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**The Tetrazole VT-1161 Is a Potent Inhibitor of  
*Trichophyton rubrum* through its Inhibition of *T. rubrum*  
CYP51**

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**Running title:** *VT-1161 inhibition of T. rubrum CYP51.*

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specificity.

*VT-1161 inhibition of T. rubrum CYP51*

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Prior to characterization of antifungal inhibitors that target this enzyme, *Trichophyton rubrum* CYP51 was expressed in *Escherichia coli*, purified and characterized. *T. rubrum* CYP51 bound lanosterol, obtusifoliol and eburicol with similar affinities ( $K_d$  values 22.7, 20.3 and 20.9  $\mu\text{M}$ ), but displayed substrate specificity insofar as only eburicol was demethylated in CYP51 reconstitution assays (turnover number  $1.55 \text{ min}^{-1}$ ,  $K_m$  value 2  $\mu\text{M}$ ). The investigational agent VT-1161 bound tightly to *T. rubrum* CYP51 ( $K_d = 242 \text{ nM}$ ) with similar affinity as clotrimazole, fluconazole, ketoconazole and voriconazole ( $K_d$  values 179, 173, 312, and 304 nM, respectively), and with lower affinity than itraconazole ( $K_d = 53 \text{ nM}$ ).  $\text{IC}_{50}$  determinations using 0.5  $\mu\text{M}$  CYP51 showed VT-1161 was a tight-binding inhibitor of *T. rubrum* CYP51 activity yielding an  $\text{IC}_{50}$  value of 0.14  $\mu\text{M}$  compared to 0.26, 0.4 and 0.6  $\mu\text{M}$  for itraconazole, fluconazole and ketoconazole, respectively. When tested against 34 clinical isolates, VT-1161 was a potent inhibitor of *T. rubrum* growth with  $\text{MIC}_{50}$ ,  $\text{MIC}_{90}$ , and geometric mean MIC values of  $\leq 0.03$ , 0.06, and 0.033  $\mu\text{g ml}^{-1}$ , respectively. With its selectivity versus human CYP51 and drug metabolizing CYPs having already been established, VT-1161 should prove safe and effective in combating *T. rubrum* infections in patients.

Infections of the ascomycete fungi *Trichophyton* spp. (e.g., onychomycosis or nail fungus, tinea pedis or athlete's foot, tinea corporis or ringworm) are some of the oldest human dermatological afflictions. Whilst not life-threatening, these infections can be of significant annoyance to the sufferer. *T. rubrum* is the most common dermatophyte infection in healthy individuals, accounting for up to 70% of skin infections (1) and up to 90% of nail infections (2, 3). Nail infections caused by *T. rubrum* affects around 10% of the population and are frequently intractable and prone to relapse upon termination of antifungal therapy (4, 5). *T. rubrum* infections of hair, skin and nails have increased over the past 70 years, especially in the elderly and in some countries also in children (6-8). Chronic skin infections caused by *T. rubrum* can become sites for secondary infection by other microorganisms, such as *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp. and *Staphylococcus aureus*, which can become life-threatening in immunocompromised and immunosuppressed patients if the secondary infection becomes systemic (9-12).

Current therapeutic treatments against *T. rubrum* infection include azole antifungal agents, allylamines and thiocarbamates (all inhibiting ergosterol biosynthesis) administered orally or applied topically in creams and lotions. In chronic invasive and systemic fungal infections, especially amongst immunocompromised patients, amphotericin B (which disrupts fungal cell membranes) can be utilized intravenously. These antimycotic agents are most effective against the growing organism but are often ineffective against static phases of the organism, such as *T. rubrum* conidia, leading to reinfection unless

prolonged treatment regimens are adopted. Recently photodynamic treatments have been developed using photosensitizers in combination with UVA-1 radiation (340-400 nm) to kill both the mycelial form and conidia of *T. rubrum* (13) in topical dermal infections. The most commonly used antifungal agents against *T. rubrum* are ketoconazole, fluconazole, terbinafine and flucytosine (13). The prolonged treatment regimens often required have led to the emergence of azole resistant *T. rubrum* strains, especially against fluconazole (14-17).

