REVIEW ARTICLE

RECORDS OF PHARMACEUTICAL AND BIOMEDICAL SCIENCES

Insights into Novel Drug Targets in Mycobacterium tuberculosis: Where Do We Stand and Where Do We Go from Here?

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Abstract

Tuberculosis (TB) is the ninth leading cause of death worldwide and the leading cause from a single infectious agent, ranking above HIV/AIDS with 6.3 million new cases of TB reported in 2016. TB is an air-borne disease associated with the aerobic bacterium Mycobacterium tuberculosis (Mtb), which mainly infects the lungs. Aerosolization of diseased pulmonary secretions, by coughing, sneezing and speaking, discharge the Mtb bacilli into the atmosphere. Infected aerosol droplet nuclei sized 1-10 μm are largely trapped in the upper nasal passages or are expelled into the pharynx by the mucociliary mechanism of the lower respiratory tract and are harmlessly swallowed and digested. Infected persons may overcome the initial TB infection, resulting in the development of asymptomatic latent TB. About 10% of individuals may develop the active disease after infection; where the bacteria undergo more rapid growth and overcome the host immune system. In cases of multi-drug resistant (MDR) strains, and extreme drug-resistant (XDR) strains, treatment fails, and the bacteria propagate and attack the host, leading to death from systemic infection. Due to the increased spread of TB worldwide, both the academic and industrial communities have initiated intensive research to develop new therapeutics targeting new enzymes such as cytochrome P450s in Mtb.

Keywords: *Mycobacterium tuberculosis*, Cytochrome P450s, Therapeutics

1. Introduction

Mycobacterium tuberculosis (*Mtb*) belongs to the Mycobacterium genus, characterised by its ability to synthesise mycolic acids as constituents of its cell wall. This unique envelope surrounds these bacteria, and is composed of an inner cytoplasmic membrane, an intermediate cell wall with a mixture of sugar molecules, and an exterior wall containing mycolic acid-based lipids, as well as surface signalling and transmembrane proteins. The presence of this cell wall in mycobacteria also aids in the resistance of *Mtb* to external stresses such as macrophage environments and anti-TB drugs (Figure 1) (Bhamidi et al., 2008). The mycobacterial outer membrane is important in giving the mycobacteria their pathogenic features. The outer membrane is comprised of 40% mycolic acids, covalently linked to arabinose and galactose subunits called arabinogalactans that are themselves linked to the peptidoglycan middle layer (Brennan et al., 1995). Lipoarabinomannan lipids of the outer layer contribute to the structural integrity.

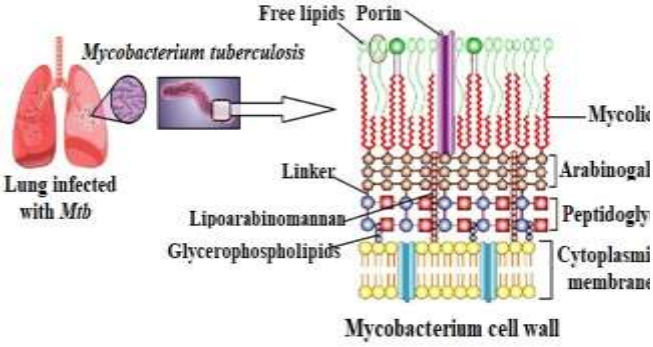

Figure 1. The Mycobacterium Cell wall: the envelope is subdivided into three layers. The mycobacterial outer membrane, which is comprised of mycolic acids and various lipids such as branched and capped portions of lipoarabinomannans. The middle layer or the periplasmic cell wall consists mainly of peptidoglycans and galactans, as well as some lipomannan portions of lipoarabinomannans. There are also significant amounts of phosphatidylinositol mannosides that link the periplasmic cell wall to the inner third layer, the cytoplasmic phospholipid bilayer. This layer consists mainly of basic plasma membrane phospholipids as well as some polyprenyl sugars.

The porin proteins present in the outer layer are responsible for trafficking molecules across the outer membrane, and the acquisition of nutrients to aid metabolism (Niderweis, 2008).

2. Treatment of TB

The use of anti-TB drug combinations, such as isoniazid, *para*-amino-salicyclic acid and rifampicin, was approved as an ideal chemotherapeutic treatment against TB infections (Laurenzi et al., 2007; Yew et al., 2011). The standard therapeutic regimens involve the combination of four first-line drugs, often isoniazid, rifampicin, pyrazinamide and either streptomycin or ethambutol, depending upon whether there is a latent or an active infection (McLean et al., 2008). Figure 2 shows the chemical structures of the following described drugs.

