X-ray Structure of 4,4′-Dihydroxybenzophenone Mimicking Sterol Substrate in the Active Site of Sterol 14α-Demethylase (CYP51)*§∗

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A universal step in the biosynthesis of membrane sterols and steroid hormones is the oxidative removal of the 14α-methyl group from sterol precursors by sterol 14α-demethylase (CYP51). This enzyme is a primary target in treatment of fungal infections in organisms ranging from humans to plants, and development of more potent and selective CYP51 inhibitors is an important biological objective. Our continuing interest in structural aspects of substrate and inhibitor recognition in CYP51 led us to determine (to a resolution of 1.95 Å) the structure of CYP51 from Mycobacterium tuberculosis (CYP51Mt) co-crystallized with 4,4′-dihydroxybenzophenone (DHBP), a small organic molecule previously identified among top type I binding hits in a library screened against CYP51Mt. The newly determined CYP51Mt-DHBP structure is the most complete to date and is an improved template for three-dimensional modeling of CYP51 enzymes from fungal and prokaryotic pathogens. The structure demonstrates the induction of conformational fit of the flexible protein regions and the interactions of conserved Phe-89 essential for both fungal drug resistance and catalytic function, which were obscure in the previously characterized CYP51Mt-estriol complex. DHBP represents a benzophenone scaffold binding in the CYP51 active site via a type I mechanism, suggesting (i) a possible new class of CYP51 inhibitors targeting flexible regions, (ii) an alternative catalytic function for bacterial CYP51 enzymes, and (iii) a potential for hydroxybenzophenones, widely distributed in the environment, to interfere with sterol biosynthesis. Finally, we show the inhibition of M. tuberculosis growth by DHBP in a mouse macrophage model.

Sterol 14α-demethylase (CYP51)2 is a cytochrome P450 (P450, CYP) heme thiolate containing enzyme involved in biosynthesis of membrane sterols, including cholesterol in animals, ergosterol in fungi, and a variety of C24-modified sterols in plants and protozoa in most organisms in biological kingdoms from bacteria to animals (1). CYP51 has been a therapeutic target for several generations ofazole antifungal agents including fluconazole, voriconazole, itraconazole, ravuconazole, and posaconazole (2). These drugs inhibit microbial growth by disrupting biosynthesis of ergosterol, a major component of fungal membrane. Protozoa share with fungi the requirement of ergosterol and ergosterol-related sterols for cell viability and proliferation (3). Inhibition of sterol biosynthesis has been proven to be effective in trypanosomatids (3–5) and Leishmania spp (6), which cause such tropical diseases as African sleeping sickness, Chagas disease, and leishmaniasis.

Although mammalian CYP51 enzymes perform the same catalytic reaction (7) as their fungal and protozoan orthologs (1), they share relatively modest overall sequence identity (within 30%) with them. This accounts for the reduced sensitivity of mammalian CYP51 to azole and triazole drugs. Despite the lack of the full sterol biosynthetic pathway in Mycobacterium tuberculosis (8), and hence, de novo sterol biosynthesis, CYP51 encoded by this organism (CYP51Mt) performs in vitro the same catalytic reaction as its mammalian, fungal, and protozoan counterparts (9). CYP51Mt has served as a homology model for the marginally soluble microsomal representatives of the CYP51 protein family since its discovery (10, 11) and characterization (9) and the determination of the crystal structures for ligand-free (PDB ID codes 2BZ9 and 1H52) (12), inhibitor-bound (PDB ID codes 1E9X, 1EA1, 2C10, 2C1B) (12, 13), and substrate-analog-bound PDB ID 1X8V (14) forms. The CYP51Mt structure is widely used for homology modeling of CYP51 enzymes from human (15), pathogenic fungi including Candida albicans (15–22), Candida krusei (17), Aspergillus fumigatus (18–20, 23), and Penicillium digitatum (24), and the protozoan Trypanosoma cruzi (25). Similarly, the CYP51Mt

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2 The abbreviations used are: P450, CYP, cytochrome P450 monoxygenase; CYP51, sterol 14α-demethylase; CYP51Mt, sterol 14α-demethylase from M. tuberculosis; PAH, polycyclic aromatic hydrocarbon; DHBP, 4,4′-dihydroxybenzophenone; EPBA, α-ethyl-N-4-pyridinyl-benzeneacetamide; BSPPA, 2-(benzol[o]-2,1,3-thiadiazole-4-sulfonyl)-2-amino-2-phenyl-N-(pyridinyl-4)-acetamide; PDB, Protein Data Bank.
structure has assisted in the evaluation and prediction of the binding mode of existing antifungal drugs and the de novo design of CYP51 inhibitors that have potential to become therapeutic drugs (22, 26–30).

