between the FLDs, the MIC for RIF was assessed with three different mycobacterial strains which, included- *Mycobacterium smegmatis* strain mc²155, H37Rv Johannesburg H37Rv(J), and H37Rv Sasseti Rv(S). As seen in Figure 24 A, the MIC for *M. smegmatis* was 1.5625 µg/ml, as previously reported by Shaku et al (personal communication, unpublished). Growth of H37Rv(J) was inhibited at 0.003 µg/ml (Figure 24 B), as reported by Kana et al., 2010 (42), whilst the MIC for H37Rv(S) – the strain used in this study - was 0.000375 µg/ml. This concluded that the RIFs efficacy was variable between strains and that the problem was narrowed down to the strain of *M.tb* used in setting up the chequerboard assays.



Since the H37Rv(S) strain was used in this study and based on the above findings, the starting concentrations for RIF and INH were lowered further to 0.12 and 0.05 µg/ml, respectively. At these concentrations, the chequerboard assay resulted in an MIC of 0.0075 µg/ml for RIF and 0.025 µg/ml for INH (Figure 25).

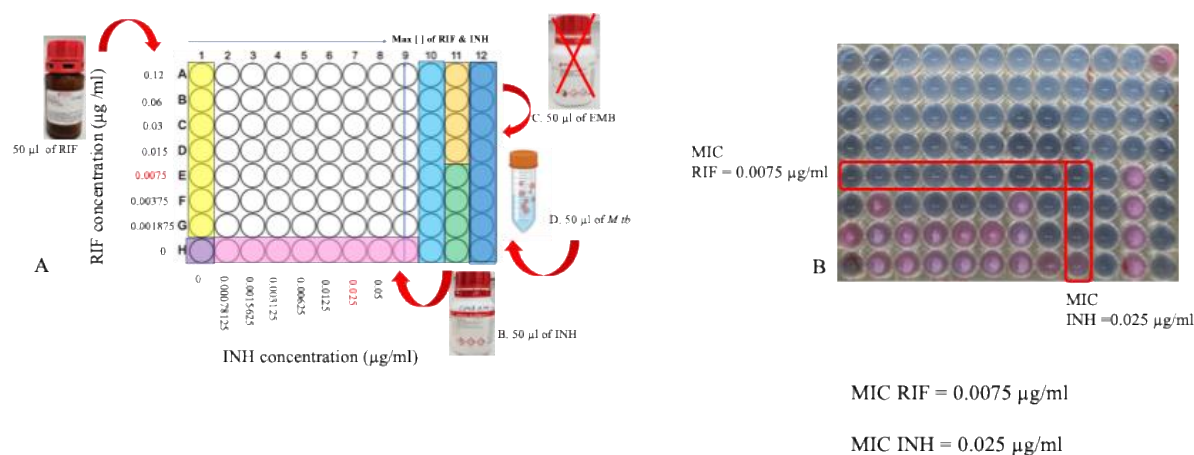


Figure 25. The optimization and setting up of a chequerboard assay using lowered starting concentrations for RIF and INH at 0.12 µg/ml and 0.1 µg/ml respectively.

A) Chequerboard assay plate layout setup with lowered starting concentrations of RIF (in yellow wells), lowered starting concentrations of INH (in pink wells), and a combination of RIF and INH, (in white wells). Empty wells in blue were filled with water, the positive control is represented in the green wells, and the negative control in the orange wells. The red boxes show the expected MIC ranges previously reported. **B)** Chequerboard assay plate after 14 days of incubation and the addition of AlamarBlue®. This experiment was carried out three times.

At each time when this experiment was carried out, it produced varying results for RIF, ranging between 0.00375-0.0075 µg/ml, but with a constant MIC of 0.025 µg/ml for INH. Based on these data the starting concentration for RIF was lowered to 0.03 µg/ml in the chequerboard assay, and the single drugs were tested individually to ensure that the drug had the expected potency. This experiment yielded MICs for RIF at 0.00375 µg/ml and INH at 0.025 µg/ml in the chequerboard plate (Figure 26 B) whilst the MICs for the single drugs was 0.003 µg/ml for RIF and 0.05 µg/ml for INH. These MICs were used to calculate the synergy of the combination of RIF and INH drugs. The Fractional Inhibitory Concentration Index (FICI) indicates the type of interaction between the drugs.

$$\begin{aligned} \text{FIC of Drug A} &= \text{MIC of RIF in combination} / \text{MIC of RIF A alone} \\ &= 0.00375 / 0.003 \\ &= 1.25 \end{aligned}$$

$$\begin{aligned} \text{FIC of Drug B} &= \text{MIC of INH in combination} / \text{MIC of INH alone} \\ &= 0.025 / 0.05 \\ &= 0.5 \end{aligned}$$

$$\begin{aligned}
 \text{FIC Index (FICI)} &: \text{FICI} = \text{FIC (RIF)} + \text{FIC (INH)} \\
 &= 1.25 + 0.5 \\
 &= 1.75
 \end{aligned}$$

The synergy of drug combinations can be assessed using the Fractional Inhibitory Concentration Index (FICI), where $\text{FICI} < 0.5$ indicates synergy (drugs work better together than individually), FICI between 0.5 and 4 suggests an additive or indifferent effect (drugs perform similarly alone or in combination), and $\text{FICI} > 4$ signifies antagonism, where the drugs interfere with each other, reducing the effectiveness (114).

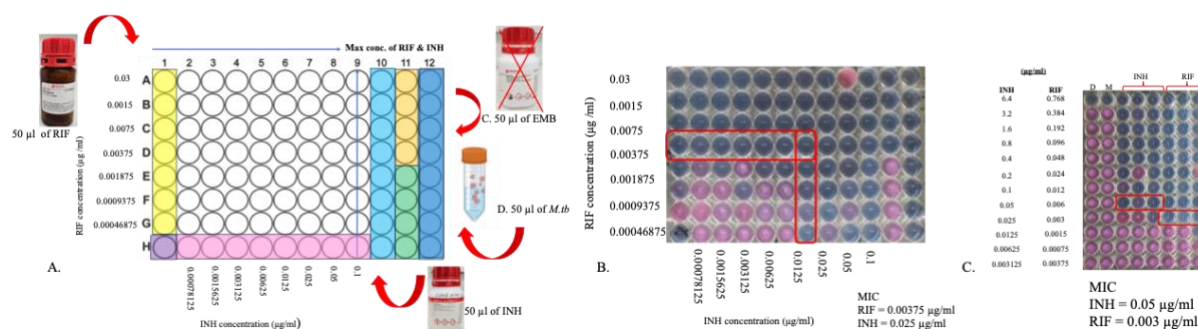


Figure 26. The optimization and setting up of a chequerboard assay.

The starting concentrations for RIF and INH at 0.03 µg/ml and 0.1 µg/ml, respectively. **A)** Chequerboard assay plate layout setup with lowered starting concentrations of RIF (in yellow wells), lowered starting concentrations of INH (in pink wells), and a combination of RIF and INH, (in white wells). Empty wells in blue were filled with water, the positive control is represented in the green wells, and the negative control in the orange wells. The red boxes note the expected MIC ranges previously reported. **B)** Chequerboard assay plate after 14 days of incubation and the addition of AlamarBlue® **C)** A plate with the single drugs was set up as a control. The experiment was carried out in triplicate.

4.3 Mycoplasma detection of THP-1 cells for viability assay

Before any infection experiments, the sterility of the THP-1 cell line was verified to prevent interference or false results. Mycoplasma contamination was assessed using Mycostrips on cell suspensions prior to the infection experiments. As shown in Figure 27, both culture 1 and culture 2 tested negative for mycoplasma when compared to the positive control strip.

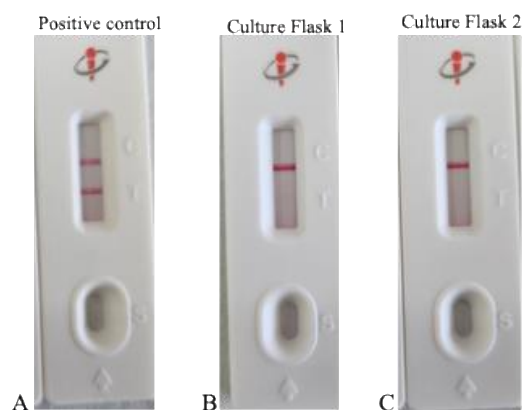


Figure 27. A MycoStrip™ detection assay to test for common cell contaminants like Mycoplasma in the THP-1 cell line culture.

- A)** A MycoStrip™ positive control that was provided in the kit, that contains mycoplasma DNA.
- B)** A MycoStrip™ for culture flask 1, which yielded a negative result, negative for Mycoplasma.
- C)** A MycoStrip™ for culture flask 2, which yielded a negative result, negative for Mycoplasma.

4.4 Viability assay using MTT

Figures 28 to 33 present an analysis of THP-1 cell viability at varying drug concentrations (1x, 5 x, and 10 x MIC) over 4 to 72 hours. The primary objective was to determine the MIC at which cytotoxicity induces cell death. Establishing the optimal concentration was essential to ensure that subsequent experiments involving bacterial infection of THP-1 cells would reflect effects due to the infection rather than excessive drug exposure. The MTT assay was performed in three independent biological replicates, each conducted over one week. Cells underwent 72-hour drug exposure followed by a 4-hour MTT incubation before absorbance measurement; accordingly, the full series spanned three weeks with one replicate per week. Each 24-well plate was prepared with each drug condition represented in duplicate technical wells.

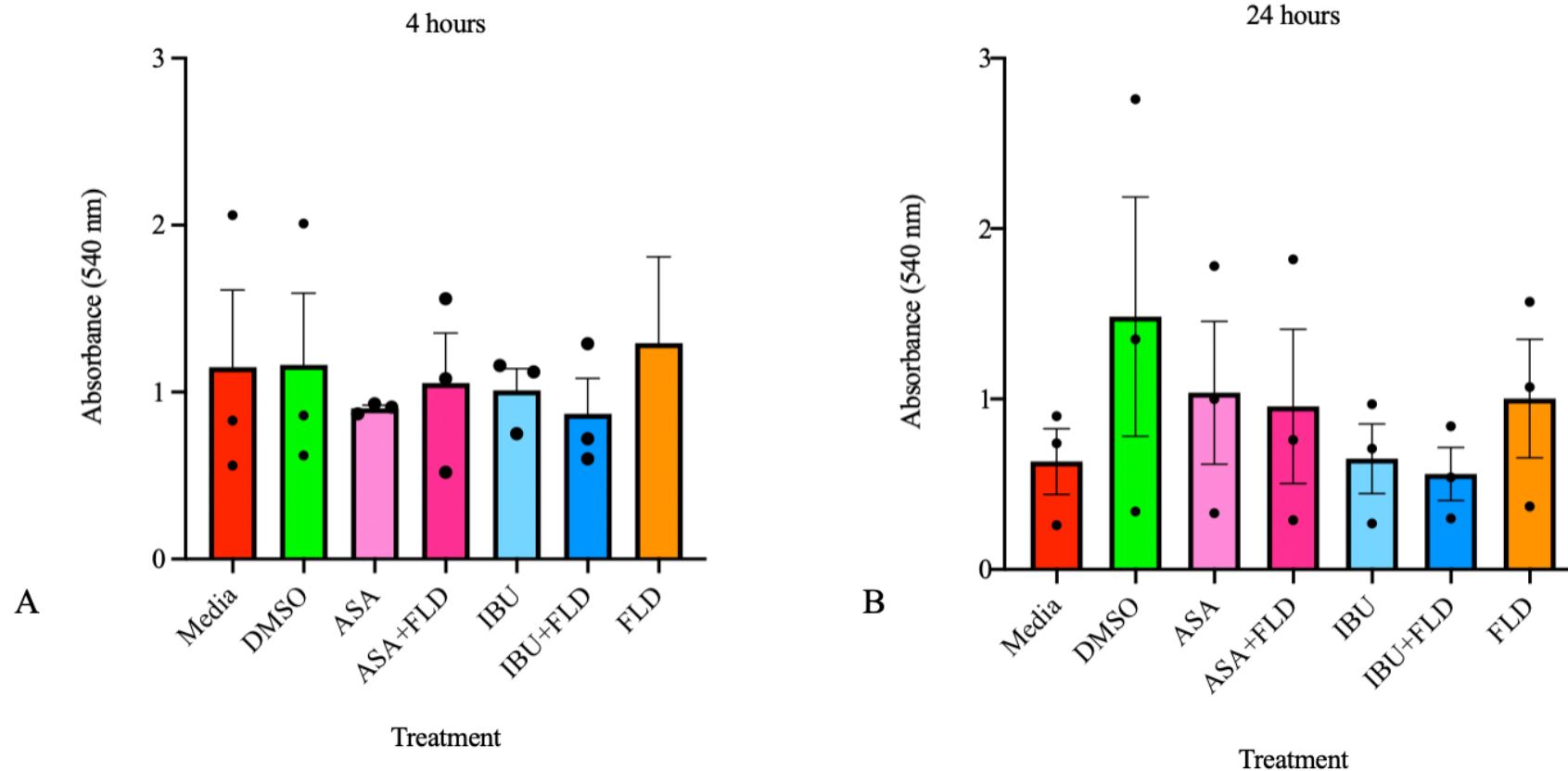


Figure 28. A viability assay using MTT at a drug concentration of 1 x MIC after 4 and 24 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 1x MIC at the A) 4-hour timepoint and B) 24-hour timepoint. Media and DMSO served as controls, NSAIDs (ASA and IBU) were tested individually and in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. No significant cell death was noted at these time points with the NSAIDs or FLD. The experiment was carried out three times.

