The Investigational Drug VT-1129 Is a Highly Potent Inhibitor of *Cryptococcus* Species CYP51 but Only Weakly Inhibits the Human Enzyme

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Cryptococcosis is a life-threatening disease often associated with HIV infection. Three *Cryptococcus* species CYP51 enzymes were purified and catalyzed the 14 α -demethylation of lanosterol, eburicol, and obtusifolol. The investigational agent VT-1129 bound tightly to all three CYP51 proteins (dissociation constant [K_d] range, 14 to 25 nM) with affinities similar to those of fluconazole, voriconazole, itraconazole, clotrimazole, and ketoconazole (K_d range, 4 to 52 nM), whereas VT-1129 bound weakly to human CYP51 (K_d 4.53 μ M). VT-1129 was as effective as conventional triazole antifungal drugs at inhibiting cryptococcal CYP51 activity (50% inhibitory concentration [IC_{50}] range, 0.14 to 0.20 μ M), while it only weakly inhibited human CYP51 activity (IC_{50} , ~600 μ M). Furthermore, VT-1129 weakly inhibited human CYP2C9, CYP2C19, and CYP3A4, suggesting a low drug-drug interaction potential. Finally, the cellular mode of action for VT-1129 was confirmed to be CYP51 inhibition, resulting in the depletion of ergosterol and ergosta-7-enol and the accumulation of eburicol, obtusifolone, and lanosterol/obtusifolol in the cell membranes.

Cryptococcosis is the most common systemic fungal infection in immunocompromised patients with HIV infection/AIDS and is caused by the opportunistic basidiomycete yeast pathogen *Cryptococcus neoformans* (1), which infects the lungs and brain. Meningoencephalitis is the most lethal manifestation of cryptococcosis, with life expectancy being less than a month if the infection is left untreated (2). Pathogenic *Cryptococcus* species cause disease in almost 1 million people and over 620,000 deaths annually, and a third of all deaths among patients with HIV infection/AIDS are attributable to *Cryptococcus* species infection (1). Current treatment options are limited to a few drugs, namely, initial induction therapy with a combination of amphotericin B and fluconazole followed by a maintenance regimen of fluconazole (2). Even after administration of the recommended treatment, 3-month mortality rates of 10 to 20% are common (3, 4). In addition, adoption of such treatment is costly and often impractical (amphotericin B requires intravenous administration), especially in developing countries, where mortality rates can approach 100% (5, 6).

Three main *C. neoformans* varieties are observed in clinical infections. *C. neoformans* var. *grubii* (primarily serotype A), which is ubiquitous in the environment, especially soil, is globally distributed and is responsible for almost all cryptococcal infections in patients with HIV infection/AIDS (6–8). *C. neoformans* var. *neoformans* (primarily serotype D) is less likely to cause severe infection and is more commonly found in Europe (4). *C. neoformans* var. *gattii* (primarily serotypes B and C), a tree-dwelling basidiomycete yeast that is primarily located in the tropics and subtropics but localized outbreaks of which are found in the northeastern United States, is now considered a separate species (*Cryptococcus gattii*) and is predominantly a primary pathogen that infects healthy (immunocompetent) individuals but that also infects immunocompromised patients if the opportunity arises (9). Most *Cryptococcus* infections of humans and nearly all infections of pa-

tients with HIV infection/AIDS are caused by *C. neoformans* var. *grubii*, with the most prevalent strain being H99, although *C. gattii* infection is increasing in prevalence, especially in North America and Africa (9). The taxonomy of *Cryptococcus* species is still evolving, with Hagen et al. (10) proposing that *C. neoformans* var. *neoformans* and *C. neoformans* var. *grubii* are separate species and that *C. gattii* consists of five distinct species based on phylogenetic analysis of 11 genetic loci.

Azole resistance, especially fluconazole resistance, among *Cryptococcus* species in the clinic can be problematic due to prolonged maintenance treatment regimens (11). Increased azole tolerance in *Cryptococcus* species has been attributed to point mutations in CYP51, including G484S and Y145F (12, 13), increased expression levels of CYP51 and the transporter protein AFR1 (14), and the genome plasticity of *Cryptococcus* species postinfection (15). Recently, an *in silico* three-dimensional model of *C. neoformans* CYP51 has been published (16) with the aim of aiding new drug design. Because many of the marketed azole drugs are limited by a low therapeutic index (17), a drug with a higher therapeutic index might be able to combat resistant pathogens at plasma concentrations still below toxic levels.

In this study, we compared the potency of the novel tetrazole

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FIG 1 Chemical structures of the azole antifungals used for IC₅₀ studies. The chemical structures of fluconazole (molecular weight, 306), voriconazole (molecular weight, 349), VT-1129 (molecular weight, 513), and itraconazole (molecular weight, 706) are shown.

antifungal VT-1129 (18, 19) (Fig. 1) with that of clinical azole antifungal drugs. We also compared their selectivity of binding to and inhibition of three recombinant cryptococcal CYP51 enzymes with their selectivity of binding to and inhibition of human CYP51 and human CYPs that are critical xenobiotic-metabolizing enzymes. In addition, the *in vivo* mode of action of VT-1129 was demonstrated through sterol profile analysis.