Although a paradigm for the CYP51 family of sterol 14α-demethylases, the biological and molecular functions of CYP51Mt in M. tuberculosis remain poorly understood. The requirement of M. tuberculosis for host cholesterol to be taken up by macrophages and for subsequent intracellular survival (31) suggests that in vitro sterol-modifying CYP51Mt may be involved in cholesterol-mediated cell entry (32). However, cyp51 has not been identified among cholesterol catabolic genes of Rhodococcus sp. RHA1 or M. tuberculosis H37Rv (33), leaving open the question of the biological function of CYP51Mt.

To date, CYP51Mt has been co-crystallized with a number of “type II inhibitors” (34), including the clinical drug fluconazole (13), as well as two compounds newly discovered by high-throughput screening: α-ethyl-N-4-pyridinyl-benzenacacetamide (EPBA) and 2-(benzo[b]-2,1,3-thiadiazole-4-sulfonyl)-2-amino-2-phenyl-N-(pyridinyl-4)-acetamide (BSPPA) (12). In contrast, only one “type I ligand” (35), the substrate-analog estriol, has so far been co-crystallized with CYP51Mt (14). The estriol-bound CYP51Mt structure revealed active site residues, defined the orientation of the sterol substrate in the active site, and established structural determinants for discrimination between the 4α-methylated and 4α,β-dimethylated CYP51 sterols (14).

Based on the distribution of temperature factor values (B-factors) in previously reported CYP51Mt structures (12–14), an assumption was made that the enzyme undergoes conformational changes when the substrate binds and that these changes are dominated by the remodeling of the C helix and the BC-loop, facilitated by the flexibilities of the remote GH- and HI-loops. These highly dynamic regions are enclosed in mutational hot spots in azole-resistant clinical isolates of C. albicans (13, 36, 37), indicating their either direct or indirect (via protein dynamics) involvement in the substrate or inhibitor binding. A critical mutation hot spot, the BC-loop (36), could not be fully defined in previously reported structures due to insufficient electron density in this region.

Here, we report determination of the crystal structure of the CYP51Mt-4,4’-dihydroxybenzophenone (DHBP) complex to a resolution of 1.95 Å. DHBP was identified previously as a top type I binding hit in a library of small synthetic organic molecules screened against CYP51Mt (12). The newly determined structure confirms a potential for the benzophenone scaffold to mimic steroids and suggests its utility for the development of a novel class of inhibitors targeting flexible regions and dynamic states of CYP51 to overcome drug resistance acquired by fungi toward azole and triazole drugs. The structure reveals previously obscure interactions and highlights the role of the conserved aromatic residue Phe-89 (analogous to the mutational hot spot residue Tyr-132 in C. albicans), thus making a more accurate template for modeling the three-dimensional structures of fungal and prokaryotic orthologs.

**EXPERIMENTAL PROCEDURES**

**Spectroscopic Binding Assays—**CYP51Mt double C37L/C442A mutant with improved expression and purification qualities (14) was prepared as described elsewhere (12). Spectroscopic characterization of DHBP (ABCR GmbH & Co.KG) binding was conducted in a 1-ml quartz cuvette containing 2.5 μM CYP51Mt in 50 mM Tris-HCl, pH 7.5, and 10% glycerol. DHBP dissolved in Me2SO at 25 mM concentration was added in 0.5-μl aliquots, resulting in a concentration increase from 12.5 to 125 μM in 12.5-μM increments. The same amounts of Me2SO alone were added to a reference cuvette followed by recording of difference spectra. Concentration of CYP51Mt was determined from the difference spectra between the carbon monoxide-bound ferrous and water-bound ferric forms using the extinction coefficient of 91,000 M⁻¹ cm⁻¹ (38). To determine the dissociation constant (Kd), titration data were fitted using program GraphPad PRISM (Graphpad Software Inc.) to the rectangular hyperbola (Aobs = Amax(S/(Kd + S)), where Aobs is the absorption shift at 420 nm determined at any ligand concentration; Amax is the maximal absorption shift obtained at saturation; Kd is the dissociation constant for the protein-ligand complex; and S is the ligand concentration.

