IN SILICO STUDY AND SYNTHESIS OF FOLIC ACID-ISONIAZID COMPLEX AS A POTENTIAL ANTITUBERCULOSIS AGENT

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by

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LIST OF EQUATIONS

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LIST OF SYMBOLS

α	Alpha
Δ	Change
©	Copyright
°C	Degree Celsius
=	Equal
γ	Gamma
>	Greater than
≥	Greater than or equal to
<	Less than
≤	Less than or equal to
π	Pi
®	Registered
σ	Sigma
™	Trademark

LIST OF ABBREVIATIONS

ALA	Alanine
AMK	Amikacin
ANN	Artificial neural network
API	Active pharmaceutical ingredient
ARG	Arginine
ASN	Asparagine
ASP	Aspartic acid
ATP	Adenosine triphosphate
BPG	1,3-bisphosphoglycerate
CAP	Capreomycin
CASP	Computer aided synthesis planning
CD4	Cluster of differentiation 4
CD44	Cluster of differentiation 44
CIP	Ciprofloxacin
COVID-19	Coronavirus Disease of 2019
DCM	Dichloromethane
EMB	Ethambutol
EPR	Enhance permeability & retention
EtOH	Ethanol
FA	Folic acid
G3P	Glycerol-3-phosphate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLU	Glutamic acid
GLY	Glycine

GTP	Guanosine triphosphate
HIS	Histidine
HIV	Human immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
ILE	Isoleucine
INF	Interferon
INH	Isoniazid
KAN	Kanamycin
LBDD	Ligand based drug design
LBP	Ligand based pharmacophore
LIP	Liposomes
MDR	Multidrug-resistant
MDR	Molecular dynamic
MeOH	Methanol
MOX	Moxifloxacin
MTB	Mycobacterium Tuberculosis
MTC	Mycobacterium Tuberculosis Complex
MW	Molecular weight
NAD+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
OFX	Ofloxacin
PAS	*para*-amino salicylic acid
PbPK	Physiology based Pharmacokinetics
PHE	Phenylalanine
PLGA	polylactic-co-glycolic
PMN	Polymorphonuclear cell
PRO	Proline

prPK	Predictive pharmacokinetics
PZA	Pyrazinamide
QSAR	Quantitative structure–activity relationship
RBP	Receptor based pharmacophore
RIF	Rifampicin
RNA	Ribonucleic acid
SAR	Structure activity relationship
SBDD	Structure based drug design
SigH	Sigma Factor H
SM	Streptomycin
SMILES	Simplified molecular-input line-entry system
SVM	Support vector machine
SWCNTs	Single wall carbon nanotubes
TB	Tuberculosis
Tf	Transferrin
TFRs	Transferrin receptor
THR	Threonine
TNF	Tumor necrosis factor
XDR	Extensively drug-resistant

LIST OF APPENDICES

KAJIAN IN SILICO DAN SINTESIS KOMPLEKS ASID FOLIK-ISONIAZID SEBAGAI AGEN ANTITUBERKULOSIS BERPOTENSI

ABSTRAK

Kajian ini bertujuan untuk mereka bentuk sebatian konjugasi novel, konjugat asid folik-INH (FA-INH), menggunakan reka bentuk ubat berasaskan ligan dan pendekatan komputasi, disahkan oleh pengedokan molekul, dan mengoptimumkan substrat utama untuk sintesis konjugat melalui pendekatan konvensional dan pendekatan dengan bantuan gelombang mikro. Daripada 80 ligan yang berpotensi, hanya dua sebatian, C1 dan C2, telah dipilih dengan kebarangkalian sintesisnya yang tinggi ($p > 0.8$). Kedua-dua C1 dan C2 menjalani penilaian pemprofilan ubat dan pengedokan molekul terhadap GAPDH (ID PDB: 6IEP) pada pH 7.4 dan pH 6.0, serta katG (ID PDB: 6ZJI) dalam kedua-dua jenis liar dan bentuk mutasinya. Sintesis berlaku dalam empat fasa, dengan Fasa 1 dan Fasa 2 dikaji dan dioptimumkan. Kedua-dua C1 dan C2 menunjukkan potensi sebagai calon ubat, dengan risiko ADMET (skor = 9/22) dan risiko ketoksikan (skor = 1.5/6.0) yang rendah. Interaksi pengikatan antara C1 dan C2 dengan GAPDH dan KatG adalah memberangsangkan (ΔG C1 GAPDH pH 7.4 = -6.20 kcal/mol, ΔG C2 GAPDH pH 7.4 = -6.37 kcal/mol, ΔG C1 katG = -6.19 kcal/mol, ΔG C2 katG = -8.99 kcal/mol) dengan pH mempengaruhi interaksi ikatan terhadap GAPDH (ΔG C1 GAPDH pH 6.0 = -6.98 kcal/mol, ΔG C2 GAPDH pH 6.0 = -6.63 kcal/mol). Sementara itu pada katG, C1 menunjukkan pertalian yang lebih baik untuk S315T (ΔG C1 S315T = -6.61 kcal/mol) dan R418L (ΔG C1 katG = -7.02 kcal/mol). Pendekatan menerusi bantuan gelombang mikro memberikan hasil tertinggi bagi produk Fasa 1 dan 2 (82.10 ± 0.291 % hingga 85.23 ± 0.495% untuk produk Fasa 1 dan 64.4 ± 0.183% hingga 67.3 ± 1.711% untuk produk Fasa 2)

berbanding dengan pendekatan konvensional (62.98 ± 0.383% hingga 67.44 ± 0.133% untuk produk Fasa 1 dan 59.86 ± 0.924% hingga 61.58 ± 0.160% untuk produk Fasa 2). Kehadiran mangkin juga meningkatkan sintesis Fasa 2 melalui kaedah konvensional (67.72 ± 0.733%). Fasa 1 dan 2 telah dioptimumkan lagi menerusi pendekatan dengan bantuan gelombang mikro (62.98 ± 0.383% untuk produk Fasa 1 dan 59.39 ± 2.486% untuk produk Fasa 2) dan pendekatan konvensional (82.10 ± 0.291% untuk produk Fasa 1 dan 62.50 ± 0.924% untuk produk Fasa 2). Penggunaan pendekatan dengan bantuan gelombang mikro telah meningkatkan hasil bagi Fasa 1 (+16.83 hingga 22.41%) dan Fasa 2 (+3.11%) berbanding kaedah konvensional. Kesimpulannya, sebatian C1 dan C2, menunjukkan janji sebagai calon ubat dan sintesis dapat dioptimumkan melalui pendekatan dengan bantuan gelombang mikro.

