Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2016, 8(6):179-188
(http://derpharmachemica.com/archive.html)

Mycobacterial CYP121 as a target for anti-TB drug discovery

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ABSTRACT

Despite the introduction of the first line treatment regimen forty years ago and the continuous trials since that time to introduce new regimens, tuberculosis (TB) is considered to be the cause of considerable mortality worldwide. Recent research highlighted the Mycobacterium tuberculosis (Mtb)CYP450s as potential drug targets. This article reviews mycobacterial CYP121 as a target for anti-TB drug discovery.

Key words: Mtb CYP121, Mycobacterium tuberculosis, Dicyclotyrosine derivatives.

INTRODUCTION

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*). [1] *Mtb* is a large rod-shaped non-motile bacterium related to *Actinomycetes.* And an obligate aerobe that is always found in the well aerated upper lobes of the lungs. The bacterium is an intracellular facultative parasite, usually of macrophages, and has a slow generation time of 15-20 hours, which is a physiological characteristic that may contribute to its virulence. [2]

Upon inhalation of a tubercle bacilli droplet, it reaches the alveoli of the lungs such that the majority of these bacilli are destroyed or inhibited by lung macrophages. A small number may multiply intracellularly and are released when the macrophages die. Bacilli that are not destroyed may spread through lymphatic channels or the bloodstream to more remote tissues and organs. [3] Immune cells (macrophages) ingest and surround the tubercle bacilli forming a barrier shell, called a granuloma this keeps the bacilli contained and under control and is defined as latent TB infection (LTBI). Once there is immune deficiency, the bacilli begin to multiply rapidly causing TB disease. [3, 4]

Tuberculosis still remains a major cause of human mortality and the emergence of strains of Mtb, the causative agent, resistant to the frontline antitubercular drugs increases the urgency for the development of new therapeutic agents. According to the World Health Organization (WHO) estimations a marked increase in TB infections resulted in 1.5 million deaths reported in 2014. Moreover, a marked increase of 6% in the TB incidence was reported in 2014 compared with the numbers reported in 2013. [5]

Drug-resistance can be either primary or secondary; primary resistance occurs when a patient is exposed to an infected person with resistant organisms while secondary resistance, or acquired resistance, develops during TB therapy, either because the patient did not follow the prescribed regimen appropriately or was treated with an

inadequate regimen or because of the other conditions that led to low drug serum levels such as drug malabsorption or drug-drug interactions. [6] Drug resistant TB can be also classified as: multidrug resistant TB (MDR TB), which is caused by organisms resistant to first-line drugs (isoniazid and rifampicin) used in the treatment of TB disease; [4] excessive drug resistant TB (XDR TB), which is caused by organisms resistant to both isoniazid and rifampin and any fluoroquinolone plus at least one of three injectable second-line drugs (e.g.amikacin, kanamycin, or capreomycin); rifampicin-resistant TB (RR-TB) is caused by organisms resistant to rifampicin, with or without resistance to other drugs. Both MDR-TB and XDR-TB are forms of RR-TB; [7] total drug resistant TB (TDR-TB) is the term used to describe TB caused by *Mtb* strains that are resistant to all available first-line and second-line TB drugs.

An estimated 480,000 people developed MDR-TB in 2014 and it is the development of drug resistance and the HIV epidemic that are the two major threats to global TB control. [5, 8]

1. Cytochromes as drug targets

Sequencing of the *Mtb* genome revealed the existence of twenty CYP450 enzymes, [9] whereas CYP450 enzymes are relatively rare in other bacterial genomes. For instance, *E. coli* has none while *Bacillus subtilis* has seven CYP450s, which is the next largest bacterial complement of CYP450s before the determination of the *Mtb* genome sequence. [10]