**Crystallography, Data Collection, and Determination of Crystal Structure—**Crystals of the CYP51Mt-DHPB complex could not be obtained under conditions promoting crystal growth for the other previously characterized CYP51Mt forms. Therefore, ab initio screening of crystallization conditions was performed using the nanoliter drop setter Mosquito (TTP LabTech) (hanging drop crystallization protocol) and high throughput screening kits purchased from both Hampton Research and Qiagen. Protein from the 1.2 mM frozen stock stored at −80 °C in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, and 0.5 mM EDTA was mixed with DHBP dissolved in Me2SO at 10 mM stock concentration to final concentrations of 0.2 mM for protein and 2 mM for the ligand. The volume of the mixture was adjusted with 10 mM Tris-HCl, pH 7.5. The crystallization conditions generating small needle-shaped crystals were identified and optimized further in the 24-well crystallization plates. Larger needles of the diffraction quality were finally obtained by hanging drop vapor diffusion method from 1.2 M lithium sulfate, 0.1 M HEPES, pH 7.5, and 2% isopropyl alcohol. Prior to data collection, the crystals were briefly washed in the well solution supplemented with 20% glycerol and then flash-frozen in liquid nitrogen. Diffraction data were collected to a resolution of 1.9 Å at 100–110 K at beamline 8.3.1, Advanced Light Source, Lawrence Berkeley National Laboratory. The images were integrated, and the intensities were merged by using the HKL2000 software suite (39). Diffraction statistics are shown in Table 1.

The structure was determined by molecular replacement using coordinates of BSPPA-bound CYP51Mt (Protein Data Bank ID 2CIB) as a search model. The final atomic model (Table 1) was obtained after a few iterations of refinement using REFMAC5 (40) and CNS (41) and model building using the COOT (42) and O (43) programs. The quality of the structure (Table 1) was assessed by the program PROCHECK (44).

**Macrophage Assay for DHBP Inhibitory Activity—**Bone marrow-derived macrophages were extracted from C57/BL6 mice and seeded in culture dishes (~5 × 10⁶ cells/dish). Macrophages were allowed to differentiate from 5 to 6 days in Dulbecco’s modified essential medium containing 10% heat-inacti-
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| TABLE 1 |
|---|
| **Crystallographic data and statistics** |
| **Parameter** | **Value(s)** or determination (PDB ID 2VKU) |
| Wavelength, Å | 1.11587 |
| Resolution, Å | 1.95 |
| Unique reflections | 40591 |
| Redundancy | 5.2 (5.3) |
| Completeness, % | 100.0 (100.0) |
| Space group | P2₁,2₁,2₁ |
| Cell dimensions (a, b, c), Å | 77.7, 82.1, 85.9 |
| Molecules in asymmetric unit | 1 |
| Solvent content, % | 53 |
| Rcryst, % | 7.9 (41.4) |
| l/σ | 23.4 (4.1) |

- Numbers in parentheses correspond to the highest resolution shell.
- Rcryst = \( \sum |F_o| - |F_c| / \sum |F_o| \), where \( F_o \) is the intensity of the observed reflection, and \( F_c \) is the mean intensity of reflection.
- Rcryst = \( \sum |F_o| - |F_c| / \sum |F_o| \), calculated with the working reflection set. Rcryst is the same as Rcryst but calculated with the reserved reflection set.
- r.m.s. = root mean square.
- Program PROCHECK (44), portions of the protein residues in most favored/additional allowed/generously allowed/disallowed regions.

