Universidade de Lisboa
Faculdade de Farmácia

Past, present and future of drug-resistant tuberculosis treatment

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Monografia orientada pela Professora Doutora Maria João Catalão, Professora Auxiliar

Mestrado Integrado em Ciências Farmacêuticas

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Resumo

Globalmente, a tuberculose é uma das principais causas de morte por doenças infeciosas. A Organização Mundial da Saúde estima que em 2020 cerca de 10 milhões de pessoas adoeceram com tuberculose e cerca de 1,5 milhões morreram da mesma doença. A crescente prevalência de resistência aos antibióticos utilizados no tratamento da tuberculose evidenciou a necessidade de desenvolvimento de novos esquemas de tratamento, mais seguros e eficazes, uma vez que atualmente o tratamento da tuberculose multirresistente é prolongado e engloba mais fármacos no esquema terapêutico quando comparado com os esquemas utilizados para o tratamento da tuberculose suscetível a medicamentos, o que pode levar a maiores efeitos adversos e interações medicamentosas. Assim, é de extrema importância que os regimes disponíveis para o tratamento da tuberculose sejam eficazes e bem tolerados.

A Organização Mundial da Saúde define quatro regimes de tratamento para: i) tuberculose resistente à isoniazida; ii) tuberculose multirresistente ou resistente apenas à rifampicina; iii) regime mais prolongado para tuberculose multirresistente ou resistente apenas à rifampicina; iv) tuberculose multirresistente com resistência adicional a fluoroquinolonas. O facto destes esquemas serem compostos maioritariamente por medicamentos administrados por via oral é essencial para contribuir para o sucesso da terapêutica.

Existem vários ensaios clínicos a decorrer, como o ZeNix ou o SimpliciTB, que estudam a combinação de vários medicamentos com o objetivo de criar esquemas terapêuticos de menor duração e maior eficácia. Também estão a decorrer ensaios clínicos, cujos resultados disponíveis parecem ser bastante promissores, para o estudo de novas moléculas para o tratamento de formas resistentes de tuberculose. Embora os antibióticos β-lactâmicos raramente sejam usados no tratamento da tuberculose, tem sido demonstrado que a associação de carbapenemos com inibidores de β-lactamases pode ser benéfica para o tratamento de formas resistentes desta doença. O uso de medicamentos inicialmente aprovados para o tratamento de outras patologias também tem demostrado resultados encorajadores, o que os pode vir a tornar numa opção terapêutica para o tratamento da tuberculose.

Embora ainda exista um longo caminho a percorrer para erradicar a tuberculose, os estudos e descobertas recentes têm-se mostrado bastante promissores, o que representa uma mais-valia para atingir este importante objetivo.

Palavras-chave: *Mycobacterium tuberculosis*; tuberculose multirresistente; tratamento; mutações genéticas; ensaios clínicos.

Abstract

Globally, tuberculosis is one of the leading causes of death from infectious diseases. The World Health Organization estimates that in 2020 about 10 million people fell ill with tuberculosis and about 1.5 million died from the same disease. The increasing prevalence of resistance to antibiotics used to treat tuberculosis has highlighted the need to develop new, safer and more effective treatment regimens since currently the treatment of multidrug-resistant tuberculosis is prolonged and has more drugs in the therapeutic regimen when compared to regimens used for the treatment of drug-susceptible tuberculosis, which can also lead to greater adverse effects and drug interactions. Therefore, it is of utmost importance that the regimens available to treat this infectious disease are effective and well-tolerated.

The World Health Organization defines four treatment regimens for: i) tuberculosis resistant to just isoniazid; ii) multidrug-resistant or rifampicin-resistant tuberculosis; iii) longer regimen for multidrug-resistant or rifampicin-resistant tuberculosis; iv) multidrug-resistant tuberculosis with additional resistance to fluoroquinolones. The fact that these regimens are mainly composed of drugs administered orally is essential to contribute to the success of the therapy.

There are several clinical trials underway, such as ZeNix or SimpliciTB, which study the combination of drugs to create therapeutic regimens of shorter duration and greater effectiveness. Clinical trials are also underway to study new molecules for the treatment of resistant forms of tuberculosis, which have shown promising results. Furthermore, even though β-lactam antibiotics are rarely used to treat tuberculosis, it has been shown that the association of carbapenems with β-lactamase inhibitors may be beneficial for the treatment of resistant forms of this disease. Additionally, the use of drugs initially approved to treat other pathologies has also shown promising results and may also become an option for the treatment of tuberculosis.

Although there is still a long way to go to eradicate tuberculosis, recent studies and discoveries have shown to be quite promising, which is an asset to achieve this important goal.

Keywords: *Mycobacterium tuberculosis*; multidrug-resistant tuberculosis; treatment; genetic mutations; clinical trials.

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Abbreviations

ADP - Adenosine diphosphate

AG - Arabinogalactan

Ag85v- Antigen 85 complex

ATP - Adenosine triphosphate

BPaL - Bedaquiline, pretomanid and linezolid

BPaMZ - Bedaquiline, pretomanid, moxifloxacin and pyrazinamide

BTZ - Benzothiazinones

CDC - Centers for Disease Control and Prevention

CW - Cell wall

DGS - *Direção-Geral da Saúde*

DPA - Decaprenylphosphoryl arabinose

DprE1 - Decaprenylphosphoryl-β-d-ribose oxidase

DST - Drug susceptibility testing

Eis - Enhanced intracellular survival

EMA - European Medicines Agency

EMB - Ethambutol

ETH - Ethionamide

FDA - Food and Drug Administration

GlcNAc - *N*-acetylglucosamine

HDTs - Host-directed therapies

hERG - Human Ether-à-go-go-Related Gene

HIV - Human Immunodeficiency Virus

IFN - Interferon

IL - Interleukin

INH - Isoniazid

Ldts - L,D-transpeptidases

LPAs - Line Probe Assays

LTBI - Latent tuberculosis infection

MA - Mycolic acids

mAGP - Mycolyl-arabino-galactan-peptidoglycan

MDR/RR-TB - Multidrug-resistant TB or rifampicin-resistant TB

MDR-TB - Multidrug-resistant TB

MfpA - Mycobacterium fluoroquinolone resistance protein A

MIC - Minimum inhibitory concentration

Mtb - *Mycobacterium tuberculosis*

mTOR - Mammalian target of rapamycin

MurNAc - *N*-acetylmuramic acid

PAS - Para-aminosalicylic acid

PBPs - Penicillin-binding proteins

PBTZ - Piperazine-containing benzothiazinones

PCR - Polymerase chain reaction

PG - Peptidoglycan

Pi - phosphates

pre-XDR-TB - pre-extensively drug-resistant TB

PZA - Pyrazinamide

QRDR - Quinolone-resistance-determining region

QTc - QT corrected

RIF - Rifampicin

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RRDR - Rifampicin resistance-determining region

RR-TB - Rifampicin-resistant TB

TB - Tuberculosis

WGS - Whole-Genome Sequencing

WHO - World Health Organization

XDR-TB - Extensively drug-resistant TB

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1 Introduction

1.1 Tuberculosis and *Mycobacterium tuberculosis* pathogenesis

Tuberculosis (TB) is a respiratory infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*) that usually affects the lungs, causing millions of new infections and deaths every year. *Mtb* is transmitted through airborne particles, called droplet nuclei (1). So, transmission occurs when a person inhales droplet nuclei containing *Mtb*, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs (1,2). Phagocytosis of *Mtb* by alveolar macrophages and dendritic cells initiates a cascade of events involving the production of pro-inflammatory cytokines and chemokines, which stimulate the activation of phagocyte anti-microbial activities and recruit blood leukocytes into the tissue to the site of infection (2,3). The accumulation of these leukocytes around infected cells leads to the formation of a macrophage-rich cell mass known as granuloma (3). The role of the granuloma is to control the growth of intracellular *Mtb* and to limit bacillary dissemination (figure 1) (3,4).

There are two TB-related conditions: latent tuberculosis infection (LTBI) and active TB disease (5). In LTBI, the macrophages form a granuloma that keeps the bacilli contained and under control (1,2,6). In this case, people are asymptomatic and cannot spread the infection. In some of these patients, especially in those who are immunosuppressed, the bacteria become active, multiply, and cause active TB disease (1,2). In this case, the immune system cannot keep the tubercle bacilli under control and the bacilli begin to multiply rapidly and are released when the macrophages die (5). This process can occur in different areas of the body, such as the lungs, kidneys, brain, or bone (1,5).

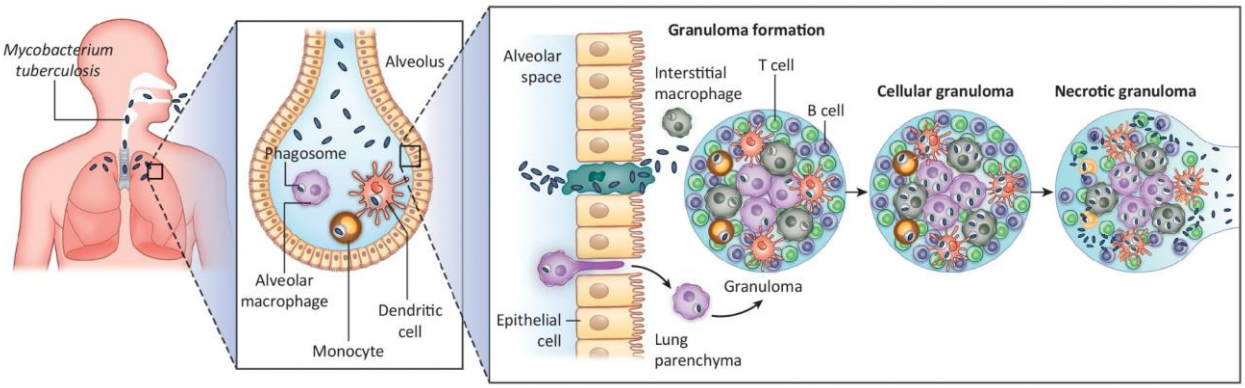

Figure 1: **Pathogenesis mechanisms of *Mycobacterium tuberculosis.*** Adapted from (7).

