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Trypanosoma cruzi CYP51 Inhibitor Derived from a Mycobacterium tuberculosis Screen Hit

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Abstract

Background: The two front-line drugs for chronic Trypanosoma cruzi infections are limited by adverse side-effects and declining efficacy. One potential new target for Chagas’ disease chemotherapy is sterol 14α-demethylase (CYP51), a cytochrome P450 enzyme involved in biosynthesis of membrane sterols.

Methodology/Principal Finding: In a screening effort targeting Mycobacterium tuberculosis CYP51 (CYP51Mt), we previously identified the N-[4-pyridyl]-formamide moiety as a building block capable of delivering a variety of chemotypes into the CYP51 active site. In that work, the binding modes of several second generation compounds carrying this scaffold were determined by high-resolution co-crystal structures with CYP51Mt. Subsequent assays against the CYP51 orthologue in T. cruzi, CYP51Tc, demonstrated that two of the compounds tested in the earlier effort bound tightly to this enzyme. Both were tested in vitro for inhibitory effects against T. cruzi and the related protozoan parasite Trypanosoma brucei, the causative agent of African sleeping sickness. One of the compounds had potent, selective anti-T. cruzi activity in infected mouse macrophages. Cure of treated host cells was confirmed by prolonged incubation in the absence of the inhibiting compound. Discrimination between T. cruzi and T. brucei CYP51 by the inhibitor was largely based on the variability (phenylalanine versus isoleucine) of a single residue at a critical position in the active site.

Conclusions/Significance: CYP51Mt-based crystal structure analysis revealed that the functional groups of the two tightly bound compounds are likely to occupy different spaces in the CYP51 active site, suggesting the possibility of combining the beneficial features of both inhibitors in a third generation of compounds to achieve more potent and selective inhibition of CYP51Tc.

Introduction

The drug development pipeline targeting diseases caused by trypanosome parasites is sparse [1]. Despite significant advances in its control over the last 15 years [2], Chagas’ disease, caused by the parasitic protozoan Trypanosoma cruzi [3], remains a major public health concern in Latin America, with an estimated total of 8 million people infected [4]. Nifurtimox and benznidazole, the two principal drugs for treatment of Chagas’ disease, were launched in 1967 and 1972 respectively, and suffer from the twin liabilities of serious side-effects and reduced efficacy in chronic T. cruzi infections [2]. A potential new target for Chagas’ disease chemotherapy is sterol 14α-demethylase (CYP51) [3], a cytochrome P450 heme thiolate-containing enzyme which is involved in biosynthesis of membrane sterols in all biological kingdoms from bacteria to animals [6]. T. cruzi sterols are similar in composition to those in fungi, with ergosterol and ergosteroïd-like sterols the major membrane components [7]. Clinically employed antifungal azoles [8,9] inhibit ergosterol biosynthesis in fungi and are partially effective against Leishmania and Trypanosoma parasites [10–12]. Azoles block CYP51 activity, resulting in decline of the normal complement of endogenous sterols and accumulation of various 14α-methyl sterols with cytostatic or cytotoxic consequences [11]. Aside from the compounds optimized for antifungal therapy, other CYP51 inhibitors with strong anti-T. cruzi activity have also been reported [13–15].

Mammalian CYP51 shares relatively modest overall sequence identity – below 30% – with its fungal and protozoan counterparts, but within the active site the amino acid residues are far more conserved. Based upon crystal structures of CYP51 of M. tuberculosis (CYP51Mt) [16–20], three of the thirteen active site residues, Y76, F83, and H259 (numbering according to CYP51Mt), are invariant throughout the cyp51 gene family. Two residues, F78 and P255, are specific to the methylation status of the C-4 atom in the sterol nucleus [18,21], and amino acid identities of seven other
Author Summary

Enzyme sterol 14α-demethylase (CYP51) is a well-established target for anti-fungal therapy and is a prospective target for Chagas’ disease therapy. We previously identified a chemical scaffold capable of delivering a variety of chemical structures into the CYP51 active site. In this work the binding modes of several second generation compounds carrying this scaffold were determined in high-resolution co-crystal structures with CYP51 of Mycobacterium tuberculosis. Subsequent assays against CYP51 in Trypanosoma cruzi, the agent of Chagas’ disease, demonstrated that two of the compounds bound tightly to the enzyme. Both were tested for inhibitory effects against T. cruzi and the related protozoan parasite Trypanosoma brucei. One of the compounds had potent, selective anti-T. cruzi activity in infected mouse macrophages. This compound is currently being evaluated in animal models of Chagas’ disease. Discrimination between T. cruzi and T. brucei CYP51 by the inhibitor was largely based on the variability of a single amino acid residue at a critical position in the active site. Our work is aimed at rational design of potent and highly selective CYP51 inhibitors with potential to become therapeutic drugs. Drug selectivity to prevent host-pathogen cross-reactivity is pharmacologically important, because CYP51 is present in human host.