Plates were incubated at 37 °C for 3 weeks followed by the enumeration of colony-forming units. Data representing the averaged numbers of bacteria in the triplicate cultures plotted against compound concentration were fitted using program GraphPad PRISM.

**RESULTS**

**Binding of DHBP in the Active Site of CYP51Mt—DHBP binding was addressed by manual spectroscopic binding assays (Fig. 1), and a \( K_D \) of 29.5 ± 1.6 μM was determined for the CYP51Mt-DHBP complex, indicating 3-fold higher binding affinity for DHBP when compared with a non-substrate sterol estriol (\( K_D = 100 \) μM) (14).**

**Determination of the Crystal Structure—**The CYP51Mt-DHBP complex crystallized under different conditions than those favoring crystallization of other CYP51Mt forms (12–14). Crystals diffracted in the P2₁,2₁,2₁ space group having different unit cell dimensions, and hence, different packing interactions, apparently resulting from reshaping protein surface caused by the DHBP binding. Structure was determined to a resolution of 1.95 Å, and the coordinates were deposited with the Protein Data Bank (PDB ID code 2VKU).

**Overall Structure of the CYP51Mt-DHBP Complex—**Unlike other structurally defined CYP51Mt forms, the CYP51Mt-DHBP structure is represented by a continuous electron density for all protein regions including the BC-loop and the C helix, which makes it the most complete CYP51Mt structure determined to date. For comparison, 10 residues (85–94) within the BC-loop are disordered in the CYP51Mt-estriol complex (PDB ID code 1X8V), 20 residues (85–104) in both the CYP51Mt-EPBA and the CYP51Mt-BSPPA complexes (PDB ID codes 2C10 and 2CIB, respectively), and 10 residues (91–100) in the ligand-free CYP51Mt (PDB ID code 1H5Z). Superposition of the CYP51Mt-DHBP complex with the other CYP51Mt forms (Fig. 2) maps differences between the structures to the region comprising the BC-loop, the C helix, the H helix and its adjacent loops, and the C terminus of the G helix. No significant differences were observed in the FG-region, confirming that the substrate/inhibitor access to the active site in CYP51Mt occurs...
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![Image](49x429 to 301x547)

**FIGURE 2. Overall structure of the CYP51Mt-DHBP complex.** Stereo of the superimposed CYP51Mt forms bound to DHBP (green) or to 4-phenylimidazole (gray) (PDB ID code 1E9X) is shown. The arrows indicate the directions of the movement for the most mobile structural elements upon binding DHBP. Relocation distances are shown in Angstroms. The helices C, G, and H are represented by tubes. Heme is in orange, and DHBP in cyan. Images here and in Fig. 4 are generated using the VMD software (76).

![Image](49x621 to 301x734)

**FIGURE 3. DHBP binding in the CYP51Mt active site.** A stereo view of DHBP bound in the active site of CYP51Mt is shown. C atoms are colored in cyan (DHBP) or green (protein), N atoms are shown in blue, O atoms are shown in red, and S atoms are shown in yellow. Amino acid side chains within 4 Å from DHBP are labeled in black, and Phe-89 (within 7.3 Å) is labeled in blue. Fragments of the 2Fo-Fc electron density composite omit map contoured at 1.0 σ (pale cyan and cyan) and 0.6 σ (red) are shown as mesh. To avoid excessive cluttering, heme (orange) was excluded from the map calculation. The image was generated using PyMOL (77).

between the BC-loop and the C helix in the direction parallel to the heme plane and orthogonal to that in other P450 enzymes.

The ~150° I helix kink persists in the CYP51Mt-DHBP complex, although as a less destructive and smoother bend, largely preserving the H-bonding pattern of the α-helix. The short H helix, which connects G and I helices via the adjacent loops, is relocated up to 5.0 Å closer to the protein core (when compared with the 4-phenylimidazole-bound CYP51Mt (PDB ID code 1E9X) having root mean square deviation with the CYP51Mt-DHBP structures of 0.85 Å), thus transmitting the 2.3 Å motion of the I helix N terminus to the C terminus of the G helix, which is displaced 5.2 Å (Fig. 2). The BC-loop relocates up to 5.5 Å when DHBP binds in the active site.

