

The Clinical Candidate VT-1161 Is a Highly Potent Inhibitor of *Candida albicans* CYP51 but Fails To Bind the Human Enzyme

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The binding and cytochrome P45051 (CYP51) inhibition properties of a novel antifungal compound, VT-1161, against purified recombinant *Candida albicans* CYP51 (*ERG11*) and *Homo sapiens* CYP51 were compared with those of clotrimazole, fluconazole, itraconazole, and voriconazole. VT-1161 produced a type II binding spectrum with *Candida albicans* CYP51, characteristic of heme iron coordination. The binding affinity of VT-1161 for *Candida albicans* CYP51 was high (dissociation constant [K_d], ≤ 39 nM) and similar to that of the pharmaceutical azole antifungals (K_d , ≤ 50 nM). In stark contrast, VT-1161 at concentrations up to 86 μ M did not perturb the spectrum of recombinant human CYP51, whereas all the pharmaceutical azoles bound to human CYP51. In reconstitution assays, VT-1161 inhibited *Candida albicans* CYP51 activity in a tight-binding fashion with a potency similar to that of the pharmaceutical azoles but failed to inhibit the human enzyme at the highest concentration tested (50 μ M). In addition, VT-1161 (MIC = 0.002 μ g ml⁻¹) had a more pronounced fungal sterol disruption profile (increased levels of methylated sterols and decreased levels of ergosterol) than the known CYP51 inhibitor voriconazole (MIC = 0.004 μ g ml⁻¹). Furthermore, VT-1161 weakly inhibited human CYP2C9, CYP2C19, and CYP3A4, suggesting a low drug-drug interaction potential. In summary, VT-1161 potently inhibited *Candida albicans* CYP51 and culture growth but did not inhibit human CYP51, demonstrating a >2,000-fold selectivity. This degree of potency and selectivity strongly supports the potential utility of VT-1161 in the treatment of *Candida* infections.

Candida albicans is responsible for a wide range of human fungal infections ranging in severity from relatively minor but disruptive mucosal infections, such as vaginitis and oral candidiasis, to potentially life-threatening systemic candidemia. The azole class of drugs targeting fungal cytochrome P45051 (CYP51) is widely used as a first-line treatment for fungal infections or as preemptive treatment. Relative to amphotericin B, the approved azoles, such as fluconazole, itraconazole, and voriconazole, have fewer side effects. However, several side effects do indeed exist and are predominantly linked to inhibition of off-target human cytochromes P450 (CYPs). All marketed azoles have multiple drug-drug interactions (DDIs) due to inhibition of such human CYPs as CYP3A4, CYP2C9, and CYP2C19 (1). Because many patients with fungal infections are on therapy for an underlying disease, these DDIs are problematic and contribute to the need for monitoring plasma drug levels. The well-documented visual disturbances caused by voriconazole have been postulated to be due to inhibition of human CYP46 (2). Warnings against the use of voriconazole, itraconazole, and fluconazole during pregnancy result from inhibition of endocrine biosynthetic enzymes, such as CYP19 (3). The mechanism of hepatotoxicity, with itraconazole causing the most pronounced occurrence, resulting in a 10% discontinuation rate (4), is not as well understood but may be in part due to interaction with liver CYPs. Fungal CYP51 inhibitors with greater selectivity for fungal CYP51 than off-target human CYPs could overcome these limitations and would thus be a significant advancement in the field of fungal therapy.

Additionally, the widespread use of azole antifungals, especially for prolonged treatment periods, has led to the emergence of azole-resistant strains of *Candida albicans* and other *Candida* species, especially with immunocompromised patients (5–12). Thus, there is a growing need to develop new effective antifungal drugs to combat the increasing occurrence of *Candida* resistance, espe-

cially for the treatment of deep systemic infections. Because many of the marketed azole drugs are limited by a low therapeutic index (13), 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 novel antifungal VT-1161 (14) with four clinical azole antifungal drugs in terms of its potency and selectivity of binding to and inhibition of recombinant *C. albicans* CYP51 (CaCYP51) compared to human CYP51, wild-type azole-susceptible *C. albicans* in cellular growth assays, and three critical human xenobiotic-metabolizing CYPs (CYP2C9, CYP2C19, and CYP3A4). Using a combination of ligand binding spectroscopy techniques, CYP enzymatic assays, and analysis of cellular sterol pathway components, VT-1161 was demonstrated to be of therapeutic interest because of its high *C. albicans* CYP51 binding affinity, potent *C. albicans* growth inhibition, and minimal interactions with human CYP51 and key drug-metabolizing CYPs.