1.1. Crystal structure of Mycobacterium Cytochromes

Generally the X-ray crystallography characterization of bacterial CYP450 enzymes revealed that bacterial cytochromes exhibit certain similarities in sharing a basic tertiary conserved core, which is associated with binding of the heme prosthetic group. This core is a decapeptide at the start of the L-helix that has been generally regarded as the CYP450 signature motif for identifyingCYP450 sequences in genome mapping. The general formula of this core is FxxGxxxCxG where x is any amino acid residue, F phenylalanine, G glycine and C cysteine (represents the proximal heme ligand). Although the x residues can refer to any amino acid there is a certain preference that the amino acid residue two positions upstream of cysteine is usually basic in character and might be either histidine or arginine as shown in the majority of bacterial CYP450s investigated thus far. [11] Besides the decapeptide signature, there are other conserved regions in CYP450 sequences related to the maintenance of tertiary structure or related to redox partner binding sites. [12] Most bacterial CYP450s utilize an iron-sulphurredoxin for electron transfer from NADH and the types of redox partner involved in the catalytic cycle can vary from one CYP450 to another. [13,14] A conserved threonine residue is present in most CYP450s for the activation and binding of dioxygen. This threonine residue is considered to act co-operatively with a preceding acidic residue in forming a proton-transfer channel within the CYP450 active site, which would be hydrated when the substrate is absent. [15]

2.2. Mycobacterium CYP121 as a drug target

The physiological role for CYP121 remains uncertain as the highest similarities observed between the CYP121 amino acid sequence and other CYP450 sequences in protein databases was 34% identity, thus too low to predict a function for CYP121 confidently. However, one of its closest relatives is CYP450eryF (CYP107A1), involved in erythromycin biosynthesis. Accordingly, it is thought that CYP121 might play a role in polyketide synthesis. [16] The gene encoding the CYP121 *rv2276* was shown to be essential for *Mtb* viability, this gene was found to be associated in an operon-like structure *rv2275* indicating that the two corresponding proteins might be involved in the same metabolic pathway. [17] Moreover, more than 90% of the cyclodipeptides synthesized by recombinant *E. coli* expressing *rv2275* are cyclo(L-Tyr-L-Tyr) (cYY). These findings indicated that cYY was the main product of Rv2275. [16] Cyclodipeptide synthases are a family of tRNA-dependent peptide bond-forming enzymes. [18] In 2003 the atomic structure of CYP121 was solved at a resolution of 1.06 Å, which is considered as the highest resolution structure for any CYP450 enzyme [19], then in 2009 the X-ray structure of cyclo(L-Tyr-L-Tyr)-bound to CYP121 was published [16] and in turn opened the way for design of more selective and potent *anti-Mtb* drugs. CYP450s generally catalyze the insertion of an oxygen atom, however CYP121 exhibits a novel diketopiperazine (DKP) modifying activity, catalyzing the formation of a C-C bond between the two tyrosyl side chains of cYY resulting in a novel chemical entity called mycocyclosin. [16] **Figure 1**

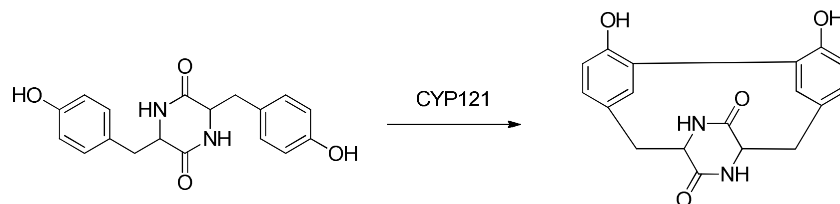


Figure 1 Formation of mycocyclosin

2.2.1. Crystal structure of CYP121

The atomic structure is composed mainly of α helices. The heme (yellowspace fill in Figure 2) is sandwiched between the two major domains of the structure (an α helix rich domain and smaller β sheet-rich domain **Figure 2**

