Biophysical Screening of a Focused Library for the Discovery of CYP121 Inhibitors as Novel Antimycobacterials

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The development of novel antimycobacterial agents against Mycobacterium tuberculosis (Mtb) is urgently required due to the appearance of multidrug resistance (MDR) combined with complicated long-term treatment. CYP121 was shown to be a promising novel target for inhibition of mycobacterial growth. In this study, we describe the rational discovery of new CYP121 inhibitors by a systematic screening based on biophysical and microbiological methods. The best hits originating from only one structural class gave initial information about molecular motifs required for binding and activity. The initial screening procedure was followed by mode-of-action studies and further biological characterizations. The results demonstrate superior antimycobacterial efficacy and a decreased toxicity profile of our frontrunner compound relative to the reference compound econazole. Due to its low molecular weight, promising biological profile, and physicochemical properties, this compound is an excellent starting point for further rational optimization.

Introduction

Tuberculosis belongs to the most lethal infectious diseases caused by bacteria. According to the WHO Global Tuberculosis Report,[1] 1.5 million people died in 2013 due to infections caused by Mtb. This goes along with an estimated amount of 9 million new cases of Mtb infections arising each year. Despite a broad spectrum of first- and second-line antimycobacterial drugs, there is an antibiotic gap for the treatment of infections with multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB).[1] Additionally, alarming reports have been published describing totally drug-resistant TB (TDR-TB).[2, 3] Moreover, tuberculosis still requires long-term treatment leading to an increased probability for noncompliance, which impairs therapeutic outcome.[4] Hence, there is an urgent need for new antimycobacterial agents with novel modes of action, which, in the best case, could also lead to shorter treatment periods.

Driven by the elucidation of the Mtb genome in 1998,[5] new potential drug targets were identified.[6] Interestingly, Mtb exhibits an unusual high number of twenty P450 enzymes in contrast to other bacteria. Further studies have revealed some of them to be essential for viability, survival and/or pathogenicity.[7] Out of these, CYP121 was shown to be essential for bacterial growth by an in vitro knock out study.[8] Moreover, the deficient strain could be revived by a complementary plasmid.[8, 9] The first evidence of CYP121 function in Mtb was derived from its gene position which is located in an operon harboring two enzymes involved in the formation of cyclo-d-tyrosine (cYY).[10] In vitro studies provided proof, that CYP121 catalyzes the reaction of cYY to mycocyclosin with high substrate specificity.[10, 11] The role of its substrate and product in the cellular setting remains to be elucidated. However, the variety of biological functions of diketopiperazines is well described for example, as quorum sensing signals.[12] Thus, besides development of antimycobacterials targeting CYP121, a small selective molecule with in cellulo efficiency may help to understand the precise function of CYP121.

Due to the fact that CYP121 is a potential target for Mtb treatment, some efforts have been undertaken to identify potent inhibitors. Hudson et al.[13] and Kavanagh et al.[14] published several compounds designed for selective CYP121 binding and inhibition. However, none of them were shown to be effective against Mtb. Fonvielle et al. described a CYP121 inhib-
Kavanagh et al. designed compounds based on substrate fragmentation with micromolar affinity and selectivity over other Mtb P450’s without data concerning biological activity. Regarding compounds with cellular activity, it was shown that azole antifungals bind tightly to CYP121 and exhibit it in vitro and in vivo activity against Mtb. Furthermore, the binding to the enzyme was in good correlation with the antymycobacterial effect. As the azole antifungals are active on Mtb cells and effective in mice infection models they display a valuable reference for antymycobacterial CYP inhibitors.

The essential role of CYP121 for Mtb survival and our expertise in developing potent and selective human steroidogenic CYP enzyme inhibitors motivated us to identify novel CYP121 inhibitors with increased efficiency and improved properties relative to the azole antifungals.

Potential antymycobacterial activity could provide further evidence of target validity, druggability and stimulate development of respective inhibitors toward new therapeutic agents bearing the potential to treat MDR and XDR Mtb infections. For these reasons, we established a screening strategy based on in vitro and cell-based assays (Scheme 1). By the use of a small highly diverse library composed of our CYP-inhibitors, we could identify a CYP121 inhibitor with increased antymycobacterial potency relative to positive control econazole. This compound possesses desirable physicochemical properties, low toxicity toward human cells and high antibacterial selectivity against Mtb, rendering it an appropriate candidate for further optimization.

Results and Discussion

Library generation

For hit discovery we selected 139 compounds from our in-house CYP-inhibitor library designed for inhibition of CYP17, CYP19, CYP11B1 and B2 (Supporting Information [SI]). The screening library is composed of six different scaffolds to ensure a broad structural diversity (Figure 1). Additionally, known pharmacological profiles, drug-likeness, and established synthetic routes of the compounds provide an ideal starting point for future optimization. As a reference compound we chose econazole which was shown to have the highest reported affinity to CYP121 (UV/Vis heme P450 binding assay) and the strongest inhibition of mycobacterial growth in the class of azole antifungals.