IN SILICO STUDY AND SYNTHESIS OF FOLIC ACID-ISONIAZID COMPLEX AS A POTENTIAL ANTITUBERCULOSIS AGENT

ABSTRACT

This study aimed to design a novel conjugated compound, folic acid-isoniazid (FA-INH) conjugate, using ligand-based drug design and computational approaches, validated by molecular docking, and optimizing the key substrates for the conjugate synthesis through conventional and microwaved-assisted approaches. Out of 80 potential ligands, two compounds, C1 and C2, were chosen due to their high synthesis probability ($p > 0.8$). Both C1 and C2 underwent drug profiling and molecular docking assessments against GAPDH (PDB ID: 6IEP) at pH 7.4 and pH 6.0, as well as katG (PDB ID: 6ZJI) in both its wild type and mutant forms. The synthesis of C1 and C2 occurred in four phases, with Phase 1 and Phase 2 being further investigated and. Both C1 and C2 showed promise as drug candidates, with low ADMET (score = 9/22) and toxicity risks (score = 1.5/6.0). The binding interactions between C1 and C2 with GAPDH and KatG were favorable (ΔG C1 GAPDH pH 7.4 = -6.20 kcal/mol, ΔG C2 GAPDH pH 7.4 = -6.37 kcal/mol, ΔG C1 katG = -6.19 kcal/mol, ΔG C2 katG = -8.99 kcal/mol) with pH affecting binding strength towards GAPDH (ΔG C1 GAPDH pH 6.0 = -6.98 kcal/mol, ΔG C2 GAPDH pH 6.0 = -6.63 kcal/mol). Meanwhile on katG, C1 demonstrated better affinity for S315T (ΔG C1 S315T = -6.61 kcal/mol) and R418L (ΔG C1 katG = -7.02 kcal/mol). The microwaved-assisted approach gives highest yield of Phase 1 and 2 products (82.10 ± 0.291 % to 85.23 ± 0.495% for Phase 1 and 64.4 ± 0.183% to 67.3 ± 1.711% for Phase 2) compared to conventional approach (62.98 ± 0.383 to 67.44 ± 0.133 for Phase 1 and 59.86 ± 0.924% to 61.58 ± 0.160% for Phase 2). The presence of catalyst also improves the synthesis of Phase 2 through

conventional method (67.72 ± 0.733%). However, considering the cost-benefit, Phase 1 and 2 can be further optimized and produced good yield with considerable condition for microwaved-assisted approach (62.98 ± 0.383% for Phase 1 and 59.39 ± 2.486% for Phase 2) and conventional approach (82.10 ± 0.291% for Phase 1 and 62.50 ± 0.924%). The application of microwaved-assisted method has improved yield for Phase 1 (+16.83 to 22.41%) and Phase 2 (+3.11%) over conventional method. In conclusion, C1 and C2 show promise as drug candidates and the synthesis able to be optimized through microwaved-assisted method.

CHAPTER 1

INTRODUCTION

1.1 Mycobacterium tuberculosis: An Ancient Bacteria.

When science revealed the microscopic level of life at the end of the nineteenth century, scientifical bacterial taxonomy developed. Since Aristotle, 350 BC, macroscopic have been categorised using the standard taxonomic levels, which include *Species, Genus, Family, Order, Class, Phylum,* and *Kingdom*, with *Domain* was added as the eighth rank in modern taxonomy (Kubica & Wayne, 1984; Woese et al., 1990).

The tuberculosis causative agent was first isolated by Robert Koch in 1882, and Zopf suggested the name Bacillus tuberculosis in 1883, which was later changed to *Mycobacterium tuberculosis* (MTB) in 1907 (Azé et al., 2015) (Table 1). MTB is a member of the *Mycobacterium tuberculosis* complex (MTC), which is made up of bacteria that cause tuberculosis (TB) or TB-like diseases (Mostowy & Behr, 2005). As it has afflicted humans for decades, there is no question that MTB has high adaptive capability that ensure its sustenance and continuous infectivity in human (Chai et al., 2018). The etiological agent of TB in humans is MTB while *Mycobacterium bovis* causes tuberculosis in cows and indirectly in humans through consumption of unpasteurized milk. Extrapulmonary TB (ETB) can be transmitted *via* this path that leads to the collapse of the vertebrae, causing hunchback due to bone deformity (Kenneth Todar, 2020).

Table 1.1 The taxonomy classification for MTB

Taxonomy	**Classification**
Kingdom	Bacteria
Phylum	Actinobacteria
Order	Actinomycetales
Suborder	Corynebacterineae
Family	Mycobacteriaceae
Genus	*Mycobacterium*
Species	*Mycobacterium tuberculosis*

The whole-genome sequence information for many species, including MTB H37Rv, MTB CDC1551, MTB 210, *M. microti* OV254, *M. bovis* 2122, and *M bovis bacille Calmette-Guerin* (BCG) Pasteur, has offered a wealth of genomic insight for the Mycobacterium genus (Mostowy & Behr, 2005). MTB strain H37Rv was 100% related to one strain of *M. bovis* and the BCG strain of *M. bovis* (Copenhagen strain) was based on this DNA homology (Baess, 1979). One strain of *M. leprae* showed 21% relatedness to *MTB* strain TMC 102 and 25% relatedness to *M. bovis* strain BCG (TMC1011), while another strain of *M. lepraemurium* showed 27% relatedness to *M. bovis* strains ATCC 19210 and 14% relatedness to *M, tuberculosis* strain TMC102 (Athwal et al., 1984). These results supported the theory that MTB and *M. bovis* belong to the same genus which

belongs under the Actinobacteria phylum, that is also increasingly linked to drug resistance due to mutations and rearrangements in its single circular chromosome.

1.2 Morphology and structure: Mycobactrium tuberculosis (MTB)

MTB is a non-motile rod-shaped bacterium, similar to the Actinomycetes but they have different suborder to each other. The rods have a length of 2-4 μm and a width of 0.2-0.5 μm. Many non-pathogenic mycobacteria are present in dry and oily environments and are a part of human natural flora. In classic cases of TB, MTC are often located in the well-aerated upper lobes of the lungs since it is an aerobic bacterium. It is a facultative intracellular parasite that most commonly infects macrophages. It has a slow generation period of 15-20 hours, which can contribute to its virulence. MTB is neither Gram-positive nor Gram-negative bacteria because it lacks the chemical characteristics of either, even though the bacteria's cell wall contains peptidoglycan (murein). However, MTB may stain very weakly Gram-positive when stained with a Gram dye. Due to their impermeability to some dyes and stains, MTB is known as acid-fast bacteria. Regardless, acid-fast bacteria that have been stained can preserve dyes when heated and treated with acidified organic compounds through a process called the Ziehl-Neelsen staining process (Kenneth Todar, 2020).