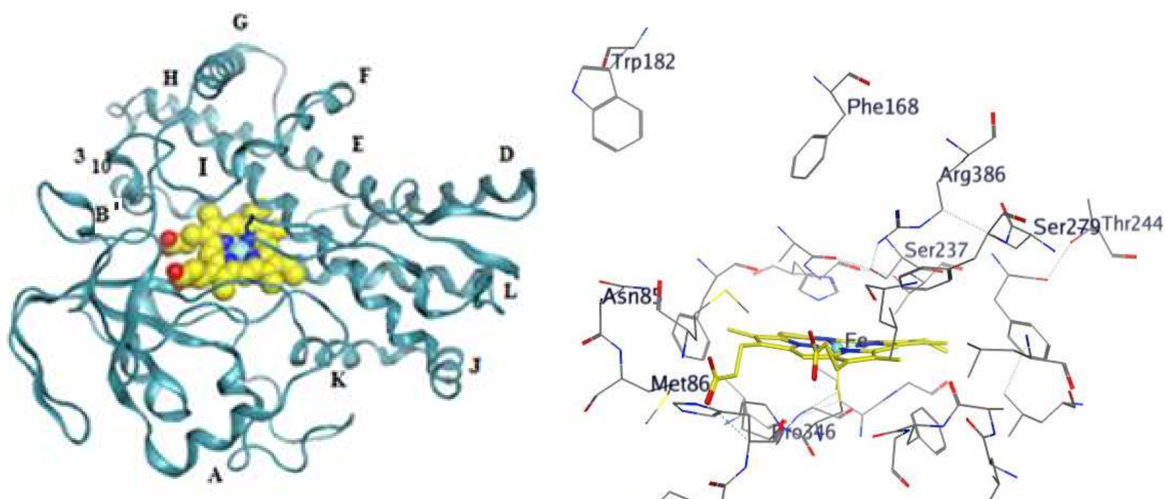


Figure 2The overall fold of *Mtb* CYP121 (left) and crystal structure CYP121 (right) (pdb code: 3G5F)

Arg386 dominates the active site close to the heme. The most distinguished interaction of Arg386 is with Ser237, which is hydrogen bonded to a water molecule. This water molecule provides the distal axial ligand to the heme iron. The Arg–Ser motif could act as a docking site for negatively charged functional groups. The heme is bound in two orientations, related to one another by a 180° rotation about an axis of symmetry across the CHα–Fe–CHδ atoms of the heme. [20] The heme is pushed by 30° upward from the heme plane because of the interaction of the side chain of Pro346. [21] **Figure2**.

Residue Ser279 acts as a channel for protons delivered from either of two aqueous channels from the protein surface involving Thr244 and Glu310 as it occupies two distinct conformations in CYP121 [19**] Figure3**.

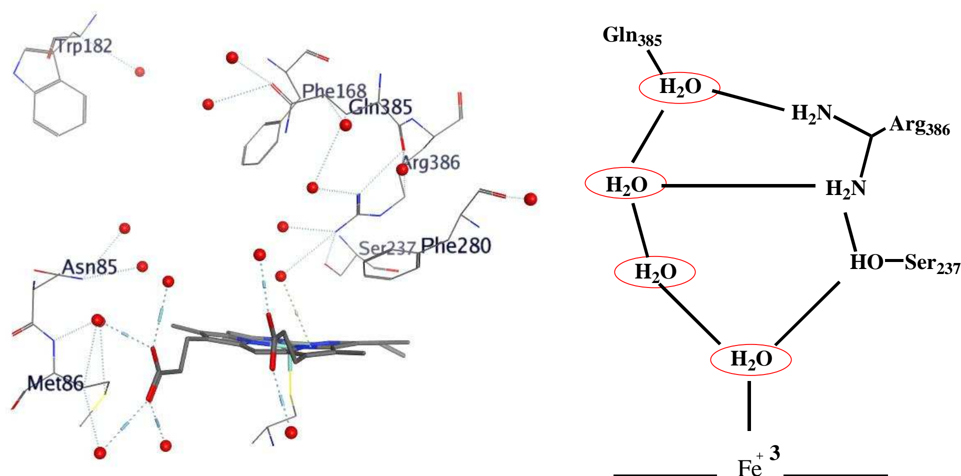


Figure 3 Hydrogen bonding patterns between CYP121 heme iron and surrounding amino acids (pdb code: 3G5F)

Spectroscopic characterization of CYP121

UV–visible absorption spectroscopy is considered to be the main technique for characterization and recognition of CYP450 enzymes. The oxidized form of pure *Mtb* CYP121 shows spectral properties distinctive for members of the CYP450 enzyme class, with the major (Soret or g) band appearing at 416.5 nm, and smaller a and b bands appearing at 565 and 538 nm, respectively. When the pure enzyme is reduced with sodium dithionite, the Soret band shifts to 450 nm and diminishes in intensity. However, there is a slight difference between CYP121 and CYP450cam in the spectral maxima for both the oxidized and reduced forms, as CYP121 spectral maxima are at slightly shorter wavelengths (approximately 2 nm). [22]

Electron Paramagnetic Resonance (EPR) analysis

The CYP121 EPR spectrum is virtually identical to those reported previously for well characterizedCYP450cam, as the major signals in the spectrum are a rhombic trio of g-tensor elements at g 2.47, 2.25 and 1.90 typical for low ferric heme iron. [23]