1.2 The problematic of drug-resistant tuberculosis: types of resistance and epidemiology

TB is one of the leading causes of death by infectious diseases and despite the effort put into treating TB, resistance to first-line drugs is a recognized public health problem. According to a report from the WHO, in 2020, an estimated 10 million people fell ill with TB worldwide and 1.5 million people died from TB in the same year (8). In 2019, Portugal had an incidence rate of 19 per 100.000 habitants and the global average of the incidence rate was 130 per 100.000 population.

Over the last century, TB incidence and mortality have been decreasing worldwide, due to medical advancements in the field such as the development of BCG, the first live-attenuated vaccine, and the discovery and use of anti-TB agents. However, drug-resistant TB is a major contributor to antimicrobial resistance worldwide and continues to be a public health threat. Drug resistance is a formidable obstacle to TB care and prevention globally, making it harder and longer to treat, often with poorer outcomes for patients (8,10). The World Health Organization (WHO) divides the different forms of TB resistance into five categories: isoniazid-resistant TB, rifampicin-resistant TB (RR-TB), multidrug-resistant TB (MDR-TB) which means that exists resistance to at least both isoniazid (INH) and rifampicin (RIF), two of the first-line drugs used to treat TB (8). Recently the WHO's Global TB Programme, highlighting the seriousness of these forms of TB, defined pre-extensively drug-resistant TB (pre-XDR-TB) for the first time, which implies fulfilling the definition of multidrug-resistant and rifampicin-resistant TB (MDR/RR-TB) plus resistant to any fluoroquinolone (8,9,10). On the other hand, the definition of extensively drug-resistant TB (XDR-TB) has been revised and implies a TB infection caused by *Mtb* strains that fulfill the definition of MDR/RR-TB and which are also resistant to any fluoroquinolone and at least one additional Group A drug (levofloxacin, moxifloxacin, bedaquiline and linezolid) (table 1) (9,10).

According to a report from the WHO, worldwide in 2019, close to half a million people developed RR-TB, of which 78% had MDR-TB (figure 2) (11). Globally, although treatment success rates have increased, almost 15% of MDR/RR-TB patients die from the disease, and 26% of those deaths are in patients with XDR-TB (12). In 2012, the treatment success rate for MDR/RR-TB was only 50% and in 2018 it rose to 59%, which demonstrates the potential of the newly approved drugs, however, this therapeutic success rate is still far below expectations (8).

The ambitious goal of WHO includes ending the TB epidemic by 2030. The "End TB Strategy" defines milestones (for 2020 and 2025) and targets (for 2030 and 2035) for the reduction of TB cases and deaths. The milestones for 2020 were a 35% reduction in the

number of TB deaths and a 20% reduction in the TB incidence rate. The strategy also included a 2020 milestone in that no TB patients and their households faced catastrophic costs as a result of TB disease. The targets for 2030 are a 90% reduction in the number of TB deaths and an 80% reduction in the TB incidence rate compared with the same levels in 2015 (8, 13).

Figure 2: Estimated incidence of multidrug- and rifampicin-resistant tuberculosis in 2019, for countries with at least 1000 incident cases. Adapted from (11).

1.3 Diagnosis

In cases where there are signs and symptoms consistent with TB, prompt clinical evaluation is essential to ensure a rapid diagnosis. This diagnosis is supported by clinical history and physical examination.

The definitive diagnosis of TB requires the isolation of the etiologic agent, and the gold-standard test is the isolation of *Mtb* in a cultural examination (14,15). Respiratory tract specimens, such as bronchial secretions, are the most frequently analyzed product when pulmonary TB is suspected (14,15). Despite its high sensitivity and specificity, it is a very time-consuming process, which may require several weeks to obtain a definitive result due to the slow growth of bacteria. However, it allows the identification of the mycobacterial species, testing antibiotic sensitivity and monitoring response to therapy (15).

Acid-fast bacilli smear microscopy is a rapid technique that plays an important role in the presumptive diagnosis of TB (15). This method is based on the characteristics of the cell wall (CW) of mycobacteria, which have a high content of lipids, the mycolic acids (MA), which makes them acid-fast organisms, remaining red at the end of Ziehl–Neelsen staining (15). This

only allows a presumptive diagnosis since other nontuberculous mycobacteria are also acid-fast organisms (15).

Another option for rapid diagnosis are nucleic acid amplification tests, the most common being the polymerase chain reaction (PCR) (15). These tests emerged to provide a faster result, between 24 and 48 hours, allowing an early diagnosis of TB and immediate initiation of therapy. However, it is still always necessary to obtain confirmation of the diagnosis through a cultural exam (15).

Drug resistance is suspected when after an adequate treatment regimen, the disease remains active and there is no improvement in symptoms, there is a worsening of the disease, or even when the patient in question had contact with another patient with drug-resistant TB.

Furthermore, the *Direção-Geral da Saúde* (DGS) establishes that drug susceptibility testing (DST) for first-line drugs must be performed in all cases of *Mtb* isolation, both in new cases and in previously treated patients (16-18). On the other hand, DST should also be repeated if a patient has positive cultures after three months of treatment, if they develop positive cultures after a period of negative cultures, or when MDR- or XDR-TB is suspected (16-18). Whenever cases of MDR-TB are identified, according to the circular of the DGS, DST must still be carried out on second-line drugs (17,18).

These diagnoses can be made using phenotypic or genotypic methods (19). The phenotypic methods evaluate the growth of an *Mtb* isolate in media containing an anti-TB drug to assess the minimum inhibitory concentration (MIC) (19). This assay is performed with a critical concentration of the antibiotic, which is defined as 'the lowest concentration of an anti-TB agent in vitro that will inhibit the growth of 99% of phenotypically wild-type strains of MTB complex' (19,20).

On the other hand, genotypic methods are faster, easier and safer since the manipulation of live mycobacteria is not necessary (19). However, the performance of phenotypic tests cannot be excluded. These methods detect specific genetic mutations associated with resistance to anti-TB drugs. The Xpert® MTB/RIF assay allows both the diagnosis and detection of RIF resistance and is one of the most used genotypic assays (20-22). Thus, more recently, the Xpert® MTB/RIF Ultra assay has emerged, which has been shown to have more sensitivity but slightly less specificity in diagnosing TB and equal capacity in detecting resistance when compared to its predecessor (23). Another type of genotypic assay widely used are the Line Probe Assays (LPAs), being widely used to detect mutations that confer resistance to RIF, INH, fluoroquinolones and injectable drugs (24). The LPAs together with the Xpert® MTB/RIF Ultra assay allow the detection of most of the mutations that cause the most frequently associated antibiotic resistance (19).

It is essential to be able to identify all the mutations that cause antibiotic resistance so that an adequate therapy can be instituted in patients with multiple resistances. However, none of the methods described above can do it when used alone. As such, *Mtb*'s Whole-Genome Sequencing (WGS) allows the identification of all known antibiotic resistance mutations for all classes of anti-TB drugs, including the newly approved antibiotics (25). It has already been shown that this contemporary method can generate results about a month earlier than traditional phenotypic methods, which is extremely important because the faster the proper treatment is started, the more favourable the prognosis will be (26). This method has been increasingly used in the investigation of TB outbreaks since it can also monitor the emergence of new mutations and minimize the empirical treatment of patients (27). However, the high cost of the WGS has proved to be the main barrier to its more widespread implementation (27).

2 Objectives

Currently, TB is the second leading cause of death from infectious diseases (after SARS-CoV-2) and the number of patients infected with *Mtb* strains that contain resistance is increasing. For this reason and because for many decades new drugs have not been approved for the treatment of resistant forms of this disease, the scientific community has shown itself to be very concerned about this public health problem.

The aims of this work include, but are not limited to, answering the following research questions:

- How does resistance appear in *Mycobacterium tuberculosis*?

- What are the main mutations responsible for causing resistance to each of the drugs?

- Which mechanisms can cause resistance to several drugs at the same time?

- Which therapeutic regimens are recommended to treat the different forms of resistant tuberculosis?

- What drugs are under development and in clinical trials for the treatment of drug-resistant tuberculosis?

- What treatment regimens could be approved in the future?

Furthermore, this monograph aims to evaluate the most recent progress in the area of tuberculosis treatment as well as to understand the future perspectives on the treatment of this infectious disease.

3 Methods

According to the objectives of this monograph, the research for its writing was carried out using several browsers such as PubMed, ScienceDirect and Google Scholar in which the most used keywords were: "tuberculosis", "*Mtb*", "resistance", "clinical trials", "treatment", "multidrug-resistant tuberculosis", "extensively drug-resistant tuberculosis". From this research, were selected several articles relevant to the topics to be addressed in the thesis and considering the date of publication.

Guidelines and reports from the World Health Organization (WHO), the Centers for Disease Control and Prevention (CDC) and *Direção-Geral da Saúde* (DGS) were also consulted, as well as some websites of important companies in the development of drugs and regimens against TB, such as the TB Alliance and the clinical trial database ClinicalTrials.gov, from the National Library of Medicine.

4 Evolution of tuberculosis treatment

Since the discovery of TB, by Robert Koch in 1882 (28), and until the first half of the 20th century, there were no drugs to treat this infectious disease since the antibiotics developed until then (penicillins and sulfonamides) did not demonstrate activity against *Mtb* (29). However, the discovery of streptomycin in 1943 brought hope for the treatment of TB, since until this time the current standard treatment was bed rest (30). The British Medical Research Council carried out the first clinical trial with this antibiotic, where patients aged 15 to 30 years old were selected to undergo treatment with streptomycin and compared with the control group (only on bed rest) (31). During the first three months of treatment, there was a decrease in mortality and radiological improvements in the group treated with streptomycin, however, in the subsequent six months, these differences were less pronounced (31). Mainly after the fourth month of treatment, it was possible to verify the beginning of the development of resistance to this new antibiotic, and it was realized that treatment with streptomycin alone would not be indicated to cure patients infected with TB, mainly due to the emergence of resistance due to the use of streptomycin alone (31,32).