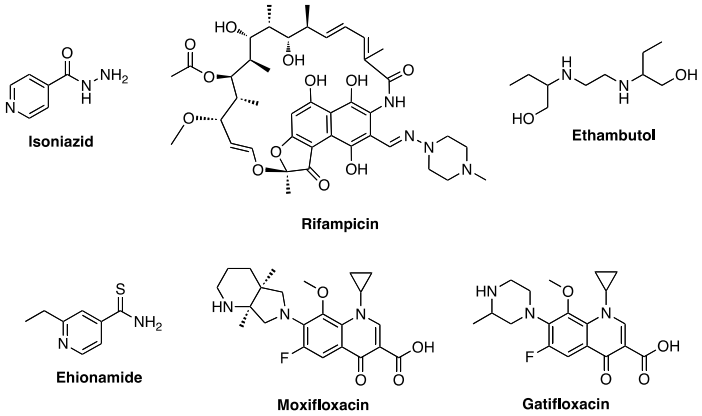

Figure 2. The chemical structures of the common current anti-TB drugs

2.1.Isoniazid

Although isoniazid was first characterised in 1912, it was not released into clinical trials prior to 1952. Isoniazid is a pro-drug that is converted by the enzyme catalase/peroxidase into an activated radical form, which reacts with NAD(P)H resulting in the formation of an isonicotinic acyl-NAD(P)H complex. The complex binds covalently to the NAD(H) recognition site of the gene encoded NADH-dependent enoyl-(acyl carrier protein) reductase. This enzyme is involved in the biosynthetic pathway of mycolic acids, and hence isoniazid inhibits mycolic acid synthesis, which is a critical component of the *Mtb* cell wall (Scior et al., 2002).

2.2. Rifampicin

Rifampicin was first described in 1967 and its specific inhibition of the *Mtb* DNA-directed RNA polymerase enzyme explains its efficiency as an anti-TB drug (Yew et al., 2011). The conformational change, induced by the binding of rifampicin to the β-subunit of the polymerase, prevents the initiation of RNA synthesis and thus inhibits the process of transcription (Janin, 2007). Resistance to rifampicin arises from mutations in the *rpoB* gene that encodes the RNA polymerase β-subunit. These mutations produce major effects on a specific region of the bacterial RNA polymerase, causing alterations to the drug-binding site, but not adversely affecting enzyme function (McLean et al., 2008).

2.3. Ethambutol

Ethambutol is a classical bacteriostatic anti-TB drug acting by the inhibition of arabinose biosynthesis, which is critical for the *Mtb* cell wall structure (McLean et al., 2008).

2.4. Ethionamide

Ethionamide is another pro-drug, like isoniazid, which is used in the chemotherapy of MDR-TB. The *Mtb* flavin-containing monooxygenase enzyme causes the oxidative activation of ethionamide (McLean et al., 2008). Activated ethionamide reacts with nucleophilic residues on target protein(s), forming covalent adducts in an analogous manner to isoniazid.

2.5. Fluoroquinolones

Fluoroquinolones are potent inhibitors of DNA gyrase and thus interfere with bacterial chromosomal replication (Field et al., 2012), exhibiting mycobactericidal properties, as well as showing concentration-dependent killing of *Mtb* at lower Minimum Inhibitory Concentrations (MICs) than those of other first-line drugs (Donald et al., 2008). Therefore, fluoroquinolones are crucial second-line drugs in the treatment of MDR-TB (Field et al., 2012).

2.5.1. Moxifloxacin

This 4th generation fluoroquinolone drug is a broad-spectrum 8-methoxy fluoroquinolone with anti-bacterial activity against both Gram-positive and Gram-negative bacteria, as well as against anaerobes acting specifically as an inhibitor of DNA gyrase. In rifampicin-resistant *Mtb* strains, moxifloxacin shows the greatest sterilizing activity, and thus it may currently be the most potent drug for the treatment of TB in such strains (Lalloo et al., 2010).

2.5.2. Gatifloxacin

Gatifloxacin has the same inhibitory mechanism as moxifloxacin in blocking DNA gyrase enzyme activity (Boogaard et al., 2009), and has demonstrated a high potential for the rapid clearance of the bacterial burden from sputum of infected patients (Yew et al., 2011). However, USA FDA recommendations have resulted in the cessation of the manufacture of gatifloxacin in 2006, owing to adverse effects causing the condition dysglycemia, as well as other disturbances to glucose homeostasis (Nuermberger et al., 2010). Figure 3 describes the mechanism of action of some anti-TB drugs.