**CYP51Mt-DHBP Interactions in the Active Site**—DHBP binds CYP51Mt utilizing hydrophobic and H-bonding interactions with the active site residues. The electron density for DHBP is unambiguously defined (Fig. 3). Eighteen amino acid residues, including Glu-72, Tyr-76, Phe-78, Met-79, Phe-83, Arg-96, Ser-252, Phe-255, Ala-256, His-259, Leu-321, Ile-322, Ile-323, Leu-324, Met-325, Met-433, Val-434, and Val-435, are within 6 Å from the ligand (Fig. 4). All adopt the same conformations as in the CYP51Mt-estriol complex but are slightly shifted (<1 Å) in one or another direction. This observation indicates that a large portion of the CYP51Mt active site is a rather rigid formation, whereas the BC-loop and the C helix have their structures induced or stabilized by the bound ligand.

The most distinct feature of the bound DHBP is that one of its two 4-hydroxylated phenyl rings (Fig. 4A, ring A) coincides with the 3-hydroxylated ring of estriol, suggesting for the polar hydroxyl group a role of a substrate recognition landmark, which apparently is neither sterol-specific nor CYP51-specific, because all CYP51 sterol substrates possess this same feature (Scheme 1A), and no contacts with any of the amino acid side chains of the protein are involved in the interactions. Thus, one of the 4-hydroxyl group of the DHBP is bound in the hydrophilic pocket formed by the backbone amide groups of Ile-322 (via a water molecule) and Ile-323 and the carbonyl group of Ile-323, whereas the side chains of the Tyr-76, Phe-78, Leu-321, Leu-324, and Val-434 stabilize ring A via hydrophobic contacts (Fig. 4). The oxygen atom of the carbonyl group connecting the rings A and B H-bonds (distance 2.9 Å) to the side chain of His-259. Ring B approaches the heme iron within 8 Å and is stabilized by π-π interactions with the side chains of Tyr-76, Phe-83, Met-79, and Phe-255, whereas its 4-hydroxyl group points toward the hydrophilic and/or charged groups of Gln-72, Arg-96, and the heme propionate moiety, the latter being bent toward the ligand.

**Roles of the Conserved Active Site Residues**—Three of the eighteen active site residues, Tyr-76, Phe-83, and His-259, are invariant in the CYP51 protein family (supplemental Fig. S1). Moreover, Phe-83 is followed by glycine in all CYP51 proteins identified to date. The Phe-78 and Phe-255 are strictly specific for 14α-demethylation of the sterol substrates carrying a single C-4 methyl group in α-configuration. Phe-78 was shown to impose steric constrains on binding sterol substrates having a methyl group in β-configuration (14), and therefore, is a key
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The reduction in the bacterial colony-forming unit (CFU) count at 1–5 days after macrophage infection with *M. tuberculosis* H37Rv as a result of macrophage treatment with increasing concentrations of DHBP (A); (■) non-treated, (▲) 50 μM DHBP, (▼) 100 μM DHBP or EPBA (B); (△) non-treated, (×) 10 μM EPBA, (○) 30 μM EPBA is shown.

FIGURE 5. Inhibitory effects of DHBP (A) and EPBA (B) in mouse macrophages. The reduction in the bacterial colony-forming unit (CFU) count at 1–5 days after macrophage infection with *M. tuberculosis* H37Rv as a result of macrophage treatment with increasing concentrations of DHBP (A); (■) non-treated, (▲) 50 μM DHBP, (▼) 100 μM DHBP or EPBA (B); (△) non-treated, (×) 10 μM EPBA, (○) 30 μM EPBA is shown.

discriminator between CYP51 sterol substrates single- and double-methylated at the C-4 atom (14, 45). The “humanized” F78L CYP51Mt mutant does not bind DHBP (12), suggesting an essential role for Phe-78 in the recognition of benzophenone scaffold.