Preparation of CYP51Tc

Design of the CYP51Tc expression vector was based on an entity in the NCBI data bank [ID:AY283022 [22]], which was modified by replacing the first 31 residues upstream of Pro32 with the fragment MARKTSSKGL from the CYP2C3 sequence [23] (CYP2C3 residues marked in bold) to improve protein solubility, and by inserting a His10-tag at the C-terminus to facilitate purification. This coding sequence (kindly provided by M. Waterman in the form of the pET vector) was subsequently sub-cloned into pCWori vector [24] between the NdeI and HindIII restriction sites and in this form used to transform Escherichia coli strain HMS174(DE3).

Transformants were grown for 5 h at 37°C and 250 rpm agitation in Terrific Broth medium supplemented with 1 mM thiamine, 50 μg/ml ampicillin, and trace elements. CYP51Tc expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 0.2 mM) and 8-aminolevulinic acid, a precursor of heme biosynthesis (final concentration 1 mM). Following induction, temperature was decreased to 25°C and agitation to 180 rpm. After 30 hours the cells were harvested and lysed by sonication. Insoluble material was removed from crude extract by centrifugation (30 min at 35,000 rpm). The supernatant was subjected to a series of chromatographic steps, including nickel-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN), followed by Q-Sepharose (Amersham Biosciences) in the flow-through regime, and then by S-Sepharose (Amersham Biosciences). From the S-Sepharose, protein was eluted in a 0.2 to 1.0 M NaCl gradient and observed by means of a 12% SDS-PAGE to be virtually homogeneous. Fractions containing P450 were combined, concentrated using a Centriprep concentrating device (Millipore), and stored at −80°C. Twenty mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, and 1 M DTT were maintained throughout all chromatographic steps. Spectral characteristics of CYP51Tc are shown in Figure 1A.

Preparation of CYP51Tb

The expression vector for CYP51Tb (ID: EAN79583) was generated using T. brucei genomic DNA and upstream GCCGCGCATATGGCTCTTGAAGTTGGCC and downstream CGCAAGCTTCTAAGTGATGTTAGTGATGTTAGTGA-GCAAGCTTGCCGCCCTTTCC primers. The underlining denotes an NdeI restriction cloning site in the upstream primer and HindIII restriction cloning site in the downstream primer followed by the stop codon. The bold sequence in the upstream primer highlights second codon replaced with alanine to optimize expression in E. coli cells [24]. The boldface in the downstream primer indicates the His10 tag. The original genomic DNA contained internal NdeI site at 345 base pair which was removed by introducing a silent mutation via the quick-change mutagenesis protocol (Stratagene). DNA amplification reaction was carried out as follows: 5 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C, for 30 cycles, followed by extension for 10 min at 72°C. The purified 1.5 kb PCR product was ligated into the pCR 2.1 TA cloning vector (Invitrogen). Insert was subsequently cleaved with NdeI and HindIII and ligated into pCWori vector digested with the same restriction enzymes and treated with alkaline phosphatase. The identity of the resulting vector was confirmed by DNA sequencing.