MATERIALS AND METHODS

Construction of pCWori⁺:CneoCYP51, pCWori⁺:CgruCYP51, and pCWori⁺:CgatCYP51 expression vectors. The *C. neoformans* var. *neoformans* CYP51 gene (*CneoCYP51*; UniProtKB accession number Q5KQ65), the *C. neoformans* var. *grubii* CYP51 gene (*CgruCYP51*; UniProtKB accession number Q09GQ2), and the *C. gattii* CYP51 gene (*CgatCYP51*; UniProtKB accession number E6QZS1) were synthesized by Eurofins MWG Operon (Ebersberg, Germany). An NdeI restriction site was incorporated at the 5' end of the genes, a HindIII restriction site was incorporated at the 3' end of the genes, and the entire construct was cloned into the pBSISK⁺ plasmid. In addition, the first 8 amino acids were changed to MALLAVF (20) and a four-histidine extension (CATCACCATCAC) was inserted immediately before the stop codon. The cryptococcal CYP51 genes were excised by NdeI/HindIII restriction digestion, followed by cloning into the pCWori⁺ expression vector. Gene integrities were confirmed by DNA sequencing.

Heterologous expression and purification of recombinant cryptococcal CYP51 proteins. The pCWori⁺:CYP51 constructs were transformed into competent *Escherichia coli* DH5α cells and expressed as previously described (21). Recombinant CYP51 proteins were isolated according to the method of Arase et al. (22), except that 2% (wt/vol)

sodium cholate was used in the sonication buffer and Tween 20 was omitted. The solubilized CYP51 proteins were purified by affinity chromatography using Ni²⁺-nitrilotriacetic acid (NTA) agarose as previously described (21, 23) prior to characterization. Human CYP51 with a deletion of 60 amino acids from the N terminus (Δ60 truncated human CYP51) was expressed and purified as previously described (24) and was shown to be comparable to the full-length human CYP51 in terms of binding to azole antifungal drugs. Protein purities were assessed by SDS-polyacrylamide gel electrophoresis.

Cytochrome P450 protein determinations. Reduced carbon monoxide difference spectroscopy was performed (25), with carbon monoxide being passed through the cytochrome P450 solution prior to addition of sodium dithionite to the sample cuvette (light path, 10 mm). An extinction coefficient of 91 mM⁻¹ cm⁻¹ (26) was used to calculate cytochrome P450 concentrations from the difference in the absorbance (ΔA) between the absorbance at 447 and that at 490 nm. Absolute spectra were determined between 700 and 300 nm (light path, 10 mm). All spectral determinations were made using a Hitachi U-3310 UV/visible spectrophotometer (San Jose, California).

Ligand binding studies. Stock solutions (2.5 mM) of lanosterol, eburicol, and obtusifolol were prepared in 40% (wt/vol) (2-hydroxypropyl)-β-cyclodextrin (HPCD) using an ultrasonic bath. Sterol was progressively titrated against 5 μM CYP51 protein in a quartz semimicrocuvette (light path, 4.5 mm) by addition of equivalent amounts of 40% (wt/vol) HPCD to the reference cuvette, which also contained 5 μM CYP51. The difference in the spectrum between the absorbance at 500 nm and that at 350 nm was determined after each incremental addition of sterol (up to 75 μM). The sterol saturation curves were constructed from the difference spectra (difference in the A₃₉₀ and A₄₂₅). The substrate dissociation constants (*K_d*) were determined by nonlinear regression (Levenberg-Marquardt algorithm) using the Michaelis-Menten equation.

Studies evaluating the binding of clotrimazole, fluconazole, voriconazole, itraconazole, ketoconazole, and VT-1129 to the cryptococcal CYP51 proteins were performed as previously described (21, 27) using split cuvettes with a 4.5-mm light path. Stock 0.1-mg·ml⁻¹ solutions of the azole antifungal drugs were prepared in dimethyl sulfoxide (DMSO) and progressively titrated against 2 μM CYP51 in 0.1 M Tris-HCl (pH 8.1) and 25% (wt/vol) glycerol. The difference spectra between 500 and 350 nm were determined after each incremental addition of azole, and binding saturation curves were constructed from the difference in the absorption at the peak and the absorption at the trough (ΔA_{peak-trough}) against the azole concentration. The properties of VT-1129 binding with 5 μM recombinant human CYP51 were also determined (24). The *K_d* of the enzyme-azole complex were determined by nonlinear regression (Levenberg-Marquardt algorithm) using a rearrangement of the Morrison equation for tight ligand binding (28, 29). Tight binding occurs when the *K_d* for a ligand is similar to or lower than the concentration of the enzyme present (30).