Enzyme expression, characterization and initial SPR screening

As starting point for SPR screening we expressed CYP121 in a heterologous host (E. coli K12 BL21) and purified the protein by ion affinity chromatography (IMAC). Notably, addition of 1% Triton X-100 into the lysis buffer during purification in-
creased the protein yield by about 10-fold. The purity of the enzyme was determined by SDS-PAGE (SI, section 2, Figure S1). To ensure active protein conformation we conducted enzymatic in vitro activity tests. A first experiment to gain information about activity of P450 enzymes is the determination of CO-binding spectra. 50% of the expressed enzymes showed the typical P450 band of CO-bound heme after dithionate reduction (SI, section 3, Figures S2 and S3). Using the same experimental conditions but replacing sodium dithionate, we were able to identify Etp1fd (516–618) as ferredoxin and Arh1_A18G as ferredoxin reductase, two proteins of the fungus/fission yeast *Schizosaccharomyces pombe* as suitable heterologous electron-transfer system for CYP121. Additionally, using the latter system, we could show conversion of cYY to mycoclosin proving enzymatic activity of CYP121 (SI, section 4, Figures S4 and S5).

Surface plasmon resonance spectroscopy (SPR) is a modern yet well-established biophysical methodology that allows the detection of binding events between an immobilized target (e.g., enzyme) and a solvated analyte. However, interactions detected by SPR can also occur outside the enzymes active site. Hence, this SPR-based primary screening filter was used to distinguish between binders and non-binders.

For SPR immobilization of the protein we used the biotin-streptavidin interaction. Prior to immobilization we conjugated a biotin tag to CYP121. To confirm applicability of the SPR method, we determined a response curve of econazole to the target protein (SI, section 5, Figure S6). The SPR signal of econazole, measured in response units (RU), was set to one (R \(_{\text{pos}}\)). The binding event of library compounds (R) was referenced to the positive control and declared as R/R \(_{\text{pos}}\). We defined R/R \(_{\text{pos}}\) > 0.5 as the threshold for hits from SPR screening procedure. Using this approach, we identified 44 binders out of 139 compounds with representatives from all of the six classes (Figure 2). Notably, we found 17 compounds with higher responses than econazole (SI, section 6, Table S1).

**Binding mode and affinity characterization by UV/Vis heme binding assay**

The 44 SPR binders were investigated for their ability to interact with the iron(II)–heme by monitoring the shift of the characteristic absorbance band at 416 nm of CYP121 (Figure 3). In addition to the 44 SPR hits, we also took two weak SPR binders into consideration (I:1 and I:33, R/R \(_{\text{pos}}\) < 0.5) to conduct a retrospective evaluation of the reliability of our SPR screening. McLean et al. reported that econazole has a tight-binding profile to CYP121 with a \(K_D = 0.02\ \mu\text{M}\). However, we observed a \(K_D\) of 3 \(\mu\text{M}\). This discrepancy could be due to a difference in UV/Vis spectrometric devices used and, thus, limited sensitivity. To provide a higher throughput employing 96-well plates, we were limited to a higher enzyme concentration which impairs measurement in lower nanomolar ranges. Compounds were initially tested at a concentration of 100 \(\mu\text{M}\) to identify iron–heme interactions and distinguish between type I (water-bridged iron interaction) and type II (direct iron interac-
The most affine binder \( K = 5 \) and \( K = 1 \) shown a type II binding behavior. The latter compounds appear in class I, II, III and IV indicating that the catalytic center accepts imidazolyl and pyridinyl moieties for iron–heme coordination. I:1 and I:33 (weak SPR binders) did not coordinate the iron heme as they do not bind to the heme iron which emphasizes the suitability of our SPR screening procedure. The identified type II binders were further investigated regarding their \( K_0 \). For eight binders an affinity better than 15 \( \mu \)M was observed (Si, section 6, Table S1). Interestingly, this subset of compounds only arose from classes I and II. Pyridinyl (class II) as well as imidazolyl (class I) motifs were tolerated as heme coordinators while the imidazolyl ligands showed higher affinities (Si, section 6, Table S1). With regard to class I the highest affinity could be found for compounds decorated with hydrophobic and space-demanding moieties connected to the benzimidazole substructure. This is also a structural trend in the class of antimycobacterial azoles (e.g., econazole, clotrimazole). The most affine binder I:16 showed improved \( K_0 \) relative to econazole (\( K_0 = 3 \) \( \mu \)M; Si, section 6, Table S1). Furthermore, two linearized, para-substituted biphenyl compounds of this class (I:47 and I:48) possessed a CYP121 affinity similar to that of econazole. As mentioned before, molecules with linear biphenyl units bearing an \( N \)-methylenebenzimidazolyl moiety instead of an unsubstituted benzylimidazolyl scaffold did not bind to the heme (I:33). Additionally, replacement of the interconnecting phenyl group within this class by pyridinyl resulted in inactive compounds (I:1). Moreover, the analysis of all regioisomers of benzimidazolyl scaffolds substituted with phenyl revealed that the \( \text{para} \) (I:32) and \( \text{meta} \) (I:30) position lead to similar affinities. A phenyl group at the \( \text{ortho} \) position (I:15) impairs binding. The \( \text{para} \)-benzodioxine substituent of I:48 and the \( \text{para} \)-benzodioxole substituent of I:47 increase the affinity by about two- to threefold (Si, section 6 Table S1). However, compared with econazole our most active compounds showed similar (e.g., I:47 5 \( \mu \)M and I:48 5 \( \mu \)M) or slightly better \( K_0 \) (I:16 1 \( \mu \)M; Si, section 7, Figure S7).