In 1948 para-aminosalicylic acid (PAS) was developed (33), for which it was also found that its administration alone also led to the development of resistance (34). Subsequently, it was discovered that taking the two new drugs at the same time made the treatment more effective and reduced the development of resistance (35-37).

After the discovery of INH in 1952, a 24-month treatment regimen consisting of streptomycin, PAS and INH was instituted (37,38). However, the development of resistance to all these drugs was increasing and there was a need to develop more effective drugs (38). Later, in the 1960s, after the discovery of ethambutol (EMB), this new drug replaced PAS in the previous regimen and the regimen lasted 18 months (39,40). Subsequently, RIF was added to the treatment regimen, which lasted for 9-12 months (41,42) and in the 1980s streptomycin was replaced by pyrazinamide (PZA) (43) and this all-oral regimen lasts for 6 months and has been shown to be effective, with a cure rate of about 95% of patients, which is currently the most used regimen for the treatment of drug-susceptible TB (37,44-46). That is, the treatment of TB in a patient who does not have any type of resistance is divided into two phases: an initial phase, of attack, which lasts for 2 months in which treatment is carried out with INH, RIF, EMB and PZA, followed by a maintenance phase with only RIF and INH, which usually lasts for 4 months, but can be extended if cultures remain positive or in the presence of extrapulmonary TB (46). The discovery of all the drugs mentioned above is represented in figure 3.

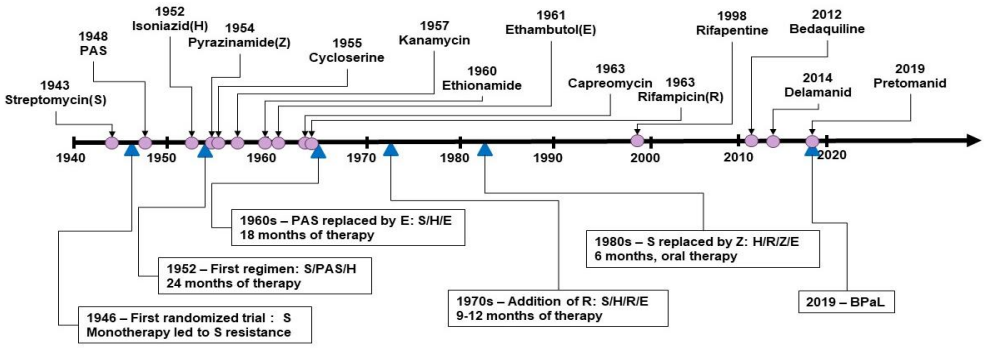

Figure 3: Pipeline with the discovery of drugs used in the treatment of tuberculosis (top) and main therapeutic regimens (bottom). Adapted from (47).

5 Mechanism of resistance

Generally, drug resistance is a major challenge for public health. However, in the case of TB, it may be more worrying, as there has not been much progress in this area in recent years. This is partly because, compared to other diseases, TB does not have such significant funding for research (48).

In TB, there are two main types of drug resistance: (i) primary or intrinsic resistance, which occurs when resistant strains are transmitted to a new host, and (ii) secondary or acquired resistance, which occurs through the acquisition of mutations that confer resistance to the drugs (49-51). In other bacteria resistance is generally mediated by horizontal gene transfer, however, in *Mtb* resistance occurs mainly through spontaneous genetic mutations (52). The mechanisms responsible for *Mtb* resistance to different antibiotics are essentially related to mutations in genes that encode drug targets or enzymes responsible for the activation of these drugs (49,50,53). Since the treatment is prolonged and, until recently, also composed of injectable drugs, more often leads to non-adherence to therapy which causes patients with susceptible TB or mono-resistant TB to rapidly progress to MDR- or XDR-TB. Although extremely effective drugs exist, *Mtb* is also able to resist these drugs through various mechanisms such as overexpression of efflux pumps, compensatory mechanisms, or cell envelope impermeability (51).

5.1 Drug-relatable resistance

5.1.1 RIF-resistant TB

RIF is a bactericidal agent and one of the most effective anti-TB drugs because it is active against metabolically-active replicating and non-growing bacilli (54). RIF binds to the β-subunit of the DNA-dependent ribonucleic acid (RNA) polymerase, interrupting the elongation of messenger RNA and consequently, inhibiting the RNA synthesis (55,56).

Most of the strains that are responsible for RR-TB have mutations in the *rpoB* gene, the gene that codes for the β-subunit of RNA polymerase (55,56). When mutated, this subunit will change conformation and decrease the affinity for RIF, therefore preventing the drug from binding to its target (56). About 96% of these resistance mutations are in an 81 base pair zone called the RIF resistance-determining region (RRDR), spanning codons 507-533 of the *rpoB* gene, that encode 27 amino acids (57-59). Mutations mainly occur due to amino acid substitutions, which lead to the conformational change in the *rpoB* gene described above, and the most common are at codons 516, 526 and 531 (56,60,61). Although less common, mutations can also occur outside the RRDR of *rpoB* genes (62).

5.1.2 INH-resistant TB

INH is a prodrug and unlike RIF, is only active against metabolically-active replicating bacilli (63,64). As a prodrug, INH is activated, intracellularly, by the mycobacterial catalase-peroxidase enzyme KatG, encoded by the *katG* gene (65). Once activated, INH is converted into isonicotinoyl, which binds to NAD^+ and $NADP^+$ to form the INH-NAD(P) complex, that inhibits InhA, a NADH dependent enoyl-ACP-reductase, a key enzyme in the synthesis of the MA, involved in the elongation of fatty acids in the MA synthesis (66), negatively affecting the CW integrity and leading to cell death (67-70).

The conversion of INH in its active metabolite also generates high levels of reactive oxygen species (ROS) that contribute to its high bactericidal activity (71).

The most frequent mutations responsible for INH resistance are associated with the *katG* and *inhA* genes or the promoter region of the *inhA* gene (50). However, the most common gene mutation has been identified as S315T in *katG*, where serine is replaced by threonine at codon 315, resulting in a reduced ability to activate INH (57,72). This mutation has been associated with high-level INH resistance (MIC of 5–10 µg/mL), due to the reduced catalase activity, and MDR strains (50,73).

Resistance to INH can also occur by mutations in the promoter region of *inhA* which cause overexpression of InhA, the most prevalent being found at position -15 (66,74). Less frequently, mutations can also occur in the *inhA* coding region, leading to a conformational change in InhA that reduces its binding affinity to the INH-NAD adduct (75). These mutations are associated with low-level INH resistance (MIC < 1 µg/mL) (66,76).

There has also been reported cross-resistance to a structurally related drug, ethionamide (ETH), once this drug shares with INH the same target, *inhA* (66,77). A previous study also reported that the presence of a mutation in the *inhA* regulatory region together with a mutation in the *inhA* coding region can lead to the development of high-level INH resistance and cross-resistance to ETH (78). These mutations, if isolated, generally cause low-level INH resistance. However, when both mutations exist, they lead to high-level INH resistance and cross-resistance to ETH (66,78).

5.1.3 Fluoroquinolone-resistant TB

According to the most recent recommendations from the WHO for the treatment of MDR-TB, a fluoroquinolone should be included in the treatment regimen, and the most used are moxifloxacin and levofloxacin (10).

Fluoroquinolones are potent bactericidal antibiotics that prevent transcription during cell replication. This class of drugs inhibits the DNA gyrase, also known as topoisomerase II,

and the topoisomerase IV, responsible for catalyzing DNA supercoiling (79), and for untangling newly replicated DNA and allowing the segregation of daughter chromosomes (80), respectively, being both essential for bacterial viability. *Mtb* only has DNA gyrase, which is the only target of fluoroquinolones (81). DNA gyrase is a tetramer consisting of two α subunits and two β subunits, encoded by *gyrA* and *gyrB*, respectively (82).

In *Mtb*, the main resistance mechanisms occur in a region known as the quinolone-resistance-determining region (QRDR) of *gyrA* or *gyr*B (82,83). Mutations in *gyrB* are rarer (84) and, therefore, the most frequent mutations are associated with *gyrA* being most commonly found at codons 90, 91, and 94, and aspartic acid 94 is generally the most frequently mutated (85,86). Higher levels of resistance exist when there are two mutations in *gyrA* or when there are simultaneously a mutation in *gyrA* and a mutation in *gyrB* (82,85).

In contrast, it has been demonstrated that the simultaneous presence of T80A and A90G mutations in *gyrA* leads to a hypersusceptibility to the action of several fluoroquinolones such as moxifloxacin and levofloxacin, with MIC up to 14-fold lower than the values for the wild type strains (87).

Although in most cases, there is cross-resistance between fluoroquinolones (88), the MIC of moxifloxacin seems to remain lower than the others (89,90), making it a particularly effective drug and one of the most recommended by the WHO (10).

Another mechanism responsible for causing resistance to this class of antibiotics is molecular mimicry, a mechanism used by *Mtb* to annul the action of fluoroquinolones. Mycobacterium fluoroquinolone resistance protein A (MfpA) closely resembles the DNA double helix, both in size and shape (91-93). Due to these similarities, it has been suggested that fluoroquinolones bind to MfpA in the cytoplasm of mycobacteria rather than to the DNA structure, allowing transcription to continue to occur (92,93).

5.1.4 Bedaquiline-resistant TB

Bedaquiline is the only drug approved for the treatment of TB that acts on energy metabolism (94-96). This drug targets mycobacterial adenosine triphosphate (ATP) synthase, inhibiting bacterial respiration (94-96). ATP synthase is found in the inner membrane of bacterial mitochondria and is composed of two domains: the F_0 domain located inside the membrane with three subunits (a, b2 and c10-15) and the F_1 domain located in the cytoplasm with five subunits (α3, β3, γ, δ and ε) (97-99).

The c10-15 subunit of the F_0 domain is arranged in the form of helices that, when rotating, will activate the β subunit of the F_1 domain, which has catalytic activity and combines adenosine diphosphate (ADP) with phosphates (Pi) to form ATP (97-99).

Bedaquiline can bind between the a and c subunits of the F_0 domain of ATP synthase, blocking the rotation of the helices and, consequently, the entire process of ATP synthesis (94,95).

