Identification, characterization and azole-binding properties of *Mycobacterium smegmatis* CYP164A2, a homolog of ML2088, the sole cytochrome P450 gene of *Mycobacterium leprae*

Andrew G.S. Warrilow,¹ Colin J. Jackson,¹ Josie E. Parker,¹ Timothy H. Marczylo,²
Diane E. Kelly,¹ David C. Lamb,¹ and Steven L. Kelly¹*

Institute of Life Science, Swansea University, Swansea, Wales, UK,¹ Cancer Biomarkers and Prevention Group, RKCSB, Leicester Royal Infirmary, Leicester, UK.²

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* Corresponding author. Mailing address: Institute of Life Science, Swansea University, Swansea SA2 8PP. Phone: +44 1792 292207 Fax: +44 1792 503430 Email: s.l.kelly@swansea.ac.uk
The genome sequence of *Mycobacterium leprae* revealed a single ORF, ML2088 (CYP164A1), encoding a putative full-length cytochrome P450 monooxygenase and twelve pseudogenes. We have identified a homolog of ML2088 in the *Mycobacterium smegmatis*, and report here the cloning, expression, purification and azole-binding characteristics of this cytochrome P450 (CYP164A2).

*CYP164A2* is 1245 bp long encoding a protein 414 amino acids in length of molecular weight 45 kDa. CYP164A2 has 60% identity with *Mycobacterium leprae* CYP161A1 and 66 to 69% identity to eight other mycobacterial CYP164A1 homologs with 3 highly conserved motifs being identified. Recombinant CYP164A2 has the typical spectral characteristics of a cytochrome P450 monooxygenase predominantly in the iron (III) low-spin state. Unusually, the spin state was readily modulated by increasing ionic strength at pH 7.5 with 50% high-spin occupancy achieved with 0.14 M NaCl. CYP164A2 bound clotrimazole, econazole and miconazole strongly ($k_d$ 1.2 to 2.5 µM), however, strong binding with itraconazole, ketoconazole and voriconazole was only observed in the presence of 0.5 M NaCl. Fluconazole did not bind to CYP164A2 at pH 7.5 with no discernable type II binding spectrum observed.
Leprosy has afflicted humanity down the ages, and remains today a serious and disfiguring condition in communities throughout the developing world. Immunization (18) and multidrug treatment (MDT) programs have been effective in reducing morbidity and mortality due to lepromatous leprosy, but the incidence of new infections, at 680,000 per annum (36), remains high. Genome sequencing projects have been completed for several mycobacterial species, including both *Mycobacterium leprae*, the etiologic agent of leprosy, and *Mycobacterium tuberculosis*. A common objective of these schemes is to identify potential targets for the development of novel antimycobacterial compounds. Unusually for bacteria, the actinomycetes such as mycobacteria and streptomycetes contain substantial numbers of genes encoding for cytochromes P450 (CYPs), or P450 pseudogenes (6, 23). These enzymes carry out a wide range of monoxygenation reactions involved in biocatalysis, secondary metabolism, and detoxification. CYPs are ubiquitous throughout the eukaryotes, but are relatively uncommon in prokaryotes with most containing no CYP genes. The unexpected discovery in *M. tuberculosis* of an ortholog of CYP51 (3), the sterol 14α demethylase of eukaryotes (19), proved a major catalyst for investigations into the CYP complements (CYPomes) of mycobacteria as CYP51 is the target for azole antifungal drugs which have been shown to possess antimycobacterial properties (1, 5, 15, 35).

Most azole antifungal compounds show selective targeting of CYP51, having an 8- to 128-fold higher affinity for fungal CYP51s relative to the orthologous enzyme present in the host (29). These compounds act by inhibiting the fungal sterol biosynthesis pathway which does not exist in mycobacteria. The abundance of P450 proteins in mycobacterial species has raised the possibility that azoles could have either a specific or...
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general inhibitory activity against mycobacteria through inhibition of CYP activity, even if the currentazole antifungals are not effective. Several recent publications have supported the validity of this hypothesis (1, 5, 14, 16, 35). Ketoconazole, when used in conjunction with isoniazid, pyrazinamide and rifampicin significantly improved the treatment of pulmonary TB in mice in comparison to using the same drug regimen minus ketoconazole (5). Orally administered econazole used in combination with moxifloxacin and rifampicin was effective in totally clearing TB from the organs of mice after 8 weeks (1). Recently, 4,4'-dihydroxybenzophenone (DHBP) has been co-crystalised with CYP51 from M. tuberculosis (11). Treatment with 100 µM DHBP reduced the number of M. tuberculosis colony forming units by 40 to 70% isolated from mouse macrophage cells.