E. coli HMS174(DE3) strain was co-transformed with this vector and the pGRO7 plasmid (Takara) encoding the E. coli chaperones GroES and GroEL. Double transformants were selected on agar plates containing both ampicillin and chloramphenicol. One liter of Terrific Broth medium supplemented with 1 mM thiamine, 100 μg/ml ampicillin, 40 μg/ml chloramphenicol, and trace
Following induction, temperature was decreased to 15°C (0.3 mM), and the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 0.3 mM) was used to induce CYP51Tc, with the qualification that S-Sepharose was used in the purification. Growth continued at 27°C and 180 rpm until OD reached 0.6. Then CYP51 Tb expression was induced by addition of arabinose. Growth continued at 37°C and 250 rpm agitation until OD 600 reached 0.3. At that point expression of chaperones was induced with 0.2% arabinose. Growth continued at 27°C and 180 rpm until OD reached 0.6. Then CYP51Tb expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, final concentration 0.3 mM), and δ-aminolevulinic acid (1 mM). Following induction, temperature was decreased to 15°C. After 48 hours the cells were harvested and lysed by sonication. Purification was conducted similarly to as described above for CYP51Tc, with the qualification that S-Sepharose was used in the flow-through regime, while the protein was bound to and eluted with 0.2% arabinose. Growth continued at 27°C and 180 rpm.

Analysis of spectral characteristics of CYP51Tc (A) and CYP51Tb (B). Main panel shows the absolute protein spectrum, while insert shows CO-bound reduced difference spectrum. doi:10.1371/journal.pntd.0000372.g001

Crystal Structure

Crystallization, Data Collection, and Determination of Crystal Structure

Five compounds (Fig. 2), purchased from ChemDiv (San Diego, California) were used for co-crystallization with the CYP51Mt C37L/C442A double mutant. Compared to the wild type, this construct has superior propensity for crystallization. Compound numbering is according to the order in which they were received in our laboratory, with number 7 being the first used in the current work. Ligands were dissolved in Me2SO at ≤100 mM stock concentration, and brought to final concentrations ranging from 1 to 5 mM in the crystallization mix, depending on ligand solubility. Protein concentration was 0.2 mM. A narrow crystallization screening grid (15–30% PEG 4000, 2–12% isopropanol, 0.1 M HEPES, pH 7.5), previously devised to obtain CYP51M crystals [16,18,19] was utilized for co-crystallization of complexes by the vapor diffusion hanging drop method. Four co-crystal forms were obtained, all diffracted to resolutions between 1.56 to 1.60 Å. Diffraction data were collected at 100–110 K at the Southeast Regional Collaborative Access Team (SER-CAT) 22ID beamline, Advanced Photon Source, Argonne National Laboratory using SER-CAT mail-in data collection program (Table 1). The images were integrated and the intensities merged with the HKL2000 software suite [25]. The structures were determined by molecular replacement using coordinates of estriol-bound CYP51Mt (Protein Data Bank ID 1X8V) as a search model. The final atomic models were obtained after a few iterations of refinement using REFMAC5 [26] and model-building using the COOT graphics modeling program [27]. The quality of the structures was assessed by the program PROCHECK [28]. One residue, A46, was found in the generously allowed region of the Ramachandran plot in all structures where, together with the adjacent G47, it enables a sharp turn between two β strands.

Spectroscopic Binding Assays

Spectroscopic binding assays were performed at room temperature in 1-mL quartz cuvette containing 1 μM or 2 μM CYP51 in 50 mM Tris-HCl, pH 7.5, and 10% glycerol using a Cary UV-visible scanning spectrophotometer (Varian). Concentration of CYP51 was determined at 450 nm from the difference spectra between the carbon monoxide-bound ferrous and water-bound ferric forms, with an extinction coefficient of 91,000 M⁻¹ cm⁻¹ [29]. In the first round, compounds dissolved in Me2SO at 10 mM concentration were added to the 2 μM protein solution in 0.5 μl aliquots, resulting in concentration increases from 5 μM to 50 μM in 5 μM increments. The same amounts of Me2SO alone were added to the protein in the reference cuvette, followed by recording the difference spectra. In the second round, compounds with high affinities were diluted to 100 μM by Me2SO and titrated into 1 μM protein solution in 1 μl aliquots to increase compound concentration from 0.1 μM to 2 μM in 0.1 μM increments. To determine the Kd, we used the GraphPad PRISM software (Graphpad Software Inc.) to fit titration data to either rectangular or quadratic hyperbolas to correct for the bound ligand fraction, according to the functions ΔA = (Amax[S]/Kd+S) or ΔA = (Amax/2[E])([Kd+[E]+[L])−([Kd+[E]+[L])−4[E][L])/3], respectively, where E is total enzyme and L total ligand concentration, A0max the maximal absorption shift at saturation, and KD the apparent dissociation constant for the enzyme-ligand complex.