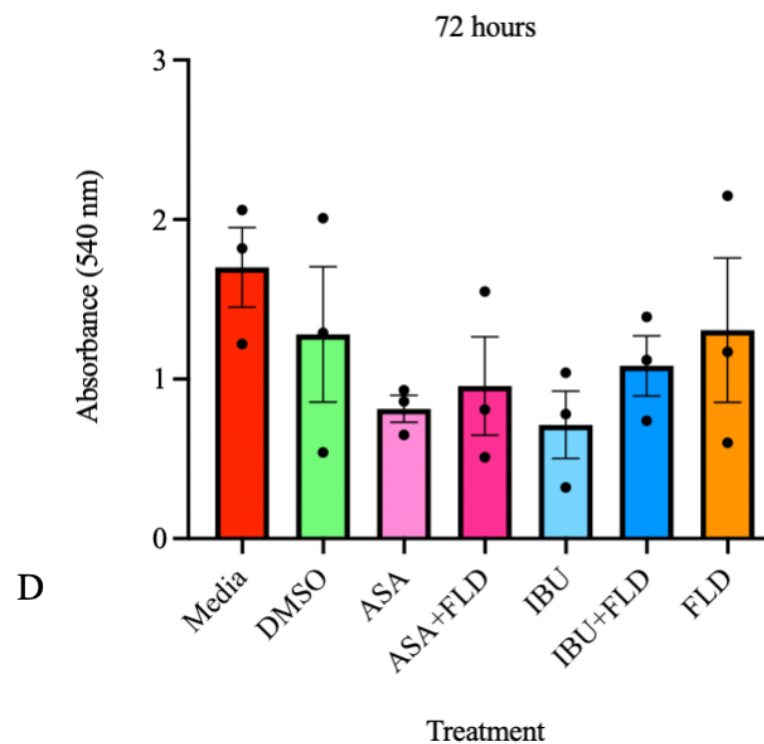
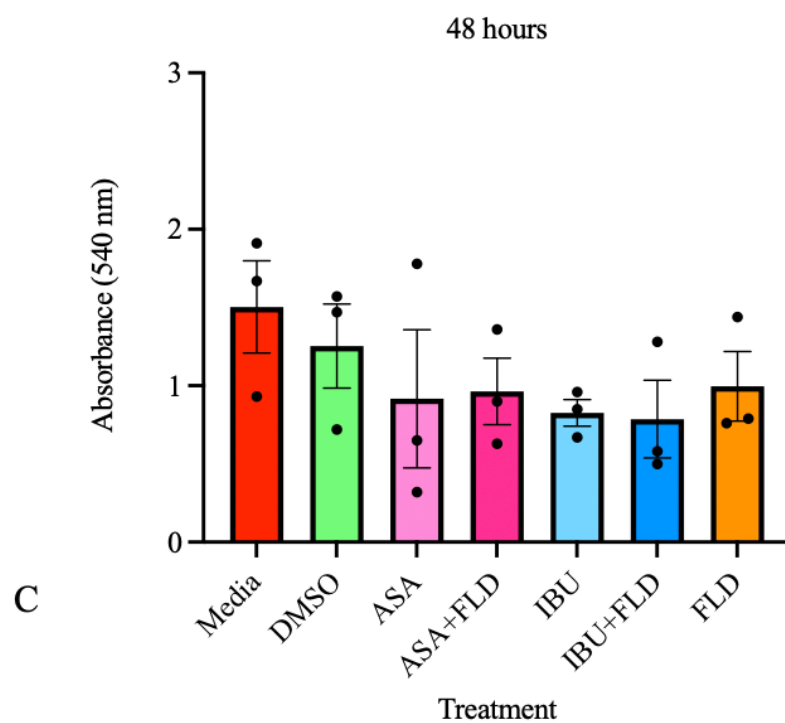


Figure 29. A viability assay using MTT at a drug concentration of 1 x MIC after 48 and 72 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 1x MIC at the A) 48-hour timepoint. B) 72-hour timepoint. Media and DMSO served as controls, NSAIDs alone (ASA and IBU) were tested individually and then in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. No significant cell death was noted at these time points. The experiment was carried out three times.

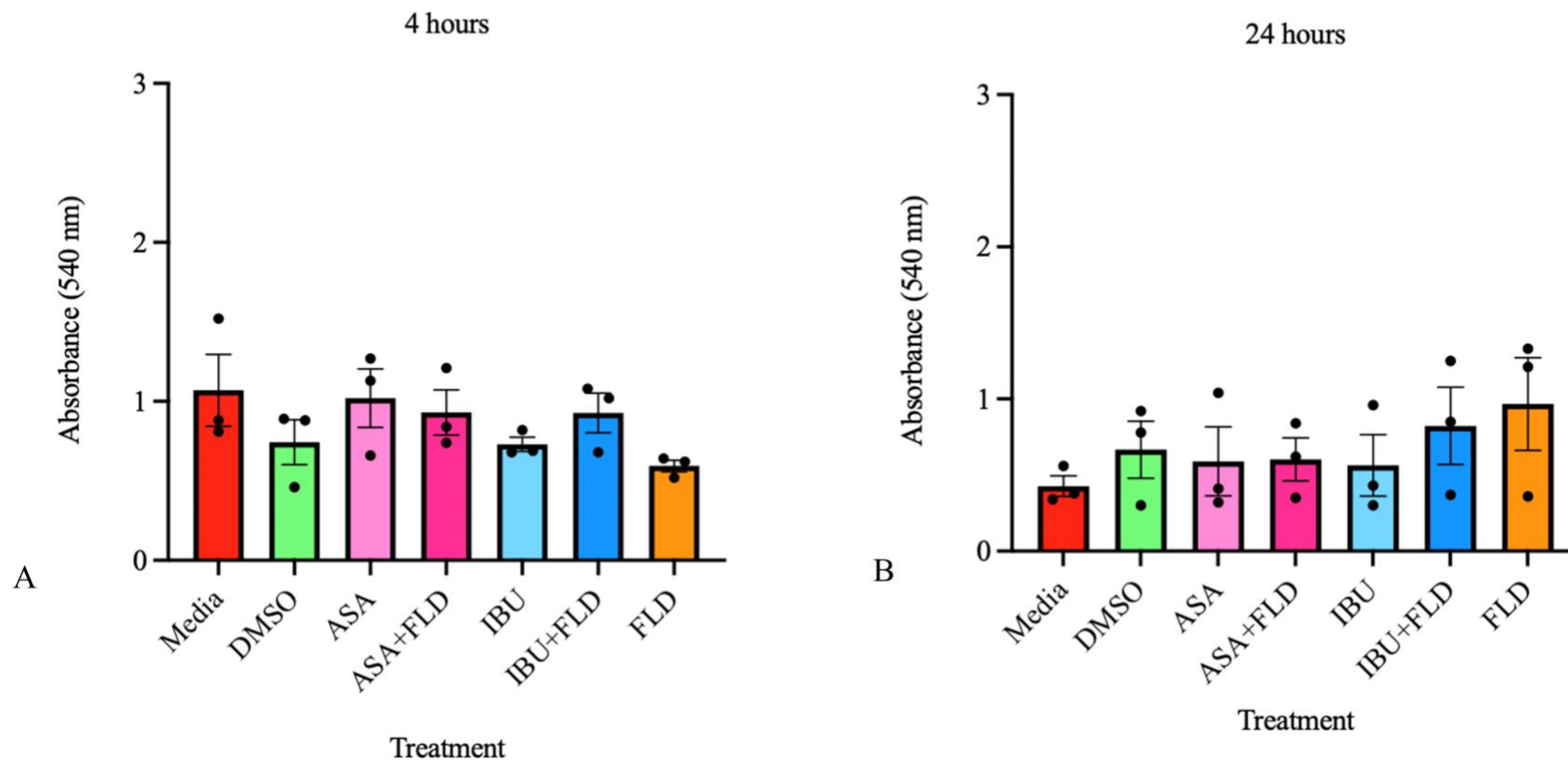


Figure 30. A viability assay using MTT at a drug concentration of 5 x MIC for 4 and 24 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 5 x MIC **A**) 4-hour timepoint **B**) 24-hour timepoint. Media and DMSO served as controls, NSAIDs alone (ASA and IBU) were tested individually and then in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. No significant cell death was noted at these time points. The experiment was carried out three times.

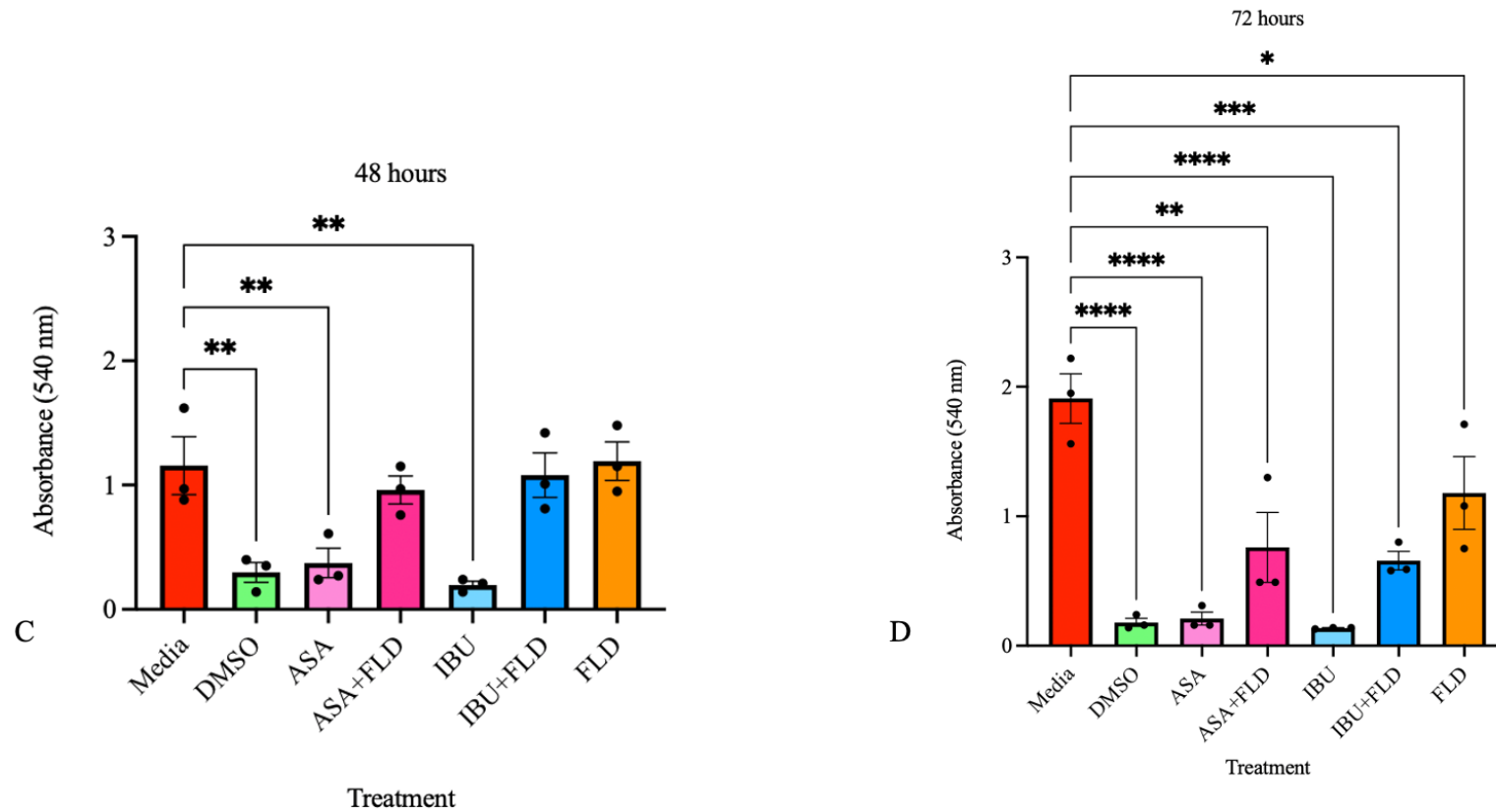


Figure 31. A viability assay using MTT at a drug concentration of 5 x MIC for 48 and 72 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 5x MIC at the A)48-hour timepoint B)72-hour timepoint. Media and DMSO served as controls, NSAIDs alone (ASA and IBU) and then in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. No significant cell death was noted at these time points. The experiment was carried out three times.

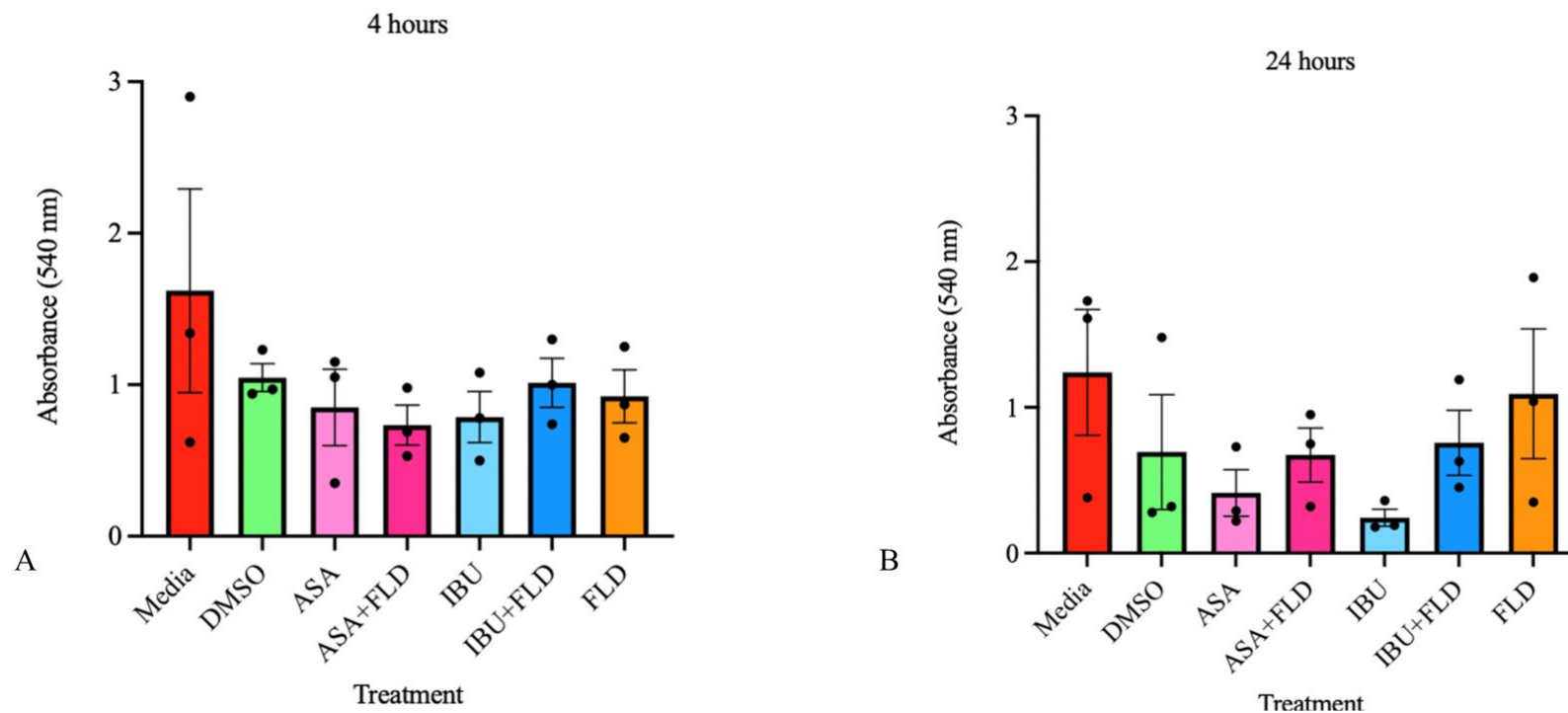


Figure 32. A viability assay using MTT at a drug concentration of 10 x MIC for 4 and 24 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 10 x MIC at the **A)** 4-hour timepoint **B)** 24-hour timepoint.

Media and DMSO served as controls, NSAIDs alone (ASA and IBU) and then in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. No significant cell death was noted at these time points. The experiment was carried out three times.