MATERIALS AND METHODS

Cloning, expression, solubilization, purification, and characterization of CaCYP51 and $\Delta 60$ HsCYP51 enzymes. All methods were performed as previously described (15). In *Homo sapiens* CYP51 with a 60-codon gene

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truncation ($\Delta 60\text{HsCYP51}$), the N-terminal transmembrane domain upstream of Pro-61 was replaced with the N-terminal MAKKTSSKGKL sequence from CYP2C3 (16, 17). The first eight amino acids of the full-length CaCYP51 construct were altered to MALLAVF (ATGGCTCTGT TATTAGCAGTTTTT) to facilitate expression in *Escherichia coli* (18). Both gene constructs included a 12-base insertion (CATCACCATCAC) encoding a four-histidine tag immediately in front of the stop codon to facilitate protein purification by Ni^{2+} -nitrilotriacetic acid (NTA) agarose affinity chromatography. Previously, we have shown that the properties of binding of the pharmaceutical azole antifungal drugs to the $\Delta 60\text{HsCYP51}$ protein were comparable to those of binding to full-length HsCYP51 (15). Ni^{2+} -NTA agarose-purified CaCYP51 and $\Delta 60\text{HsCYP51}$ enzymes were used for all subsequent spectrophotometric binding studies and CYP51 reconstitution assay 50% inhibitory concentration (IC_{50}) determinations.

Antifungal agent binding properties. Binding assays with 5 μM CaCYP51 and $\Delta 60\text{HsCYP51}$ proteins were performed as previously described (19) using quartz cuvettes with a 4.5-mm light path and stock 1-, 0.5-, 0.2-, and 0.1-mg ml^{-1} antifungal agent solutions prepared in dimethyl sulfoxide (DMSO). Antifungal agents were titrated against 5 μM CYP51 proteins in 0.1 M Tris-HCl (pH 8.1) and 25% glycerol at 22°C, with equivalent volumes of DMSO also being added to the CYP51-containing compartment of the reference cuvette. The absorbance difference spectra between 500 and 350 nm were determined after each incremental addition of compound, with binding saturation curves being constructed from the change in the absorbance at 430 nm and that at 412 nm ($\Delta A_{430-412}$) against the antifungal agent concentration. The dissociation constant (K_d) of the enzyme-antifungal agent complex for tight ligand binding was determined by nonlinear regression (Levenberg-Marquardt algorithm) using a rearrangement of the Morrison equation (20, 21). Each binding determination was performed in triplicate. Binding studies using clotrimazole, fluconazole, itraconazole, and voriconazole with 5 μM CaCYP51 and $\Delta 60\text{HsCYP51}$ were performed previously (15).

In addition, absolute spectra between 700 and 300 nm for 5 μM CaCYP51 and $\Delta 60\text{HsCYP51}$ in the absence and presence of 10 or 40 μM antifungal agent were measured using 10-mm-light-path semimicro-quartz cuvettes, and the difference spectra were calculated by subtracting the resting-state absolute CYP51 spectra from the azole-treated absolute CYP51 spectra. The chemical structures of the antifungal agents used in this study are shown in Fig. 1.

CYP51 reconstitution assays and IC_{50} determinations. Lanosterol (15 μg) and dilaurylphosphatidylcholine (DLPC; 100 μg) were dissolved in 0.25 ml of chloroform prior to the addition of 0.2 ml water. The mixture was vortexed to form a white emulsion while the chloroform was simultaneously evaporated off under nitrogen to produce aqueous sterol-DLPC micelles. The CYP51 reconstitution system consisted of 0.2 ml of sterol-DLPC micelles diluted to 0.8 ml with 2.5 nmol CYP51 protein, 10 nmol *Saccharomyces cerevisiae* cytochrome P450 reductase truncated by 33 amino acids (22), 3 U *S. cerevisiae* glucose-6-phosphate dehydrogenase, 2 μmol disodium glucose-6-phosphate, and 100 μmol potassium phosphate (pH 6.6). The mixture was preincubated at 37°C for 5 min prior to reaction initiation by the addition of 0.1 ml 20 mM β -NADPH- Na_4 in 0.2% NaHCO_3 , followed by further incubation at 37°C for 60 min with shaking. The final pH of the CYP51 reconstitution assay mixture was 7.0 to 7.2. Addition of 2 ml of 15% KOH in ethanol terminated the reaction, and subsequent incubation at 85°C for 90 min saponified the lipid fraction. Hexane extraction recovered the sterol metabolites, which were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) prior to analysis by gas chromatography (GC)-mass spectrometry (MS) (23). The IC_{50} s for antifungal agents were determined by varying the concentration of the antifungal compound in 5 μl of DMSO, which was added to the assay mixture prior to preincubation at 37°C and addition of β -NADPH- Na_4 .

