New antitubercular prodrugs with novel mechanisms of action

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Abstract

Tuberculosis (TB) is a contagious and deadly disease caused by *Mycobacterium tuberculosis* bacillus, which has reached pandemic proportions. In 2015, it was estimated globally that there were 10.4 million new cases of TB and 1.8 million deaths, 400,000 of whom were HIV-positive. In recent years, the control of TB is hampered by the emergence and the spread of *M. tuberculosis* drug-resistant strains (MDR-TB, XDR-TB, TDR-TB). Considering this extremely dangerous threat to public health, it is urgently needed the development of antitubercular compounds with novel mechanisms of action and new cellular targets.

Recently, in this direction, three new antitubercular promising prodrugs, the thienopyrimidine TP11126053 (TP053) and the thiophenecarboxamides 7947882 and 7904688, have been identified.

TP053, with an MIC in vitro of 0.125 μg/mL, is active against both replicating and non-replicating *M. tuberculosis* bacilli. By microbiological, genetic and biochemical studies, it was characterized as a prodrug activated by the reduced form of the DsbA-like mycoredoxin Rv2466c.

7947882 and 7904688, both with an MIC value of 0.5 μg/mL, emerged from the phenotypic screening of a National Institute of Allergy and Infectious Diseases (NIAD) set of chemical compounds. They showed an efficient inhibitory activity against *M. tuberculosis* H37Rv in its growing, latent and intracellular conditions. With a multidisciplinary approach, 7947882 and 7904688 were characterized as prodrugs activated by the monooxygenase EthA that affect the CTP synthetase PyrG. The active metabolite of 7947882 was identified and characterized as direct inhibitor of PyrG and PanK.

In this work we studied in depth the mechanism of action and of resistance of these three prodrugs.

Firstly, it was demonstrated that the *M. tuberculosis* Rv0579 protein is involved in the mechanism of resistance to TP053 by microbiological and biochemical approaches, even if its physiological role still remains unclear.

In addition, the mechanism of action of TP053 was clarified by metabolomic, meta-proteomic, biochemical and microbiological
methodologies. In fact, the activated compound globally affects the mycobacterial growth by NO release mainly affecting the protein synthesis.

For the last two prodrugs, 7947882 and 7904688, it was demonstrated that the pantothenate kinase PanK represents their second target, as well as PyrG. Consequently, these molecules are two multitargeting compounds targeting PyrG and PanK. In order to find further multitargeting compounds, it has been developed a phenotypic target-based screening leading to the identification of two further promising agents, affecting both targets.

Finally, two putative active metabolites of 7904688 have been identified by a metabolomic approach and their characterization is still under investigation.
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**Abbreviations**

ATP: Adenosine diphosphate  
CoA: Coenzyme A  
CTP: Cytidine triphosphate  
**DR-TB:** Drug-resistant tuberculosis  
EMB: Ethambutol  
**EPTB:** Extrapulmonary TB  
ETH: Ethionamide  
FDA: Food and Drug Administration  
HIV: Human immunodeficiency virus  
INH: Isoniazid  
LPZ: Lansoprazole  
**MDR-TB:** MultiDrug-Resistant Tuberculosis  
**MIC:** Minimal Inhibitory Concentration  
**NADH:** Nicotinamide adenine dinucleotide  
**NADPH:** Nicotinamide adenine dinucleotide phosphate  
OD: Optical Density  
PAS: *para*-Amino salicylic acid  
**PBTZ:** Piperazine-containing benzothiazinone  
**PTB:** Pulmonary TB  
PZA: Pyrazinamide  
**RIF:** Rifampicin  
**ROS:** Reactive Oxygen Species  
**SDS-PAGE:** Sodium Dodecyl Sulphate-PolyAcrilamide Gel Electrophoresis  
**TB:** Tuberculosis  
**TDR-TB:** Totally Drug-Resistant Tuberculosis  
**UTP:** Uridine triphosphate  
**WHO:** World Health Organization  
**XDR-TB:** Extensively Drug-Resistant Tuberculosis
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1. Introduction

1.1 Mycobacterium tuberculosis: an ancient enemy

Tuberculosis (TB) is a contagious and lethal infectious disease caused by *Mycobacterium tuberculosis* bacillus. This pathogen is a Gram-positive rod-shaped bacterium without flagella and capsule that does not produce spores and toxins (Ducati *et al.*, 2006). This aerobic bacillus has a width of 0.3-0.6 μm and a height of 1-4 μm; it is characterized by a complex cellular envelope, markedly slow growth and genetic homogeneity (Cole *et al.*, 1998).

*M. tuberculosis* cells spread from person to person through the air and the disease usually affects the lung (pulmonary TB, PTB); in 10-42% of cases TB can also affect other parts of the body such as brain, intestines, kidneys, bones or the spine (extrapulmonary TB, EPTB). Ethnic background, age, sex, immune status, presence or absence of other diseases and genotype of *M. tuberculosis* strain represent the main determinants of TB disease (Zaman, 2010; Caws *et al.*, 2008).

*M. tuberculosis* infection can result in active tuberculosis or, in more than 90% of cases, in latent tuberculosis, a condition in which infected individuals are asymptomatic and non-contagious (Zumla *et al.*, 2013b; Matteelli *et al.*, 2017). Latent TB affects about 2 billion people globally and the risk of developing the active disease is 5-15% within 2-5 years after initial infection, depending on microbial, host and environmental factors (WHO, 2016; Getahun *et al.*, 2015). In particular, *M. tuberculosis* infection can result in the elimination of the pathogen, either because of innate immune responses or because of acquired T cell immunity. Some individuals retain a strong memory T cell response and, if the pathogen is not eliminated, bacteria persist in a quiescent or latent state. Patients with active TB disease present symptoms such as cough, fever and weight loss (Pai *et al.*, 2016; Fig. 1). The TB latent form represents the main resource of new cases, considering mostly low burden countries, and, consequently, it is the main target to strike in order to fight the disease (Matteelli *et al.*, 2017).


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![Image]

**Figure 1. M. tuberculosis infection.**
A. latent infection; B. Active disease (Pai et al., 2016).

TB is an ancient plague that probably emerged about 70 thousand years ago and that has afflicted humankind through different historical ages, reaching pandemic proportions in Europe and North America during 18th and 19th centuries (Daniel, 2006; Comas et al., 2013).

Thanks to the introduction of the chemotherapy in the mid-20th century, the number of deaths caused by TB underwent an extreme reduction, which remained almost stable for many years (Ducati et al., 2006). However, the World Health Organization (WHO) declared TB as a global emergency in 1993, recognizing it as the single infectious pathogen responsible for most human deaths (Almeida Da Silva and Palomino, 2011; Ducati et al., 2006). Several anthropic factors promoted the return of TB (the so-called “TB resumption”), such as: the HIV/AIDS co-infection; the emergence of drug-resistant *M. tuberculosis* strains; the degradation of health care systems; the aging of population; the increase of international
immigration; and the changes in social structures (Fäkteneuer et al., 1999). For Europe and North America, *M. tuberculosis* represented a re-emerging pathogen, whilst for Brazil, South-East Asia and Africa was much more a long-lasting public health problem (Ruffino-Netto, 2002).

### 1.2 Global epidemiology of tuberculosis

TB is still one of the top ten causes of death worldwide (Dheda et al., 2016). According to WHO, in 2015 *M. tuberculosis* killed 1.4 million people and 0.4 million HIV-positive people and it was estimated globally that there were 10.4 million new TB cases (Fig. 2). Among the new cases, the 56% were estimated to be men, 34% women, 10% children and 1.2 million people were HIV-positive (Fig. 3) (WHO, 2016). Most of new TB cases have focused in Asia (61%) and African Region (26%); in particular, India, Indonesia, China, Nigeria, Pakistan and South Africa represent the six countries with the biggest number of new cases in 2015. The remaining 13% of new cases occurred in the Eastern Mediterranean Region (7%), Europe (3%) and the Region of Americas (3%) (WHO, 2016).

The cost of TB chemotherapy, the striking spread of drug-resistant TB and the impact of HIV co-infection highlight the need to increase TB prevention and control (Glaziou et al., 2015). Global targets for the reduction of the burden of TB have been set up for the period 2016-2035 and are reported in the Sustainable Developmental Goals (SDGs) and in the WHO’s End TB Strategy. First goals to achieve by 2020 are the following: an increase of 4-5% of the decline of TB incidence rate (the rate of TB incidence decreased only by 1.5% from 2014 to 2015) and a reduction of 10% of the global TB mortality. Looking at 2035, the targets are the reduction of 90% of TB deaths and the reduction of 80% of TB incidence (10 new TB cases per 100.000 population per year), compared with 2015 data (WHO, 2016).
Figure 2. Estimated TB incidence in 2015 (WHO, 2016).
Figure 3. Estimated HIV prevalence in new TB cases (WHO, 2016).
1.3 Current antitubercular therapy

The antitubercular therapy, in order to be effective, has to fulfill its precise tasks: to avoid the risk of developing complications and of relapse of the disease; to prevent the mortality and the diffusion of *M. tuberculosis* drug-resistant strains. To overcome these challenges, the therapy is characterized by two essential phases: an induction phase, which aims to drastically reduce the mycobacterial infection; and a consolidation phase, which aims to completely eradicate the disease (WHO, 2010; Al Matar *et al.*, 2017).

The WHO classifies the current antitubercular drugs as first-line, second-line and third-line drugs, relying mainly on availability and efficacy (Lalloo and Ambaram, 2010).

The **first-line drugs** are bactericidal and are characterized by a high efficacy degree and a relative low toxicity. They include: isoniazid, rifampicin, pyrazinamide and ethambutol (Fig. 4).

The **second-line drugs**, instead, are bacteriostatic, generally low efficient and more toxic. They include: fluoroquinolones, streptomycin, ethionamide, *para*-aminosalicylic acid, aminoglycosides, and cycloserine (Fig. 5).

Lastly, the **third-line drugs** (such as linezolid, thiacetazone, thioridazine, rifabutin and nitroimidazoles) are still undergoing clinical development, are more toxic and more expensive (Lalloo and Ambaram, 2010; Goodman *et al.*, 1996).
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Figure 4. First-line antitubercular drugs (Chetty et al., 2017).

Figure 5. Second-line antitubercular drugs (Chetty et al., 2017).
1.3.1 First-line antitubercular agents

Isoniazid (INH) is one of the most important drugs used in tuberculosis treatment and is highly effective against several species of Mycobacterium genus, including M. tuberculosis and Mycobacterium bovis. It has a bactericidal action against mycobacteria in fast-growing phase, while it has a bacteriostatic action against mycobacteria in slow-growth or latency phase (Fernandes et al., 2017; Global Alliance for TB Drug Development, 2008). Pyrazinamide (PZA) is a unique antitubercular drug, introduced in the early 1950s, able to kill the most persistent non-replicating mycobacteria. For this peculiarity, it is essential to shorten TB therapy and for drug combination both in drug-sensitive and drug-resistant TB (Zhang et al., 2014).

These two first-line compounds will be discussed in detail in the section “Old antitubercular prodrugs”.

Rifampicin (RIF) is a bactericidal compound, classified as a lipophilic ansamycin, introduced in 1972. It has a very efficient antimicrobial activity, inhibiting both replicating and non-replicating M. tuberculosis bacilli, and, therefore, together with isoniazid, is considered the basis of short-course TB treatment (Rattan et al., 1998). Rifampicin targets the β-subunit of RNA polymerase, physically blocking the transcription of mRNA. Mutations in 81 bp region of rpoB gene, encoding the β-subunit of RNA polymerase, are responsible for more than 97% of cases of RIF-resistance (Goldstein, 2014; Telenti et al., 1993).

Ethambutol (EMB) is a bacteriostatic agent introduced for the first time in TB treatment in 1966, used in case of active M. tuberculosis infection. It inhibits the arabinosyl transferase, an enzyme involved in the polymerization of arabinogalactan (AG), which is an essential component of mycobacterial cell wall (Yakrus et al., 2016; Sreevatsan et al., 1997). Structural changes in EMB-binding site of arabinosyl transferase, due to mutations in the coding embB gene, are the main mechanism of resistance to this antitubercular drug (Almeida Da Silva and Palomino, 2011).

1.3.2 Second-line antitubercular agents

Ethionamide (ETH) is an oral agent recommended for the treatment of M. tuberculosis multi-drug resistant (MDR) strains and is the most frequent drug used for pediatric TB therapy (Laurenzo and Mousa, 2011; Thee et al.,
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2011). As INH and PYR, ETH will be discussed in detail in the section “Old antitubercular prodrugs”.

Streptomycin was the first antibiotic used to cure TB, introduced in clinical practice in 1948. It is an aminocyclitol glycoside, extracted from the soil bacterium *Streptomyces griseus*. Streptomycin binds to 16S rRNA, blocking the initiation of protein translation and, consequently, inhibiting the protein synthesis (Chetty et al., 2017). Mutations in two genes, *rrs* and *rspL*, are responsible for the majority of cases of streptomycin-resistance (Sreevatsan et al., 1996; Almeida Da Silvia and Palomino, 2011).

Fluoroquinolones, defined as fluorine-containing nalidixic acid derivatives, were introduced in TB treatment regimen in 1980s. Their target is the mycobacterial type II topoisomerase (DNA gyrase), a heterotetrameric enzyme, that catalyzes the supercoiling of DNA. The subunits of DNA gyrase are encoded by *gyrA* and *gyrB* genes (Drlica, 1999; Takiff et al., 1994). Due to their high bactericidal activity and limited side effects, the use of fluoroquinolones has increased in recent years. However, this has led to a simultaneous increase in fluoroquinolone-resistance (Ginsburg et al., 2003). Mutations in the so-called quinolone resistance-determining region (QRDR) of *gyrA* and *gyrB*, which lead to amino acid substitutions in the DNA binding site of the gyrase, have been associated with fluoroquinolone-resistance (Almeida Da Silva and Palomino, 2011; Takiff et al., 1994).

*para*-Amino salicylic acid (PAS) was the first antimicrobial agent used in a combinatorial drug regimen, in association with isoniazid and streptomycin. Its mechanism of action has never been fully clarified. It was proposed that PAS is a prodrug that acts as a competitive inhibitor of dihydropteroate synthase, a fundamental enzyme in folate synthesis. However, 37% of *M. tuberculosis* PAS-resistant strains showed mutations in *thyA* gene, which encodes the thymidylate synthase A, suggesting the existence of different mechanisms of action for this anti-TB drug (Rengarajan et al., 2004; Zheng et al., 2013).

Kanamycin, amikacin and capreomycin are second-line drugs used in the treatment of MDR-TB. Kanamycin and amikacin are aminoglycoside agents, while capreomycin is a cyclic peptide. Even if they are classified in different antibiotic families, they inhibit the protein translation and share a common mechanism of resistance, which has been associated with an
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A1401G mutation in the ribosomal RNA 16S rrs gene (Sowajassatakul et al., 2014; Almeida Da Silva and Palomino, 2011).

Cycloserine is an analogue of D-alanine that exerts its inhibitory activity on two main targets: the D-alanine:D-alanine ligase (Ddl), an enzyme involved in the biosynthesis of peptidoglycan; and the D-alanine racemase (Alr), which converts L-alanine into D-alanine, the substrate of Ddl. The mechanism of resistance to this drug has been attributed to the overexpression of alr gene (Zhang, 2005; Desjardins et al., 2016).

1.3.3 Tuberculosis treatment regimen: certainties and new challenges

The drug treatment currently recommended by WHO is at least 6-month long, with a risk of relapse of 5-8%. Relapse can occur within the 12 months following the end of the therapy, indicating that TB was not completely eradicated (Tuberculosis Trials Consortium, 2011; Nunn et al., 2010). The standard treatment of 6 months consists of 2 months of induction phase with the four drugs isoniazid, rifampicin, pyrazinamide and ethambutol, followed by 4 months of consolidation phase with at least isoniazid and rifampicin. This kind of therapeutic regimen is adopted for cases of pulmonary TB and for most of cases of extrapulmonary TB. For latent TB, instead, a preventive treatment of at least 6 months with the only administration of isoniazid is required to kill the non-replicating M. tuberculosis bacilli (WHO, 2010; Zumla et al., 2013a).

In case of HIV-associated TB, there is the high risk of reactivation of latent TB and the rapid spread of drug-resistant TB, with consequent high mortality rates (Swaminathan et al., 2010). Optimal treatment regimen requires the simultaneous administration of antitubercular and antiviral agents, minimizing drug-drug interactions and overlapping toxicity. Unfortunately, currently available combinations are limited (Breen et al., 2006). For instance, rifampicin is a strong inducer of the hepatic cytochrome P450 (CYP) responsible for the metabolism of several xenobiotics. Among them, the protease inhibitors (PIs) and the non-nucleoside reverse transcriptase inhibitors (NNRTIs) represent two of the main classes of drugs used in the highly active antiretroviral therapy (HAART). Thus, the CYP induction by rifampicin can cause sub-therapeutic plasma concentrations of the antiviral agents, particularly of PIs. An alternative to rifampicin can be
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the rifabutin, which has similar efficacy, but less effect on CYP induction (Breen *et al.*, 2006; Narita *et al.*, 2000; Gonzalez-Montaner *et al.*, 1994).

However, TB therapy has to face several issues: drug intolerance and toxicity, which lead to treatment suspension; drug-drug interactions mostly with antiretroviral therapy (ART) in HIV-positive people; adherence to antitubercular regimen, considering its long duration (Zumla *et al.*, 2013a).

Drug-resistant TB (DR-TB) is another big challenge to the TB global control and is a major public health problem in several countries (WHO, 2015). Globally, in 2015 there were an estimated 580,000 new cases of multi-drug resistant TB (MDR-TB) (Fig. 6) and, among these, the 9.5% were extensively-drug resistant TB (XDR-TB) ones (WHO, 2016). Moreover, in the last few years some totally-drug resistant TB (TDR-TB) cases have been reported (Brouqui *et al.*, 2013; Velayati *et al.*, 2009).

WHO defines MDR as *M. tuberculosis* strain resistant to isoniazid and rifampicin, the two main first-line drugs.

XDR is defined as MDR strain resistant also to any fluoroquinolone and at least one of the three second-line injectable drugs kanamycin, amikacin and capreomycin.

Lastly, TDR is defined as strain resistant to all available first-line and second-line drugs (Caminero *et al.*, 2010; Udwadia *et al.*, 2012).
Figure 6. New MDR-TB cases in 2015 (WHO, 2016).
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The strategies for the treatment of DR-TB are based on systemic review and analysis of the best data available (WHO, 2015; Falzon et al., 2011). Before starting a therapeutic treatment, suspected DR-TB cases should be proved using the drug-susceptibility testing: this is a crucial step to decide whether to choose a standardized or an individualized treatment. A standardized treatment is based on the data from representative patient populations. Instead, an individualized drug treatment is designed both on the result of the drug susceptibility testing and on the history of TB treatment of the patient (Zumla et al., 2015).

For the new MDR-TB cases, the recommended drug regimen is characterized by an intensive phase of at least 8 months and by a consolidation phase of 12 months. In presence of previous MDR treatment, the antibiotic therapy lasts 18 months. Anyway, the duration of the therapy should be modified according to the response of the patient to the treatment. The regimen should include at least four potential active second-line drugs (such as late-generation fluoroquinolones and the injectable aminoglycosides) and first-line drugs to which the M. tuberculosis isolate is susceptible (Zumla et al., 2015; Zumla et al., 2013a). Unfortunately, the successful rate of MDR therapy is only of 65%, mainly because of the severe side effects of the used drugs and, consequently, the lower patient adherence (Weiss et al., 2014).

The regimen for XDR-TB treatment has not been yet determined because it depends on several factors, such as the condition of the immune system of the patient, the drug-resistance profile of M. tuberculosis isolate and the kind of tissue damage (Zumla et al., 2015). Generally, XDR-TB required a longer therapy than MDR-TB and the used of third-line drugs, which have several side effects and are more expensive than the other available drugs (Zumla et al., 2013a). Sometimes, a strong therapy can lead to a cure rate of 60% for XDR-TB in HIV-negative patients. Unfortunately, the mortality rate in patients co-infected with HIV can reach the 100%, if the diagnosis of XDR-TB does not occur in a short period (Jacobson et al., 2010; Dheda et al., 2010).

It is noteworthy that in order to fight tuberculosis there is an urgent need of new antitubercular drugs that must have the following features:

- to reduce the duration of the therapy;
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- to minimize the side effects in order to increase the patient’s adherence;
- to have novel mechanisms of action in order to be active against DR-TB;
- to avoid the drug-drug interactions in HIV-positive patients (Koul et al., 2011).

1.4 Prodrugs

Prodrug design is a promising and well-known molecular modification strategy used to optimize the physicochemical, pharmacological and pharmacokinetic properties of drugs (Jornada et al., 2015). This strategy, exploited both in drug discovery and in development, aims to solve the possible undesirable drug features like low solubility, low selectivity, chemical instability, toxicity and, consequently, to improve the absorption, distribution, metabolism and excretion (Huttunen et al., 2011; Stella, 2010).

The term “prodrug” was first introduced by Adrien Albert in 1958 to describe a poorly active or an inactive compound that undergoes chemical or enzymatic biotransformation in vivo to release the parent drug as a pharmacologically active molecule at efficacious level (Albert, 1958; Zawilska et al., 2013).

However, the history of prodrugs began in the second half of 19th century. The first two prodrugs introduced in medical practice were the acetanilide by Cahn and Hepp in 1867 and the phenacetin by von Mering in 1887. They are two analgesic drugs, whose prodrug nature was determined only later on (Zawilska et al., 2013).

Schering, instead, was the first to synthesize intentionally a prodrug, the methenamine, in 1899. Methenamine is an antibacterial agent used in the treatment of urinary tract infection, which releases formaldehyde and ammonium ions in urine acidic environment (Testa, 2004; Stella et al., 2007).

In the same year, aspirin was introduced in clinical use as an anti-inflammatory agent, in substitution of the more irritating salicylic acid by Bayer. Aspirin is an acetyl derivative of salicylic acid and remains subject of debate in scientific world: it is an irreversible inhibitor of cyclooxygenase, the key enzyme for formation of prostaglandins and thromboxanes, but the salicylic acid is a weak reversible inhibitor of the
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The aforementioned enzyme. For this reason, aspirin cannot be classified as a prodrug. On the other hand, it is rapidly metabolized in the intestinal wall and liver, as salicylic acid in blood, indicating that aspirin works as a prodrug (Vane and Botting, 2003; Testa and Mayer, 2003).

Bayer introduced an antibiotic agent, the sulphonamide prontosil, in 1935 but, as for acetanilide and phenacetin, has been described as a prodrug only one year later, by identifying the para-aminophenylsulfonamide as the active component. Thanks to prontosil, it was discovered that the sulfanilamide moiety is a good linker in prodrug design, leading to the beginning of the second generation of sulphonamide prodrugs (Bentley, 2009).

The use of the prodrug design has grown enormously from 1960s to the beginning of 21st century, when this strategy prevailed in the discovery and in the development of new drugs (van De Waterbeemd et al., 2001).

1.4.1 Classification of prodrugs

Prodrugs are classified generally in two main classes: the carrier-linked prodrugs and the bioprecursor prodrugs (Huttunen et al., 2011).

The carrier-linked prodrugs are characterised by an active molecule (the drug) and a carrier (the promoiety) that are temporary linked through a bioreversible covalent linkage. Biotransformation inside the cells allows the release of the two components (Zawilska et al., 2013). This type of prodrugs can be further divided in: bipartite prodrugs, when the active molecule is directly linked to the carrier; and tripartite prodrugs, when a spacer connects the carrier to the active molecule (Abu-jaish et al., 2014). The major groups of carrier-linked prodrugs are: esters, amides, carbamates, carbonates, ethers, imines, phosphates, oximes and N-Mannich bases (Fig. 7) (Zawilska et al., 2013; Testa, 2009; Rautio et al., 2008). Mutual prodrugs (or multiple prodrugs) are carrier-linked prodrugs in which there are two active compounds linked together in a single molecule and one part acts as a carrier for the other one (Das et al., 2010).
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![Diagram of prodrug transformation](image)

**Figure 7. In vivo biotransformation of prodrugs** (Jornada et al., 2015).

Bioprecursors are compounds that do not contain a carrier, but they are rapidly transformed into active molecules through metabolic or chemical reactions, such as hydration, oxidation or reduction (Rautio et al., 2008; Graf and Lippard, 2012; Kokil and Rewatkar, 2010).

The site of conversion of the prodrugs into the active molecules is the basis for another kind of classification of prodrugs. **Type I prodrugs** are metabolized intracellularly; type IA are metabolized at the cellular targets of their inhibitory activity, whilst type IB are metabolized by the liver. Instead, **type II prodrugs** are metabolized extracellularly; type IIA are converted into the gastrointestinal fluid, type IIB in the circulatory system and type IIC near or inside the targets.

Prodrugs that share properties of more than one group and that do not follow the established classification are so called **mixed-type prodrugs** (Wu, 2009; Zawilska et al., 2013).
1.4.2 Old antitubercular prodrugs

It is estimated that about 10% of all globally available medicines can be classified as prodrugs (Huttunen et al., 2011). Looking at the current antitubercular treatment regimen, INH, PZA and ETH are prodrugs that must be activated by mycobacterial enzymes to exert their inhibitory activity (Vale et al., 2013).