**Figure 3.** Binding of I:47 to CYP121 as determined from heme coordination assay: a) UV/Vis spectra of the enzyme were recorded in the presence of I:47 (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10 \( \mu \)M; dotted lines) and in the absence of I:47 (solid line). b) Corresponding difference spectra. c) The \( K_0 \) value of I:47 was derived by nonlinear fitting of the data using Equation (1) and the difference in absorbtion at 430 and 410 nm. Error bars represent the standard deviation of three replicate measurements.

**MIC determination in BCGT and Mtb**

For investigating cellular activity we focused on those compounds with a \( K_0 \) lower than 15 \( \mu \)M, but also added selected compounds showing low affinity to CYP121 as negative controls (Si, section 6, Table S1). In this setting, econazole was used as described antimycobacterial reference compound.

For initial screening on mycobacterial growth inhibition we used the bovine strain BCGT. The strain serves as a suitable substitute for Mtb as it carries a copy of CYP121 in its genome with an overall amino acid identity of 100\% in comparison with its Mtb congener (Si, section 8, Figure S8). Regarding the more complex situation in the cellular context, the six identified classes have to be discussed separately. In case of subset III to VI, we could only detect weak growth inhibition (MIC\(_\text{BCGT}\) > 10 mg L\(^{-1}\); Si, section 6, Table S1). In class II three compounds were found to have a \( K_0 \) value below 15 \( \mu \)M but none of them had MIC\(_\text{BCGT}\) below 10 mg L\(^{-1}\) (II:20, see Si, section 6, Table S1). For econazole, we observed a MIC\(_\text{BCGT}\) = 5.4 mg L\(^{-1}\) which is in good correlation to previous findings. Most active compounds were observed in class I (Si, section 9, Figures S9 and S10). The best heme binder to CYP121 (I:16) with a threefold increased affinity relative to econazole showed a MIC\(_\text{BCGT}\) of 1.6 mg L\(^{-1}\). For I:47 and I:48 we could determine MIC\(_\text{BCGT}\) = 0.3 mg L\(^{-1}\) and 2 mg L\(^{-1}\) which renders I:47 to be the most potent antimycobacterial compound in this subset. The MIC tests of negative controls out of class I (I:1 and I:33) showed no significant growth inhibition. Within class I results of the MIC assay are in good correlation to the \( K_0 \) values on the target enzyme CYP121 (Table 1).

To test the potency of the most effective antimycobacterial compounds against the human pathogen Mtb, we used the MABA assay system. For MIC\(_\text{Mtb}\) determination we chose the common laboratory strain H\(_\text{37Rv}\). In several studies the MIC\(_\text{Mtb}\) value of econazole was determined ranging from 0.12 mg L\(^{-1}\) to 8 mg L\(^{-1}\). To facilitate comparability of the MIC\(_\text{Mtb}\) values, we referenced them to results made in our assay system where a MIC\(_\text{Mtb}\) for econazole of 4.2 mg L\(^{-1}\) was determined previously. The most effective compounds were I:47 with MIC\(_\text{Mtb}\) = 0.3 mg L\(^{-1}\) followed by I:16 (MIC\(_\text{Mtb}\) = 1.9 mg L\(^{-1}\)) and I:48 (MIC\(_\text{Mtb}\) = 3.5 mg L\(^{-1}\); see Table 1). Notably, in terms of cellular efficiency metrics, I:47 has an AE = 0.39 and hence, a
To compare cellular toxicity of econazole (0.24) and rifampicin (0.16) (SI, section 10, Table S2).

Toxicity on human cell lines

The azole antifungals are known to attenuate growth of several human cell lines.\textsuperscript{36,37} To compare cellular toxicity of econazole with our three most promising hits we used HEK293 cells in a MTT-based assay\textsuperscript{10} 6.0 mg L\textsuperscript{-1} of econazole killed 50% of HEK293 cell population after 48 h. Notably, the toxicity of our most active antimycobacterial compounds was lower than that of the azole antifungal drug (I:16 LD\textsubscript{50} = 6.1 mg L\textsuperscript{-1}; I:47 LD\textsubscript{50} = 18.6 mg L\textsuperscript{-1}; I:48 22.3 mg L\textsuperscript{-1}). For comparability reasons, we calculated the toxicity factor for I:47 (MIC\textsubscript{spa}/LD\textsubscript{50}), which revealed a 44-fold improvement over econazole (Table 2). One of the most prominent undesirable effects ofazole antifungals is their hepatotoxicity observed in mice.\textsuperscript{33,34} For this reason, we also conducted toxicity experiments employing HepG2 cells.

We could observe an approximate twofold increased toxicity for econazole (3.1 mg L\textsuperscript{-1}) and I:16 (3.9 mg L\textsuperscript{-1}) relative to HEK293 cells. The LD\textsubscript{50} of I:47 was 17.1 mg L\textsuperscript{-1} which is close to the toxicity observed in HEK293 cells (see above, SI, section 11, Table S3).