M. leprae has one CYP gene, ML2088, encoding a P450 and this represented the first member of the CYP164 cytochrome P450 family and was named CYP164A1 (http://drnelson.utmem.edu/CytochromeP450.html) (7). In common with many other genes, numerous cytochromes P450 have been lost recently from the genome associated with evolution towards a host-dependent life cycle. Pseudogenes with homology to ten of the twenty M. tuberculosis CYPs, as well as for two without homology to M. tuberculosis CYPs, are also present. Interestingly, there is no leprosy pseudogene corresponding to a lost CYP51. The characterization of the ML2088 CYP of M. leprae presents difficulties, as this bacterium cannot be cultured in vitro. Standard approaches to express ML2088 as heterologous protein in E. coli using pET vectors failed to yield correctly folded protein, as indicated by a reduced carbon monoxide difference spectral peak for the hemoprotein around 450 nm (unpublished observation). However, studies in our laboratory on the CYPome of M. smegmatis (16, 19) uncovered a CYP (A0R5U2) with close identity to
M. leprae P450 homolog that encoded by ML2088. It was assigned as CYP164A2, having greater than 55% amino acid identity with M. tuberculosis CYP164A1. We report here the isolation, characterization andazole binding properties of this novel mycobacterial CYP.
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MATERIALS AND METHODS

Identification and cloning of a ML2088 homolog from M. smegmatis ATCC

700084. The predicted protein translation of the leprosy P450 gene ML2088, encoding for CYP164A1 (Q9CBE7), was used to perform a TBLASTN search of the National Center for Biotechnology Information (NCBI) microbial genomes database [http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/genom_table_cgi.]. The best alignment was produced with a translated product from M. smegmatis.

The P450 gene was amplified from genomic DNA of M. smegmatis strain ATCC 700084. The forward primer, MSLEPF (5’-AGCTCATATGCAACAATGGGTGGAT-3’) and reverse primer, MSLEPHIS (5’-GCATAAGCTTTCAATGGTGGTTGTAACCGCGATGGATAACG-3’) (MWG-Biotech, Ebersberg, Germany) were constructed to amplify the 1245 bp P450 gene. MSLEPF was designed from residues 841237-841254 and MSLEPHIS from the reverse complement of residues 842464-842481 of contig 3312 from the M. smegmatis genome sequencing project. The primers incorporated NdeI and HindIII restriction sites (underlined) to facilitate subcloning into the Novagen expression vector pET17b. The stop codon was removed from the reverse primer MSLEPHIS, and 4 histidine codons (double underlined) followed by a new stop codon were inserted after the 3’ end of the downstream coding sequence for ease of purification of the recombinant protein. The MSLEP DNA and protein sequences have subsequently been deposited in the UniProKM/TrEMBL database as accession number A0R5U2 (see footnote).

Bioinformatic analyses. A BLASTP search was performed using the CYP164A2 amino acid sequence against both the UniProtKB/TrEMBL and NCBI databases. This
A BLASTP search of the *M. marinum* genome project database (http://www.sanger.ac.uk/Projects/M_marinum/) identified one CYP164A2-like protein. These nine CYP proteins were *M. leprae* CYP (Q9CBE7), *M. gilvum* (A4T681), *Mycobacterium sp.* strain JLS (A3Q7G4), *Mycobacterium sp.* strain MCS (Q1B233), *Mycobacterium sp.* strain KMS (A1UN16), *M. vanbaalenii* (A1TGP4), *M. paratuberculosis* (Q744J7), *M. avium* (A0Q9Q7) and *M. marinum* (MM5268). The online BLOCKMAKER program (http://blocks.fhcrc.org/blocks/make_blocks.html) was used to identify conserved ungapped 'MOTIF' segments between the ten mycobacterial CYP proteins. Sequence identities were determined using the on-line BLAST2 sequence comparison tool (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

**Heterologous expression in E. coli and isolation of recombinant CYP51 and CYP164A2 proteins.** The *CYP51*-pET17b and *CYP164A2*-pET17b constructs were transformed into *E. coli* strain BL21(DE3).pLys using ampicillin selection. *M. smegmatis* CYP51-pET17b (16) was used as a control to allow a comparison of the azole-binding properties. Overnight cultures (10 ml) of transformants were used to inoculate one litre volumes of Terrific Broth supplemented with 20 g.l⁻¹ peptone and 0.1 mg.ml⁻¹ sodium ampicillin. Cultures were grown at 37°C, 230 rpm for 6 hours prior to induction with 1 mM IPTG and expression at 25°C, 190 rpm for 18 hours in the presence of 1 mM 5-aminolevulenic acid. Recombinant CYP51 and CYP164A2 proteins were isolated according to the method of Arase et al (2) except that 2% (w/v) sodium cholate and no Tween20 were used in the sonication buffer. The solubilized CYP51 and CYP164A2 proteins were purified by affinity chromatography using Ni²⁺-NTA agarose as previously
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described (3) with the modification that 0.1% (w/v) L-histidine in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol was used to elute non-specifically bound *E. coli* proteins after the salt washes and elution of P450 protein was achieved with 1% (w/v) L-histidine in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol. Protein purity was assessed by SDS polyacrylamide gel electrophoresis (20) and specific P450 content.