In this study, we characterize the catalytic properties of recombinant *T. rubrum* CYP51 and compare the novel antifungal VT-1161 (18, 19) with clinical azole antifungal drugs in terms of potency and selectivity of binding to and inhibition of recombinant *T. rubrum* CYP51 and in inhibition of fungal growth in broth microdilution assays.

## RESULTS

**Expression and purification of Trub51.** Following heterologous expression in *E. coli*, Trub51 protein was extracted by sonication in 2% (wt/vol) sodium cholate which yielded 240 ( $\pm 80$ ) nmoles per liter culture as determined by carbon monoxide difference spectroscopy (20). Purification by Ni<sup>2+</sup>-NTA agarose chromatography resulted in an 84% recovery of native Trub51 protein yielding a stock 48  $\mu$ M solution after dialysis. SDS polyacrylamide gel electrophoresis confirmed the purity of the Ni<sup>2+</sup>-NTA agarose purified Trub51 to be greater than 90% when assessed by staining intensity.

**Spectral properties of Trub51.** The absolute spectrum of the resting oxidized form of Trub51 (Fig. 1A) was typical for a low-spin ferric cytochrome P450 enzyme (21, 22) with  $\alpha$ ,  $\beta$ , Soret ( $\gamma$ ) and  $\delta$  spectral bands at 567, 540, 420 and 361 nm. Reduced carbon monoxide difference spectra for Trub51 (Fig. 1B) gave the red-shifted heme Soret peak at 447 nm, characteristic of P450 enzymes, indicating the Trub51 protein was isolated in the native form. The formation of the reduced CO-P450 complex with Trub51 was rapid ( $t_{0.5} = 0.18 \pm 0.06$  min) although did not proceed to completion (hump visible at 422 nm).

**Sterol binding properties of Trub51.** Progressive titration of Trub51 with lanosterol, eburicol and obtusifoliol gave type I difference spectra with a peak at 388 nm and a trough at 421 nm (Fig. 2). 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 (22). The intensity

( $\Delta A_{\max}$ ) of the type I binding spectra obtained with lanosterol was 7-fold lower than that obtained with eburicol and 3-fold lower than that obtained with obtusifoliol, suggesting that eburicol was the preferred substrate. However,  $K_d$  values of  $20.3 \pm 1.2 \mu\text{M}$ ,  $22.7 \pm 3.6 \mu\text{M}$  and  $20.9 \pm 0.3 \mu\text{M}$  were obtained for eburicol, lanosterol and obtusifoliol, respectively, indicating all three sterols bound with similar affinity.

**CYP51 reconstitution assays.** Trub51 did not catalyze the  $14\alpha$ -demethylation of lanosterol under the stated assay conditions. GC traces for TMS-derivatized CYP51 assay metabolites show lanosterol and eburicol emerging from the GC column after 35.65 and 38.25 minutes (Fig. 3A), whereas the  $14\alpha$ -demethylated product of eburicol emerged after 39.15 minutes. Confirmation of the identity of product 'P' was obtained by the mass fragmentation pattern (Fig. 3B) as TMS-derivatized C14-demethylated eburicol ( $M^+$  496). Trial Trub51 assays using  $50 \mu\text{M}$  obtusifoliol yielded no detectable metabolites (data not shown). This is only the second time that such strict substrate specificity has been observed for a fungal CYP51 enzyme, with *Mycosphaerella graminicola* CYP51 previously being shown to demethylate eburicol but not lanosterol *in vitro* (23).