Isoniazid is a synthetic antimicrobial agent that still represents one of the main drugs of the antitubercular therapy. Since its introduction in 1952, INH has become a milestone of the tuberculosis treatment regimen thanks to its great potency and high selectivity against *M. tuberculosis*, even if its mechanism of action was characterized only in recent years (Fernandes et al., 2017). INH enters the mycobacterial cell by passive diffusion; it is a prodrug activated by the mycobacterial enzyme KatG, a multi-functional catalase-peroxidase whose enzymatic role probably contributes with *M. tuberculosis* persistence in vivo (Bardou et al., 1998; Zhang et al., 1992; DeVito and Morris, 2003). The activation of INH produces reactive oxygen species (ROS), like superoxide, hydrogen peroxide, peroxynitrite and the isonicotinoyl radical. These ROS bind to a NAD molecule, resulting in a INH-NAD adduct that inhibits the enoyl-ACP reductase InhA of the fatty acid synthase type II system (FASII) (Fig. 8) (Vilchêze and Jacobs, 2007; Timmins and Deretic, 2006). The inhibition of InhA leads to an accumulation of long-chain fatty acids, blocking the synthesis of mycolic acids and, consequently, affecting the cell wall biosynthesis (Marrakchi et al., 2000). Moreover, it has been hypothesized that the production of ROS may affect also the synthesis of DNA, proteins and lipids, thus explaining the strong bactericidal activity of INH (Wengenack and Rusnak, 2001; Timmins et al., 2004). The most common mechanisms of resistance to INH are related with mutations in *katG* and *inhA* genes. Mutations in *katG* gene prevent the activation of INH, consequently the formation of INH-NAD adduct, while mutations in *inhA* gene generally reduce the affinity for the cofactor NADH, thus also for the adduct (Fernandes et al., 2017).

Other mutated genes seem to be involved in the mechanism of resistance to INH, such as *ndh*, *ahpC*, and *nat. ndh* codes for NADH dehydrogenase of the respiratory chain; consequently, mutations in *ndh* lower the rate of NADH oxidation and increase the intracellular NADH/NAD+ ratio. An increased amount of NADH may prevent the
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formation of isonicotinic acyl NADH or may promote displacement of adduct from InhA (Miesel et al., 1998). The enzyme AhpC (alkyl hydroperoxidase) coded by \textit{ahpC} gene is responsible for the reduction of substrate peroxides. Mutations in the \textit{ahpC} promoter region cause increased expression of the AhpC protein, which could compensate for loss of KatG/CP activity (Sherman et al., 1996). \textit{nat} gene encodes the arylamine N-acetyltransferase (NAT) enzyme, which acetylates the nitrogen group of INH avoiding KatG activation (Upton et al., 2001).

Looking at the mechanism of action of INH, it could be useful to develop new antitubercular agents with improved INH features, such as:

- compounds that naturally produce the isonicotinoyl radical in order to kill \textit{katG} mutants resistant to INH;
- compounds that increase mycobacterial levels of NAD$^+$ in co-administration with INH to achieve a more potent action of the INH-NAD adduct;
- compounds obtained exploiting the structure of INH-NAD adduct to specifically inhibit InhA (Timmins and Deretic, 2006).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Mechanism of action of INH (Vilchène and Jacobs, 2007).}
\end{figure}
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Pyrazinamide (PZA) is an unconventional drug due to its antimicrobial nature, which mainly inhibits non-growing mycobacteria persisting in acidic environment inside macrophages (Zhang *et al.*, 2014; Mitchison, 1985). PZA is a structural analog of nicotinamide (Fig. 9A) and a prodrug activated by the pyrazinamidase (PZase; encoded by *pncA* gene) enzyme, leading to the formation of the active metabolite pyrazinoic acid (POA) (Scorpio and Zhang, 1996). PZA enters *M. tuberculosis* cells through passive diffusion and is converted to POA by the cytoplasmic PZase enzyme. Thanks to the passive diffusion and to a weak efflux mechanism, the active metabolite arrives at cellular surface, where extracellular acid environment allows the conversion of a small amount of POA into uncharged protonated acid HPOA. This molecule enters the cells and, considering that the acid-facilitated POA influx usually exceeds the POA efflux, the high accumulation of POA inside the mycobacterial cell principally results in the disruption of membrane energy and function, inhibition of trans-translation and possibly inhibition of pantothenate and CoA biosynthesis. Moreover, HPOA brings protons inside the cells, which acidify the cytoplasm and affect key enzymes of the cellular metabolism (Fig. 9B) (Zhang *et al.*, 1999; Zhang *et al.*, 2014).

Cases of PZA resistance are attributed mostly to mutations in *pncA* gene. However, other mutated genes have been identified: *rpsA*, encoding RpsA (ribosomal protein S1) which is involved in the process of trans-translation, and *panD*, encoding the pantothenate synthase which is involved in synthesis of β-alanine, a precursor for pantothenate and CoA biosynthesis (Shi *et al.*, 2011; Zhang *et al.*, 2013). Nevertheless, novel mechanisms of resistance to PZA need to be further discovered because some researchers described PZA-resistant mutants that did not show any mutations in the aforementioned genes (Jure’en *et al.*, 2008; Zhang *et al.*, 2014; Njire *et al.*, 2016).
Ethionamide is a structural analog of INH and they share an identical mechanism of action, even if ETH shows a different mode of activation (Vale et al., 2013). ETH is a prodrug activated by EthA, an NADPH-specific FAD-containing monooxygenase encoded by \textit{ethA} gene that oxidizes the compound. The activated form of ETH binds to NAD$^+$ forming an ETH-NAD adduct that inhibits InhA (Fig. 10), as for INH (Brossier et al., 2011; Morlock et al., 2003; Wang et al., 2007). Unfortunately, ETH has several side effects, such as hepatitis and gastrointestinal symptoms, and a high toxicity, due to the production of S-oxides during its activation, that
Introduction

limit its use in tuberculosis therapy (Francois et al., 2009; Flipo et al., 2011). For this reason, ETH is classified as a second-line drug and is used only in case of MDR- and XDR-TB (Wolff and Nguyen, 2012). The main mutated genes that confer ETH resistance are \textit{ethA}, \textit{ethR}, and \textit{inhA}. The most common mutations in \textit{ethA} involve its open reading frame affecting the enzymatic activity of the encoded protein, thus reducing the activation of ETH and, consequently, the level of inhibition of the cellular target InhA (Brossier et al., 2011; DeBarber et al., 2000). \textit{ethR} encodes a repressor of \textit{ethA} transcription and mutations in this gene leading to the over-production of EthR protein reduce the synthesis of EthA, thus the activation of ETH (Baulard et al., 2000; Brossier et al., 2011). Lastly, there are two main types of \textit{inhA} mutations: mutations inside the gene reduce the binding affinity to the activated ETH, while mutations in \textit{inhA} promoter lead to an over-expression of the protein, thus achieving an excess of target in comparison with the activated ETH-NAD adduct (Brossier et al., 2011; Morlock et al., 2003; Banerjee et al., 1994). Moreover, even if ETH and INH share the same cellular target, a cross-resistance with these two prodrugs has been observed only in the 13% of cases, suggesting that ETH and INH affect different sites of the same protein InhA (Baulard et al., 2000; Schaaf et al., 2009).
**Introduction**

![Chemical Reaction Diagram](image)

**Figure 10. Mode of activation of ETH** (adapted from Vilchèze and Jacobs, 2014).

### 1.4.3 New potential antitubercular prodrugs among the novel antituberculars under development

The main difficulty related to the use of antitubercular prodrugs is the emergence of drug-resistant *M. tuberculosis* strains, in which the gene encoding the activator is mutated, and the possibility of unexpected toxicity of metabolites, resulting from the activation of the compound. Understanding better the mechanism of action of the old prodrugs, it could be possible to develop new and improved antitubercular prodrugs (Mori *et al.*, 2017; de Campos *et al.*, 2014), that could be used against DR-TB.

After several decades of near inactivity of TB drug development, in the last years a promising pipeline emerged, showing a number of new compounds and repurposed drugs in the different stages of pre-clinical and clinical development (Zumla *et al.*, 2013a) (Fig. 11).
Figure 11. Current pipeline of new antitubercular drugs in preclinical and clinical development (From: http://www.newtbdrugs.org/pipeline.php).

For the first time since the introduction of rifampin in the late 1960s, among the new drugs under development, two new antituberculars, bedaquiline and delamanid, became available in 2013 and 2014, respectively. They are used for the treatment of MDR tuberculosis under certain conditions in several countries due to the serious adverse effects observed upon treatment (Hoagland et al., 2016; Zignol et al., 2016).

Bedaquiline belongs to a new class of drugs, diarylquinoline, and inhibits the ATP-synthetase (Andries et al., 2005).

The bicyclic nitroimidazole PA-824 (premanad) is a new prodrug active against both actively replicating and hypoxic non-replicating M. tuberculosis bacilli. The deazaflavin (cofactor F420)-dependent nitroreductase (Ddn), encoded by Rv3547 gene, is responsible for the intracellular activation of PA-824. It converts PA-824 into three main metabolites, among which the des-nitroimidazole (des-nitro) is the most important one (Fig. 12) (Manjunatha et al., 2009; Manjunatha et al., 2006). Studies on the mechanism of action of this antitubercular prodrug revealed that PA-824 exerts its inhibitory activity in two different ways. Replicating M. tuberculosis cells are killed by the inhibition of mycolic acid biosynthesis. In fact, PA-824 blocks the production of ketomycoclates leading to the accumulation of hydroxymycoclates, which are the major constituents of mycobacterial cell wall (Stover et al., 2000; Manjunatha et
*Introduction*

*al., 2009). Since non-replicating bacilli do not widely produce the cell wall mycolic acids under anaerobiosis, it was discovered that the killing mechanism in non-replicating condition is the intracellular nitric oxide (NO) release (Boshoff and Barry, 2006; Singh *et al.*, 2008). In particular, the quantity of metabolite des-nitro is related directly with an increased inhibitory activity of the compound: in fact, metabolite profile of PA-824 revealed that the compound acts as a NO donor during its activation, thus generating nitric oxide and other reactive nitrogen species, the effectors of the anaerobic activity (Singh *et al.*, 2008).

![Chemical Diagram](image)

**Figure 12.** Nitroimidazole reduction by deazaflavin-dependent nitroreductase (Ddn) and the three main PA-824 metabolites (Singh *et al.*, 2008).
**Introduction**

The dihydro-imidazooxazole **delamanid** (Fig. 13), previously known as **OPC-67683**, is a new antitubercular compound, which is active against drug-resistant tuberculosis (Rustomjee and Zumla, 2015). Its antimicrobial activity primary affects the synthesis of two main components of the mycobacterial cell wall, the methoxymycolic and ketomycolic acids, probably *via* a radical intermediate (Xavier and Lakshmanan, 2014; Szumowski and Lynch, 2015). As the related compound PA-824, delamanid is a prodrug activated by the enzyme deazaflavin-dependent nitroreductase (Ddn), and it is active against both replicating and non-replicating *M. tuberculosis* bacilli (European Medicines Agency, 2013; Gurumurthy *et al.*, 2012).

![Delamanid structure](image)

**Figure 13. Delamanid structure** (Szumowski and Lynch, 2015).

Among the drugs under development, **PBTZ169** (2-piperazino-benzothiazinone 169) is a piperazine-containing benzothiazinone (PBTZ) (Fig. 14), which has been synthetized starting from the antitubercular class of 1,3-benzothiazin-4-ones (BTZs) (Makarov *et al.*, 2009; Makarov *et al.*, 2014). The lead compound of BTZs is the nitroaromatic prodrug BTZ043 (Fig. 14A), whose cellular target is the flavoprotein decaprenylphosphoryl-beta-D-ribose oxidase DprE1. This enzyme, which works in concert with DprE2, catalyzes the epimerization of decaprenylphosphoryl-beta-D-ribose, the only source of the decaprenylphosphoryl-beta-D-arabinose, essential for the biosynthesis of the cell wall components arabinogalactan and lipoarabinomannan (Makarov *et al.*, 2014). Moreover, DprE1 is responsible for the reduction of the essential nitro group of BTZ043 into a nitroso group, thus activating the compound (Fig. 14B) (Trefzer *et al.*, 2012; Trefzer *et al.*, 2010). Unfortunately, BTZ043 showed both a lower efficacy than expected in mouse model and a hydrophobicity, suggesting the need of improvement of the pharmacological properties. To this purpose, it was introduced a piperazine group into the BTZ scaffold, getting the PBTZ
Introduction

derivatives. The mechanisms of activation and action of the prodrug PBTZ169 are the same as BTZ043, but this new compound shows better characteristics (Makarov et al., 2014). Compared to BTZ043, PBTZ169 is more potent in lower concentrations in mouse model of chronic TB and is less toxic, showing a good solubility. The lack of chiral centers makes its synthesis less expensive and its quality control more accurate. In addition, it has been demonstrated a full compatibility of PBTZ169 with other drug candidates, in particular bedaquiline (BDQ) and PZA. This data is of great interest, considering that this new drug combination seems to fight the mycobacterial infection more rapidly than the standard therapeutic treatment (INH, RIF, PZA) (Makarov et al., 2014). Currently, PBTZ169 is entered Phase II clinical trials in Russian Federation and it will enter soon in Europe (Makarov V., personal communication) (Fig. 11).

A.

B.

Figure 14. Class of Benzothiazinones.
A. Chemical structures of BTZ043 and PBTZ169 (Makarov et al., 2014);
B. Mechanism of activation of BTZs (Mori et al., 2017).
**Introduction**

*Lansoprazole (LPZ)*, gastric proton pump inhibitor (PPI), is a compound that selectively affects the $\text{H}^+\text{K}^+$-ATPase of the gastric parietal cell and that it is widely used in the treatment of acid-related diseases of the stomach (Welage, 2003). A recent and innovative high-throughput screening of a panel of FDA-approved drugs revealed that LPZ is a prodrug with an efficient action against MDR-TB (Rybniker *et al.*, 2014; Rybniker *et al.*, 2015). In case of gastric therapy, the acid environment of gastric gland lumen allows the activation of LPZ; firstly, it is converted into a sulfenic acid intermediate and, then, into a sulfenamide that is able to bind and to inhibit the gastric $\text{H}^+\text{K}^+$-ATPase (Fig. 15). In case of tuberculosis treatment, instead, LPZ undergoes an intracellular sulfoxide reduction to lansoprazole sulfide in the cytoplasm of *M. tuberculosis*-host cells (Fig. 15). This mechanism of activation avoids the inhibition of the gastric $\text{H}^+\text{K}^+$-ATPase, providing a high specificity against the pathogen and making LPZ a safe antitubercular compound (Rybniker *et al.*, 2015). The target of the active metabolite is the $\beta$-subunit of the cytochrome $bc_1$ complex (complex III), that being required for ATP production, leads to the inhibition of the mycobacterial respiratory chain. Thus, this novel screen provided both a new well-defined lead compound and a new tool to test novel potential antitubercular compounds (Rybniker *et al.*, 2015).
The thiophenecarboxamide derivatives 7947882 and 7904688 are two promising prodrugs identified through a phenotypic screening, that showed antitubercular activity against *M. tuberculosis* in its replicating, non-replicating and intracellular conditions (Fig. 16) (Mori et al., 2015). Both compounds are activated by EthA, through the formation of S-oxide and S-dioxide metabolites (Mori et al., 2015). The activated prodrugs inhibit a novel drug target, the CTP synthetase PyrG, an essential enzyme involved in *de novo* pyrimidine biosynthesis. Thus, PyrG inhibition affects the nucleotide metabolism and also other metabolic processes that require nucleotides, such as fatty acid, carbohydrate and amino acid biosynthesis, cell wall biosynthesis, and cAMP- and c-di-AMP-dependent signaling (Mori et al., 2015; Meng et al., 2004).
Introduction

Figure 16. Chemical structures of 7947882 and 7904688 (Mori et al., 2015).

The thienopyrimidine derivative TP1126053 is a new potential antitubercular compound that kills replicating and non-replicating *M. tuberculosis*, with a Minimum Inhibitory Concentration (MIC) *in vitro* of 0.125 μg/mL. TP053 is a prodrug activated by the reduced form of the mycothiol-dependent reductase Rv2466c, a member of the mycoredoxin cluster belonging to the DsbA family.

It was demonstrated that the Rv2466c subunits present a canonical thioredoxin-fold with a Cys\(^{19}\)-Pro\(^{20}\)-Trp\(^{21}\)-Cys\(^{22}\) (CPWC) active-site motif and an insertion consisting of a four α-helical bundle and a short α-helical hairpin (Albesa-Jové et al., 2014; Albesa-Jové et al., 2015; Rosado et al., 2017). Rv2466c undergoes severe redox-dependent conformational changes, including partial unfolding of an α-helical subdomain, in order to catalyse the activation of TP053. In particular, this α-helical subdomain works synergistically with the redox state of the thioredoxin domain: Rv2466c assumes an open inactive conformation when Cys\(^{19}\) and Cys\(^{22}\) are bonded, whilst the enzyme comes back to its closed active conformation after reduction (Fig. 17) (Albesa-Jové et al., 2015). This chaperone-like mechanism of conformational changes promotes the TP053 reduction (so its activation) through a dithiol-disulfide mechanism, receiving electrons from the mycothiol/mycothione reductase/NADPH (MSH/Mtr/NADPH) pathway (Fig. 18) (Albesa-Jové et al., 2015; Rosado et al., 2017).
The structure of TP053 is characterized by a NO₂ group and it has been hypothesized that the toxic effects of the activated compound could be due to the reduction of this chemical group. This putative mechanism of action has been elucidated for other antimicrobial prodrugs containing a NO₂ group or other highly reactive compounds like nitroso derivatives. The resulting toxicity is able to globally affect the microbial cells, harming protein, lipid and DNA synthesis (as previously described for the antitubercular nitroimidazole drugs PA-824 and delamanid) (Albesa-Jové et al., 2014; Kohanski et al., 2007). Further investigations on the mechanism of action of TP053 are object of this thesis.

The prodrug strategy is becoming an important element of the drug design and delivery process, in order to improve the efficacy of the
compounds and to reduce the side effects of the parent molecules. Indeed, the number of new approved prodrugs is increasing every year (Fig. 11). Nevertheless, more efforts are needed, mainly in the early stages of drug discovery, in order to achieve the desired goal and to definitely impose prodrugs in modern pharmacotherapy (Zawilska et al., 2013; Piplani et al., 2016; Rautio et al., 2008).
2. Aims of the work

Tuberculosis (TB) is an ancient disease that has affected humankind for centuries and it is still threatening the public health worldwide, killing an enormous number of people every year. Considering that TB is globally one of the top causes of death from a single infectious agent, there is an urgent need for novel antitubercular compounds.

This research is part of the project “More Medicines for Tuberculosis (MM4TB)”, funded by the European Commission (EC-VII Framework program, 2011-2016), whose aim was to validate at least five new drug targets and to identify at least one class of new potential antitubercular compounds. It evolved from the highly successful project “New Medicines for Tuberculosis (NM4TB)”, that delivered a candidate drug for clinical development two years ahead of schedule. Within this project, the Molecular Microbiology Laboratory at the University of Pavia has identified the molecular targets of several compounds, already available and proven to be effective in models of active or latent tuberculosis.

In this context, the thesis research project is focused on the study in depth of the mechanism of action of three prodrugs, highly active against *M. tuberculosis* H37Rv in its replicating, non-replicating and intracellular states.

The first prodrug is the thienopirimidine (TP) derivative TP11126053, which has been demonstrated to be activated by the reduced form of the DsbA-like mycoredoxin Rv2466c. The other two compounds, 7947882 and 7904688, have been characterized as prodrugs activated by the monooxygenase EthA targeting the CTP synthetase PyrG.

The first part of this thesis aimed to characterize the mechanisms of action and of resistance of TP11126053, because only the mode of activation was known.

The second part, instead, had the goal to better elucidate the mechanism of action of both 7947882 and 7904688 prodrugs and to identify the active metabolite(s) of 7904688, after EthA-activation, taking advantage of a metabolomic approach.
3. Materials and Methods

3.1 Bacterial strains

Bacterial strains utilized in this work are listed in Table 1. *Escherichia coli* XL1 Blue was used for cloning experiments, whilst *E. coli* BL21(DE3) for expression studies.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td><em>endA1 gyrA96 (nai^B</em>) thi-1 recA1 relA1 lac glnV44 F^[:=Tn10 proAB^lacI^A(lacZ)M15] hsdR17(rK mK*)*</td>
<td>Stratagene</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>F-ompThsdSB (rB- mB-)</td>
<td>Laboratory</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>dcm(DE3)</td>
<td>collection</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>F-ompThsdSB (rB^- mB^-) dcm gal</td>
<td>Laboratory</td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>λ(DE3) pLysSCm^f</td>
<td>collection</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>Wild type strain</td>
<td>Laboratory</td>
</tr>
<tr>
<td>mc^155</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium bovis</em></td>
<td>Wild type strain</td>
<td>Laboratory</td>
</tr>
<tr>
<td><em>BCG</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mycobacterium</em></td>
<td>Wild type strain</td>
<td>Laboratory</td>
</tr>
<tr>
<td><em>tuberculosis</em> H37Rv</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Bacterial strains used in this work.

3.2 Growth media and conditions

*E. coli* cells were grown aerobically at 37°C with orbital shaking at 200 rpm either in Luria-Bertani (LB) broth or onto LB agar (Table 2). When necessary, antibiotics were added at the following concentrations: ampicillin, 100 μg/mL; and kanamycin, 50 μg/mL.

*Mycobacterium smegmatis* strains were grown aerobically at 37°C with orbital shaking at 200 rpm, whilst *Mycobacterium bovis* BCG and *M. tuberculosis* strains were grown aerobically at 37°C either in Middlebrook...
**Materials and Methods**

7H9 medium without shaking or onto Middlebrook 7H11 agar, both supplemented with 10% OADC Middlebrook Enrichment (Table 2).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LB broth</strong></td>
<td>Tryptone</td>
<td>10 g/L</td>
</tr>
<tr>
<td></td>
<td>Yeast Extract</td>
<td>5g/L</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>10 g/L</td>
</tr>
<tr>
<td></td>
<td>Sterile Water to</td>
<td>to 1L</td>
</tr>
<tr>
<td><strong>Middlebrook 7H9 broth</strong></td>
<td>Ammonium sulfate</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td>(Difco)</td>
<td>L-Glutamic acid</td>
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</tr>
<tr>
<td></td>
<td>Sodium citrate</td>
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</tr>
<tr>
<td></td>
<td>Pyridoxine</td>
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</tr>
<tr>
<td></td>
<td>Biotin</td>
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</tr>
<tr>
<td></td>
<td>Disodium phosphate</td>
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<tr>
<td></td>
<td>Monopotassium phosphate</td>
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</tr>
<tr>
<td></td>
<td>Ferric ammonium citrate</td>
<td>0.04 g/L</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulfate</td>
<td>0.05 g/L</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride</td>
<td>0.5 mg/L</td>
</tr>
<tr>
<td></td>
<td>Zinc sulfate</td>
<td>1 mg/L</td>
</tr>
<tr>
<td></td>
<td>Copper sulfate</td>
<td>1 mg/L</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>to 900 mL</td>
</tr>
<tr>
<td><strong>Middlebrook 7H11 agar</strong></td>
<td>Pancreatic digest of casein</td>
<td>1.0 g/L</td>
</tr>
<tr>
<td></td>
<td>Monopotassium phosphate</td>
<td>1.5 g/L</td>
</tr>
<tr>
<td></td>
<td>Disodium phosphate</td>
<td>1.5 g/L</td>
</tr>
<tr>
<td></td>
<td>Monosodium glutamate</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td></td>
<td>Ammonium sulfate</td>
<td>0.5 g/L</td>
</tr>
<tr>
<td></td>
<td>Sodium citrate</td>
<td>0.4 g/L</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.04 g/L</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>0.05 g/L</td>
</tr>
<tr>
<td>Zinc sulfate</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Copper sulfate</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>1 mg/L</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.5 mg/L</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.5 mg/L</td>
</tr>
<tr>
<td>Malachite green</td>
<td>0.25 mg/L</td>
</tr>
<tr>
<td>Agar</td>
<td>13.5 g/L</td>
</tr>
<tr>
<td>Water</td>
<td>to 900 mL</td>
</tr>
<tr>
<td>Middlebrook OADC enrichment</td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>0.6 mL/L</td>
</tr>
<tr>
<td>Bovine albumin (fraction V)</td>
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</tr>
<tr>
<td>Dextrose</td>
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</tr>
<tr>
<td>Catalase</td>
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<tr>
<td>Sodium chloride</td>
<td>8.5 g/L</td>
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</tbody>
</table>

Table 2. Media used in this work.

#### 3.3 MIC determination

Minimal Inhibitory Concentration (MIC) values were determined by means of the micro-broth dilution method. A single colony of each *M. tuberculosis* strain was inoculated in complete Middlebrook 7H9 medium. The cell cultures were grown at 37°C until late exponential growth phase was reached (~10⁸ CFU/mL), corresponding to an OD₆₆₀ value of 0.8 - 1.0. After dilution to a final concentration of ~10⁷ CFU/mL, 1 μL of the mycobacterial cell cultures was streaked onto complete Middlebrook 7H11 agar containing a range of drug concentrations. Plates were incubated at 37°C for about 21 days, and the growth was visually evaluated. The lowest drug dilution at which visible growth failed to occur was taken as the MIC value. Results were expressed as the average of at least three independent determinations.
Materials and Methods

3.4 Isolation of *M. tuberculosis* spontaneous mutants resistant to TP11126053

The isolation of *M. tuberculosis* resistant mutants was performed by plating ~10^{10} cells from an exponential growth phase wild-type culture, onto 7H11 medium containing different concentration of TP11126053 (TP053) ranging from 4 to 20-fold the MIC for the wild-type strain. Plates were incubated at 37°C for at least 21 days. The phenotype of the resistant colonies was confirmed by determining the MIC values and the experiment was repeated three times.