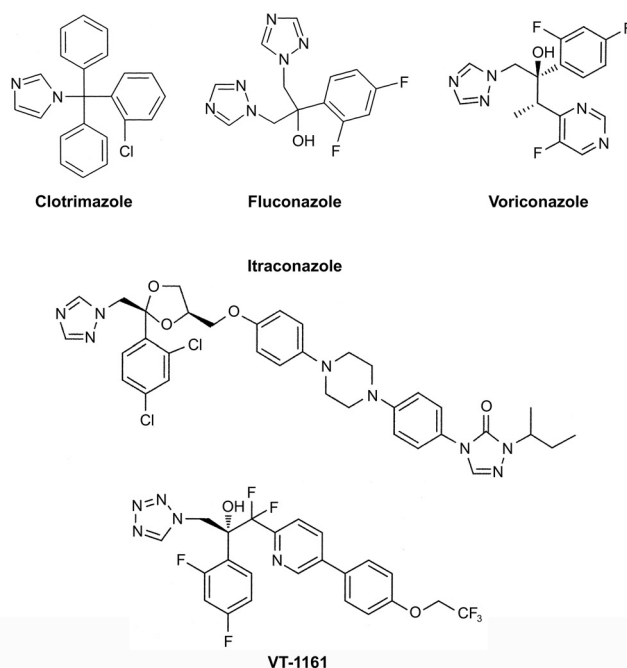


FIG 1 Chemical structures of azole antifungal agents. The chemical structures of clotrimazole (molecular weight, 345), fluconazole (molecular weight, 306), itraconazole (molecular weight, 706), voriconazole (molecular weight, 349), and VT-1161 (molecular weight, 527) are shown.

Antifungal susceptibility testing. The susceptibilities of the SC5314 and CA14 *C. albicans* wild-type strains to all antifungals were determined using the standardized CLSI M27-A3 broth dilution method (24).

***C. albicans* sterol composition determinations.** Single colonies were used to inoculate 15-ml volumes of RPMI 1640, which were incubated for 18 h at 37°C and 180 rpm in the presence of 0.004 $\mu\text{g ml}^{-1}$ antifungal agent or an equivalent amount of DMSO (untreated control colonies). Cells were harvested by centrifugation and washed with sterile water prior to sterol extraction as previously described (25). Cell pellets were suspended in 2.5 ml methanol, 1.5 ml potassium hydroxide (60%, wt/vol), and 1 ml methanol-dissolved pyrogallol (0.5%, wt/vol), and the suspension was heated at 90°C for 2 h. Nonsaponifiable sterols were extracted into glass vials with three sequential 2-ml volumes of hexane. Extracts were evaporated to dryness using a centrifugal evaporator (Heto Maxi dry plus) and derivatized (BSTFA-TMCS [99:1] plus 50 μl anhydrous pyridine, 70°C) for 2 h. TMCS-derivatized sterols were analyzed and identified using GC-MS with reference to the retention times and fragmentation spectra for known standards (see Fig. S1 in the supplemental material). GC-MS data files were analyzed using Agilent software (Mass Selective Detector Enhanced ChemStation; Agilent Technologies Inc.) to determine the sterol profiles for SC5314 and CA14 and for derivation of integrated peak heights. In addition to the analysis of untreated RPMI 1640-grown SC5314 and CA14, the sterol compositions of both isolates following treatment with a fixed azole concentration of 0.004 $\mu\text{g ml}^{-1}$ were determined using GC-MS.

Strains and media. Wild-type (azole-sensitive) *Candida albicans* strain SC5314 (ATCC MYA-2876) (26) and wild-type clinical isolate CA14 (27) were maintained at 37°C on yeast extract peptone dextrose (YEPD) agar containing 2% glucose, 2% Bacto peptone, 1% yeast extract, and 2% agar (Formedium). RPMI 1640 medium (Sigma) buffered with 0.165 M MOPS (morpholinepropanesulfonic acid; pH 7) was used to culture the isolates for sterol analyses and antifungal susceptibility testing.

Inhibition of human liver CYP enzymes. *In vitro* studies determined the IC_{50} s of the test compounds for CYP2C9, CYP2C19, and CYP3A4