Mild substrate inhibition was evident from the eburicol velocity curve obtained for Trub51 (Fig. 4) with calculated  $K_m$  and  $K_i$  values for eburicol of  $2 \mu\text{M}$  and  $225 \mu\text{M}$ , respectively. The maximum eburicol turnover number was  $1.55 \text{ min}^{-1}$ . The observed substrate inhibition suggests the presence of two distinct eburicol binding sites or binding orientations in Trub51 with one binding site /

orientation being catalytically productive whilst the other leads to the formation of an unproductive dead-end complex. However, no allosterism was observed in the eburicol type I difference binding spectra (Fig. 2B), suggesting eburicol binds in only one conformation that causes the displacement of the axial ligated heme water molecule responsible for the low- to high-spin state transition.

**CYP51 inhibitor binding properties of Trub51.** All five marketed imidazole and triazole antifungal agents and the novel tetrazole VT-1161 produced type II binding spectra (24) with Trub51 (Fig. 5). Ligand saturation curves (Fig. 6) confirmed azole binding was tight with the rearranged Morrison equation providing the best fit to the data (25, 26). Trub51 bound itraconazole the tightest with a  $K_d$  value of 53 ( $\pm 29$ ) nM whilst clotrimazole, fluconazole, voriconazole, ketoconazole and VT-1161 all apparently bound less tightly to Trub51 with similar  $K_d$  values of 179 ( $\pm 83$ ), 173 ( $\pm 53$ ), 304 ( $\pm 64$ ), 312 ( $\pm 36$ ) and 242 ( $\pm 99$ ), respectively.

**CYP51 inhibitor  $IC_{50}$  determinations.**  $IC_{50}$  determinations (Fig. 7) confirmed that fluconazole, itraconazole, ketoconazole and VT-1161 all inhibited *T. rubrum* CYP51 activity *in vitro*. VT-1161 caused the strongest inhibition ( $IC_{50}$  0.14  $\mu$ M), followed by itraconazole ( $IC_{50}$  0.26  $\mu$ M), then fluconazole and ketoconazole ( $IC_{50}$  values 0.4 and 0.6  $\mu$ M). Given the concentration of CYP51 used in this assay was 0.5  $\mu$ M, the expected  $IC_{50}$  value for an extremely tight-binding azole antifungal would be 0.25  $\mu$ M. Therefore, both VT-1161 and itraconazole bound extremely tightly to Trub51 whilst fluconazole and ketoconazole bound less tightly.

**CYP51 inhibitor MIC determinations.** MIC determinations (Table 1) confirmed the potency of VT-1161, as the MICs ranged from less than or equal to the lowest concentration tested ( $0.03 \mu\text{g ml}^{-1}$ ) to the highest MIC values of  $0.06 \mu\text{g ml}^{-1}$ . VT-1161's MIC<sub>50</sub>, MIC<sub>90</sub> and geometric mean values of  $\leq 0.03$ ,  $0.06$ , and  $0.033 \mu\text{g ml}^{-1}$  were slightly less than those for itraconazole ( $0.06$ ,  $0.06$ , and  $0.052 \mu\text{g ml}^{-1}$ , respectively), and both of these CYP51 inhibitors were significantly more potent than fluconazole ( $2$ ,  $16$ , and  $2.3 \mu\text{g ml}^{-1}$ , respectively). The GM MICs of VT-1161 and itraconazole were both significantly lower than that of fluconazole ( $p = 0.0018$  for both comparisons) but were not significantly different between each other.

**Phylogenetic comparison of fungal CYP51 enzymes.** The primary amino acid sequence of *T. rubrum* CYP51 contained all twenty-three conserved CYP51 residues previously identified by Lepesheva and Waterman (27) in addition to the conserved heme-binding cysteine residue (supplementary figure S1). The degree of conservation between the six-substrate recognition sites (SRSs) (28) varied (supplementary figure S1) with SRS-1 being the most conserved and SRS-6 the least conserved. Both *T. rubrum* and *M. graminicola* CYP51 enzymes can turnover eburicol but not lanosterol (this study; 23). *A. fumigatus* CYP51 isoenzymes A and B turnover both eburicol and lanosterol, albeit with a 4- to 7-fold preference for eburicol in terms of measured velocity using purified proteins (29) or more than an 18-fold preference for eburicol using membrane fractions. *C. albicans*, *C. neoformans* and *M. globosa* CYP51 enzymes, on the other hand, all readily turnover both eburicol and lanosterol (29-

31). Analysis of the amino acid sequences of the six SRSs between the seven fungal CYP51 enzymes did not identify any residue changes that could be directly linked to the change in substrate specificity observed in the *T. rubrum* and *M. graminicola* CYP51 enzymes.