CYP51 reconstitution assays. Cryptococcal CYP51 reconstitution assay mixtures (31, 32) contained 0.5 μM CYP51, 1 μM *Aspergillus fumigatus* cytochrome P450 reductase (AfCPR1; UniProtKB accession number Q4WM67), 50 μM C-14 methylated sterol substrate (lanosterol, eburicol, obtusifolol), 50 μM dilaurylphosphatidylcholine, 4% (wt/vol) HPCD, 0.4 mg ml⁻¹ isocitrate dehydrogenase, 25 mM trisodium isocitrate, 50 mM NaCl, 5 mM MgCl₂, and 40 mM MOPS (morpholinepropanesulfonic acid; pH ~7.2). Assay mixtures were incubated at 37°C prior to initiation of the reaction with 4 mM β-NADPH Na₄, followed by shaking at 37°C for 15 min. Human CYP51 reconstitution assays were performed as described above, except that 0.5 μM soluble human CYP51 (24) and 2 μM human cytochrome P450 reductase (UniProtKB accession number P16435) were used and the reaction time was reduced to 5 min at 37°C. Sterol metabolites were recovered by extraction with ethyl acetate, followed by derivatization with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and tetramethylsilane (TMCS), prior to analysis by gas chromatography (GC)-mass spectrometry (MS) (33).

Determinations of the 50% inhibitory concentrations (IC_{50} s) were performed using 50 μ M lanosterol as the substrate to which various concentrations of fluconazole, itraconazole, voriconazole, and VT-1129 in 2.5 μ l DMSO were added prior to incubation at 37°C and addition of β -NADPH Na_4 .

Cryptococcus sterol analysis. *C. neoformans* var. *neoformans* (strain ATCC MYA-565), *C. neoformans* var. *grubii* (strain ATCC 208821), and *C. gattii* (strain ATCC MYA-4071) were grown in MOPS-buffered RPMI (0.165 M MOPS), pH 7.0, at 37°C and 200 rpm. MOPS-buffered RPMI, pH 7.0, without drug (1%, vol/vol, DMSO control) or with fluconazole or VT-1129 was inoculated at a final concentration of 2.5×10^4 cells ml^{-1} . *C. neoformans* var. *neoformans* was grown in the presence of 0.2 μ g ml^{-1} fluconazole or 0.0039 μ g ml^{-1} VT-1129, *C. neoformans* var. *grubii* was grown in the presence of 0.4 μ g ml^{-1} fluconazole or 0.0039 μ g ml^{-1} VT-1129, and *C. gattii* was grown in the presence of 0.4 μ g ml^{-1} fluconazole or 0.0078 μ g ml^{-1} VT-1129. The cultures were grown for 2 days at 37°C and 200 rpm, and nonsaponifiable lipids were extracted as previously reported (34).

Sterones were derivatized with methoxyamine-HCl by the addition of 200 μ l of methoxyamine-HCl (2%, wt/vol, in anhydrous pyridine) and incubation for 30 min at 70°C. Samples were mixed with 2 ml of saturated NaCl, and the lipids were extracted in three sequential 2-ml volumes of ethyl acetate. The combined ethyl acetate fractions were washed with 2-ml volumes of NaCl-saturated 0.1 M HCl, saturated NaCl, NaCl-saturated 5% (wt/vol) sodium bicarbonate solution, and saturated NaCl. The samples were then dried over anhydrous magnesium sulfate and evaporated using a vacuum centrifuge. Sterols in the dried extracts were derivatized with 0.1 ml BSTFA-TMCS (99:1) and 0.3 ml anhydrous pyridine (2 h at 80°C) prior to analysis by GC-MS (33). Individual sterols and sterones were identified by reference to the retention times, mass ions, and fragmentation patterns of sterol and sterone standards. The sterol composition was calculated using peak areas.

Inhibition of human liver CYP enzymes. *In vitro* studies determined the IC_{50} s of the test compounds for CYP2C9, CYP2C19, and CYP3A4 (with either midazolam or testosterone as the substrates) in intact human liver microsomes. A separate series of incubation mixtures was prepared with each test compound at final concentrations in the reaction mixture ranging from 0.0128 to 200 μ M. Each incubation mixture contained pooled human liver microsomes at an assay concentration of 1 mg ml^{-1} microsomal protein (Life Technologies, Grand Island, NY) and metabolic substrates of isozymes for CYP2C9, CYP2C19, and CYP3A4 (diclofenac, omeprazole, and midazolam or testosterone, respectively) at their experimentally determined K_m concentrations. Active control wells contained microsomes, a substrate(s), and the test compound diluent (i.e., DMSO-acetonitrile-phosphate buffer, 5:5:190) substituted for test compound solutions. The reaction was initiated by addition of an enzyme cofactor source (NADPH-regenerating solution; BD Biosciences, San Jose, CA), and the mixtures were incubated at 37°C. After 10 min, the incubation mixtures were quenched with acetonitrile, mixed, and centrifuged. The supernatant was analyzed by high-performance liquid chromatography-tandem MS for the hydroxy metabolite of the substrates. Each product peak area was normalized to be represented as a percentage of that of the average for the enzyme control. The IC_{50} of each test compound was determined by fitting a 4-parameter logistical fit to the dose-response data and graphically determining the inhibitor concentration at 50% of the maximal enzymatic response.