2.2.2. MtbCYP121 natural substrate (cYY)

cYY, the natural substrate, is a small molecule occupying partially (300 Å) of a large cavity
(1350 Å) of CYP121, thus splitting it into two cavities filled with water. The binding of cYY to CYP121includes a direct hydrogen bond interaction between the carbonyl of cYY and nitrogen of Asn85 and a water mediated hydrogen bonding pattern. Hydrophobic interactions are also demonstrated on the other side chain. **Figure 4**.[16]

2.2.3. MtbCYP121 inhibitors

Various azole-class drugs bind with high affinity to the *Mtb*CYP450heme and are potent *Mtb* antibiotics [22], these azole drugs coordinate tightly to the heme iron of Mtb CYP51B1, CYP130A1 and CYP121A1.

Binding to CYP450 may be either type I: (substrate like binding) as the substrate displaces the axial water disturbing the water network around the heme iron causing a shift to high spin and the soret peak will be shifted to lower λ max, or type II: caused by inhibitors that most likely coordinate by a heteroatom to the heme iron through a lone pair of electrons causing a shift to low spin state and the soret peak will be shifted to higher λ max.

Azole antifungals have high binding affinities to CYP121, which act by type II azole heme coordination. [24] The fact that azole drugs coordinate tightly to the heme iron of *Mtb* CYP51B1, CYP130A1, CYP125A1, CYP121 and CYP144 can help in the design of new inhibitors in addition to the knowledge of the binding interactions of the natural CYP121 substrate. [20, 25]

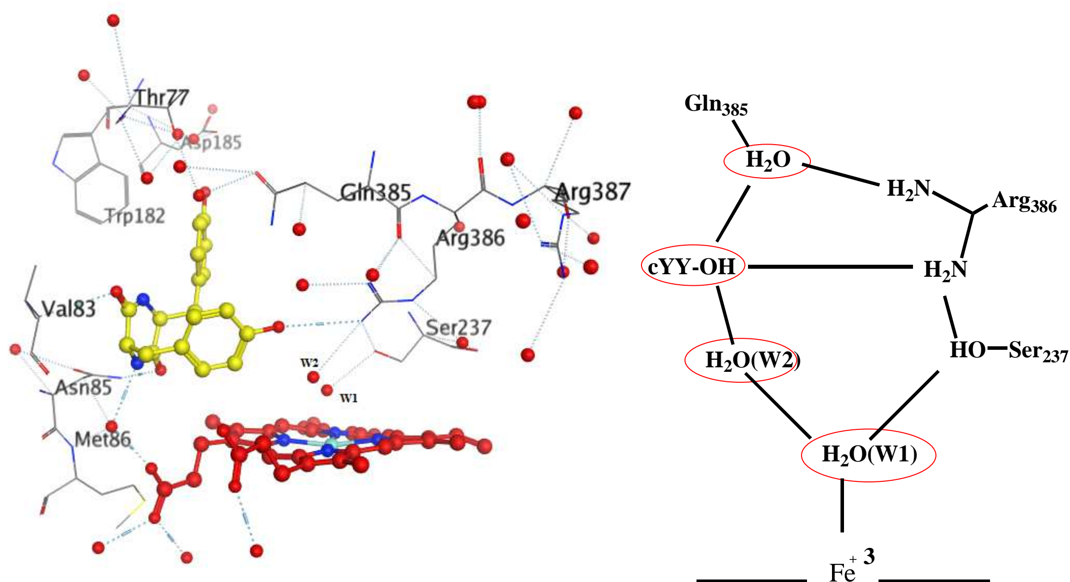


Figure 4 Crystal structure of cYY-bound to CYP121 (left). Hydrogen bonding patterns between cYY and CYP121 heme iron (right). (pdb code:3G5H)

Azole antifungal drugs (clotrimazole, econazole, fluconazole, ketoconazole and miconazole), **Figure 5** were found to bind tightly to CYP121 inducing a shift of the soret peak to423.5 nm. The Kd (dissociation constant) values for clotrimazole, econazole and miconazole are very low (<0.2 μM) indicating tight binding, fluconazole showed weaker binding with a Kd value 9.7±0.2 μM while ketoconazole showed higher affinity than fluconazole with a Kd value 3.3±0.3μM.
[26]