**Determination of cytochrome P450 protein concentrations.** Cytochrome P450 concentration was determined by reduced carbon monoxide difference spectra according to Estabrook *et al* (12). An extinction coefficient of 91 mM$^{-1}$cm$^{-1}$ (27) was used to calculate P450 concentrations. Absolute spectra for both *M. smegmatis* CYP51 and CYP164A2 were determined as previously described (3) in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol (binding buffer). The oxidized spectra of both proteins were determined in the presence 0, 0.5 M and 4 M NaCl in binding buffer. The low-spin state spectra of CYP164A2 and CYP51 were determined in 60% (v/v) ethylene glycol in 50 mM Tris-HCl, pH 8.2. Calculation of the low-spin fraction was performed as previously described (25). All spectral determinations were made using a Hitachi U-3310 UV/VIS spectrophotometer (San Jose, California) with Ni$^{2+}$-NTA agarose purified CYP51 (2.44 µM) and CYP164A2 (2.62 µM) proteins. Protein concentrations were determined by the Coomassie Blue R250 dye-binding method (BioRad, Hemel Hempstead, UK) using bovine serum albumin standards.

**Azole-binding spectral determinations.** Binding of azole antifungal agents to CYP51 and CYP164A2 were performed as previously described (21, 22) using split-cuvettes, except that dimethylsulphoxide (DMSO) was also added to the cytochrome P450-containing compartment of the reference cuvette. Stock 0.5 mg.ml$^{-1}$ solutions of
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clotrimazole, econazole, fluconazole, itraconazole, ketoconazole, miconazole and voriconazole were prepared in DMSO. Azole was progressively titrated against Ni$^{2+}$-NTA agarose purified CYP51 (2.44 µM) and CYP164A2 (2.62 µM) to a maximum DMSO concentration of 2.5% (v/v) with the spectral difference determined after each incremental addition of azole. All spectral determinations were performed in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol, both in the presence and absence of 0.5 M NaCl. Voriconazole binding to CYP164A2 was investigated at several NaCl concentrations from 0 to 2 M. In addition, the binding of clotrimazole to CYP51 and CYP164A2 was determined in the presence of 0.1% (w/v) Triton X-100. The dissociation constant of the enzyme-azole complex ($k_d$) for each azole was determined by non-linear regression (Levenberg-Marquardt algorithm) of $\Delta A_{\text{peak-trough}}$ against azole concentration using the Hill equation [$\Delta A = \Delta A_{\text{max}} / (1 + k_d / [\text{Azole}]^n)$] (28) and the Michaelis-Menten-Henri equation [$\Delta A = (\Delta A_{\text{max}}. [\text{Azole}]) / (k_d + [\text{Azole}])$] where allosterysm was not observed.

**Data analysis.** Analysis of the DNA and protein sequences were performed using the computer programs Chromas version 1.45 (http://www.technelysium.com.au/chromas14x.html), ClustalX version 1.8 (ftp://ftp-igbmc.u-strasbg.fr/pub/) and BioEdit version 5.0.6. (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Curve-fitting of data were performed using ProFit 5.01 (QuantumSoft, Zurich, Switzerland).

**Chemicals.** All chemicals, including azole antifungals except voriconazole, were obtained from Sigma Chemical Company (Poole, UK). Voriconazole was supplied by
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Discovery Fine Chemicals (Bournemouth, UK). DIFCO growth media were obtained from Becton Dickinson Ltd (Cowley, UK).
RESULTS AND DISCUSSION

Bioinformatic analysis. Phylogenetic analysis of *M. smegmatis* CYP164A2 (A0R5U2) against nine other closely related mycobacterial CYPs (CYP164A1 homologs) indicated that CYP164A2 had greatest homology towards *M. gilvum* (A4T681) with 69% identity, followed by *Mycobacterium sp.* strain JLS (A3Q7G4), *Mycobacterium sp.* strain MCS (Q1B233), *Mycobacterium sp.* strain KMS (A1UN16) and *M. vanbaalenii* (A1TGP4), with 68% identity. *M. smegmatis* CYP164A2 shared 67% identity to both *M. paratuberculosis* (Q744J7) and *M. avium* (A0Q9Q7) along with 66% identity with *M. marinum* (MM5268). *M. smegmatis* CYP164A2 shared 60% identity (249/415) and 75% similarity (313/415) with *M. leprae* CYP164A1 (ML2088) in a 415 amino acid overlap (BLASTP score e-130). Homology extended across all regions of the proteins, with only two introduced gaps. In contrast, the closest *M. tuberculosis* homolog was CYP140, this gave only 38% identity (145/379) and 51% similarity (196/397) in a 397 bp overlap, with thirty introduced gaps (BLASTP score 3e-58). Regions of homology occured in sequences containing conserved P450 motifs, such as the heme-binding domain. CYP164A2 shared only 24% identity with *M. smegmatis* CYP51 and 23% identity with *M. tuberculosis* CYP51. The closely related *Streptomyces avermitilis* CYP107P2 (Q82ES4) shared 39% identity with *M. smegmatis* CYP164A2 and *Saccharopolyspora erythraea* CYP107A1 (P450eryF – Q00441) shared 36% identity with CYP164A2.

The *M. smegmatis* CYP164A2 gene is 1245 bp in length and encodes for a protein that is 414 amino acids long of 45.0 kDa molecular weight and theoretical pI 5.09 in comparison to *M. leprae* CYP164A1 (Q9CBE7) which was 1305 bp long encoding a protein 415 amino acids in length of 47.2 kDa molecular weight and theoretical pI 5.16.
The other mycobacterial CYP164A1 homologs were 405 to 442 amino acids in length with predicted molecular weights of 43.5 to 48.7 kDa and predicted pI values of 4.8 to 5.2. *M. smegmatis* CYP164A2 and *M. smegmatis* CYP51, in common with most bacterial CYPs, contain no identifiable N-terminal signal or membrane anchor sequences and are expressed in the cytosol unlike fungal CYPs which contain a membrane anchor sequence and are located in the endoplasmic reticulum.