Table 2. Comparison of human cellular toxicity and anti-Mtb effect.

| Compd | MIC\textsubscript{spa} [mg L\textsuperscript{-1}] | LD\textsubscript{50} [mg L\textsuperscript{-1}] \textsuperscript{[a]} | TF: MIC\textsubscript{spa}/LD\textsubscript{50} \textsuperscript{[b]} |
|-------|-----------------|-----------------|-----------------|
| Eco   | 4.2             | 6.0             | 1.4             |
| I:16  | 1.9             | 6.1             | 3.2             |
| I:47  | 0.3             | 18.6            | 62.0            |
| I:48  | 3.5             | 22.3            | 6.4             |

\textsuperscript{[a]} 50% lethal dose toward HEK293 cells. \textsuperscript{[b]} Toxity factor = (MIC\textsubscript{spa})/(LD\textsubscript{50,HEK293}); this was used to enhance the comparability of compounds with regard to their antimycobacterial effect.
Physicochemical and selectivity profile of I:47

The aforementioned compounds were originally designed as inhibitors of human CYP17, CYP19 and CYP11B1/2 known to be involved in steroid biosynthesis. I:47 was initially synthesized as an inhibitor of CYP17. The compound showed only a low activity on CYP17 relative to other inhibitors with an IC_{50} = 3.1 μm. Additionally, only a 48% inhibition of aromatase at a concentration of 25 μm was observed.\(^{[40]}\) Regarding physicochemical properties suitable for permeation through the cellular membranes, one has to differentiate between biological barriers of human and mycobacterial origin. For humans, a guidepost for appropriate physicochemical properties is the Lipinski’s rule of five for oral bioavailability of drugs (< 5 hydrogen bond donors, ≤ 10 hydrogen bond acceptors, M_r < 500 Da, logP ≤ 5).\(^{[41]}\) Our frontrunner compound I:47 fulfils all four criteria (0 hydrogen bond donors, 3 hydrogen bond acceptors, M_r = 278 Da, logP = 3.1, SI, section 13). To the best of our knowledge, similar correlations for physicochemical properties with mycobacterial membrane passage have not yet been established. Thus, a respective guide for compound development is still missing.\(^{[42]}\)

Molecular modeling studies on the binding mode of I:47

As a type II binding profile was observed for I:47, we set up a constrained docking protocol to predict its binding mode to the heme center of CYP121. Docking to the active site of CYP121 was restricted by two essential pharmacophore features reflecting the direct interaction between the coordinated iron and I:47 (type II binding). The resulting docking poses were sorted by their predicted binding energies and the best scored docking pose was chosen for further studies (Figure 4). This modeling approach revealed new possibilities for further derivatization or rigidification. For instance, intramolecular linking of the methylene bridge at the imidazolyl unit with the ortho position of phenyl using for example, a five-membered ring should be tolerated by CYP121 and increase affinity through a decrease in entropic penalties upon binding. Furthermore, the central hydrophobic aromatic moiety shows van der Waals contacts to flanking hydrophobic amino acids Phe168 and Met62. Regarding steric factors, the ring could easily rotate in this position. This degree of rotatable freedom might be necessary to place the aforementioned phenyl in a suitable position to grant access to a large flat sub-pocket (composed of Met61, Asn84 and the backbone of Asn83) which could then be reached by substituents at its 2-position. The 1,3-benzodioxole moiety was placed in a hydrophilic sub-pocket, formed by Arg72, Asn74 and Thr65, which is in good accordance with the low lipophilicity of this motif (cLogP = 0.27). Moreover, the 5-position of 1,3-benzodioxole holds great potential for further enlargement of the molecule, as it directly points to another sub-pocket which is decorated with several hydrophobic amino acids, namely Leu73, Phe280, Leu284, and the side chain of Gln385.

![Figure 4. Molecular docking of I:47 against the prepared co-crystal structure of CYP121. Heme coordination was a prerequisite for the docking process which was achieve by placing a pharmacophore feature on the interacting metal and ligand position. Resulting docking poses were sorted by score (E_refine), and the highest scored pose is depicted as a) a 3D model and b) a 2D-interaction chart. I:47 shows close van der Waals contacts to surrounding amino acids but also possibilities for compound enlargement (b). The grey surface in panel (a) represents the van der Waals surface of the protein, which is also shown as dotted lines in panel (b).](image)

Conclusions

Despite the fact that CYP121 had been reported to be a potential target for the treatment of Mtb infections, not many inhibitors with cellular activity had been discovered. Herein we have presented a rational screening approach to address CYP121 by a small library focused on privileged scaffolds for CYP enzyme inhibition. The identified compounds could help to clarify the hitherto unknown role of CYP121 in Mtb metabolism and provide a good starting point for a drug optimization program.