T. cruzi Assay

Irradiated (1000 rads) J774 mouse macrophages were plated in 12-well tissue culture plates 24 h prior to infection with 10⁵ T. cruzi Y strain trypomastigotes for 2 h at 37°C. Cultures were maintained in RPMI-1640 medium with 5% heat-inactivated fetal calf serum and 5% CO2 with the addition of 10 μM compound 8 or 10. Untreated controls, controls treated with the inhibitor K11777 (10 μM) [30,31], and uninfected macrophage controls were also included. All cultures were in triplicate and medium was replaced every 48 h. Treatment with CYP51 inhibitors continued for up to 27 days. Subsequently, treated cultures were maintained without inhibitor for an additional 13–
15 days to confirm inhibitor effectiveness and cure of infected cells. Cultures were monitored daily by contrast phase microscopy to determine presence of *T. cruzi* infected cells and free infectious trypomastigotes (Table 2).

**IC50**

To determine IC50, mouse J774 macrophages were irradiated (1000 rads) to deter growth and plated onto 12-well tissue culture plates. Cells were infected with 10^7 tissue culture trypomastigotes of the Y strain of *T. cruzi* for 2 h at 37°C, as described above. Next, medium was replaced with the addition of compound 10 at 0, 1 nM, 10 nM, 100 nM, 500 nM, 1 μM, 5 μM, and 10 μM; these cultures were incubated for 52 h at 37°C. Controls with 10 μM K11777 and 10 μM compound 8 were also included. All treatments were performed in triplicate to ensure statistical validity. Cultures were then fixed in 4% paraformaldehyde in PBS for 2 h at room temperature and stained with DAPI (10 nM) in PBS. One hundred cells and their intracellular parasites were quantified as previously described to estimate the mean number of parasites/cell [32]. Mean P/cell data were plotted against compound concentration to estimate the IC50.

**Mammalian Cell Toxicity Assay**

Toxicity was evaluated in bovine muscle cells (BESM), mouse J774 macrophages, and human Huh7 hepatocytes against compound 10 at 10 μM, 50 μM and 100 μM concentrations. After 48 h in culture at 37°C, cells were stained with 10% Tripan Blue and the number of live versus dead cells was quantified (Table 3).

**T. brucei Assay**

Trypanosomes were grown in complete HMI-9 medium containing 10% FBS, 10% Serum Plus medium (Sigma Inc. St. Louis Mo. USA) and 1x penicillin/streptomycin. Trypanosomes were diluted to 1.0×10^7/ml in complete HMI-9 medium. Diluted trypanosomes were aliquoted in Greiner sterile 96-well flat white opaque culture plates using a WellMate cell dispenser (Matrix Tech., Hudson, NH, USA). Compounds 8 and 10 were serially diluted in Me 2SO. Trypanosomes were incubated with the compounds for 48 h at 37°C with 5% CO_2 before monitoring viability. Trypanosomes were then lysed in the wells by adding 50 μl of CellTiter-GloTM (Promega Inc., Madison, WI, USA). Lysed trypanosomes were placed on an orbital shaker at room temperature for 2 min. The resulting ATP-bioluminescence of the trypanosomes in the 96-well plates was measured at room temperature using an Analyst HT plate reader (Molecular Devices, Sunnyvale, CA, USA).