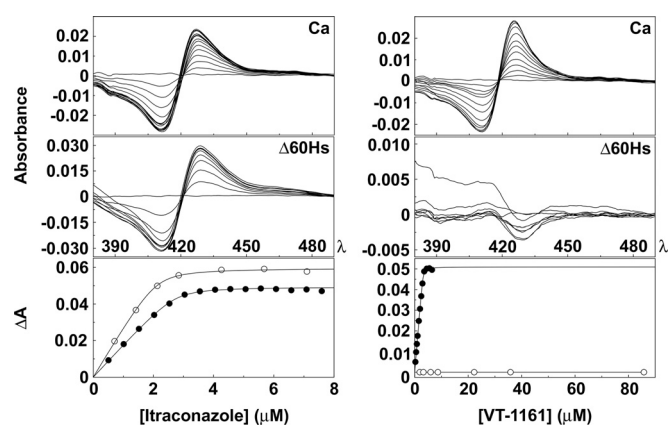


FIG 2 Azole binding properties of CaCYP51 (Ca) and $\Delta 60\text{HsCYP51}$ ($\Delta 60\text{Hs}$). VT-1161 and itraconazole were progressively titrated against 5 μM CaCYP51 and 5 μM $\Delta 60\text{HsCYP51}$, and binding saturation curves were constructed from the change in absorbance ($\Delta A_{430-412}$) against the antifungal concentration for CaCYP51 (filled circles) and $\Delta 60\text{HsCYP51}$ (hollow circles). All spectral determinations were performed in triplicate, although only one replicate is shown.

(with either midazolam or testosterone as the substrates) in intact human liver microsomes. A separate series of incubation mixtures was prepared, with the final concentrations of each test compound in the reaction mixture ranging from 0.0128 to 200 μM . 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, 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-pressure liquid chromatography–MS/MS for the hydroxy metabolite of the substrates. Each product peak area was normalized to be represented as a percentage of the average for the enzyme control. The IC_{50} for 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. Curve fitting of ligand binding data was performed using the computer program ProFit (version 6.1.12; QuantumSoft, Zurich, Switzerland). Spectral determinations were made using quartz semi-microcuvettes with an Hitachi U-3310 UV-visible spectrophotometer (San Jose, CA).

Chemicals. All chemicals, including all azole antifungals except voriconazole, were obtained from Sigma Chemical Company (Poole, United Kingdom). Voriconazole was supplied by Discovery Fine Chemicals (Bournemouth, United Kingdom). VT-1161 was supplied by Viامت Pharmaceuticals, Inc. (Durham, NC).

RESULTS

VT-1161 binding properties. VT-1161 bound tightly to CaCYP51 ($K_d \leq 39$ nM), producing a strong type II difference spectrum (Fig. 2) with an affinity similar to the affinities of the pharmaceutical antifungal agents clotrimazole, fluconazole, itraconazole, and voriconazole (K_d , near or below 50 nM) (Table 1). Type II binding spectra with a maximum absorbance at 429 to 431 nm and a trough absorbance at 411 to 413 nm arise through the triazole N-4 nitrogen (fluconazole, itraconazole, and voriconazole) or the imidazole ring N-3 nitrogen (clotrimazole), coordinating as the sixth ligand with the heme iron (28, 29) to form a low-spin CYP51-azole complex. The interaction of VT-1161 with the heme ferric ion is through a terminal (N-3 or N-4) tetrazole nitrogen atom (Fig. 1). The N-4 nitrogen was found to be more nucleophilic in heats of formation experiments (data not shown).

In contrast, VT-1161 was unique as an azole-based antifungal agent, giving no discernible binding spectrum with $\Delta 60\text{HsCYP51}$ at concentrations up to 86 μM , indicating no disturbance of the heme environment. These data suggest that VT-1161 either binds to $\Delta 60\text{HsCYP51}$ in an orientation that does not perturb the heme environment or does not interact with $\Delta 60\text{HsCYP51}$ at this concentration. Clotrimazole and itraconazole, however, bound tightly to $\Delta 60\text{HsCYP51}$ (Table 1), producing characteristic type II binding spectra (Fig. 2 and 3) with K_d values of 55 and 92 nM, respectively, while voriconazole and fluconazole bound weakly to $\Delta 60\text{HsCYP51}$ (K_d values, 2.3 and 30 μM , respectively). These results were in agreement with previous findings for truncated human CYP51 (30), where clotrimazole bound tightly ($K_d < 0.1$ μM) and fluconazole bound weakly (K_d , 70 μM). In addition, we have previously shown that full-length human CYP51 bound pharmaceutical azole antifungal agents with affinities similar to those of the truncated $\Delta 60\text{HsCYP51}$ enzyme (15).

TABLE 1 Binding affinity for CaCYP51 and $\Delta 60\text{HsCYP51}$ and MICs for wild-type *C. albicans*^f

Antifungal	K_d for antifungal (nM) ^a		Fold difference in K_d between $\Delta 60\text{HsCYP51}$ and CaCYP51 ^a	MIC for antifungal ($\mu\text{g ml}^{-1}$ [nM])	
	CaCYP51	$\Delta 60\text{HsCYP51}$		SC5314 ^d	CA14 ^e
Clotrimazole	26 (6)	55 (5)	2.1		
Fluconazole	56 (4)	30,400 (4,100) ^b	543	0.125 (408)	1.0 (3,268)
Itraconazole	19 (5)	92 (7)	4.8		
Voriconazole	10 (2)	2,290 (120) ^b	229	0.004 (11)	0.0625 (179)
VT-1161	39 (2)	No binding ^c	>2,000 ^c	0.002 (4)	0.002 (4)

^a The values of the parameters were previously determined (15) for all antifungals except VT-1161.