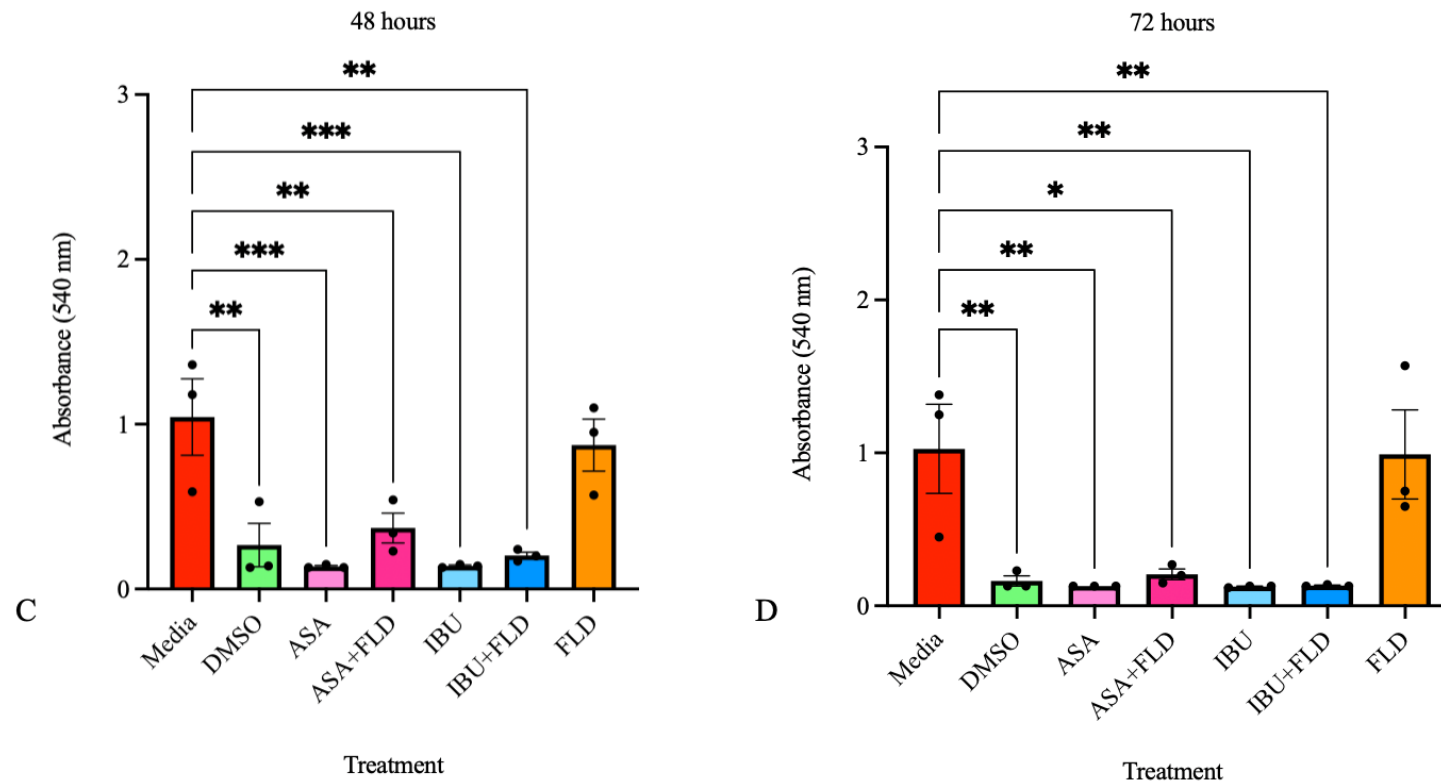


Figure 33. A viability assay using MTT at a drug concentration of 10 x MIC for 48 and 72 hours.

Cell viability was measured after THP-1 cells were incubated with NSAIDs and FLD concentrations as 10 x MIC at the **A)**48-hour timepoint **B)**72-hour-timepoint. Media and DMSO served as controls, NSAIDs alone (ASA and IBU) and then in combination with FLDs (RIF and INH), absorbance was measured at 540 nm. Significant cell death was noted at these time points, $p < 0.05$. The experiment was carried out three times.

Figure 28 A, at 4 hours, no major differences were observed between groups. Figure 28 B, 24 hours, IBU and IBU+FLD showed reduced viability compared to other treatment group. Figures 29 C and D; At 48 and 72 hours, respectively. Media and DMSO controls consistently maintained higher absorbance, whereas ASA, ASA+FLD, IBU, and IBU+FLD showed lower levels, with FLD maintaining intermediate values. Overall, these differences were not statistically significant.

Figure 30 A, at 4 hours, viability was comparable across all groups, with Media and ASA+FLD showing slightly higher values, while FLD alone displayed the lowest. Figure 30 B shows that at 24 hours, variation between treatments became more evident, with Media showing the lowest viability, whereas FLD and IBU+FLD demonstrated higher viability. Overall, no statistically significant differences were detected between treatments.

Figures 31 C, at 48 hours, DMSO, ASA, and IBU significantly reduced viability compared to the media control, indicating decreased viability. ASA+FLD, IBU+FLD, and FLD alone maintained higher absorbance, suggesting better cell viability compared to ASA or IBU alone. Figures 31 D, at 72 hours, the reductions were more pronounced: DMSO, ASA, and IBU showed strong inhibition of viability, when compared to media. FLD alone and drug combinations showed moderate viability.

Figure 32 A, no major differences between treatments and media; viability remained relatively stable across all groups. Figure 32 B, DMSO, ASA, and IBU, showed a decreased viability compared to the media. FLD alone and drug combinations showed moderate viability.

Figures 33 C, at 48 hours, DMSO, ASA, IBU, and their combinations with FLD significantly reduced absorbance compared to the media, indicating reduced viability. FLD alone preserved viability, with higher absorbance than the other treatments. Figure 33 D, FLD alone again showed the highest absorbance, suggesting it best maintained cell viability.

Based on these results, a 1 x MIC was used for the subsequent experiment

4.5 Spoligotyping of strains

The CTAB-extracted gDNA from the three *M. tuberculosis* strains is shown in Figure 34A, with purity and concentration summarized in Figure 34 B. Although the DNA concentration for LAM was comparable to Bj, initial spoligotyping of LAM was unsuccessful (Figure 34 C). A frozen stock of LAM was plated on agar for gDNA extraction; RNA contamination was observed (Figure 34 D), and a repeat extraction showed DNA shearing (Figure 34 E). Cells from a frozen LAM culture were also plated on 7H11, and while plate-derived DNA appeared sheared (Figure 34 F), the frozen sample remained intact, with a concentration comparable to Bj (Figure 34 G). Final spoligotyping of H37Rv, Bj, and LAM is shown in Figure 34 H.

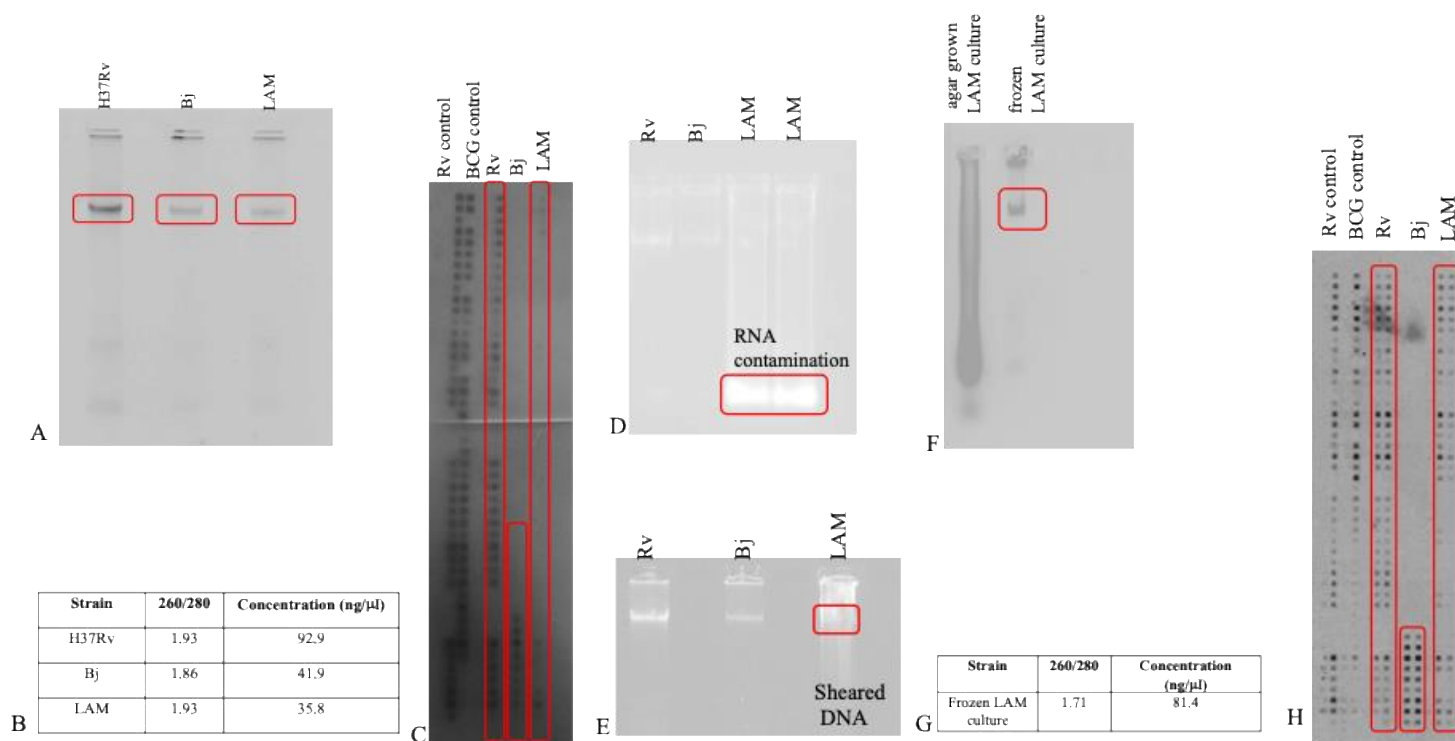


Figure 34. Illustrating the quantification of gDNA on an agarose gel and the NanoDrop for spoligotyping.

A) 1% electrophoresis agarose gel, with DNA from H37Rv strain, Bj strain, and LAM strain. The DNA is indicated by the red boxes **B)** Table with DNA 260/280 indicating the purity of DNA yielded from the CTAB extraction and the concentration of each strain. **C)** Spoligotyping x-ray film with H37Rv and BCG controls in lanes 1 and 2, respectively, H37Rv strain in Lane 3, Bj in Lane 4 and LAM strain detected very faintly. **D)** LAM was extracted again in duplicate and assessed on an agarose gel but RNA contamination was seen as indicated by the red boxes. **E)** An agarose gel showing the gDNA after RNase cleanup was done on the RNA-contaminated DNA, but sheared DNA was noted as indicated in the red boxes. **F)** LAM gDNA from cells plated on 7H11 agar and allowed to grow for 4-6 weeks, and gDNA, was extracted from a frozen aliquot. Only the frozen culture yielded DNA. **G)** Table with DNA 260/280 ratio, indicating the concentration. **H)** Spoligotyping x-ray film with H37Rv and BCG controls in Lane 1 and 3, respectively. In lanes 4 and 5 are confirmed strains of H37Rv, lanes 6 and 7 are confirmed strains of Bj, and LAM is confirmed in lanes 7 and 8.

Once the strains were confirmed through spoligotyping, they were deemed suitable for subsequent infection of THP-1 cells.

4.6 THP-1 infections

The established drug concentrations in the MIC, chequerboard assay, and MTT experiments were used to set up the THP-1 infection experiment. In Figure 35 A it can be seen that all NSAIDs alone or in combination with FLDs have no effect on the growing H37Rv bacteria within the macrophages over 72 hours. In B, the extracellular CFU/ml (represented by the bright green bar) appears slightly higher than the intracellular CFU/ml (lighter green bar). A paired t-test was performed to compare the bacterial burden between the extracellular and intracellular environments, revealing no statistically significant difference ($p = 0.3414$).

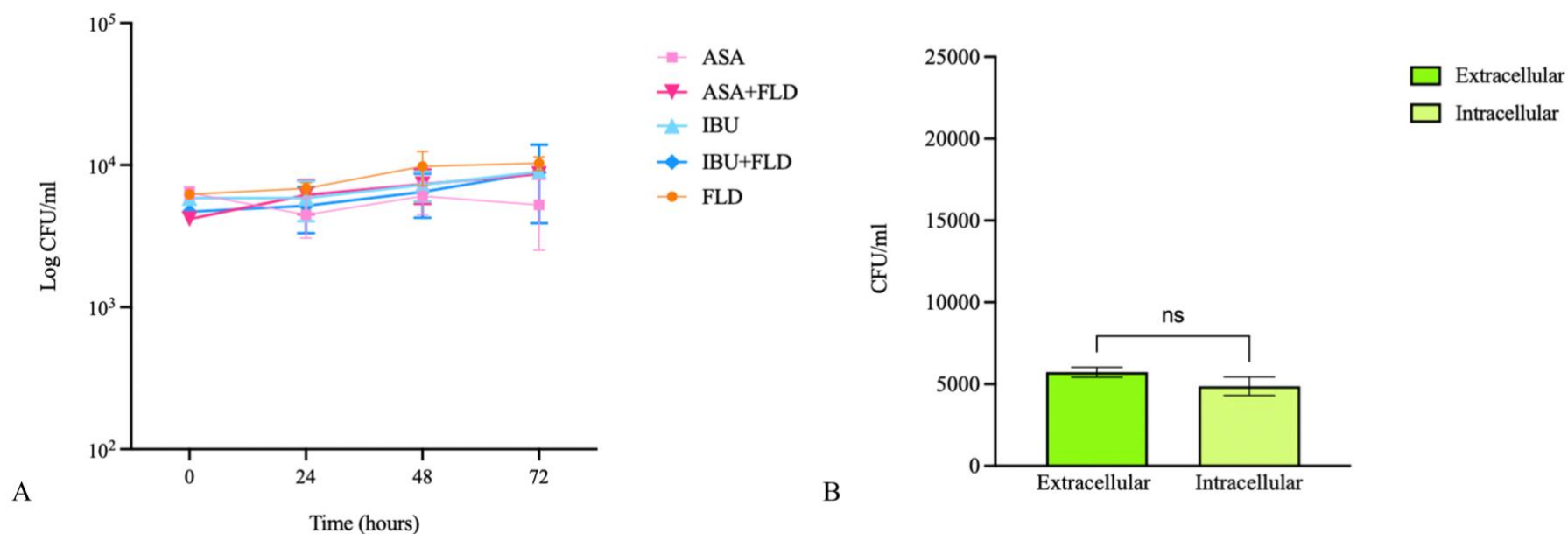


Figure 35. THP-1 infected with *Mycobacterium tuberculosis* H37Rv.

A) THP-1 cells were infected and treated with NSAIDs alone, NSAID+FLD, and FLDs alone for 24 hours, 48 hours, and 72 hours and the Log_{10} CFU/ml was plotted.

B) At T_0 , the extracellular and intracellular bacteria were plated, and the CFU/ml was counted after 4 weeks of incubation. This was carried out in three independent experiments.