Data analysis. All ligand binding experiments were performed in triplicate, and curve fitting of the data was performed using the computer program ProFit (version 6.1.12; QuantumSoft, Zurich, Switzerland). GC-MS data were analyzed using Thermo Xcalibur (version 2.2) software.

Chemicals. VT-1129 was provided by Viamet Pharmaceuticals, Inc. (Durham, NC, USA). All other chemicals were obtained from Sigma Chemical Company (Poole, United Kingdom). Growth media, sodium ampicillin, IPTG (isopropyl- β -D-thiogalactopyranoside), and 5-aminolevulinic acid were obtained from Foremedium Ltd. (Hunstanton, United

Kingdom). The Ni^{2+} -NTA agarose affinity chromatography matrix was obtained from Qiagen (Crawley, United Kingdom).

RESULTS

Expression and purification of cryptococcal CYP51 proteins.

Following heterologous expression in *E. coli*, CneoCYP51, CgruCYP51, and CgatCYP51 were extracted by sonication with 2% (wt/vol) sodium cholate (22), which yielded 240 ± 90 , 160 ± 50 , and 290 ± 80 nmol per liter culture, as determined by carbon monoxide difference spectroscopy (25). Purification by chromatography on Ni^{2+} -NTA agarose resulted in 70%, 54%, and 45% recoveries of native CneoCYP51, CgruCYP51, and CgatCYP51 proteins, respectively. SDS-polyacrylamide gel electrophoresis confirmed that the purity of the cryptococcal CYP51 proteins eluted on Ni^{2+} -NTA agarose was greater than 90% when assessed by staining intensity, with the apparent molecular weights being 55,000 to 58,000; the predicted molecular weights when the N-terminal modifications and 4His C-terminal extensions are included were 62,708 for *C. neoformans* var. *neoformans*, 62,310 for *C. neoformans* var. *grubii*, and 62,689 for *C. gattii*.

Spectral properties of cryptococcal CYP51 proteins. The absolute spectra of the resting oxidized forms of all three CYP51 proteins (Fig. 2A) were typical for a low-spin ferric cytochrome P450 enzyme (23, 35) with α , β , Soret (γ), and δ spectral bands appearing at 566, 536, 418, and 360 nm, respectively. Reduced carbon monoxide difference spectra (Fig. 2B) gave the red-shifted heme Soret peak at 447 nm, characteristic of P450 enzymes, indicating that all three CYP51 proteins were expressed in the native form.

Sterol binding properties of cryptococcal CYP51 proteins.

Progressive titration with lanosterol, eburicol, and obtusifolol gave characteristic type I difference spectra for all three CYP51 proteins with a peak at 390 nm and a trough at 425 nm (Fig. 3). Type I binding spectra occur when the substrate or another molecule displaces the water molecule coordinated as the sixth ligand to the low-spin hexa-coordinated heme prosthetic group, causing the heme to adopt the high-spin penta-coordinated conformation (35). The cryptococcal CYP51 proteins had similar affinities for the three sterols (Table 1), with K_d values being 16 to 18 μ M for lanosterol, 12 to 16 μ M for eburicol, and 12 to 21 μ M for obtusifolol. This result suggests that all three 14 α -methylated sterols are potential substrates for the cryptococcal CYP51 proteins.

The sterol binding affinities of the three cryptococcal CYP51 proteins (K_d range, 12 to 21 μ M) were similar to those reported for other CYP51 proteins. For example, the K_d values for lanosterol and eburicol were 11 to 16 and 25 to 28 μ M, respectively, with *Candida albicans* CYP51 (21) and 11 and 13 μ M, respectively, with *Mycosphaerella graminicola* CYP51 (36); and the K_d values were 0.5 to 18 μ M for lanosterol with human CYP51 (24, 37, 38). However, the sterol K_d values obtained were 10- to 20-fold higher than those obtained for lanosterol with *Mycobacterium tuberculosis* CYP51 (1 μ M) (23) and for lanosterol and eburicol with *Trypanosoma cruzi* CYP51 (1.9 and 1.2 μ M, respectively) (32).

CYP51 reconstitution assays. CYP51 assays using 50 μ M sterol gave turnover numbers of 1.2 to 1.9 min^{-1} for lanosterol, 3.7 to 7.6 min^{-1} for eburicol, and 3.5 to 4.5 min^{-1} for obtusifolol (Table 1), confirming that all three cryptococcal CYP51 proteins readily catalyzed the 14 α -demethylation of these three sterols. Both CneoCYP51 and CgruCYP51 displayed a substrate preference for eburicol over obtusifolol and lanosterol, while Cgat-

FIG 2 Absolute and reduced carbon monoxide spectra of cryptococcal CYP51 proteins. Absolute spectra in the oxidized resting state (A) and reduced carbon monoxide difference spectra (B) were determined using 5 μ M solutions of purified CneoCYP51 (line 1), CgruCYP51 (line 2), and CgatCYP51 (line 3). Spectral determinations were made using quartz semimicrocuvettes with a path length of 10 mm.