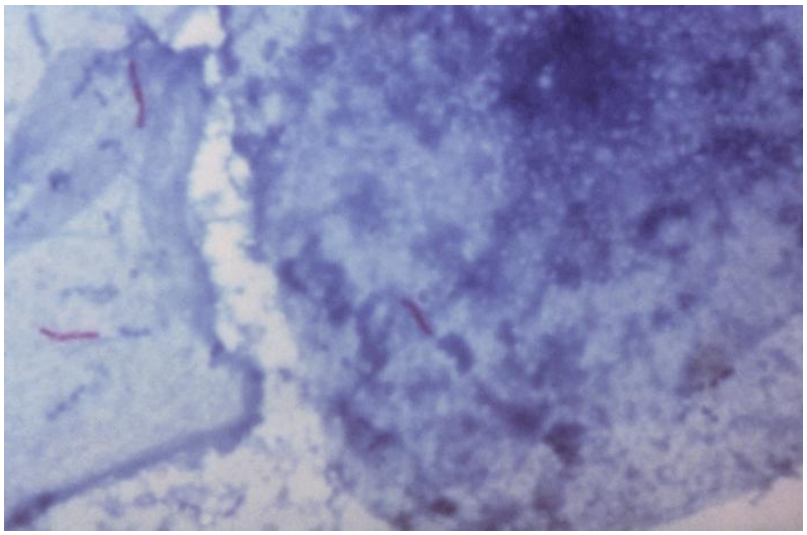

Figure 1.1 Acid-fast staining of MTB through Ziehl-Neelsen staining process. Magnification: 1000x. By Centre of Disease Control (CDC), 2011. PHIL Photo ID#5790.

The MTB cell wall structure deserves a special attention because of its uniqueness in comparison to the other procaryotes and the role it plays in the bacterium's virulence. The cell wall complex contains peptidoglycan, but it is mostly made up of complex lipids. Lipids make up more than 60% of the mycobacterial cell wall. Mycolic acids, cord factor, and wax-D are the three major components of the lipid fraction in MTB cell wall. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and influence the permeability properties at the cell surface. They are thought to be an important determinant of MTB virulence and they play an important role in shielding the mycobacteria from cationic proteins, lysozyme, and oxygen radicals in the phagocytic granule, besides protecting the extracellular mycobacteria from complement deposition in serum. The serpentine cording described above is caused by Cord Factor. Cord factor is toxic to mammalian cells and a polymorphonuclear cell (PMN) migration inhibitor. Cord factor is most prevalent in MTB strains that are virulent. Freund's full adjuvant is

primarily composed of wax-D in the cell envelope. The high concentration of lipids in the cell wall of MTB has been linked to the bacterium's properties which are (i) stain and dye impermeability, (ii) responsible in the development of resistance against antibiotics, (iii) responsible in its resistance against acidic and alkaline compounds, (iv) complement deposition provides resistance to osmotic lysis, and (v) aid in survival within macrophages and resistance to lethal oxidations (Kalscheuer et al., 2019; Kenneth Todar, 2020).

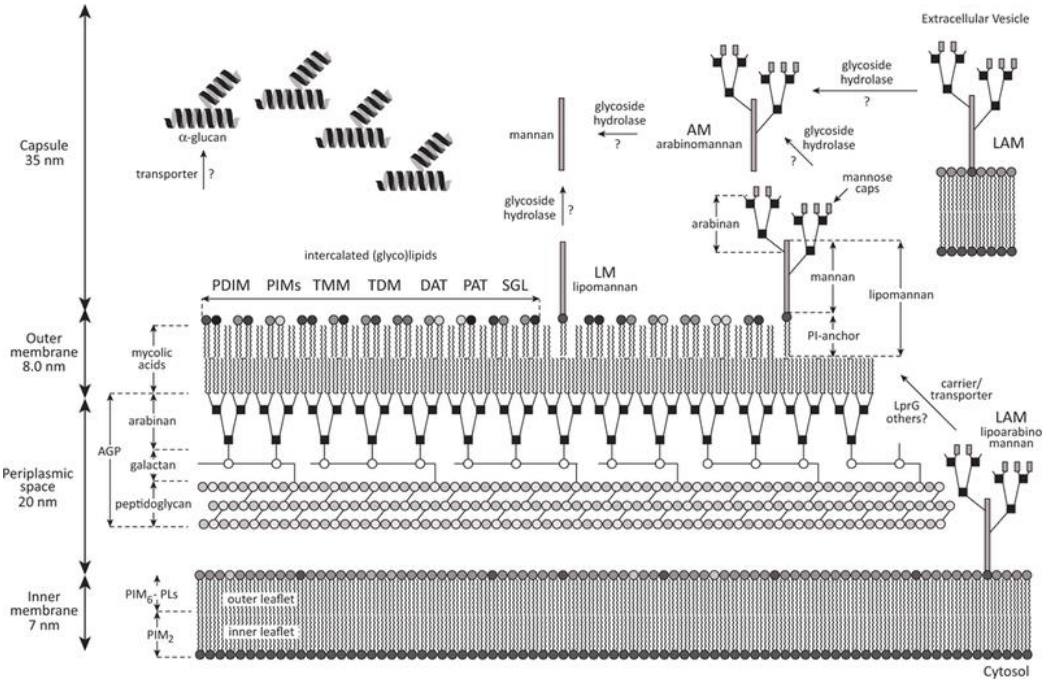

Figure 1.2 The MTB cell envelope by Kalscheuer, R., 2019. The *Mycobacterium tuberculosis* capsule: a cell structure with key implications in pathogenesis. Biochemical Journal, 476(14), 1995-2016.

1.3 Tuberculosis prevalence

Its already known that TB is an infectious airborne disease caused by the MTB complex of species which is a successful human pathogen that is old and extremely destructive (Rojano et al., 2019). Through its epidemiology, TB possibly existed in

animals as an endemic disease well before affecting humans (Steele & Ranney, 1958). World Health Organization (WHO) has reported that up to 2018, it is one of the main causes of death due to infectious diseases in the Asia-Pacific region reported (WHO, 2018). However, the COVID-19 pandemic began in 2019 and continues to have a negative influence on tuberculosis detection and treatment, as well as the disease burden. The decrease in reported tuberculosis cases globally in 2020 and 2021 indicates a rise in undiagnosed and untreated TB cases, leading to higher mortality rates and increased transmission within communities. This pattern eventually resulted in a delayed increase in the number of individuals developing TB. The countries primarily responsible for the reduction in 2020 were India, Indonesia, and the Philippines, which continued to contribute significantly to the global decrease compared to 2019, even with partial recoveries in 2021 (WHO, 2022).