In Figure 36 A it is evident that neither NSAIDs, administered individually nor in combination with FLDs, exhibited any measurable effect on the proliferation of intracellular B_j bacteria within macrophages over the 72-hour observation period. In Figure 38 B, following cell lysis and plating, the extracellular bacterial load was observed to be higher than the intracellular bacterial burden, suggesting that the bacteria were growing and lysing the cells, rather than being killed by drugs.

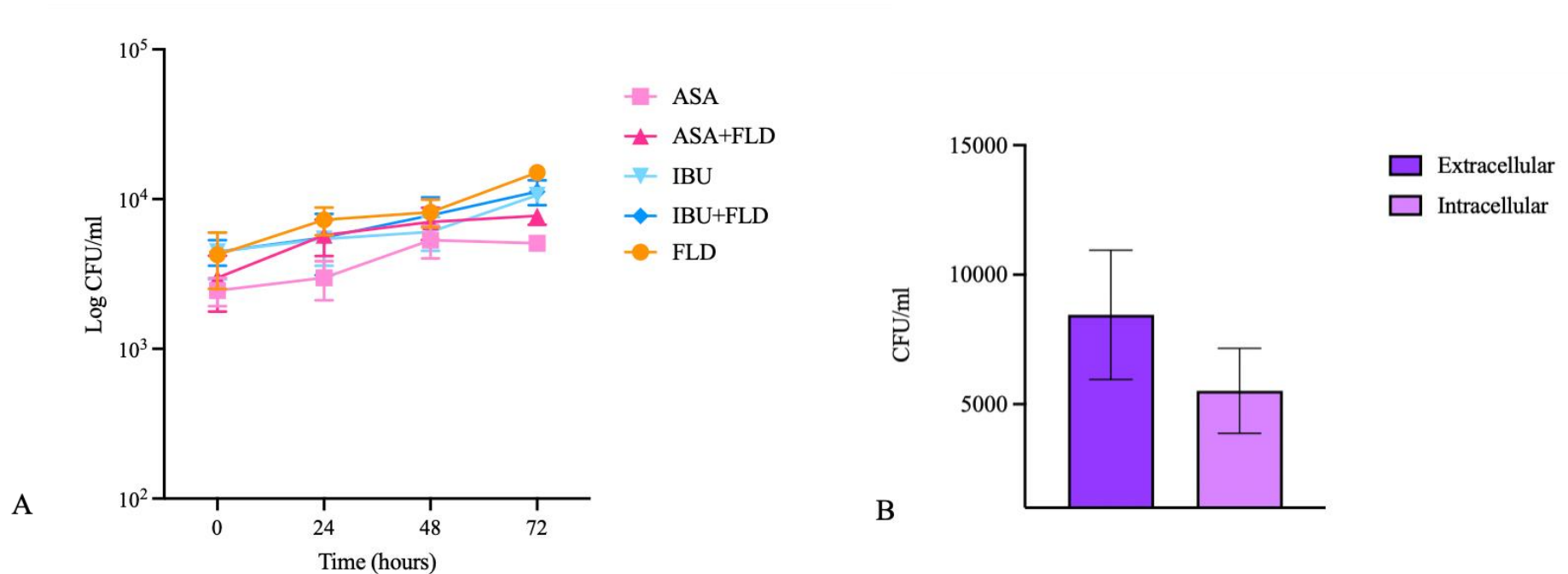


Figure 36. THP-1 infected with *Mycobacterium tuberculosis* B_j

A) THP-1 cells were infected and treated with NSAIDs alone, NSAID+FLD, and FLDs alone for 24 hours, 48 hours, and 72 hours and the Log₁₀ CFU/ml was plotted.

B) At T₀, the extracellular and intracellular bacteria were plated, and the CFU/ml was counted after 4 weeks of incubation.

In Figure 37 A, the combination of drugs (likely ASA, IBU, and FLDs) had no significant effect on bacterial killing, whether used alone or together. Additionally, In Figure 37 B, LAM bacilli were found to be more present in the extracellular lysate than intracellularly, indicating that the bacteria were not efficiently cleared or killed within the THP-1 cells.

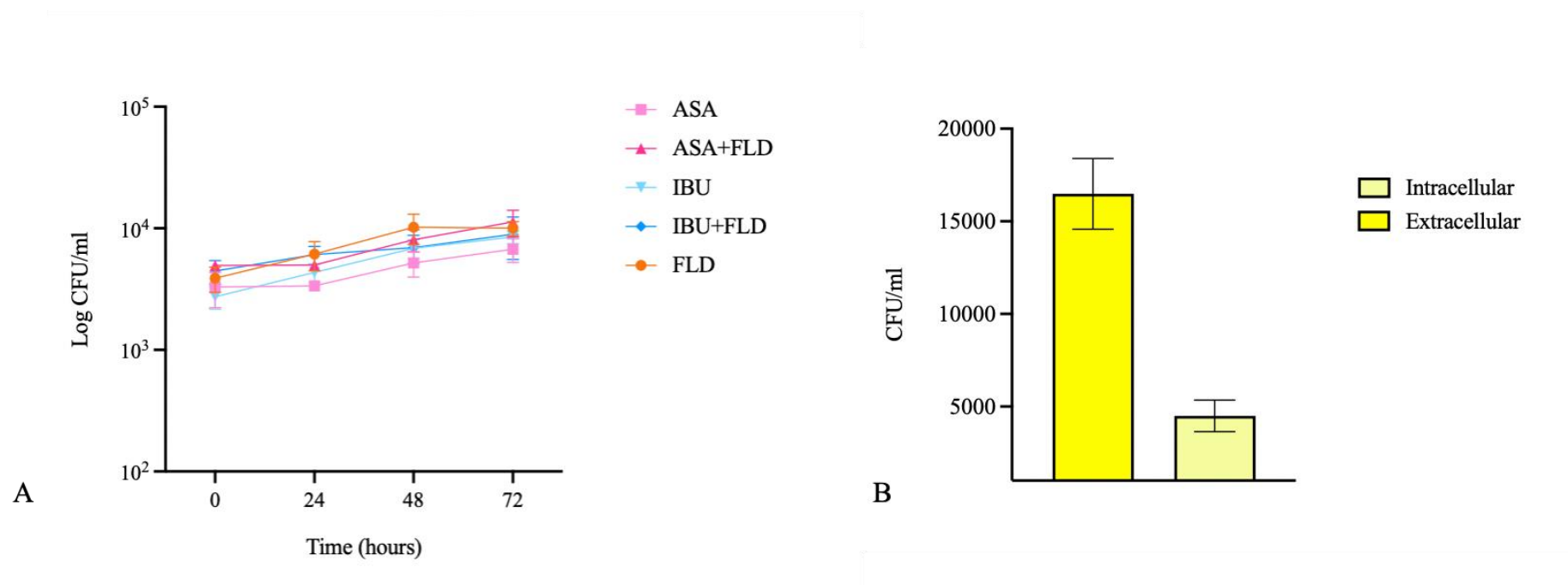


Figure 37. THP-1 infected with *Mycobacterium tuberculosis* LAM

A) THP-1 cells were infected and treated with NSAIDs alone, NSAID+FLD, and FLDs alone for 24 hours, 48 hours, and 72 hours and the Log₁₀ CFU/ml was plotted.

B) At T₀, the extracellular and intracellular bacteria were plated, and the CFU/ml was counted after 4 weeks of incubation. This was carried out in three independent experiments.

From Figures 35–37, it's clear that neither NSAIDs (ASA and IBU) alone, nor their combinations with FLDs, had a significant effect on bacterial killing in THP-1 cells infected with different strains of *M.tb*. However, a key observation is that FLDs alone were also not effective in killing the bacteria as expected. This prompted an exploration of higher drug concentrations for FLDs alone, possibly aiming to determine whether increasing the dose would have a more pronounced effect on bacterial killing, addressing the unexpected lack of efficacy at the previously tested concentrations. This line of inquiry suggested that the current doses of FLDs may have been insufficient to achieve the desired bactericidal effect. Figure 38 A illustrates that 10 x MIC of FLD shows better bacterial killing when compared to 5 x MIC, which suggests that increasing the concentration of FLDs leads to enhanced bactericidal activity. A higher MIC was chosen to ensure a more effective response in killing the bacteria. Figure 38 B, A multiple comparison between 5 x and 10 x MIC of FLD was done and there is no statistical difference between drug concentrations. However, as expected when compared to the infected no drug control, there is a significant difference. This finding supports the idea that higher concentrations of FLDs are necessary to achieve the desired therapeutic effect. The THP-1 infection was carried out again at 10 x MIC, which is seen in Figures 39-41.

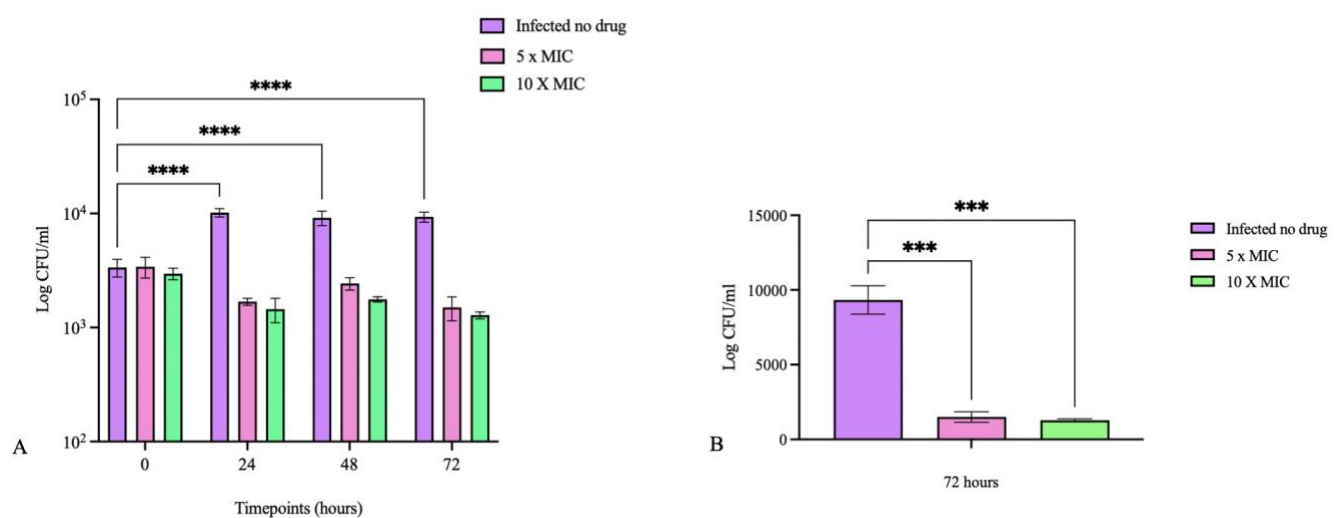


Figure 38. An optimization of FLDs at 5 x, and 10 x MIC in the THP-1 cell line.

A) THP-1 Cells were infected with H37Rv and treated with FLDs for 24 hours, 48 hours, and 72 hours, and the Log₁₀ CFU/ml was determined and plotted. An uninfected cell with no drug treatment was included as a control, which was statistically different ($p < 0.05$) as the cells grew over the 72 hours. The cells treated with 5 x MIC and 10 x MIC of FLD showed a decrease in CFU count, which were not significantly different from each other. B) The bacterial burden at 72 hours post-infection and treatment with 5 x and 10 x MIC drug concentrations were similar but a significance is noted when compared to the infected no drug control, * $p < 0.05$.

In Figure 39 A, at time-point 0, all groups show the initial bacterial loads for H37Rv. The exclusion of the 24-hour time point and the extension of the experiment to 96 hours revealed that bacterial clearance was observed with FLDs when THP-1 cells were infected for a longer time with H37Rv. At 96 hours, a comparison of ASA+FLD, IBU+FLD, and FLD alone showed statistical significance with ASA+FLD and FLD, while IBU+FLD inhibited the activity of FLDs. This finding suggests that FLDs were primarily responsible for the bacterial clearance observed and the NSAIDs did not enhance the bacterial killing. In Figure 39 B, it can be seen that there was more bacteria present in the extracellular supernatant when compared to the intracellular supernatant. However, this difference was not statistically significant, as determined by the t-test analysis.

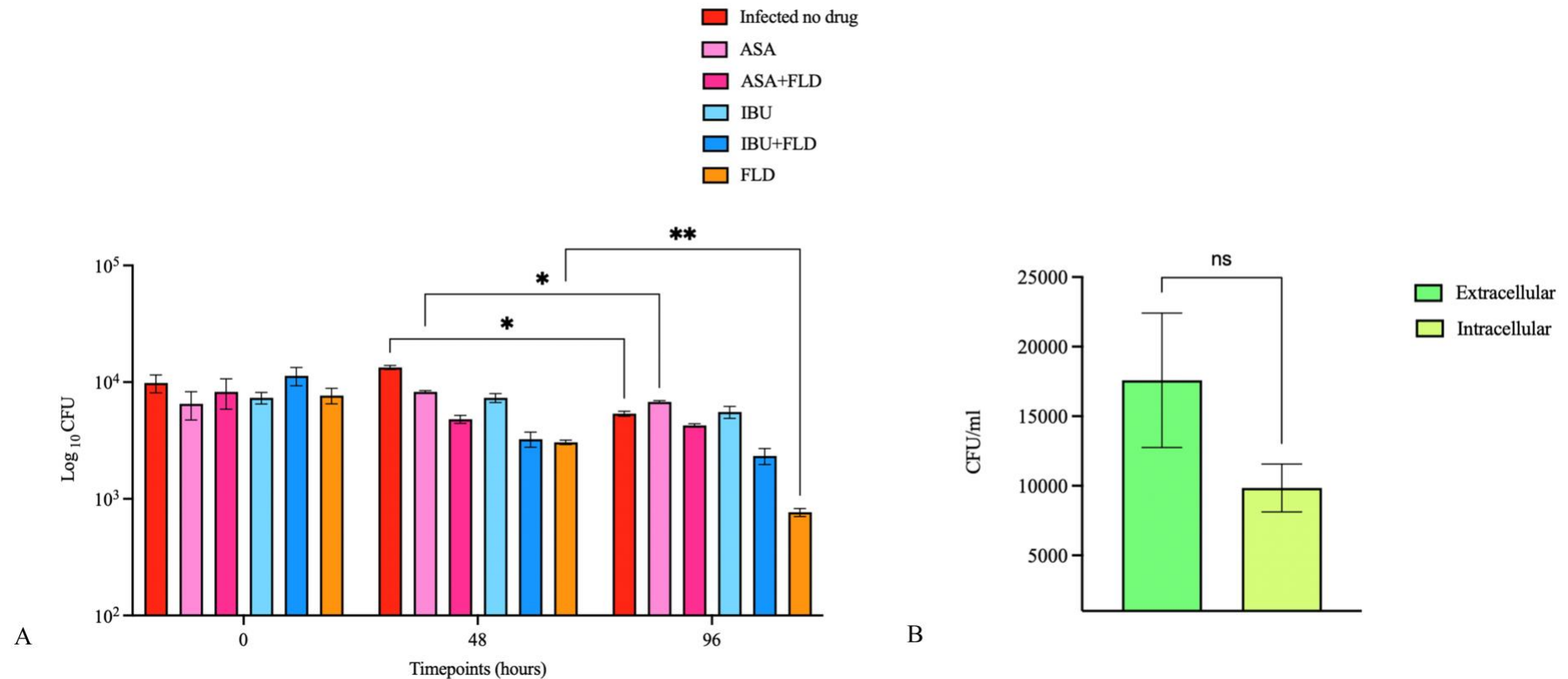


Figure 39. THP-1 infected with *Mycobacterium tuberculosis* H37Rv

A) THP-1 cells were infected and not treated with drugs as a control (red bar), treated with ASA alone (light pink bar), ASA + FLDs (dark pink bar), IBU alone (light blue bar), IBU+FLDs (dark blue bar), and FLDs (orange bar) NSAID+FLD and FLDs alone for 24 hours, 48 hours, and 96 hours and the Log₁₀ CFU/ ml were analysed.