CYP51 displayed a substrate preference for obtusifolol over eburicol and lanosterol. The ability of CgatCYP51, in particular, to readily demethylate obtusifolol indicates a preference for a C-24-methylated sterol substrate.

Azole binding properties of CYP51 proteins. All five medical azole antifungal agents and the agent being investigated, VT-1129, bound tightly to all three cryptococcal CYP51 proteins, producing

type II binding spectra. The binding spectra and saturation curves obtained for fluconazole and itraconazole (Fig. 4) and for VT-1129 (Fig. 5) are shown with a peak at ~ 429 nm and a trough at ~ 412 nm. Type II binding spectra are caused by the triazole ring N-4 (fluconazole, itraconazole, and voriconazole) or the imidazole ring N-3 (clotrimazole, ketoconazole) coordinating as the sixth ligand with the heme iron (39) to form the low-spin CYP51-azole complex, resulting in a red shift of the heme Soret peak. The interaction of VT-1129 with the heme ferric ion is through a terminal (N-3 or N-4) tetrazole nitrogen atom. CneoCYP51 bound the azole antifungal agents the strongest, with apparent K_d values of 4 to 11 nM (Table 1), followed by CgatCYP51 with apparent K_d values of 5 to 24 nM, and CgruCYP51 bound the azole antifungal agents the weakest, with apparent K_d values of 14 to 52 nM. None of the cryptococcal CYP51 enzymes appeared to be inherently resistant to azole antifungal agents, as the range of K_d values observed (4 to 52 nM) was similar to that observed with *C. albicans* CYP51 (10 to 56 nM) (24), whereas *Aspergillus fumigatus* CYP51A appeared to be inherently resistant to fluconazole with an apparent K_d value of 11.9 μ M (40). The affinity of VT-1129 binding to all three cryptococcal CYP51 proteins was strong (K_d range, 11 to 25 nM) and similar to that of the other five clinical azole antifungal agents examined, suggesting that VT-1129 would be effective as a therapeutic agent against *Cryptococcus* species infections. The

FIG 3 Sterol binding properties of cryptococcal CYP51 proteins. (A) Absorbance difference spectra were measured during the progressive titration of 5 μ M CYP51 proteins with lanosterol, eburicol, and obtusifolol. (B) Sterol saturation curves were constructed for lanosterol (filled circles), eburicol (hollow circles), and obtusifolol (crosses) with the CYP51 proteins from the difference between the A_{390} and A_{425} of the type I binding spectra observed and were fitted using the Michaelis-Menten equation.

TABLE 1 Ligand binding affinities, azole IC₅₀s, and turnover numbers for CYP51 proteins

CYP51	<i>K_d</i> (nM)								
	Sterols			Azoles					
	Lanosterol	Eburicol	Obtusifoliol	Clotrimazole	Fluconazole	Itraconazole	Ketoconazole	Voriconazole	VT-1129
CneoCYP51	16,300 ± 2,800	13,000 ± 1,200	16,800 ± 2,100	4 ± 3	9 ± 5	7 ± 3	6 ± 2	4 ± 2	11 ± 5
CgruCYP51	17,300 ± 900	11,700 ± 600	12,200 ± 3,000	44 ± 18	52 ± 15	42 ± 11	32 ± 15	14 ± 9	25 ± 4
CgatCYP51	17,500 ± 1,900	15,800 ± 1,300	20,600 ± 1,000	11 ± 4	24 ± 9	6 ± 2	5 ± 2	19 ± 1	24 ± 10
HsapCYP51 ^a	18,400 ± 1,500 ^b			55 ± 5 ^b	30,400 ± 4,100 ^b	92 ± 7 ^b	42 ± 16 ^a	2,290 ± 120 ^b	4,530 ± 300

^a HsapCYP51, *Homo sapiens* CYP51.
^b Values were taken from Warrilow et al. (24).
^c Thirteen percent inhibition was observed in the presence of 150 μM VT-1129.

similar azole binding properties of the three cryptococcal CYP51 proteins agree with their close sequence homology with CneoCYP51, which shares 98% and 96% amino acid sequence identity with CgruCYP51 and CgatCYP51, respectively.

In contrast, VT-1129 bound relatively weakly to human CYP51 (Fig. 5) with an apparent *K_d* of 4.53 μM (Table 1). The interaction of VT-1129 with human CYP51 was atypical, as it gave rise to a red-shifted type I difference spectrum (peak at 410 nm and trough at 426 nm) rather than the expected type II difference spectrum

normally observed for the interaction of azole antifungal agents with CYP51 proteins. This suggests that the mode of interaction of VT-1129 with the human CYP51 was different from that observed with the three cryptococcal CYP51 proteins. VT-1129 still perturbs the heme environment of human CYP51, as a difference spectrum was observed, though it was not through the azole nitrogen directly coordinating with the heme ferric ion. This altered interaction of VT-1129 with human CYP51 resulted in very weak inhibition of CYP51 activity in the CYP51 reconstitution assay (see below). The *K_d* values obtained for VT-1129 with the cryptococcal CYP51 enzymes were 180- to 410-fold lower than the *K_d*