Our search for new inhibitors of CYP121 started with an initial SPR screen of the aforementioned focused library. As the compounds were designed for P450 inhibition we observed a high number of binders (32%). The identified compound classes differed highly with regard to their structures. Clearly, the large pocket of CYP121 (1350 Å^3) which is necessary for the sterically demanding enzyme reaction can accept a large variety of differently shaped molecules.\(^{[8, 10, 43, 44]}\) However, it has to be noted that the SPR method does not exclude compound attachment outside the enzymes active site.
For rational design approaches it is of high interest to clarify the binding mode and affinity of our hit compounds. A common method for P450 enzymes to address this issue is the heme coordination assay. All SPR binders from classes III, IV, V and VI had only weak affinity to the heme iron ($K_D > 100 \mu M$). IV:13 is the only compound from this subset that could be titrated and gave a $K_D$ of 62 $\mu M$ (SI, section 6, Table 1). Therefore, we conclude that most of the compounds from these classes do not bind directly to heme but address another unknown site. This information could, however, be valuable for fragment-linking approaches at a later stage of drug development. For classes I and II we identified eight compounds with $K_D$ values below 15 $\mu M$. Notably, the best compounds of class II (II:20 and II:34) contained a space-demanding trityl moiety. It was discussed for a crystal structure of CYP121 (PDB ID: 1N40) that Arg386 may restrict access of voluminous moieties to the iron–heme. Nevertheless, we observed that the enzyme can accommodate space-demanding molecules at the heme site as shown by our UV/Vis experiments. Binders with the best affinity were found in class I. A comparison of compound structures and binding efficiencies within this class gave first evidence for properties needed to gain affinity toward CYP121. Imidazolyl head group linked by an methylene bridge to a hydrophobic core can be considered as an important basic structure for a good binding efficiency (I:16, I:47 and I:48). In case of the line- arized compounds, an N-methylenbenzimidazoyl head group (I:33), ortho-substituted biphenyl system (I:15), and an inter- connecting pyridinyl (I:1) ring had unfavorable binding properties. In a hit optimization process these structural characteristics should be avoided. In contrast to this observation, a para- benzodioxine substituent (I:48) and a para-benzodioxole substituent (I:47) linked to the biphenyl system increases affinity. This might provide a possible position for further derivatiza- tion. Our docking study supports this result as this motif was predicted to be placed in a sub-pocket having a great potential for new interactions.

A straightforward approach for target validation is to corre- late on-target potency and cellular activity. Although it has to be noted, that such a correlation can be flawed by the fact that compounds might also be inefficient due to poor mem- brane penetration, for example. We hypothesized that class II might be a prime example for compounds that poorly permeate the membrane of mycobacteria and, thus, cannot reach their intracellular target. This could be an explanation for the lack of in cellulo activity although a moderate affinity to the target was measured.

Class I is the most remarkable of the six classes showing reason- able affinity toward CYP121 and, more importantly, also high activity in cellulo against Mtb and BCGT. Furthermore, the on-target affinity of class I compounds directly correlates with their activity on mycobacteria which provides further evidence of a CYP121-dependent effect. In detail, on-target inactive compounds like I:1 and I:33 had no activity against BCGT, while moderate binders for example, I:15, I:30 and I:32 had low antimycobacterial effects. Finally, compounds with highest affinity (I:16, I:47, I:48) were the most potent in the cellular setting. Especially, compounds I:16, I:47, and I:48 are even more effective on mycobacteria than the positive control, econ- nazole, although no optimization has been undertaken, yet (Table 1). In terms of antibacterial efficiency (AE), I:47 is superior to econazole and the first-line drug rifampicin indicating an excellent optimization potential of this novel inhibitor class. Moreover, we could provide data that I:47 does not only bind to CYP121 but does also inhibit the enzyme reaction (SI, section 4). The correlation between MIC and $K_D$ bares minor inconsis- tences, which might be due to poor penetration through the mycobacterial cell wall of some compounds (see for example, I:47 and econazole). A highly lipophilic molecule (e.g., econ- nazole $cLogP = 5.3$ versus I:47 $cLogP = 3.1$) might be trapped in this lipophilic barrier containing mycotic acids and slowly or only partially released into the mycobacterial cytoplasm. This results in lower cellular activity than expected from on-target affinities. A second explanation for the differences in MIC and $K_D$ at least for econazole, is its promiscuous behavior in differ- ent growth inhibition assays. This suggests that there are addi- tional targets for econazole. An explanation for the antibacteri- al activity of econazole against E. coli and S. aureus was already provided before. In these studies econazole was described as an inhibitor of flavohemoglobin. Further possible target systems of azole antifungals within Mtb metabolism have also been described. However, evaluation of I:47 and I:48 leads to the conclusion that these novel structures are of im- proved selectivity toward Mtb with a good correlation of CYP121 affinity and antimycobacterial activity. Furthermore, the two compounds possess lower toxicity against human cells than determined for econazole. Although toxicity to hepcato- cytes was low, it is of high interest to clarify potential inhibi- tion of metabolizing CYP enzymes (e.g., CYP3A4). These results further underline the target-based mycobacterial specificity of our compounds, at least in the subset of bacterial and human cells tested. Taken together the in vitro and cell-based studies con-ducted herein, CYP121 is most certainly the major target of I:47 and I:48.