The remaining 13 residues overlap among phyla. Among them, position 96 is the most phylum-specific, being occupied largely by arginine in bacteria and plants (supplemental Fig. S1, highlighted in yellow), methionine in protists (highlighted in pink), phenylalanine in animals (highlighted in green), and leucine (highlighted in orange) in most fungi, with an exception being phenylalanine in *Schizosaccharomyces pombe*, *Cunninghamella elegans*, and *Ustilago maydis*.

Nonspecific DHBP Binding—Three other DHBP molecules bound to the protein surface were identified. The first, having two alternative conformations, binds in the same crevice between the H and I helices as the second estriol molecule in the CYP51Mt-estriol structure (14). The second one binds to a crevice formed by the cysteinyl loop on the proximal (in respect to heme) surface of the protein, and a third one binds between the A′ and the F′ helices. Both the second and the third DHBP molecules mediate protein-protein contacts, which might have facilitated crystallization of the CYP51Mt-DHBP complex. Binding of multiple molecules may suggest the propensity for DHBP for nonspecific interactions with the proteins.

DHBP-BC-loop Interactions—A remarkable finding provided by the crystal structure of the CYP51Mt-DHBP complex is elucidation of the function of residue Phe-89, which is important for substrate binding and conversion in CYP51Mt (46). Well conserved Phe-89 (supplemental Fig. S1) was reported to be involved in fungal drug resistance, inhibitor binding, and the catalytic function of CYP51 in *C. albicans* (Tyr-132, according to *C. albicans* numbering) (36, 47–51), *Histoplasma capsulatum* (Tyr-136, according to *H. capsulatum* numbering) (52), and the causative agents of zygomycosis in humans, *Rhizopus oryzae* and *Absidia corymbifera* (53). Phe-89 interactions were obscure in the CYP51Mt-estriol complex due to the missing electron density for this portion of the loop. In the CYP51Mt-DHBP structure, the electron density for the BC-loop backbone and the majority of the side chains, including Phe-89, is defined, revealing no direct contacts of Phe-89 with the ligand. Instead, Phe-89 serves to maintain the loop architecture via the interactions with Phe-83, Arg-96, and Ser-252 (Figs. 3 and 4), supporting the function of Arg-96, which apparently recognizes the sterol side chain attached to the C-17 atom of the sterol nucleus (Scheme 1A). The position of Arg-96 suits for the interactions with the side chain of lanosterol docked in the active site based on the molecular dynamics simulations (37) (Fig. 4B).

Inhibitory Effect of DHBP and EPBA—Tuberculostatic activity of DHBP and EPBA was examined in the *M. tuberculosis* infection model using mouse primary bone marrow-derived macrophages. Both compounds inhibited *M. tuberculosis* growth, albeit to different degrees. A slight inhibitory effect of DHBP was observed at 50 μM, which increased markedly as DHBP concentration increased to 100 μM (Fig. 5A). Four days after infection, DHBP induced from 40 to 70% reduction in bacterial colony-forming units, when compared with the untreated control, whereas EPBA induced only 30% reduction (Fig. 5B). It should be noted that the highest EPBA concentra-
tion tested was 30 μM due to the compound toxicity at higher concentrations. On the contrary, no cell toxicity was detected for DHBP up to 200 μM. The higher inhibitory effect of DHBP in macrophage model agrees with the results previously obtained in broth culture using the Alamar blue assay (12).

**DISCUSSION**

A soluble ortholog of its eukaryotic membrane-associated counterparts, CYP51<sub>Mt</sub> serves as a template for three-dimen-sional modeling of CYP51-inhibitor interactions in microbial pathogens. CYP51<sub>Mt</sub> undergoes extensive remodeling of the BC-loop and C helix, the closed conformation for the BC-loop in the CYP51<sub>Mt</sub>-DHBP complex having been observed for the first time here. A screen of synthetic organic molecules against CYP51<sub>Mt</sub> (12) identified three type I hits (affinity range 25–50 μM): DHBP, the estrogen metabolite 11-ketoestrone, and 2,7-dihydroxy-9-fluorenone (Scheme 1B). Although structurally different, all three compounds have certain similarities of size, shape, and hydroxylation pattern.