3.5 Recombineering-mediated point mutagenesis strategy

Recombineering is a recently developed technique for efficient genetic manipulation of bacteria and is facilitated by phage derived recombination proteins; moreover, it takes advantage of using DNA substrates with short regions of homology (Marinelli *et al.*, 2012). It was developed initially as a powerful system for mutagenesis in *E. coli* and, subsequently, it was successfully employed in other Gram-negative bacteria, such as *Salmonella* spp. and *Shigella* spp. (Ranallo *et al.*, 2006; Swingle *et al.*, 2010). Mycobacterial Che9c encodes for homologues of both RecE and RecT, the products of genes 60 and 61 respectively, and they can be exploited to develop recombineering systems for both fast- and slow-growing mycobacteria (van Kessel *et al.*, 2008). Thus, the desired mutation can be transferred into *M. tuberculosis* cells expressing Che9c gp61 (thanks to the recombinant plasmid pJV62) using the phage Che9c recombination functions.

To this purpose, a specific oligonucleotide carrying the studied mutation was designed to target the lagging strand of the *Rv0579* wild type gene (Table 3).
Materials and Methods

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Point mutation</th>
<th>Amino acidic mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv0579</td>
<td>AGTTAGCTGGTCCCGCAGTCG TTCGACGA**GCGAACCAACC GTGCGTGGTGGATCCCCGG</td>
<td>c718g</td>
<td>V240L</td>
</tr>
</tbody>
</table>

Table 3. Oligonucleotide used in the recombineering method.

In particular, the aforementioned oligonucleotide sequence was used to transform by electroporation *M. tuberculosis* cells containing the recombineering plasmid pJV62 (Figure 19). This plasmid carries a kanamycin-resistance marker and its importance is due to the only expression of the RecT function at high levels from the acetamidase promoter, in such a way that the rate of the oligo-mediated recombineering is four orders of magnitude higher than the spontaneous rate of mutation (van Kessel et al., 2008).

*M. tuberculosis* transformant colonies were isolated at high TP053 concentrations and the introduced mutation in Rv0579 gene was confirmed for all of them by PCR amplification and sequencing of Rv0579 locus.
Materials and Methods

3.6 Real Time-PCR reaction (RT-PCR)

The quantitative Real-Time PCR experiments were performed using RNA extracted from *M. tuberculosis* H37Rv WT and Rv0579 mutant strains, both treated or not with TP053 using the Direct-zol™ RNA MiniPrep Kit (Zymo Research). Then, the treatment with DNase (Ambion) to remove any genomic DNA contaminations was done. All samples were tested by PCR amplification to exclude any residual DNA contaminations. The cDNA was obtained from about 1 µg of total RNA by the Quantitect reverse transcription kit (Qiagen). The RT-PCR experiments were
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performed using the kit QuantiTect SYBR Green PCR Master Mix (Qiagen) and the thermocycler "Rotor Gene 6000" (Qiagen). When SYBR Green binds to DNA molecules, it emits a fluorescent signal, avoiding synthesis of marked primers.

The reaction was carried out in a final volume of 15 µL and contained: 7.5 µL of 2X SYBR Green Buffer (supplied by the kit), cDNA, primers (7.5 pmol) and RNase-free water. All reactions were repeated in triplicate and the mean value was considered. The primers used to assess the transcriptional analysis of sigA and Rv0579 genes are present in Table 4.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>sigAF</td>
<td>GATGACGACGAGGAGAT</td>
</tr>
<tr>
<td>sigAR</td>
<td>GCCGATCTGTGTGAGGTA</td>
</tr>
<tr>
<td>0579rtF</td>
<td>GACGTCAACCTCGGCCAG</td>
</tr>
<tr>
<td>0579rtR</td>
<td>GAATCGCTGCTCGCCCA</td>
</tr>
</tbody>
</table>

Table 4. RT-PCR primers.

The sigA gene, coding for the sigma factor, is an essential housekeeping gene, constitutively expressed in all mycobacterial strains, and was chosen as a reference standard. Expression data were calculated with the -2∆∆Ct method (ΔCt = Ct sample – Ct control) and were reported as n-fold change in gene expression of the sample (mutant strain) normalized to the invariant gene (sigA) relative to the control (wild-type strain).

3.7 Cloning procedures

DNA manipulation was performed according to standard procedures described by Sambrook and Russel (2001).

The purification of PCR fragments of genes from agarose gel was done using Wizard SV Gel and PCR Clean-Up System (Promega) and the ligation into the correspondent vector utilizing T4 DNA ligase (Promega). E. coli electrocompetent cells were transformed by electroporation, utilizing Bio-Rad Gene Pulser. The primers and the plasmid used in this work are reported in Table 5 and Figure 20.
### Materials and Methods

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0579SumoFor</td>
<td>ATGGTCGGCTATGTTGGA</td>
</tr>
<tr>
<td>0579SumoRev</td>
<td>TCAGGTCCAGTAGTTAGAG</td>
</tr>
</tbody>
</table>

Table 5. Primers used for cloning into pET-SUMO vector.

The pET-SUMO (Invitrogen) (Fig. 20) is an expression vector for *E. coli* that allows the expression and purification of recombinant proteins, fused with a small ubiquitin-like modifier (SUMO). Fusion with SUMO increases the solubility of expressed proteins and their expression level. Moreover, the SUMO tertiary structure is recognized and cleaved by SUMO protease, an ubiquitin-like protein-processing enzyme, resulting in the production of a native protein.

**Figure 20.** pET-SUMO vector.

pET-SUMO vector was used for the expression and purification of *M. tuberculosis* Rv0579 in *E. coli*. 

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3.8 Expression and purification of recombinant Rv0579

A 2 mL pre-inoculum of *E. coli* BL21(DE3) One Shot® cells transformed with pET-SUMO/Rv0579 recombinant plasmid was inoculated in 100 mL of LB (50 μg/mL kanamycin) at 37°C to OD_{600} value of 0.6. Then protein expression was induced by addition of 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG), followed by overnight incubation at 25°C with orbital shaking at 160 rpm. The bacterial cells (2 L of culture) were harvested and re-suspended in 150 mL of Buffer A (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 0.5 mM dithiothreitol (DTT), 5% glycerol, 2 mM MgCl₂) containing 1 mM PMSF and 100 μL of DNase (2 mg/mL). Cells were disrupted by sonication (30 sec for ten cycles) and centrifuged for 50 minutes at 10000 g. The supernatant was loaded on HisTrap Crude column (1 mL), washed with 50 mL of lysis buffer containing 50 mM ImOH, until the OD_{280} value returned to zero. The protein elution was performed using scalar concentrations (50 mM, 100 mM, 200 mM and 500 mM) of ImOH in buffer A. The fractions were checked by SDS-PAGE, and those containing Rv0579 protein were pooled, and excess of imidazole was removed using a HiPreP Desalting 26/10 column (equilibrated in buffer B: 50 mM Tris-HCl pH 7.5, 50 mM ImOH, 100 mM NaCl, 0.5 mM DTT, 2 MgCl₂, 5% glycerol). Desalted protein was incubated at 4°C in the presence of 15 μL SUMO-protease and then re-loaded on HisTrap Crude column, equilibrated in the desalting buffer. 15% SDS-PAGE was performed afterwards to control the purification. The purified protein was concentrated to 5 mg/mL, and stored at -80°C. The concentration of Rv0579 was determined by its specific absorbance at 280 nm (ε: 21680 M⁻¹cm⁻¹).

3.9 Expression and purification of recombinant Rv2466c

The recombinant Rv2466c protein (221 residues), obtained using *E. coli* pET29a/Rv2466c recombinant plasmid, is characterized by an additional peptide of 14 amino acids (1-MHHHHHHHMYFQG-14) at the N-terminus, that includes a histidine tag, and the optimal tobacco etch virus protease (TEV) cleavage site (ENLYFQG) (Albesa-Jové et al., 2014). *E. coli* BL21(DE3)pLysS cells transformed with pET29a/Rv2466c were grown in 3000 mL of 2xYT medium (25 μg/mL kanamycin and 35 μg/mL chloramphenicol) at 37°C. When the culture reached the OD_{600} value of 0.8,
**Materials and Methods**

Rv2466c expression was induced by adding 1 mM IPTG. After about 16 h at 18°C, cells were harvested and resuspended in 40 mL of Solution A (20 mM imidazol, 50 mM Tris-HCl pH 7.5, 500 mM NaCl), containing protease inhibitors (Complete EDTA-free, Roche). Cells were disrupted by sonication (five cycles of 1 min) and the suspension was centrifuged for 20 min at 10000 g. The supernatant was applied to a HisTrap Chelating column (5 mL, GE HealthCare) equilibrated with Solution A. The column was washed with the same solution until no absorbance at 280 nm was detected. Elution was performed with a linear gradient of 20-500 mM imidazole in 40 mL of solution A and fractions containing Rv2466c were pooled. In order to remove the histidine tag, Rv2466c (0.1 mg/mL) was mixed with the TEV protease (30:1 mass ratio; prepared as described by Lucast et al., 2001) and incubated at 18°C during 16-24 hours in Solution B (50 mM Tris-HCl pH 7.5, 50 mM NaCl). The completeness of the enzymatic digestion reaction was checked by SDS-PAGE analysis. The mixture was directly applied to a HisTrap Chelating column (1 mL, GE HealthCare) equilibrated with Solution A, and the flow through containing cleaved Rv2466c, was recovered. The protein was diluted ~10 folds with 50 mM Tris-HCl pH 7.5, and applied onto a Q-Sepharose column (GE Healthcare) equilibrated in the same buffer. The enzyme was eluted in one-step with Solution C (50 mM Tris-HCl pH 7.5, 500 mM NaCl). The fractions containing Rv2466c were pooled and loaded into a HiLoad 26/60 Superdex 200 column (GE15HealthCare) equilibrated with Solution D (50 mM Tris-HCl pH 7.5, 150 mM NaCl). The purified Rv2466c was checked by SDS-PAGE, concentrated to 10-50 mg/mL in 10 mM Tris-HCl pH 7.5 and stored at -80°C. The concentration of Rv2466c was determined via its specific absorbance at 280nm (ε: 39085 M⁻¹cm⁻¹).

**3.10 NO release assay**

**3.10.1 In vitro reaction**

For the in vitro NO release assay, TP053 (200 μM) has been incubated at 37°C with Rv2466c (1 mg/mL), in Na-phosphate buffer 50 mM pH 7.5, DTT 1 mM, 2% of methanolic *M. smegmatis* cell extracts (Albesa Jove et al., 2014), in a final volume of 1 mL. To determine the release of NO, after 1, 2, 3 and 4 hours, samples were centrifuged, diluted 1:1 with Griess
reagent (Sigma Aldrich) and the absorbance at 540 nm was measured. 
HNO$_2$ was evaluated considering a standard curve using sodium nitrite. As 
controls, the same reaction was performed in absence of TP053 or in 
absence of DTT and *M. smegmatis* cell extracts, to prevent Rv2466c 
activation.

### 3.10.2 In mycobacterial cell cultures

Four independent *M. bovis* BCG cell cultures were grown until late 
exponential phase (OD$_{600}$ value of 1.0-1.2) and then they were treated as 
follows:

- one culture was treated with 1 (4X MIC) and 8 µg/mL (4X MIC) of 
  TP053 compound;
- one culture, used as negative control, was treated with 1 and 8 
  µg/mL of 11426026 (026) compound (a nitro compound which is 
  known to act with a mechanism of action different from NO 
  release);
- one culture, used as positive control, was treated with 1 (4X MIC) 
  and 8 µg/mL (4X MIC) of the nitroimidazole PA-824 (compound 
  acting with NO release);
- the last culture was treated with DMSO (negative control).

Cells were read after 4, 8, 24, 32, and 48 hours. At each point, 1 mL of 
culture was centrifuged and the supernatant was used to determine NO 
release by measuring nitrite with Griess reagent as above.

### 3.11 Click chemistry

Click chemistry is a growing area of chemical biology, useful for the 
identification of protein binding partners and of the target of studied 
compounds in live cells (Mamidyla and Finn, 2010).

To this purpose, a TP053 derivative, named 11526119 (or 6119), was 
synthetized by Dr. Vadim Makarov, by adding an alkyne group. This 
derivative was used to perform azide-alkyne cycloaddiction reaction with 
Azide-PEG3-biotin conjugate (Sigma Aldrich), to form an adduct (called 
6119-biotin) which can be immobilized on a Streptavidin Agarose resin 
(Fig. 21).
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Figure 21. Cycloaddiction reaction of 6119 compound with Azide-PEG3-biotin conjugate.

To this purpose, in a final volume of 500 μL, 6119 (200 μM) was incubated overnight at room temperature in the dark with 2-fold excess of Azide-PEG3-biotin conjugate, in the presence of 250 μM of CuSO4 and 5 mM Na-ascorbate. Then, the reaction was incubated 1 hour with Streptavidine Agarose resin (Sigma Aldrich) and, next it was washed three times with PBS to remove any unbound material.

3.11.1 In vitro reaction

To demonstrate a direct interaction within Rv0579 and TP053 moiety, 6119 compound- Azide-PEG3-biotin conjugate complex was used.

The resin bound with the adduct was incubated with Rv0579 (0.5 mg/mL) in a final volume of 200 μL, in the presence of Rv2466c (1 mg/mL), DTT (1 mM) and M. smegmatis extracts (2%) at 37°C for 30 min in order to allow the activation of the compound. After the incubation, the resin was washed five times with PBS to remove unbound proteins and then
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the resin was resuspended in 20 μL of loading buffer for SDS-PAGE analysis.

As a negative control, the same reaction was performed using TP053, which is not able to interact with the Azide-PEG3-biotin conjugate, or in absence of mycothiols, to avoid Rv2466c activation.

3.11.2 Target fishing experiment in M. bovis BCG cells

Three independent M. bovis BCG 100 mL cultures were grown until exponential phase (OD₆₀₀ = 0.6). Then, the three cultures were treated as follows:

1) one culture was treated with 0.25 μg/mL of 6119 (2X MIC of the wild type strain) and incubated for 24 h at 37°C.

2) The other two cultures were used as controls:
   - one culture was treated with 0.25 μg/mL of TP053 (2X MIC of the wild type strain) and incubated for 24 h at 37°C;
   - the last culture was treated with DMSO and incubated for 24 h at 37°C.

At the same time, the Streptavidine Agarose resin was prepared as previously described, incubating it with:
   - the 6119 compound- Azide-PEG3-biotin conjugate adduct;
   - TP053 and Azide-PEG3-biotin conjugate mixture;
   - only with Azide-PEG3-biotin conjugate.

The cultures were harvested, and cells resuspended in 3 mL of PBS, disrupted by sonication, and centrifuged for 20 min at 12000 rpm at 4°C.

The M. tuberculosis cell free extracts were incubated with 50 μL of the corresponding prepared Streptavidine Agarose resin for 1 h at room temperature. The resins were then centrifuged for 2 min at 3000 rpm, and the supernatant, containing unbound proteins, was discarded. The resins were washed three times with PBS to remove other possible unbound proteins, freezed at -80°C and submitted to the proteomic analysis (in collaboration with Prof. E. Marengo, University of Piemonte Orientale, Alessandria, Italy).
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3.12 Preparation of *M. tuberculosis* cell extracts treated with TP053 compound for proteomic analysis

Four independent 120 mL *M. tuberculosis* H37Rv cultures (TB1, TB3, TB5 and Ctrl) were grown until OD$_{600}$ value of 0.6 and then treated with a final TP053 concentration of 0.25 µg/mL (2X MIC of the wild type strain) for 24, 48 and 72 hours at 37°C.

At each time, cells were harvested, washed with PBS and the supernatant was discarded. The obtained pellets were resuspended in 1 mL of PBS, lysed using 0.1 µm of zirconia beads and centrifuged for 30 min at 12000 rpm at 4°C, recovering the supernatant.

Proteins were precipitated with trichloro acetic acid (TCA) 10% for 3 hours in ice and then centrifuged for 30 min at 12000 rpm at 4°C. The obtained pellets were recovered, washed twice with 1 mL of tetrahydrofuran (THF) and dried under vacum, and sent for the proteomic analysis (in collaboration with Prof. E. Marengo, University of Piemonte Orientale, Alessandria, Italy). Total protein content was determined by Bradford.

3.13 Preparation of *M. tuberculosis* cell extracts treated with 7904688 compound for metabolomic analysis

120 mL cultures of *M. tuberculosis* H37Rv were grown up to an OD$_{600}$ value of 0.6 and treated with 7904688 to a final concentration of 1 µg/mL (2X MIC of the wild type strain). The cultures were grown for at least one generation (24 h) at 37°C and harvested at different time (24, 48 and 72 hours). The cells were washed twice with 20 mL of PBS, centrifuged for 15 min at 4000 rpm at 4°C and the supernatant was discarded. Then the pellets were resuspended with 2 mL of ACN and 1 mL of MeOH and lysed with 0.1 µm of zirconia beads. The cellular extracts were centrifuged for 30 min at 12000 rpm at 4°C. The supernatants were filtered with PTFE membrane filters 0.2 µm, dried in speed-vac and sent for metabolomic analysis (in collaboration with Prof. E. Marengo, University of Piemonte Orientale, Alessandria, Italy).
4. Results and discussion

Part I: The thienopyrimidine (TP) derivative TP11126053

Tuberculosis (TB) is a contagious and deadly disease that spreads through the air, which has reached pandemic proportions (Zignol et al., 2016). Unfortunately, in recent years the control of tuberculosis is hampered by the emergence of *Mycobacterium tuberculosis* drug-resistant strains, which represent an extremely dangerous threat to public health. Consequently, new antitubercular drugs are urgently needed (Almeida Da Silva and Palomino, 2011; Velayati et al., 2013; Koul et al., 2011).

The Molecular Microbiology Laboratory of the University of Pavia, which was a member of the European project “More Medicines for Tuberculosis (MM4TB; FP VII)”, is involved in the fight against drug-resistant tuberculosis.

Among the studied compounds, the TP11126053 (TP053 thereafter, Fig. 22), a new thienopirimidine (TP) derivative synthetized by Dr. Vadim Makarov, is able to kill both replicating and non-replicating *M. tuberculosis* bacilli, with an MIC *in vitro* of 0.125 μg/mL. TP053 has been characterized as a prodrug activated by the reduced form of the DsbA-like mycoredoxin Rv2466c (Albesa-Jové et al., 2014; Rosado et al., 2017).

![Figure 22. TP053 chemical structure](image-url) (Albesa-Jové et al., 2014).
Results and discussion

4.1 Understanding the mechanism of resistance to TP053

In order to provide a new insight into the mechanism of action of TP053, a first attempt in the isolation of \textit{M. tuberculosis} spontaneous mutants resistant to TP053 led to the identification of a single mutant, named 53.8, with a MIC of 2.5 \(\mu\)g/mL (20X the MIC of the wild type strain) (Table 6). Whole genome sequencing analysis revealed the presence of mutations in three different genes: \textit{Rv0579}, encoding for a conserved hypothetical protein of unknown function; \textit{Rv0158}, encoding for a putative transcriptional regulator (possibly TetR-family); and \textit{esxD}, encoding for a ESAT-6 like protein EsxD (Table 6). At the same time, no alterations were found in \textit{Rv2466c} gene, coding the TP053 activator.

<table>
<thead>
<tr>
<th>\textit{M. tuberculosis} strains</th>
<th>MIC to TP053 ((\mu)g/mL)</th>
<th>Mutation (aa change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>0.125</td>
<td>-</td>
</tr>
</tbody>
</table>
| 53.8                             | 2.5 (20X MIC)               | \textit{Rv0158} (V48A)  \
|                                  |                             | \textit{esxD} (M41R)  \
|                                  |                             | \textit{Rv0579} (L240V) |

Table 6. Features of \textit{M. tuberculosis} 53.8 mutant resistant to TP053.

4.1.1 Isolation of \textit{M. tuberculosis} TP053 resistant mutants

Considering the presence of the three afore mentioned mutations in a single mutant, a second attempt in the isolation of \textit{M. tuberculosis} TP053 resistant mutants was performed starting from a \textit{M. tuberculosis} culture over-expressing the \textit{Rv2466c} activator (\textit{M. tuberculosis/pSODIT-Rv2466c}). Two \textit{M. tuberculosis} TP053 spontaneous resistant mutants, named 2466.3 and 2466.4, were isolated. As for the 53.8 mutant, the genome profile of these mutants displayed no alterations in \textit{Rv2466c} (both on the genome and on the plasmid). Interestingly, both mutants harboured only the mutation (C718G) previously found in \textit{Rv0579} gene, corresponding to the amino acid substitution Leu240Val (Table 7).
Results and discussion

<table>
<thead>
<tr>
<th></th>
<th>MIC to TP053 (μg/mL)</th>
<th>Mutation in <em>Rv2466c</em> (aa change)</th>
<th>Mutation in <em>Rv0579</em> (aa change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>On genome</td>
<td>On plasmid</td>
</tr>
<tr>
<td>*M. tuberculosis/*pSODIT-Rv2466c</td>
<td>0.125</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2466.3 mutant</td>
<td>0.5 (4X MIC)</td>
<td>NO</td>
<td>NO</td>
</tr>
<tr>
<td>2466.4 mutant</td>
<td>1 (8X MIC)</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

Table 7. Features of new *M. tuberculosis* mutants resistant to TP053.

Thanks to obtained data, it was hypothesized that Rv0579 could be responsible for the resistance to TP053.

4.1.2 Investigation of the role of Rv0579 in TP053 resistance

*Rv0579* gene is 759 bp long and encodes for a conserved hypothetical protein of unknown function, which is not essential for *M. tuberculosis* growth. Bioinformatic analysis revealed that Rv0579 is characterized by two conserved domains:

1. an Ubiquitin Mut7-C domain at the N-terminal region, which probably act as an RNA-binding domain;
2. a Mut7-C RNase domain (a putative RNase domain) at the C terminus region, where the L240V substitution is located (Fig. 23).

![Figure 23. Putative domains of *M. tuberculosis* Rv0579 protein](from NCBI, gene database).
**Results and discussion**

To investigate the role of the Rv0579 protein in the mechanism of TP053 resistance, a recombineering-mediated point mutagenesis strategy was used. Recombineering is a powerful technique developed for genetic manipulation of bacteria, with the possibility to construct gene knockout or replacement mutants. Its biggest peculiarity is to increase incredibly the recombination efficiencies, compared to homologous recombination observed with linear dsDNA allelic exchange substrates (AESs), taking advantage of phage derived recombination proteins and DNA substrates with short regions of homology (van Kessel *et al.*, 2008).

The oligonucleotide (reported in “Materials and Methods”, Table 3), carrying the C718G transition, specifically designed to target the lagging strand of Rv0579 wild type gene, was used to transform *M. tuberculosis* H37Rv cells containing the recombineering plasmid pJV62.

Five *M. tuberculosis* transformant colonies were isolated at the following TP053 concentrations: 1.25 μg/mL and 5 μg/mL (10X and 40X the MIC of the wild type strain, respectively). For all colonies, the presence of the mutation in Rv0579 gene was confirmed by PCR amplification and Sanger sequencing of Rv0579 locus. The TP053 resistance profile of new Rv0579 mutants was confirmed by determining the MIC values (Table 8).

<table>
<thead>
<tr>
<th>TP053 (μg/mL)</th>
<th>Number of resistant colonies</th>
<th>Mutation in Rv0579 (nucleotidic change)</th>
<th>MIC to TP053 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>4</td>
<td>C718G</td>
<td>2.5 (20X MIC)</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>C718G</td>
<td>2.5 (20X MIC)</td>
</tr>
</tbody>
</table>

*Table 8. MIC to TP053 of M. tuberculosis recombineering mutants.*

These results confirmed that the identified C718G point mutation in Rv0579 gene confers resistance to TP053, strongly indicating a role of Rv0579 in this mechanism of resistance.

For this reason, the possible effects of TP053 on Rv0579 expression levels were investigated. To this purpose, RT-PCR experiments were performed on both *M. tuberculosis* H37Rv wild type (WT) and mutant strain carrying the mutated Rv0579 gene, treated with TP053. As reported in
**Results and discussion**

Table 9, no significant difference in the expression of Rv0579 was found in all *M. tuberculosis* strains, thus excluding an influence of TP053 in the Rv0579 expression levels.

<table>
<thead>
<tr>
<th><strong>M. tuberculosis strains</strong></th>
<th><strong>Rv0579 expression levels</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>1.00 ± 0.028</td>
</tr>
<tr>
<td>H37Rv + 0.06 μg/mL (0.5X MIC) TP053</td>
<td>0.93 ± 0.011</td>
</tr>
<tr>
<td>Rec5 (Rv0579 mutant)</td>
<td>1.33 ± 0.11</td>
</tr>
<tr>
<td>Rec5 (Rv0579 mutant) + 0.06 μg/mL (0.5X MIC) TP053</td>
<td>0.73 ± 0.17</td>
</tr>
</tbody>
</table>

Table 9. Expression levels of Rv0579 in *M. tuberculosis* H37RV WT and Rv0579 mutant strains.

**4.1.3 Expression and purification of recombinant Rv0579 protein**

In order to better understand the role of Rv0579 in the TP053 resistance, the Rv0579 protein was over-expressed in *E. coli* BL21(DE3) cells, using the Champion™ pET SUMO Protein Expression System. All cloning procedures are described in the section “Materials and Methods”.

To obtain a significant protein expression, several conditions were tested and the best result was obtained in presence of IPTG 0.5 mM at 25°C overnight. This condition was exploited also to perform large-scale purification trials.

Then, the recombinant protein was purified to homogeneity by affinity chromatography on HisTrap crude column, and SUMO-protease digestion followed by a second affinity column (Fig. 24).
4.1.4 Target fishing experiment \textit{in vitro}\n
Successively, the achieved Rv0579 recombinant protein was used to understand if there is a direct interaction between it and the activated TP053.