## DISCUSSION

In preparation for studying antifungal inhibitors of this enzyme, we have fully characterized CYP51 from the most prevalent fungus causing human dermatophytosis, *Trichophyton rubrum*. The *T. rubrum* CYP51 (Trub51)  $K_d$  values for sterol substrates of 20 to 23  $\mu\text{M}$  were comparable with CYP51 enzymes from *Candida albicans* (11 to 28  $\mu\text{M}$ ) (32), *Mycosphaerella graminicola* (11 to 13  $\mu\text{M}$ ) (33), *Aspergillus fumigatus* CYP51B (9 to 23  $\mu\text{M}$ ) (29), *Cryptococcus neoformans* (12 to 21  $\mu\text{M}$ ) (30) and *Malassezia globosa* (23 to 32  $\mu\text{M}$ ) (31). However, the  $K_d$  values for lanosterol and eburicol with *Trypanosoma cruzi* CYP51 were lower at 1.9 and 1.2  $\mu\text{M}$  (34), and the  $K_d$  values for lanosterol with *H. sapiens* and *Mycobacterium tuberculosis* CYP51s were lower than Trub51 at 0.5 to 6  $\mu\text{M}$  (28, 35) and 1  $\mu\text{M}$  (21), respectively. However, Trub51 only catalyzed the 14 $\alpha$ -demethylation of eburicol and not that of lanosterol and obtusifoliol and mirrors that previously observed for *Mycosphaerella graminicola* CYP51 (23). This narrow substrate specificity is in contrast to the broad substrate specificity observed previously for CYP51 enzymes from *Candida albicans*, *Mycobacterium tuberculosis*, *Homo sapiens*, *Trypanosoma cruzi*, *Cryptococcus neoformans*, and *Malassezia globosa*, (30, 31, 36). Additional CYP51 enzymes that exhibit narrow substrate specificities include obtusifoliol-specific *Trypanosoma brucei* CYP51 and plant CYP51 enzymes such as *Sorgham bicolor* CYP51 (36), whilst *Aspergillus fumigatus* CYP51A and CYP51B isoenzymes have a strong preference for eburicol (29). The Trub51  $K_m$  for eburicol of 2  $\mu\text{M}$  was comparable to the substrate  $K_m$  values previously obtained for CYP51

enzymes from *C. albicans* and *Saccharomyces cerevisiae* (32, 37, 38), but was 5- to 30-fold lower than those determined for CYP51 enzymes from *Leishmania infantum*, *Homo sapiens*, *Mycosphaerella graminicola*, and *Malassezia globosa*, (23, 31, 36, 39). The strict eburicol substrate specificity of Trub51 could not be directly attributable to changes in the primary amino acid sequence of the six substrate recognition sites (28) relative to fungal CYP51 enzymes that readily demethylate both eburicol and lanosterol (supplementary figure S1).

It has been long recognized that fungal CYP51 inhibitors derive much of their binding potency through an “azole”/heme iron interaction (40), and that this binding can be directly measured spectroscopically (41). Therefore, as expected, Trub51 bound imidazole-based ketoconazole and clotrimazole, triazole-based fluconazole, voriconazole, and itraconazole, and the novel tetrazole-based VT-1161. Each compound displayed a type II binding spectra caused by the interaction of a heterocyclic ring nitrogen coordinating as the sixth ligand with the heme iron (24) to form the low-spin CYP51-azole complex resulting in a 'red-shift' of the heme Soret peak. Whereas, the specific nitrogen is known for the triazole inhibitors (N-4) (42) and imidazole inhibitors (N-3) (28), the interaction of VT-1161 with the heme ferric ion is through either the tetrazole's N-3 or N-4 nitrogen. The N-4 nitrogen was found to be more nucleophilic in heats of formation experiments (data not shown), and would therefore be the most likely atom to interact with the CYP51 heme iron.