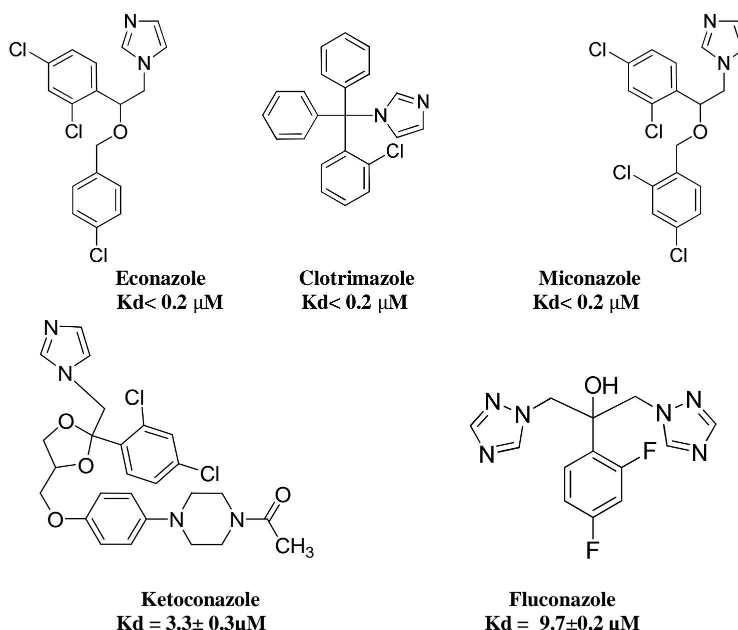


Figure 5 Structure and Kd values of econazole, clotrimazole, miconazole and fluconazole

In contrast to structurally characterized CYP450 azole complexes, where the azole nitrogen directly coordinates the heme iron [27], the crystal structure of *Mtb* CYP121 in complex with fluconazole revealed a new ligation mode.

Fluconazole does not displace the aqua sixth heme ligand but a fluconazole triazole group binds to the water sixth heme ligand in addition to forming a hydrogen bond with a second water molecule that is, at the same time hydrogen-bonded to a heme propionate [21] **Figure 6.** The remainder of the fluconazole molecule is mainly bound via hydrophobic interactions. A minority of CYP121-fluconazole complex molecules showed direct ligation of fluconazole triazole to the heme iron. Even though there are significant differences in the position and conformation of the fluconazole ligand by comparison with other CYP450 azole complexes, the triazole plane was predicted to be perpendicular with the porphyrin macrocycle and the ligating triazole nitrogen predicted to be linearly arranged with the iron and cysteine sulfur atom. However, the ligating triazole nitrogen atom is 0.7 Å away from a position along the iron-sulfur axis, while the triazole cycle is at a 25° angle with that axis. occupying a near-ideal ligation pattern. [21]

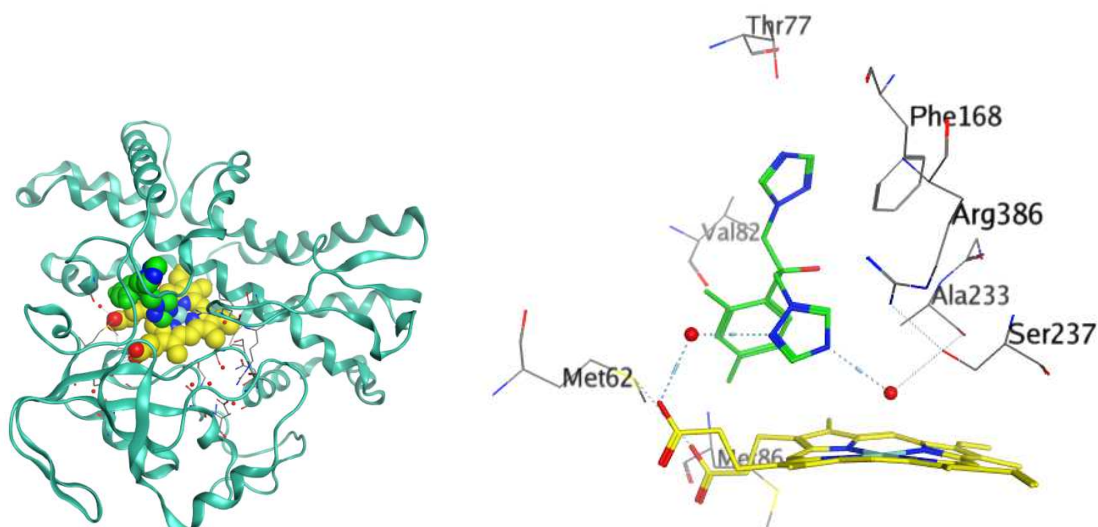


Figure 6 Structural topology of CYP121 bound to fluconazole (left) and the fluconazole binding site of *Mtb* CYP121 (PDB code: 2IJ7) (right)