To this purpose, a target fishing experiment was performed using a TP053 derivative (the 6119 compound) suitable for click-chemistry (Fig. 21). The 6119 compound was conjugated with an Azide-PEG3-biotin molecule; then the achieved adduct was firstly incubated with Rv0579, in presence of Rv2466c activator, followed by incubation with the Streptavidin Agarose resin. After several washing steps, the resin was analyzed by SDS-PAGE (Fig. 25). As control, the same reaction was performed in the absence of mycothiols (from \textit{M. smegmatis} cell extracts), to avoid Rv2466c activation, or using TP053, which is not able to interact with the Azide-PEG3-biotin.
Results and discussion

![Figure 25. Target fishing experiment in vitro.](image) SDS-page analysis of the click chemistry reaction. -: reaction without mycothiols; +: reaction with mycothiols; r: resin; w: wash.

These data demonstrated that not only 6119 binds to Rv2466c activator as expected, but also to Rv0579, upon Rv2466c activation. This result indicated that there is a direct interaction between the activated TP053 and Rv0579, thus confirming that this protein has a role in the mechanism of TP053 resistance.

However, being the physiological function of Rv0579 still unknown, further investigations are required to better elucidate the role of this protein in TP053 metabolism.

4.2 Understanding the mechanism of action of TP053

4.2.1 Isolation of the putative TP053 active metabolite from *M. tuberculosis* H37Rv cells over-expressing Rv2466c

In order to elucidate the mechanism of action of TP053 prodrug, the identification of its active form is mandatory. Previously, it was demonstrated that the NO₂ group of TP053 is essential for its activity, and that the Rv2466c activator acts by reducing this moiety (Albesa-Jove *et al*., 2014). However, the TP053 active metabolite is still unknown, as well as its cellular target.
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In this context, our collaborator Dr. Vadim Makarov analyzed by mass spectrometry the cellular extracts of the *M. tuberculosis* strain over-expressing *Rv2466c*, grown in the presence of the compound. The *M. tuberculosis* H37Rv culture (transformed with pSODIT-2 alone) was used as control. This analysis revealed the presence of a peak with m/w of 261, absent in the control, which could correspond to a TP053 metabolite (Fig. 26A). This metabolite could be a 2-(4-mercapto-6-(methylamino)-2-phenylpyrimidin-5-yl)ethan-1-ol derivative. It could arise from the reduction of the nitro moiety and consequent release of nitric oxide (Fig. 26B). Moreover, the presence of the –SH and –OH functional groups, which easily react with biological substrates, could explain why it is so difficult to isolate this unstable TP053 active metabolite.

![Image of mass spectra and chemical structure](image)

Figure 26. Identification of the putative TP053 active metabolite.  
A. Mass spectra of the analyzed metabolite obtained from *M. tuberculosis* cells over-expressing *Rv2466c*; B. Chemical structure of the putative metabolite.

4.2.2 The mechanism of TP053 action involves NO release

Thanks to data obtained by our collaborator Dr. V. Makarov and considering that TP053 is characterized by a NO\textsubscript{2} moiety, it was
hypothesized that NO release could represent one of mechanism of action of the activated TP053.

In order to demonstrate the aforementioned hypothesis, firstly the NO release was evaluated in the in vitro reaction of Rv2466c enzyme with TP053. NO\textsubscript{x} release was determined by using Griess reagent and in presence of DTT and \textit{M. smegmatis} extracts, to allow the TP053 activation. To demonstrate that NO\textsubscript{x} release is only due to the activated drug, as control, the same reaction was performed in absence of TP053, or in absence of DTT and \textit{M. smegmatis} extracts which prevent Rv2466c activation. Interestingly, during the Rv2466c in vitro reaction, there was a production of NO\textsubscript{x}, which correlates with consumption of the TP053 substrate of the enzyme, thus confirming that Rv2466c activation causes a release of nitric oxide (Fig. 27).

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure27.png}
\caption{Determination of NO\textsubscript{x} released by in vitro reaction of Rv2466c with TP053.}
\end{figure}

For this reason, the cytotoxic effects of TP053 related to a release of nitric oxide driven by Rv2466c-activation, have been investigated using \textit{M. bovis} BCG cell extracts. In this experiment, \textit{M. bovis} BCG cells were treated with 1 \textmu g/mL (Fig. 28A) or 8 \textmu g/mL (Fig. 28B) of TP053 and NO\textsubscript{x} release in the culture broth was evaluated by Griess reagent.
Results and discussion

Figure 28. NOx determination in *M. bovis* BCG cultures treated with TP053, 026 and PA-824 compounds.
A. Treatment with 1 μg/mL of each compound; B. Treatment with 8 μg/mL of each compound.
Results and discussion

The same reaction was performed also using the 026 compound with a different mechanism of action, as negative control, and the nitroimidazole PA-824, as positive control.

These data demonstrated that TP053 metabolism is correlated with NO release (Fig. 28). However, the observed release of NO\textsubscript{x} is lower than that found with PA-824, thus suggesting that other mechanisms of action could occur.

4.2.3 The cytotoxic effects of TP053 globally affect the mycobacterial growth

To point out the pathways affected by the TP053 action, a meta-proteomic approach was used in collaboration with Prof. E. Marengo (University of Piemonte Orientale, Alessandria, Italy). This strategy had the goal to evaluate the changes in the proteomic profile in \textit{M. tuberculosis} cell extracts treated with TP053. Data obtained by proteomic analysis were examined using the database “NCBI \textit{Mycobacterium tuberculosis} RefSeq (3910 proteins)”. It was shown that the activated TP053 causes a global perturbation of mycobacterial metabolism, rather than affecting a specific cellular target, further confirming that TP053 has different mechanisms of action (Fig. 29). In particular, it was found that the protein synthesis is the main pathway affected, but the amino acids, RNA and mycolic acids biosynthesis were also influenced. It remains to be clarified if and which protein(s) directly bind(s) to the activated TP053.
Results and discussion

4.2.4 30S ribosomal protein S13 directly interacts with the activated TP053

In order to evaluate if there are some protein(s) that, beyond Rv0579, directly interact(s) with the activated TP053, a target fishing experiment, using 6119 (0.25 μg/mL, 2X MIC), was performed in M. bovis BCG cells. M. bovis BCG cell extracts were treated with the 6119 compound-Azide-PEG3-biotin conjugate adduct (see “Materials and Methods”), and incubated with the Streptavidin Agarose resin. Data obtained by the proteomic analysis (in collaboration with Prof. E. Marengo, University of Piemonte Orientale, Alessandria, Italy) of the reaction products revealed that, among the identified proteins, only the 30S ribosomal protein S13 was constantly found in three independent replicates (Fig. 30). This result suggested a direct interaction between this protein and the activated TP053. Moreover, this result is in support of the previous observation that the protein synthesis is the main pathway affected by the toxic effect of TP053.
Results and discussion

Further ongoing experiments aim to clarify TP053 metabolism, and to understand better the role of 30S ribosomal protein S13 in the mechanism of action of TP053.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0A0K2I1L2_MYCBI</td>
<td>30S ribosomal protein S13</td>
</tr>
</tbody>
</table>

Figure 30. Target fishing experiment.

Part II: The thiophenecarboxamide derivatives 7947882 and 7904688

4.3 Background

The 594 compounds belonging to NIAID (National Institute of Allergy and Infectious Diseases) library were tested for their activity against *M. tuberculosis* H37Rv growth. Two hit compounds were selected: the 5-methyl-N-(4-nitrophenyl)thiophene-2-carboxamide (7947882) and a 3-phenyl-N-[(4-piperidin-1-ylphenyl) carbamothioyl]propanamide (7904688) (Figure 16). These two thiophenecarboxamide derivatives, both with an MIC value of 0.5 μg/mL, showed to be active against *M. tuberculosis* H37Rv in its growing, latent and intracellular states and to be not toxic (Mori et al., 2015). The isolation of *M. tuberculosis* resistant mutants revealed the presence of mutations in two main genes:
Results and discussion

- different point mutations in *ethA* (Rv3854c) gene, coding the monooxygenase EthA, responsible for the activation of ethionamide (ETH) (Mori *et al.*, 2015; Baulard *et al.*, 2000);
- and/or the mutation T557G (Val186Gly) in *pyrG* (Rv1699), an essential gene coding the CTP synthetase PyrG, responsible for the ATP-dependent amination of UTP in CTP (Mori *et al.*, 2015; Endrizzi *et al.*, 2004; Sassetti *et al.*, 2001).

Biochemical and genetic studies demonstrated that the EthA monooxygenase is the activator of the two prodrugs 7947882 and 7904688. In particular, EthA performs the oxygenation of the thiophene moiety of 7947882, producing S-dioxide and S-monoxy derivatives. The S-dioxide metabolite of 7947882, named 11426026 (Fig. 31), has been isolated and identified by mass spectrometry analysis. The re-synthesized 11426026 showed inhibitory activity against PyrG, behaving as a competitive inhibitor toward the ATP-binding site of the enzyme (Mori *et al.*, 2015). Moreover, through a metabolomic approach, it was demonstrated that the inhibition of PyrG led to a global deregulation of the nucleotide metabolism, establishing PyrG as a novel antitubercular drug target (Mori *et al.*, 2015).

![Figure 31. EthA-activation of 7947882 with the production of the active metabolite 11426026 (Mori *et al.*, 2015).](image)

4.3.1 Validation of the pantothenate kinase PanK as second cellular target of 7947882 and 7904688 prodrugs

In order to better characterize the mechanisms of action and of resistance of 7947882 and 7904688, new resistant mutants of *M. tuberculosis* have been isolated, showing cross-resistance to both compounds and to the active metabolite 11426026 (Table 10). Their Illumina whole genome sequencing analysis revealed the presence of the same mutation A620G (Q207R) in *coaA* gene, coding the pantothenate kinase PanK. This enzyme is responsible for the conversion of the
pantothenate in 4’-phosphopantothenate, which is the first step of the Coenzyme-A biosynthesis (Jackowski and Rock, 1981; Evans et al., 2016). One resistant mutant harbored an additional mutation in ethA gene (82.21 mutant; Table 10).

<table>
<thead>
<tr>
<th>M.tuberculosis strains</th>
<th>MIC (µg/mL)</th>
<th>Mutations (aa change)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7947882</td>
<td>7904688</td>
</tr>
<tr>
<td>H37Rv</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>82.21</td>
<td>&gt; 40</td>
<td>&gt; 40</td>
</tr>
<tr>
<td>88.1</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>88.2</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>88.3, 88.4, 88.5, 88.9, 88.11, 88.12</td>
<td>2.5</td>
<td>10</td>
</tr>
<tr>
<td>81.10*</td>
<td>&gt; 40</td>
<td>&gt; 40</td>
</tr>
</tbody>
</table>

Table 10. Features of M. tuberculosis mutants resistant to 7947882 and 7904688. *Laboratory collection.

These results suggested an involvement of PanK in the mechanism of resistance to both prodrugs, probably acting as a second target.

To demonstrate the aforementioned hypothesis, by an enzymatic assay it was shown that the active metabolites of both prodrugs (included 11426026) are able to bind to PanK, like PyrG. In addition, by a docking experiment it was demonstrated that the active metabolite 11426026, as well as the activated prodrugs, inhibit PanK by binding at its ATP binding site, as for PyrG (Chiarelli et al., submitted).

Finally, by microbiological, biochemical and in silico techniques, it was shown that PanK is a second target of 7947882 and 7904688 prodrugs and of the active metabolite 11426026, establishing them as multitargeting compounds targeting both PyrG and PanK (Chiarelli et al., submitted).
4.3.2 Identification of new multitargeting antitubercular compounds

Taking into account our finding of new multitargeting compounds targeting PyrG and PanK, we would like to develop a phenotypic target-based screening in order to select other multitargeting agents affecting both targets. To do it, two different approaches were used:

- *in silico* virtual searching;
- target-based screening

For the virtual screening, a similarity search based on the 4-nitroanilide moiety of 11426026 was performed on the CDD (Collaborative Drug Discovery) database. Twelve potential molecules with known antitubercular activity were selected as potential ligands of PyrG (Mori *et al.*, 2015). Among them, CDD-823953 only showed to be active against PyrG and to be a weak inhibitor of PanK (Fig. 32). The CDD-934506 showed a moderate inhibitory activity only against PanK (Fig. 32).

![Figure 32. IC₅₀ determination against PanK for the novel inhibitors. CDD-823953 (▼), CDD-934506 (●) and GSK-735826A (■).](image)

For the target-based screening, the GSK (GlaxoSmithKline) TB-set library of antitubercular compounds (Ballell *et al.*, 2013) was exploited. Among these compounds, three (GSK735826A, GSK1570606A,
Results and discussion

GSK920684A) were found to inhibit PyrG, with a good activity against *M. tuberculosis* H37Rv growth (Table 11). Unfortunately, it was demonstrated that only GSK735826A is also an efficient PanK inhibitor (Fig. 32).

In order to better characterize the mechanism of action of these three GSK compounds, several attempts in the isolation of *M. tuberculosis* spontaneous mutants resistant to them were performed, without success.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> strain</th>
<th>MIC (µg/mL)</th>
<th>GSK735826A</th>
<th>GSK1570606A</th>
<th>GSK920684A</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Rv</td>
<td>0.5</td>
<td>5</td>
<td>2.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 11. MIC values of the three GSK compounds identified from the GSK TB-set.

Thanks to all collected data, CDD-823953 and GSK735826A can be considered as new multitargeting compounds affecting both PyrG and PanK. These two enzymes can be used as a valid tool in order to identify other new promising antitubercular multitargeting compounds.

4.3.3 Identification of the putative active metabolite(s) of the 7904688 prodrug

Differently from the 7947882 compound, it was not possible to identify the *in vitro* EthA active metabolite(s) of 7904688. Thus, a metabolomic approach, using *M. tuberculosis* cell extracts treated with 7904688 (1 µg/mL, 2X MIC) was used. The *M. tuberculosis* cell extracts were analyzed by metabolomics using the GC-MS (full scan) technology with liquid injection (in collaboration with Prof. E. Marengo, University of Piemonte Orientale, Alessandria, Italy).

From the metabolomic analysis, it emerged that 7904688 is presumably an unstable compound. Indeed, the analysis of the *in vitro* reaction containing only the molecule led to the identification of two different catabolites: the phenyl-proprionamide and the amino-phenyl-piperidine (Fig. 33).
Results and discussion

Moreover, from the complete metabolomic reaction in presence of *M. tuberculosis* cell extracts, two possible metabolites, which could derive from EthA activation of the original 7904688, were identified: the acrylamide-NN-methyl ester (Fig. 34) and the proprionamide (Fig. 35).

However, further studies are required in order to demonstrate that one of them (or both) is (are) the active metabolite(s) of 7904688. The experiments in order to test their antitubercular activity and their inhibitory action against the two targets PyrG and PanK are in progress.

Figure 33. Catabolites of 7904688 prodrug.
Figure 34. The putative active metabolite acrylamide-NN-methyl ester. A. Ctrl; B. Drug Metabolite; C. Drug; D. Chemical structure of acrylamide-NN-methyl ester.
Figure 35. The putative active metabolite propionamide. 
A. Ctrl; B. Drug Metabolite; C. Drug; D. Chemical structure of propionamide.
5. Conclusion

Tuberculosis (TB) has critically influenced the human population throughout its history (Hopewell et al., 2016). Despite the introduction of the four-drug (INH, RIF, PZA and ETH) treatment regimen forty years ago, TB is still cause of high morbidity and mortality worldwide (Zumla et al., 2013a). Moreover, the HIV epidemic, the spread of M. tuberculosis drug resistant strains and the need to kill the bacillus both in its latent and active conditions have emphasized the urgency of novel antitubercular compounds (Glaziou et al., 2015; AlMatar et al., 2017).

An efficacious and promising strategy to achieve these goals is represented by the prodrug design. This strategy allows overcoming the several limitations that a drug may have, like poor aqueous solubility, toxicity, high first pass metabolism, poor bioavailability, chemical instability and incomplete absorption (Piplani et al., 2016). In addition, the prodrug design depicts a faster strategy than searching for a completely new therapeutic chemical agent with desired ADMET features (Jornada et al., 2015; Huttunen et al., 2011).

In recent years the number of antitubercular prodrug candidates in preclinical trials has grown incredibly. The antimycobacterial prodrugs can be activated through mycobacterial metabolic activation or through host activation (Mori et al., 2017; Vale et al., 2013). The mycobacterial bioactivated prodrugs have the advantages to improve bioavailability and solubility and to reduce toxicity. On the other hand, the host bioactivated prodrugs have the great advantage to improve most of the ADMET properties (Mori et al., 2017). However, both prodrug classes present some limitations. First of all, mutations in the gene coding for the activator of the mycobacterial bioactivated prodrugs confer drug-resistance, representing the biggest problem related to these compounds (de Campos et al., 2104). Secondly, both types of prodrugs have a risk of toxicity related to the production of unexpected metabolites (Vilchèze and Jacobs, 2014). Nevertheless, their importance in the fight against tuberculosis remains uncontested.

The main aim of this thesis is focused on the study of the mechanism of action and of resistance of three new and promising mycobacterial
bioactivated prodrugs: the thienopyrimidine TP11126053 and the thiophenecarboxamides 7947882 and 7904688.

TP053 is a new thienopyrimidine derivative activated by the mycoredoxin Rv2466c (Albesa-Jové et al., 2014; Albesa-Jové et al., 2015). This activator is a homodimer, which has been recently characterized as a DsbA-like mycoredoxin essential for mycobacterial persistence under oxidative stress (Rosado et al., 2017). Despite the thorough characterization of Rv2466c, its precise mechanism for TP053 bioactivation is still unknown, as well as the actual active form of the compound. It has obviously complicated the understanding of the mechanism of action of TP053.

In order to discover the mechanism of action of the compound, the isolation of M. tuberculosis TP053 resistant mutants was performed. The sequencing of the genome of a single mutant, named 53.8, led to the identification of mutations in three different genes: Rv0579, Rv0158 and extxD. A second attempt in the isolation of drug-resistant mutants, using a strain over-expressing the activator Rv2466c, confirmed only the presence of the same mutation (Leu240Val) in Rv0579. This gene encodes a conserved hypothetical protein of unknown function, that is not essential for mycobacterial growth. Moreover, a recombineering-mediated point mutagenesis strategy further confirmed that the aforementioned mutation in Rv0579 gene is associated with TP053 resistance.

To characterize the role of Rv0579 in TP053 resistance and to unveil its physiological role, the recombinant Rv0579 protein was expressed and purified in Escherichia coli. Rv0579 protein was used to perform a target fishing experiment in vitro, demonstrating that Rv0579 directly interacts with the Rv2466c-activated TP053. However, Rv0579 role in TP053 resistance remains unknown. The lack of information about its physiological function makes more difficult the understanding of its possible role into TP053 metabolism. For this reason, further studies are needed to clarify its function.

The discovery of the active metabolites of a prodrug is another fundamental step to elucidate its mechanism of action and, in a second time, to improve its chemical properties.

The identification of the putative TP053 active metabolite performed by our collaborator Dr. V. Makarov suggested that NO release could be the
main mechanism of action of this compound. Combining biochemical, metabolomic and proteomic approaches, we demonstrated that the activated TP053 globally affects the mycobacterial growth by NO release. Indeed, it emerged that the principal influenced pathway is the protein synthesis, supported by the finding that the 30S ribosomal protein S13 presumably interacts directly with the activated TP053. Other affected pathways are the amino acids, RNA and mycolic acids biosynthesis. The involvement of the protein synthesis could partially explain the role of Rv0579 in TP053 resistance. In fact, this protein is characterized by an RNase domain, which could probably act affecting the protein metabolism, representing a secondary effect of the activated TP053.

Further ongoing experiments should shed light into the precise mechanism of action of this promising antitubercular agent.

The last two produgs 7947882 and 7904688, object of this work, came from the phenotypical screening of NIAID library. They are activated by EthA monooxygenase and targeted the CTP synthetase PyrG.

EthA is a NADPH-specific flavin adeninedinucleotide (FAD)-containing monooxygenase, encoded by Rv3854c gene (Vannelli et al., 2002; Fraaije et al., 2004). This enzyme catalizes a typical oxidation reaction, the Baeyer-Villiger oxidation reaction (Fraaije et al., 2004). Although the physiological function of EthA is still not clear, it has been observed that the enzyme possesses a wide cluster of substrates. EthA is known to be the activator of the prodrug ethionamide (ETH), as well as of the thiocarbamide-containing drugs like thiacetazone, thiocarlide, prothionamide, thiobenzamide, and isothionicotinamide(Vannelli et al., 2002; Fraaije et al., 2004).

In the mechanism of action of 7947882 and 7904688, some points needed to be better elucidated. For this reason, a new attempt in the isolation of M. tuberculosis mutants resistant to both produgs was performed, revealing the presence of the same mutation in coaA gene. This finding suggested an additional role of the panthotenate kinase PanK in the mechanism of resistance and action of these two produgs. Biochemical and in silico tecniques demonstrated that PanK is the second target of 7947882 and 7904688, bringing to light that these produgs are two multitargeting compounds.
Conclusion

The importance of this result must be attributed to the fact that the multitargeting drugs (known as single compound that inhibits simultaneously more than one target) represent a valid tool in the control of drug resistance. In particular, this kind of compounds showed to be effective in monotherapy, thus avoiding the need of combinatory treatment (Silver, 2007; Morphy, 2012). The mechanism of action of the multitargeting drugs can include the following different types of inhibition:

- “series inhibition” means that the targets belong to the same metabolic pathway;
- “parallel inhibition” means that the targets do not belong to the same pathway, but the drug is able to reproduce a common substrate;
- “network inhibition” means that many series and/or parallel targets are affected by the compound (Fig. 36) (Li et al., 2014).

![Different types of inhibition of multitargeting compounds](image)

**Figure 36. Different types of inhibition of multitargeting compounds** (Li et al., 2014).

Therefore, a phenotypic target-based screening was developed with the aim to identify new multitargeting agents affecting both PyrG and PanK. Exploiting the use of *in silico* virtual searching and target-based screening approaches, a number of potential multitargeting compounds have been identified. Among them, the CDD-823953 and GSK735826A have been validated as multitargeting compounds affecting both PyrG and PanK activities. In this way, these two mycobacterial enzymes have been established as a precious “double-tool” in the discovery of new antitubercular drugs.
Conclusion

Finally, we took advantage of a metabolomic approach to attempt the identification of the active metabolite(s) of 7904688. This technology helped us to understand that most probably this prodrug has an unstable chemical structure, explaining why it was so difficult until now to isolate its active metabolite(s). Moreover, two putative active 7904688 metabolites, the acrylamide-NN-methyl ester and the propionamide, have been identified, even if their antitubercular activity and their enzymatic inhibition still must be proven.

In conclusion, this work demonstrated the enormous potentiality of the prodrug design as a powerful strategy in the fight against tuberculosis. Nevertheless, further steps are needed to improve this tool in order to obtain novel efficient antitubercular compounds, which are able to arrest the spread of *M. tuberculosis* drug-resistant strains and to kill the tubercle bacillus in its active and non-growing states.
6. References


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References


References


References


References

References

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References


References


References

7. List of originals manuscripts

Full papers


Papers obtained from collaboration with other research lines

List of original manuscripts


### Posters


List of original manuscripts


New and Old Hot Drug Targets in Tuberculosis

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Department of Biology and Biotechnology Lazzaro Spallanzani, University of Pavia, Pavia, Italy

Abstract: Tuberculosis is an infectious disease caused by the bacterium Mycobacterium tuberculosis. The World Health Organization publishes global tuberculosis reports annually in order to provide the latest information in the surveillance of drug resistance. Given the alarming rise of resistance to antibiotic drugs worldwide, finding new cellular targets and developing new analogues or new compounds with greater potency against already known targets is both important aspects in fighting drug-sensitive and drug-resistant M. tuberculosis strains. In this context, the introduction of the phenotypic screens is an efficient tool for the identification of active compounds. For tuberculosis drug discovery has improved the possibility to find new effective targets.

With this review we describe the state of art of the currently well validated antibacterial drug targets as well as the advances in discovery of new ones. The main targets will be discussed starting from the oldest such as the enoyl reductase InhA which is currently superposed with new inhibitors, through the well assessed targets like the gyrase, the ATP synthetase or the RNA polymerase, up to the hot promising targets deeply phosphoryl D-ribosyl oxidase DpoEi and the mycophenolic acid transporter MepA3, or the newly validated and promising targets like the CTP synthetase.

Keywords: Tuberculosis, antibacterial drugs, repurposed targets, promiscuous targets, phenotypic screening, drug design.

1. INTRODUCTION

Tuberculosis (TB), the second most damaging among the human infectious diseases, is still a major concern to global public health. In 2014, TB killed 1.5 million people and 9.6 million people were affected by TB worldwide [1, 2]. Moreover, the World Health Organization (WHO) reported that the 3.3% of new tuberculosis cases and the 20% of the previously treated cases had multidrug resistant TB (MDR-TB).

The recommended regimen for drug-sensitive tuberculosis was established four decades ago and is still highly effective, it includes first-line drugs such as isoniazid and rifampicin for 6 months, together with pyrazinamide and ethambutol for the first 2 months. Nevertheless, the major threat is represented by the spread of MDR-TB, resistant at least to rifampicin and isoniazid. These cases are treated with second-line drugs such as new fluoroquinolones (e.g. moxifloxacin or levofloxacin), in combination with an injectable drug (aminosalen, kanamycin, or capreomycin) [1]. Notwithstanding, 9.7% of MDR-TB has been reported as extensively drug resistant TB (XDR-TB), resistant to first-line drugs and to at least one fluoroquinolone and to one second line injectable drug in 105 countries [1, 2]. The need of new antibacterial drugs is thus considered a priority and new agents have entered clinical trials after a long period of stagnation (Table 1).