**Results**

**Crystal Structures of CYP51<sub>Mt</sub>-Inhibitor Complexes**

Co-crystals were obtained for compounds 8, 9 and 11. Compound 10 failed to generate any crystals with CYP51<sub>Mt</sub>. Compound 7 was not found in the CYP51<sub>Mt</sub> active site in the
crystal, which is consistent with lack of spectrally detectable binding (Fig. 2). Compounds 8 [3-[(4-methylphenyl)sulfonyl]amino]propylpyridin-4-ylcarbamate], 9 (cis-4-methyl-N-[[(1S)-3-(methylsulfonyl)-1-]pyridin-4-ylcarbamoyl]propyl)cyclohexanecarboxamide], and 11 [N-[(1S)-2-methyl-1-pyridin-4-ylcarbamoyl]-propyl)cyclohexanecarboxamide], were observed bound in the CYP51Mt active site as predicted, through the coordination of the heme iron via a lone pair of aromatic nitrogen electrons of the N-4-pyridyl-formamide moiety (highlighted in gray in Fig. 2) and interactions with the invariant residues Y76 and H259 (Fig. 3). Functional groups other than the N-4-pyridyl-formamide moiety in compounds 9 and 11 either were accommodated in the species-specific cavity or else protruded through the opening of the active site toward bulk solvent. H259 hydrogen-bonded to the carbonyl oxygen in both compounds, while interactions with Y76 were mediated by two similarly positioned water molecules (Figs. 3A and 3B). The residual F,F,S electron density map suggested two alternative conformations for compounds 11 and 9, designated by pink and cyan respectively in Figures 4A and 4B. In the CYP51Mt-compound 11 complex, the cyclohexane ring protruded toward the bulk solvent (Fig. 3A), barely interacting with the protein in two alternative conformations (Fig. 4A). Together with the limited interactions of the isopropyl moiety, this lack of contact explains the low binding affinity of 11. In the CYP51Mt-compound 9 complex, the methylcyclohexane moiety protruded toward bulk solvent, while the methylsulfonyl group loosely bound in the species-specific cavity (Fig. 3B) in two alternative conformations (Fig. 4B). The side chain of M433 also adopted two alternative conformations. In both complexes, a portion of the BC-region was disordered and missing from the electron density map. Although racemic mixtures were used for co-crystallization, only one enantiomer of each compound was found in the active site.

A different binding mode was revealed for compound 8. Its flexible backbone allowed it to fold head-to-tail over the heme plane to bring the methylphenylsulfonylamide group into intramolecular stacking interactions with the pyridinyl moiety and also with the heme macrocycle (Fig. 3C, 4C). Folding minimized the nonpolar surface of compound 8 by exposing the sulfonylamide group to interactions with Q72, K97, and the heme propionate side chain. The hydrophobic side chain of K97 aligned along the methylphenyl moiety. A similar folding of the benzothiadiazolylamido group has been observed in previous work for 2-[[2,1,3-benzothiadiazol-4-sulfonylamido]-2-phenyl-N-pyridin-4-acetamide (BSPPA) [19]. Mutually stabilizing protein-ligand interactions involving the BC-loop residues including F78 result in increased binding affinity of the CYP51Mt-compound 8 complex and in unambiguous electron density both for compound 8 (Fig. 3C) and for the entire BC-region. In the CYP51Mt-compound 8 complex, H259 directly H-bonded to the amide nitrogen of compound 8, whereas Y76 interacted hydrophobically with the compound’s flexible backbone (Fig. 3C).

### Table 1. Crystallographic data and statistics.

| Compound # | Compound 8 | Compound 9 | Compound 11 |
|------------|------------|------------|-------------|
| ChemDiv #  | 4596-0233  | C155-0065  | C155-0337   |
| PDB ID     | 2W0B       | 2W09       | 2W0A        |
| **Data collection** |          |            |             |
| Wavelength, Å | 1.0000     | 1.0000     | 1.0000      |
| Resolution, Å | 1.56       | 1.57       | 1.60        |
| Unique reflections | 60321      | 59811      | 53704       |
| Redundancy | 5.5 (4.7)  | 5.4 (3.4)  | 5.1 (3.9)   |
| Completeness, % | 99.5 (99.1) | 95.8 (79.4) | 93.8 (77.7) |
| Space group | P212121    | P212121    | P212121     |
| Cell dimensions (a, b, c), Å | 46.7, 84.8, 110.3 | 46.4, 85.1, 110.9 | 44.7, 85.7, 110.9 |
| Molecules in asymmetric unit | 1          | 1          | 1           |
| Solvent content, % | 40         | 40         | 40          |
| R cryst, % | 16.4/20.1  | 18.8/22.2  | 18.9/23.2   |
| I/σ | 32.1 (4.3)  | 45.5 (4.4)  | 32.5 (3.4)  |
| **Refinement** | 62962      | 59743      | 53638       |
| Reflections used in refinement |            |            |             |
| R cryst (R free), % | 16.4/20.1  | 18.8/22.2  | 18.9/23.2   |
| No. of atoms | 3989       | 3753       | 3726        |
| Protein | 3542       | 3409       | 3376        |
| Heme | 43         | 43         | 43          |
| Substrate | 24         | 24         | 22          |
| Water | 380        | 277        | 285         |
| Wilon plot B-values, Å² | 15.2       | 22.6       | 18.7        |
| Mean B-factor, Å² | 14.6       | 25.5       | 20.3        |
| Protein | 14.0       | 24.9       | 19.7        |
| Substrate | 9.6        | 22.6       | 21.0        |
| Water | 22.8       | 32.6       | 27.8        |
| r.m.s. deviations |            |            |             |
| Bond length, Å | 0.010      | 0.013      | 0.013       |
| Bond angles, ° | 1.3        | 1.4        | 1.4         |
| Ramachandran (%) | 91.97/9.0/0.3 | 91.77/8.0/0.3 | 91.58/2.0/0.3 |

*Numbers in parentheses correspond to the highest resolution shell.