^b Where ligand binding was no longer tight, the Michaelis-Menten equation was used to determine K_d values.

^c No discernible ligand binding spectrum was obtained for VT-1161 with $\Delta 60\text{HsCYP51}$ at drug concentrations up to 86 μM .

^d Laboratory wild-type *C. albicans* strain (26).

^e Wild-type clinical *C. albicans* isolate (27).

^f Binding studies were performed using 5 μM CYP51 proteins. K_d values were calculated using a rearrangement of the Morrison equation for tight-binding ligands (20, 21). Mean K_d values of three replicates are shown, and the associated standard errors are given in parentheses. MICs were determined using the CLSI broth dilution methodology (24) after 48 h growth at 37°C and 180 rpm.

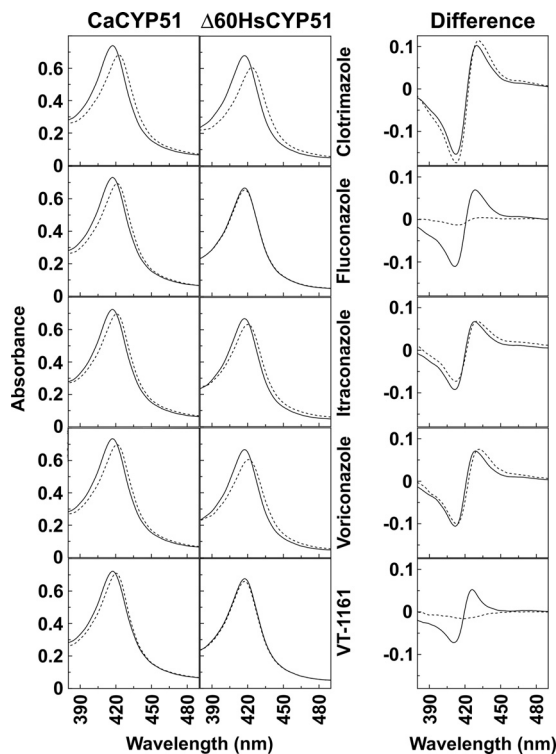


FIG 3 Red shift of the heme Soret peak induced by azole ligand binding. Absolute spectra of 5 μM CaCYP51 and 5 μM $\Delta 60\text{HsCYP51}$ were measured in the absence (solid lines) and presence (dashed lines) of 10 μM antifungal compound. The 380- to 490-nm region of the spectra is shown to highlight any red shift of the Soret peak in response to ligand binding. The difference spectra for CaCYP51 (solid lines) and $\Delta 60\text{HsCYP51}$ (dashed lines), obtained by subtracting the resting-state absolute CYP51 spectra from the +10 μM azole-treated absolute CYP51 spectra, are also shown.

The differences in antifungal agent binding potency observed between CaCYP51 and $\Delta 60\text{HsCYP51}$ using ligand binding difference spectroscopy were confirmed by measuring the absolute spectra of the CYP51 proteins in the absence and presence of 10 μM compound (Fig. 3). When bound to CaCYP51, VT-1161 and the four pharmaceutical azoles induced a red shift of the 417-nm Soret peak to 420 nm with VT-1161, 423 nm with clotrimazole, and 421 nm with fluconazole, itraconazole, and voriconazole. These shifts are consistent with basicity governing the magnitude, with the most basic imidazole giving the greatest shift, followed by triazole and then tetrazole (31). Subtraction of the resting-state absolute spectrum for ferric CaCYP51 from the antifungal-bound absolute spectra gave the characteristic type II difference spectra (Fig. 3). In contrast, the addition of 10 μM or 40 μM VT-1161 to $\Delta 60\text{HsCYP51}$ did not cause any shift of the heme Soret peak, which explained the lack of any type II difference spectrum during the titration of VT-1161 with $\Delta 60\text{HsCYP51}$ (Fig. 2). Clotrimazole, itraconazole, and voriconazole, however, induced a red shift of the Soret peak of $\Delta 60\text{HsCYP51}$ from 417 nm to 423, 420, and 421 nm, respectively (Fig. 3). In contrast, fluconazole caused only a 1-nm red shift of the Soret peak of $\Delta 60\text{HsCYP51}$ to 418 nm, confirming the weak type II difference spectrum previously observed for fluconazole with $\Delta 60\text{HsCYP51}$ (15).