Despite being approved by the Food and Drug Administration (FDA) only about ten years ago, and in Europe shortly thereafter, resistance to bedaquiline is emerging (100,101). A recent study reported the acquisition of resistance to bedaquiline in more than 15% of patients with MDR-TB taking this drug (102). This resistance is associated with mutations that occur in the *atpE* gene, the gene that encodes the c subunit of the F_0 domain of ATP synthase, being the most common mutations found at aspartate at position 28th and alanine at position 63rd, that are replaced by proline and valine, respectively (100,101). So, bedaquiline cannot exert its action on the mutated protein and, consequently, ATP synthesis cannot be interrupted.

However, in a study in which 53 bedaquiline-resistant mutants were analyzed, mutations in the *atpE* gene were only found in 15 of them, suggesting that there may be alternative mechanisms that confer resistance to this drug (103).

5.2 General mechanisms of drug resistance

5.2.1 Compensatory mutations

To resist anti-TB drugs, *Mtb* contains mutations that affect essential processes for the survival of bacilli. The bacteria can only survive by simultaneously possessing secondary mutations that act in a compensatory way, minimizing the consequences of the original mutations (104-106). These secondary mutations usually occur in genes or proteins involved in the affected mechanism (104,105).

In KatG-negative INH-resistant strains, the lack of KatG catalase-peroxidase activity does not allow INH activation. However, the expression of KatG is quite important for *Mtb* as it allows the protection of the bacilli against the toxic effects of organic peroxides (107). As such, compensatory mutations have been observed in the promoter region of *ahpC*, a gene that encodes an alkyl hydroperoxide reductase implicated in resistance to reactive oxygen intermediates (108). That is, this mutation leads to a hyperexpression of AhpC in the absence or decrease of the catalase-peroxidase activity, functioning as a compensatory mechanism and allowing the survival of bacilli (107,109).

Some studies demonstrate that in the presence of a mutation in the *rpoB* gene, in strains that are resistant to RIF, the *rpoA* and *rpo*C genes, which code for the α and β'-subunits of the RNA polymerase, respectively, act as compensatory mechanisms, thus allowing the growth of *Mtb* (110-112).

5.2.2 Efflux-mediated resistance

Efflux pump systems have the ability to expel drugs to the outside of the cells, being an obstacle for the treatment of TB since they are highlighted as a resistance mechanism in *Mtb* (113,114). When occurs an overexpression of efflux pumps, there is a greater expulsion of the antibiotic to the outside of the cell, where it cannot exert its effect (115,116).

In a study, MDR-TB strains were included and the expression of 20 efflux pumps as well as the most common mutations responsible for causing resistance to RIF and INH were evaluated (117). When comparing MDR isolates that had mutations with those that were wild type for *katG*, *inhA* and *oxyR-ahpC*, it was found that most of the efflux pump genes were overexpressed in the wild type isolates (117). A MDR isolate that had a mutation in the *rpoB* gene, a mutation that confers resistance to RIF, was also analyzed, but no mutation associated with INH was found, which suggests that INH-resistance may be caused only by the overexpression of efflux pumps (117).

There may also be cross-resistance associated with the overexpression of efflux pumps. An example of this, that has also been reported and studied, is the cross-resistance between bedaquiline and clofazimine, a drug whose mechanism of action is the release of ROS (100,118-120). The mutation most frequently associated with this resistance phenomenon occurs in *rv0678*, a gene that encodes for a transcriptional repressor of the MmpS5-MmpL5 efflux system, so mutations in this gene cause an increase in the expression of efflux pumps (100,119-121). Mutations in *rv0678* have been reported to lead to a 2 to 16-fold increase in the MIC of bedaquiline (100,120,122).

Simultaneous use of efflux pump inhibitors, such as reserpine, carbonyl cyanide, m-chlorophenylhydrazone and verapamil, has been shown to lead to an inhibition of this drug expulsion system, thereby decreasing the MIC and increasing the susceptibility of *Mtb* to the antibiotic (123,126). Verapamil, a calcium channel blocker, has shown promising effects as adjuvant therapy in the treatment of TB, since when administered to mice infected with a RIF-resistant strain of *Mtb*, it allowed a significant increase in the susceptibility to the anti-TB drug (127). More recently, it was shown that verapamil could also have similar effects in the treatment of bedaquiline-resistant strains, as it allowed an 8 to 16-fold decrease in the MIC (128). Also, the MIC of clofazimine decreases about 8-fold in the presence of verapamil (128), demonstrating the potential of this drug to increase the susceptibility of strains resistant to bedaquiline and/or clofazimine.

5.2.3 Drug degradation

Bacteria also degrade drugs as a survival mechanism. An example of this is what happens with β-lactam drugs in *Mtb*. This class of antibiotics exerts its effect by binding to and inhibiting penicillin-binding proteins (PBPs), proteins that are essential for the formation of the complex peptidoglycan (PG) network, interrupting bacterial CW synthesis and, consequently, leading to cell death (129). The presence of β-lactamases, enzymes that hydrolyze the β-lactam ring, contribute to the ineffectiveness and resistance of this class of drugs (129,130). Mycobacterial β-lactamases are less effective compared to other bacteria (129,130). However, the high impermeability of the *Mtb* CW causes β-lactams to penetrate slowly and, in this way, β-lactamases can perform their function effectively, inactivating this class of antibiotics (130,131).

Mtb encodes at least four β-lactamases, with BlaC being the most important and the most widely studied (132,133). Carbapenems, that are also β-lactams, are generally resistant to β-lactamases in other bacteria. However, BlaC from *Mtb* can also hydrolyze them, but this hydrolysis occurs slowly, allowing some carbapenems to be effective against these bacteria (134).

5.2.4 Drug modification

Mycobacteria can also chemically modify drugs, inactivating them and increasing *Mtb* resistance to antibiotics. An example of this is what happens to aminoglycosides, which despite being no longer recommended by the latest WHO's guidelines (10), were widely used for many decades to treat resistant forms of TB. *Mtb* secretes an acetyltransferase known as enhanced intracellular survival (Eis) that can inactivate aminoglycosides such as kanamycin. It has been shown in *in vitro* studies that Eis exerts its action through the acetylation of amine groups in this class of drugs (135,136). Houghton *et al.* demonstrated that this protein can also acetylate and inactivate capreomycin (137).

Eis has been shown to protect *Mtb* from host immunity by modulating autophagy, cell death, and inflammation through ROS inhibition (138,139).

5.2.5 Impermeability of cell envelope

The mycobacterial envelope is unique, and it has three major components: a conventional cytoplasmic membrane, a notable and atypical CW and an outermost layer, also called capsule in the case of pathogenic species (figure 4) (140).

The CW core structure, commonly mentioned as the mycolyl-arabino-galactan-peptidoglycan (mAGP) complex, is a giant tripartite, low permeable complex composed of a

very cross-linked and modified network of PG, a highly branched arabinogalactan (AG) polysaccharide and a characteristic long-chain of MA (140-142). Many drugs against *Mtb* target the mAGP complex, which constitutes an important barrier against the surroundings, protecting *Mtb* from dehydration, osmosis and drugs, much contributing to the inherent resistance to anti-TB agents (140). There is still a periplasmic space between the cell membrane and the PG, which also contributes to impermeability to antibiotics.

MA are formed by a long chain of fatty acids, (about 70 to 90 carbon atoms per chain, which is longer than normal), that are the main determinant for CW permeability since they form a highly hydrophobic barrier preventing the penetration of hydrophilic molecules (1140,143). Nonetheless, *Mtb* has porins, inserted in the outer membrane, responsible for the uptake of small molecules and nutrients, and some hydrophilic molecules can also cross through these porins, which can reduce the resistance to some anti-TB drugs (144). However, hydrophobic molecules, despite being able to cross, penetrate slowly due to the thickness and reduced fluidity of the mycobacterial CW (143).

Liu and Nikaido demonstrated for the first time in a mycolate-deficient strain of *Mycobacterium smegmatis* that could not correctly synthesize MA, an increase in absorption, and consequently in sensitivity, to RIF and erythromycin, two hydrophobic antibiotics, thus demonstrating the influence of the MA in resistance to anti-TB drugs (145).

Furthermore, there is a family of mycolyltransferases, also known as the antigen 85 complex (Ag85) which includes 3 proteins (Ag85A, Ag85B and Ag85C) involved in the coupling of MA and AG (146,147). With the deletion of these proteins, was demonstrated a decrease in the levels of trehalose dimycolate, an important component for the integrity of the CW, therefore increasing the sensitivity to antibiotics, suggesting that inhibitors of Ag85 may be an effective strategy for the treatment of TB (147).

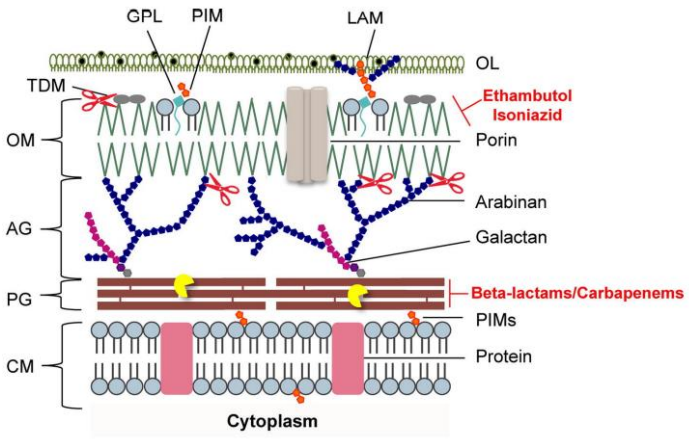

Figure 4: Schematic representation of the mycobacterial cell envelope. Adapted from (148)

The mycobacterial cell envelope includes a cytoplasmic membrane (CM), the mAGP complex and an outermost layer (OL). The mAGP complex includes the peptidoglycan (PG), the arabinogalactan (AG) layer and the outer membrane (OM), where the mycolic acids (MAs) are included. AG, arabinogalactan; GLP, glycolipids; LAM, lipoarabinomannan; PIMs, phosphatidylinositol mannosides; TDM, trehalose dimycolate.