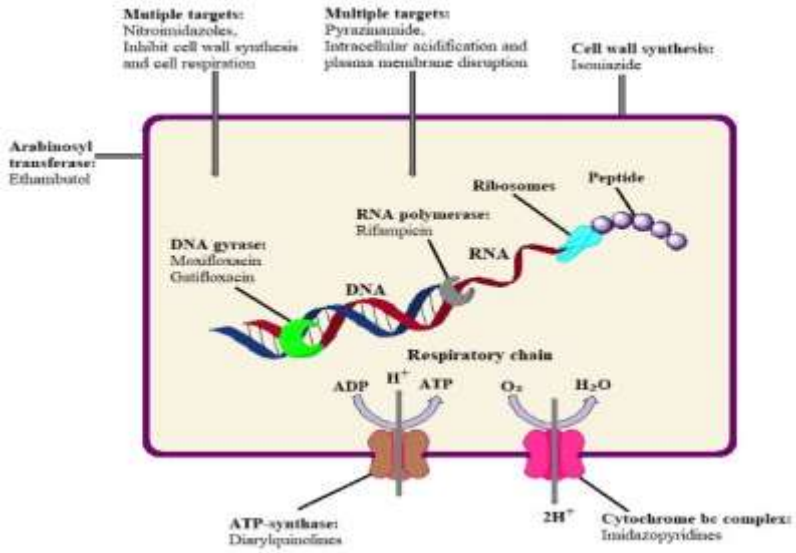

Figure 3. Mechanism of action of some anti-TB drugs

Among the more recently developed novel compounds as anti-TB drugs are diarylquinolines, phenothiazines and nitroimidazopyrans (Diacon et al., 2009; Makarov et al. 2009; Pethe et al., 2013). Most of these drugs are now in the preclinical stage of drug development (Figure 4).

2.6.Diarylquinolines

The FDA approved TMC207 (Bedaquiline) as a suitable and effective drug to treat MDR-TB in December, 2012 as a part of a four drug combination therapy (Svensson et al., 2013).
Bedaquiline is the first diarylquinoline drug to enter phase II human clinical trials. Bedaquiline inhibits the

mycobacterial ATP synthase enzyme, thus reducing adenosine triphosphate (ATP) levels, and shows some effects on other bacteria but is not effective on the homologous ATPase enzyme in eukaryotes (Andries et al., 2005; Haagsma et al., 2009; De Jonge et al., 2007; Koul et al., 2008; Field et al., 2012). Bedaquiline was reported to be effective against streptomycin, pyrazinamide, ethambutol and moxifloxacin resistant strains (Lienhardt et al., 2010; Boogaard et al., 2009; Field et al., 2012). Recent studies indicated that bedaquiline is effective against both macrophage-engulfed and extracellular *Mtb* (Dhillon et al., 2010).

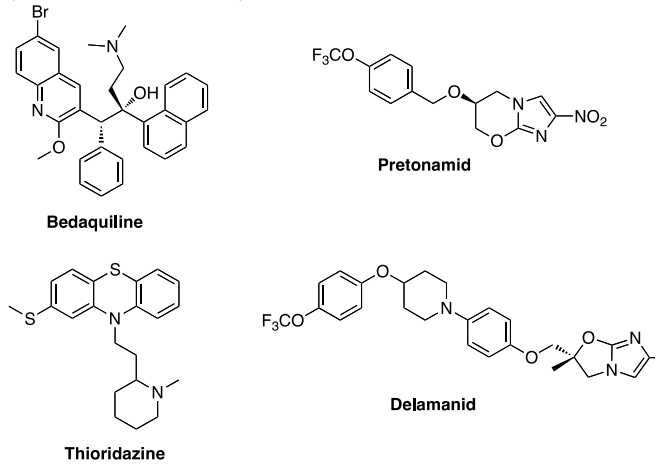

Figure 4. The chemical structures of recent anti-TB drugs

2.7.Phenothiazines

Thioridazine has activity against *Mtb* by inhibiting several crucial biosynthetic pathways involved in respiration, antibiotic resistance and bacterial efflux mechanisms in *Mtb* (Sharma et al., 2011; Amaral et al., 2008). Thioridazine inhibits microaerobic respiration by inhibiting *Mtb* respiratory chain enzymes, specifically the type II nicotinamide adenine dinucleotide dehydrogenase and inhibits calcium-calmodulin binding and can reverse antibiotic resistance in certain cases through symbiotic relationships with other co-administered anti-TB drugs (Sharma et al., 2011; Field et al., 2012) Thioridazine also inhibits bacterial efflux mechanisms, hence enhancing the sensitivity of *Mtb* to anti-TB agents (Amaral et al., 2008). Thioridazine has a potential therapeutic treatment regimen for XDR-TB infected individuals, although there are still some questions regarding its toxicity (Amaral et al., 2010; Field et al., 2012).