### Table 2. T. cruzi infection in vitro.

| Treatment | Host cell survival | T. cruzi development |
|-----------|--------------------|----------------------|
| Untreated control | 5 days | 5 days |
| Compound 8 (10 μM) | 5 days | 5 days |
| Compound 10 (10 μM) | 40 days | No |
| K11777 control | 40 days | No |

*Table 1. Crystallographic data and statistics. Table 2. T. cruzi infection in vitro.*
for inhibitory effects against both T. cruzi and T. brucei. In a mouse macrophage assay, T. cruzi completed its intracellular development in 5 days in untreated controls, resulting in death of host macrophages and abundant trypomastigotes in culture supernatant (Table 2). As anticipated, the control compound K11777 [30] cured T. cruzi infection. No parasites survived a treatment regime of 27 days with compound 10. Cure of host cells was confirmed by incubation of the cultures for an additional 15 days in the absence of inhibitor. In contrast, and similarly to untreated controls, T. cruzi completed its development in 5 days in cultures treated with compound 8.

An IC50 of ~1 nM concentration for compound 10 (Fig. 6) was estimated for T. cruzi intracellular amastigotes. T. cruzi developed well intracellularly in untreated macrophages with a final mean number of 3.57±0.5 P/cell (0% inhibition). As determined previously, 10 μM compound 10 was deleterious for T. cruzi, with a mean of 0.25±0.01 P/cell (100% growth inhibition). Ten μM of control compound K11777 was also parasiticidal for T. cruzi with a mean of 0.25±0.01 P/cell (IC100) [30], while compound 8 was not parasiticidal at this concentration with a mean of 1.22±0.1 P/cell (data not shown).

Toxicity for mammalian cells was addressed by treating the three different cell types with increasing concentrations of compound 10 (Table 3). No toxicity was observed at 10 μM compound 10, while 50 μM was mildly toxic for muscle cells. One hundred μM compound 10 was toxic for all mammalian cells tested, especially muscle cells.

Consistent with the spectral binding assays, neither compound 8 nor 10 had any inhibitory effects against cultured T. brucei even at the highest tested concentration of 10 μM.
Protein Data Bank Accession Numbers

The atomic coordinates and structure factors determined in this study (Protein Data Bank IDs 2W09, 2W0A, and 2W0B) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

Discussion

We explored sterol 14α-demethylase (CYP51) as a potential target for trypanosomiasis chemotherapy by probing CYP51Mt, CYP51Tc, and CYP51Tc with second generation compounds that contain a universal building block, the N-[4-pyridyl]-formamide moiety, which is capable of delivering small molecule compounds to the CYP51 active site. The affinities of the N-[4-pyridyl]-formamide-derivative compounds that we tested against CYP51Mt were lower than that of EPBA (Fig. 2), from which the formamide building block was derived. Affinities of all compounds examined were much higher toward CYP51Tc than to CYP51Mt. Strikingly large increases in binding affinities – 300 and 500 fold – were observed for compounds 8 and 10. Although compound 10 did not produce crystals with CYP51Mt, based on the binding modes

Figure 4. Stereo view of compounds in the active site. Compounds 11 (A), 9 (B), and 8 (C) are shown surrounded by the CYP51 active site residues. The fragments of the electron density 2Fo-Fc map (gray mesh) are cut at 1.2 σ. Different conformers in (A) and (B) are highlighted in pink and cyan. Images were generated using PYMOL [36].

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Figure 5. Spectroscopic binding of compounds. (A) Type II spectral responses of CYP51_Tc to increasing concentrations of compound 10. The concentration dependence of compound 10, fluconazole (B), and compound 8 (C) binding were deduced from the difference absorption changes obtained from the titration of CYP51_Tc with increasing concentrations of the inhibitor. The concentration dependence of fluconazole (D) was deduced from the difference absorption changes obtained from the titration of CYP51_Tb.