Among these, many are new inhibitors of old repurposed targets, such as new fluoroquinolones and new rifamycins, targeting DNA gyrase and RNA polymerase, respectively (Table 1) [2]. However, new drug
Table 1. New antimycobacterial drugs in preclinical and clinical trials.

<table>
<thead>
<tr>
<th>Preclinical development</th>
<th>CLP technology</th>
<th>Phase I</th>
<th>Phase II</th>
<th>Phase III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB4-166 (Eptifibatide)</td>
<td></td>
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<tr>
<td>QVD-151 (Capanevacat)</td>
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<td>SQ461 (Capanevacat)</td>
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<td>1699 (Specticamide)</td>
<td></td>
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<tr>
<td>SQ29 (Markelamide)</td>
<td></td>
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Clinical development

<p>| | | | |</p>
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</tr>
</thead>
</table>

- Semalunol (Semalunol)
- Bepotastone NMC-207 (Bepotastone) for MDR-TB
- SQ116
- Enzyme for EB-TB
- High-dose of D-Ergotoxin for Drug Resistant-TB
- Delamanid OPC-67483 (Delamanid) for MDR-TB
- Bedaquiline (Bedaquiline-Pyrroloquinoline Oxidoreductase-Pyrazinamide combination)
- Pretomanid (Pretomanid)
- Bedaquiline (Bedaquiline-Pyrroloquinoline Oxidoreductase-Pyrazinamide combination)
- Levofloxacin for MDR-TB


candidates should be characterized by new mechanisms of action in order to prevent cross resistance.

Recently, new drug targets were identified and some of their inhibitors have entered clinical development. Some of these drug candidates comprised benzothiazolones (PF232443 and PBT21099) and SQ299, inhibiting the two promiscuous targets DprE1 and ManL3, respectively [3-5], and bedaquiline/TMC 207, a dihydrofolate reductase in 7B phase of clinical trials which inhibits ATP synthase [6]. Based on the results of phase 2, bedaquiline received an accelerated approval for MDR-TB by Food and Drug Administration in 2012 [7]. Unfortunately, its use in the treatment of MDR- and XDR-TB is linked to an increased number of unexplained deaths and to an abnormal heartbeat (Long QT syndrome) [2, 3]. Another drug which received conditional approval for MDR-TB by the European Medicines Agency in 2014 is delamanid/OPC 67483, a nitramidazole inhibiting mycolic acid biosynthesis [8]. However, delamanid has been related to prolonged QT and to potential central nervous system (CNS) toxicity; when used in combination with isoniazid or fluoroquinolones during MDR-TB therapy [2, 10]. In addition, mutations in M. tuberculosis genome causing resistance to bedaquiline and delamanid have been recently documented [11].

For these reasons and even though new antimycobacterial drugs are available, TB drug discovery research must continue in order to fight the spread of drug resistance. In this context, particular attention should be given not only to established targets, but also to new targets that have recently emerged.

Herein, we present a review of the main known cellular targets inhibited by antimycobacterial compounds, going from the first ones characterized (e.g. InhA), up to the latest discovered (e.g. PyrD), with particular focus on their use in drug design strategies.

2. OLD REPURPOSED TARGET

Among the new compounds under clinical evaluation, some of them were developed as derivatives of already known antimycobacterial drugs and have now been repurposed for TB treatment because of the essentiality and the vulnerability of their targets: InhA, RNA polymerase and DNA gyrase.

Isoniazid (isonicotinic hydrazide, INH) is a drug whose activation is mediated by the M. tuberculosis catalase-peroxidase KatG enzyme, leading to the formation of an INH-NAD adduct. This adduct is able to inhibit the NADH-dependent αketoacyl-ACP reductase (encoded by rkaD gene) of the fatty acid synthase type II system, involved in mycolic acid biosynthesis. InhA is considered an ideal drug target, since its inhibition directly results in cellular death [12]. M. tuberculosis INH-resistant strains harbour mutations in the katG or rkaD genes. Consequently, pan- InhA inhibitors that do not require KatG activation are needed.
Rifampicin (RIF) is another first-line multitubercular compound active against both replicating and non-replicating M. tuberculosis strains. It targets the β subunit of the RNA polymerase encoded by rpoB gene [13].

Among the second-line drugs, the most used are the fluoroquinolones class of compounds (e.g., moxifloxacin and gatifloxacin), they target the DNA gyrase, a tetramer of two A and two B subunits, encoded by gyrA and gyrB genes, respectively [14].

InhA, RNA polymerase and DNA gyrase cellular targets are treated in detail in the next paragraphs.

2.1. Trans-2-Methyl-2-Butyl Carrier Protein Reductase (InhA)

InhA is an essential enzyme identified as the cellular target of INH and of the prodigiosin antibiotic (ETAX) (Fig. 1) [15]. INH and ETH inhibit mycolic acids biosynthesis causing accumulation of long-chain fatty acids; inhibition of C24 and C26 monounsaturated fatty acid bioynthesis and cell death [10]. INH is activated by the KatG peroxidase, thus forming an imidazolyl anion or radical [17] that reacts with NAD⁺ producing the INH-NAD adduct, which in turn binds inhA [18]. InhA belongs to the fatty acid synthase type II (FASII), which elongates fatty acids up to 56 carbons to form mycolic acids, and catalyzes the NADH-dependent reduction of 2-trans-alkyl-ACP molecules with 16 or more carbons during the last step of fatty acid elongation [19].

The INH-NAD adduct inhibits InhA, blocking mycolic acid biosynthesis then causing cell death.

Whereas the majority of the M. tuberculosis INH-resistant mutants show mutations in the inhA gene, encoding for the activator, about 25% of the M. tuberculosis INH-resistant clinical isolates display Ser944Ala and Ser657Thr mutations in InhA. These mutations cause INH resistance by decreasing the affinity of InhA for the NADH and for the INH-NAD complex [16].

The first InhA crystal structure was solved in complex with NADH (Fig. 2A) [19]. The InhA enzyme is composed of seven β-strands and eight α-helices and it has a core structure similar to the dinucleotide-binding fold of many dehydrogenases. The NADH cofactor binding site is in a pocket between the back and the side of the InhA enzyme and its structure suggests that recognition of NADH is mediated by interactions with an array of polar amino acids and backbone atoms. In the InhA wild-type structure, the NADH phosphate forms hydrogen bonds with the main-chain nitrogen atom of the leu21 and with a well-ordered water molecule. The crystal structure of the Ser944Ala mutant revealed that the water molecule is disordered and forms only one hydrogen bond with the NADH phosphate, resulting in a fivefold reduced affinity for NADH compared to that of the wild-type strain, thus accounting for INH resistance [19].

ETH (Fig. 1) is a prodigiosin activated by the NADPH-specific flavin adenine dinucleotide-containing monooxygenase EthA [20]. ETH active form reacts with NAD⁺ yielding an ETH-NAD adduct, which in turn binds inhA, causing the inhibition of mycolic acid bioynthesis [21]. Similarly to INH, mutations in the gene encoding for the EthA activator conferred...
high level of resistance to this drug [22]. In order to solve this problem, new drugs targeting InhA and avoiding Rv0605c or EnoA activation are required.

Some of the new InhA inhibitors were obtained starting from thiocoumarin (TRC), an uncompetitive InhA inhibitor with a low in vitro activity against M. tuberculosis (Minimum Inhibitory Concentration, MIC = 3.10 mg/ml) [23]. Among the obtained derivatives, chelate chemistry allowed the synthesis of 1-dioctyl-4-phenyl-1H-1,2,3-triazole by replacing the phenolic moiety with 1,4-dihydroxybenzene. This derivative displayed MIC values against M. tuberculosis H37Rv lower than 2 mg/ml [24]. A new series of triazole derivatives have been synthesized showing analogues with MIC values [25]. All of them inhibited M. tuberculosis growth and had a good activity against InhA enzyme, even if the best compound (MIC = 0.6 mg/ml) was totally inactive against InhA. Recently, new TRC analogues were obtained with specific modifications in its 5 and 4 positions and seven of the obtained derivatives were highly active against M. tuberculosis, compared to TRC. Among them, the most active, the 4-[(4-butyryl)-1,2,3-triazol-1-yl]pyridone, had an MIC of 0.6 µg/ml and inhibited InhA activity [26].

Pyridoxycin (Fig. 1) is one of the most interesting and promising among the new InhA inhibitors. It is a natural compound produced by Erythrosporangium sphaericum, very active against M. tuberculosis growth (MIC = 0.31 mg/ml). M. tuberculosis pyridoxycin resistant mutant harbour the ArgL-Arg26 mutation in InhA, but it is not cross-resistant to INH and ETH. In the same way, the M. tuberculosis INH resistant mutant carrying the Ser94Asp mutation is susceptible to pyrdoxycin. This finding suggested that INH and pyridoxycin bind the InhA active site in different ways. In fact, structural studies demonstrated that pyridoxycin inhibits InhA binding the core of the enzyme active site, simultaneously blocking part of the NADH and fatty acyl lipid binding pockets. These findings allowed the discovery of a new druggable pocket in the InhA enzyme [27], thus giving hope in pyridoxycin development. In 2013 new pyridoxycin derivatives were synthesized, lacking the characteristic enol ether moiety of the hit compound, and leading to a four-fold more active compounds [28]. Through in vivo screening based on the succinamidone core fragment, a new promising pharmacophore moiety was identified in the 3-[3R-(4-fluorophenyl)-8-yl]pyridin-3-yl]pyridazino-3,5-dione (Fig. 1) [29]. These new derivatives displayed an interesting activity against InhA, with the most active 3.5-dichlorophenyl-3,5-dione (MIC = 4.7 µM) being also effective against M. tuberculosis INH resistant clinical isolates [29].

Another class of direct InhA inhibitors was identified in the methyl triazolone scaffold. The best compounds of this new triazolone series showed potent enzyme inhibition (InhA IC50 = 0.003 µM) and good MIC against M. tuberculosis both in vitro (MIC = 0.19 µM) and in vivo (MIC = 1 µM), although being less potent than INH (Fig. 1). The InhA inhibition mechanism was investigated. Most of the InhA inhibitors, like TRC bind the NAD-InhA complex, whilst this last class of compounds binds tightly (Kd = 13 ± 7 nM) to the NADH-bound enzyme forming a ternary complex [30, 31]. Structural studies of InhA in complex with a methyl triazolone revealed new interactions between the compound and the enzyme active site [30]. Precisely, the compound forms hydrogen bonds between the thiazole ring and the Met98, and between the triazolone ring and the ribose moiety of NADH [30]. This information has been exploited to produce improved analogues.

Among the new classes of direct InhA inhibitors, the 4-hydroxy-3-pyridones were recently identified through a phenotypic high-throughput whole-cell screening [33]. These compounds had potent bactericidal activity against several M. tuberculosis INH resistant clinical isolates. They do not require Rv0605c activation, and they have an in vitro frequency of spontaneous resistant mutants 100 times lower than that of INH resistant mutants. The NITD-916 lead compound (MIC = 0.05 µM; Fig. 1) preferentially binds the NADH-InhA complex, similarly to methyl triazolone. Since InhA has a higher affinity for NADH rather than for NAD, the inhibitors binding the NADH-InhA complex are likely to be more efficient. NITD-916 shows good efficacy in vivo, paving the way for further development of inhibitors of a well-assessed old target [32].

2.2. DNA Polymerase (RNAP)

Rifampicin (RIF) (Fig. 3), the major frontline antimycobacterial agent together with INH [33], belongs to the rifamycin class of DNA-dependent RNA-synthesising inhibitors. RIF is one of the most potent and broad spectrum antibiotic, it blocks DNA transcription through the inhibition of the RNA-polymerase (RNAP) with a very high affinity for bacterial enzymes (binding constant about 10⁻⁶ vs 10⁻¹⁰ M of herpamylov RNA-polymerase) [34].

Bacterial RNAP has a σ70 subunit composition organized in a shape that is similar to that of a crab claw (Fig 4), with the two "pinchers" formed by the
larger subunits \( \beta' \) and \( \beta \) defining the active site [35]. Structural studies demonstrated that RNAS can adopt several conformations, from a fully open clamp to a fully closed conformation which allows or prevents DNA entry and exit into the active site during the different stages of transcription [36, 37].

![Crystal structure of M tuberculosis INHb in complex NADH (PDB 4DRR).](image)

All the mutations conferring resistance to RIF map in rpoB, an essential gene encoding the \( \beta \) subunit of the RpoB enzyme. These mutations mainly involve the amino acid residue going from position 530 to 535 [38, 39]. Kinetic analysis demonstrated that RIF does not impair the binding of the promoter to the RNAS, the formation of the open complex, or the formation of the first phosphodiester bond, but it blocks the formation of the second or third phosphodiester bond depending on whether the transcription initiated with a nucleoside tri-phosphate or with a nucleoside mono-phosphate instead [40]. RIF does not impair the substrate binding or the catalytic activity, but it was suggested that it simply acts as a steric block of the RNA elongation [40], as demonstrated by the crystallography studies performed on the Thermus aquaticus RpoB [13]. Precisely, RIF binds in a pocket of the RNAS \( \beta \) subunit. This pocket is surrounded by those amino acids that confer resistance to the antibiotics when mutated. When RIF binds to the \( \beta \) subunit, it is in contact with almost 25% of the possibly mutated amino acids, mainly through van der Waals or hydrogen interactions [13]. It is noteworthy that among the clinical isolates resistant to RIF about 80% of the mutations affect only three residues: Asp516, His526 and Ser531 [39].

RIF is commonly used in therapy, but it shows several disadvantages, such as the high frequency of spontaneous resistant mutants and the serious side effects, such as hepatotoxicity at high doses. Moreover, this compound is a strong cytochrome P450 inducer, thus leading to several drug-drug interactions, precluding its use in HIV-infected people [41]. Two further rifamycin compounds, rifapentine and rifabutin, have been approved for TB treatment (Fig. 8).

![Diagram of rifapentine structure.](image)

Although rifapentine shows cross-resistance with RIF, it has a better pharmacokinetic profile with a longer half-life, it is more potent in vivo and useful for shortening TB therapy [42, 43]. Similarly, rifabutin shows improved potency and better pharmacokinetic compared to RIF. It is a very weak cytochrome P450 inducer, thus reducing drug-drug interactions [44] and being suitable also for HIV-infected patients [45]. The most interesting among the newer RIF derivatives is the benzoxazinomycins rifaxan, [46], which combines an improved potency with a very reduced cytochrome P450 induction and greatly low toxicity in early rodent studies [47, 48]. However, clinical trials evidenced adverse effects causing the suspension of the development of rifaxan for TB treatment [41, 49].

The antibiotic mycoxoprin was the first one identified as inhibitor of the "switch region" of RNAS. This region is a structural element at the base of the RNAS clamp comprising five segments (switch 1 to 5) which can adopt different local conformations, serving as a hinge for their opening and closure (Fig. 4). Different residues of the switch region, particularly switch 1, 2 and 3, interact with the DNA, thus coordinating the clamp closure and DNA binding [35].

Structural studies demonstrated that mycoxoprin inhibits transcription by binding a pocket encompass-
ing the switch 1 and switch 2 regions, through interaction with both α' and β subunits, thus locking RNAP in one conformation [37]. The same study demonstrated that two other antibiotics, cordycepin and rifampicin, shared this same target and mechanism of action. All these compounds do not show cross-resistance with rifampicin, since their binding site region does not overlap, thus providing the basis for the development of new different inhibitors of a well assessed target [30].

Soon after, lipiarnycin (also known as tiamadinic B, or fidaxomicin) (Fig. 5) was identified as a RNAP inhibitor targeting the switch region [31, 32]. It has been demonstrated that lipiarnycin inhibits RNAP by blocking the open conformation of the clamp with a mechanism similar to that of the myxopyronin. However, lipiarnycin interacts with the switch region of RNAP by binding the switch 2 and 3, thus only partially overlapping the myxopyronin binding site [32]. For this reason, lipiarnycin does not show cross-resistance neither with myxopyronin nor with rifampicin [30].

Fig. (5) Structure of lipiarnycin compound targeting the RNAP switch region.

Lipiarnycin has been approved for the treatment of Clostridium difficile. It is very active also against M. tuberculosis but it actually shows very low oral bioavailability thus limiting its use in the systemic tuberculosis infection [53]. Moreover, despite its isolation in 1975 [54], no SAR studies have been performed and no total synthesis of lipiarnycin have been reported for several years. In spite of that, synthesis of aglycone lipiarnycin has been recently published, finally giving the possibility to develop RNAP switch region inhibitors [53-57].

2.3. DNA Gyrase

Bacterial topoisomerases are still very attractive targets for drug discovery, and fluoroquinolones are one of the most successful classes of antibacterials targeting type II and type IV topoisomerases [38]. Unlike many other bacterial species, M. tuberculosis only has topoisomerase II, also known as DNA gyrase. This enzyme is an essential heterotetrameric protein consisting of two A and two B subunits, encoded by the gyrA (320 bp) and gyrB (375 bp) genes, respectively [59]. These two subunits control the DNA topological state catalys-
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ing its negative supercoiling through a transient double strand DNA break, thanks to ATP hydrolysis. Precisely, GyrA contains the tyrosine active-site involved in DNA cleavage and in the formation of the protein-DNA covalent bond, whilst GyrB contains the ATPase active site [59]. Fluoroquinolones inhibit topoisomerase by converting the transient double strand DNA breaks into a covalent enzyme-DNA adduct, forming the so-called “cleaved complex”. The two most active fluoroquinolones, levofloxacin and gatifloxacin (Fig. 6), are currently under evaluation as promising first-line therapeutics, being already used for MDR-TB treatment [2, 60, 61]. Most of the mutations associated with fluoroquinolone resistance map in the conserved quinolone resistance determining region (QRDR), comprising both gyrA and gyrB genes. Specifically, the most common mutations identified in M. tuberculosis fluoroquinolone-resistant strains involve the substitution of the residues Asn90, Ser91 and Arg94 in GyrA, or Asn499, Thr500 and Gly501 in GyrB (Fig. 7) [14, 59, 62].

The crystallographic structures of the M. tuberculosis gyrA cleavage core, complexed with DNA and five fluoroquinolones, has been recently described for the first time [63] (Fig. 7). A biochemical assay demonstrated the importance of the fluoroquinolone-derivative complex stability in determining the efficacy of the compound. An efficacy ranking among the different fluoroquinolones has been defined with levofloxacin and its C8 methyl derivative being the most potent among the five analyzed. These new fluoroquinolones were followed by the most potent gatifloxacin, and the less efficient ciprofloxacin and levofloxacin [65]. However, the crystal structures did not evidence specific interactions between the different fluoroquinolones and the gyrase, thus explaining the differences in their in vivo antibacterial activities.

Recently, new drugs targeting DNA gyrase have been discovered. Among these, the amino-pyrazinamides (Fig. 6) were identified from a high throughput screening against the recently explored GyrB ATPase domain of Mycobacterium smegmatis protein [64]. These compounds were shown to specifically inhibit mycobacterial GyrB, with a very weak activity against other Gram-positive or Gram-negative bacteria. Structural studies confirmed the unique and specific interaction present between the compound and the GyrB active site. It differs from that of other known GyrB inhibitors, such as novobiocin, thus rendering amino-pyrazinamides highly pathogen specific [64]. The amino-pyrazinamides class of compounds showed a re-

Fig. 6. Compounds targeting DNA gyrase.
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bust SAR and they were found to be highly bactericidal against both replicating and non-replicating M. tuberculosis. For these reasons, they are considered as a new class with a great potential for further optimization [64].

Another new promising scaffold suitable for ATPase inhibition of Gyrb ATPase is aminopyridine derivatives [65] (Fig. 6). Like the other Gyrb ATPase inhibitors, these compounds are very active also against M. tuberculosis drug-resistant strains, including quinolone-resistant ones, as they target different DNA gyrase activities. The best derivatives, showing antibacterial activities below 1 μM concentration (MIC < 0.1 μM) are efficacious in vivo in an acute murine model of tuberculosis [65].

Starting from thiazolopyridazine urea scaffold, the thiazolopyridazines were designed in order to allow hydrophobic interactions also in the pouch pocket of the Gyrb ATP binding region [66]. Resulting compounds showed good activity in vitro, but were affected by a low solubility, thus needing further optimization [66].

Since Gyrb mutations conferring resistance to fluoroquinolones do not confer resistance to the ATPase inhibitors, the interest in new drugs acting through inhibition of the Gyrb ATPase activity increased. New series were then developed using different strategies: the thiazolo-aminopyridine and the quinolone-aminopyridine, developed by molecular hybridization [67, 68], the pyrrolidinones, obtained by drug-design [69, 70] and the aminobenzimidazole, optimized using structure-guided design and structure-activity relationship (SAR) studies of potency against both Gram-positive and some Gram-negative bacterial species [71, 72] (Fig. 6). Pyrrolidinones optimization led to compounds with significant bactericidal activity against M. tuberculosis in vitro and ex vivo, and moderately active in vivo [69].

The last optimization of the aminobenzimidazole metabolic profile led to the identification of VX-486 [72] VX-486 was very effective against M. tuberculosis drug-sensitive and drug-resistant isolates in vitro (MICs of 0.03 to 0.30 μg/mL and 0.08 to 5.48 μg/mL, respectively), ex vivo, and in vivo. Moreover, this compound showed bactericidal activity also against M. tuberculosis drug-resistant bacterial strain VX-486 was then improved using a phosphate ester prodrug approach. The obtained prodrug (pVX-486) was more potent in vivo and when used in combination with other antibacterial drugs (rifampicin-pyrazinamide and bedaquiline-pyrazinamide) was effective like moxifloxacin [72].

For all these characteristics pVX-486 could be considered a promising starting point for future drug development.

Finally, new classes of gyrases inhibitors are emerging. All of them are characterized by different mechanisms of action and they do not show cross resistance with fluoroquinolones. Among these, the N-linked aminopyridine compounds developed by ActivaZyme exert their inhibition through a single strand cleavage complex [74]. These compounds have a good in vitro and in vivo efficacy, a good pharmacokinetic profile and show sufficient structural diversity for further optimization [74].

Recently, GS1 has identified new topoisomerase II inhibitors characterized by zero mechanism of action. These compounds are called MGIs (Fig. 6) and they showed good in vitro and in vivo antibacterial activity without cross-resistance with fluoroquinolones [73]. The isolated MGIs resistant mutants had mutations in either GyrA or Gyrb, suggesting that the binding to the enzyme occurs in proximity of the fluoroquinolones binding site. This hypothesis has been confirmed by DNA gyrase supercoiling and the cleavage complex assay [75]. These recent compounds further confirm the usefulness of the gyrases as well assayed targets for the development of new antibacterial drugs.

3. NOVEL PROMISCUOUS TARGETS

After the first complete sequencing of M. tuberculosis genome [76] and the introduction of next generation sequencing platforms, whole genome sequencing
(WGS) was widely and intensively used for identification of the cellular target of novel compounds. The screening of several chemical libraries led to a number of new potential drug targets, some of which inhibited by more than one compound or chemical entity [77, 78]. For this reason, these targets are defined as "promiscuous" [78]. The two more representative M. tuberculosis promiscuous targets, the decaprenylphosphoryl-β-D-ribose 2'-oxidase (DprE1) and the transmembrane transporter of trehalose monomycolate (ManL3), are here described.

3.1 Decaprenylphosphoryl-β-D-Ribose 2'-Oxidase (DprE1)

Among the M. tuberculosis drug targets, DprE1 is probably the most promiscuous, being defined also as a "magic drug target" [79]. It has been recognized to be inhibited by more than ten different classes of compounds with antituberculosis activity [4, 80–91], most of them discovered through independent whole cell screening of different compound libraries.

DprE1 is an essential mycobacterial enzyme which works in concert with DprE2 (decaprenylphosphoryl-D-2-keto erythrose reductase) to catalyze the epimerization of decaprenylphosphoryl-β-D-ribose (DPR) to decaprenylphosphoryl arabinose (DPA), a precursor for arabinogalactan biosynthesis [92, 93]. Specifically, DprE1 catalyzes the first part of the reaction, the oxidation of DPR into decaprenylphosphoryl-2-keto-β-D-erythro-pentofuranose (DFX), which can then be reduced by DprE2 into DPA [94].

DprE1 is usually reported as an oxidase since it catalyzes a flavin adenine dinucleotide (FAD)-dependent oxidation, but it should be considered a dehydrogenase instead [95]. In fact, although the enzyme can use molecular oxygen to reoxidize the FAD cofactor, it has been observed that it can also use several organic compounds as electron acceptors [95].

Besides its physiological relevance, the ability of DprE1 to reduce organic compounds for the reoxidation of its FAD cofactor explains the peculiar mechanism of action of its covalent inhibitors.

The first DprE1 inhibitors were the 1,3-benzothiazin-4-ones (BTZs), a class of compounds belonging to a series of derivatives of the antibacterial dicaflamidithiocarbamates [4]. The lead compound from this series, the 8-nitro benzothiazinone BTZ043, is one of the most active antibacterial agents known to date (MIC = 1 ng/ml). BTZ043 is characterized by the presence of an 8-nitro group that is essential for its activity, since the substitution of this group with an amino (BTZ384) or hydroxylamine (BTZ040) led up to 5000-fold loss in potency [4] (Fig. 8A).

The BTZ043 IC50 against DprE1 is only in the micromolar range, but its outstanding antibacterial activity resides in a peculiar mechanism of action. The compound is effectively a pro-drug which, upon the reduction of the nitro to a nitroso group, reacts with a cysteine (Cys387) of DprE1 forming a semicarbazide covalent adduct, irreversibly blocking the enzyme activity [96].