A study of the inhibitory potency of azole derivatives found a strong correlatation (R = 0.99) with their lipophilic character, as described by the log D 7.4 value (D 7.4 is the distribution coefficient at pH 7.4). Increasing the log D 7.4 values leads to increase in -log MIC.[15]

A non-denaturing nano-electrospray ionization mass (nanoESI MS) study on both ligand-free and ligand-bound states of CYP121 revealed that isolated unbound CYP121 is a predominantly dimeric protein, with a minor monomeric form. However, high affinity azoles (e.g. clotrimazole, and miconazole) cause the dissociation of dimeric CYP121 into a monomer, whereas weaker binding azoles (e.g. fluconazole and itraconazole) induce partial dimer dissociation or do not significantly destabilize the dimer. Unlike with the azoles, CYP121 forms a stable complex with its natural substrate cYY that does not undergo gas phase dissociation. On the basis of binding to the monomer and/or CYP121 dimer it was possible to determine the relative order of their CYP121 binding affinities. [21]

A fragment screening approach for drug design is based on structure-guided design and synthesis of inhibitory molecules that target a specific enzyme. [28] An example of this approach was implemented to discover novel *Mtb* CYP121 inhibitors starting with a library of 665commercially available fragments, the majority were benzene or heterocyclic aromatic fragments. The fragment library was a first-generation Rule of three compliant set [29] through an initial fragment-screening cascade involving fluorescence based thermal shift [30], NMR spectroscopy and X-ray crystallography in an attempt to identify new *Mtb* CYP121 inhibitory molecules. [31]

Four fragments were determined to bind to *Mtb* CYP121 and binding affinity was determined using the isothermal titration method (ITC). [31] **Figure7**.

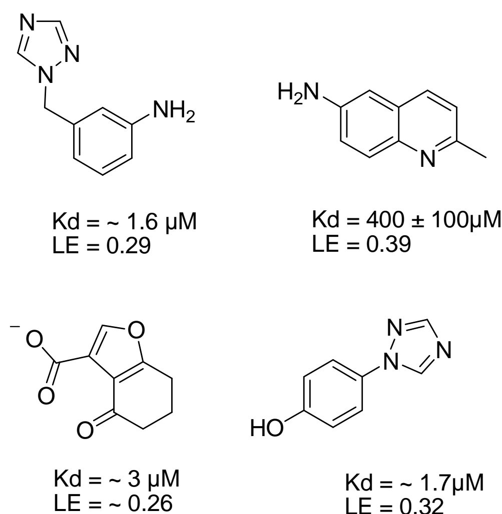
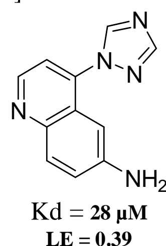


Figure 7 Fragments showing binding to *Mtb* CYP121
LE (ligand efficiency) = - ΔG of binding/non-hydrogen atoms in the ligand, Kd = dissociation constant

Several fragments were synthesized through merging of the four fragments having *Mtb* CYP121 binding affinity. A lead aminoquinoline fragment (**Figure 8**) with high ligand efficiency (LE) and Kd value of28 µM selectivity provided an excellent scaffold for competitive *Mtb* CYP121 inhibitors. The lead aminoquinoline coordinates the heme iron through its arylamine nitrogen atom. [31]