A comparison of the CYP164A1 homologs using "BLOCKMAKER" identified eight conserved 'MOTIF' regions (Table 1). Blocks A, C and E are highly conserved with 65, 52 and 67%, respectively, of the amino acid residues conserved among all ten mycobacterial species. Blocks B, G and H are moderately conserved (46, 47 and 41%, respectively) and Blocks D and F were poorly conserved (37 and 24%). Block G contained the cysteine residue that forms the fifth ligand of the heme prosthetic group and the main C-terminal heme-binding domain. The high degree of conservation in the conserved motifs identified (Table 1), especially in blocks A, C and E, suggests a possible role in defining the substrate access channel and substrate-binding pocket for the mycobacterial CYP164A1 homologs.

**Heterologous expression and purification of recombinant CYP51 and CYP164A2 proteins.** Both *M. smegmatis* CYP51 and CYP164A2 proteins were expressed in *E. coli*. Protein isolation by cholate extraction using sonication (2) gave yields of 1.5 and 0.6 µmoles per litre culture for CYP51 and CYP164A2, respectively. Purification by Ni²⁺-NTA agarose affinity chromatography gave 4.1- and 16.8-fold increases in purity for CYP51 and CYP164A2, respectively, with specific activities of
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10.1 and 18.7 nmol.mg\(^{-1}\) for CYP51 and CYP164A2 based on the reduced CO-P450 assay of Estabrook et al (12).

The purity of Ni\(^{2+}\)-NTA agarose purified CYP51 and CYP164A2 was determined as being 63% and 79%, respectively, using the UTHSCSA ImageTool 3.0 (http://ddsdx.uthscsa.edu/dig/itdesc.html) to analyse pixel gray-scale density and coverage area of the stained SDS-polyacrylamide gel. This compared with purities of 57% and 84% for CYP51 and CYP164A2 obtained from CO-P450 difference spectra and protein determinations using the BioRad Coomassie-Blue dye binding assay. Apparent molecular weights of 50.2 and 44.7 kDa were obtained for CYP51 and CYP164A2 which were close to the predicted values of 51,563 and 44,955 Da.

**Spectral properties of recombinant CYP51 and CYP164A2 proteins.** Absolute spectra (Fig. 1A and 1B) and reduced CO-P450 difference spectra (Fig. 1C) of M. smegmatis CYP51 and CYP164A2 were characteristic of a cytochrome P450 enzyme (3, 17) indicating that both enzymes were expressed in their native form. CYP51 displayed the typical spectral properties of a ferric-P450 with a low-spin state heme iron Soret \(\gamma\) band at 418 nm in addition to \(\alpha\), \(\beta\) and \(\delta\) bands at 570, 535 and 355 nm, respectively. CYP164A2 gave a similar absolute spectrum in the oxidized low-spin ferric form with a Soret \(\gamma\) band at 415 nm in addition to \(\alpha\), \(\beta\) and \(\delta\) bands at 566, 534 and 358 nm, respectively. The \(\delta\) band of CYP164A2 was just distinguishable as a shoulder on the leading-edge of the \(\gamma\) band. Dithionite one electron reduction caused a small blue-shift of the Soret \(\gamma\) band from 418 to 416 nm and from 415 to 412 nm for CYP51 and CYP164A2, respectively and the \(\alpha\) and \(\beta\) bands to merge with absorption maxima at 549 and 545 nm for CYP51 and CYP164A2, respectively.
Binding carbon monoxide to dithionite-reduced ferrous-P450 resulted in a typical red-shift of the Soret $\gamma$ band from 416 to 448 nm and from 412 to 448 nm for CYP51 and CYP164A2, respectively, in the formation of the CO-ferrous P450 complex. For *M. smegmatis* CYP51, a significant amount of P420 was also formed when carbon monoxide bound, suggesting instability in the presence of dithionite. The carbon monoxide difference spectra obtained (Fig. 1C) showed no significant P420 present when dithionite was added last just prior to recording the absorbance spectrum.

The spin-state of *M. smegmatis* CYP164A2 was highly sensitive to NaCl concentration at pH 7.5 (Fig. 2). In the absence of NaCl, nearly 75% of CYP164A2 molecules occupying the low-spin state (25% high-spin), as calculated using the method of Lange et al (25). The addition of 0.5 M and 4 M NaCl caused 86% and 100% of the CYP164A2 molecules to occupy the high-spin state. The low- to high-spin state transition of CYP164A2 was characterized by the blue-shift of the $\gamma$-Soret band from 417 to 393 nm. The calculated 50% high-spin occupancy point for CYP164A2 was 0.14 M NaCl in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol. The spin state of *M. smegmatis* CYP51, in contrast, was relatively insensitive to NaCl concentration at pH 7.5. In the absence of NaCl, 94% of the CYP51 molecules occupied the low-spin state (6% high-spin), with the addition of 0.5 M and 4 M NaCl only causing 12% and 23% occupancy of the high-spin state, respectively. The complete modulation of CYP164A2 spin state by ionic strength, especially in the absence of substrate, is a rare phenomenon amongst cytochrome P450 enzymes. The resting state of most cytochrome P450s is predominantly the low-spin iron (III) state (34) where water is co-ordinated to the heme prosthetic group as the sixth ligand. Displacement of water as the sixth ligand, for example by substrate, induces a
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change in spin state from low- to high-spin, suggesting that increasing ionic strength, either directly or indirectly, causes the progressive dissociated of water as the sixth ligand of the heme moiety in CYP164A2. Previous studies investigating the effects of ionic strength on the CYP spin state have used sub-zero temperatures, varying pH, presence of co-solvents, presence of substrates and elevated pressures (8, 9, 10, 13, 24, 25, 31, 32, 37, 38) due to the relative insensitivity of the spin state of many CYPs to modulation by ionic strength in the absence of substrate. We are currently performing further investigations on the modulation of CYP164A2 spin state by ionic strength.