This activation has been demonstrated to be catalyzed by DprE1 itself [95, 96], which uses the nitro group of the BTZ043 to reoxidize its FAD cofactor, thus reducing it to a nitroso. The nitroso group can then readily react with the nearby cysteine 387 of DprE1 active site, forming the covalent adduct (Fig. 8B).

Fig. (8A) Structure of 1,3-benzothiazin-4-ones. (B) Mechanism of activation of BTZ043 by DprE1 itself, with subsequent formation of the covalent semicarbazide adduct between Cys384 and BTZ043.

BTZ043 could also be activated by the cysteine itself through a non-enzymatic reduction of the nitro
group induced by the thioureas [97]. However, the fact that the BZ2043-DpsE1 complex adduct does not occur in the absence of the substrate, is in favor of an enzymatic activation [98].

The broad specificity for the different electron acceptors explains the number of the different chemical entities found to inhibit DpsE1. In the 3 years following the discovery of DpsE1 as the target of the BZs, three independent whole cell screenings of different chemical libraries identified new compounds targeting this enzyme (Fig. 9) [80-82]. All these compounds were characterized by a tri-nitro acridine group and, although less potent than the BZ2043, behave as covalent inhibitors thus showing a similar mechanism of action [95, 99].

Crystalllographic studies finally demonstrated that BZ2043 forms a covalent adduct with the cystine residue of the active site of DpsE1. The crystal structures of M. smegmatis DpsE1, in apo form and in complex with BZ2043 [95] and the crystal structure of the M. tuberculosis enzyme in complex with the nitroanalog CT335 [100] have been published at the same time. In both cases, BZ2043 was found to be allocated in front of the P450 cofactor, forming the expected semicarbazide adduct with the Cys387 (Fig. 10). An additional interaction was found between the side chain of the Lys418, a residue essential for DpsE1 enzymatic activity, and the semicarbazide hydroxyl group [95]. Further key interactions are formed between the tri-nitroacetyl group of BZ2043 and a pocket formed by the His132, Gly135, Lys134, Ala165 and Lys187 residues. In contrast, no particular interactions are present with the tri-nitroacetyl piperidine group of BZ2043. Effectively, S.A.R. studies evidenced the essentiality of the nitro group (R, Fig. 11) and the tri-nitroacetyl group (R2) as well as the sulfamoyl (R3) and oxygen in the diazine ring for the antimicrobial activity of BZ, whereas the spiroacetyl piperidine group (R4) seemed more suitable for discrimination [3, 4].

Despite the outstanding in vitro potency of BZ2043, its efficacy in a mouse model of TB was relatively low, mainly due to the poor solubility of the compound. Moreover, BZ2043 was found to be the substrate of microorganisms, such as the M. smegmatis NADH enzyme, that can transform the compound into its active hydroxylamine or amine form [101]. Further S.A.R. studies are therefore needed in order to improve the P.K.P.D. parameters.

As previously mentioned, all strategies designed to modify the nitro group [4, 102, 103] led to a 500- to 1000-fold decrease in potency. Similarly, a loss of potency with a consequent increase in the MIC values results from modifications of the tri-nitroacetyl group [4], or from modification of the sulfur atoms into sulfonyl or sulfone [104] (Fig. 11). Because of that, all the major efforts used to improve BZs properties have been concentrated in the modification of the tri-nitroacetyl piperidine moiety (R4) (Fig. 11) [3, 105, 106].

PBTZ169, the most attractive DpsE1 inhibitor emerged among the numerous studies performed, was obtained upon the introduction of a cyclohexylmethylpiperazine substituent in the BZ scaffold (Fig. 11). This compound was more effective than BZ2043 in a TB mouse model [3]. The compound improved in vivo potency was mainly attributed to the higher affinity for DpsE1, reflected in a 10-fold reduced MIC, combined with a lower susceptibility to microorganisms [3]. PBTZ169 is also less toxic and is a good candidate to enter clinical trials. It was in fact on December 2015 that the Ministry of Health of the Russian Federation gave permission for Phase I clinical trials of PBTZ169 in Russia. In 2016, NEARMEDIC is planning to conduct human clinical trials (http://www.nearmedic.ru/ en/node/690).

The high tractability of DpsE1 as a drug target is further corroborated by a number of non-covalent, reversible antituberculosis inhibitors found in recent years. In fact, the enzyme has been found to be the target of several non-nitro compounds (Fig. 12), some of them deriving from S.A.R. studies of BZA [99, 102, 103]. However, the majority of these were identified through whole cell screening [84-87], from target based whole cell screening [90], or from structure based approaches [99].

Crystalllographic analysis [81, 87] and molecular docking [93, 85, 86, 89, 107] demonstrated these compound to interact with DpsE1 in a similar way to that of...
the BTZs, despite their structural differences. The crystal structure of DpsE1 in complex with Ty38C [Fig. 13 A] [87] and in complex with TCA1 (Fig. 13 B) [84] showed how the compounds bind the enzyme in the active site, in front of the FAD cofactor, mostly involving the same residues participating in BTZ binding, such as the backbone of the residues 132-134, and the side chain of Asn365 and Lys411. As expected, since there is no formation of covalent adducts, mutations in the Cys334 residue of DpsE1 do not confer resistance to the non-covalent inhibitors. Interestingly, mutations leading to the substitution of the Lys411 were recurrent among the isolated M. tuberculosis mutants resistant to one of the non-covalent DpsE1 inhibitors. These compounds showed very high affinity for DpsE1, with IC₅₀ in the nanomolar range and MIC values higher than that of the BTZs [55-58, 107]. However, none of these DpsE1 inhibitors showed any efficacy in vivo, with the exception of TCA1 and of the aminoles [84, 85]. In fact, differently from covalent inhibitors, for non-covalent inhibitors to be effective in vivo DpsE1 inhibition should be characterized by a prolonged occupancy of the active site [108].

Although a very high number of different classes of compounds are active against DpsE1, this is not sufficient to fully explain the high promiscuity of this enzyme. It has been recently demonstrated that DPA biosynthesis occurs outside the mycobacterial plasma membrane, in the periplasm [109]. Therefore, despite the lack of known export signals, DpsE1 is not located in the cytoplasm but in the periplasmic space, thus avoiding the action of efflux pumps or potential cytosolic inactivation mechanisms. DpsE1 cellular localization makes the enzyme more accessible to the drugs, thus explaining, together with its particular enzymology, the great potential of this drug target.

3.2. Mycobacterial Membrane Protein Large 3 (MmpL3)

Another “promiscuous” drug target is the trehalose monomycolate transporter MmpL3. This 12-transmembrane domain protein (Fig. 14) belongs to the mycobacterial membrane protein large (MmpL) family, a class of the resistance-nodulation-division (RND) efflux pump [102] transporters exporting substrates through a proton export mechanism [110]. Among the 13 mmpL genes identified in M. tuberculosis, mmpL3 is the only one that is essential, whilst mutations in mmpL4, mmpL5, mmpL7, mmpL8, mmpL10, and mmpL11 lead to growth impairment [111]. MmpL3 is involved in the export of trehalose monomycolate (TMMO) [5] and it also has a role in iron uptake being involved in heme transport together with MmpL11 [112].

The first compound described to target MmpL3 is BMD212 (Fig. 15), a 1,5-disoxopyrrole derivative active against several multidrug-resistant M. tuberculosis clinical isolates [113]. Genomic library screening and WGS of M. tuberculosis, M. bovis and M. smegmatis spontaneous resistant mutants lead to the identification of different mutations in mmpL3 gene, thus indicating MmpL3 as target of BMD212 [114, 115]. Moreover, uptake-efflux experiments excluded that the phenotype was associated to BMD212 efflux, thus confirming that the transporter is the target of the compound [114].

From this initial scaffold, several studies were conducted in order to improve the antimycobacterial potency as well as the pharmacokinetic properties [116, 117], leading to improved analogs active in a murine tuberculosis infection model and still targeting MmpL3 [113].

Nearly at the same time, MmpL3 was identified as the target of other two unrelated compounds: the 1,2-diamine SQ109 (Fig. 15) [3], and the thiaminyl urea AT71235 [118, 119]. SQ109 was selected using combinatorial chemistry by screening a chemical library designed around ethambutol (EUB), with the aim to re-
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Fig. (11). Most significant DpyEL inhibitors derived from SAR studies of BT2045.

Fig. (12). Most representative non-coupled DpyEL inhibitors.

Fig. (13). Details of the crystal structure of DpyEL in complex with Ty3BC (A) and 7CA1 (B) molecules (PDB-4P8K and 4KWS).
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![Diagram](image)

Fig. (14). Predicted topology of the MmpL3 protein and localization of the mutations responsible for the resistance to multiple inhibitors or to their analogs. (IC indolenin-bacitracin, AU amikacin and avea, SP sparfloxacin).

visit this well-established drug [120]. Although designed to be an analog of EMB, SQ109 had a different mechanism of action, being also active against M. tuberculosis EMB-resistant strains [121]. SQ109 showed to have and in vivo efficacy, as well as a pharmacokinetic profile better than EMB [121]. At present, SQ109 is in Phase II clinical trials (http://www.newhtdrug.org/glipaline.php) [122], despite its mechanism of action remained uncharacterized for several years.

The isolation of M. tuberculosis spontaneous SQ109 resistant mutants was in all cases unsuccessful. It was only by using less potent analogs of the compound that cross-resistant mutants could be isolated. The WGS of five of these isolated mutants revealed mutations in mmpL3 gene leading to G40R, L50F and A700T mutations, Fig. (14), indicating MmpL3 as the target. Moreover, coexpression biochemical studies demonstrated that SQ109 disrupts the cell wall assembly, leading to an intracellular accumulation of TBM, thus demonstrating the physiological function of the transporter [5]. Metabolic labeling studies and WGS of M. tuberculosis resistant mutants allowed identification of MmpL3 as the target of AC1235 and further confirmed its role in the export of TBM [118].

The high drugability of MmpL5 was demonstrated by a number of further unrelated inhibitors described within the following three years, such as the benzimidazole C215 [82], tetrahydropyrazole[1,5-a]pyrimidine-3-carboxamide (THPP) [123], imidazoles [124, 125] and spironolactones [126] (Fig. 15). Many of them are active in Tb mouse model of infection. MmpL3 mutations causing resistance to multiple compounds are numerous and widely distributed along the protein, demonstrating a large cross-resistance among MmpL3 inhibitors. Noteworthy most of these mutants were present in residues within or near the transmembrane helices 4, 11 and 12, and close to those amino acids probably involved in the proton gradient necessary for the translocation activity of the pump [127].

The resistance levels conferred by these mutations are very different and in several cases quite low, such as 4- to 8-fold increase in MIC for SQ109 [5], up to 16-fold for BM212 [114, 115], 4- to 70-fold for THPP [112] or 8- to 70-fold for indolethiophenes [124]. Moreover, SQ109, BM212 and THPP also showed activity against different pathogens lacking mycolic acids. However, being a lipophilic amine, SQ109 probably leads to membrane disruption and loss of membrane polarization, possibly causing nonspecific host cell toxicity. Finally, BM212, SQ109 and spironolactones are active against non-replicating M. tuberculosis, differently from the other compounds targeting cell wall biosynthesis [128], suggesting for them a different mechanism of action [127, 129].

Recently, it has been recently shown that SQ109 and its analogs are multianalyzing compounds [129]. In addition to MmpL3, they also inhibit MstA and MstQ, two enzymes involved in menaquinone biosynthesis. Moreover, these compounds were shown to act as uncouplers, thus interfering with the pH gradient (σpH) and membrane potential (σm), thus collapsing the proton motive force (PMF) [129].

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Recently, a series of SQ109 analogues in which the ethylenediamine linker was replaced by one, this, or heterocyclic species, was tested against different microorganisms including *M. tuberculosis* and *Trypanosoma cruzi* [130]. Although none of the analogues showed improved potency against *M. tuberculosis*, they were demonstrated to affect both ΔpH and Δψ, targeting also the mitochondrial membrane potential of T. brucei [130]. These additional SQ109 activities explain why this compound is active against non-replicating *M. tuberculosis*, as well as against pathogens lacking mycolic acids. Moreover, the accumulation of TMD should be related to the disruption of PMF, necessary for the activity of the MmpL pump. These details suggest that MmpL3 is only a secondary target of these compounds, accounting for the low level of resistance conferred by MmpL3 mutation to SQ109.

Further studies demonstrated that BMD218, ALU1215 and the indolocarbazoles behave as uncompetitors, disrupting the PMF and having the same proposed mechanism of action [127]. Nevertheless, the findings seem to be in contradiction with the consistent isolation of spontaneous *M. tuberculosis* resistant mutants carrying mutations in MmpL3. The authors suggested that the MmpL3 mutations represent the first response to the compounds, in order to counteract its early toxic effects [127].

Considering these last results, the notion that these compounds target exclusively MmpL3 should be revisited; although a direct inhibition of MmpL3 cannot be completely excluded [127, 129].

4. NEW PROMISING DRUGGABLE TARGETS

Even though several drugs and targets are already available, the worldwide increase in resistance to the first and second-line TB drugs suggests that further drug targets are needed. Several efforts have now led to new potential antibacterial targets, which encompass different metabolism or processes, such as the biosynthesis of coenzyme A or of nucleotides, cellular division or energetic metabolism. A selection of these promising drug targets is here described.

4.1. Pantothene Synthetases (PncC)

Pantothene biosynthetic pathway is essential in prokaryotes, but absent in mammals, and it has been recognized as a promising drug target for the development of antimicrobials [131]. The biosynthesis of the essential precursor of coenzyme A is carried out by four enzymes, PncB and PncE, that realize the D-pantate synthetase, PncD, which participates in the synthesis of β-alanine, and the pantolactone synthetase PncC [131]. The latter of these enzymes is essential for *M. tuberculosis* growth and has been extensively exploited in drug discovery.

The importance of the pantothene biosynthetic pathway has been demonstrated in murine models, where a pantothene auxotroph of *M. tuberculosis*, defective in the *de novo* pantothene synthetase, results in a significantly reduced pathogenicity, protecting mice from the infection [135]. This evidence suggests that pantothene acid biosynthesis is a valuable drug.
target, despite the existence of panthothenate salvage pathways. The growth of this atoxic strain was unimpeded in vitro when enough exogenous panthothenate was added in high amount, thus highlighting the need of using physiological concentration of metabolites in such screens [131]. Nevertheless, the phosphotriester intermediates of the CoA pathway and CoA itself cannot be utilized by most of the bacteria when supplied exogenously. This pathway is therefore likely to be essential not only for the viability of M. tuberculosis but also for that of many more bacteria [131]. Moreover, since this pathway is absent in mammals, PanC has been considered an attractive target for new antituberculosis drug development [131].

The product of PanC, panthothenate, besides being a critical precursor of CoA, is also involved in the synthesis of the acyl carrier protein, essential for fatty acids synthesis, as well as for cell signaling, nonribosomal peptides and polyketides synthesis [131]. PanC catalyzes a double-step reaction to condense D-pantoate with β-alanine in an ATP-dependent manner [133], firstly, D-pantoate reacts with ATP forming a pantoyl-adenylate intermediate, with a consequent pyrophosphate release; then PanC catalyzes the fission of β-alanine with the pantoyl-adenylate intermediate, thus forming ATP and pantotheate [133].

The crystal structure of the enzyme from M. tuberculosis has been solved, showing that PanC consists of two subunits, each of them made by two well-defined domains. The active site is positioned at the N-terminus of the protein, whilst the C-terminus partially masks the active site cavity [134]. Additionally, crystal structures of PanC in complex with AMPCP, an ATP analog, with D-pantoate and with the reaction intermediate pantoyl-adenylate have been determined [134], allowing rational drug design approaches.

There are several strategies that could be used to identify PanC inhibitors [135]. Among them, these are the most identification by high-throughput screening (HTS) [136], fragment-based approaches [137], energy-based pharmacophore modeling [138], Group Efficiency (GE) analysis [139] and synthesis of pantoyl-adenylate analogues, based on the knowledge of the strong interactions present between the intermediate and the enzyme active site [134].

In this context, the last strategy is the most frequently employed. However, despite the interest in PanC, no effective inhibitors of the M. tuberculosis enzyme have been obtained by rational design, and only weak inhibitors have been found by HTS [136, 140, 141]. The first effective PanC inhibitors reported (IC50 0.22 μM) are sulfinylmethyl adenine derivatives (Fig. 16). The crystal structure of PanC in complex with these compounds was solved, allowing elucidation of its binding mode mimicking the pantoyl-adenylate intermediate in the enzyme catalytic site (Fig. 17) [140].

For this reason, the sulfinylmethyl adenine scaffold was considered a useful tool for the design of new inhibitors through rational design and fragment-based approaches. However, neither these compounds, nor their further derivatives are effective against M. tuberculosis growth [135].

![Fig. 16. Sulfinylmethyl adenine derivative effective against PanC activity.](image)

Based on the fact that a non-reactive pantoyl adenylate analog could be an effective inhibitor, several compounds emerged from virtual HTS, molecular levitation and rational design, such as 2-methylimidazole, 1,2,3-triazolines, 1,3-carboxamides [142], thiazolidine derivatives [155], tetrahydrothiophene [153], 1,3-cyclopentanes, 2-carboxylic acids [144], 1-phenyl-4-5,6,7-tetrahydro-1-H-pyrazol-4-3-cyclohexene derivatives [142] (Fig. 18). The majority of these compounds are not active against M. tuberculosis growth, even if some of them are effective against a M. tuberculosis strain with downregulated panC expression [144].

However, all these PanC inhibitors could serve as scaffolds for new inhibitors identification, in order to increase their potency and hopefully to improve their inhibitory effects against bacterial growth [144].

One explanation of the low or non-existent antimycobacterial activity of most of these compounds could be represented by the presence of several effect systems in the bacteria that allow survival of the pathogens in the presence of several drugs. Based on this hypothesis, new hits inhibiting PanC have been characterized by firstly exploiting "energy-based pharmacophore modelling" strategy. The best among these derivatives was thiazolidine, with IC50 0.35 μM and moderate activity against M. tuberculosis growth (MIC 1.55 μM) (Fig. 10) [138]. It is noteworthy that the thiazolidines are considered promising compounds, also known as PanA assay Interference Compounds (PAICs), as they appear as frequent hits in many biochemical high-throughput screens [146, 147].
Fig. (17). Detail of the crystal structure of PanC showing the active site occupied by the sulfonyl adenine derivative (PDB 3COY).

Afterward, the same compounds were assayed against *M. tuberculosis* growth in combination with known efflux pumps inhibitors [138]. Almost all molecules increased the inhibitory power against *M. tuberculosis* growth, with a decrease of MIC values of two- to six-fold. Particularly, the best compounds of this series displayed an MIC of 0.38 μM, demonstrating that inhibition of efflux pumps significantly improves the efficacy of novel antimycobacterial compounds [138].

Very recently, interesting progress has been made by utilizing the Group Efficiency approach to improve already existing PanC inhibitors [148]. The purpose of this technique is to dissect these compounds to evaluate each single chemical group binding contribution, consequently introducing modifications in the molecules in order to improve binding efficiency. Through this approach, sulfonamide derivatives have been obtained (Fig. 18), not only active against *M. tuberculosis* PanC, but also having effects against bacterial growth [148].

Similarly, new isoindol[2,1-b]thiazole derivatives have been recently reported, showing moderate activity against growing and latent *M. tuberculosis*, and also active in a relevant model of *Mycobacterium avium* infection [149].

In conclusion, despite the fact that no PanC inhibitors have been tested in vivo in mammalian models, PanC still remains an interesting target.

4.2. DNA Topoisomerase I (Top I)

DNA topoisomerases are an essential class of enzymes whose role is to maintain topological homeosta-

sis during a variety of DNA transaction processes such as replication, transcription, chromosome segregation and recombination [150, 151]. Topoisomerases are divided into two different groups, type I and type II, based on their structure and mechanism of action [152]. The type I group includes all those enzymes that cleave and rejoin only one strand of DNA. There is a further classification into type IA subfamily, if topoisomerases bind to a 5'-phosphate, and type IB subfamily, if topoisomerases bind to a 3'-phosphate. The type II group includes all those enzymes that cleave and rejoin both strands of DNA. Type II topoisomerases have been divided into type IIA and type IIB subfamilies on the basis of the discovery of a novel type IIB enzyme from hyperthermophilic archeon *Sulfolobus solfataricus*, representing the prototype of IIB subfamily [153]. The reaction catalyzed by topoisomerases includes the formation of a phosphotriester covalent adduct and a DNA single- or double-strand break during the two transfectional reactions [154].

![Diagram](image_url)

Fig. (18). PanC inhibitors showing good antimycobacterial activity.

Type I topoisomerases, responsible for relaxing negatively supercoiled DNA, are present in all bacteria.
and belong to type IA subfamily [153, 156]. M. tuberculosis, differently from E. coli and other bacteria, possesses only one DNA type I topoisomerase, Mitop, a protein of 934 amino acids encoded by top1 gene (Rv3646c) [76].

Recently, the crystal structure of Mitop was solved [157]. The structure of this enzyme is composed of two different parts: a core D1-D4, which contains all conserved motifs (Fig. 19) forming the active site of the protein, and a C-terminal end D5, which is the most variable in size and sequence among species. The D1 N-terminal end includes a TOPRIM (TopoisomeraseFRIMase) domain, which harbors the metal ion Mg\(^2\)+ coordination motif App-X-App-X Gln, essential for the enzyme activity [158, 159], whilst the D5 has several residues thought to interact with DNA.

![Crystal structure of Mitop](image)

A new expression and purification protocol for M. tuberculosis DNA topoisomerase I [156] allowed a better characterization of the enzyme and the DNA cleavage by Mitop has been characterized for the first time. Based on a careful comparison with E. coli DNA topoisomerase I (Etopol), Mitop has been shown to possess the same enzymatic efficiency of Etopol in initial removal of negative supercoils, but the efficiency is lower in removing the remaining negative supercoils. The only specificity for the DNA cleavage sites of Mitop is a C nucleotide in the +4 position, like most of the bacterial DNA topoisomerases [156].

Because of its sensitivity in many crucial biological pathways, Mitop represents an ideal target for the discovery of novel antimycobacterial compounds, causing DNA lesions and cytotoxicity [159, 160]. The main inhibitors of M. tuberculosis DNA topoisomerase I are m-AMS, imipramine, norclomipramine and hydroxy-camphothecin (Fig. 20) [150, 159, 161].

m-AMS is a tisomer of amitriptyline (Fig. 20), an antidepressive derivative able to act as a submicroscopic topoisomerase II poison, known for its potent anti-neoangiogenic activity [162]. m-AMS was identified as a possible M. tuberculosis DNA topoisomerase I inhibitor through in silico screening which revealed a favorable docking score for this drug. Its inhibitory potential was evaluated against M. tuberculosis, M. smegmatis and E. coli DNA topoisomerase I [161]. The activity of Mitop and Mitopol was completely inhibited at 10 \( \mu \)M, while the inhibition of Etopol was between 10 and 25 \( \mu \)M. This result clearly indicated that the enzyme activity is inhibited in a concentration-dependent manner and that m-AMS is able to inhibit topoisomerase I of different bacteria, probably binding a region that is shared between the enzymes of different species. Furthermore, two crucial characteristics of the m-AMS mechanism of action were understood: the first one is that the compound must interact either with the enzyme or the DNA before the formation of the topo-DNA complex for inhibition of the reaction; otherwise its efficiency could be severely compromised. The second characteristic revealed that the microcalcification of the drug to the DNA is required for its activity, like its mechanism of action against eukaryotic topoisomerase II [161]. Unfortunately, considering its powerful activity against eukaryotic topoisomerase II, m-AMS cannot be introduced in M. tuberculosis therapy. However, these two features represent a starting point to develop new molecules with a specific activity against mycobacterial topoisomerase I [161].

Imipramine and norclomipramine are two well-known compounds: the first one is clinically used as a tricyclic anti-depressant and the second one is the active metabolite of the tricyclic antidepressant clomipramine (Fig. 20) [163, 164]. These compounds were identified as mycobacterial topoisomerase I inhibitors by in silico screening using a homology model of the enzyme. The inhibitory activities of both compounds were tested against DNA topoisomerase I from M. tuberculosis, M. smegmatis and E. coli. The DNA relaxation assay revealed a complete inhibition of Mitop and Mitopol at 0.1 \( \mu \)M while there was no inhibition of Etopol, clearly indicating that imipramine and norclomipramine are specific inhibitors of mycobacterial topoisomerase I [159].
To understand their mechanism of action, assays on individual steps of the DNA relaxation cycle of topoisomerase I were performed. Both imipramine and norclomipramine are able to bind the enzyme, the DNA or the enzyme-DNA complex, but their favorite target is the pre-formed enzyme-DNA complex. In particular, they interact with the TOPRIM domain of the enzyme near the Gln112 residue of the metal coordination motif. In addition, it was demonstrated that imipramine and norclomipramine act as cytotoxic agents inducing protein-mediated DNA breaks and affecting cell growth. Finally, it was shown that it is possible to combine the use of imipramine and mexiteline, a norclomipramine, a mycobacterial gyrase inhibitor. This latter discovery is a desirable result considering the alarming spread of M. tuberculosis resistant strains [159].

![Diagram](image)

**Fig. 19. Principle antibacterial Mtopol inhibitors.**

The screening of a chemical library, in which also smaricine was included, led to the isolation of hydroxycaquelinotin (Fig. 20). This compound showed the best inhibitory activity with an IC50 of 6.23 μM. Docking study of hydroxycaquelinotin suggested some important details about its structure and interaction with Mtopol, where the hydroxyl group of the compound is facing towards the opening of the active site. At this site, the residues Ala41, Pro181, Thr184 and Ala193 are involved in hydrophobic interaction with the compound, while the residues Arg115 and Ser485 interact with the compound for hydrogen bonding [150].