Kd = **28 µM**
LE = 0.39

Figure 8 Aminoquinoline fragment acting as *Mtb* CYP121 inhibitor

A novel series of potent and selective *Mtb* CYP121 inhibitors have been developed using synthetic merging and optimization of a triazol-y-l phenol fragment (Kd = 1.7 mM, LE = 0.32) that was identified to bind to *Mtb* CYP121. The initial optimization strategy of thetriazol-y-l phenol fragment yielded the triphenolpyrazole amine lead compound with 100-fold improvement in the binding affinity but with low LE (Kd = 15µM, LE = 0.23). Deconstruction of the triphenolpyrazole amine lead compound into its component retrofragments, including the mono and biphenolpyrazoles, allowed the assessment of the binding contribution or group efficiency (GE) of the aromatic rings that are attached to the aminopyrazole core of the triphenolpyrazole amine lead compound**.** The retrofragment scaffolds were incorporated with a metal binding pharmacophore to target binding hotspots in the active site and to afford selectivity against human CYP450 enzymes. Extensive characterization of the synthesized analogues by X-ray crystallography, UV−vis spectroscopy, and native mass spectrometry resulted in a low nanomolar (Kd = 15 nM) *Mtb*CYP121 inhibitor having good selectivity against human CYP450 enzymes. However, although binding affinity was promising, the compounds designed by this approach were all devoid of any activity against *Mtb* itself.[32]

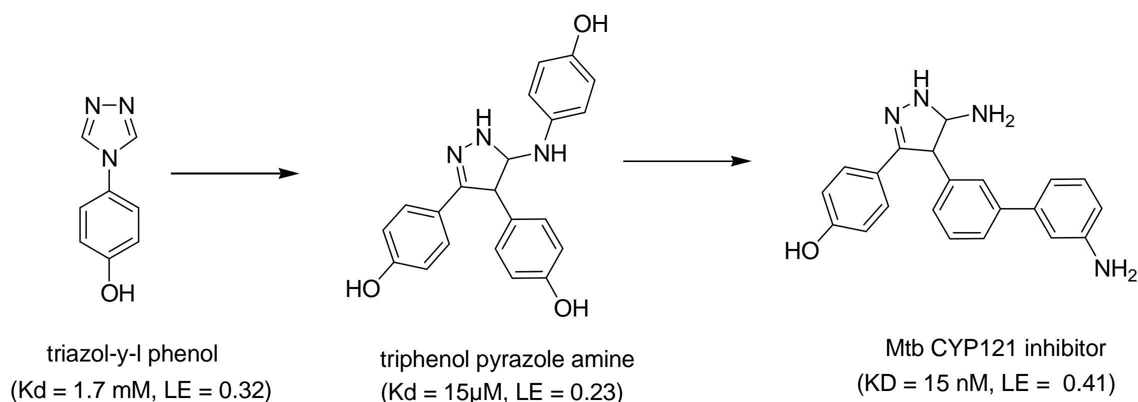


Figure 9 Synthetic optimization steps to explore Mtb CYP121 inhibitor

Belin *et al*. studied the binding of different cyclodipeptides (cYY analogues) to *Mtb* CYP121. [33] The cyclodipeptides were composed of a tyrosine chain and a second amino acid side chain other than tyrosine, and the cyclodipeptide comprised at least one L-amino acid **Figure 10**. Results indicated that binding of (L-DOPA-L-Tyr) and (L-Tyr-L-Trp) to CYP121 induced low to high spin transition indicating type I binding with binding constant 14.7 ± 1.5 µMand63.4 ± 8.8 µM respectively. Unlike cYY no transformation was detected when (L-DOPA-L-Tyr) was incubated with CYP121 and an electron transport chain plus NADPH at 30°C for 60 minutes, however a small amount of (L-Tyr-L-Trp) was transformed.

The conversion of cYY into product was achieved in less than 30 minutes at 30 °C when incubated in presence of exogenous electron transport chain and NADPH. However, addition of (L-DOPA-L-Tyr) or (L-Tyr-L-Trp) inhibited the cYY transformation.

From the crystal structure of the cYY analogues shown in **Figure 10** in complex with *Mtb* CYP121, common binding patterns were observed: (1) The DKP ring establishes contact with the residue of the B helix and one propionate chain of the heme either directly or through water; (2) The hydroxyls of the tyrosyl moieties are localized nearly at the same position between helix F and G.

From the binding patterns of the cYY analogues a set of interactions with CYP121 were identified that could help in the design of CYP121 inhibitors including the heme, the B helix region (Pro79, Pro80, Glu81, Val82, Val83), Phe168 and/or Trp182 and/or Phe280, and Asn385 and/or Arg386.The design of *Mtb* CYP121 inhibitors can also include adding functional groups to covalently link the DKP to *Mtb* CYP121 active site or to interact directly with the heme iron through conjugation with an electron pair of an azole molecule.