2.8. Nitroimidazopyrans

The two important classes of nitroimidazopyran compounds in clinical trials are the nitroimidazoxazine (PA-824, Pretomanid) and the nitroimidazoxazole (OPC-67683, Delamanid) (Field et al., 2012).
Pretomanid is a pro-drug, activated by the mycobacterial enzyme glucose-6-phosphate dehydrogenase, leading to the production of microbicidal molecules such as nitric oxide and other reactive nitrogen intermediates that damage/inhibit the *Mtb* cytochrome oxidase enzyme. Activated pretomanid also inhibits vital mycobacterial processes such as protein and mycolic acid synthesis (Field et al., 2012). Pretomanid is effective against both actively replicating and non-replicating *Mtb* strains (Lienhardt et al., 2010).
Delamanid is also a pro-drug that requires metabolic activation for its activity (Mukherjee et al., 2011; Field et al., 2012). Delamanid produces reactive nitrogen intermediates, which inhibit the synthesis of protein and mycolic acids. The MICs reported for delamanid range from 0.006 to 0.024 μg/mL (Field et al., 2012).

3. Mtb P450s

Following the unravelling of the genome sequence of the virulent *Mtb* H37Rv strain (Cole et al., 1998), twenty different cytochrome genes coding for P450s were revealed. This large number of cytochrome genes was uncommon for a bacterium and, as a result, studies over the past ten years have focused on characterising these *Mtb* P450s. Some of the recently studied *Mtb* P450s are:

3.1. CYP51B1

The CYP51 family is a substrate-specific enzyme that catalyses the 14α-demethylation of sterols (Ortiz

de Montellano, 2005). In *Mtb*, CYP51B1 catalyses the conversion of lanosterol and closely related 14α-methyl sterols to 14α-desmethyl derivatives (Bellamine et al., 1999; Bellamine et al., 2001; McLean et al., 2006). Whether CYP51B1 is a suitable target for drug development or not is still questionable because *Mtb* does not have a functional sterol biosynthetic pathway as the genes coding for squalene epoxidase and oxidosqualene cyclase, two key enzymes required for construction of the sterol skeleton, are absent in *Mtb*, in addition to the fact that CYP51B1 is not required for mycobacterial growth (Sassetti et al., 2003; Sassetti et al., 2001; McLean et al., 2010).

3.2. CYP126A1

The crystal structure, the biophysical properties (UV-Vis, Electron Paramagnetic Resonance, and Magnetic Circular Dichroism spectra) and the iron redox potentials in the substrate-free and ligand bound forms of CYP126A1 have been determined (Chenge et al., 2017). Fragment-approaches revealed that chlorophenol derivatives and nitro compounds with three aromatic rings act as ligands for CYP126A1 but are poor substrates for the enzyme (Chenge et al., 2017). The role and importance of CYP126A1 are still unclear, making it uncertain whether it has potential as a drug target or not.

3.3. CYP130A1 and CYP141A1

CYP130A1 and CYP141A1 are absent from the *Mycobacterium bovis* genome. These findings suggested that CYP130A1 and CYP141A1 are important to the pathogenic process in the human host, although they are not essential for the viability of the bacterium (Ouellet et al., 2008). Screening efforts with a diversity of cytochrome P450 substrates, including conventional P450 probes, fatty acids, and steroids, did not lead to identification of a substrate for CYP130A1 (Chenge et al., 2017). Furthermore, a spectroscopic screen of 20,000 compounds in a search for enzyme ligands failed to identify any compounds giving rise to a Type I spectral shift (i.e. potential substrates) but could identify a range of compounds that gave the Type II spectral shifts associated with enzyme inhibitors (Ouellet et al., 2008). Polycyclic arylamines were identified as novel inhibitors for CYP130A1 (Podust et al., 2009).

3.4. CYP124A1

CYP124A1 catalyses the terminal hydroxylation of methyl-branched hydrocarbon chains such as those of phytanic acid and farnesol (Johnston et al., 2009), cholesterol and related sterols (Johnston et al., 2010; Johnston et al., 2012), and vitamin D_3 (Vasilevskaya et al., 2017). Strains of *Mtb* lacking functional CYP124A1 grow readily in culture (Johnston et al., 2010), indicating that this enzyme is not essential for growth, although it could play a role in the more complex context of an *in vivo* infection. The viability of CYP124A1 as a potential target for anti-TB drug development remains uncertain.