According to these studies and considering its non-cytotoxicity, hydroxycaquelinotin was derivatized substituting the terminal hydroxyl group with different hydrophobic moieties, in order to understand the influence of these elements in the compound activity. The best compound, harboring a nitro-phenyl substituent, was very active also against XDR and non-replicating M. tuberculosis and, being also active in an in vivo model of M. marinum infection, it represents the most promising Mtopol inhibitor for the development of antibiotic drugs.

4.3. Cytochrome bc Complex – QrB

Cytochrome bc complex, or ubiquinone-cytochrome c oxidoreductase, is an energy-transducing enzyme playing an essential role in the energy conversion machinery of respiratory and photosynthetic electron transfer chains [165, 166]. In bacteria, this multi-subunit complex is located in the plasma membrane and it catalyzes the reduction of ubiquinone by cytochrome bc complex, reducing a c-type cytochrome and promoting the translocation of protons across the membrane [165, 166]. This mechanism is known as Q cycle and it contributes to the proton motive force used for ATP synthesis [167]. The catalytic core of the complex is composed by three redox-active subunits: cytochrome b (cyt b), which has two cofactors heme b1 and b2 and two reactive sites named the ubisemiquinone (UbQ) and the ubiquinone reduction (Qr) sites, cytochrome c1 (cyt c1) which has the cofactor heme c1 and the Raske iron-sulfur protein (FeS), which contains 2Fe–2S cluster [165].

An interesting number of studies have confirmed the importance of cytochrome bc complexes for mycobacterial growth, thus providing a novel and excellent opportunity to target M. tuberculosis [168, 169]. Among the main inhibitors of cytochrome b (QrB) of bc complex, there are the class of imidazoles [1,2,4] pyridines (IP), Q203 and Lamprazole (LPZ) [169-171].

The imidazoles [1,2,4] pyridines (IP) compounds (Fig. 21) were identified as potent class of inhibitors of M. tuberculosis subunit b of cytochrome bc complexes. All of them are active against M. tuberculosis growth and are non-cytotoxic antibacterial compounds. Two of them showed metabolic stability both in vitro and in murine pharmacokinetic model, one of them revealed a bacteriostatic behavior in vivo models. QrB was identified as the cellular target of IP compounds by WGS of resistant mutants and by the over-expression of qrb gene [170].

Q203 compound is an imidazoquinazoline amide (IPA) derivative (Fig. 21) [171] synthesized in one sequential amide-coupling step with an imidazoquinazoline acid and as pantethinebenzyl amine. This method allows a large-scale synthesis and low production costs, a feature that is a considerable advantage since M. tuberculosis mainly affects people in poor countries [171]. The compound efficacy is enhanced by the introduction of a
In *M. tuberculosis*, lamivudine sulfone (LPZS) was identified as the stable and active metabolite of LPZ [169]. LPZS active metabolite was very active against *M. tuberculosis* growth and also against resistant strains but, unfortunately, it showed additive effects when tested in combination with some first- and second-line anti-TB drugs (rifampicin, isoniazid, moxifloxacin, bedaquiline and BTZ063). Finally, as observed for Q305 compound, LPZS inhibits simultaneously QcrB activity, binding at the ubiquitin Qcr site, and the ATP synthesis. LPZ represents a good example of a new activity found for an old drug using an innovative screen [169].

IF, Q303 and LPZ are promising antitubercular compounds and, considering the essentiality of QcrB target, it is possible that they could be active against both replicating and non-replicating *M. tuberculosis*, an important feature for future anti-tubercular drugs.

4.4. ATP Synthase

Bacterial ATP synthase is an ubiquitous enzyme involved in energy metabolism. It utilizes the electrochemical transmembrane ion gradient (H+ or Na+) for production of ATP, to satisfy cell demand [172]. It is a macromolecular, membrane-embedded protein complex also known as F$_{0}$-F$_{1}$-ATPase, where F$_{0}$ and F$_{1}$ represent the hydrophobic and the membrane portions, respectively (Fig. 22). F$_{0}$ domain consists of the subunits α$_{2}$β$_{2}$γ$_{2}$δ, whilst the F$_{1}$ domain is composed in subunits α$_{3}$β$_{3}$γ$_{1}$δ$_{3}$ε, building a structural connection between the two F$_{0}$-F$_{1}$ domains that is crucial for their functional coupling [Fig. 22] [173-175].

Recently, an innovative host cell-based high-throughput screen has been developed to select compounds that protect MRC-5 lung fibroblasts from *M. tuberculosis* cytotoxicity. In this way, lamivudine (LPZ) (Fig. 21), a genetic proton-pump (H$^{+}$-K$^{+}$-ATPase) inhibitor (PPi), has been identified as an antitubercular compound able to protect fibroblasts [169].
adenine triphosphate (ATP) [173-175]. Pathogenic bacteria such as M. tuberculosis can reside in the human host for many years, entering a so-called dormant state, which renders this mycobacterium poorly susceptible to most of the currently used antibiotics [176-178]. ATP synthase in bacteria such as M. tuberculosis is responsible for facilitating survival under particular conditions found within the host, e.g., low oxygen tensions and/or nutrient limitation [179]. ATP synthase is reported to be essential in M. tuberculosis for optimal growth and in the non-pathogenic model strain M. smegmatis. ATP synthase activity is completely blocked by the diarylglycinamide class of drugs [6, 180-182]. Among the antibacterial compounds belonging to this chemical class, the bedaquiline TMC207 (or R207910) is a promising agent in the fight against TB, directly targeting the rotor ring of the M. tuberculosis ATP synthase [6, 183, 184]. Bedaquiline has two chiral centers leading to four stereoisomers, where the R207910 (R,S) stereoisomer (Fig. 23) is the most active, with minimal concentration required to inhibit 50% of M. tuberculosis isolates (MICc) of 0.06 μg/ml, and with a strong affinity to the c-subunit of the M. tuberculosis ATP synthase [181, 185].

Bedaquiline has been proposed to bind in the c-subunit transmembrane region of the M. tuberculosis ATP synthase [6, 185]. The c-ring from the non-pathogenic Mycobacterium phlei shares 83.7% of sequence identity with its M. tuberculosis homolog and displays MIC values very close to that of M. tuberculosis (0.05 μg/ml and 0.06 μg/ml, respectively). For these reasons a quite identical mode of interaction between the drug and the rotor ring of these two species has been suggested, and the M. phlei c-ring has been thus selected as a model system and co-crystallized with Bedaquiline [6, 184-186]. The ATP synthase c-ring has a membrane-exposed ion-binding site, in which the Bedaquiline molecule can be accommodated upon conformational changes of the c-ring, which provides a hydrophobic space for the drug. Several specific molecular interactions will then be formed thanks to changes in the conformation of Bedaquiline itself, e.g., one example is an ionic intermolecular H-bond between the dihydropyrimidine (DHP) moiety group and Glo65 (Fig. 24). This Glo65-DHP conformational change blocks the c-ring rotation and, consequently, an exchange at P1, resulting in halting ATP synthesis and causing subsequent mycobacterial death [184, 187-191]. According to this mechanism, the binding of one single Bedaquiline molecule is enough to completely stop ATP synthesis [184].

Bedaquiline has been approved by the U.S. Food and Drug Administration for the treatment of both drug-sensitive and drug-resistant TB, but with a specific attention for the treatment of multidrug-resistant TB (http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm313505.htm). Clinical trials confirmed that, in patients receiving the diarylglycinamide bedaquiline, sputum cultures turned from positive to negative. Likewise, bacterial clearance occurred earlier in those patients receiving bedaquiline in combination with the first-line antibiotic pyrazinamide, thus shortening the treatment of patients with MDR-TB [192-194]. Diarylglycinamides are characterized by a dual bactericidal activity, being able to inhibit both replication and dormant M. tuberculosis bacilli. This unique feature distinguishes this class of drugs from all the presently used antimycobacterials, such as DHP and RIF [192]. Moreover, they display bactericidal activity attaining the effects of actual first-line antimycobacterial antibiotics, as demonstrated by in vitro experiments in mouse models [192] [6].

Although bedaquiline has remarkable potential for shortening the tuberculosis therapy duration, several side effects such as nausea, chest pain and/or headache have been observed, thus suggesting further chemical exploration of new ATP synthase inhibitors without any possible adverse effect [7].

Taking advantage of the diarylglycinamide structure, novel quinoline derivatives with significant in vitro bactericidal activity on M. tuberculosis have been synthesized [195, 196]. Several efforts led to the development of more potent, orally bioavailable and ATP synthase inhibitors, the quinolone class of arylsulfonamides compounds [197]. SAR studies identified
low micromolar FzZ inhibitors of the \( M. \) \text{smegmatis} ATP synthase with IC\(_50\) values ranging from 0.36 to 5.45 \( \mu \)M. Among these, the 4-di-chlorophenyl valeryl compound (Fig. 2C) displayed IC\(_50\) value of 0.51 \( \mu \)M and led to promising results in mice infected with \( M. \) \text{tuberculosis} \_H37Rv\ [19]. The binding mode of the compound at the active site of homology modeled \( M. \) \text{tuberculosis} ATP synthase revealed I-bonds formation similar to that of bedaquiline [19]. The in vitro efficacy of this compound disabled that of ethambutol, and its activity against both replicating and dormant \( M. \) \text{tuberculosis} bacilli is comparable to that of bedaquiline [19]. For these reasons, this lead compound is considered a candidate molecule for deeper preclinical investigations.

![Fig. 2C](image)

**Fig. 2C**: Bend view of the \( \alpha\)-ring containing side showing the interaction of bedaquiline with FzZ5 and Gbd5 (PDB: 3C4X).

### 4.5. FzZ

Filament-forming temperature-sensitive genes “Fz” were identified in 1980 in \( E. \) \text{coli} as genes encoding for proteins involved in septum formation [19]. The bacterial cell division machinery remained unexplored for therapeutic purposes for several years, and it was only in 1991 that the FzZ protein was discovered to be involved in the initiation of cell division [199, 200]. When GTP is present, FzZ proteins cooperatively polymerize on the inner membrane at the center of the cell, growing bidirectionally and stacking in a head to tail fashion, with GTP located between two FzZ subunits, thus forming a highly dynamic helical structure called the “Z-ring”. Moreover, a link between GTP hydrolysis and polymerization dynamics was first shown when the formation of FzZ polymers in solution was found to be coupled with GTP hydrolysis, since polymers loss co-occurred with GTP depletion [201, 202]. FzZ was shown to form straight filaments when bound to GTP, while FzZ bound to GDP forms highly curved filaments [203]. This transition from straight filaments to curved conformation suggests that GTP hydrolysis provide the energy for generating the mechanical force for cell division [203]. Other cell division proteins are then recruited, causing Z-ring contraction, septum formation and eventually cell division [204–208].

With FzZ inactive, septum formation is impaired (Fig. 2D). Accordingly, FzZ represents a very promising target for new antimicrobial drug discovery because of its known biochemical activity and its central role in cell division [199]. Being a novel drug target, new compounds targeting FzZ would not be affected by already known drug-resistance mechanisms caused by the use of current anti-TB drugs [209].

**Fig. 2D**: Z-ring formation is impaired in the presence of FzZ inhibitors.

The identification of the short amino acid sequence \( \text{GGGTG} \) from the crystal structure of the Methanococcus jannaschii FzZ leads to the identification of FzZ as a homologue of tubulin, being virtually identical to the motif sequence, \( \text{G(G)}\text{G(G)}\text{G} \), found in all \( \alpha, \beta, \text{ and } \gamma \) tubulins [210].

Although sequence similarity is limited to 10%, FzZ and tubulin share a common fold made up of two domains linked by a \( \alpha\)-helix [211, 212]. Both tubulin and FzZ polymerize in the presence of GTP into protofilaments while depolymerization following GTP hydrolysis [206]. GTP hydrolysis generates the major rate-limiting step, and phosphate release rapidly follows. Polymers bound to GDP tend to curve, leading to constriction of the Z ring in cell division. GDP release from the polymer might be partially rate limiting. Depolymerization and GDP release then occur, followed by molecule exchange in the monomers [213, 214].
Structural and functional homology suggests that drugs affecting the assembly of tubulin into microtubules can be used as lead targeting FtnZ assembly. Furthermore, the very low sequence homology at the protein level gives the opportunity to investigate drugs that are FtnZ specific with limited cytotoxicity to eukaryotic cells [208].

FtnZ protein from M. tuberculosis (MtFtnZ) crystallized as a tightly associated dimer in solution, with the A and B subunits associated to form an x-shaped dimer (Fig. 26) [215-217]. The GTPase domain is located in the N-terminal domain, which is connected to the C-terminal dimer by a central helix H10. The structures of the two subunits are quite identical, except for an unexpected secondary structural switch at the subunit interface: in subunit A, the H1 helix adopts a helical conformation, whilst the H1 helix of subunit B assumes a b-strand conformation instead. This secondary structural switch is located in the GTPase domain, forming most of the dimer interface [216].

FtnZ is pathogen specific, essential and highly conserved in mycobacteria, thus potential FtnZ inhibitors may be developed as broad-spectrum antibacterial agents to which acquiring resistance by mutations in the protein may be demanding for bacteria [218, 219]. Drug development is still at an early stage, nevertheless several classes of compounds have already been found effective against M. tuberculosis FtnZ, some of these being classified as promising leads.

Several benzimidazole, pyridopyrazine and pyridazine-based FtnZ inhibitors have been screened and identified as potential powerful antibacterials [209, 216, 220-224]. Other reported antibacterial agents are benzimidazole derivatives, responsible for a delay in the M. tuberculosis cell division process, but very limited is known so far (Fig. 27) [225].

Zunin Z3 (Fig. 27) was discovered through a high-throughput screening of inhibitors of the GTPase activity of FtnZ. It was shown to be uniquely active and selective in its inhibition of FtnZ when compared to other molecules. It has no effecting electrophilic or phenolic functionality and is the best in terms of overall performance against the protein from multiple species of bacteria under a variety of conditions. Ideally, SAR studies will allow identification of a compound with significantly better inhibition for both cell-based studies and crystallography [226]. Recent SAR studies showed that the introduction of a smaller quinazoline ring, instead of the benzimidazolequinoline, retain the potency of the compound. Whisk the incorporation of a small and positively charged side chain improved activity [227].

Taking into account the structural similarity of the pyridopyrazine moiety, pyridazine moiety, chloroacetamide, and thiabendazole, it has been hypothesized that the benzimidazole scaffold would be a good starting point for the development of novel FtnZ inhibitors, with good activity against both drug-sensitive and drug-resistant M. tuberculosis strains [228]. Specifically, the activity of benzimidazole derivatives (Fig. 27) against M. tuberculosis clinical strain has been reported [229]. Docking, synthesis, structure elucidation, in vivo antibacterial activity and cytotoxicity assay against VERO cells of benzimidazole derivatives led to compounds with good in vitro antibacterial activity [223]. The potency, selectivity and low cytotoxicity of these compounds make them valid leads for improved antibacterial developments [223].

However, investigations of the benzimidazole family molecules led to the development of PC190723 (Fig. 27) [229], which was the first non-metabolite inhibitor to be co-crystallized with FtnZ [230]. This compound activates the GTPase activity and alters the cooperativity of the FtnZ monomers, as demonstrated by crystallographic studies. Being also active in vivo, PC190723 is considered the best inhibitor of FtnZ to date, although actually limited to lipophilic reservoirs.

4.6. CTP Synthetase (PyrG)

The biosynthetic and salvage pathways of pyrimidines represent a central point of interest in terms of antibacterial drug development [231]. Among the enzymes involved in this pathway, the CTP synthase is the most intriguing, being recognized as a target of antibacterial [232] and antiviral agents [233, 234] and, recently, of antimycobacterial compounds [235].
Fig. (27). PncZ inhibitors divided by their mechanism of action.

The mycobacterial CTP synthase PycG is an essential enzyme that catalyzes the production of CTP starting from UTP and glutamine in an ATP-dependent manner. It represents a key player for several biologically processes, such as DNA, RNA and phospholipids biosynthesis [236]. The crystal structure of M. tuberculosis PycG has been recently solved on a 2.6-A resolution data set, highlighting the presence of two domains that organize in a tetramer: a synthetase domain located at the N-terminus, and a glutamine amidotransferase domain located at the C-terminus [235].

Through whole cell screening of a 594 compounds chemical library, two M. tuberculosis PycG inhibitors, named 7947852 and 7904658 (Fig. 28) have been identified. These inhibitors are both produgs immediately activated by FAD-dependent monoxygenase FoaA. The FoaA mechanism of activation of these compounds has been defined and was similar to that performed by ETH activation (Fig. 29). The active 5-diol metabolite of 7947852 has been synthesized (11-260026 compound) and demonstrated to be a competitive inhibitor towards ATP (K10 μM) [235].

This behavior was in accordance with the position of the residues mutated in the resistant mutant, located in the proximity of the ATP-binding site of the enzyme.

Metabolomic studies revealed that, by blocking PycG, the nucleotide metabolism is significantly damaged, thus confirming its central role in several cellular processes where nucleotides are involved, as well as its strong potentiality as cellular target for new antituberculars [235].

Fig. (28). M. tuberculosis PycG inhibitors.

Afterwards, the virtual screening on PycG of the Collaborative Drug Discovery (CDD) compounds database identified further compounds, with already
known antimycobacterial activity, which could likely bind PyrG. Four compounds with high docking score have been identified, and one of them, the CDD-823993, resulted to be active against PyrG activity in vitro (GI of 88.9 μM) [323].

Although the CDD-823993 is a weak PyrG inhibitor, it remains a new antimycobacterial not correlated with the previous ones, that inhibits PyrG enzyme activity. All these results suggest that PyrG is a good target for new antimycobacterial compounds. Identification of inhibitors of CTP-synthetase from other organisms [212–214] makes PyrG a very promising target. Consequently, it could be useful to utilize M. tuberculosis PyrG enzyme to screen several novel chemical libraries of compound having known antimycobacterial activity, in order to find new potential compounds for antimycobacterial treatments.

CONCLUSION

There has been considerable progress in the discovery of new lead compounds for treating tuberculosis.

Among the newly identified antimycobacterial compounds, several derivatives have been synthesized starting from already known inhibitors of old target enzymes such as DNA gyrase and RNA polymerase.

A target-based approach can be a faster and more efficient method for drug identification compared to the traditional de novo drug-discovery. However, antimycobacterial compounds often show limited efficacy against M. tuberculosis, because of its thick cell wall and its efflux systems, but biochemistry and structural biology are now providing new drugs and target discoveries. For these reasons phenotypic HTS for the identification of scaffolds, followed by target identification and then optimization against the target, remains the best strategy to fight this pathogen.

Clearly, the growing reports of MDR-TB and XDR-TB strains is putting on the acute need for new cell-wall targets. Nevertheless, some of the newly identified inhibitors are considered good candidates and have now entered clinical trials. PF0124 (is one of the most effective inhibitors against the DprE1 promiscuous target and it is planned to enter human clinical trials in 2016. Phenotypic screening has allowed the characterization of new potential drug targets, highlighting the importance and essentiality of some processes, such as corrinoid A biosynthesis, nucleotide biosynthesis, cellular division and energy metabolism. Although the current situation of lead generation against TB is improved in recent years, it is still too slow and extremely challenging in success. Relevant changes are needed in order to shorten TB treatment duration and to produce novel regimens with higher efficacy against drug-resistant TB.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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A Phenotypic Based Target Screening Approach Delivers New Antitubercular CTP Synthetase Inhibitors
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Supporting Information

ABSTRACT: Despite its great potential, the target-based approach has been mostly unsuccessful in tuberculosis drug discovery, while whole cell phenotypic screening has delivered several active compounds. However, for many of these hits, the cellular target has not yet been identified, thus preventing further target-based optimization of the compounds. In this context, the newly validated drug target CTP synthetase PytG was exploited to assess a target-based approach of already known, but untargeted, antimycobacterial compounds. To this purpose the publicly available GlaxoSmithKline antimycobacterial compound set was assayed, uncovering a series of 5-(pyridin-2-yl)thiazole derivatives which efficiently inhibit the Mycobacterium tuberculosis PytG enzyme activity, one of them showing low activity against the human CTP synthetase. The three best compounds were ATP binding site competitive inhibitors, with Ki values ranging from 3 to 20 µM, but did not show any activity against a small panel of different prokaryotic and eukaryotic kinases, thus demonstrating selectivity for the CTP synthetases. Metabolic labeling experiments demonstrated that the compounds directly interfere not only with CTP biosynthesis, but also with other CTP dependent biochemical pathways, such as lipid biosynthesis. Moreover, using a M. tuberculosis pytG conditional knock-down strain, it was shown that the activity of two compounds is dependent on the intracellular concentration of the CTP synthetase. All these results strongly suggest a role of PytG as a target of these compounds, thus strengthening the value of the target-based approach for the identification of new scaffolds for drug development.

KEYWORDS: drug discovery, phenotypic screening, target-based screening, Mycobacterium tuberculosis, CTP synthetase, pyridine-thiazole

Although there has been extensive investment over the last few decades, tuberculosis (TB) still remains an urgent global health issue, with more than 10.4 million estimated new cases and about 1.8 million deaths each year.1 Moreover, the increasing spread of Mycobacterium tuberculosis (Mt) multidrug-resistant (MDR) strains (with about 400,000 cases in 2015) of which 10% have been reported as extremely drug resistant (XDR) has led to an increased requirement for new drugs, with new mechanisms of action.2 After the first complete sequencing of the Mt genome, there were great expectations derived from target-based approach for TB drug discovery, which proved to be mostly unsuccessful. High-throughput screening (HTS) has been the preferential method adopted for the identification of new hits from large compound libraries.3 In the case of Mt, whole-cell screening using HTS can provide compounds able to cross the mycobacterial cell envelope, overcoming one of the main challenges for TB drugs. The large number of phenotypic screening efforts performed until now led to the identification of several active molecules however, the mechanism of action of many of them is still not known.4 The elucidation of the cellular target is an important component of the hit to lead optimization process, enabling the assistance of biochemistry and structural biology to characterize the mechanism of action and to better assess at molecular level potential host cell...
Table 1. Compounds of the GSK TB-Set Showing Activity against M. tuberculosis PyrG

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*Enzyme assays were performed in the presence of 100 μM of each compound

Toxicities. However, for several reasons (e.g., difficulty in obtaining resistant mutants etc.), this task is not trivial. A great improvement arose from the introduction of next generation sequencing platforms, allowing wide and intensive use of genomics in combination with chemical library screening, for identification of the cellular target of novel compounds. Furthermore, it is possible to combine target-based and phenotypic screening strategies. In this context, GlassSmithKline (GSK) has identified and released the structures and activities of a large number of antimycobacterials [https://www.elt.is.uk/cchem/html/](https://www.elt.is.uk/cchem/html/) verified to be active against M. tuberculosis H37Rv cells and showing low toxicity against human cell lines. Different screens of this set allowed the identification of the cellular targets of several hits, not only by the classical genomic-based target assignment but also through different strategies. For instance, a new series of leads targeting DprE1 has been recently identified through the screening of this set against a DprE1 overexpressing strain of Mycobacterium bono BCG.

Moreover, since new drug candidates should possess a novel mechanism of action to ensure effectiveness against Mtb MDR and XDR strains, the drug discovery exploitation of the latest validated targets seems a reasonable starting point. In this respect, the novel TB drug target CTP synthetase PyrG could represent a good platform for target based screening of antibacterial compound libraries. This essential enzyme was demonstrated to be drugable, and being involved in several biochemical pathways, its inhibition has proven to affect several aspects of mycobacterial physiology. With this aim, we exploited the GSK antimycobacterial compound set (GSK TB-set) library to explore the possibility of finding new antitubercular CTP synthetase inhibitors.

**RESULTS AND DISCUSSION**

Screening of the GSK TB-Set against PyrG Affords New Inhibitors. A previous whole cell phenotypic screening of GSK compounds identified a set of 177 molecules (GSK TB-set) active against Mtb cell growth, whose targets are largely unknown. For this reason, to identify compounds targeting PyrG, the GSK TB-set was assayed against the recombinant Mtb enzyme.

The assays, performed at a final concentration of 100 μM for each compound and at submicromolar concentrations of ATP, identified 16 compounds (95% bit score) inhibiting more than 50% of enzymatic activity (Table 1).

About half of these active compounds, in particular the most active, were characterized by a (pyridin-2-yl)hydrazole group, suggesting a particular affinity of PyrG for this moiety. Among them, GSK1575006A (1), GSK920644A (3), and GSK735826A (3) (Table 1, Mtb minimal inhibitory concentration, MIC, 6, 7, and 1.4 μM, respectively) showed higher inhibitory effects against PyrG activity (IC<sub>50</sub> values of 2.9 ± 0.6, 23.3 ± 22, and 19.7 ± 2.2 μM, for compounds 1, 3, and 3, respectively) and were selected for further investigations.

First, in an attempt to gain insight into the binding between PyrG and the three inhibitors, a computational analysis of the possible poses of the compounds was performed by docking in the PyrG ATP binding site (Figure 1) using Discovery Studio 4.1 (Biosoft, San Diego, CA). The superimposition with the UTP molecule shows a partial overlap. The 4-(pyridin-2-
Figure 1. Docking GSK compounds in CTP synthetase. (A) Compound 1, Libdock score 88.19; (B) compound 2, Libdock score 92.96; (C) compound 3 Libdock score 87.23. All molecules (gray) are compared to UTP (yellow). (D) Compound 11426026, previously reported as a PyrG ATP-binding site competitive inhibitor.

The imidazole moiety is in a comparable position for 2 and 3, while 1 has a flipped orientation in the protein. However, all 3 molecules have phenyl rings that are suggested to z-stack with Arg23. This superposition is very similar to that of the 11426026 compound, a previously described PyrG inhibitor, with a IC_{50} value (35 μM) similar to that of the three GSK compounds, which has been demonstrated to be competitive toward the ATP binding site.  