FIG 4 Azole binding properties of cryptococcal CYP51 proteins. Fluconazole and itraconazole were progressively titrated against 2 μM CneoCYP51, CgruCYP51, and CgatCYP51. (A and B) The resultant type II difference spectra obtained with fluconazole (A) and itraconazole (B) are shown. (C and D) Fluconazole (C) and itraconazole (D) saturation curves were constructed from the Δ*A*_{peak-trough} of the type II binding spectra observed for CneoCYP51 (solid circles), CgruCYP51 (hollow circles), and CgatCYP51 (crosses). A rearrangement of the Morrison equation was used to fit the tight ligand binding observed. All experiments were performed in triplicate, although the results of only one replicate are shown.

FIG 5 VT-1129 binding properties of cryptococcal and human CYP51 proteins. VT-1129 was progressively titrated against 4 μM CneoCYP51, CgruCYP51, and CgatCYP51 and 5 μM human (*Homo sapiens*) CYP51 (Hsap). (A) The resultant type II difference spectra obtained with the three cryptococcal CYP51 proteins and the red-shifted type I difference spectrum with human CYP51 are shown. (B) Saturation curves were constructed from the Δ*A*_{peak-trough} of the type II binding spectra observed for CneoCYP51 (solid circles), CgruCYP51 (hollow circles), and CgatCYP51 (crosses) and the red-shifted type I binding spectrum observed for human CYP51 (solid triangles). A rearrangement of the Morrison equation was used to fit the tight ligand binding observed. All experiments were performed in triplicate, although the results of only one replicate are shown.

TABLE 1 (Continued)

IC ₅₀ (μM)					Turnover no. (min ⁻¹)		
Clotrimazole	Fluconazole	Itraconazole	Voriconazole	VT-1129	Lanosterol	Eburicol	Obtusifoliol
	0.17	0.17	0.17	0.16	1.4 ± 0.2	6.1 ± 0.5	3.5 ± 0.3
	0.2	0.19	0.2	0.18	1.9 ± 0.3	7.6 ± 0.4	3.6 ± 0.3
	0.14	0.16	0.16	0.15	1.2 ± 0.2	3.7 ± 0.4	4.5 ± 0.6
1.9	~1,300 ^b	70 ^b	112	~600 ^c	22.7 ± 4.8		

value obtained with the human homolog, confirming the high selectivity of VT-1129 for the fungal target enzyme. This compared favorably with the findings for fluconazole and voriconazole, which gave K_d values that were 370- to 1,300-fold and 120- to 570-fold lower, respectively, for cryptococcal CYP51 enzymes than for human CYP51. VT-1129 exhibited far greater selectivity than clotrimazole, ketoconazole, and itraconazole toward cryptococcal CYP51 enzymes than toward the human homolog, with clotrimazole, ketoconazole, and itraconazole exhibiting K_d values that were only 1.3- to 15-fold lower for the fungal CYP51 than for the human CYP51.

Azole IC₅₀ determinations. IC₅₀ determinations (Fig. 6) confirmed that all three cryptococcal CYP51 proteins tightly bound fluconazole, itraconazole, voriconazole, and VT-1129, giving rise to strong inhibition of the CYP51 demethylation of lanosterol. IC₅₀s of 0.14 to 0.20 μM (Table 1), which were close to half the CYP51 concentration present in the assay system, were obtained. VT-1129 proved as effective at inhibiting cryptococcal CYP51 activity as the three other azole antifungal drugs, suggesting that VT-1129 would be effective at combating *Cryptococcus* infections.

FIG 6 Azole IC₅₀ determinations for cryptococcal CYP51 proteins. The IC₅₀s of fluconazole (A), itraconazole (B), voriconazole (C), and VT-1129 (D) for 0.5 μM CneoCYP51 (filled circles), CgruCYP51 (hollow circles), and CgatCYP51 (crosses) were determined using the CYP51 reconstitution assay with 1 μM AfCPR1 as the redox partner.

In contrast, VT-1129 only weakly inhibited human CYP51 activity (IC₅₀, ~600 μM) (Fig. 7), in agreement with the weak perturbation of the heme environment of human CYP51 observed with VT-1129 (Fig. 5), whereas clotrimazole severely inhibited human CYP51 activity (IC₅₀, 1.9 μM). The IC₅₀s of VT-1129 observed for the cryptococcal CYP51 enzymes were 3,300- to 4,000-fold lower than those obtained for the human homolog (Table 1), again confirming the high selectivity of VT-1129 for the fungal target enzyme. This was comparable to the findings for fluconazole, where the IC₅₀s for the fungal CYP51 enzymes were 6,500- to 9,000-fold lower than those for human CYP51 and with the selectivity observed with fluconazole being significantly better than that observed with voriconazole and itraconazole (Table 1). The IC₅₀s of VT-1129 were more potent than the K_d values for binding to cryptococcal CYP51 enzymes, suggesting that the K_d values calculated by the Morrison equation were an overestimate, in part due to the relatively high CYP51 protein concentrations required for *in vitro* binding studies.