Azole binding studies. Binding of azole antifungal agents (Table 2 and Fig. 3) identified several differences between M. smegmatis CYP51 and CYP164A2. Type II azole-binding spectra were obtained as a result of the imadazole ring nitrogen coordinating as the sixth ligand with the heme iron. Absorbance maxima were similar for both CYPs (429 to 433 nm), however the absorbance minima differed between CYP51 (409 to 412 nm) and CYP164A2 (392 to 395 nm).

Differences in azole-binding allosterism were observed with strong positive cooperativity shown for the binding of clotrimazole, econazole, itraconazole and miconazole ($n_{H,app} = 1.8$ to $2.2$) and mild positive cooperativity observed for fluconazole, ketoconazole and voriconazole ($n_{H,app} = 1.2$ to $1.3$) with CYP51. However, strong positive cooperativity was only observed for CYP164A2 with clotrimazole ($n_{H,app} = 1.7$) and no cooperativity was observed with the other azoles binding to CYP164A2 ($n_{H,app}$ below 1.2). This suggests that the size of the substrate/azole binding pocket and substrate access channel is relatively small compared to CYP51, preventing the binding of a second azole molecule.
Ouellet et al (28) suggested two possible causes for the observed positive cooperativity of azole binding to CYPs. Firstly, two or more specific azole binding sites exist on the cytochrome P450 molecule with positive cooperativity being exerted between the binding sites (multiple site cooperativity) with only one azole molecule directly coordinated as the sixth ligand of the heme prosthetic group and the other azole molecule acting as a positive allosteric effector/regulator. Secondly, cytochrome P450 molecules aggregate in vitro to form oligomers which then display positive cooperativity between the monomers during azole binding (multimer cooperativity). For M. tuberculosis CYP130 (28) the allosteric binding of econazole was due to protein-protein interactions as the allosterism was disrupted by the addition of 50 mM KCl, whilst 1:1 stoichiometry for bound azole was maintained. The addition of 0.5 M NaCl did not reduce the allosterism observed (Table 2) for azole binding with M smegmatis CYP51, or the binding of clotrimazole to CYP164A2, suggesting that the allosterism observed was not due to electrostatic protein-protein interactions. The inclusion of the detergent Triton X-100 (Table 2) at 0.1% (w/v) during the binding of clotrimazole to CYP51 and CYP164A2 resulted in the elimination of the positive cooperativity previously observed with the apparent Hill number falling to 1.0 and the $k_d$ for clotrimazole increasing 5-fold for CYP51 and 13-fold for CYP164A2, indicating reduced affinity for the azole. Increasing the detergent concentration to 1% (w/v) caused further increases in $k_d$ and a progressive reduction in $\Delta A_{\text{max}}$ (data not shown). Therefore the allosterism observed for clotrimazole binding to CYP51 and CYP164A2 appears to be mediated by hydrophobic interactions between CYP monomers which are disrupted by the inclusion of detergent. The increase in $k_d$ for clotrimazole binding was probably due to detergent molecules adhering to
hydrophobic regions of the CYP molecules, inhibiting clotrimazole access to the
substrate/azole binding pocket.

*M. smegmatis* CYP51 bound clotrimazole, econazole and miconazole with a 2- to
3-fold higher affinity than CYP164A2 (Table 2), in contrast to itraconazole, ketoconazole
and voriconazole where CYP164A2 and CYP51 had similar affinities for these azoles.
CYP164A2 binding affinities for clotrimazole, econazole and miconazole ($k_d$ 1.2-2.5 µM)
were similar to those previously determined with *M. tuberculosis* CYP51 ($k_d$ 0.2 to 5 µM
– 3, 16, 26, 28), *M. smegmatis* CYP51 (16), *M. avium* CYP51 (30) and *M. tuberculosis*
CYP130 (28) with the exception of clotrimazole ($k_d$ 13.3 µM). The affinity of CYP164A2
for these three azoles was 17- to 84-fold less than *M. tuberculosis* CYP121 (26).