There were 10 million cases of (new and recurrent) TB worldwide in 2018, including 1.2 million deaths among HIV negative people worldwide. More than 40% of cases worldwide were estimated to occur in India, Indonesia and Pakistan (WHO, 2020). However, in 2021, fatalities from TB among HIV-negative persons were expected to be 1.4 million, while deaths among HIV-positive individuals were estimated to be 187,000, for a total of 1.6 million deaths. This implies an increase from the expected numbers of fatalities in 2020 (1.5 million) and 2019 (1.4 million), reverting to the level experienced in 2017. The overall reduction in TB fatalities from 2015 to 2021 was 5.9%, which is almost one-sixth of the way to achieving the first milestone of the WHO's end TB plan (WHO, 2022). As a global public health epidemic, TB poses new challenges, especially at a time of growing risks to certain groups of patients due to COVID-19, human immunodeficiency virus (HIV) infection, multidrug-resistant (MDR) and extensively

drug-resistant (XDR) MTB strains. Also, to counteract and reverse the negative impacts of the COVID-19 pandemic on TB, there is an urgent need for increased efforts supported by increased financial resources. Because of the continuing war in Ukraine, as well as other global conflicts and issues such as the petroleum crisis and possible risks to food security, which are expected to worsen the underlying elements contributing to TB, this requirement has become even more crucial.

The World Health Assembly aims to minimise TB casualties by 95% and reduce the occurrence of TB by 95% by year 2035. However, the efficiency of case reporting is of concern. Based on year 2004 results of infection and disease prevalence surveys, assessments of the effectiveness of surveillance systems and death registrations, it was found that there were approximately 8.9 million new cases of TB, of which less than half were reported to public health authorities and the WHO. Sputum-smear, the most infectious form of the disease, was positive in about 3.9 million cases (WHO, 2020).

Most of these cases of TB and deaths occur disproportionately among males, but the burden of women's disease is also high as it remains among the world's top three killers. If diagnosed early and given the correct treatment, most cases of TB are curable, thus reducing the onward transmission of infection. The rate of incidence among young adults is highest and most cases are due to recent infection or reinfection. The case load shifts to older adults as transmission falls, and a higher proportion of cases are attributable to latent infection reactivation. Indigenous patients with TB, therefore, tend to be of older age in the countries of Western Europe and North America, which now have low incidence rates, while patients who are immigrants from high-incidence countries tend to be young adults. Although the per capita incidence rate of TB appears to be increasing

worldwide, the trends of case reporting are decreasing if not maintained at a steady rate in most regions in the world (WHO, 2018, 2020).

Table 1.2 The data on tuberculosis (TB) cases in the Asia-Pacific region from WHO 2020.

Country	TB Cases (millions)	Case Notification Rate (per 100,000)	Incidence Rate (per 100,000)
India	2.69	-	-
China	0.86	-	-
Indonesia	0.84	-	-
Philippines	0.59	> 300	> 500
Pakistan	0.56	-	-
Korea	-	> 300	> 500
Papua New Guinea	-	> 300	-
Australia	-	< 15	-
New Zealand	-	< 15	-
Japan	-	< 15	-

From the table 1.2, we can observe that India, China, Indonesia, the Philippines, and Pakistan have a considerable number of TB cases in the Asia-Pacific area, adding

considerably to the worldwide burden. With high case notification and incidence rates, the Philippines and Korea stand out, showing a crucial need for targeted treatments and enhanced TB control strategies. In comparison, nations such as Australia, New Zealand, and Japan have a comparably lower TB case burden (WHO, 2020).

TB was the number one cause of death in Malaysia in the early 1940s and 1950s and there were several sanatoriums built in different parts of the country for TB patients. During the time, TB were managed by surgical means before TB chemotherapy became accessible. TB remained the second most frequent communicable disease in Malaysia after malaria until 1987. However, according to WHO, TB will continue to be the second leading cause of mortality due to a single infectious illness through 2020 (Arokiasamy J. T., 1990; WHO, 2020). A preliminary study carried out by Elamin E. I. *et* al. in 2004 in Penang shows that majority TB patients were male, smoker, non-alcoholic, vaccinated with BCG, had secondary education, and not working (Elamin et al., 2004). This demographic statistic data is being studied and researched further by Bhatti Z. et al., on 2021, which appears to reveal an essentially identical pattern of demography of TB patients.

Table 1.3 The demographic of TB patients recorded in Penang in 2004 and 2021.

Demographic Characteristic	TB Patients in Penang - 2004	TB Patients in Penang - 2021
Number of patients	207	337
Ethnicity	Chinese (55.5%); Malay (33.8%); Indian (9.7%)	Chinese (45.2%); Malay (40.6%); Indian (11.0%)
Gender	Male (72.9); Female (27.1%)	Male (68.55); Female (35.5%)
Smoking Status	Smoker (49.8%)	Smoker (55.7%)
Alcohol Consumption	Non-alcoholic (69.6%)	N/A
BCG Vaccination	Vaccinated (53.6%)	N/A
Education Level	Secondary (52.2%)	<12 years (79.5%)
Employment Status	Not Working (52.7%)	Employee (72.5%)

1.4 Anti-TB treatment

Typically, anti-TB treatment consists of an initial treatment phase of four medications; isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for a period of two months followed by a continuation phase of INH and RIF therapy for another four months (Table 1.4). This treatment, also referred to as DOTS (direct observed therapy), is the safest therapy for TB and is recommended by the WHO

to treat patients with susceptible TB. Most cases of TB are curable, thereby reducing the onward spread of infection. However, this lengthy course of treatment is often a challenge to be completed and adhered to by patients. This stimulates the attempt to create shorter medication regimens (Pai & Sotgiu, 2016), besides the need to overcome the occurrence of drug resistance TB that will lead to the selective survival of the drug-resistant mutated cells following treatment with the conventional anti-TB drugs (Rojano et al., 2019).

Table 1.4 Dosages of first-line anti-tuberculosis drugs. Adopted by World Health Organization. Treatment of Tuberculosis Guidelines Fourth Edition.

Drug*	Recommended dose			
	Daily		3 times per week	
	Dose (range) in mg/kg body weight	Maximum in mg	Dose (range) mg/kg body weight	Daily maximum in mg
Isoniazid (H)	5 (4 - 6)	300	10 (8 - 12)	900
Rifampicin (R)	10 (8 - 12)	600	10 (8 - 12)	600
Pyrazinamide (Z)	25 (20 - 30)	2000	35 (30 - 40)*	3000*
Ethambutol (E)	15 (15 - 20)	1600	30 (25 - 35)*	2400*
Streptomycin (S)	15 (12 - 18)	1000	15 (12 - 18)*	1500*

Pyridoxine 10 - 50 mg daily needs to be added if isoniazid is prescribed.
* Daily treatment is the prefered regimen.