Cryptococcus sterol content. The treatment of *Cryptococcus* spp. with 0.2 to 0.4 μg ml⁻¹ fluconazole and 0.0039 to 0.0078 μg ml⁻¹ VT-1129 resulted in the accumulation of eburicol (Table 2), obtusifolione, and lanosterol/obtusifoliol. The accumulation of CYP51 substrates is indicative of direct CYP51 inhibition in treated cells. Both azole treatments resulted in the depletion of the post-CYP51 sterol metabolites ergosta-7,22-dienol and ergosta-7-enol and the partial depletion of ergosterol levels (Table 2), showing CYP51 inhibition. In these cellular experiments, VT-1129 was significantly more potent than fluconazole, as VT-1129 caused greater inhibition of cryptococcal CYP51 activity at a 50-fold

FIG 7 IC₅₀ determinations with human CYP51 for clotrimazole, voriconazole, and VT-1129. The CYP51 reconstitution assay contained 0.5 μM human CYP51 and 2 μM human cytochrome P450 reductase as the redox partner in the presence of clotrimazole (filled circles), voriconazole (hollow circles), and VT-1129 (crosses) at concentrations ranging from 0 to 150 μM.

TABLE 2 Sterol profiles of *Cryptococcus* spp.

Sterol	Sterol composition (%) with the indicated treatment ^a								
	<i>C. neoformans</i> var. <i>neoformans</i>			<i>C. neoformans</i> var. <i>grubii</i>			<i>C. gattii</i>		
	DMSO	FLUC	VT-1129	DMSO	FLUC	VT-1129	DMSO	FLUC	VT-1129
Ergosta-5,7,22,24(28)-tetraenol		1.2 ± 0.3	5.0 ± 0.5	2.5 ± 1.4	2.1 ± 1.6	2.3 ± 0.3		1.8 ± 0.2	4.8 ± 1.0
Ergosta-5,8,22-trienol		1.0 ± 0.0	3.7 ± 0.3						3.5 ± 0.2
Ergosterol	60.6 ± 2.5	42.2 ± 0.7	11.5 ± 3.9	43.9 ± 4.3	34.1 ± 3.6	18.7 ± 0.6	49.3 ± 9.7	52.1 ± 5.5	23.2 ± 2.9
Ergosta-7,22-dienol	7.4 ± 0.4			9.3 ± 1.1			10.8 ± 6.0		
Fecosterol [E8,24(28)]							1.0 ± 0.9		
Ergosta-8-enol		1.6 ± 0.4							3.8 ± 0.5
Ergosta-5,7-dienol							3.0 ± 0.6	1.8 ± 0.4	
Ergosta-7-enol	25.3 ± 1.0			28.8 ± 0.5			30.2 ± 6.6		1.1 ± 0.2
Eburicone									1.9 ± 0.3
Lanosterol/obtusifolol		3.7 ± 0.9	4.4 ± 0.2	1.7 ± 1.1	10.9 ± 2.1	4.9 ± 0.0		7.2 ± 1.8	6.6 ± 0.2
4-Methyl fecosterol				2.5 ± 0.4					
Obtusifolione		35.9 ± 1.8	17.1 ± 1.0		22.1 ± 1.5	24.5 ± 0.6		20.0 ± 6.0	39.4 ± 2.5
Eburicol	1.5 ± 0.8	12.8 ± 0.7	55.8 ± 5.7	6.8 ± 2.0	30.0 ± 2.1	49.1 ± 1.1	3.1 ± 2.5	13.6 ± 0.7	15.4 ± 1.6
4,4-Dimethyl-ergosta-8,24(28)-dienol				4.0 ± 0.8					

^a Mean values from three replicates ± standard deviations are shown. FLUC, fluconazole.

lower concentration than fluconazole (relative to the results obtained with the DMSO control, VT-1129 caused greater reductions in ergosterol levels than fluconazole at a 50-fold higher concentration, and in all cases, the accumulation of the 14-methylated product showed that CYP51 was inhibited in the cells; Table 2).

Inhibition of human liver drug-metabolizing CYPs. The inhibition of three critical xenobiotic-metabolizing CYPs by the four approved azole drugs and VT-1129 is shown in Table 3. The IC₅₀s of the marketed agents available in the literature (41–43) agree well with those measured in this study. The imidazole-containing agent clotrimazole was the most potent CYP inhibitor, inhibiting the activities of all CYPs at sub- or low-micromolar concentrations. The three triazole-containing agents had variable inhibitory potencies, with itraconazole potently inhibiting CYP3A4 with either substrate (IC₅₀s, 0.08 and 0.13 μM), voriconazole inhibiting the activities of all CYPs with a relatively tight range of potencies (IC₅₀s, 4 to 13 μM), and fluconazole showing a slightly broader range (IC₅₀s, 6 to 34 μM). In contrast, VT-1129 weakly inhibited the activities of each of these enzymes (IC₅₀s, 79 to 178 μM).