9-Fluorenone, a non-hydroxylated precursor of 2,7-dihydroxy-9-fluorenone, is an oxidation product of fluorene, a major component of fossil fuels and a by-product of energy-related industries. Fluorene is one of the 31 compounds on the U.S. Environmental Protection Agency Priority Chemicals list of pollutants (www.epa.gov/wastemin/chemlist.htm). It is structurally related to other chemicals of concern such as carbazoles, dibenzothiophenes, dibenzofurans, and dibenzodioxins. In the environment, fluorene undergoes biodegradation by a variety of bacterial strains that use polycyclic aromatic hydrocarbons as a source of carbon and energy, including Arthrobacter sp (54), Sphingomonas spp (55, 56), Pseudomonas spp (57, 58), Staphylococcus auricularis (59), and Mycobacterium vanbaalenii (60–63). In Mycobacterium vanbaalenii RYP-1, P450 monooxygenase systems have been implicated in polycyclic aromatic hydrocarbon degradation (62).

Hydroxybenzophenones are industrial bulk products used as UV stabilizers in plastic surface coatings on food packaging. Hydroxybenzophenone derivatives are used as ingredients in sunscreen and other cosmetics to absorb UV light. These compounds are absorbed through human skin, and bioaccumulation may occur in wildlife and humans (64–66). An inhibitory effect of benzophenones on CYP51 has not to our knowledge been reported previously. However, hydroxybenzophenones, including DHBP, have been found elsewhere to inhibit squalene cyclase, another enzyme in cholesterol biosynthesis (67), and have been identified as estrogen receptor ligands exhibiting estrogenic and antiandrogenic activities in a human breast cancer cell line (68, 69). Further, a biochemical evaluation of non-steroidal benzophenone-containing analogs of cholesterol suggests that the benzophenone scaffold can successfully replace the sterol tetracyclic nucleus in apolipoprotein-dependent cellular sterol efflux (70).

DHBP binds to CYP51<sub>Mt</sub>, with about the same affinity as to the estrogen receptor (68). The 4-hydroxyl group of DHBP, which is essential for hormonal activities of benzophenone derivatives (69), mimics the sterol C-3 hydroxyl group in the active site of CYP51<sub>Mt</sub>. Two DHBP 4-hydroxyphenyl rings positioned in an angular arrangement contact the same amino acid residue as the tetracyclic estriol (Fig. 4A) but, unlike estriol, they rearrange the BC-loop, revealing the interactions of the conserved aromatic functionality of Phe-89. Similar to the other mutation hot spot residues in CYP51, Phe-89 is not involved in direct interactions with the ligand bound in the active site, supporting our previous assumption that fungal resistance toward azole drugs related to CYP51 modifications evolved via alteration of CYP51 dynamics rather than through immediate protein-ligand contacts.

Lastly, tuberculosis is becoming increasingly difficult to treat as a result of the growing incidence of multidrug and extensively drug-resistant *M. tuberculosis* strains (71). Thus, the need for new therapeutic agents has become imperative.

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In summary, the binding mode of the aromatic compound DHBP to CYP51 was validated by determination of the x-ray structure of the CYP51<sub>Mt</sub>-DHBP complex. DHBP binding triggers rearrangement of the protein BC-loop, allowing elucidation of the previously obscure role of the conserved Phe-89, reported elsewhere to be essential for substrate binding, catalytic conversion, and fungal drug resistance. Structurally different from sterols, DHBP binds CYP51<sub>Mt</sub> via a type I mechanism, suggesting a possibility of exploiting a DHBP scaffold to design a novel class of CYP51 inhibitors targeting regions including the flexible BC-loop and the C helix. Such inhibitors may help to overcome the fungal drug resistance to antifungal azoles and triazoles, which has developed over the years.

Further, our study is the potential of hydroxybenzophenones to interact with steroid 14α-demethylases and probably to affect sterol biosynthesis. Additionally, the interactions of benzophenone and fluorenone scaffolds with CYP51<sub>Mt</sub> suggest the possibility of alternative catalytic functions for bacterial CYP51 enzymes, such as biodegradation of polycyclic aromatic hydrocarbons. In this regard, a precedent has recently been reported of CYP51 being recruited for production of antimicrobial plant defense compounds (75).

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