When THP-1 cells were infected with Bj in Figure 40, at timepoint 0, all groups show the initial bacterial loads for Bj. Over time, at 48 and 96 hours, the infected no drug (red) group maintains or increases bacterial counts over time. The combination treatments (ASA + FLD and IBU + FLD) show no enhanced bacterial clearance. The FLD-only group (orange) shows a significant reduction in bacterial load by 96 hours. The data presented in Figure 40 B indicate that bacterial counts were higher in the extracellular supernatant compared to the intracellular supernatant. However, statistical analysis using a t-test revealed that this difference was not significant (ns).

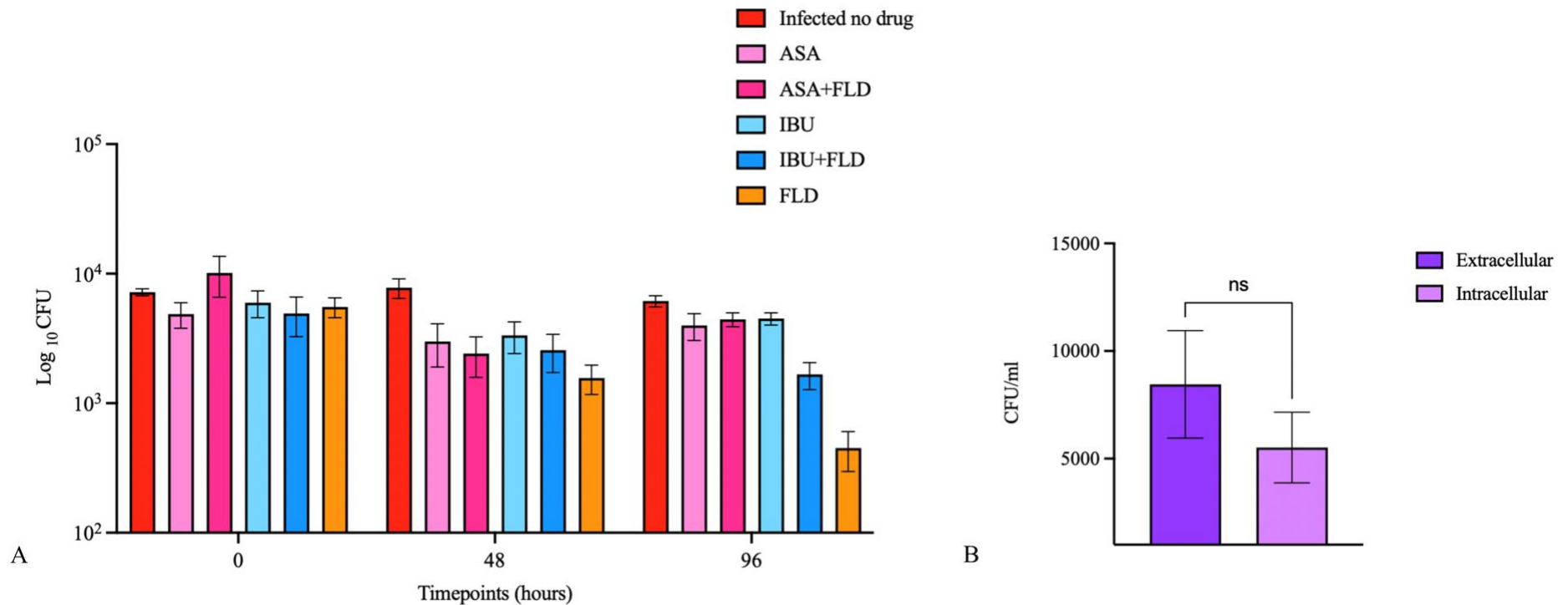


Figure 40. THP-1 infected with *Mycobacterium tuberculosis* Bj

A) THP-1 cells were infected and not treated with drugs as a control (red bar), treated with NSAIDs alone, NSAID+FLD and FLDs alone for 24 hours, 48 hours, and 96 hours and the Log₁₀ CFU/ml was plotted.

B) At T₀, the extracellular and intracellular bacteria were plated, and the CFU/ml was counted after 4 weeks of incubation. This was carried out in three independent experiments.

In Figure 41 A, at timepoint 0, all groups show the initial bacterial loads. Then at 48 and 96 hours, ASA + FLD and IBU + FLD treatments show a reduction in bacterial load compared to ASA or IBU alone, as we know, FLD is effective in TB treatment. The FLD-only group (orange) shows a substantial reduction in bacterial load by 96 hours, indicating the effectiveness of FLDs. In Figure 41 B, the extracellular bacterial count is significantly higher than the intracellular bacterial count. A t-test was performed, and it was found to be statistically (ns) significant. Collectively, these data show that regardless of the type of *M.tb* strains, NSAIDs have no killing effect on the bacilli, as it has an inflammatory effect, rather than an antibiotic effect (42).

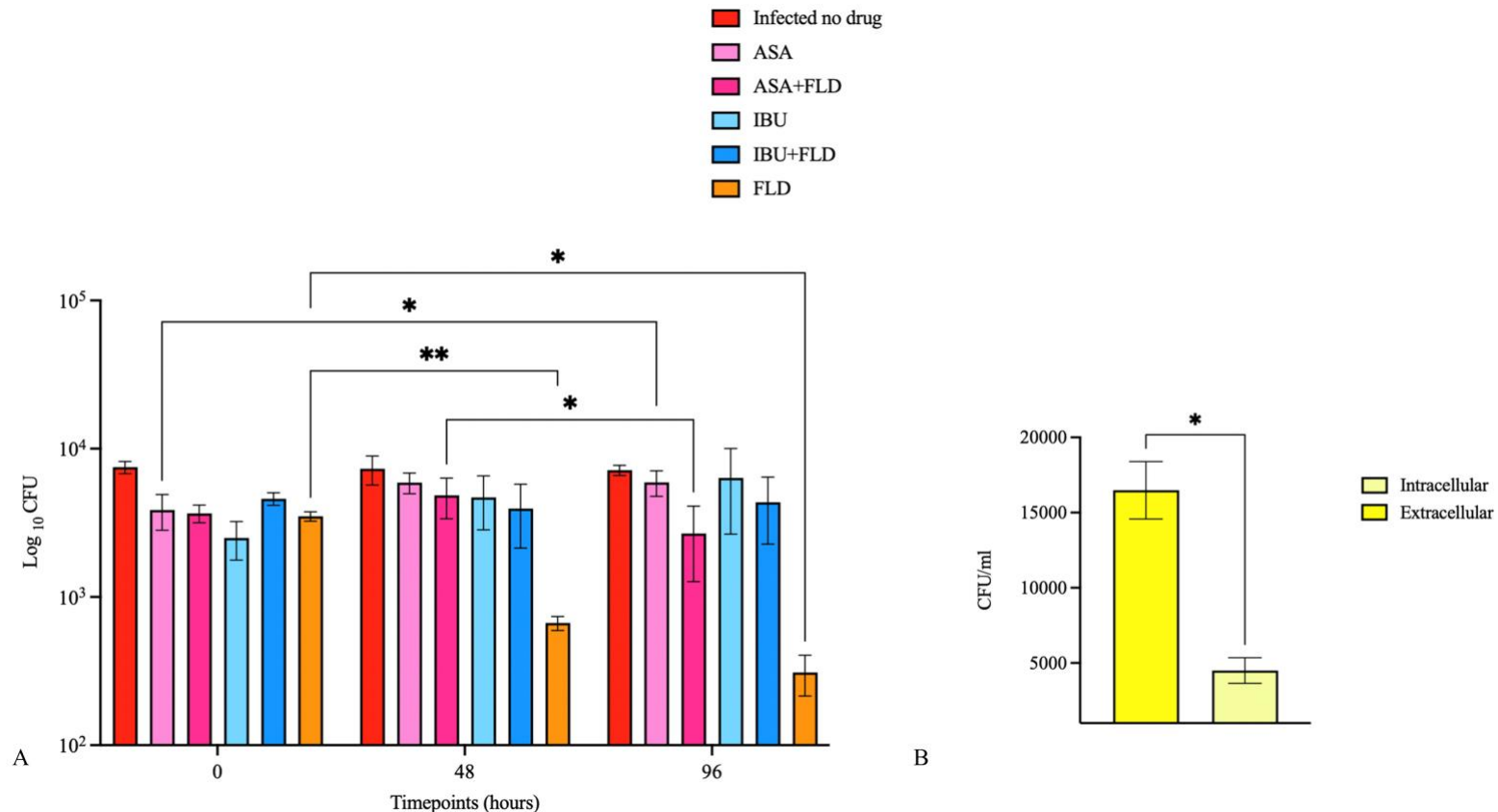


Figure 41. THP-1 infected with *Mycobacterium tuberculosis* LAM

A) THP-1 cells were infected and not treated with drugs as a control (red bar), treated with ASA alone (light pink bar), ASA + FLDs (dark pink bar), IBU alone (light blue bar), IBU+FLDs (dark blue bar), and FLDs (orange bar) NSAID+FLD and FLDs alone for 24 hours, 48 hours, and 96 hours and the Log₁₀ CFU/ ml were analysed.

B) At T₀, the extracellular and intracellular bacteria were plated, and the CFU/ ml was counted after 4 weeks of incubation. This experiment was done in triplicate.

4.7 Mycobacterial Growth Inhibition Assay (MGIA)

A retrospective data analysis was conducted using data from the ‘Pilot Study to Estimate the Potential Efficacy and Safety of Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis,’ which was carried out between 2017 and 2019 in Tbilisi, Georgia. Ethical approval for this study was obtained from the HREC and is documented in Appendix B. The participants were enrolled into two equal groups of 10 individuals each. The control group received FLD TB treatment, while the treated group received the FLD TB treatment with IBU. PBMC samples were isolated from whole blood and stored at three-time points: Baseline, Month 2, and Month 6, with 10 participants in each subgroup. The cohort sampling is shown in Figure 42.

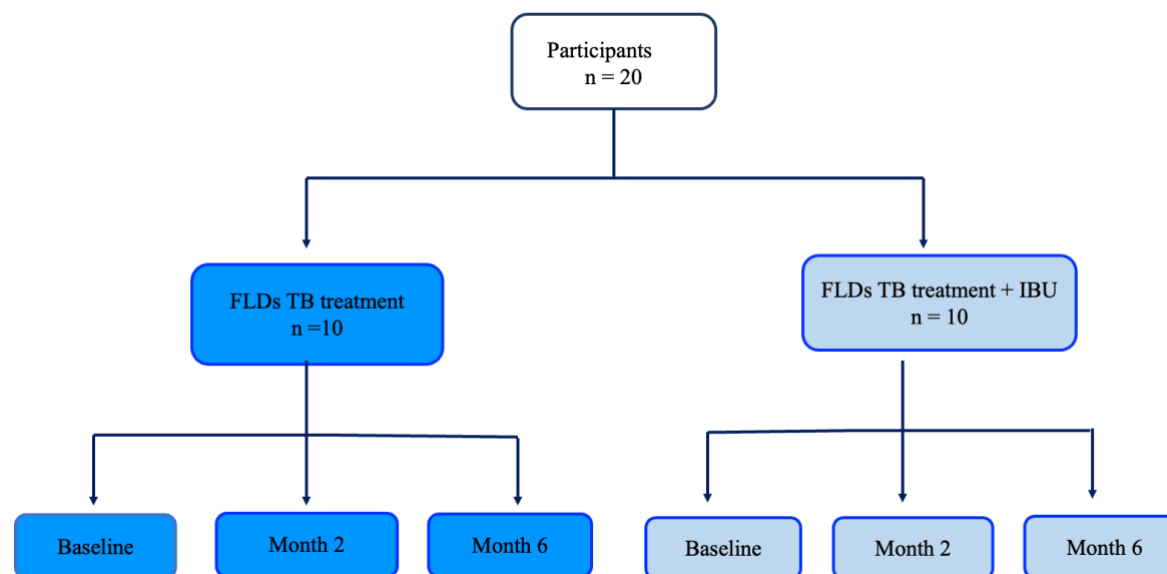


Figure 42. A schematic representation of the sample cohort and groups that were used for MGIA assay.

60 samples were collected from 20 participants at three different visits, Baseline, Month 2, and Month 6. The participants were randomized into a FLDs TB treatment group and FLDs TB treatment + IBU group.

From the raw data that was shared for the MIGIA assay, a standard curve was calculated by plotting the TTP (hours) and converting it to log₁₀ CFU by using the equation of the line. Regression analysis was used to obtain the equation, by inserting the TTP for any sample, the log₁₀ CFU was calculated. BCG was used for the standard curve (Figure 43) as a safer option than that of *M.tb* bacteria as this was carried out in the biosafety laboratory 2 (BSL2).

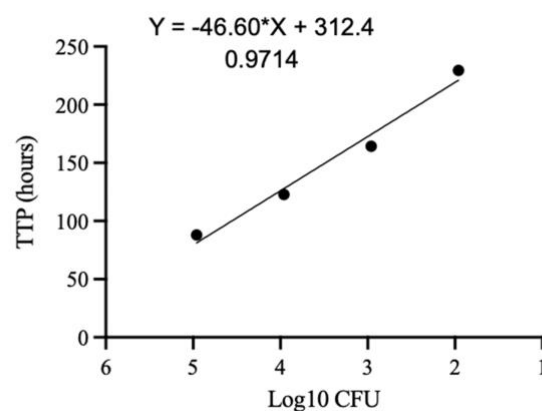


Figure 43. A standard curve was generated, TTP was plotted relative to CFU for each input volume, and regression analysis was performed to derive the equation of the curve.

A) CFU was determined by fitting a semi-logarithmic line and performing linear regression on the log₁₀ CFU. The equation of the line was expressed as $Y = A \cdot X + B$, where X represents the sample TTP, and A is the slope while B is the y-intercept. The corresponding CFU was calculated by solving the equation $X = (Y - B) / A$, where Y represents the TTP for any sample.