DISCUSSION

Sionov et al. (14) demonstrated that *C. neoformans* strains are heteroresistant to fluconazole, with each strain yielding a subpopulation that can survive in the presence of fluconazole concentrations well above the MIC values through disomy of chromosome

1, which duplicates the CYP51 and AFR1 transporter genes. The disomy of chromosome 1 coupled with reported G484S and Y145F CYP51 mutations (12, 13) increased CYP51 and AFR1 expression levels (14), and the genome plasticity postinfection (15) may explain the divergent range of MIC values of fluconazole of 0.5 to 64 μg ml⁻¹ reported for *Cryptococcus* spp. (44–47). The MIC values reported for voriconazole (0.008 to 0.5 μg ml⁻¹), itraconazole (0.015 to 0.5 μg ml⁻¹), and posaconazole (0.008 to 0.5 μg ml⁻¹) were lower and less variable than those reported for fluconazole (44–47), indicating the therapeutic efficacy of these triazole antifungals and their potential for use should fluconazole tolerance become problematic. However, as previously observed with *Candida* spp. and *Aspergillus* spp., it can be anticipated that tolerance against current triazole therapeutics will emerge in *Cryptococcus* spp.

New antifungal drug candidates for the treatment of systemic *Cryptococcus* infection which target CYP51 should ideally have high potency against the intended cryptococcal CYP51 target enzymes and minimal interaction with human CYP51 and other critical CYP enzymes, such as those that metabolize xenobiotics. VT-1129 meets both these criteria by binding tightly to cryptococcal CYP51 enzymes (*K_d* range, 11 to 25 nM) with a high affinity similar to that of other pharmaceutical azole antifungal agents (*K_d* range, 4 to 52 nM) while binding weakly to the CYP51 of the human host *in vitro* (*K_d*, 4.53 μM). Binding studies (Fig. 4 and 5) provide useful preliminary information on a cyclized nitrogen-containing antifungal drug candidate's likely effectiveness at inhibiting CYP51 activity. However, only IC₅₀ determinations using a CYP51 reconstitution assay system can determine the functional activity of each compound as a CYP51 inhibitor. IC₅₀ determinations confirmed that VT-1129 is a strong inhibitor of cryptococcal CYP51 activity, consistent with tight binding inhibition, but only weakly inhibits human CYP51 (13% inhibition at 150 μM VT-1129). The selectivity of VT-1129 for the cryptococcal CYP51 protein over the human homolog was ~3,300-fold in terms of inhibiting CYP51 catalysis, and VT-1129 was as effective as conventional triazole antifungal drugs at inhibiting cryptococcal CYP51 activity. VT-1129's selectivity for inhibiting cryptococcal CYP51 was similarly high compared to its selectivity

TABLE 3 Inhibition of human liver CYPs by fungal CYP51 inhibitors

Inhibitor	IC ₅₀ (μM) ^a			
	CYP2C9	CYP2C19	CYP3A4 ^b	CYP3A4 ^c
Clotrimazole	1.4 (0.1)	0.6 (0.2)	0.03 (0.01)	0.045 (0.001)
Fluconazole	34 (10)	13 (9)	32 (5)	6 (2)
Itraconazole	80 (28)	78 (31)	0.08 (0.02)	0.13 (0.06)
Voriconazole	10 (5)	10 (4)	13 (4)	3.8 (0.2)
VT-1129	87 (21)	110 (80)	79 (23)	178 (31)

^a Values are averages of 2 to 4 separate determinations, and standard deviations are provided in parentheses.

^b Testosterone as the substrate.

^c Midazolam as the substrate.

for inhibiting key human xenobiotic-metabolizing CYPs, suggesting a low potential for clinical drug-drug interactions.

Sterol profile analysis confirmed that VT-1129 inhibited cryptococcal CYP51 activity in whole cells, resulting in the depletion of ergosterol and ergosta-7-enol from the cell membranes and the accumulation of the 14-methylated compounds eburicol, lanosterol/obtusifolol, and obtusifolione. In a separate study measuring a large number of *Cryptococcus* species isolates and using 50% inhibition as the endpoint, the MIC₉₀ of VT-1129 was 0.060 µg ml⁻¹ for 180 isolates of *C. neoformans* and 0.25 µg ml⁻¹ for 321 isolates of *C. gattii* (19), confirming that VT-1129 is a potent inhibitor of *Cryptococcus* growth. In both studies, VT-1129 was a more potent inhibitor of *Cryptococcus* CYP51 than fluconazole. In addition, VT-1129 retained all or most of its antifungal potency against 50 Ugandan clinical isolates of *C. neoformans* with elevated fluconazole MIC values (48). This potency coupled with its excellent selectivity for fungal rather than human CYP enzymes shown here supports VT-1129 as a good candidate for the treatment of systemic *Cryptococcus* infections. Given the unmet need for more potent drugs for the treatment of cryptococcosis, especially in sub-Saharan Africa, further assessments in clinical trials are warranted, with VT-1129 phase 1 studies with healthy volunteers now being under way.

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