1.5 Isoniazid (INH): An important part of TB treatment

INH also known as isonicotinic acid hydrazide, is one of the important drugs in anti-TB treatment. It has a relatively basic chemical structure made up of a pyridine ring and a hydrazine group connected to the pyridine nitrogen in *para* position (Y. , Zhang et al., 1992) (Figure 1.3).

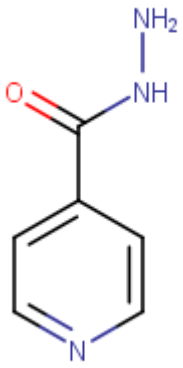

Figure 1.3 The molecular structure of INH.

The discovery of INH as an effective anti-TB drug marked the beginning of the new era in TB chemotherapy. In 1951, a remarkable clinical coincidence began when pharmaceutical experts from Hoffmann-LaRoche and Squibb in the United States and Bayer Chemical in Germany simultaneously "discovered" INH at nearly the same time and started using it. INH represented a monumental development in that it was the most potent drug launched to date while still being cheap, well absorbed, and effective. It was so persuasive that it was initially thought that INH alone will be successful, but patients subsequently relapsed, with approximately three-fourths of them having species resistant to INH, showing once again that multiple drug therapy was needed. The commanding and long-lasting result was known as "triple therapy", which include oral INH in combination with *para*-aminosalicylic acid (PAS) for 18 to 24 months and intramuscular streptomycin for the first 6 months. For about 15 years, “triple therapy” was the traditional treatment for all types of TB (Robitzek & Selikoff, 1952) before the current four-drug regimen was

introduced in 1974Currently, INH is still part of the first-line treatment due to its excellent minimal inhibitory concentration (MIC) of ≥1.0 μg/mL against MTB (Scior et al., 2002).

INH penetrates the mycobacterial cell wall through passive diffusion, and although it is not toxic to the bacteria, it functions as a prodrug that is activated by the mycobacterial enzyme katG (Bardou et al., 1998; Y. , Zhang et al., 1992). KatG activates INH through peroxidation to generate intracellular toxic INH-derived isonicotinoyl radical species that will bind covalently to a NAD molecule, causing the FASII enoyl-ACP reductase InhA to be inhibited. This enzymatic complex is essential in the biosynthesis of mycolic acid, and its inhibition causes an accumulation of long-chain fatty acids, which inhibits cell wall synthesis and eventually leads to cell death (Timmins & Deretic, 2006a; Vilchèze & Jacobs, 2007).

Mutations in the *M. tuberculosis* inhA gene and the katG enzyme are the most common mechanisms of resistance to INH. As the inhA gene is responsible for mycolic acid biosynthesis, mutation in the katG enzyme will prevent INH from interacting with the binding site in the inhA complex (Shekar et al., 2014). Selective point mutations have also been reported that result in a partially active protein that maintains some of KatG's bacterial survival-supporting activities while minimising INH-induced toxicity to the MTB cells (e.g., S315T).

1.6 Targeted Drug Delivery Approach

Targeted drug delivery, also known as smart delivery of drugs, is a therapeutic strategy that is achieved through two methods: passive targeting and active targeting. The drug is delivered either into the targeted cells or to the surrounding tissues using drug

delivery vehicles (Tewabe et al., 2021). Passive targeting relies heavily on the disease's physiological environment to allow for preferential concentration at the site of interest and the limitation of non-specific dissemination, and this targeting method involves long-circulating drug delivery systems that remain in the blood to allow for site-specific accumulation. Meanwhile, in active targeting, the delivery of drug to specified areas is based on molecular recognition using specific ligands conjugated to the drug that bind on relevant receptors expressed by cells at the targeted region, allowing drugs to be administered at a lower effective dose. There will be less drug molecules transported unnecessarily to other sites, lowering the number of pharmaceuticals required to achieve the intended effect in comparison to the same treatment without a targeting ligand (Khairul et al., 2021; Torchilin, 2000). Using the active targeting approach, it is also possible to design new therapeutic agents with the potential to prevent resistance by identifying the resistance development process.

1.7 Problem statement

There is a very limited number of studies reported in the literature on the application of active targeting against MTB. Most studies would use passive targeting with nanoparticles as the main approach of TB targeting research, likely due to the state of understanding on the MTB itself. In 2017, a paper by Malhotra H. et. al., described a cell surface receptor discovered on MTB called glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Malhotra et al., 2017). This discovery has paved the way for the development of a cell-targeted anti-tuberculosis medication that will boost the potency of the therapy and reduce the risk of resistance development.

In 2021, Noh et al. announced the development of a possible GAPDH receptor targeting ligand, one of which is folic acid (FA) with a high binding affinity (Noh et al., 2021). As a result, it piques our interest to conduct research into using this technology to improve the delivery of the current drug, INH, and convert it into active drug delivery. The purpose of this study is to design and synthesize novel drug components with some modifications using FA as a ligand for our active drug, INH, which targets the GAPDH carrier protein of MTB.

1.8 General Objectives

For this study, we want to design new lead (FA-INH conjugates) that potential to be used as active targeting approaches directing the MTB GAPDH-iron carrier and katG protein, as well as highlights the potential of microwaved-assisted method that may improve the efficiency of synthesis for the initial substrate of the new lead.

1.8.1 Specific objectives

1. To design novel FA-INH conjugates through ligand-based drug design and computational approaches.
2. To evaluate the binding interaction and affinity of the designed FA-INH conjugates on GAPDH and KatG through *in silico* study.
3. To synthesize and optimize azido-spacer molecule (Phase 1) and INH – alkyl (Phase 2) through conventional and microwave-assisted techniques.

CHAPTER 2

LITERATURE REVIEW

2.1 MTB epidemiology and pathophysiology

MTB is a human pathogen responsible for TB. It employs a range of mechanisms to survive and sustain in host lesions and avoid immune response (Zhai et al., 2019). The understanding of the dynamic relationships between the pathogen and human as its host is crucial to design effective therapeutics that is needed to combat the emergence of the multidrug resistant strains, MDR-TB and extensive drug resistance strains, XDR-TB.