IC_{50} determinations with CaCYP51 and $\Delta 60\text{HsCYP51}$. VT-1161 potently inhibited the sterol 14 α -demethylase activity of iso-

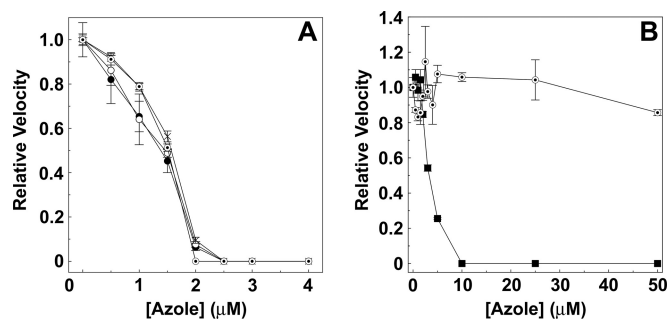


FIG 4 IC_{50} determinations for antifungal agents with CaCYP51 and $\Delta 60\text{HsCYP51}$. CYP51 reconstitution assays containing either 2.5 μM CaCYP51 (A) or 2.5 μM $\Delta 60\text{HsCYP51}$ (B) were performed. For CaCYP51, the concentrations of fluconazole (filled circles), itraconazole (hollow circles), voriconazole (crosses), and VT-1161 (bullets) were varied from 0 to 4 μM . For $\Delta 60\text{HsCYP51}$, the concentrations of clotrimazole (filled squares) and VT-1161 (hollow squares) were varied from 0 to 50 μM . The DMSO concentration was kept constant at 0.5% in all assays. Mean relative enzyme velocities from three replicate data points along with the associated standard error bars are shown. Relative velocities of 1.0 correspond to actual velocities of 0.32 ± 0.04 nmol min^{-1} for CaCYP51 and 0.69 ± 0.18 nmol min^{-1} for $\Delta 60\text{HsCYP51}$.

lated CaCYP51 (Fig. 4A), as did fluconazole, itraconazole, and voriconazole, with IC_{50} s that were approximately half the CaCYP51 concentration present (1.4 to 1.6 μM). This behavior is typical of tight-binding enzyme inhibitors. VT-1161, however, caused no significant inhibition of $\Delta 60\text{HsCYP51}$ activity even at a concentration of 50 μM (Fig. 4B), again consistent with VT-1161 not binding to $\Delta 60\text{HsCYP51}$. In contrast, clotrimazole strongly inhibited $\Delta 60\text{HsCYP51}$ sterol 14 α -demethylase activity (IC_{50} , 3.2 μM) *in vitro*. Previously, we have shown that fluconazole only weakly inhibited $\Delta 60\text{HsCYP51}$ activity (15) with an IC_{50} of $\sim 1,300$ μM and that itraconazole also inhibited $\Delta 60\text{HsCYP51}$ relatively poorly ($\text{IC}_{50} \sim 70$ μM) in comparison to clotrimazole.

Antifungal agent susceptibility of wild-type *C. albicans* strains. Fluconazole and voriconazole inhibited both wild-type *C. albicans* strains. However, the CA14 wild-type clinical strain was 8-fold less susceptible toward fluconazole and 16-fold less susceptible toward voriconazole (Table 1) than the wild-type SC5314 laboratory strain. In contrast, VT-1161 was equally effective against the wild-type strains, with a MIC value of 0.002 $\mu\text{g ml}^{-1}$ (or 3.8 nM) (Table 1). The mode of action of VT-1161 as a sterol 14 α -demethylase inhibitor was confirmed by the sterol composition of VT-1161-treated SC5314 *C. albicans* cells, which was compared to that of untreated or fluconazole- and voriconazole-treated cells (Fig. 5 and Table 2). The untreated cell sterol fraction contained $\sim 92\%$ ergosterol, indicating that sterol biosynthesis was fully functional. The fluconazole-treated cell sterol fraction contained only marginally less ergosterol ($\sim 88\%$), as the fluconazole concentration used (0.004 $\mu\text{g ml}^{-1}$) was ~ 30 -fold lower than the MIC value (Table 1). The voriconazole-treated cell sterol fraction contained far less ergosterol ($\sim 16\%$), increased levels of lanosterol ($\sim 36\%$) and eburicol ($\sim 16\%$), and an accumulation of the fungistatic metabolite 14 α -methylergosta-8,24(28)-dien-3 β ,6 α -diol ($\sim 23\%$). This metabolite is the end product of the sequential action of ERG25, ERG26, ERG27, and ERG3 enzymes on eburicol, indicative of the disruption of the sterol biosynthesis pathway by inhibition of CYP51 activity (27, 32, 33). VT-1161 caused the potent disruption of sterol biosynthesis in *C. albicans*, resulting in a dramatic reduction ($\sim 95\%$) in the ergosterol con-