In summary, we have reported a biophysical screening pro- cedure employing a focused library of privileged scaffolds, which ultimately lead to the discovery of novel CYP121 inhibi- tors. From this process, I:47 turned out to be the most promis- ing hit compound pairing convincing antimycobacterial activity and bacterial selectivity with a good toxicity profile. Further- more, this compound exhibits a fragment-like molecular weight and preferable physiochemical properties that fulfil the Lipinski rules for oral bioavailability (SI, section 13). Thus, I:47 is an excellent starting point for rational structure-based drug discovery. Our in silico studies revealed several possible modifi- cations to be investigated in future optimization steps. Addi- tionally, the inhibitor might be a suitable candidate for an in vivo proof-of-concept study toward validation of CYP121 as a drug target.

**Experimental Section**

**Bacterial strains and growth conditions:** Bacterial strains used in this study were Mycobacterium bovis DSM-43990 (BCGT), Mycobac- terium tuberculosis H$_3$Rv (Mtb), Escherichia coli TolC acr A/B defi-
cient, *Staphylococcus aureus* (Newman strain) and *E. coli* K12 BL21. Mammalian cell lines for cytotoxicity evaluation were HEK293 (human embryonic kidney) and Hep2G (human liver carcinoma cells). Mycobacteria were cultured in 7H9GC-Tween[52] or Middlebrook 7H9 broth complemented with ADC Enrichment (Middlebrook). *E. coli* TolC and *S. aureus* tests were performed in lysogenic broth (LB) and LB plus ADC Enrichment.

**Chemical synthesis and analytical characterization:** Chemicals were purchased from commercial suppliers and used without further purification. Column flash chromatography was performed on silica gel (40–63 μm), and reaction progress was monitored by TLC on TLC Silica Gel 60 F<sub>254</sub> plates (Merck, Darmstadt, Germany). All moisture-sensitive reactions were performed under nitrogen atmosphere using anhydrous solvents. 1H and 13C NMR spectra were recorded on Bruker Fourier spectrometers (300 MHz) at ambient temperature with the chemical shifts recorded as δ values in ppm units by reference to the deuteromethane residues of deuterated solvent as internal standard. Coupling constants (J) are given in Hertz (Hz), and signal patterns are indicated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet, br, broad signal. The purity of the final compounds was > 95% measured by HPLC with UV detection at 254 nm. The SpectraSystem LC system consisted of a pump, an autosampler, and a UV/Vis detector (ThermoFisher, Dreieich, Germany). Mass spectrometry was performed on an LC-coupled Surveyor MSQ electrospray mass spectrometer (ThermoFisher, Dreieich, Germany). The system was operated by the Xcalibur software package. An RP C<sub>18</sub> NUCLEODUR ec 100-5 m 125×3 mm 5 μm column (Macherey-Nagel GmbH, Düren, Germany) was used as the stationary phase. All solvents were HPLC grade. In a gradient run, the percentage of acetonitrile (containing 0.1% trifluoroacetic acid) was increased from an initial concentration of 0% at 0 min to 100% at 15 min and kept at 100% for 5 min. The injection volume was 10 μl, and flow rate was set to 800 μl min<sup>-1</sup>. MS analysis was carried out at a spray voltage of 3800 V, a source CID of 10 V and a capillary temperature of 350 °C. Spectra were acquired in positive mode from 100 to 1000 m/z, cYy and mycocyclosin were synthesized as described[51,52]. Experimental details on modification of cYy and mycocyclosin synthesis and analytical data can be found in the Supporting Information (SI, section 4). The synthesis of library compounds has been described previously: class I[36,56]–class III[59]–class IV[90]–class V[91] and class VI.[52]

**Protein expression, purification and biotinylation:** *E. coli* K12 BL21 (DE3) cells were transformed with plasmid harboring cyp121 gene (pHAT2/cyp121).[52] The previously described[28] enzyme expression and purification method was slightly modified: His<sub>6</sub>-tagged CYP121 (H<sub>6</sub>-CYP121) was expressed in *E. coli* K12 BL21 and purified using a single affinity chromatography step. Briefly, *E. coli* K12 BL21 cells containing the pHAT2/cyp121 were grown in terrific broth medium containing 100 μg mL<sup>-1</sup> ampicillin at 37 °C until an OD<sub>600</sub> of approximately 0.8 units was reached, followed by induction with 0.5 mM IPTG and 0.5 mM 3-aminolevulinic acid for 36 h at 25 °C and 200 rpm. The cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C), and the cell pellet was resuspended in 100 mL of binding buffer containing 1% Triton X-100 (50 mM tris-HCl, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.2) and lysed by sonication for a total process time of 2.5 min. Cellular debris was removed by centrifugation (18500 rpm, 30% 2-harm, 40 min, 4 °C), and the supernatant was filtered through a syringe filter (0.2 μm). The clear lysate was immediately applied to a Ni-NTA affinity column, washed with binding buffer, and eluted with a one-step gradient of 500 mM imidazole. The protein containing fractions were buffer-exchanged into storage buffer (140 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> and 10% glycerol (v/v), pH 7.2), using a PD10 column (GE Healthcare, Little Chalfont, UK) and judged to be pure by SDS-PAGE analysis. Then protein was stored in aliquots at −80 °C in a final concentration of 50 μM.[29]