Among the reported mechanisms include (1) suppressing phagolysosome maturation and acidification by limiting the build-up of vacuolar adenosine triphosphate (ATP) and guanosine triphosphate (GTP) by bacterial proteins such as early secretory antigen-6/culture filtrate protein and ATP1/2 (Z. Chen et al., 2015; Rohde et al., 2007), (2) inhibiting the oxidative stress and the function of reactive oxygen and reactive nitrogen intermediates by MTB's ribonucleic acid (RNA) polymerase sigma-H factor (SigH or σ^H) (Sharp et al., 2016), (3) inhibiting apoptosis by overexpressing miR-30A, nitrous oxide and pro-inflammatory cytokines which suppress the elimination of intracellular MTB and inhibit tumour necrosis factor (TNF)-α-induced apoptosis (Z. Chen et al., 2015; Czabotar et al., 2014), (4) structural antigenic mutation response which alter the recognition of MTB from immune system (Ernst, 2017), (5) targeting DC-SIGN, a type II transmembrane protein under C-type lectin family to suppress cellular immune responses (Geijtenbeek et al., 2003), (6) increasing the expression of the peroxisome proliferator-activated receptor γ (PPAR- γ) which leads to an increase in lipid droplet production and a decrease in macrophage responsiveness (Almeida et al., 2012), (7)

inhibiting the transcription of genes expressing MHC class, reducing the amount of CD8+ T lymphocytes in the spleen, liver, and lungs by Pro-Pro-Glu 38 and PPE38 (Danelishvili et al., 2010) and (8) reducing the interaction between MTB rv0431 gene with macrophage receptors and thus limiting the immune stimulating factor synthesis and subsequently lowering MTB elimination (Rath et al., 2013).

These complex mechanisms led to their high drug tolerance, which is a huge problem especially with the widespread of MDR-TB and XDR-TB (Koul et al., 2011). The advent of MDR-TB has made the development of new molecular scaffolds a priority to discover new anti-TB agents. In addition, to gain successful regulation, the current situation also requires re-engineering and repositioning of certain clinically available drugs into more effective anti-TB agents (Koul et al., 2011; Smith et al., 2012).

In 2020, TB claimed the lives of 1.5 million individuals, including 214,000 patients who also had HIV. TB is the world's 13th leading cause of death and, after COVID-19, the second leading infectious killer, surpassed only by HIV/AIDS. By 2020, an estimated 10 million individuals had contracted TB, including 5.6 million men, 3.3 million women, and 1.1 million children (WHO, 2021a, 2022). The 30 countries with the highest TB burden are responsible for 86% of new cases, with India leading the way, followed by China, Indonesia, the Philippines, Pakistan, Nigeria, Bangladesh, and South Africa (Figure 2.1). India alone accounts for two-thirds of the total. MDR-TB remains a public health emergency and security issue, and only around one-third of patients with drug-resistant TB receive treatment. Globally, the incidence of TB is decreasing at a rate of roughly 2% per year, with a total reduction of 11% between 2015 and 2020 (WHO, 2021a).

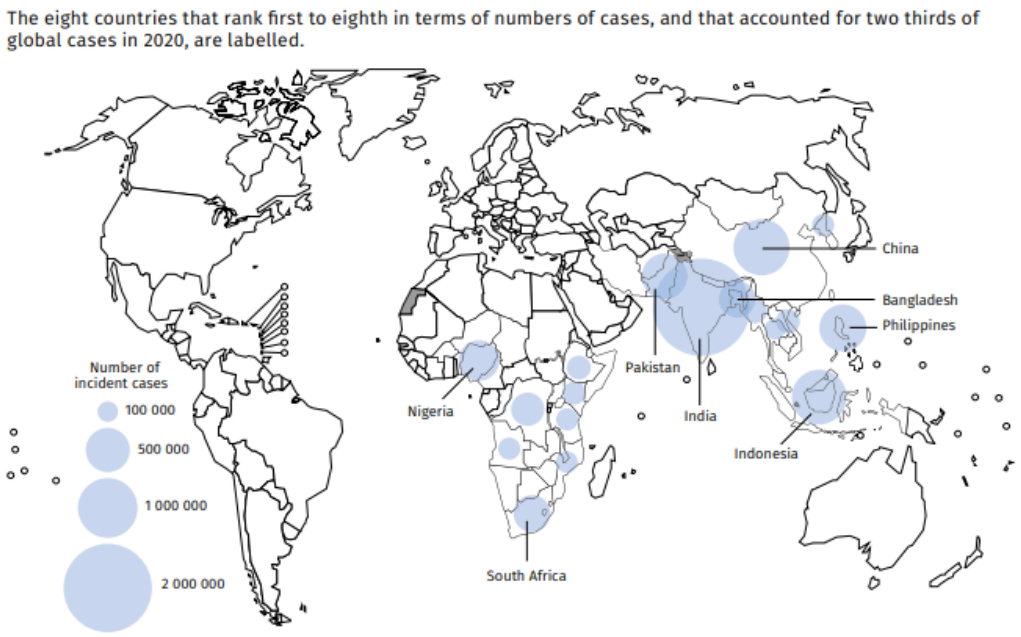

Figure 2.1 The estimated number of TB incidents for top eight countries with at least 100,000 incident cases.

The WHO South-East Asia (SEA) region, which is home to 26% of the world's population and has a 43% TB incidence rate, estimates that nearly 4.3 million people will contract TB in 2020, with an estimated 700,000 deaths from the disease (excluding HIV+ TB mortality). This accounts for more than half of the global TB deaths of 1.3 million. Despite this, treatment has proven successful in 85% of new and relapsed TB patients (Arinaminpathy et al., 2019; WHO, 2021b).