No discernable type II binding spectra with fluconazole could be obtained using
CYP164A2 either in the presence or absence of 0.5 M NaCl. Fluconazole only bound
weakly to CYP51 ($k_d$ 19 µM). *M. avium* CYP51 (30) also bound fluconazole extremely
weakly. The failure of CYP164A2 to bind fluconazole is probably not due to an inability
to enter the substrate/azole binding pocket as larger azoles such as ketoconazole and
itraconazole successfully bind to the CYP164A2 heme moiety. Therefore it is likely that
fluconazole is sterically hindered within the substrate/azole binding pocket from
obtaining a favourable conformation to coordinate with the heme moiety of CYP164A2
as the sixth ligand. Previous studies have demonstrated binding of fluconazole to *M. tuberculosis* CYP51, *M. tuberculosis* CYP121 and *M. smegmatis* CYP51, albeit with
lower affinity ($k_d$ 6 to 23 µM) than other azole antifungals (3, 16, 26), whilst a $k_d$ value of
70 µM was obtained for human CYP51 (4).
CYP164A2 binding affinity for ketoconazole ($k_d$ 5.7 µM) was similar to those previously determined with *M. tuberculosis* CYP51 (3, 16, 26), *M. smegmatis* CYP51 (16) and *M. tuberculosis* CYP121 (26). However, CYP164A2 bound ketoconazole with 2-fold greater affinity than *M. avium* CYP51 (30), 8-fold greater affinity than *M. tuberculosis* CYP130 (28) and 3-fold greater affinity than *M. tuberculosis* CYP51 as reported by Ouellet et al (28). This compares with a $k_d$ value of 0.11 and 0.32 µM for ketoconazole obtained with human and bovine CYP51 (33), indicating ketoconazole would not be theazole of choice for intravenous treatment of mycobacterial infections. Voriconazole and itraconazole were found to bind poorly to *M. avium* CYP51 (30).

The intensity of the azole-binding spectra varied with strong spectra ($\Delta A_{\text{max}}$ above 0.05) being obtained with clotrimazole, econazole, ketoconazole and miconazole for CYP51 and fluconazole, itraconazole and voriconazole producing moderately strong spectra ($\Delta A_{\text{max}}$ above 0.02). In contrast, only clotrimazole gave strong azole-binding spectra with CYP164A2 in the absence of NaCl, econazole and miconazole gave moderately strong spectra and itraconazole, ketoconazole and voriconazole gave weak binding spectra ($\Delta A_{\text{max}}$ below 0.01) with CYP164A2.

The presence of 0.5 M NaCl had little effect on the azole-binding properties of CYP51, with the exception of fluconazole where the observed $\Delta A_{\text{max}}$ increased by 2-fold and the $k_d$ fell by 3-fold (Table 2). In contrast, 0.5 M NaCl had a dramatic effect on the azole-binding properties of CYP164A2, increasing the observed spectral binding intensity ($\Delta A_{\text{max}}$) by 2- to 7.5-fold (especially itraconazole, ketoconazole and voriconazole), accompanied by a 1.3- to 6-fold increase in $k_d$ value. This suggests the binding of azoles to the high-spin state form of the heme moiety is favoured over the low-
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spin form for CYP164A2 and for the binding of fluconazole to M. smegmatis CYP51.

Increasing the ionic strength with 0.5 M NaCl caused a blue-shift of the observed type II spectral maxima and minima with CYP164A2 by -6 nm for clotrimazole, econazole, miconazole and voriconazole, -2 nm for itraconazole and only -1 nm for ketoconazole. The observed blue-shift was caused by the increased proportion of the high-spin state form (86%) of CYP164A2 in the presence of 0.5 M NaCl (17). No corresponding blue-shift was observed with M. smegmatis CYP51.

Voriconazole saturation studies with CYP164A2 (Table 3) indicated that increasing the ionic strength between 0 and 0.3 M NaCl increased the intensity of the observed type II binding spectra with a commensurate 4-fold increase in $\Delta A_{\text{max}}$. However, increasing the NaCl concentration above 0.5 M resulted in a weakening of the intensity of the observed type II binding spectrum (reduction in $\Delta A_{\text{max}}$) suggesting optimal voriconazole binding occurs between 0.3 and 0.5 M NaCl. However, the $k_d$ initially increased 12-fold, reaching a maximum at 0.3 M NaCl then decreasing at higher NaCl concentrations. This suggests that the affinity of CYP164A2 for voriconazole decreases with increasing NaCl concentration up to 0.3 M, above which affinity for voriconazole once again increases, even though the high ionic strength appears to no longer favour the formation of the [CYP164A2-voriconazole] complex as observed by the decreasing $\Delta A_{\text{max}}$ values. NaCl concentrations above 0.5 M either progressively alter the 3D structure of CYP164A2 to restrict access to the substrate/azole binding pocket or high ionic strength increasingly dissociates the azole molecule from binding sites within the substrate/azole binding pocket of the enzyme. Reduced solubility of azole antifungal drugs at high NaCl concentrations, especially for the larger hydrophobic azoles, could
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also be an important factor in the apparent reduced binding of azoles to CYP164A2 at high NaCl concentrations.