The antifungal agents tested in this study bound Trub51 somewhat less tightly than to other fungal CYP51 enzymes, with the possible exception of

itraconazole. The relative differences observed in the  $K_d$  values, however, did not translate into equally large differences in  $IC_{50}$  values, with only a 4-fold increase in  $IC_{50}$  value being observed between VT-1161 and ketoconazole, and the  $IC_{50}$  for VT-1161 being numerically but not significantly lower than that for itraconazole. Therefore, for Trub51, the CYP51 reconstitution assay proved to be better at assessing CYP51-inhibitor potency than direct ligand binding to aqueous purified enzyme and was in agreement with the intrinsic antifungal potency measured in broth microdilution assays which ranked VT-1161 as the most potent *T. rubrum* inhibitor, closely followed by itraconazole, with fluconazole being the least potent (Table 1).

The performance of the drug candidate VT-1161 against *T. rubrum* CYP51 and *T. rubrum* itself was encouraging. We have shown biochemically that VT-1161 bound to the heme iron in the active site of Trub51 and strongly inhibited Trub51 activity through tight ligand binding. VT-1161 cellular potency against *T. rubrum* ranged from  $\leq 0.03$  to  $0.06 \mu\text{g/ml}$ , slightly more potent than for itraconazole ( $\leq 0.03$  to  $0.12 \mu\text{g/ml}$ ) and significantly more potent than for fluconazole ( $0.5$  to  $>64 \mu\text{g/ml}$ ). This MIC potency range for VT-1161 compares favorably to published MIC values for *T. rubrum* of  $0.03$  to  $256 \mu\text{g ml}^{-1}$  for fluconazole,  $0.008$  to  $0.25 \mu\text{g ml}^{-1}$  for itraconazole,  $0.06$  to  $2 \mu\text{g ml}^{-1}$  for ketoconazole and  $0.06$  to  $1 \mu\text{g ml}^{-1}$  for voriconazole (43-46). In addition, VT-1161 was as effective as itraconazole in treating *T. mentagrophytes*-induced dermatophytosis in guinea pig when treatments were orally administered daily and superior to itraconazole when administered weekly (47).

*VT-1161 inhibition of T. rubrum CYP51*

Equally important, the use of the tetrazole has allowed for the engineering of a more selective fungal CYP51 inhibitor relative to key human CYP enzymes (IC<sub>50</sub> values against CYPs 3A4, 2C9, 2C19, and 51 ranging from 65 to ~600 μM) (19). This greater selectivity coupled with at least maintaining if not improving antifungal potency should translate into a greater clinical therapeutic window, which in turn could allow for higher doses and possible greater efficacy. To this end, VT-1161 has achieved proof-of-concept efficacy (48) in a Phase 2a study in treatment of tinea pedis (NCT01891305), and has just completed a Phase 2b study in the treatment of onychomycosis (NCT02267356) with interim data demonstrating antifungal and clinical efficacy in conjunction with an excellent safety profile (49). Phase 3 studies are currently being planned to support registration approval of VT-1161 as a novel agent to treat onychomycosis.

## **MATERIALS AND METHODS**

**Construction of the pCWori<sup>+</sup>:Trub51 expression vector.** The *T. rubrum* CYP51 gene (Trub51 – UniProt accession number F2SHH3) was synthesized by Eurofins MWG Operon (Ebersberg, Germany) incorporating an *Nde*I restriction site at the 5' end and a *Hind*III restriction site at the 3' end of the gene cloned into pBSIISK<sup>+</sup> plasmid. In addition, the first eight amino acids were changed to 'MALLLAVF' (50) and a four-histidine extension (CATCACCATCAC) was inserted immediately before the stop codon. The Trub51 gene was excised by *Nde*I / *Hind*III restriction digestion followed by cloning into the pCWori<sup>+</sup> expression vector. Gene integrity was confirmed by DNA sequencing.