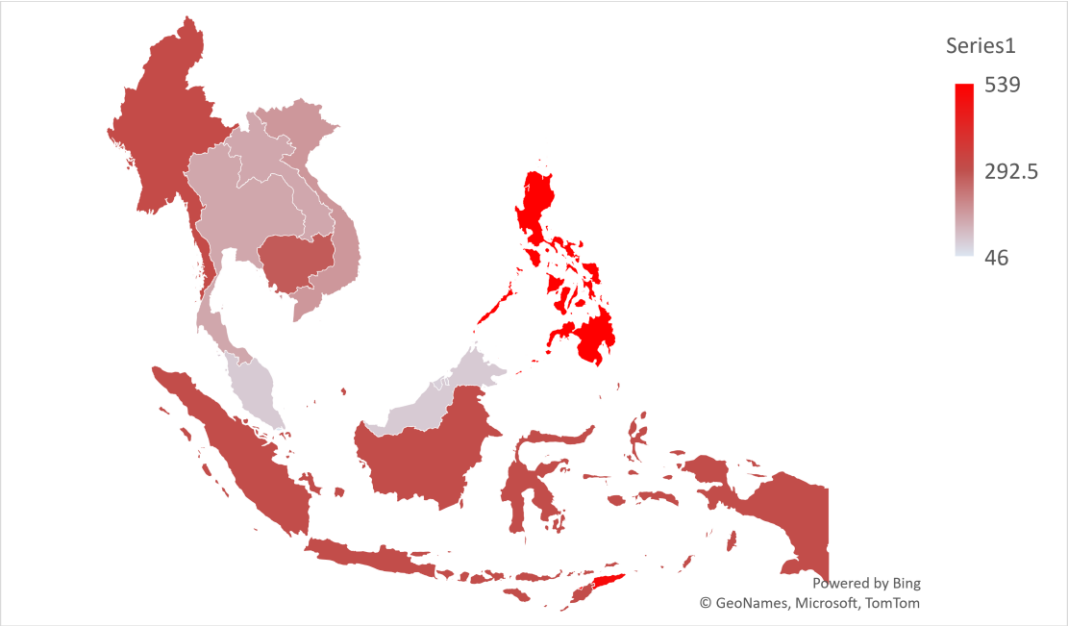

Figure 2.2 The number of TB incidents in South-East Asia countries (SEA) with at least 100,000 incident cases.

In 2020, Malaysia had a TB incidence rate of 92 out of 100,000 people, with the majority of reported cases affecting Malay (54%), followed by Chinese (25%), Indians (7.5%), indigenous communities in Sabah and Sarawak (11.8%), and others (1.7%) (MOH, 2020). The goal is to reduce the incidence to fewer than 10 per 100,000 people by 2035, with less than 85 fatalities by 2035 (MOH, 2020, 2021). However, according to NIH, Malaysia has an expected total TB incidence of 97 (ranging from 79 to 106) per 100,000 people in 2021, placing it in the intermediate group in terms of TB burden (Ab Rashid et al., 2023).

The pathophysiology of TB begins with the transmission of MTB through the inhalation of droplets nuclei produced by a person with pulmonary or laryngeal TB through coughing, sneezing, or talking, which can result in one of the four outcomes: (1) quick clearance of the organism, (2) latent infection, (3) the beginning of active disease

(primary disease), or (4) reactivation of TB (Wani, 2013). The transmission of TB is influenced by various factors such as the number of bacilli in the droplets, bacilli virulence, bacilli exposure to UV radiation, degree of ventilation, and opportunities for aerosolization (ATS, 2000; Knechel, 2009; Lee, 2004). The infectious droplets settle throughout the airways after being inhaled. Most of the bacilli will be trapped in the upper airways, where mucus-secreting goblet cells are present. The produced mucus traps foreign substances, and the cilia on the cell surface continually beat the mucus and its captured particles upward for elimination. Bacteria in droplets that enter the alveoli without passing through the mucociliary system are rapidly engulfed and destroyed by alveolar macrophages (ATS, 2000; Frieden et al., 2003).

These macrophages, which are part of the innate immune system, are the human body's next line of defence, and they help to eliminate invading mycobacteria and prevent infection. If the host's defence system fails to remove the infection, the bacilli multiply inside alveolar macrophages and can ultimately lead to the death of the human host. To prevent this, infected macrophages release cytokines and chemokines that attract additional phagocytic cells such as monocytes, other alveolar macrophages, and neutrophils, which ultimately form the tubercle, a nodular granulomatous formation (Wani, 2013). Macrophages are readily available phagocytic cells that combat a wide range of infections without the need for prior pathogen contact (Figure 2.3). Lipoarabinomannan from mycobacteria is an important ligand for a macrophage receptor and the complement system also plays a role in bacterial phagocytosis. The subsequent phagocytosis by macrophages sets off a chain of events that leads to either effective infection management, followed by latent TB, or progression to active disease, known as primary progressive TB. The outcome is primarily determined by the quality of the host

defence system and the balance between the host defences and the invading mycobacteria (Ferguson et al., 2004; Frieden et al., 2003; Y. J. Li et al., 2002; Nicod, 2007). Mycobacteria continue to multiply slowly after being consumed by macrophages, with bacterial cell division occurring every 25 to 32 hours. Regardless of whether the infection is suppressed or progresses, macrophages will produce proteolytic enzymes and cytokines in an attempt to destroy the bacteria. T lymphocytes, which make up cell-mediated immunity, are attracted to the site by the released cytokines. T lymphocytes are then presented with mycobacterial antigens on the surface of macrophages. This first immunological phase lasts two to twelve weeks, during which time the bacteria multiply to enough to fully trigger the cell-mediated immune response, which may be confirmed by a tuberculin skin test (TST) (Knechel, 2009).

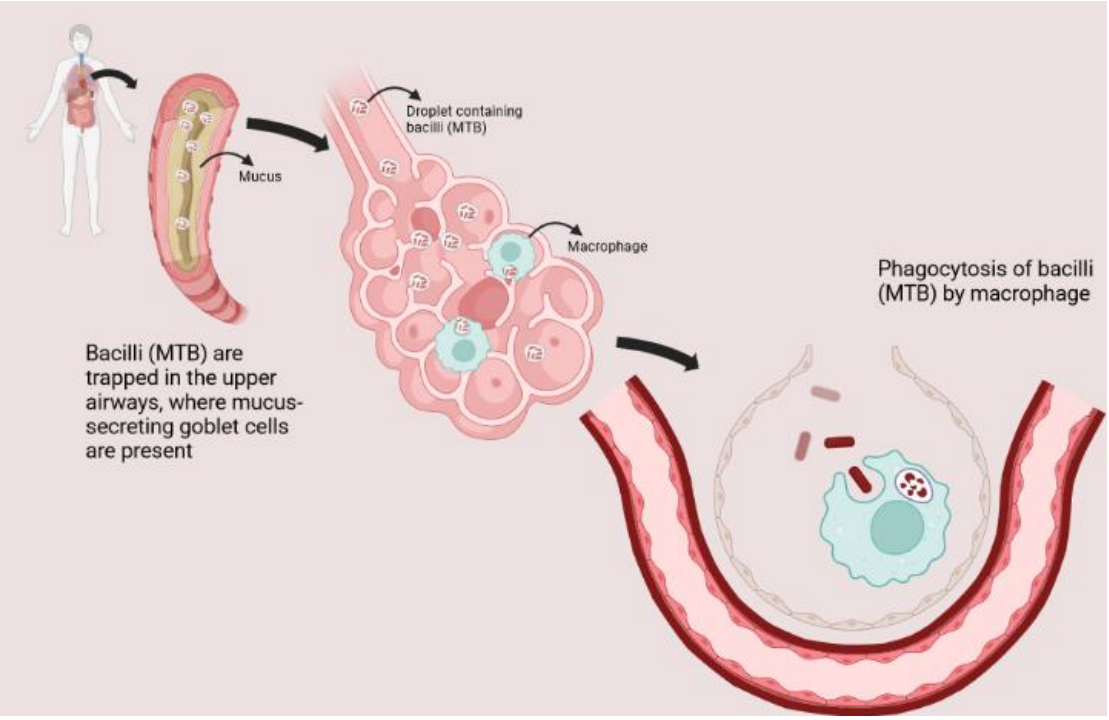

Figure 2.3 The migration of droplet containing MTB into the lung.