6 Present of drug-resistant tuberculosis treatment

Treatment of drug-resistant TB should be done with at least four drugs, to improve efficacy and reduce the likelihood of additional resistance developing and whenever possible, resistance to the drugs used in the treatment regimen should be ruled out. It is important to strike a balance between the number of drugs needed for a treatment regimen to be effective and the likelihood of drug-drug interactions, pill burden and associated adverse effects. The WHO recommends four treatment regimens related to the type of resistance that exists and its guidelines are the most valued and used worldwide. The fact that all regimens currently recommended by the WHO are composed of generally well-tolerated and totally oral drugs will likely improve adherence to therapy and, consequently, treatment outcomes.

6.1 Regimen for isoniazid-resistant tuberculosis

In patients with confirmed RIF-susceptible and INH-resistant TB, it is recommended treatment with RIF, EMB, PZA and levofloxacin for 6 months (10). Treatment duration was determined based on the outcomes of patients who received treatment for six months or more than six months. Comparing both treatment durations it was found that the success rate was similar and no significant differences were found between both groups, so it was decided that the treatment regimen for six months was more favourable (10). Furthermore, it was found that the probability of the patient acquiring additional resistance to RIF was higher in patients treated with this regimen for more than six months (10). The duration of treatment with PZA was also evaluated due to its potential to cause hepatotoxicity, and it was found that when this drug is used for less than three months, there is a worse outcome, so it was decided to maintain its use for the entire regimen (10). However, patients treated with PZA should be tested each month for levels of aspartate aminotransferase, given the hepatotoxic potential of prolonged PZA use (10,154,155).

The previous recommendations did not include a fluoroquinolone in the regimen, however, treatment success rates were higher when fluoroquinolones were added to regimens (10,149). Therefore, adding a fluoroquinolone to the regimen can reduce the number of deaths and the probability of acquiring additional resistance with progression to MDR-TB (10). The fluoroquinolone recommended is levofloxacin for various reasons. It is the fluoroquinolone with the safest profile because is better characterized than the others, has fewer drug interactions in comparison to moxifloxacin, the other fluoroquinolone frequently used for TB treatment, and there are no contraindications for its use with antiretroviral agents, except lamivudine (10,150-153).

Additionally, in patients with INH-resistant TB, it is not recommended to add streptomycin or other injectable agents to the treatment regimen because streptomycin can decrease treatment success and does not reduce mortality significantly (10,156-158). It is also associated with dose-related adverse effects, in particular, ototoxicity and nephrotoxicity (10,156-158). Before starting this regimen, it is imperative that RIF resistance is excluded and, ideally, fluoroquinolone resistance should also be ruled out so that the treatment regimen is as effective as possible. Therefore, in patients where resistance to fluoroquinolones cannot be excluded or if resistance is known or suspected, the patient should withdraw the fluoroquinolone from the regimen and be treated with the remaining drugs for six months. The same applies during pregnancy or breastfeeding and in patients with prolonged QT interval (10). If the patient is found to be unresponsive to treatment, resistance to RIF and, if possible, to PZA and fluoroquinolones should be retested.

6.2 Shorter regimen for multidrug- or rifampicin-resistant tuberculosis

For patients with MDR/RR-TB, the WHO suggests an all-oral regimen of 9-12 months with bedaquiline during 6 months (400 mg once a day for the first 2 weeks, followed by 200 mg three times per week for 22 weeks), in combination with levofloxacin or moxifloxacin, ETH, EMB, INH (high-dose), PZA and clofazimine for 4 months (or 6 months if the patient remains sputum smear positive at the end of the fourth month), followed by 5 months of treatment with levofloxacin or moxifloxacin, clofazimine, EMB and PZA (figure 5) (10).

There are only included in this treatment regimen patients with confirmed MDR/RR-TB, with resistance to fluoroquinolones ruled out, exposure to previous treatment with second-line medicines used in this regimen for no more than one month, not pregnant, no severe extrapulmonary TB and/or extensive TB disease and, if, a child, aged less than 6 years (10). In patients also infected with the Human Immunodeficiency Virus (HIV), the taking of efavirenz and protease inhibitors should be avoided, because they lead to a decrease and an increase, respectively, in the concentration of bedaquiline (10,159-161).

There were some concerns about instituting a 9-12 month all-oral regimen containing bedaquiline to treat MDR/RR-TB cases. To address these concerns, this regimen was compared to a shorter regimen containing an injectable, a longer regimen containing bedaquiline, and regimens based on the WHO guidelines from 2016 also longer but without recently approved drugs (10). After analyzing the treatment success rates related to each of these regimens, it was found that the 9-12 month all-oral regimen containing bedaquiline was shown to have superior results and lower loss to follow-up (10). The same results apply to HIV-positive patients. Thus, this new treatment regimen, in addition to presenting better efficacy, is

a completely oral recommendation, which leads to greater adherence to therapy and better outcomes.

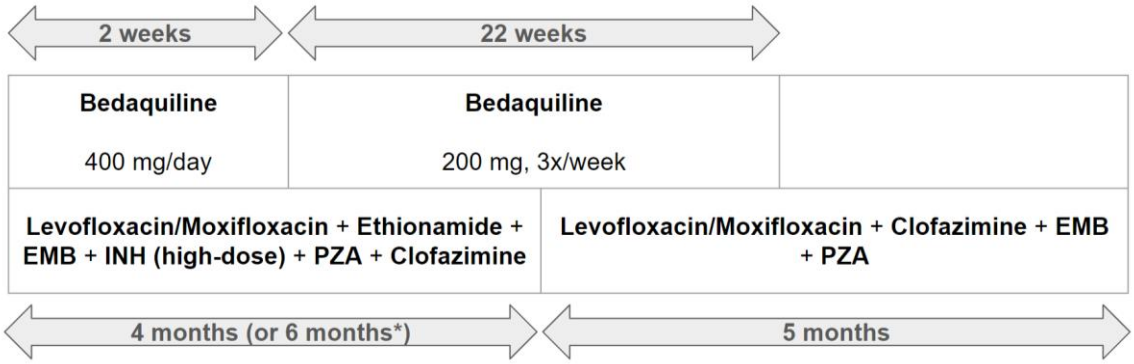

Figure 5: Shorter treatment regimen for MDR/RR-TB. Adapted from (10).

The regimen is composed of bedaquiline for six months, with 400 mg per day for the first two weeks and 200 mg per week for the next 22 weeks. Simultaneously, for four (or six) months, levofloxacin or moxifloxacin, ethionamide, ethambutol (EMB), high-dose isoniazid (INH), pyrazinamide (PZA) and clofazimine are also administered, and for the following five months only levofloxacin or moxifloxacin, clofazimine, ethambutol and pyrazinamide.

*if the patient remains with a positive culture at the end of the fourth month.

6.3 Longer regimen for multidrug- or rifampicin-resistant tuberculosis

For patients with MDR/RR-TB who have been exposed to treatment with second-line TB medicines including bedaquiline for more than one month, patients in whom resistance to fluoroquinolones has not been excluded, and patients with extensive TB or severe extrapulmonary TB, there is an alternative regimen also all-oral, but longer (10). This regimen lasts an average of 18-20 months, and it is composed of all three group A antibiotics (table 1) and at least one group B agent should be included (10). When it is required, group C drugs can be added to the regimen to complete it or when group A or B agents cannot be used, because this treatment should be started with at least four TB drugs plausible to be effective and finished with at least three if bedaquiline is stopped, since there are only safety studies for its use during six months (10,162). This division into three groups (A, B and C), was based on the relative benefits and risks associated with each of the agents, with group A antibiotics being considered effective and highly recommended for inclusion in the regimen of all patients, unless contraindicated for any reason (10). Group B agents are recommended as a second option and group C includes all other drugs that may be included when the regimen cannot be complete with only group A and B agents (10).

Regarding group C drugs, amikacin can be included in the regimen in patients over 18 years, but only after susceptibility has been demonstrated and the correct measures have been taken to monitor the possible emergence of adverse effects (10). If amikacin is not available, it can be replaced by streptomycin under the same conditions (10). According to the WHO guideline, clavulanic acid, an inhibitor of β-lactamases, should be included in the regimen as an adjuvant to carbapenems (imipenem-cilastatin and meropenem) and should be administered whenever carbapenem is administered (10). Like bedaquiline, delamanid, a drug in group C, can currently only be administered for six months (163).

On the other hand, linezolid, despite belonging to group A, has been associated with serious adverse effects such as peripheral neuropathy and myelosuppression, which may limit its use. However, its use for periods of less than six months is related to worse outcomes, which is why this drug should, whenever possible, be included in the treatment for as long as possible, since its use throughout the period of treatment showed very satisfactory results (10,164).

Table 1: Treatment groups for the longer regimen for multidrug- or rifampicin-resistant tuberculosis. Adapted from (10)

Groups	Steps	Medicine
A	Include all three medicines	Levofloxacin or moxifloxacin Bedaquiline Linezolid
B	Add one or both medicines	Clofazimine Cycloserine or terizidone
C	Add to complete the regimen and when medicines from groups A and B cannot be used	Ethambutol Delamanid Pyrazinamide Imipenem–cilastatin or meropenem Amikacin (or streptomycin) Ethionamide or prothionamide P-aminosalicylic acid

6.4 Regimen for multidrug-resistant TB with additional fluoroquinolone resistance

Lastly, for MDR-TB patients with TB that are also resistant to fluoroquinolones, it is recommended a 6-9 months regimen composed of bedaquiline, pretomanid and linezolid (BPaL) (10). However, can just be included in this regimen patients who have never been treated with bedaquiline or linezolid for more than two weeks (10,165). The BPaL regimen was studied in a phase III clinical trial, the Nix-TB study, in which 109 patients with MDR-TB intolerant or unresponsive to treatment or XDR-TB (pre-2021 classification) participated (165,166).