**Heterologous expression and purification of recombinant Trub51 protein.** The pCWori<sup>+</sup>:*Trub51* construct was transformed into competent DH5 $\alpha$  *E. coli* cells and expressed as previously described for *Candida albicans* CYP51 (32). Recombinant Trub51 protein was isolated according to the method of Arase *et al* (51) except that 2% (wt/vol) sodium cholate was used as sole detergent in the sonication buffer with the addition of 0.1 mM phenylmethylsulfonylfluoride. The solubilized Trub51 protein was purified by affinity chromatography using Ni<sup>2+</sup>-NTA agarose as previously described (21, 32) followed by dialysis against 20 mM Tris-HCl (pH 8.1) and 10% (wt/vol) glycerol. Protein purity was assessed by SDS polyacrylamide gel electrophoresis.

**Cytochrome P450 protein determinations.** Reduced carbon monoxide difference spectroscopy was performed (20) 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  $91 \text{ mM}^{-1} \text{ cm}^{-1}$  (52) was used to calculate cytochrome P450 concentrations from the absorbance difference between 447 and 490 nm. Absolute spectra were determined between 700 and 300 nm (light path 4.5 mm). All spectral determinations were made using a Hitachi U-3310 UV/VIS spectrophotometer (San Jose, California).

**Sterol binding properties of Trub51.** Stock  $2 \text{ mg ml}^{-1}$  solutions of lanosterol, obtusifoliol and eburicol were prepared in 40% (wt/vol) (2-hydroxypropyl)- $\beta$ -cyclodextrin (HPCD). Sterol was progressively titrated against  $5 \mu\text{M}$  Trub51 in a quartz semi-micro cuvette (light path 4.5 mm) with equivalent amounts of 40% (wt/vol) HPCD added to the reference cuvette which also contained  $5 \mu\text{M}$  Trub51. The absorbance difference spectrum between 500 and 350 nm was determined after each incremental addition of sterol (up to  $75 \mu\text{M}$ ). The sterol saturation curves were constructed from  $\Delta A_{388-421}$  derived from the difference spectra. The substrate dissociation constants ( $K_d$ ) were determined by non-linear regression (Levenberg-Marquardt algorithm) using the Michaelis-Menten equation.

**Azole binding properties of Trub51.** Binding clotrimazole, fluconazole, voriconazole, itraconazole, ketoconazole and the drug candidate VT-1161 to Trub51 was performed as previously described (32, 53) using 4.5 mm light-path quartz split-cuvettes. Stock  $0.05$ ,  $0.1$  and  $0.2 \text{ mg ml}^{-1}$  solutions of the azoles were prepared in dimethylsulfoxide and progressively titrated against  $2 \mu\text{M}$  of Trub51 in  $0.1 \text{ M}$  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  $\Delta A_{\text{peak-trough}}$  against azole concentration. The dissociation constants of the enzyme-azole complex ( $K_d$ ) were determined by non-linear regression (Levenberg-Marquardt algorithm) using a rearrangement of the Morrison equation for tight ligand binding (25, 26). Tight binding is normally observed where the  $K_d$  for a ligand is similar or lower than the concentration of the enzyme present (54).