After the PBMC-MIGIA assay was carried out, the TTP that was generated was then converted to $\log_{10}$ CFU and this was used to the clinical samples, at the three time-points, baseline, Month 2, and Month 6.

Since PBMCs play a crucial role in immune function, and given the generally poor health outcomes of pre-XDR-TB and XDR-TB patients, it was essential to establish a reliable reference or control group. To achieve this, PBMCs from healthy volunteers were utilized, as their cell count and viability were expected to be better when compared to those of clinical patients. This approach ensured a good comparison when assessing immune responses or cellular characteristics in TB patients. Healthy volunteer (HV) PBMCs were isolated and used to optimize the experiment. All three HVs cell counts yielded $\log_{10}$ CFU of 5 as seen in Figure 44. The use of the HV samples allowed for a better understanding of the assay before the clinical samples were used, which many of which had fewer than the average cryovials stored.

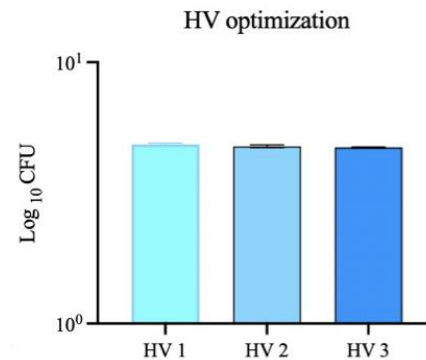


Figure 44. Healthy volunteer PBMCs were utilized to optimize the MGIA assay.

PBMC counts were $\sim 3 \times 10^6$ cells were infected with BCG and the MGIA 4 day assay was carried out. After 96 hours of incubation, the lysate was introduced into the BACTEC machine. The TTP was recorded and subsequently converted to $\log_{10}$ CFU using the standard curve.

The MGIA results indicate that the $\log_{10}$ CFU measured at baseline, Month 2, and Month 6 show no significant difference between the control arm and the treated IBU arm, Figure 45. This suggests that treatment with IBU did not result in any noticeable change in bacterial load over time when compared to the control group. IBU may not have had a meaningful impact on inhibiting bacterial growth or reducing bacterial burden in the treated group.

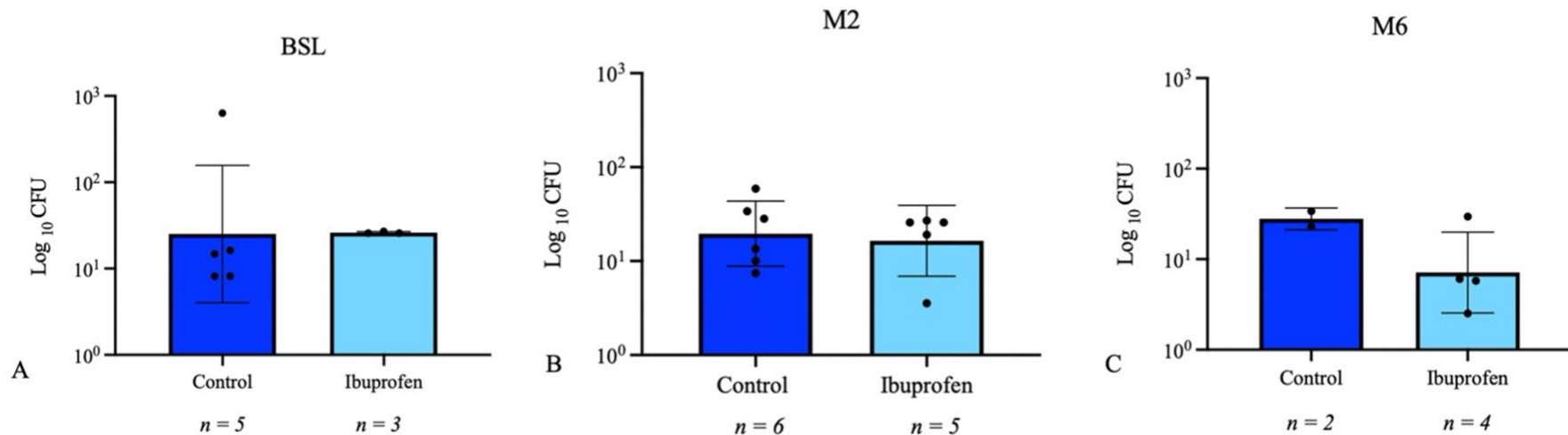


Figure 45. An analysis of clinical PBMC samples from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrating MGIA results.

PBMC samples that were collected between 2017-2019 at baseline (BSL), month 1 (M1), and month 6 (M6). Control patients received FLDs only while the treated group received both FLDs + IBU. The PBMCs were challenged with BCG and incubated for 96 hours, and thereafter lysed and the lysate was introduced into MGIT tubes and placed onto the BACTEC system until TTP was detected. With the aid of the standard curve, the TTP was converted to $\log_{10}$ CFU and plotted.

- A) BSL, the control, and treated group
- B) M2, the control and treated group
- C) M6, the control and treated group

The available clinical data were collected under the XDR-TB study carried out in Tbilisi, Georgia, and the key demographic and anthropometric aspects of the cohort, including age, height, weight, and body mass index (BMI) are presented as either the median with interquartile range (IQR) or the mean with standard deviation (SD), on the basis of the distribution of the variables, Table 5.

Table 5. Demographics of clinical characteristics of participants in the XDR-TB study

Variables	Female n=7	Male n=21
Age (years): median, IQR	52 (13.8)	35 (13.8)
Height (cm): mean, SD	165 (5.4)	170 (14.1)
Weight (kg) : mean, SD	60 (16.7)	66 (10.8)
BMI (kg/cm): median, IQR	19.4 (10.8)	21.6 (3.7)

The median age varied between the two gender groups, with females having an older median age of 52 years compared to 35 years in the males. The mean height and weight were slightly higher in the males, with a mean height of 170 cm and weight of 66 kg, compared to 165 cm and 60 kg in females. The median BMI is lower in the females (19.4 kg/cm²) compared to the males (21.6 kg/cm²).

This analysis looked at the age of individuals in the control and treated groups who were categorized as middle-aged, young adults, and old age. A majority of middle-aged individuals were enrolled in both groups, and a small percentage of participants were classified as elderly, indicating that older adults constituted the minority of the study population.

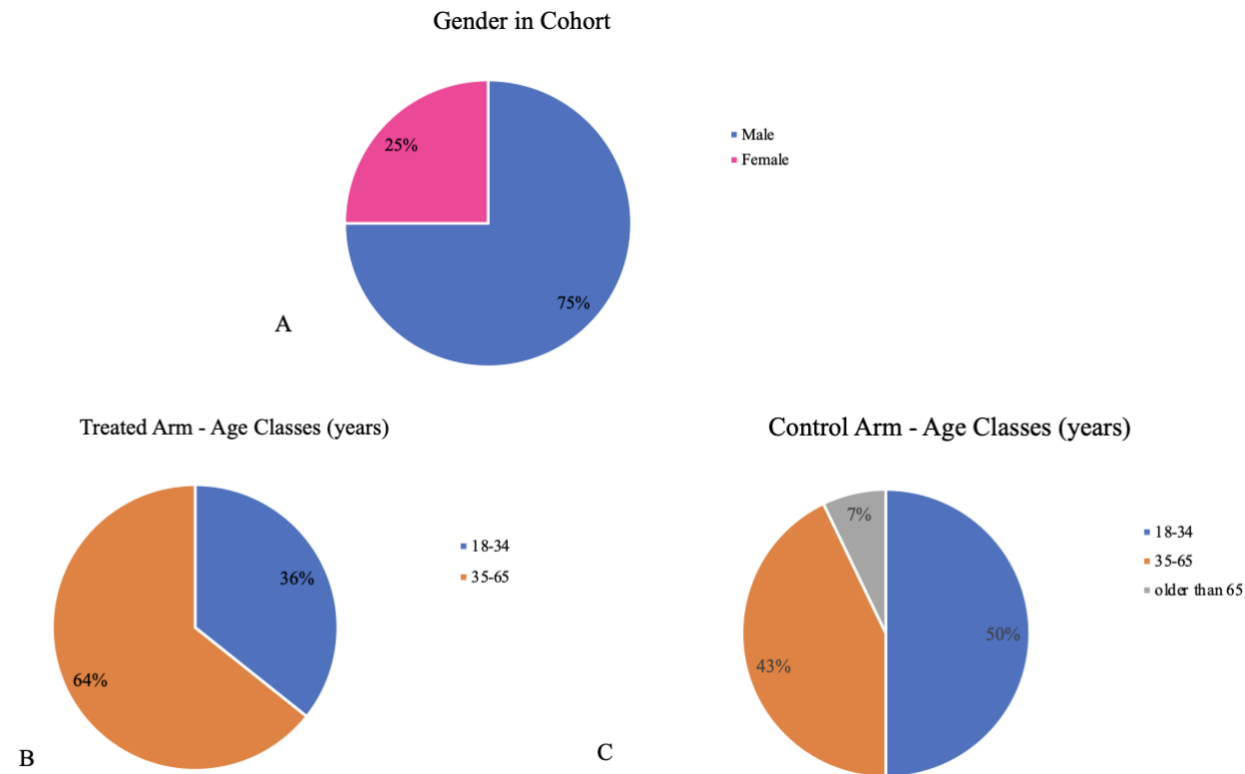


Figure 46. An analysis of clinical data from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates gender and age.

- A) A pie chart representation of the gender in the cohort.
- B) A pie chart that shows the treated arm age class groups.
- C) A pie chart that shows the control arm age class groups.

TB disease progression and treatment outcomes are influenced by multiple host-related and clinical factors. Among these, hepatitis C virus (hep C) co-infection appears to be a significant factor affecting patient outcomes. Figure 47 illustrates the prevalence of hep C infection in both the treated and control arms of the study. The chart shows that the percentage of individuals with hep C infection was equal in both arms, suggesting that the distribution of hep C infection was balanced between the two groups at the start of the study. The data also indicates that smoking and alcohol misuse are prevalent in more than half of the participants in each arm of the cohort.

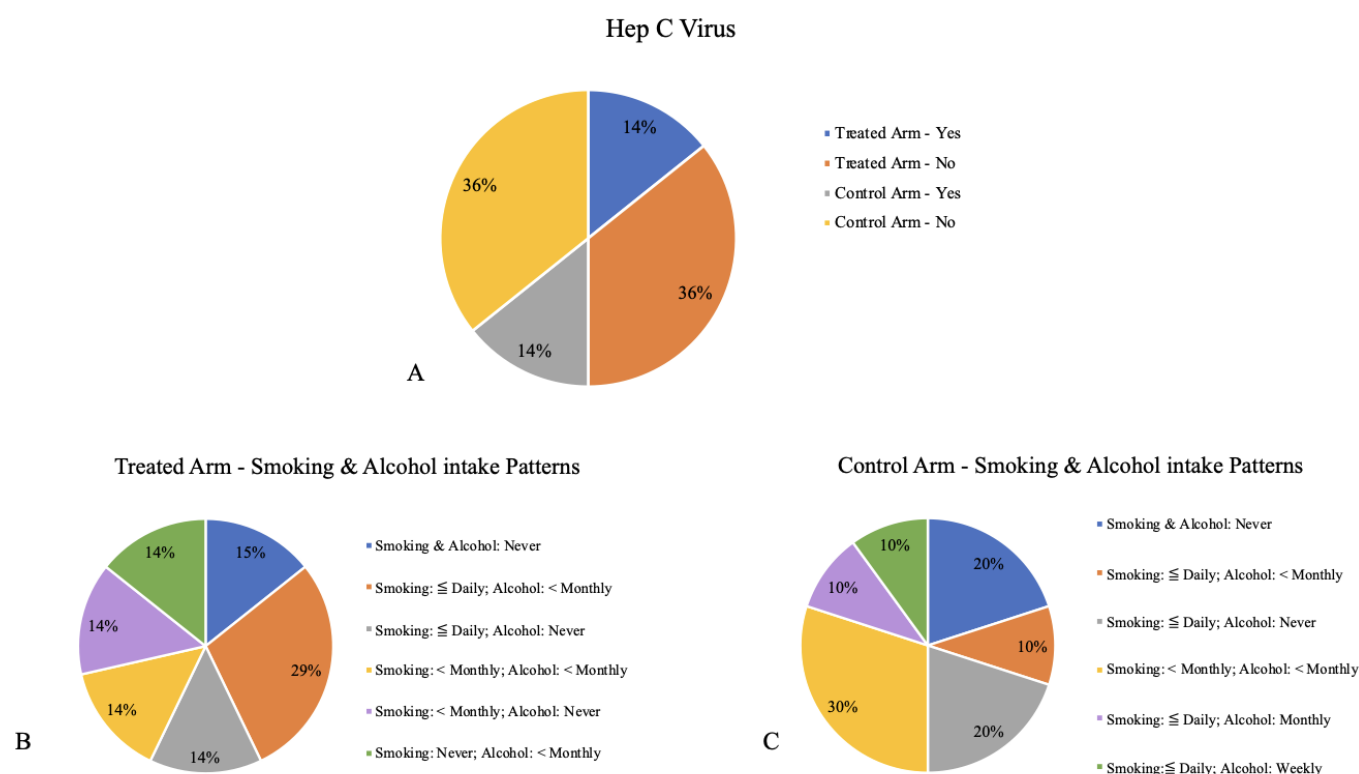


Figure 47. An analysis of clinical data from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates hep C and alcohol intake patterns.

- A) A pie chart representation of hep C infection in the cohort.
 B) A pie chart that shows the treated arm smoking and alcohol intake patterns.
 C) A pie chart that shows the control arm smoking and alcohol intake patterns.

In Figures 48 A and B, it can be seen that the prevalence of DR-TB in the study population was high, with XDR-TB resistance as the most common one. In Figures 48 C and D, 36% of participants in the treated arm were lost to follow-up compared to 29% in the control arm. Interestingly, it can be noted that there are no participants that were microbiologically cured, even with IBU as an adjunctive treatment. In the control arm, the cure rate was recorded , with only 7% of participants achieving a microbiological cure. IBU shows no benefit or enhancement in curability in TB treatment.