To confirm the hypothesis that the compounds bind to the ATP site, PyrG steady-state kinetic analysis was performed in the presence of different concentrations of the compounds. The analysis revealed that all the compounds behave as competitive inhibitors toward the ATP binding site, showing apparent K_i values of 3.5 ± 0.4 μM (1), 22.0 ± 0.6 μM (2), and 16.3 ± 0.5 μM (3) (Figure 2). By contrast, all of them were uncompetitive toward the pyrimidine nucleotide binding site (Figure S1).

Finally, as the CTP synthetase physiologically uses glutamine as an ammonia donor to fulfill the catalysis, the effects of the three compounds against the glutaminase activity of PyrG were evaluated. As depicted in Figure S2, the presence of the three...
compounds leads to a decrease in the Vmax values but leaving unchanged the K_m, thus excluding a direct interaction of the compounds with the glutamine binding site.

**Effects of GSK PyrG Inhibitors against Human CTP Synthetase.** Due to the relatively large similarity between Mtb and human CTP synthetases (about 44% sequence identity), the type I human CTP synthetase (Hu-CTPS1) was obtained, to assess the compounds' specificity. The recombinant Hu-CTPS1 was produced using Pichia pastoris expression system and purified as described in Methods (Figure S3), affording about 3 mg of protein from 1 L of yeast culture, with a specific activity of 6.5 s⁻¹ and K_m values of 0.2 mM toward either UTP or ATP. The enzymatic activity assay of Hu-CTPS1 in the presence of the compounds, performed at saturating substrate concentrations, revealed that all the three 4-(pyridin-2-yl)thiazole derivatives inhibited the human CTP synthetase (Figure 3). By contrast, the 11426026 compound, a Mtb PyrG inhibitor previously identified,14 displayed a significantly higher IC₅₀ against Hu-CTPS1 (>1 mM for Hu-CTPS1 vs 13.5 ± 1.3 μM for the Mtb PyrG). However,
although compounds 2 and 3 showed IC₅₀ values against Hu-
CITPSI (3.0 ± 3.1 and 24.2 ± 8.5 μM, for 2 and 3, respectively) similar to that against Pryg, compound 4 was a more weaker inhibitor of the human enzyme with an IC₅₀ 10-fold higher (37.6 ± 2.3 μM).

The possibility to screen compound libraries against both MB and human CTP-synthetases could be a valuable "double tool" for identification of the most exclusively mycobacterial Pryg, such as 11442636. It is worth noting that, despite their activity against the human enzyme, the GSK compounds showed low toxicity against a human cell line. Moreover, the fact that compound 1 is 10-fold less active toward the human enzyme highlights the possibility to develop analogs that are more specific for the mycobacterial CTP synthetase.

Validation of PryG as a Target of the Three GSK Inhibitors. As the GSK compounds have been shown to be ATP binding site inhibitors, their specificity toward the CTP synthetase was assessed. In order to do this, the compounds were assayed against a small panel of diverse kinases or ATP binding enzymes from different sources (Echerichia coli, rabbit muscle, and human pyruvate kinase, human adenylate kinase, M. tuberculosis phosphofructokinase, pyrophosphate synthetase, human phosphoglycerate kinase, yeast histone). As shown in Table S1, none of these enzymes is inhibited by the three compounds, thus excluding a broad specificity of these compounds.

To confirm the role of PryG as a target of the compounds, the isolation of Mtb resistant mutants was attempted, to check for the presence of possible mutations in the pryG gene. Concentrations of compounds ranging from 5-fold to 20-fold the MIC were used, but despite several attempts, no resistant colonies could be isolated. The inability to obtain spontaneous resistant mutants could be related to the strict essentiality of the CTP synthetase enzyme and clearly reflects a very low mutation frequency of the pryG gene, reinforcing its significance as a drug target. However, as the three compounds were demonstrated to compete with the ATP binding site of PryG, mutations that could hamper their binding without detrimental effects on the enzyme activity could confer drug resistance. For this reason, the GSK compounds were also tested on a previously characterized Mtb mutant harboring the V186G mutation in Pryg and resistant to the already described 11442636 compound. The Mtb mutant strain did not show cross resistance with compound 3 and was only resistant to the other two compounds (MIC values 2-fold higher than that of the wild-type) (Table S2). The three compounds assayed against the recombinant PryG V186G were found to maintain activity against the mutant enzyme, showing IC₅₀ values slightly increased with respect to the wild type, thus explaining the sensitivity of the Mtb mutant strain (Figure S4).

To confirm that the intracellular mechanism of action of the compounds effectively involves the CTP synthetase, the compounds were assayed against an Mtb conditional knock-
down (KD) mutant strain (TB456), constructed using the Pip-On inducible system. In this system pryG was under the control of the inducible promoter Pₓram and its expression was induced only in the presence of the inducer (pristinamycin I). The conditional mutant TB456 was able to grow only on plates containing pristinamycin I (100 μg/mL), confirming the essentiality of the gene (Figure S5). Moreover, TB456 growth was shown to be induced in a dose-dependent manner in the presence of different amounts of pristinamycin I in liquid media. Staining cultures of TB456 were grown using different concentrations of inducer (0 to 100 ng/mL) for 72 h, then bacteria were diluted to OD₅₆₀ 0.05 in the same media conditions. After 72 h, it was clearly visible that the mutant growth depended on pristinamycin concentration. After the second refresh, the growth of bacteria exposed to the lowest concentrations of pristinamycin I was arrested (Figure S6).

Then, TB456 cultures grown in different pristinamycin I concentrations were exposed to the compounds that are the object of the study and to 11442636, which is known to target Pryg, as control (Table 2). Actually, the MIC for compound 2 did not show any variation in function of pristinamycin I amount in which the bacteria were grown. In contrast, the MRCs for compound 1, as well as to 11442636, were halved at lower concentrations of the inducer, increasing only at the highest pristinamycin I concentration. Furthermore, in the presence of compound 3, the TB456 strain did not grow at minimal pristinamycin I concentrations, showing a four-fold lower MRC value even at the highest inducer. The dependence of the effects of compound 1 and particularly of compound 3 on the Pryg intracellular level demonstrate its role in the mechanism of their action, thus confirming the hypothesis that PryG is an intracellular target of these two compounds.

GSK PryG Inhibitors Cause Several Metabolism Alterations in M. tuberculosis. The role of the CTP synthetase in the mechanism of action of these compounds was also examined by metabolic labeling of the non-pathogenic model strain M. tuberculosis H37Ra (MIC of 20.8, 15.2, and 0.28 μM, for compound 1, 2, and 3, respectively) with [¹³C]Glut. Within mycobacterial cells, [¹³C]Glut is incorpor-
ated into [¹³C]UMP by uracil phosphoribosyltransferase (Upt) from the primodine salvage pathway, thus entering further metabolism to the whole range of nucleotides. Mtb H37Ra cells were grown in the absence or presence of the compounds (5 X MIC) for 1 h, then [¹³C]Glut was added and radiothermal labeling continued for 3 h. The radiolabeled nucleotides were then extracted from cell pellets with 9% formic acid and separated by thin layer chromatography. As depicted in Figure 4A, addition of the compounds to the media during cultivation of mycobacteria led to significant increase in the ratios of radioactivity incorporated to [¹³C]UTP and [¹³C]CTP compared to the control, pointing to inhibition of PryG. A similar trend was found in the positive control 11442636, while for bromodimethane BTZ 0.44), the drug with an unrelated mode of action targeting cell wall enzyme DprEII, the [¹³C]UTP/ [¹³C]CTP ratio was comparable to the untreated control cells (Figure S3).

Clear inhibition of CTP synthetase activity would affect numerous catalytic and anabolic reactions in mycobacteria, due to the requirement of CTP by the bifunctional CoA/CoM enzyme involved in the biosynthesis of coenzyme A. This enzyme was
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Figure 4. Examination of effects of 1, 2, and 3 on M. tuberculosis H37Rv cells by metabolic labeling. Bacterial cells were pretreated with 8 fold MBC of the drug for 1 h, followed by 3 h of radiolabeling with [3H]uracil (A) or [3H]acetate (B, C). (A) Ratios of radiotracer incorporated in [3H]UDP and [3H]CTP extracted from [3H]uracil labeled cells. Macrolide extracts were separated by TLC on silica gel plate. Columns and error bars represent mean ± SD of triplicate measurements of radioactivity in the [3H]UDP and [3H]CTP spots cut from TLC plates corresponding to two separate experiments; from each experiment two plates were analyzed. The autoradiograph is representative image from three separate experiments. (B) TLC analysis of the lipids from [3H]acetate labeled cells. Lipids were extracted as in Methods; aliquots corresponding to the same amounts of extracted material (as shown in panel C) were loaded on a silica gel TLC plate and separated in Solvent I CHCl3/CH3OH/H2O (65:25:3.5:6), Solvent II CHCl3/CH3OH/H2O (90:5:5), and Solvent III petroleum ether/ethyl acetate (96:4) The plates were exposed to an autoradiography film at −80°C for 6 days. TDM, tuberculostearic acid; TMM, tuberculostearic acid; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIM, phosphatidylinositol mannosides; CL, cardiolipin; TAG, triacylglycerol. Autoradiographs are representative images from these separate experiments. (C) Visualization of the lipid bodies by cryo-substrate staining.

recently shown as a validated bactericidal target in M. tuberculosis. It was critical for lipid metabolism also for producing of activated CDP-derivatives required for biosynthesis of phospholipids. We thus investigated the effects of the compounds on lipid biosynthesis by [3H]acetate metabolic labeling in the same conditions as described for [3H]uracil. Analysis of lipids extracted from the radiolabeled cells revealed that incorporation of [3H]acetate was decreased in the cells treated with compounds 1, 2, and 11.26/26. In contrast, the presence of BT2045 resulted in an increase of the radiolabel
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inclusion due to accumulation of TDM, as expected (Figure 4B,C). These experiments confirm that the effects of the compounds are pleiotropic, as expected, but it is not possible to conclude whether they are the result of only PyrG inhibition or due to targeting additional enzyme(s).

CONCLUSION

The use of whole cell phenotypic screening in combination with target based assay has been recently demonstrated a useful strategy for the identification of new leads.17 With this aim, an antibacterial compound library was screened against CTP synthetase PyrG, identifying a new series of 4-(pyridin-2-yl)thiazole compounds able to inhibit the enzyme activity. These compounds, which have been shown to be quite specific for the CTP synthetases, confirm the high druggability of PyrG. Indeed, several lines of evidence, which emerged from the different approaches used, point to PyrG as a main target of this series, although additional targets could not be completely excluded, demonstrating the effectiveness of each screen against novel validated targets, to identify novel scaffolds for drug development. Moreover, the parallel screen against the corresponding human enzyme CTPS-I could help in defining the selectivity of the compounds. Indeed, among the three hit molecules identified, compound 1 was more selective for the mycobacterial enzyme and represents a useful scaffold for further target-assisted structure—activity relationship studies in order to improve its efficacy.

METHODS

Compound Library Screening against PyrG Enzyme Activity. Wild-type and mutant M. tuberculosis PyrG recombinant proteins were obtained in E. coli, as previously reported.16 The publicly available GlaxoSmithKline antimycobacterial compound set (GSK TB-set), consisting of 177 compounds active against Mtb growth,18 was screened against PyrG activity, at a final concentration of 100 μM for each compound. PyrG enzyme activity was determined in a final volume of 100 μL at 37 °C by continuous spectrophotometric assay following the rate of increase in absorbance at 291 nm due to the conversion of UTP to CTP (ε = 1340 M⁻¹ cm⁻¹).19 The reaction mixture contained 50 mM HEPES, pH 8.0, 10 mM MgCl₂, 1 mM UTP, 0.2 mM ATP, 0.3 μM PyrG, 1 μL of compound (10 mM in dimethyl sulfoxide, DMSO), and the reaction was started by the addition of 100 mM NADH. Blank reactions were performed by adding 1 μL of DMSO. All assays were performed in triplicate.

The compounds inhibiting more than 75% of PyrG activity in these conditions and selected for further investigation were repurchased from MedPort (Riga, Latvia): GSK3750069A (2-(4-fluorophenyl)-N-(4-(2-methylpyridine-3-yl)thiazole-2-yl)acetamide, MedPort003-158-205), GSK3753826A (N-(4-(2-methylpyridine-3-yl)thiazole-2-yl)-4-(1H-purazol-4-yl)-4H-benzo[1,2,3-cd]azine-6-amine, MedPort003-038-940), GSK5206664A (2-(3-fluorophenyl)-N-(2-(3-methylpyridine-3-yl)thiazole-2-yl)acetamide, MedPort004-106-239). PyrG inhibition was reconfirmed, and then IC₅₀ and Kᵢ values were determined. For IC₅₀ determinations, the enzyme activities were measured in the presence of compound, and values were estimated according to eq 1, where A₀ is the enzyme activity in inhibitor concentration [I] and Aᵢ is the enzyme activity without inhibitor.

The inhibition constant (Kᵢ) values were determined by analyzing the PyrG enzymatic activity at different substrate and compound concentrations. The assays were performed at a UTP final concentration of 2 mM when ATP was the variable substrate and at 2 mM ATP when UTP was the variable substrate. Kᵢ values were calculated using an adapted equation for competitive inhibition (eq 2) with Origin 8 software.

\[
f = \frac{[S]}{Kᵢ + [S] + \frac{1}{V_{max}}}
\]

PyrG glutaminase activity was assayed by using a glutamate dehydrogenase (GDH, Sigma-Aldrich) coupled assay, which converts the produced glutamic acid into α-ketoglutarate acid through the reduction of NAD⁺. Thus, the activity was determined measuring the increase in absorbance at 340 nm of the produced NADH (ε = 6220 M⁻¹ cm⁻¹). Assays were performed at 37 °C in 50 mM HEPES, pH 8.0, 10 mM MgCl₂, 1 mM NAD⁺, 100 μM GDP, 0.2 mM ATP, 2 mM UTP, 1 μM GDH, PyrG 1 μM, and reaction was started by adding the glutaminase solution. Steady state kinetics toward Glu were performed at 3 and 6 μM for compound 1 and 20 and 40 μM for compounds 2 and 3.

Screening of the Compounds against Different Kinases. E. coli pyruvate kinase, human R type pyruvate kinase, human adenylate kinase, M. tuberculosis phosphoribosyl pyrophosphate synthetase, and human phosphoglycerate kinase were prepared, and their activity was assayed, as previously reported.20,21 Rabbit muscle pyruvate kinase and yeast hexokinase were purchased from Sigma-Aldrich, and their activity was assayed according to the manufacturer. Enzyme activities were determined at three substrate concentrations, ATP concentrations in the presence and in the absence of 100 μM of compound 1, 2, or 3.

Minimal Inhibitory Concentration Determinations and Isolation of M. tuberculosis Spontaneous Resistant Mutants. The MIC of the compounds was determined in solid medium. A single colony of each Mtb strain was inoculated in complete Middlebrook 7H9 supplemented with 10% OADC Middlebrook Enrichment and grown at 37 °C until exponential growth phase (~10⁶ CFU/mL). Dilutions to the final concentration of ~10⁴ CFU/mL were performed, and about 1 μL of cell culture was streaked onto plates containing 1.5-μl serial dilutions of appropriate compound. MIC values were assigned as the lowest drug concentrations inhibiting bacterial cell growth. All experiments were performed in triplicate. The isolation of Mtb mutants was attempted by plating ~10⁵ cells from an exponential growth phase wild-type culture onto 7H11 medium containing different concentrations of compounds, ranging from 5 to 200 μg/mL. MICs of the compounds for M. tuberculosis H37Ra were established by resazurin microplate assay (REMA).

Construction and Characterization of a pyrG Conditional Knock-down Mutant. A Mtb pyrG conditional knockdown mutant was constructed using the PIP-CGN system.22 At this purpose, the first 694 by nucleotides of the pyrG coding sequence were amplified with RP1690 (5'-ttagatgatcagacgacccagagct-3') and RP1610 (5'-ttttattcagtaaacggtggttcca-3') primers and cloned into a suicide plasmid
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in frame with the priminacin-inducible promoter, P
. The
obtained plasmid pG11 was then electroporated into an HStre-
dervative containing the pJG gene integrated into the L5 stiff
site obtaining TB456. To characterize the growth of the pgyt
mutant, TB456 was grown to mid log phase and then
diluted to a theoretical OD600 of 0.05 in medium with different
amounts of priminacin (0, 5, 7.5, 25, 50, 75, and 100 mg/
ml). Serial dilutions of standing culture of TB456 grown in
M63 broth 719 in the absence of priminacin for 24–48 h were
drawn onto solid media with and without priminacin (100
mg/ml).

MIC Determinations in pgyt Conditional Knock-down
Mutant: Compound sensitivity of the Mh pgyt knockdown
mutant was determined in the presence of different
priminacin concentrations using the resazurine microplate
assay (REMA) as previously described.27

Docking. The PyoGT protein was prepared as described
previously,16 for docking using the default settings of the
“prepare protein” protocol in Discovery Studio 4.1 (Biorvax,
San Diego, CA). The PyoGT protein (PDB ID 4ZJF)16 was used
for docking using LDoDock.27 The protocol included 100 hotspots
docking tolerance (0.25). The FAST conformation
method was also used along with steepest descent minimization
with CHARMM

Metabolic Labeling of M. tuberculosis H37Ra: The
effects of the PyoGT inhibitors on the mycobacterial metabolism
were assessed through metabolic labeling of Mtb H37Ra with
stationary in 7H9 medium supplemented with 10% ADC and
0.5% Tween 80 until OD600 = 0.25–0.35. The culture was then
diluted to 5 ml aliquots and the compounds 1, 2, and 3
dissolved in DMSO were added at 8x MIC control
dose 1143063 and 1772043 were added at 16 or 0.64 mg/ml,
respectively. Final concentration of DMSO in all cultures was
1%. After 1 h drug treatment, [1]C]tartrate (American Radionu-
cide Chemicals, specific activity 53 mCi/mmol) was added to
a final concentration 0.1 or 1 mg/ml, or [1]C]acetate (American
Radionuclide Chemicals, specific activity 106 mCi/mmol)
was added to a final concentration 0.1 or 1 mg/ml, and the
incubation continued for next 3 h. Inhibition of mycobacterial
growth by all tested compounds after 4 h treatment was
between 2% and 10% based on OD600 measurement. Extraction
of [1]C]labeled nucleotides with ice-cold 9% formic acid
was carried out as previously reported.15 For TLC analysis,
the extracts were evaporated under vacuum, mixed with
10 nol each of UTP and CTP, and loaded on PEI Cellulose E plate
(Millipore). After 15 min soaking in CH3OH, the air-dried
TLC plate was developed in 0.75 M H3PO4, pH adjusted to
3.5 with 0.75 M H2PO4 for 3 h. Spots corresponding to UTP
and CTP were visualized under UV (λ = 254 nm); then they
were cut from the plate and placed in scintillation vials with
EcoLite scintillation liquid for determination of radiocity. Bacteria
labeled with [14]C]tartrate were subjected to extraction
with 1.5 ml of CHCl3/CH3OH (1:2), followed by extraction
with 1.5 ml of CHCl3/CH3OH (2:1). Each extraction was
carried out for 2 h at 36°C; the extracts were combined, dried,
and subjected to fractionation in CHCl3/CH3OH/H2O
(4:2:1). Upper (water) phase was discarded and bottom
(organic) phase was dried and dissolved in Solvent 1 (see
below) in the ratio 75 µL/OD600 0.52 ml of the culture.
Alcohols (5 µL) of the lipid extracts were analyzed by TLC on
silica gel plates (Merck) in Solvent I, CHCl3/CH3OH/NH4OH/H2O
(6:2:5:0.5:4), Solvent II, CHCl3/CH3OH/H2O
(20:40:5:0.5), and Solvent III, petroleum ether/ethyl acetate
(98:2, 3 times). After chromatography, the plates were
transferred to autoradiography film (BioMax MR) at ~80°C. For
visualization of the cold lipids, the plates were sprayed with
ceric tetrathionate (10% in 100% ethanol/2% ammonium
hydroxide) and incubated at 30°C for 2–3 days until colonies
appeared.

Cloning, Expression, and Purification of the Human
CTPS1. Recombinant human CTP-synthetase 1 (HClatps1-
T) was expressed in P. pastoris KM71H (lentrogen).31
The full length Hucatps1 cDNA was purchased from
Dharmacon. The cDNA was inserted into Biolm/Not
restriction sites of the pPICZ-B-EGFP-6His vector, affording
the pPICZ-B-Hucatps1-eGFP-6His to produce the recombinant
protein fused with C-terminal enhanced green fluorescent
protein (eGFP) and 6X histidine tags. The pPICZ-B-eGFP plasmid
was a modified pPICZ-B vector (Invitrogen), in which the
PreScission protease recognition sequence and the eGFP-
6His tag sequence were inserted at the 3’ of the mult cloning
site. P. pastoris KM71H transformation was performed according
to Lin-Cereghino et al.32 using the pPICZ-B-Hucatps1-cGFP-
6His plasmid linearized with SacI (Promega). Transformed
cells were plated on YPD (2% Bacto-Yeast Extract, 2% dextrose,
1% peptone) agar supplemented with 100 µg/ml B conclusions
and incubated at 30°C for 2–3 days until colonies appeared.

In order to identify the clone showing the best protein
expression, each colony was inoculated in a 24-well plate
containing 2 ml of BMMY medium (100 mM HEPES, pH
6.0, 6.4% yeast nitrogen base without amino acids, 1% glucose, 4 x 10^-3 M biotin), incubated at 30°C
shaking at 280 rpm for 60 hours. Successively, for protein
expression induction, the medium was exchanged with BMM
(100 mM HEPES, pH 6.4, 6.4% yeast nitrogen base with
ammonium sulfate without amino acids, 4 x 10^-3 M biotin, 0.5%
methanol), and eGFP fluorescence was measured after 24, 48,
and 72 h using the Claristor plate reader (BMG Labtech,
excitation 489 nm, emission 509 nm), thus identifying the clone
having the highest fluorescence signal.

For large scale protein production, a promycotic of the
selected colony was grown overnight at 30°C and diluted 80-
fold in a 5 L flask containing 1 L of BMMY and 0.5% methanol added every 24 h. BMMY 50°C in 200 rpm shaking incubator. After 72 h, cells were
harvested by centrifugation and resuspended in half-volume of
BMMY medium, and 0.5% methanol was added every 24 h. Mammalian
cells were collected and resuspended in Buffer A (10 mM
sodium phosphate, pH 7.5, 500 mM NaCl, 1% glycerol, 1 mM
phospho-methyl sulfonyl fluoride (PMFS), Sigma-Aldrich),
protease inhibitors Complete EDTA-Free (Roche), and 1 mg/ml
DNase). An equal volume of zirconia beads (0.5 µm, BioSpec)
was added to the suspension, and yeast cells were
mechanically disrupted with a BioSpec Mini Bead-Beater. The
mixture was passed through a cloth mesh strainer to remove
zirconia beads, and centrifuged at 7000g for 30 min at 4°C.
The supernatant was applied on a HisTrap column (1 ml, GE
Healthcare) previously equilibrated in Buffer A, the column
was washed with 20 ml of imidazole, and then CTPS-1 was eluted
with 500 mM imidazole in the same buffer. The purified
enzyme was dialyzed against Buffer B (50 mM Tris-HCl, pH
7.5, 1 ml EDTA, 150 mM NaCl, 1% glycerol, 1 mM DT T),
digested with PreScission protease (GE Healthcare, 409 ml-
ml), and further purified by a second affinity chromatography,
followed by size exclusion chromatography on a HiLoad
Superdex 200 column (GE Healthcare). Sample purity was
checked by SDS-PAGE, and protein concentration was evaluated by Bradford reagent.15

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsome
dix7b07886.

Effects of GSK compounds against different ATP binding enzymes, MIC values of M. tuberculosis wild-type and pyrG mutant strains to GSK compounds, ratios of radioactivity incorporated to [3H]UTP and [3C]CTP of the control experiments, PyrG kinetic analysis toward UTP in the presence compounds, effects of the GSK compounds against PyrG glutaminase activity, SDS-PAGE of the purification steps of HsCPT-I, IC50 values of compounds to the wild-type and the V180G mutant PyrG enzyme, and characterization of the pyrG conditional mutant TB456 (PDF)

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**AUTHOR CONTRIBUTIONS**

M.E. and S.S. contributed equally and are considered as co-first authors to this work. M.E. and L.R.C. performed the screening of the TB-set library, provided by D.B., J.L., and L.B.; M.E. and L.R.C. performed protein expression, purification, and characterization; S.E. performed docking experiments; B.S.O. and G.M. performed MIC determinations and isolation of resistant mutants; G.D. and P.R. constructed pyrG conditional knock-down mutant and performed MIC determinations; S.S., S.H., and J.Z. performed metabolic labeling experiments; M.E. and V.P. donated the human CTPS; R.M., D.B., J.L., M.R.P., G.R., L.B., K.M., and L.R.C. analyzed data; R.M., A.M., M.R.P., G.R., K.M., and L.R.C. supervised and directed the work; S.E., R.M., K.M., and L.R.C. wrote the paper. All authors discussed the results and commented on the manuscript.

**Notes**

The authors declare the following competing financial interest(s): S.E. was a consultant for Collaborative Drug Discovery, Inc. All other authors declare no competing financial interest.

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**ABBREVIATIONS**

HEPES, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; HTS, high-throughput screening; HsCPTPSI, human CTP synthetase 1; MDR, multidrug resistant; MIC, minimal inhibitory concentration; Mtb, M. tuberculosis; PyrG, Mtb CTP synthetase; TB, tuberculosis; XDR, extremely drug resistant

**REFERENCES**