Before SPR streptavidin immobilization CYP121 was biotinylated. For biotinylation, Sulfo-NHS-LC-LC-Biotin (Thermo Science, Wal- tham, USA) was dissolved in storage buffer (140 mM NaCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> and 10% glycerol (v/v)) with CYP121 in 1:1 molar ratio. The solution was incubated on ice for 2 h and mixed carefully every 30 min. The biotinylated CYP121 was purified by size exclusion chromatography using the storage buffer and subsequently stored at −80 °C at a final concentration of 10 μM.[31]

**Spectroscopic characterization of enzyme activity:** Recombinant CYP121 from *Mycobacterium tuberculosis* as well as ferredoxin Etp1fd (516–618) and ferredoxin reductase Arh1_A18G from the fission yeast Schizosaccharomyces pombe were expressed and purified as described previously.[31,55] Functionality of CYP121 and electron transfer was assayed by the occurrence of the characteristic absorbance maximum at λ ≈ 450 nm, related to the reduced, CO-bound heme complex. The assay was conducted following the method of Omura and Sato[36] with slight modifications. CYP121 (2 μM) was reduced through the addition of a few grains of sodium dithionite or incubation with NADPH (100 μM), ferredoxin Etp1fd (516–618) (40 μM), and Arh1_A18G ferredoxin reductase (2 μM) and divided in two cuvettes to record a baseline. One of the samples was saturated with carbon monoxide for 60 s and difference spectra were recorded until the absorbance at λ = 450 nm was constant.

**SPR screening:** SPR binding studies were performed using a Reichert SR7500DC instrument optical biosensor (Reichert Technologies, Munich, Germany) and SAD500m sensor chips obtained from XenTec Bioanalytics. CYP121 was immobilized on a SAD500m sensor chip at 12 °C using standard biotin-streptavidin complexation. The surface of both channels was quenched by a 3 min injection of 0.003 mg mL<sup>−1</sup> biotin. CYP121 was immobilized at densities between 5000 and 6000 RU for binding studies.

**UV/Vis heme P450 binding assay:** Optical titration experiments were performed in 96 well plates (Greiner, Kremsmünster, Austria; transparent round bottom). The data were recorded using Tecan infinite M200Pro Nano Quant (Tecan Groupe Ltd., Männedorf, Germany). Absorbance of enzyme and enzyme-inhibitor complex was measured between 350 and 500 nm in 1 nm steps with 10 flashes. Compounds were titrated from DMSO stock solutions maintaining a final DMSO concentration of 1%. CYP121 was used in a concentration of 0.25 μM. Data were plotted as optical shift versus ligand concentration. Equation (1) was used for nonlinear regression of the resulting dose-response curves employing the Levenberg-Marquardt algorithm of Sigma Plot 12 (Systat Software GmbH, Erkrath, Germany).

\[
f = y_{\text{min}} + \frac{y_{\text{max}} - y_{\text{min}}}{1 + \left(\frac{\text{IC}_{50}}{[L]}\right)^{s}}
\]

for which \( f \) is the observed difference in absorbance at wave-lengths 410 nm and 430 nm within the difference spectrum (see Figure 3) at ligand concentration \( x \). This difference spectrum is obtained by subtracting the pure heme absorption spectrum from those with ligand present. \( y_{\text{min}} \) refers to the absorbance change at
ligand saturation, \( y_{\text{extr}} \) is the extrapolated minimal difference in absorbance; \( K_r \) refers to the dissociation constant of the CYP121 ligand complex.\(^{[22]}\)

**Determination of BCGT MIC**\(_{\text{BCGT}}\) by OD\(_{500}\) assay: A pre-culture of BCGT was grown in 7H9 medium supplemented with ADC Enrichment for 10 days. The assay was performed in 48 well plates (Greiner, Kremsmünster, Austria). Prior to culture addition, concentrations of compounds were serially diluted in DMSO to fit a final DMSO concentration of 1%. For compound susceptibility the pre-culture was diluted 1:100 with fresh medium (7H9 = ADC enrichment). After 168 h of incubation at 37°C and 80% air moisture, bacterial growth was measured by determination of OD\(_{500}\). Absorption data was recorded on a Polarstar Omega Multidetection Plate Reader (BMG LABTECH, Ortenberg, Germany). Graphs were plotted with GraphPad Prism using OneSite Log IC\(_50\) model provided by the software. MIC\(_{\text{BCGT}}\) was defined as the concentration at which 50% of growth was detected in accordance with previous methods.\(^{[24]}\) In analogy to ligand efficiency, which relates activity of compounds to their number of heavy atoms, a new metric has been introduced: antibacterial efficiency (AE).\(^{[24,40]}\) This coefficient was developed for better comparability of antimicrobial compounds differing in molecular weight [Eq. (2)].

\[
AE = -\ln\left(\frac{\text{MIC}}{\text{NHA}}\right)
\]  

In which AE refers to the antibacterial efficiency, MIC is the minimal inhibitory concentration, and NHA equals the number of heavy atoms in a given compound.