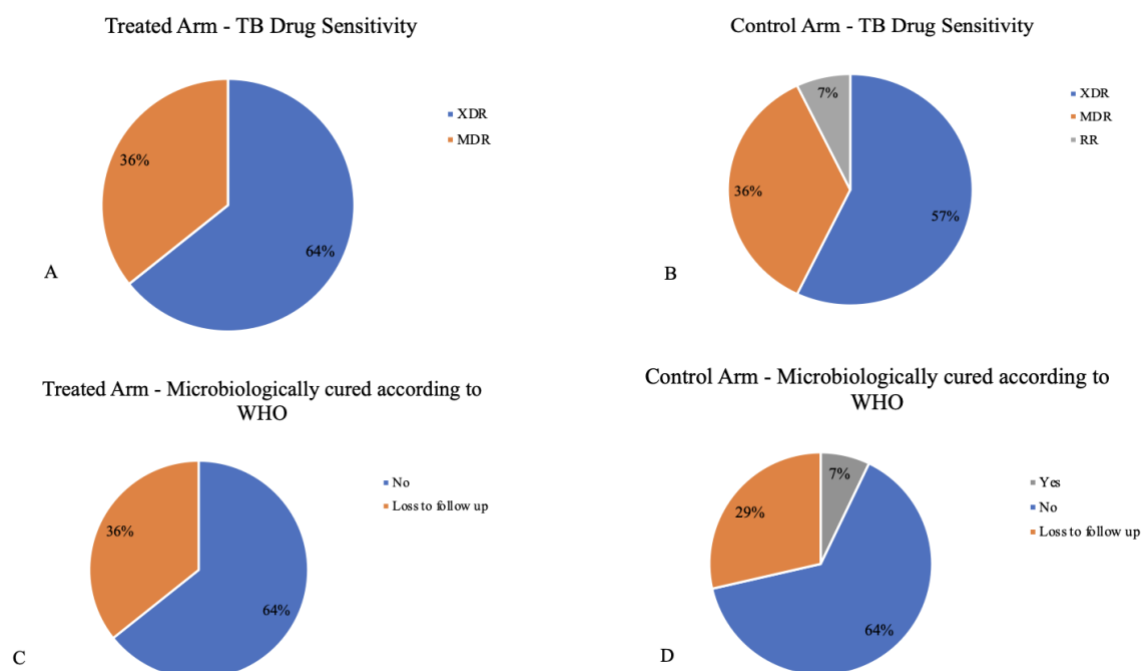


Figure 48. An analysis of clinical data from the ‘Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis’ illustrates drug sensitivity and microbial cure.

- A) A pie chart illustrating the TB drug sensitivity for the treated arm
- B) A pie chart illustrating the TB drug sensitivity for the control arm
- C) A pie chart representing the microbiologically cured according to the WHO for the treated arm
- D) A pie chart representing the microbiologically cured participants according to the WHO for the control

Figure 49 provides a comprehensive summary of the key experimental findings, integrating data from both *in vitro* and *ex vivo* models to assess the effects of HDTs on *M.tb* infection. The figure outlines the study hypothesis, objectives, and major results, including MICs, drug synergy assessments, cytotoxicity evaluations, bacterial killing kinetics in THP-1 cell infections, and retrospective PBMC-MGIA assay outcomes. These findings collectively demonstrate that ASA and IBU do not enhance TB treatment as adjunctive therapies by modulating host responses or increasing the bactericidal activity of FLDs.

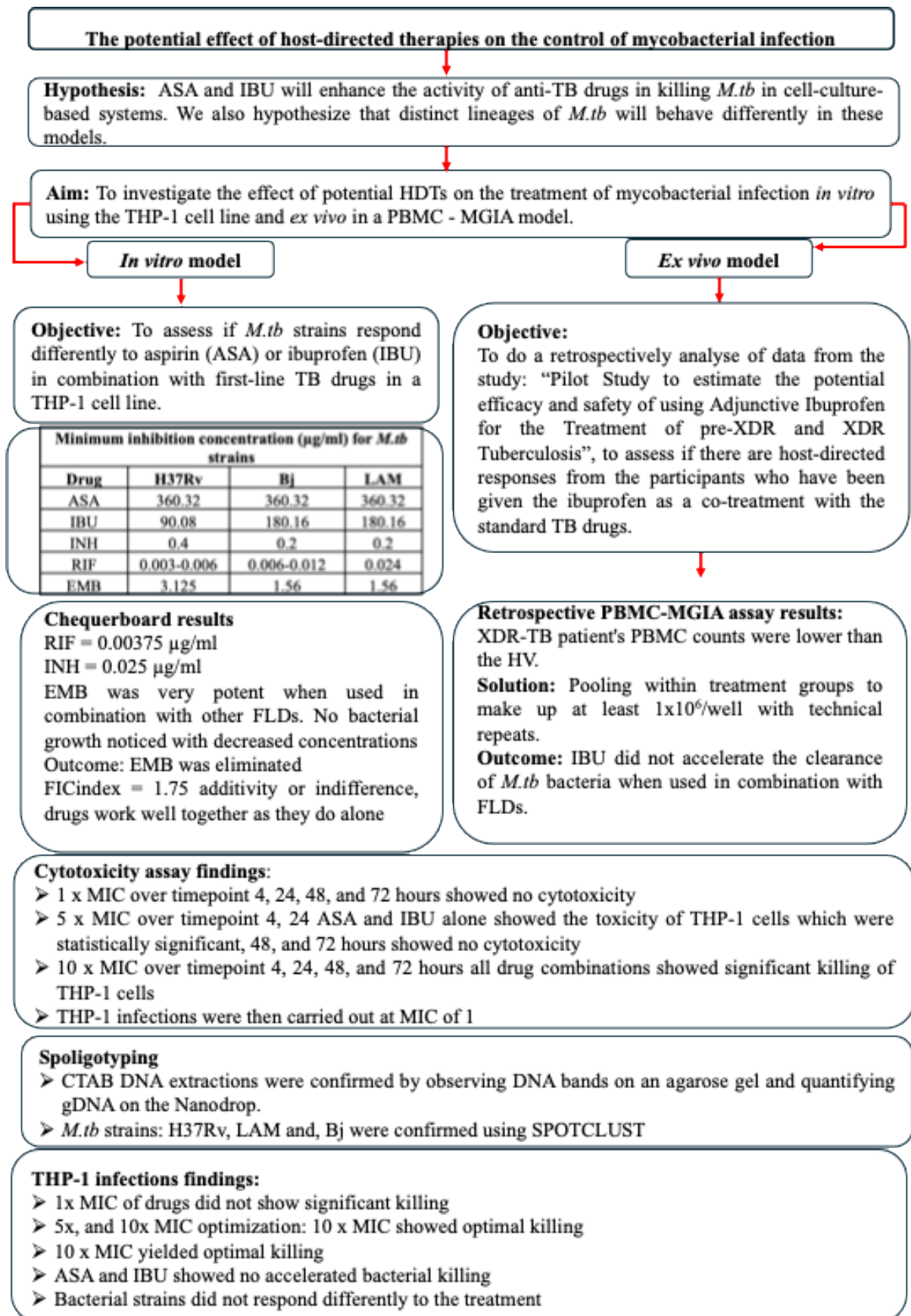


Figure 49. A flowchart summarizing the study findings on the potential effects of HDTs in controlling *Mycobacterium tuberculosis* infection. *In vitro* model findings: MICs were determined for ASA, IBU, INH, RIF, and EMB showed no bacterial growth reduction in combination with FLDs, leading to its elimination. An additive drug effect was noted with INH and RIF at 1.75. 10 x MIC showing optimal killing. The NSAIDs alone or in combination with FLDs did not enhance bacterial killing. *Ex vivo* model findings: The XDR-TB patients' PBMC counts were lower than the healthy volunteers. Conclusion: IBU did not accelerate bacterial clearance when combined with FLDs.

5. Discussion

5.1 Broth microdilution assays

5.1.1 Determination of MICs for the NSAIDs and FLD drugs

There is extensive hyperinflammation and lung damage that is associated with people living with TB, and introducing NSAIDs as an adjunct treatment with FLDs may assist in preventing permanent lung damage. Vilaplana *et al* explored this by looking at a Stratified Medicine Algorithm to predict treatment (SMA-TB) outcomes of HDT in DS-TB, and MDR-TB patients in South Africa and Georgia, Tbilisi, unpublished (115). The key objective of this study was to evaluate the therapeutic potential of ASA and IBU when used in conjunction with the standard TB treatment. However, the results demonstrated no significant difference in treatment outcomes among the HDT groups and the control group receiving only FLDs.

From extensively studying the literature, no exploration in HDT *in vitro* models has been done with NSAIDs, therefore we were unable to compare our findings to any previous studies. The experimental procedures to determine MICs for the FLDs were followed as described by Kana *et al*, 2010 (42). The MIC results for ASA were quite consistent at 360.12 µg/ml whereas IBU showed variation between biological repeats initially. Eventually, repeat experiments resulted in a MIC for IBU of 90.08 µg/ml. These results might suggest that IBU has strain-dependent activity, whereas ASA's inhibitory effects appear consistent across the strains tested.

The MICs for the FLDs correlated with those reported by Kana *et al*, who reported a MIC for RIF at 0.003-0.006 µg/ml, INH at 0.0025 µg/ml, and EMB at 1.6 -3.1 µg/ml (42). The MIC for RIF in this study was 0.006 µg/ml, INH was 0.4 µg/ml and EMB was 3.125 µg/ml. Mouton *et al* 2018, explored the optimization aspects when setting up MICs, and as per the ISO 20776-2 guidelines, which state the acceptable deviation is that of 2-fold dilutions (109). In this instance, RIF and EMB are accepted but that of INH has a 4-fold discrepancy. This could be due to the difference in drug batches or strain variability in the laboratory.

These results could indicate a potential strain-specific variation in NSAID susceptibility. The MIC for INH and EMB is uniform (0.2 µg/ml for INH and 1.56 µg/ml for EMB) across all three strains. This suggests that Bj and LAM do not show significant resistance to these FLDs compared to the standard H37Rv strain. The H37Rv strain shows the lowest MIC (0.005

µg/ml), indicating high susceptibility to RIF. Bj has a significantly higher MIC (0.024 µg/ml), indicating 4.8 times more resistance than H37Rv. LAM has an MIC range of 0.006 – 0.012 µg/ml, suggesting some variability in resistance but still generally higher than H37Rv. However, there appeared to be differences in the RIF concentrations, which were considered when setting up downstream experiments.

5.2 Chequerboard assays

5.2.1 Optimization and setting up of a chequerboard assay using the FLDs

The optimization of this assay was challenging, mostly due to the selection of the starting concentration of the drugs. The starting concentration was based on MICs by Kana., et al 2010 (42) however this resulted in growth inhibition.

RIF and INH concentrations were therefore reduced stepwise with an initial drug concentration of RIF at 1.92 to 0.48 and then to 0.12 µg/ml. INH initial drug concentration at 1.6 reduced to 0.4 and then 0.1 µg/ml, while EMB was tested at 0.5 µg/ml and eventually excluded. Despite these adjustments, inhibition across the plates persisted.

Subsequent MIC assays confirmed RIF activity against *M. smegmatis*, H37Rv(J), and H37Rv(S), yielding values consistent with published data (1.5625, 0.003, and 0.00375 µg/ml, respectively). EMB was excluded from further analysis due to its potent effect in combination with other FLDs.

Dual-drug assays with RIF and INH produced MICs of 0.003 µg/ml and 0.05 µg/ml, respectively. Synergistic activity was observed at 0.00375 µg/ml (RIF) and 0.025 µg/ml (INH), with a Fractional Inhibitory Concentration Index (FICI) of 1.75, indicating an additive interaction consistent with Odds et al (53). These findings were comparable to Bhusal et al. (2016), who reported a FICI of 0.94 for RIF–INH with H37Rv (109). However, their inclusion of EMB at higher concentrations (10–100 µg/ml) yielded stronger synergy (FICI = 0.62). The lower EMB concentrations (0.5–2 µg/ml) employed in this study likely account for the absence of a similar effect. (109). The results obtained for the 2D chequerboard were consistent with This FICI is described as additive or indifferent. However, Bhusal et al. 2016 also evaluated the in vitro synergy of RIF, INH, and EMB against *M.tb* MDR strains (109). EMB was tested at 10 µg/ml and 100 µg/ml. Their findings indicated that the combination of these three FLDs

exhibited a synergistic effect, enhancing bacterial inhibition compared to individual drugs or dual combinations. They established an FIC index of 0.62. The study demonstrated that using

5.3 Mycoplasma contamination

Mycoplasmas are the smallest free-living prokaryotic, organisms that spread easily within a laboratory (116)(117). These organisms have no cell wall, are resistant to most antibiotics, and can pass through filters. They are most commonly found in cell cultures and impact cell biology and pathogenesis (117). They can also alter the protein, DNA, and RNA of cells, affecting the signalling pathways. They change the cell metabolism by inhibiting cell proliferation and cell growth.

Considering this, it was important to test if all cell cultures were contaminant-free before any THP-1 cell infections were carried out. Moreover, these organisms can cause lymphocyte activation (62), which will trigger the release of cytokines and antibodies that would affect THP-1 macrophages and yield inconsistent laboratory findings before infection with *M.tb*. Therefore, all cell lines needed to be free of any contamination that would cause any false or inaccurate results.

5.4 Viability assay using MTT assay

The MTT assay measured the metabolic activity of THP-1 cells exposed to NSAIDs and drug concentrations (1x, 5x, and 10x MIC) over time. Absorbance indicated cell viability. Concentrations of 1x and 5x MIC, from 4 to 72 hours, did not cause significant cell death. At 10x MIC, a notable decrease in viability was observed at 48 and 72 hours for NSAIDs and NSAIDs + FLDs, but not for FLDs alone. Media-only and DMSO controls served as negative and diluent controls. Based on these results, 10× MIC NSAIDs were used for all subsequent experiments.

5.5 Quantification and visualization of genomic DNA and Spoligotyping

Spoligotyping is most commonly used in molecular epidemiology and TB research in Africa, Asia, and Eastern Europe, where the TB burden is high, particularly if it is utilized to characterize *M.tb* strains and track transmission patterns for molecular epidemiological studies. However, it is frequently combined with other genotyping methods, such as mycobacterial

interspersed repetitive units-variable number tandem repeat (MIRU-VNTR) analysis and whole-genome sequencing (WGS), to enhance resolution in surveillance and outbreak investigations in low TB prevalent areas, such as the United States and Western Europe (118, 119). Many national and international TB research institutions and public health laboratories use spoligotyping for strain identification, particularly for delineating lineages within the MTBC. The results from the extracted DNA and spoligotyping patterns were obtained and identified as H37Rv, Bj, and LAM, respectively. This allowed the next experiment to be set up with confidence in the strain type.

5.6 THP-1 infections

Bacterial killing was noted in the THP-1 infection experiments conducted with FLDs at a concentration of 1 x MIC, regardless of the *M.tb* strain type. However, as we know, FLDs are supposed to inhibit *M.tb* growth, the bacterial growth suggested suboptimal efficacy at this concentration. This observation allowed for further exploration at 5 x MIC and 10 x MIC concentrations and then comparing their effects to untreated infected control. The uninfected controls demonstrated significant proliferation over time. As anticipated, treatment at 10 x MIC resulted in greater bacterial inhibition and more pronounced killing compared to 5 x MIC., although this difference was not significant.

To further assess the impact of NSAIDs in combination with FLDs, the experimental setup included an initial infection time point (T_0) at 4 hours, followed by CFU evaluations at 48 hours and an extended incubation period of 96 hours. Data presented in Figures 32–37 indicate that no significant FLD-mediated bacterial clearance was observed at 72 hours, suggesting that either a longer incubation period or a higher drug concentration may be required for a more comprehensive evaluation of drug efficacy. Ultimately, results from the optimized experiment demonstrated that HDTs as adjunctive treatments did not enhance bacterial clearance beyond that achieved by FLDs alone.