This study took place between 2015 and 2017 in South Africa and around 50% of the patients were also infected with HIV (165,166). The cure rate of the Nix-TB study was about 95% of the participants, exhibiting the great potential of this regimen, which is why it is now recommended by the WHO (10,165). Thus, the BPaL regimen is composed of bedaquiline 400 mg once a day for the first 2 weeks, followed by 200 mg three times per week, pretomanid 200 mg daily, which has a conditional authorization and can only be used in this regimen, and linezolid 1200 mg per day (10,162,165-167). The BPaL regimen can be extendable from 6 to 9 months for patients who remained culture positive or reverted from negative to positive culture between months 4 and 6 of treatment and for those who missed doses (10).

It is important to mention that this regimen is in the context of operational research only, since more data are needed regarding its safety and efficacy (10). As such, operational research intends to generate this evidence. Ukraine, which is one of the countries in the world most affected by XDR-TB, was the first country where patients started receiving this treatment under operational research conditions to understand how it works in clinical practice settings. Preliminary results showed that 93% of patients achieved sputum culture conversion after one month of treatment and of those, 40% in just two weeks, which demonstrates the great potential of this regimen (168).

The doses of the drugs mentioned above for the treatment of adults with drug-resistant TB are represented in table 2.

Table 2: Doses of drugs for treatment of adults with drug-resistant tuberculosis. Adapted from (169)

Group	Drug	Doses for Treatment of Adults
A	Levofloxacin	750-1000 mg daily
	Moxifloxacin	400 (600-800[a]) mg daily
	Bedaquiline	400 mg daily (2 weeks) + 200 mg M/W/F (22 weeks)
	Linezolid	600 (or 1200[b]) mg daily
B	Clofazimine	100 mg daily
	Cycloserine or Terizidone	500-750[c] mg daily
C	Ethambutol	15-25 mg/kg daily
	Delamanid	100 mg twice daily
	Pyrazinamide	25-30 mg/kg daily
	Imipenem-cilastatin[d,e]	1g + 1g twice daily
	Meropenem[d,e]	1g three times a day
	Amikacin[f]	15-20 mg/kg daily
	Streptomycin[f]	15 mg/kg daily
	Ethionamide or Prothionamide	15-20 mg/kg daily[g]
	P-aminosalicylic acid	4g two or three times a day
Other	Isoniazid[h]	4-6 (or 10-15[i]) mg/kg daily
	Pretomanid[j]	200 mg daily

Abbreviations: M/W/F: Monday/Wednesday/Friday; p.o.: *per os*

a: higher doses can be used if the strain has low-level resistance.

b: for the BPaL regimen.

c: doses of cycloserine can be administered twice daily if the patient has difficult to tolerate the entire dose once daily.

d: to be used with clavulanic acid.

e: only available for intravenous administration.

f: only available for intravenous or intramuscular administration.

g: can be given at bedtime or with a meal to reduce nausea. If the patient does not tolerate can start with two divided doses, until tolerance improves.

h: pyridoxine is given with isoniazid in patients at risk (e.g. with HIV or malnutrition).

i: higher doses can be used if the strain has low-level resistance or in some treatment regimens.

j: only to be used in the BPaL regimen.

7 Future perspectives of drug-resistant tuberculosis treatment

7.1 Treatment regimens in ongoing clinical trials

After many years without drugs being approved for the treatment of TB and with the growing problem of *Mtb* resistance, in the last 10 years, three oral drugs of great interest were approved - bedaquiline, delamanid and pretomanid -, which brought hope for the future of the treatment of resistant types of this infectious disease (10,170,171).

Bedaquiline is a diarylquinoline approved by the European Medicines Agency (EMA) in 2014 through a conditional marketing authorization and is only approved as 400 mg once daily for 2 weeks followed by 200 mg three times a week for 22 weeks. However, other dosages, as well as the duration of the treatment, are being studied in clinical trials (172,173).

Bedaquiline is generally well tolerated, although, some safety concerns related to QT corrected (QTc) interval prolongation have arisen (96,162,174). Delamanid was also associated with this adverse effect, however, studies have already been carried out to assess the prevalence and severity of this cardiovascular reaction where there were no severe forms of this side effect, concluding that both drugs are generally well tolerated, even when administered in the same therapeutic regimen (175). Nevertheless, bedaquiline analogs with fewer adverse cardiovascular effects are being developed (176,177).

Delamanid is a prodrug of the nitroimidazole class that received a conditional marketing authorization from the EMA also in 2014 (163). This drug gets activated by the enzyme deazaflavin-dependent nitroreductase, and its action consists of inhibiting two components of the CW: methoxy mycolic acid and ketomycolic acid (163,178). This drug is only approved for 100 mg twice a day for 24 weeks, and like bedaquiline, there are ongoing clinical trials to study other dosages and the duration of treatment (163,179,180).

Most mutations that confer resistance to delamanid occur in genes involved in the activation of the antibiotic or in the cofactor F_{420}, also essential for its activation, and most of these mutations confer cross-resistance to pretomanid (181).

Pretomanid is also a prodrug of the nitroimidazole class, with activity in *Mtb* in active replication and under hypoxic conditions. The main mechanism of action occurs under aerobic conditions, and it is only known that this drug can inhibit the synthesis of MA, leading to cell death (182,183). However, the mechanism by which this process occurs is yet to be discovered (182,183). Under hypoxic conditions, bacteria have limited oxygen and therefore are under

conditions of oxidative stress. In these situations, pretomanid is activated by a nitroreductase enzyme, which leads to the production of several active metabolites that are responsible for inducing the production of reactive nitrogen species, including nitric oxide, which, when accumulating, leads to cell death (183).

Pretomanid was approved by EMA in 2020 and can only be used in the BPaL regimen and under the conditions described above in this dissertation (section 6.4) (167). In the Nix-TB trial, great potential for this regimen was demonstrated with 90% of patients cured after 6 months of treatment (165). However, there is still a long way to go and there are some concerns related to the toxicity of this regimen since 81% of the patients in the Nix-TB study had peripheral neuropathy and 48% had myelosuppression as an adverse effect (165). These linezolid-related adverse effects required dose reduction or discontinuation of treatment with this drug, however, there appeared to be no impairment in the treatment efficacy (165).

As such, the ZeNix clinical trial, which runs at eleven medical centers across Eastern Europe and Africa, is testing a version of the BPaL regimen with a lower dose and shorter duration of linezolid to then determine whether the effectiveness of BPaL can be maintained but with fewer associated adverse effects, namely peripheral neuropathy and myelosuppression (184). This trial is composed of four treatment arms, in all of them the administration of bedaquiline and pretomanid remains the same as in the BPaL regimen, only changing the linezolid dosage (184). The first treatment arm maintained the dosage that had already been studied with 1200 mg of linezolid per day for 26 weeks, the second treatment arm maintained the 1200 mg of linezolid but only for 8 weeks and treatment arms three and four receive 600 mg of linezolid for 26 and 8 weeks, respectively (185). The most recent results of this clinical trial, published in July 2021, show that treatment arms two, three and four have similar efficacy and higher tolerability when compared to the currently recommended BPaL regimen, and may also be considered as options for future therapies (185).

Furthermore, SimpliciTB is an ongoing study in patients with susceptible TB (4 months of treatment), MDR-TB or mono-resistance to RIF or INH (6 months of treatment), in which a regimen formed by bedaquiline, pretomanid, moxifloxacin and PZA (BPaMZ) is being studied (186). For this clinical trial there are no published results yet, however in the phase 2b study that preceded SimpliciTB, this regimen was shown to have a greater bactericidal activity to treat RR-TB when compared to the 6-month standard regimen of INH, RIF, PZA and EMB to treat susceptible TB (187). For this reason, it is now also being studied in patients with INH-resistant TB and MDR-TB.

In animal models, the BPaL regimen and the BPaMZ regimen demonstrated significantly greater bactericidal and sterilizing activity when compared to the first-line regimens (188,189).

The TB-PRACTECAL study includes TB patients with at least resistance to RIF and, like to the previous ones, it has also a duration of 6 months and is composed of three investigational arms (190). The first treatment arm consists of the BPaL regimen plus moxifloxacin but with the linezolid dosage of 600 mg daily for 16 weeks and the last 8 weeks 300 mg daily or 600 mg three times weekly, the second treatment arm is similar to the first, but moxifloxacin is replaced by clofazimine and the third treatment arm is just composed by the BPaL regimen with the same change in the linezolid dosage (190,191). According to the available results, all treatment arms were shown to be effective when compared to the control arm in which patients were on WHO-recommended standard of care treatment for 9-24 months and some patients had injectable drugs in the regimen (192). The first treatment arm proved to be the most effective, with 89% of patients cured, compared to 52% in the control arm (192,193). Interestingly, it was also the treatment arm in which patients experienced fewer adverse effects (20%) compared to the control group (59%) (193).

7.2 New drugs in clinical development

7.2.1 Oxazolidinones

Sutezolid (PNU-100480) and delpazolid (LCB01-0371) are oxazolidinones and therefore belong to the same class as linezolid. These drugs exert their action by binding to the 23S ribosomal RNA of the 50S subunit and preventing the formation of a functional 70S initiation complex, thus inhibiting protein synthesis (194).

Sutezolid was found to have greater bactericidal activity when compared to linezolid, even at lower doses (195). Furthermore, the addition of sutezolid to regimens with first-line drugs (RIF, INH, and PZA) and moxifloxacin potentially improved the activity of these regimens, suggesting that this oxazolidinone may have the potential to shorten the duration of therapy (195). As discussed above, linezolid has been associated with side effects such as myelosuppression and peripheral neuropathy, which may lead to dose reduction or treatment interruption (165,184). Sutezolid at 600 mg twice daily or 1200 mg daily does not appear to have these adverse effects and is generally well-tolerated, safe and potentially more effective than linezolid (196-198). However, this drug continues to be studied at various dosages to better determine its effectiveness (199). A study is underway in patients with susceptible TB, and if this drug proves to be an asset, it may also be recommended for the treatment of drug-resistant TB (199).

Delpazolid is also being studied in clinical trials (200,201) after demonstrating *in vitro* efficacy against *Mtb* (202). The adverse effects generally caused by linezolid such as myelosuppression were also studied for this new drug, and it was possible to verify that patients did not present this symptom (203). Therefore, this drug is generally well tolerated and due to the rare associated serious adverse effects, it could be considered as a substitute for linezolid in longer treatment regimens, such as those usually associated with resistant forms of TB (203,204).