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Figure 6. Inhibition of T. cruzi intracellular amastigotes by compound 10. T. cruzi intracellular multiplication was evaluated at 52 hr of incubation at several concentrations of the inhibitor by determining the number of parasites/cell. Intracellular parasites were counted per one hundred cells to estimate a mean number of parasites per cell. Approximation of concentration dependence of mean P/cell±SD data with a smooth curve highlights the 50% drop in parasite count at ~1 nM compound 10. SD did not exceed 14% of the mean.

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of compounds 9 and 11, we reason that the methylcyclohexane carboxamide moiety of compound 10 protrudes toward the BC-loop, suggesting that the indole ring binds in the species-specific cavity, including the space occupied in CYP51Mt by the F70 aromatic ring, which is absent from CYP51Tc, but present in CYP51Tb and CYP51Mb. Consistent with this hypothesis, compound 10 selectively bound CYP51Tb, inhibited T. cruzi growth with the IC50 value close to the Kd estimated in the spectral binding assays, and cured mouse macrophages infected with T. cruzi Y strain at 10 μM concentration without harming them.

In contrast, compound 10 failed to bind CYP51Tc, despite the identity of 12 of the 13 active site substrate binding residues, and 83% overall sequence identity between T. cruzi and T. brucei CYP51 orthologues. This result is a striking indication of the sensitivity of CYP51 to alterations of the topography of its active site at position 78. The difference in position 78 is of functional importance, because phenylalanine at this site is strictly specific to T. cruzi and other trypanosomatids, while T. brucei CYP51 lacks this specificity. This result is a striking indication of the sensitivity of CYP51 to alterations of the topography of its active site at position 78. The difference in position 78 is of functional importance, because phenylalanine at this site is strictly specific to T. cruzi and other trypanosomatids, while T. brucei CYP51 lacks this specificity. Interestingly, T. cruzi is the only protozoan where the corresponding position (position 105 according to T. cruzi numbering) is occupied by isoleucine. Consistent with this observation, CYP51Mt is catalytically more closely related to its fungal and animal orthologues, preferentially converting 4α,β-dimethylated sterol substrates [21], whereas T. brucei CYP51 is strictly specific to 4α-methylated obtusifoliol and norlanosterol [33]. The protofimbria Methylbacterium capsulatum TMB3, known to synthesize sterols from squalene [34], is the only other known organism having isoleucine in the CYP51 position corresponding to F78. Not surprising, compound 10 was inactive against T. brucei in inhibitory assays in vitro.

In humans and animals metabolizing 4α,β-dimethylated 24,25-dihydrolanosterol, position 78 is always occupied by leucine. Therefore, the F78L substitution in the CYP51Mt binding site was examined and found to slightly increase binding affinities toward compounds 8 and 10, as opposed to the rest of the compounds whose binding affinities decreased (Fig. 2). Although a single amino acid substitution does not by any means convert bacterial protein into its mammalian counterpart, this finding is consistent with lack of toxicity in mammalian cells at inhibitory concentrations, and supports the possibility of rational design of highly selective anti-protozoan CYP51 inhibitors. The latter is of particular pharmacological importance as far as host-pathogen cross-reactivity is concerned, since CYP51 is present in human host.

The increased binding affinities toward CYP51Tb of all the compounds we tested may indicate more extensive involvement of the BC-loop and C helix in protein-inhibitor interactions in CYP51Tb than in CYP51Mc. Assuming that compound 8 binds CYP51Tb, in a similarly compact donut-like shape that fills the space adjacent to the porphyrin ring, its 300-fold increase in binding affinity could be achieved solely by stabilization of the BC-region of CYP51Tb without engaging the species-specific cavity. This possibility opens the door to a rational design effort in which the beneficial features of both compounds 8 and 10 would be combined to yield third generation compounds that would more potently and selectively inhibit CYP51Tb. Toward this end compound 10 is currently being evaluated in animal models of Chagas’ disease.

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Author Contributions

Conceived and designed the experiments: PSD JHM LMP. Performed the experiments: CKC PSD LVY ZBM KKH LA. Analyzed the data: PSD ZBM LMP. Contributed reagents/materials/analysis tools: PSD JHM LMP. Wrote the paper: LMP.

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