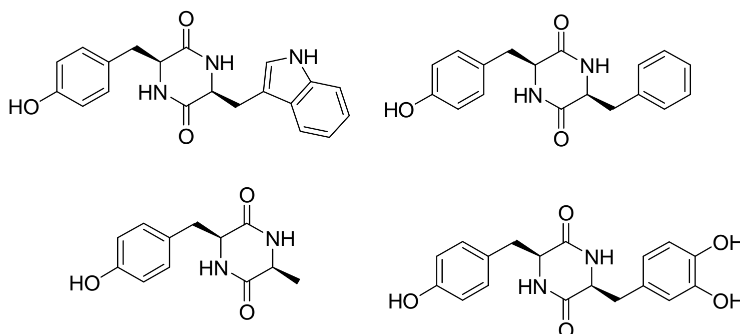


Figure 10 Different cyclodipeptides designed to act as *Mtb* CYP121 inhibitors

In 2013, Belin *et al.* continued the study on cYY analogues by investigating the effect of modifications of the side chains or the DKP ring of cYY on its activity. The study involved investigation of the interaction between *Mtb* CYP121 and the fourteen cYY analogues using UV-Vis spectroscopy, X-ray crystallography, enzymatic assays,

mass spectroscopy and solution NMR in an attempt to understand the function and catalytic mechanism of *Mtb* CYP121.

Results indicated that cyclodipeptides with a conserved DKP ring attached to two aryl side chains in the L-configuration showed significant binding to *Mtb* CYP121. However, very little binding or no binding was observed for compounds comprising one aliphatic side chain.

The new cYY analogues containing a Trp, Phe and dihydroxyphenylalanine amino acid side chain were efficiently bound to *Mtb* CYP121and the binding mode was essentially the same as that of cYY displaying contacts with Asn85, Phe168, Trp182. The tendency of cYY tyrosyl to point towards Arg386 was dependent on the presence of the DKP ring limiting the conformational freedom of the ligand. *Mtb* CYP121 did not efficiently or selectively transform any of the tested cYY analogue, indicating a high specificity for cYY. Factors that impaired binding to *Mtb* CYP121 are reduction of one keto function of the DKP ring of cYY, changing the stereochemistry of one or two Cα atoms and replacement of the DKP ring with anon-peptide or a linear pipetide scaffold. [34]

CONCLUSION

Tuberculosis has been a leading cause of human mortality for many decades [3], and despite the introduction of the first line treatment regimen forty years ago and the continuous trials since that time to introduce new regimens, tuberculosis is still a global threat. The emergence of *Mtb* strains that are resistant to the major frontline antitubercular drugs (multiple drug resistance) and more extremely resistant (excessive drug resistance and even total drug resistance) highlight the necessity of the development of new therapeutic agents. Twenty CYP450 enzymes are present in the *Mtb* genome, some of which are possible candidates for drug targeting. Recent studies including transcriptome analyses and microarray-based gene essentiality under *in vitro*, *ex vivo* and *in vivo* conditions [35] highlighted the importance of selected CYP450 isoforms for *Mtb* pathogenicity and viability thus identifying new drug targets for drug discovery.

*Mtb*CYP121 was amongst the first to be discovered within the *Mtb* genome and is exclusive to *Mtb*, so targeting this enzyme may allow a degree of selectivity.

Studies concluded that Mtb CYP 121 is highly specific enzyme acting on cYY converting it to mycocyclocin [16] **Figure 1**. Antifungal azoles are considered to act as promising Mtb inhibitors however the non-selectivity of these compounds is problematic. [24, 35, 36] Attempts to design cYY analogues was also useful and may be able to overcome the selectivity difficulties associated with the azole antifungals. Binding affinities to CYP121 in the low µM and nM range have been reported by Belin *et al*.[31, 33] for cYY derivatives and by Abell *et al.* [31] for small fragment heterocyclic inhibitors, however no inhibitory activity against the whole cell i.e. no MIC data to confirm the potency of these compound against *Mtb* has been observed. The challenge is to develop selective inhibitors of CYP121 with both potent binding affinity and potent MIC values against *Mtb* and therefore the potential as clinical candidates.

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