3.5. CYP144A1

Although CYP144A1 is not essential for growth of the mycobacteria in culture, it is suggested that this enzyme plays a role in cellular biology or in modulating resistance to azole drugs. (Carroll et al., 2015). Although the substrate and the role of CYP144A1 are unknown it may still be worth development of inhibitors of the enzyme (Chenge et al., 2017).

3.6. CYP121A1

CYP121A1 was shown to be essential for bacterial growth by *in vitro* gene knockout studies (Sassetti et al., 2001; Sassetti et al., 2003; McLean et al., 2008). The CYP121A1 gene is located in an operon harbouring two enzymes involved in the formation of cyclo-di-L-tyrosine (cYY). The first enzyme is cyclodipeptide synthase (encoded by *Rv2275*), which uses activated amino acids in the form of aminoacyl-tRNA synthetases (L-tyrosyl-$tRNA^{Tyr}$, a class Ic aa-tRNA) as substrates to catalyze the ATP-independent formation of cyclodipeptides (cyclo(L-Tyr-L-Tyr), cYY) (Gondry et al., 2009). The reaction forms a covalent intermediate between an active site Ser88 and the L-phenylalanine residue (Vetting et al., 2010).

Then, CYP121A1 (encoded by *Rv2276*) catalyses C-C crosslinking reaction between the respective carbons in the ortho position of the phenolic hydroxyl of cYY producing mycocyclosin. *Rv2276* was found to be an essential *Mtb* gene, and it was suggested that either mycocyclosin was essential or the overproduction of cYY is toxic (Belin et al., 2009; McLean et al., 2008). The C-C bond formation represents an unusual activity of the P450 enzyme superfamily.

CYP121A1 can also use cyclo(L-Tyr-L-Phe) (cYF), cyclo(L-Tyr-L-Trp) (cYW) and cyclo(L-Tyr-L-3,4-dihydroxyphenylalanine) (cY-DOPA) as substrates (Belin et al., 2009).

Table 1. Dissociation constants for the binding of selected azole drugs to *Mtb* P450s

Anti-fungal azole drug	K_D (μM)				
	CYP51B1	CYP144A1	CYP125A1	CYP142A1	CYP121A1
Econazole	2.4 ± 0.8	0.78 ± 0.29	11.7 ± 0.7	4.6 ± 0.2	< 0.2
Clotrimazole	< 0.2	0.37 ± 0.08	5.3 ± 0.6	3.8 ± 0.9	< 0.2
Miconazole	nd	0.98 ± 0.22	4.6 ± 0.4	4.0 ± 0.5	< 0.2
Ketoconazole	5.9 ± 2.7	134 ± 5	27.1 ± 0.9	21 ± 4	3.3 ± 0.3
Fluconazole	5.82 ± 0.12	nd	43.1 ± 3.8	860 ± 108	9.7 ± 0.2
Voriconazole	nd	174 ± 14	nd	nd	nd
Itraconazole	nd	nd	30.2 ± 4.3	nd	nd

nd: no data

4. Azole anti-fungal: potent inhibitors of Mtb P450s

The potency of the azole anti-fungal as anti-*Mtb* drugs is evident from their *in vitro* tight binding to *Mtb* P450s with K_D values in the low micromolar or nanomolar ranges (Driscoll et al., 2010; McLean et al., 2009; Johnston et al., 2009).

Econazole was found to be effective in treating *in vitro* and *ex vivo* latent and active *Mtb* infections in a mouse model. However, clinically effective drugs such as fluconazole and voriconazole often display quite weak binding affinity for the *Mtb* P450s, while less hydrophilic azole drugs have greater affinity (Table 1) (Driscoll et al., 2010; Johnston et al., 2009). The mode of binding of different azole drugs to several *Mtb* P450s has been established through crystallographic studies with CYP51B1, CYP121A1, CYP125A1 and CYP130A1 (McLean et al., 2009; McLean et al., 2010; Ouellet et al., 2008) Clotrimazole and econazole effectively treat latent as well as MDR-*Mtb* infections. Econazole was also shown to reduce by 90% the bacterial burden from lungs and spleen of mice infected with *Mtb* (McLean et al., 2010).

5. Conclusion

While the use of anti-TB drug combinations is effective against TB infections in standard conditions, the development of new therapeutics targeting new enzymes such as cytochrome P450s became a requirement worldwide.

In this review, we described all the available anti-TB drugs, either in the market or in the pre-clinical phase. We also described the cytochrome P450 enzymes in *Mtb* as novel drug targets for the treatment of MDR and XDR strains.

6. Acknowledgment

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