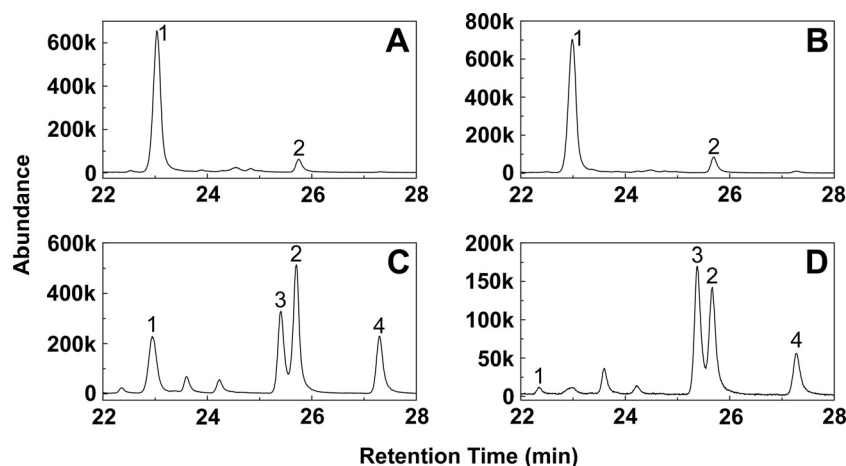


FIG 5 Gas chromatograms of the sterol fraction isolated from untreated and antifungal-treated *C. albicans*. Wild-type *C. albicans* strain SC5134 (26) was treated with $0.004 \mu\text{g ml}^{-1}$ antifungal agents and an untreated control received DMSO alone, followed by 18 h growth at 37°C and 180 rpm. The sterol content was extracted and analyzed as previously described (27). Gas chromatograms of the extracted sterols are shown for the untreated *C. albicans* cells (A) and the cells treated with the antifungals fluconazole (B), voriconazole (C), and VT-1161 (D). The four most abundant sterols present were ergosterol (peak 1), lanosterol (peak 2), 14α -methylergosta-8,24(28)-dien-3 β ,6 α -diol (peak 3), and eburicol (peak 4). The data on the abundance axis are expressed in units of 1,000 (e.g., 600k = 600,000).

tent from $\sim 92\%$ to just $\sim 3\%$ of the total sterol fraction and the accumulation of lanosterol ($\sim 33\%$), eburicol ($\sim 13\%$), and 14α -methylergosta-8,24(28)-dien-3 β ,6 α -diol ($\sim 40\%$) (Table 2).

Inhibition of human liver drug-metabolizing CYPs. The levels of inhibition of three critical xenobiotic-metabolizing CYPs by the four approved azole drugs and VT-1161 are shown in Table 3. Where available, the IC_{50} s for the marketed agents from the literature (34–36) agree well with those measured in this study. The imidazole-containing clotrimazole was the most potent CYP inhibitor, inhibiting all activities with sub- or low-micromolar potency. The three triazole-containing agents had variable inhibitory potencies, with itraconazole potently inhibiting CYP3A4 with either substrate (0.08 and $0.13 \mu\text{M}$), voriconazole inhibiting all activities with a relatively tight range of potencies (4 to $13 \mu\text{M}$), and fluconazole showing a slightly broader range (6 to $34 \mu\text{M}$). In contrast, VT-1161 weakly inhibited each of these activities (65 to $140 \mu\text{M}$).

DISCUSSION

A potential new antifungal drug candidate for the treatment of systemic *Candida* infection should ideally have high potency against the intended CaCYP51 target enzyme and minimal inter-

action with human CYP51 (and other off-target human CYP enzymes). VT-1161 meets both these criteria by binding tightly to CaCYP51 with a high affinity similar to that of other pharmaceutical azole antifungal agents and binding either extremely weakly or not at all to the host human CYP51 *in vitro*. Binding studies (Fig. 2 and 3) can provide useful preliminary information on the likely effectiveness of cyclized nitrogen-containing antifungal drug candidates inhibiting CYP51 activity. However, only IC_{50} determinations using a CYP51 reconstitution assay system can determine the functional activity of each compound as a CYP51 inhibitor.

The CYP51-antifungal agent IC_{50} determinations confirmed that VT-1161 was a strong inhibitor of CaCYP51 activity but did not significantly inhibit $\Delta 60\text{HsCYP51}$ activity at drug concentrations up to $50 \mu\text{M}$, meeting the selection criteria of high potency against the fungal drug target (CaCYP51) and minimal if any activity against the human CYP51 enzyme. Therefore, VT-1161 is a good candidate for the treatment of systemic *Candida* infections and was subjected to testing for efficacy against wild-type clinical *C. albicans* strains. According to the MICs, the activity of VT-1161 was more potent than the K_d value for binding to CaCYP51, suggesting that the Morrison equation-calculated K_d value was an

TABLE 2 Sterol composition of untreated and antifungal-treated wild-type *C. albicans* strains^a