Uncontrolled bacterial multiplication leads to the expansion of the tubercle and eventually, the bacilli will reach the local draining lymph nodes. This can result in lymphadenopathy, which is a common clinical symptom of primary TB. The Ghon complex refers to the lesion caused by the growth of the tubercle into the lung parenchyma and involvement of lymph nodes. Additionally, bacteremia may occur in conjunction with the primary infection (Wani, 2013). Granuloma development is initiated in less immunocompetent individuals, and these nodular-type lesions usually form because of an aggregation of activated T-lymphocytes and macrophages, creating a microenvironment that inhibits mycobacteria proliferation and dissemination. However, this eventually will fail to confine the bacilli. The necrotic tissue liquefies, and the fibrous wall loses its structural integrity (Figure 2.4). Droplets from the bronchus can be coughed up by infected individuals and infect others. Extrapulmonary TB is likely if there is a leakage into a vessel. Bacilli can also enter the lymphatic system and congregate in the damaged lung's tracheobronchial lymph nodes, creating new caseous granulomas (Dheda et al., 2005; Knechel, 2009).

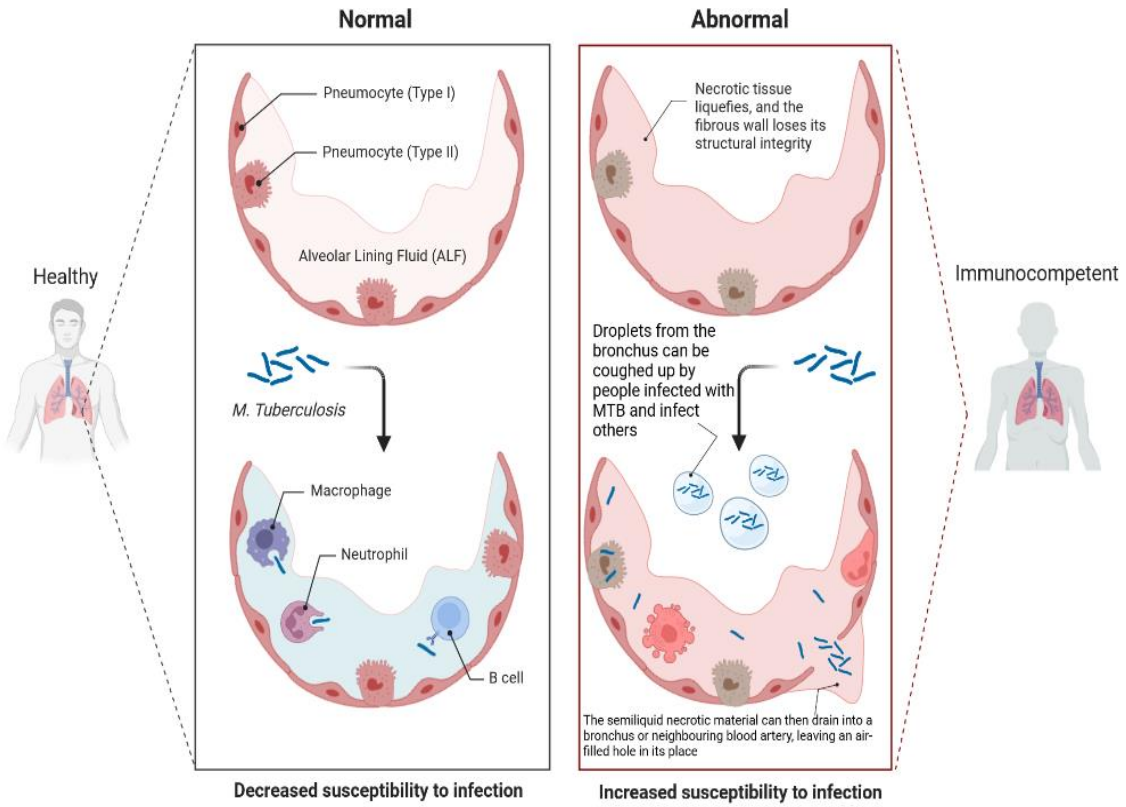

Figure 2.4 The invasion of bacilli (MTB) into the alveoli and the role of macrophage as lien of defence. Created with BioRender.com.

2.2 AntiTB drugs

2.2.1 General overview

Anti-TB drugs can be broadly classified into two groups; first-line drugs and second-line drugs (Bandodkar et al., 2020; Kanabus A., 2022). The first-line TB medications are INH, RIF, EMB, PZA, and streptomycin (SM), whereas the second-line TB treatments are classified into two groups: fluoroquinolones and injectable TB therapies. Fluoroquinolones such as ofloxacin (OFX), levofloxacin (LEV), moxifloxacin

(MOX), and ciprofloxacin (CIP), whilst injectable anti-TB medications include kanamycin (KAN), amikacin (AMK), and capreomycin (CAP) (Table 2.1).

Table 2.1 The summary of classification of anti-TB drugs

First-line TB drugs	INH (INH)	
	Rifampicin (RIF)	
	Ethambutol (EMB)	
	Pyrazinamide (PZA)	
	Streptomycin (SM)	
Second-line TB drugs	Fluoroquinolones	Ofloxacin (OFX)
		Levofloxacin (LEV)
		Moxifloxacin (MOX)
		Ciprofloxacin (CIP)
	Injectable	Kanamycin (KAN)
		Amikacin (AMK)
		Capreomycin (CAP)

The WHO has recently revised the classification of anti-TB drugs into five groups that is based on a meta-analysis and recommendations from experts (Tiberi et al., 2017). These groups include: (1) first-line oral anti-TB drugs, (2) injectable anti-TB drugs, (3) fluoroquinolones, (4) second-line bacteriostatic oral anti-TB drugs, and (5) anti-TB drugs with limited data on efficacy and long-term safety for MDR-TB and XDR-TB. This new classification is significant because it assists clinicians in prescribing an appropriate anti-TB regimen for cases of MDR-TB and XDR-TB, which may require more extensive treatment than the standard regimen. The revised classification will also aid in the development of more effective treatment regimens for these complex cases (Mazlan et al., 2021; Tiberi et al., 2017)