7.2.2 DprE1 Inhibitors

Decaprenylphosphoryl-β-d-ribose oxidase (DprE1) is a subunit of the enzyme decaprenylphosphoryl D-ribose epimerase, which produces decaprenylphosphoryl arabinose (DPA), a sugar donor essential for the synthesis of two important CW polysaccharides: arabinogalactan and lipoarabinomannan (205-207). Therefore, this enzyme is crucial for cell growth and survival of *Mtb*, as such it is a promising target for the development of new drugs such as benzothiazinones (BTZ), and it was the development of this class of drugs that led to the discovery of DprE1 (208,209). More recently, it was discovered that the location of this enzyme in the periplasm is crucial for it to be an excellent target for new drugs since the antibiotics do not need to enter the bacterial cytoplasm to exert their effect (205). Another advantage of their location is the fact that these drugs are not affected by other mechanisms responsible for causing resistance and decreasing susceptibility to drugs, such as the action of efflux pumps (205).

BTZ-043 is a drug from the BTZ class, discovered in 2009, capable of very effectively inhibiting DprE1 (208). After demonstrating activity against MDR and XDR-TB strains and superior activity to INH in mice, it is currently in a phase 2a clinical trial to assess safety, tolerability, pharmacokinetics, drug interaction and bactericidal activity (208-210).

Piperazine-containing benzothiazinones (PBTZ) such as macozinone (PBTZ-169) were later synthesized (211). This new compound when compared to BTZ-043 demonstrated higher efficacy, safety and potency in mouse and zebrafish models of TB (211-213).

Makarov *et al*. also demonstrated the existence of a marked synergy between PBTZ-169 and bedaquiline, which they suggest is caused by the better penetration of bedaquiline due to the decrease in the CW robustness provided by the inhibition of DprE1, or due to the inhibition of DprE1 there may be fewer reducing equivalents entering the electron transfer chain from $FADH_2$ (211). When PBTZ-169 was used simultaneously with bedaquiline and PZA, there was a rapid decrease in bacterial load, when compared with RIF, INH and PZA also in association (211). In a phase 2a study, macozinone was shown to be well tolerated and up to

a dose of 640 mg none of the participants experienced adverse effects considered serious (214).

Another drug capable of inhibiting DprE1 is OPC-167832, a carbostyril derivative (215). In *in vitro* studies, this compound exhibited a very low MIC for strains with mono-resistance to RIF, INH, EMB, PZA and streptomycin, demonstrating activity against *Mtb* at lower concentrations when compared to BTZ-043 and PBTZ-169 (215,216). Also, the association of this compound with delamanid, bedaquiline and moxifloxacin in mice showed very satisfactory results (180,215). Because OPC-167832 has shown such potential in *in vitro* and *in vivo* studies, this molecule is currently in phase 1b/2a clinical trials to assess its efficacy and safety in TB patients (180,215).

7.2.3 Diarylquinolines

After several decades without the approval of new drug classes, bedaquiline, a diarylquinoline, was approved and demonstrated to be effective and able to reduce the duration of TB treatment. However, some concerns have arisen regarding the prolongation of the QTc interval (217) - as it potently inhibits the cardiac human Ether-à-go-go-Related Gene (hERG) potassium channel -, the induction of phospholipidosis at higher doses, and the fact that, as it is a very lipophilic molecule (218), it has an extremely long terminal half-life (from 5 to 6 months), which can lead to its accumulation (219). In addition, highly lipophilic drugs are more likely to cause liver toxicity (220). For these reasons, and in order to avoid an accumulation of bedaquiline, after an induction phase of 400 mg per day, the remaining treatment is done only with 200 mg, three times a week (10,162). Despite the aforementioned disadvantages related to bedaquiline, this drug has been shown to be quite effective when associated with the BPaL (165) and BPaMZ (187) regimens, so the growing emergence of strains that have mutations in the *rv0678* gene and therefore reduced susceptibility to bedaquiline is of extreme concern.

For these reasons, it was necessary to develop bedaquiline analogues that are effective against bedaquiline-resistant strains, with less capacity to inhibit the hERG channel and less lipophilic, so that daily and safer ingestions can be possible.

Therefore, TBAJ-587 and TBAJ-876 were developed to combat these concerns (221-224). They have already been shown in *in vitro* assays to have greater potency and less ability to inhibit the hERG potassium channel when compared to bedaquiline, so there are possibly safer (176,177,224). Both drugs are now in phase 1 clinical trials (225,226).

Unexpectedly, when studying mice infected with a strain with the Rv0678 mutation and wild type mice, the BPaMZ regimen had similar bactericidal effects when compared to the same regimen without bedaquiline, indicating that the action of the diarylquinoline was not

affected by the mutation (176). Although further studies are needed, the authors suggest that this phenomenon may be due to the mechanism of action of PZA, which, through the interruption of the membrane potential, may reduce the function of the MmpS5-MmpL5 transporter (176,227).

In the same study, when replacing bedaquiline with TBAJ-587 in the BPaL and BPaMZ regimens, it was shown that, unlike bedaquiline, this recent analogue was effective against Rv0678 mutations in mice models (176). Furthermore, resistance to bedaquiline appeared in 100% of the mice treated with this drug alone, unlike TBAJ-587, which only provoked resistance in 5% of the mice (176). With the BPaL and BPaMZ regimens, the onset of resistance to bedaquiline was 60% and 20%, respectively (176). However, none of the mice treated with TBAJ-587 showed resistance (176). Also in *in vivo* trials, TBAJ-876 has been shown to be effective against *Mtb* strains containing the Rv0678 mutation (177). Therefore, in addition to being more effective and with fewer associated adverse effects, both TBAJ-587 and TBAJ-876 are also less likely to cause resistance, which could be very useful for the treatment of resistant forms of TB (176,177).

7.3 β-lactams to treat resistant-TB

β-lactams are the most prescribed class of antibiotics and have numerous clinical indications. They can be divided into five subclasses: penicillins, cephalosporins, monobactams, carbapenems and penems, sharing a four-sided β-lactam ring and differing in chemical composition and structure of the cyclic ring fused to the central β-lactam ring as well as in the composition of the side chains (228). These bactericidal antibiotics act by inhibiting bacterial CW synthesis by binding to PBPs, enzymes involved in the PG cross-linking. The formation of covalent bonds between the antibiotic and the PBPs inhibits the transpeptidase domain of the enzymes and prevents the cross-linking of PG, interrupting the synthesis of the CW, culminating in cell lysis (228).

The PG, as its name implies, is made of peptides and glycan strands (229). The glycan strands alternate between *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, linked in a β (1→4) configuration (229). Adjacent glycan strands are connected by short peptide stems with the sequence L-Ala-γ-D-Glu-meso-DAP-D-Ala-D-Ala and linked to the D-lactoyl residue of each MurNAc (229). Most bacteria form 4→3 bonds through D,D-transpeptidases, also known as PBPs, to cross-link PG monomers. However, in *Mtb*, about 80% of the bonds are 3→3 m-DAP-m-DAP interpeptide bridges (figure 6) formed by L,D-transpeptidases (Ldts), where most β-lactam antibiotics do not have any action, except for carbapenems (230,231). Thus, the therapeutic failure of β-lactams for the treatment of TB is explained by the non-reactivity of Ldts to most β-lactam antibiotics, as well as by the reactivity

of BlaC, an extended-spectrum β-lactamase, which is why these drugs are rarely used for the treatment of TB (134). However, this barrier can be overcome with the use of inhibitors of β-lactamases, such as clavulanic acid, or through the use of drugs resistant to the inactivation by BlaC, such as carbapenems (232,233). It has been shown that clavulanate is the only inhibitor of β-lactamases capable of irreversibly inhibiting BlaC, making *Mtb* susceptible to β-lactam antibiotics (232). As such, both solutions can be implemented by combining a carbapenem with a β-lactamases inhibitor (233). This association demonstrated bactericidal activity against *Mtb* in replicative phase and dormant forms, which led to the emergence of clinical trials that are exploring these associations (233,234).

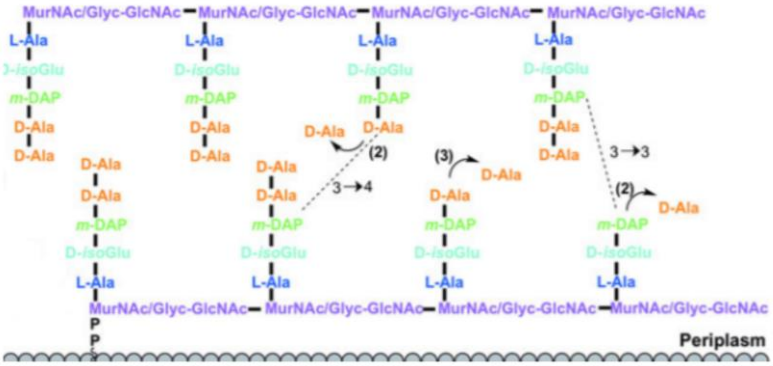

Figure 6: Schematic representation of mycobacterial peptidoglycan. Adapted from (236)

The glycan component consists of equimolar amounts of alternating residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues. The mycobacterial PG is of type A1γ, which means that the glycan strands are cross-linked by interlinked peptide branches of the sequence L-Ala-γ-D-Glu-meso-DAP-D-Ala-D-Ala. It is also represented the 3→3 m-DAP-m-DAP interpeptide bridges and the more typical 4→3 interpeptide bonds.

Meropenem, a potent carbapenem, was found to be a very weak substrate for BlaC, and its hydrolysis occurs very slowly, about five times slower when compared to ampicillin (233). The combination of this carbapenem with clavulanate is highly bactericidal *in vitro* (235). In a study with six patients infected with XDR-TB where all had a poor prognosis and there were no clinical or bacteriological improvements with standard treatment, after the introduction of meropenem-clavulanate into therapy there was a significant improvement and culture conversion after 8-20 weeks of treatment in five of the six patients (235). Furthermore, the long-term treatment was well tolerated, and no adverse reaction was attributed to meropenem-clavulanate (235). However, the small number of patients is one of the limitations of this study.