Sterol	Sterol composition (%)							
	Untreated		With fluconazole		With voriconazole		With VT-1161	
	SC5134	CA14	SC5134	CA14	SC5134	CA14	SC5134	CA14
Ergosterol	91.6	79.6	88.3	80.5	16.2	22.0	2.6	5.0
14α -Methylfecosterol					4.8	4.6	8.6	7.5
$4,14\alpha$ -Dimethylcholesta-8,24-dienol					3.9	2.5	3.0	2.5
14α -Methylergosta-8,24(28)-dien-3 β ,6 α -diol					23.1	42.9	39.8	60.2
Lanosterol	8.4	13.2	10.2	14.1	35.9	21.3	33.2	19.9
Eburicol		7.2	1.5	5.4	16.2	6.7	12.8	5.0

^a The *C. albicans* strains used included the laboratory wild-type SC5134 strain (26) and the clinical wild-type isolate CA14 (27). Both isolates were treated with antifungal agent at a concentration of $0.004 \mu\text{g ml}^{-1}$, followed by 18 h growth at 37°C and 180 rpm, and the sterol content was extracted and analyzed as previously described (27).

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-1161	99 (33)	72 (26)	65 (30)	140 (10)

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

^b With testosterone as the substrate.

^c With midazolam as the substrate.

overestimate of the *in vivo* value in part due to the relatively high CYP51 protein concentrations required for *in vitro* binding studies and also that VT-1161 was readily taken up by the *C. albicans* cells to exert a potent effect. The VT-1161-induced reduction in ergosterol content was greater than that caused by an equal concentration of voriconazole, a desirable attribute for any new antifungal drug candidate in conjunction with the extremely high selectivity observed. Using a simplified equation relating percent inhibition and IC₅₀ (37) and on the basis of the extent of inhibition (~95%) of the sterol pathway at a VT-1161 concentration of ~8 nM, the biochemical IC₅₀ for inhibiting fungal CYP51 was estimated to be ≤1 nM.

When predicting a clinical drug-drug interaction (DDI) potential on the basis of *in vitro* CYP data, it has been postulated (38) that free drug concentrations should be used in the calculations. For example, fluconazole binds plasma protein poorly and largely exists as free drug in the blood (39). Thus, using *in vitro* potencies consistent with those listed in Table 3, fluconazole was predicted to have significant DDIs with substrates of CYP2C9, CYP2C19, and CYP3A4 (38). Similarly, when the high plasma protein binder itraconazole was modeled for DDIs with CYP3A4 substrates, it too was predicted to cause DDIs due to the high potency of CYP3A4 inhibition. Importantly, there were strong correlations between these modeling predictions and documented clinical DDIs (38). Given that VT-1161 binds human plasma protein strongly (>99%) (E. P. Garvey, W. J. Hoekstra, R. J. Schotzinger, J. A. Sobel, E. A. Lilly, and P. L. Fidel, unpublished data) and given the weak *in vitro* potencies for VT-1161, no DDIs were predicted for VT-1161 for these three CYPs shown in Table 3 (or for CYP1A2, CYP2B6, CYP2C8, and CYP2D6 on the basis of *in vitro* IC₅₀ data not shown). It remains to be demonstrated in clinical DDI studies if these predictions will be borne out.

In summary, VT-1161 potently bound *C. albicans* CYP51 (K_d ≤39 nM) with a type II difference spectrum indicative of a heme interaction and inhibited *C. albicans* CYP51 sterol 14α-demethylase activity in a manner consistent with tight-binding inhibition. In *C. albicans* cellular studies, VT-1161 nearly completely inhibited the sterol biosynthesis pathway at a concentration of 0.004 μg ml⁻¹ (7.6 nM). In stark contrast, VT-1161 did not bind to human CYP51 at a concentration of 86 μM, nor did it inhibit human CYP51 activity at a concentration of 50 μM. Furthermore, VT-1161 inhibition of three critical human drug-metabolizing CYPs was weak (IC₅₀s, ≥65 μM). Using an estimated biochemical IC₅₀ of the fungal target of ≤1 nM and the very weak inhibition of human cytochrome P450 enzymes studied in this report, the selectivity of VT-1161 was ~10,000 for its target compared to that

for human liver CYPs. This degree of potency and selectivity of VT-1161 is markedly superior to that of approved CYP51 inhibitors, and the potential for a high degree of safety has been corroborated in preclinical and clinical phase I studies. The activity spectrum of VT-1161 against several pathogenic fungi (*Candida albicans* and non-*albicans Candida* species, *Cryptococcus neoformans*, endemic fungus, and mold species), including strains that possess intrinsic and acquired resistance to current azole antifungal therapeutics, has been investigated and will be reported elsewhere. VT-1161 is currently in phase IIa investigations of fungal infections and has the strong potential to significantly advance the field of fungal CYP51 therapeutics.

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