**Cyp121 in vitro enzyme inhibition assay:** The enzyme inhibition assay was performed in 200 μL PBS buffer pH 7.2. Compounds were used in a concentration of 100 μM and incubated with 1 μM CYP121 for 30 minutes at 30°C. The final DMSO concentration did not exceed 2%. After incubation the electron transfer system Arh1_A18G (5 μM), Etp1fd (15 μM) and NADPH + H\(^+\) (200 μM) was added. The reaction was started with the addition of cYY (50 μM) and stopped after 30 min by addition of 200 μL methanol with internal standard estrone (1 μM final concentration, addition included). The characterization of CYP121 activity was conducted by a UHPLC-MS/MS analysis carried out on a TSQ Quantum Access Max mass spectrometer equipped with an HESI-II source and a triple quadrupole mass detector (Thermo Scientific, Dreieich, Germany). Compounds were separated on an Accucore RS-MS 150×2.1 mm 2.6 μm column (Thermo Fisher, Waltham, US) by a methanol/water gradient (from 1.4 min – 3.5 min 50% methanol to 3.5 min – 5.0 min 90% methanol) with a flow of 550 μL min\(^{-1}\). Compounds were ionized in negative mode by electrospray ionization. Ionization was assisted by a post-column addition of 2m ammonia in methanol with an automated syringe at 1.25 μL min\(^{-1}\). Monitored ions were (mother ion [m/z]) product ion [m/z], scan time [s], scan width [m/z], collision energy [V], tube lens offset [V], polarity: mycocyclosin: 323.101, 111.100, 0.3, 0.7, 26, negative; CYY: 325.129, 113.043, 0.3, 0.7, 29, negative; internal standard (estrone): 269.153, 145.035, 0.3, 0.7, 42, negative. Samples were injected in a volume of 25 μL. Xcalibur software was used for data acquisition. For quantification, the ratios of the area under the curve of the educt and the product were used.

**Determination MIC\(_{\text{BCGT}}\) using MABA:** The assay for determination of minimal inhibition concentration against MtB was performed as previously described.\(^{[20]}\)

**MIC\(_{\text{E.coli TolC}}\) and MIC\(_{\text{S.aureus Newman}}\):** MIC\(_{\text{E.coli}}\)/MIC\(_{\text{S.aureus Newman}}\) values were performed for econazole, I:16, I:47, I:48 in E. coli TolC and S. aureus Newman. A start OD\(_{500}\) of 0.03 was used in a total volume of 200 mL in lysogeny broth (LB) + ACD enrichment containing the compounds predissolved in DMSO. Final compound concentrations were prepared from serial dilutions ranging from 1.56 to 100 μM in duplicates. The maximal DMSO concentration in the experiment was 1%. The recorded OD\(_{500}\) values were determined after addition of the compounds and again after incubation for 18 h at 37°C and 50 rpm in a 96-well plate (Sarstedt, Nürnberg, Germany) using a FLUOSStar Omega (BMG Labtech, Ortenberg, Germany). Given MIC\(_{\text{E.coli}}\)/MIC\(_{\text{S.aureus Newman}}\) values are means of two independent experiments (two different clones) and are defined as concentrations at which no bacterial growth was detectable.

**Human cytotoxicity assay:** HEK293 cells (2×10\(^3\) cells per well) were seeded in 24-well, flat-bottomed plates (Greiner Bioscience, Kremsmünster, Austria). Culturing of cells, incubations and OD measurements were performed as described previously\(^{[28]}\) with minor modifications. 24 h after seeding of the cells the incubation was started by the addition of the compounds from DMSO stock solutions to a final DMSO concentration of 1%. The living cell mass was determined 48 h after addition of the compounds and was followed by the calculation of LD\(_{50}\) values. The calculation of the LC\(_{50}\) values was performed by plotting the percent inhibition versus the concentration of inhibitor on a semi-logarithmic plot. From this, the molar concentration causing 50% reduction of the living cell mass was calculated. At least three independent experiments were performed for each compound.

**In silico binding mode of I:47:** In silico studies were performed with the X-ray co-crystal structure of a type II inhibitor and CYP121 (PDB ID: 4G44) using MOE software package (Chemical Computing Group)\(^{[29]}\). Prior to modeling, a pharmacophore model was created, placing a feature for an interacting metal on the heme iron (ML2, \( r = 1 \)) and a second feature for a metal ligand (ML, \( r = 1 \)) on the iron-coordinating nitrogen of the co-crystalized ligand. Both features were set to be essential and constrained (Atoms/Projections). Before energy minimization with LigX the solvent and the ligand was deleted from the structure. For LigX, an AMBER10:EHT forcefield with the default parameters were used but the solvation model was changed to R-Field as recommended by the manufacturer. For docking experiments the following parameters were used: Protocols = induced fit, Receptor = Receptor + Solvent, Site = Selected Atoms (these consisted of the heme and the surrounding amino acids in 4.5 Å proximity), Pharmacaphore = File (as described above), Ligand = MDB File (Database file with I:47, energy minimized with MMFF94x), Placement = Pharmacaphore, Rescoring 1 = London dG, Refinement = Forcefield, Rescoring 2 = GBVI/WSA dG. 30 poses were retained within the placement and refinement step. The resulting poses were sorted by their \( E_{\text{refine}} \) score and the first (best) pose was selected for further evaluation.

**Physicochemical properties:** Physicochemical properties were calculated using ACD/Percepta version 2012 (Build 2203, January 29, 2013), ACD/Labs.

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