Despite this, the introduction of this carbapenem combined with clavulanate may be an asset for the treatment of TB. According to the WHO recommendations, meropenem is part of the group C of the longest regimen for the treatment of MDR/RR-TB and should always be administered together with clavulanate (10).

However, there are some concerns related to carbapenems, since although they are effective for the treatment of MDR/XDR-TB, the vast majority are administered intravenously, which can be a limitation in a clinical setting and decrease adherence to treatment. In addition, they are broad-spectrum antibiotics, and their persistent use can increase the risk of resistance to other microorganisms. Thus, the development and subsequent study of oral carbapenems such as tebipenem and biapenem may be advantageous, as these have also demonstrated activity against *Mtb* (237). In a study evaluating the *in vitro* activity of various carbapenems with or without β-lactamases inhibitors on clinical isolates of MDR/XDR-TB, tebipenem in combination with clavulanate was found to have the most potent activity (237). Subsequently, biapenem was also shown to be effective both *in vitro* and *in vivo* studies (238).

Given the urgent need for therapeutic alternatives for MDR/XDR-TB, the use of these new oral carbapenems could be advantageous, however, it is necessary to move towards further clinical trials as soon as possible, as β-lactams have already been shown to be beneficial, secure and easily accessible.

On the other hand, faropenem, a β-lactam of the penem subclass, is an orally administered antibiotic that appears to be more resistant to hydrolysis by BlaC when compared to carbapenems (239, 240). Furthermore, unlike meropenem, it does not appear to need to be administered with a β-lactamases inhibitor to be more effective, as its bactericidal activity is independent of clavulanate (240). This drug is already in phase 2 clinical trials which, despite being concluded, have not yet published the respective results (234, 239).

7.4 Host-Directed Therapies

It is increasingly understood how *Mtb* interacts with the host, which is why host-directed therapies (HDTs) have been studied and developed, which can be used as adjuvants to traditional antibiotic therapy, to reduce the duration of treatment and improve outcomes, prevent permanent lung damage and decrease the mortality rate of patients with other comorbidities (241). HDTs can be used to activate specific antimicrobial pathways or inhibit host molecular factors that are important for the intracellular survival of *Mtb*, making bacteria more sensitive to host defenses (242). Furthermore, some HDTs can be used to treat TB and other comorbidities simultaneously (241, 242).

An example of this is metformin, an oral antidiabetic drug approved for the treatment of type 2 diabetes, which has also been shown to be effective in the treatment of TB. Metformin can facilitate phagosome-lysosome fusion, reduce chronic lung inflammation and increase ROS levels (243, 244). On the other hand, mice models treated with standard anti-TB drugs and metformin revealed that this oral antidiabetic drug can also potentiate the effectiveness of traditional antibiotics for the treatment of TB, such as INH, since the animals treated with INH and metformin showed a marked decrease in bacillary load when compared to mice treated with INH alone (243, 244). Furthermore, diabetes mellitus exacerbates TB symptoms and diabetic patients are more prone to infections (245). This makes metformin even more beneficial in patients who have both comorbidities. Furthermore, it was shown that mice treated with metformin, when compared to untreated mice, had a higher number of $CD4^+$ and $CD8^+$ T cells, both crucial in the control of *Mtb* infection (244). Therefore, metformin can be an adjuvant agent to improve the treatment of TB, however, further studies, including randomized clinical trials, are needed.

On the other hand, simvastatin, used in the treatment of hypercholesterolemia, is also a promising drug against *Mtb*, as it has shown to be able to increase the bactericidal effects of first-line antibiotics such as INH, RIF and PZA (246, 247). Simvastatin promotes better elimination of *Mtb* through the activation of cellular immunity by stimulating the secretion of cytokines such as interleukin (IL)-10, IL-1β and IL-12. IL-10 is an anti-inflammatory cytokine that can mitigate the damage caused by excessive inflammation. IL-1β is a pro-inflammatory cytokine that plays a crucial role in host resistance against *Mtb* infection, which was demonstrated by Krishnan *et al.,* when IL-1β knockout mice infected with *Mtb* had a higher mortality rate as well as a higher bacterial load in the lungs (248). Finally, IL-12 contributes to increased macrophage production and stimulates the synthesis of interferon (IFN)-γ (249). Therefore, the increase in the production of these cytokines favours the inhibition of *Mtb* growth in infected cells. Furthermore, simvastatin promotes macrophage apoptosis and monocyte autophagy, which will support the formation of autophagosomes, and consequently, favour the elimination of the TB-causing bacillus (247). However, it should be noted that RIF induces simvastatin metabolism and, therefore, decreases the statin serum levels. In contrast, in association with INH, the metabolism of simvastatin is inhibited and therefore increases the risk of rhabdomyolysis and myopathy, which are already known adverse effects of statins (250).

Autophagy is considered part of the innate immune response and can be used by the host to restrict *Mtb* replication and survival. This self-digestion process depends on the maturation of phagosomes into phagolysosomes, which occurs through fusion with lysosomes and consequent degradation of the engulfed components (251). However, the bacterium that

causes TB has mechanisms to prevent autophagy and maturation of phagosomes, ensuring its survival. For example, PE_PGRS47 and Eis are mycobacterial proteins that prevent the initiation of autophagy, on the other hand, Esx-1 prevents the fusion with lysosomes by limiting autophagic flux (139, 252, 253). Since *Mtb* can decrease autophagy, the use of drugs that can trigger this mechanism can be extremely important to help fight *Mtb*, especially in more complicated infections. Rapamycin is the most studied drug capable of promoting autophagy, which makes it an interesting candidate as an adjuvant in the treatment of TB (254). Rapamycin is an immunosuppressant that inhibits the mammalian target of rapamycin (mTOR), a negative regulator of autophagy (254). However, its low solubility and long intracellular half-life complicate its use as a potential candidate for HDTs in TB (254). Therefore, other molecules are being studied, such as vitamin D, which has already been shown to increase the autophagic flux in macrophages and restrict the growth of the bacillus, being one of the molecules that is mostly found in clinical trials as adjuvant therapy in the treatment of TB (255, 256). Imiquimod has also been shown in *in vitro* assays to be able to trigger the production of mitochondrial ROS, directing autophagosomes to mitochondria and promoting the elimination of infected macrophages (257). Some anticancer drugs such as imatinib or nilotinib also demonstrate the potential to induce autophagy and, therefore, continue to be studied as possible options for HDTs (258, 259). Also, non-steroidal anti-inflammatory drugs and some anticonvulsants such as valproic acid and carbamazepine are being studied as they have also shown potential in the adjuvant treatment of TB (260-262).

Despite the urgent need for new treatments for TB, the repurposing of drugs as adjuvants in the treatment is a strategy that is gaining interest within the scientific community, although more clinical trials are needed to assess the benefit-risk balance as well as the side effects associated with the use of these drugs.

8. Conclusion and future perspectives

Despite the available antibiotic treatments and the BCG vaccine, TB remains a serious public health problem, especially in cases of resistance to the antibiotics most commonly used to treat this disease. Cases of drug-resistant TB have a worse prognosis and are of extreme concern.

This resistance can be intrinsic when an individual is infected with a strain that already has resistance or acquired when genetic mutations responsible for causing resistance occurs. However, *Mtb* manages to survive through the existence of second mutations that will minimize the consequences of the original mutations, preventing the bacterium from dying. In addition, there are still other resistance mechanisms such as the overexpression of efflux pumps, which will decrease the concentration of drugs inside the cells. Furthermore, *Mtb* has an atypical structure, mostly due to its complex CW, which contributes to the virulence of this pathogen, as it makes it difficult for antibiotics to pass through it.

Although, for a long period no drugs were approved for the treatment of this infectious disease, since 2012 three of the drugs that have brought hope were approved because they have shown to be very promising: bedaquiline, delamanid and pretomanid. However, in 2018 the treatment success rate of patients with MDR/RR-TB was only 59%, which was far below expectations, since the WHO has the ambitious goal of ending the TB epidemic by 2030.

The WHO defines four main therapeutic regimens related to the type of resistance present. One such treatment regimen, the BPaL regimen, includes newly approved drugs such as bedaquiline and pretomanid and is quite effective in treating MDR-TB with additional resistance to fluoroquinolones with a cure rate of about 95% in the clinical trial that led to its recommendation. Linezolid, used in this regimen, is associated with serious adverse effects such as peripheral neuropathy and myelosuppression, which is why a clinical trial with lower doses and/or duration of treatment with linezolid is ongoing. The first results of this clinical trial showed that, with lower doses of linezolid, it was possible to maintain efficacy and reduce the adverse effects associated. There are other ongoing clinical trials such as SimpliciTB or TB-PRACTECAL, in which the study treatment regimens may be approved in the future. There are still new drugs being studied in clinical trials that have also shown very promising results.

Another strategy that may prove to be relevant will be the use of drugs such as metformin or simvastatin, which are approved for other pathologies but have properties that allow them to make *Mtb* more sensitive to host defenses. They are particularly useful in patients who have the condition for which these drugs were initially approved.

Very recently, in May 2022, the WHO made a statement declaring what the main changes will be in the next guidelines for the treatment of drug-resistant TB. Through the ZeNix study, it was concluded that the linezolid dose recommended will be 600 mg daily, since this is the dose that allows better efficacy with fewer associated adverse effects, and there is still the possibility of dose reduction in cases of toxicity or poor tolerability. The BPaL regimen will be intended for patients with MDR/RR-TB or pre-XDR-TB and if patients respond slowly to the therapy, the regimen can be extended for an additional period of three months (263).

Furthermore, the BPaLM regimen, studied in the TB-PRACTECAL clinical trial, demonstrated favourable safety and efficacy and will also be recommended as a six-month regimen for patients with MDR/RR-TB or for those who have not been previously exposed to the regimen's drugs. Both of the above regimens can be administered to patients over 15 years of age, including HIV-positive patients (263).

To conclude, over the years and with the discovery of new drugs and regimens, including those still in clinical trials, it has been possible to verify a continuous improvement in efficacy and a decrease in the duration of treatment, which brings hope to the future of treatment of resistant forms of TB.

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