**CYP51 reconstitution assays.** The reconstitution assays (34, 55) contained 0.5  $\mu\text{M}$  Trub51, 1  $\mu\text{M}$  *Aspergillus fumigatus* cytochrome P450 reductase isoenzyme 1 (AfCPR1 - UniProt accession number Q4WM67), 50  $\mu\text{M}$  sterol substrate, 50  $\mu\text{M}$  dilaurylphosphatidylcholine, 4% (wt/vol) HPCD, 0.4  $\text{mg ml}^{-1}$  isocitrate dehydrogenase, 25  $\text{mM}$  trisodium isocitrate, 50  $\text{mM}$  NaCl, 5  $\text{mM}$   $\text{MgCl}_2$  and 40  $\text{mM}$  MOPS (pH  $\sim 7.2$ ). Assay mixtures were incubated at  $37^\circ\text{C}$  prior to initiation with 4  $\text{mM}$   $\beta\text{-NADPHNa}_4$  followed by shaking at  $37^\circ\text{C}$  for 15 minutes. Sterol metabolites were recovered by extraction with ethyl acetate followed by derivatization with 0.1 ml *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) : trimethylchlorosilane (TMCS) (99:1) and 0.3 ml anhydrous pyridine (2 h at  $80^\circ\text{C}$ ) prior to analysis by gas chromatography mass spectrometry (GC/MS) (56). Trub51  $K_m$  value for eburicol was determined by varying the eburicol concentration in the CYP51 reconstitution assay between 3 and 100  $\mu\text{M}$  whilst maintaining a constant HPCD concentration of 4% (wt/vol). The single substrate inhibition equation [ $v = (V_{\text{max}} \cdot [S]) / \{K_m + [S] \cdot (1 + [S]/K_i)\}$ ] (57) was used to fit the data and to determine  $K_m$  and  $K_i$  values.

**Azole IC<sub>50</sub> determinations.** IC<sub>50</sub> determinations were performed using the CYP51 reconstitution assay detailed above in which various fluconazole, itraconazole, ketoconazole and VT-1161 concentrations in 2.5 µl dimethylsulfoxide were added prior to incubation at 37°C and addition of β-NADPHNa<sub>4</sub>. The IC<sub>50</sub> assays contained 25 µM eburicol, 0.5 µM Trub51, 1 µM AfCPR1 and 4 mM β-NADPHNa<sub>4</sub>.

**Minimum Inhibitory Concentration (MIC) determinations.** Drug preparations were prepared according to the recommendation outlined in the Clinical and Laboratory Standards Institute (CLSI) document M38-A2; this includes testing in RPMI-1640 with L-glutamine, with 0.165 M MOPS as the buffer (pH 7.0) and without bicarbonate, an inoculum size of 1-5 x 10<sup>4</sup>, and incubation at 35°C for 96 hours. The MICs were measured visually as the lowest concentrations of each antifungal agent that resulted in an 80% reduction in turbidity as compared to a drug-free, growth control wells. Stock solutions of each agent were prepared in DMSO. Further dilutions were made in RPMI-1640, and the final concentration of DMSO was 1% (vol/vol). The final testing concentrations for VT-1161 and itraconazole ranged from 0.03-16 µg ml<sup>-1</sup>, and for fluconazole from 0.125-64 µg ml<sup>-1</sup>. *Trichophyton mentagrophytes* (ATCC-MYA-4439) served as the quality control organism, as recommended by M38-A2, was used on each day of testing. Results for this control isolate were within the appropriate range for each agent test. Thirty-four clinical *Trichophyton rubrum* isolates that were submitted to the Fungus Testing Laboratory (University of Texas Health Science Center at San Antonio, San Antonio, Texas) for antifungal

susceptibility testing and/or identification were used in this study. All strains were fresh clinical strains that had not been previously frozen. All MICs were measured once.

**Phylogenetic analysis of fungal CYP51 proteins.** Selected fungal CYP51 amino acid sequences were obtained from the UniProtKB database (<http://www.uniprot.org>) and were aligned using ClustalX software version 2.0.12 (<http://www.clustal.org/clustal2/>). The fungal sequences compared were *Aspergillus fumigatus* CYP51 isoenzyme A (UniProt accession number Q4WNT5), *Aspergillus fumigatus* CYP51 isoenzyme B (Q96W81), *Candida albicans* CYP51 (P10613), *Cryptococcus neoformans* CYP51 (Q5KQ65), *Malassezia globosa* CYP51 (A8Q3I7), *Mycosphaerella graminicola* CYP51 (Q5XWE5) and *Trichophyton rubrum* CYP51 (F2SHH3).