Mycobacterial species living within human tissues will be exposed to an external environment containing 0.15 M NaCl (0.09% w/v NaCl) and a cytosolic concentration unlikely to exceed this. At this concentration approximately half of the CYP164A2 molecules would exist in the high-spin state, which appears to be the preferred spin state for azole binding in CYP164A2, leading to increased sensitivity of CYP164A2 to binding azole antifungal agents. Should azole drugs emerge as potential anti-leprosy therapies then the variation seen here for CYP164A2 may assist in drug development considerations and also for other CYP targets in drug discovery and development. As with most microbial CYPs the function of CYP164 is unknown and the functional genomic investigation is an important area of future study for CYP164, including gene deletion and metabolomic investigations. Some trials of azole compounds in treating M. leprae in model systems are also warranted.
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ACKNOWLEDGMENTS

We are grateful to the Biotechnology and Biological Science Research Council of the United Kingdom for support.

FOOTNOTE

CYP naming anomalies:

The A0R5U2 entry described the protein as a CYP107B1. However, two CYP107B1 proteins have been deposited in the UniProKB/TrEMBL database for M. smegmatis strain ATCC 700084 with the other (A0QX10) sharing only 34% identity with A0R5U2. Therefore the more appropriate name for A0R5U2 is CYP164A2, as originally assigned, because it shares 60% identity to M. leprae CYP164A1 (Q9CBE7).
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REFERENCES


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FIGURE LEGENDS

FIG. 1. Absolute and reduced carbon monoxide difference spectra. Absolute spectra of *M. smegmatis* CYP51 (A) and CYP164A2 (B) were determined for the oxidized form (line 1), dithionite reduced form (line 2) and reduced carbon monoxide form (line 3). Reduced carbon monoxide difference spectra (C) were determined independently using *M. smegmatis* CYP51 (solid line) and CYP164A2 (dashed line) as previously described by Estabrook *et al* (12) using an extinction coefficient of 91 mM⁻¹·cm⁻¹ at 448 nm (27). All spectral determinations were made using Ni²⁺-NTA agarose purified CYP51 (0.244 mg.ml⁻¹ protein) and CYP164A2 (0.140 mg.ml⁻¹ protein).

FIG. 2. Modulation of CYP164A2 spin state by NaCl. The 100% low-spin state of CYP164A2 (line 1) was obtained in the presence of 60% (v/v) ethylene glycol, 50 mM Tris-HCl, pH 8.2. The absolute spectrum of CYP164A2 was determined at pH 7.5 in the absence of NaCl (line 2), in the presence of 0.5 M NaCl (line 3) and 4 M NaCl (line 4). The γ-Soret peak region (310 to 460 nm) of the spectrum has been expanded to clearly show the change in spin state from low-spin (417 nm) to high-spin (393 nm) caused by increasing NaCl concentration.

FIG. 3. Spectral titration of clotrimazole and voriconazole against *M. smegmatis* CYP51 and CYP164A2. Azole was progressively titrated against Ni²⁺-NTA agarose purified CYP51 (2.44 µM) and CYP164A2 (2.62 µM) to a maximum DMSO concentration of 2.5% (v/v) with the spectral difference (ΔA<sub>peak-trough</sub>) determined after each incremental addition of azole. The type II binding spectra obtained using 4.4 µM clotrimazole (A) and 21.5 µM voriconazole (C) for both CYP51 (solid lines) and CYP164A2 (dashed lines) are shown as well as the saturation curves obtained using
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clotrimazole (B) and voriconazole (D) for CYP51 (hollow circles) and CYP164A2 (filled circles). Non-linear regression (Levenberg-Marquardt algorithm) of the Hill equation \[ \Delta A = \Delta A_{\text{max}} / (1 + k_d / [\text{Azole}^n]) \] (28) was used to analyse the data.
**M. leprae P450 homolog**
Fig. 2.
TABLE 1. Identification of conserved regions in mycobacterial CYP164A1 homologs.

<table>
<thead>
<tr>
<th>Block</th>
<th>Residues</th>
<th>Block Consensus Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>55</td>
<td>ANRADPYPLY AXFRXXGPLQ_LPEANLXVFS_XFXDCEVL_R HPXSXSRXK STVAQ</td>
</tr>
<tr>
<td>B</td>
<td>52</td>
<td>AAGAXRPFG PPGFLFDPD DHTRLRLVS KAFVPRVA LEPEIVSLVD XL</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>GEFDXKDLA YPLPVAICR LLGVPLED_ QFS</td>
</tr>
<tr>
<td>D</td>
<td>35</td>
<td>RXXAGXWLD _YLRLXXRR XXPGLDXXG_LIAVE</td>
</tr>
<tr>
<td>E</td>
<td>39</td>
<td>LTEDEIVATC NLLLVAGHT TVNLIANAL AMLRXPGQ_</td>
</tr>
<tr>
<td>F</td>
<td>45</td>
<td>AAL_AADPKRA XAXXETRLY DPPVQLVSR_ AAADMIGGV TIPKG</td>
</tr>
<tr>
<td>G</td>
<td>30</td>
<td>FDRPDTFDPD <em>RXXLRHLGF</em> KGAHFCLGAP</td>
</tr>
<tr>
<td>H</td>
<td>44</td>
<td>LARLEAXVAL SAVTBFPXA RLAGEPYKP _NVTLRSX _SVAX</td>
</tr>
</tbody>
</table>