The SMA-TB clinical trial was conducted in Soweto and Matlosana, South Africa, and Tbilisi, Georgia; it evaluated ASA and IBU as an adjunct treatment with the standard WHO-recommended TB drugs (114). This phase 2b randomized, double-blind, placebo-controlled clinical trial's primary objective was to compare their effects with the standard treatment. The study found no difference between clinical indicators and clinical features observed in the final stage of TB treatment in the two intervention arms and the control arm (Unpublished). This

data coincides with the retrospective data analysis done on the XDR-TB cohort, as seen in Figures 50 C and D that IBU did not enhance any clinical symptom in TB clearance, in fact, interestingly there are a few individuals who have clinically been cured in the control arm who did not receive any IBU when compared to the treated arm.

5.7 MGIA assay

No difference was observed in mycobacterial growth inhibition when PBMCs were evaluated in patients who received IBU as an adjunctive treatment compared to the control group. IBU primarily serves as an anti-inflammatory drug and is not an antibiotic, and therefore has no direct killing on *M.tb*. It reduces the pro-inflammatory cytokines by inhibiting the COX-1 and COX-2 enzymes, when these pathways are blocked, it decreases the prostaglandins which act as a key player in and signalling. Therefore, as an NSAID, IBU reduces inflammation without improving bacterial clearance, so its effect on MGIA was minimal (66) .

5.8 A retrospective data analysis on the XDR-TB pilot study

This retrospective analysis explores key demographic and clinical factors that influence XDR-TB outcomes within the study cohort. Various risk factors include: BMI, gender, age, hepatitis co-infection, smoking, alcohol misuse, TB drug sensitivity, and microbiological cure rates were examined to assess their impact on disease progression and treatment outcomes (120, 121).

5.8.1 Body mass index (BMI)

In the literature, many studies have explored the impact of nutrition on TB disease progression. The important key risk factors associated with TB development include a low body mass index (BMI) which is generally below 18.5 kg/m², smoking, alcohol misuse, HIV co-infection, and diabetes (55, 120, 121). Malnutrition and TB have a bidirectional relationship, where malnutrition increases the susceptibility to TB, and TB further exacerbates malnutrition due to increased metabolic demands and reduced nutrient intake.

This study examined BMI distribution within the cohort due to its strong link to TB risk. The median BMI was 19.4 kg/m² for females and 21.6 kg/m² for males, as shown in Table 5 of demographics. This difference may reflect variations in nutritional status, which could affect

disease progression and treatment outcomes. The lower BMI in females may suggest a greater vulnerability to TB-related complications.

5.8.2 Gender

The WHO, 2023 report indicates that TB occurs more frequently in men compared to women (36) . This trend is also seen in our data, where 75% of TB cases occurred in males and 25% in females, as illustrated in Figure 46 A.

Several factors contribute to the higher TB incidence among men:

5.8.2.1 Occupational hazards: Men are more likely to work in industries such as mining, construction, and other environments with poor ventilation and high TB exposure risk.

5.8.2.2 Healthcare-seeking behaviour: Research suggests that men are less inclined to seek timely medical care, leading to delayed diagnosis and treatment.

5.8.2.3 Substance use: Higher rates of smoking and alcohol consumption among men have been linked to an elevated risk of developing TB.

These factors align with global epidemiological data, which indicate a male-to-female prevalence of TB diagnosis at approximately 1.6:1 (122).

5.8.3 Age

Age plays a key role in TB treatment outcomes, as immune function, existing health conditions, and treatment adherence can differ across age groups. This study included individuals aged 16 and older diagnosed with pre-XDR-TB or XDR-TB. Participants were divided into a treated and a control arm, with notable differences in age distribution between the two.

In the treated arm, 36% of participants were classified as 18–34 years (young adults), while the majority (64%) were 35–65 years (middle-aged). No elderly individuals older than 65 years (old-aged) were present in this group.

The opposite distribution was seen, the control arm: 50% were young adults, 43% were middle-aged, and 7% were classified as elderly (above 65 years). As illustrated in Figures 46 B and 46 C, these variations may have implications for the interpretation of study outcomes. Age

distribution could influence treatment responses, as younger individuals may have more effective immune responses and generally better treatment adherence. While older participants also present with additional comorbidities or a higher risk of adverse drug reactions. Additionally, the treated arm, who had the majority of middle-aged individuals, may have distinct risk factors, such as prolonged TB exposure, underlying health conditions, or differing responses to treatment.

5.8.4 Hepatitis (hep C)

TB and hep C virus infections share common risk factors, including injection drug use, homelessness, and incarceration. A thorough examination and integrative analysis found that in the USA, TB is approximately 46 times more prevalent among homeless individuals compared to the general population, while hep C infection is about four times more common in this group (123). Additionally, incarceration is identified as a significant determinant for hep C infection, with the risk increasing proportionally with the length of imprisonment (124). As shown in Figure 47 A, 14% of individuals in the study had a history of hep C infection, compared to 36% who had no prior hep C infection in both study groups.

People with untreated hep C are predisposed to developing active TB. The Eastern Mediterranean Region has the highest prevalence of chronic hep C infection in the general population, recorded at 2.3%, primarily due to unsafe injection practices (10). The European Region follows as the second highest, with a prevalence of 1.5%, where transmission is largely linked to intravenous drug use. These findings highlight the overlapping contributing factors that lead to the transmission of both TB and hep C.

Notably, the prevalence of chronic hep B and hep C infections is higher among TB patients compared to the general population.

5.8.5 Smoking and alcohol misuse

Figure 47 B shows that in the treated arm, 15% never smoked or consumed alcohol, 14% smoked and never consumed alcohol, 14% never smoked but consumed alcohol, and 57% smoked and consumed alcohol. Figure 47 C illustrates the control arm where 20% never smoked or consumed alcohol, 20% smoked and never consumed alcohol, and 60% smoked and consumed alcohol. In both groups, more than half of each arm smoked and consumed alcohol. A study carried out in India found both former and current smoking, in addition to

alcohol overuse, increased the exposure of unfavourable TB treatment results. Treatment failure and recurrent TB infection were previously linked to smoking habits. When smoking was further linked, alcohol misuse exacerbated the risk of unfavourable outcomes among both past and current smokers (125) .

Another study highlighted that alcohol misuse and HIV were significant risk factors contributing to poorer treatment outcomes in MDR and XDR-TB treatment (126). Notably, smoking and alcohol use have been correlated with unfavourable therapeutic results in TB patients, including those with drug-resistant strains.

5.8.6 TB drug sensitivity

The drug susceptibility analysis of participants in both study arms highlights a significant burden of high-level DR-TB. In Figure 48 A, in the treated arm, 64% were diagnosed with XDR-TB, while 36% had MDR-TB. The control arm exhibited a slightly different distribution: 57% with XDR-TB, 36% with MDR-TB, and 7% with RR-TB, Figure 48 B.

These findings underscore the high prevalence of severe drug resistance among participants, particularly the dominance of XDR-TB, which poses significant treatment challenges. XDR-TB is associated with resistance to a minimum four of the important TB drugs, significantly limiting therapeutic options and often requiring the use of newer or revised drug therapy with longer treatment durations and potential toxicity. A methodical review and meta-assessment reported that treatment success rates for XDR-TB are substantially reduced compared to DS-TB, highlighting the difficulties in managing this condition (127). The presence of RR-TB in the control group further complicates treatment, as RR is a key indicator for DR-TB. This distribution of drug resistance has important implications for treatment outcomes, as individuals with XDR-TB often face prolonged therapy with limited efficacy, higher treatment costs, and increased risk of adverse effects. These findings highlight the urgent need for improved diagnostic strategies, early resistance monitoring, and the enhancement of more effective therapy to address the growing challenge of DR-TB.

5.8.7 Microbiologically cured

According to the WHO, a microbiological cure is defined as achieving at least three consecutive negative cultures collected at least four weeks apart, with no subsequent positive cultures recorded until the completion of TB treatment (128). As illustrated in Figures 48 C and 48 D, 64% of participants in the treated arm did not achieve a microbiological cure, while

36% were lost to follow-up. In the control arm, 7% of participants were cured, 64% were not cured, and 29% were lost to follow-up. The high number of participants lost to follow-up results in a considerable amount of missing data, potentially impacting the study's overall findings. This highlights the challenges of TB treatment, especially for XDR-TB, which remains a serious public health concern due to its limited treatment options and poor clinical outcomes.

This retrospective analysis highlights the intricate relationship between demographic, behavioural, and clinical factors in XDR-TB treatment outcomes. Investigating these dynamics is crucial for developing targeted interventions, especially for vulnerable groups such as individuals with low BMI, male patients, smokers, alcohol users, and those co-infected with hepatitis. Additionally, the high prevalence of drug resistance highlights the immediate demand for improved diagnostic tools and clinical approaches to enhance long-term TB control efforts.

6. Limitations

In vitro experiments, where THP-1 cells were used, it may have limited generalizability to humans, as HCL lacks all the environmental stimuli that shape the host immune response to infection, thereby limiting our extrapolation to clinical efficacy. For the MGIA experiments, there was a lower PBMC count when compared to the healthy control cell count. These samples were collected in 2017-2019 and stored for long periods which could have resulted in decreased cell viability. Total PBMC counts can be lower compared to healthy individuals or drug-sensitive TB patients due to systemic immune exhaustion and chronic infection. Ocaña-Guzman et al., 2021 observed alterations in XDR-TB patients' PBMC profiles and are recognized for their Key role in immune defense against TB (129).

PZA requires an acidic environment to be effective at killing and therefore was removed from the experiments. During the checkerboard assay assessing the synergistic mechanism of the FLDs; the EMB in combination with INH and RIF produced bacterial growth inhibition, and we assume that the combination was too potent. This was then removed after many different concentrations were explored.

Data collected for the XDR-TB pilot study could be skewed as the loss to follow-up status in each treatment group could have added more substantial evidence as the study aimed to evaluate if IBU contributed to better clinical outcomes.

The inherent biological heterogeneity of *M.tb* introduces variability in MIC determinations, as subpopulations within laboratory strains may differ genetically, metabolically, or in growth rate, leading to inconsistent drug responses.

7. Conclusion

There is limited clinical trial data or laboratory experimental research specifically involving ASA and IBU. Based on the limited studies available and the findings from this work, the effect of HDT on the control of mycobacterial infections does not appear to be clinically significant. Consequently, the hypothesis is considered null, as clinical strains do not exhibit differential behaviour in the THP-1 cell model. ASA and IBU did not show a meaningful impact on the clearance or control of *M.tb* infection within the chosen experimental setup used.

Further research is essential to identify NSAIDs beyond ASA and IBU, as these are primarily utilized for their anti-inflammatory properties. Exploring other antimicrobial agents could provide additional therapeutic options. Several studies have investigated the potential of natural and biologically derived compounds, such as probiotics, polyphenols, antimicrobial peptides, and IFN- γ , as alternative anti-mycobacterial agents (130-132). These molecules either possess direct bactericidal properties or demonstrate anti-inflammatory and immunomodulatory effects, thereby offering promising therapeutic avenues for developing novel treatment strategies for mycobacterial (133).

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9. Appendix

9.1 Appendix A

Table A1. PBMC cell count for HV samples.

Sample name	PBMC count (x10 ⁶)
Healthy volunteer 1	13.62
Healthy volunteer 2	10.98
Healthy volunteer 3	13.08

Table A2. PBMC cell count for clinical samples.

Sample number	PBMC count (x10 ⁶)		
	Baseline	Month 2	Month 6
1	0,44	0,88	0,56
2	0,45	1,79	0,40
3	0,27	0,33	1,00
4	0,28	0,141	0, 42
5	0,01	0, 54	1,02
6	1,08	2,14	1,44
7	0,42	0,48	1,92
10	0,41	0,09	0,74
11	0, 55	3,57	0,39
12	0,66	0,63	058
14R	0	0	0,06
15R	0,14	0	0,15
16	0,67	0,06	0,12
17	2,35	0,4	0,16
19	0,36	0,03	0,24
20	2,58	1,22	0*
21	3,25	0,06	0,08
22	1,20	0,23	1,31
23	0 *	0,22	0*
24	0,1	0,15	0,03

*0 cells were recovered after thawing of the 2 PBMC vials

Table A3. Pooled PBMC samples for experiment 1

Baseline			
New sample ID	Treatment	Samples pooled	Count x 10 ⁶
1	Ibuprofen	1+3+5+7	1.23
2	Control	2+10	1.31
3	Control	4+6	1.36
Month 2			
4	Ibuprofen	1+3	1.21
5	Control	4+10	1.04
6	Ibuprofen	5+7	1.02
7	Control	2	1.79
8*	Control	6	2.14
Month 6			
9	Ibuprofen	1+3	1.56
10	Control	2+10	1.14
11	Control	4+6	1.86
12	Ibuprofen	5	1.02
13	Ibuprofen	7	1.92

*Samples had more than 1 x 10⁶ cells therefore replicates were set-up

Table A4. Pooled PBMC samples for experiment 2

Baseline			
New sample ID	Treatment	Samples pooled	Count x 10 ⁶
1	Ibuprofen	11+15R+19	1.07
2	Control	12+16+24	1.43
3*	Ibuprofen	17	2.35
4*	Control	20	2.58
5*	Ibuprofen	21	3.25
6	Control	22	1.20
Month 2			
7*	Ibuprofen	17+19+21+23+11	(111)6
8	Control	12+16+22+24	1.67
9	Control	20	1.22
Month 6			
10	Ibuprofen	11+15R+17+19+21	1.03
11*	Control	12+14R+16+22+24	2.10

*Samples had more than 1 x 10⁶ cells, therefore, replicates were set up at a cell count of 1 x 10⁶.

9.2 Appendix B

Clearance certificate to do a retrospective data analysis.

“Pilot Study to estimate the potential efficacy and safety of using Adjunctive Ibuprofen for the Treatment of pre-XDR and XDR Tuberculosis”.


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R49 Ms ADN Pillay

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M240993

NAME: Ms ADN Pillay **DEGREE:** MSc (Med)
(Principal Investigator)

DEPARTMENT: School of Pathology
CoE in Biomedical TB Research
Faculty of Health Sciences

PROJECT TITLE: *The effect of potential host-directed therapies on the control of mycobacterial infection*

DATE CONSIDERED: Ad hoc

DECISION: Approved unconditionally

CONDITIONS:

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>

SUPERVISOR: Professor B Kana and Drs B Gordhan and Z Waja

APPROVED BY: Professor P Ruff, Chairperson, HREC (Medical)

DATE OF APPROVAL: 2024/10/14 **EXPIRY DATE:** 2029/10/13

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at <https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/>. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

Date

9.3 Turnitin report

The effect of potential HDTs on the control of M.tb
infection_dissertation.docx

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Seemi, Palesa. "Understanding Mycobacterial Genome Diversity Using a Cultureomics Approach", University of the Witwatersrand, Johannesburg (South Africa), 2025 Publication	1%
2	wiredspace.wits.ac.za Internet Source	1%
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