The online program BLOCKMAKER (http://blocks.fhcrc.org/blocks/make_blocks.html) was used to identify conserved ungapped 'MOTIF' regions of amino acid residues between *M. smegmatis* CYP164A2 and nine CYP164A1 homologs from different mycobacterial species. The mycobacterial CYPs compared were *M. smegmatis* CYP164A2 (A0R5U2), *M. leprae* CYP (Q9CBE7), *M. gilvum* (A4T681), *Mycobacterium* sp. strain JLS (A3Q74G), *Mycobacterium* sp. strain MCS (Q1B233), *Mycobacterium* sp. strain KMS (A1UN16), *M. vanbaalenii* (A1TGP4), *M. paratuberculosis* (Q744J7), *M. avium* (A0Q9Q7) and *M. marinum* (MM5268). Amino acid residues conserved between all ten sequences are underlined. Non-conserved amino acid residues, present in four or fewer of the ten CYP proteins, are represented by the letter X. The residue numbers of each block relate to the start position in the *M. smegmatis* CYP164A2 protein.
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TABLE 2. Binding constants of *M. smegmatis* CYP51 and CYP164A2 for azole antifungals.

<table>
<thead>
<tr>
<th>Azole</th>
<th>CYP51</th>
<th>CYP164A2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_d$ (µM)</td>
<td>$n_{H,app}$</td>
</tr>
<tr>
<td>Clotrimazole</td>
<td>0.62 ±0.03</td>
<td>2.20 ±0.10</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>0.43 ±0.09</td>
<td>1.80 ±0.05</td>
</tr>
<tr>
<td>+0.1% Triton$^3$</td>
<td>3.06 ±0.07</td>
<td>1.00 ±0.01</td>
</tr>
<tr>
<td>Econazole</td>
<td>0.88 ±0.01</td>
<td>1.80 ±0.06</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>1.66 ±0.08</td>
<td>2.21 ±0.08</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>19.05 ±1.52</td>
<td>1.18 ±0.07</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>5.94 ±0.27</td>
<td>1.36 ±0.04</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1.26 ±0.12</td>
<td>2.05 ±0.20</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>3.00 ±0.38</td>
<td>2.51 ±0.27</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>7.45 ±0.11</td>
<td>1.26 ±0.02</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>4.59 ±0.24</td>
<td>1.32 ±0.06</td>
</tr>
<tr>
<td>Miconazole</td>
<td>1.16 ±0.07</td>
<td>1.79 ±0.07</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>0.94 ±0.02</td>
<td>2.55 ±0.06</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>5.94 ±0.17</td>
<td>1.24 ±0.02</td>
</tr>
<tr>
<td>+0.5 M NaCl</td>
<td>4.57 ±0.07</td>
<td>1.32 ±0.01</td>
</tr>
</tbody>
</table>
1 = Standard deviations for the ΔA_{max} values did not exceed 10% of the values quoted in the table, and were typically 1 to 5% of the value quoted.

2 = The detergent Triton X-100.

nd = not determined as no reproducible type II binding spectrum could be obtained for fluconazole with CYP164A2.

Azole antifungals were titrated against *M. smegmatis* CYP51 and CYP164A2 in order to construct azole saturation curves from which *k_d* values were determined using non-linear regression of the Hill equation (\( \Delta A = \Delta A_{\text{max}} / (1 + k_d / [\text{Azole}]^n) \)). Titration was performed in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol, with and without the presence of 0.5 M NaCl. Clotrimazole binding to CYP51 and CYP164A2 was also determined in the presence of 0.1% (w/v) Triton X-100. The values quoted in the table are those obtained from curve-fitting the mean values of three replicates followed by the standard deviations generated by the curve-fitting process.
### TABLE 3. Effect of NaCl concentration on the binding constants for voriconazole with CYP164A2.

<table>
<thead>
<tr>
<th>[NaCl] (M)</th>
<th>$k_d$ (µM)</th>
<th>$\Delta A_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.74 ±0.47</td>
<td>0.0053 ±0.0002</td>
</tr>
<tr>
<td>0.05</td>
<td>8.36 ±0.62</td>
<td>0.0112 ±0.0003</td>
</tr>
<tr>
<td>0.1</td>
<td>17.91 ±2.79</td>
<td>0.0143 ±0.0011</td>
</tr>
<tr>
<td>0.2</td>
<td>20.05 ±3.77</td>
<td>0.0187 ±0.0018</td>
</tr>
<tr>
<td>0.3</td>
<td>34.04 ±9.66</td>
<td>0.0218 ±0.0041</td>
</tr>
<tr>
<td>0.5</td>
<td>19.58 ±2.07</td>
<td>0.0219 ±0.0012</td>
</tr>
<tr>
<td>1.0</td>
<td>9.85 ±2.99</td>
<td>0.0091 ±0.0012</td>
</tr>
<tr>
<td>2.0</td>
<td>13.17 ±1.37</td>
<td>0.0073 ±0.0004</td>
</tr>
</tbody>
</table>

1 NaCl concentration in 50 mM sodium phosphate, pH 7.5, 25% (w/v) glycerol.

The binding constants $k_d$ and $\Delta A_{max}$ above were determined using the Michaelis-Menten-Henri equation. Fitting the data using the Hill equation gave $n_{Hill}$ values between 0.8 and 1.2, indicating that the binding of voriconazole to CYP164A2 was not allosteric.