**Data analysis.** All ligand binding experiments were performed in triplicate and curve-fitting of data performed using the computer program QuantumSoft ProFit (version 6.1.12). Differences in geometric mean (GM) MIC values, calculated following log<sub>2</sub> transformation of individual MIC values, between VT-1161, itraconazole, and fluconazole were assessed for significance by ANOVA with Tukey's post-test for multiple comparisons. A p-value of <0.05 was considered statistically significant. For MIC values that were greater than the highest concentration tested, the next higher dilution value was used in the GM MIC calculations (e.g., fluconazole MIC >64 µg ml<sup>-1</sup>, 128 µg ml<sup>-1</sup> used). For MICs that were equal to or lower than the lowest concentration tested the lowest

concentration tested was used (e.g., VT-1161 or itraconazole MIC  $\leq$  0.03  $\mu\text{g ml}^{-1}$ , 0.03  $\mu\text{g ml}^{-1}$  used).

**Chemicals.** All chemicals, including clotrimazole, fluconazole, itraconazole, ketoconazole and voriconazole, were obtained from Sigma Chemical Company (Poole, UK). Growth media, sodium ampicillin, IPTG and 5-aminolevulinic acid were obtained from Foremedium Ltd (Hunstanton, UK). Ni<sup>2+</sup>-NTA agarose affinity chromatography matrix was obtained from Qiagen (Crawley, UK). VT-1161 was supplied by Viamet Pharmaceuticals, Inc. (Durham, North Carolina, USA).

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*VT-1161 inhibition of T. rubrum CYP51*

Table 1. MICs of VT-1161, itraconazole, and fluconazole against 34 clinical isolates of *T. rubrum*.

Drug	MIC ( $\mu\text{g ml}^{-1}$ )			
	50%	90%	Geometric mean	Range
VT-1161	$\leq 0.03$	0.06	0.033	$\leq 0.03 - 0.06$
Itraconazole	0.06	0.06	0.052	$\leq 0.03 - 0.12$
Fluconazole	2	16	2.3	0.5 - >64

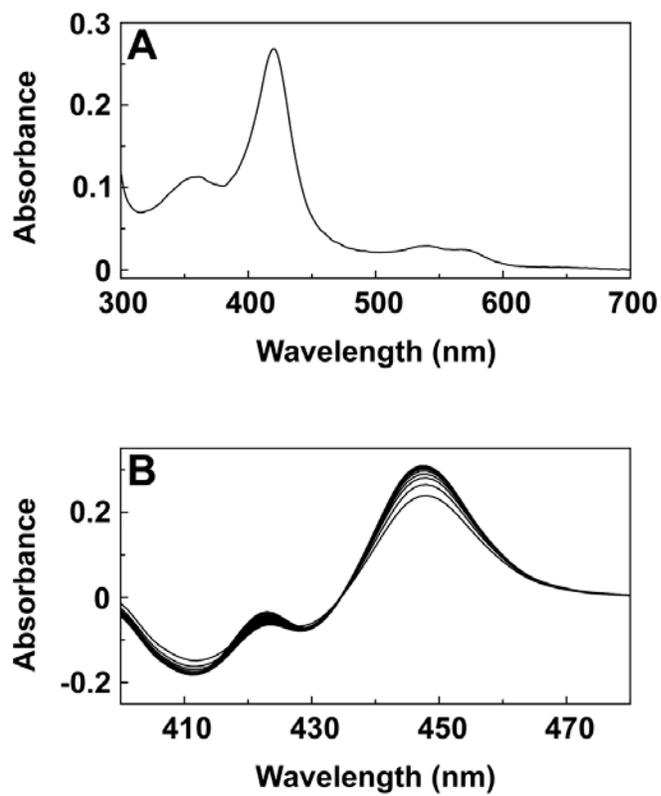


FIG. 1. *Spectral characteristics of Trub51*. Absolute spectra (A) were determined using 3  $\mu$ M purified Trub51 in the oxidised resting state. Reduced carbon monoxide difference spectra (B) were determined using 3  $\mu$ M purified Trub51 with sequential measurements made every 45 seconds.

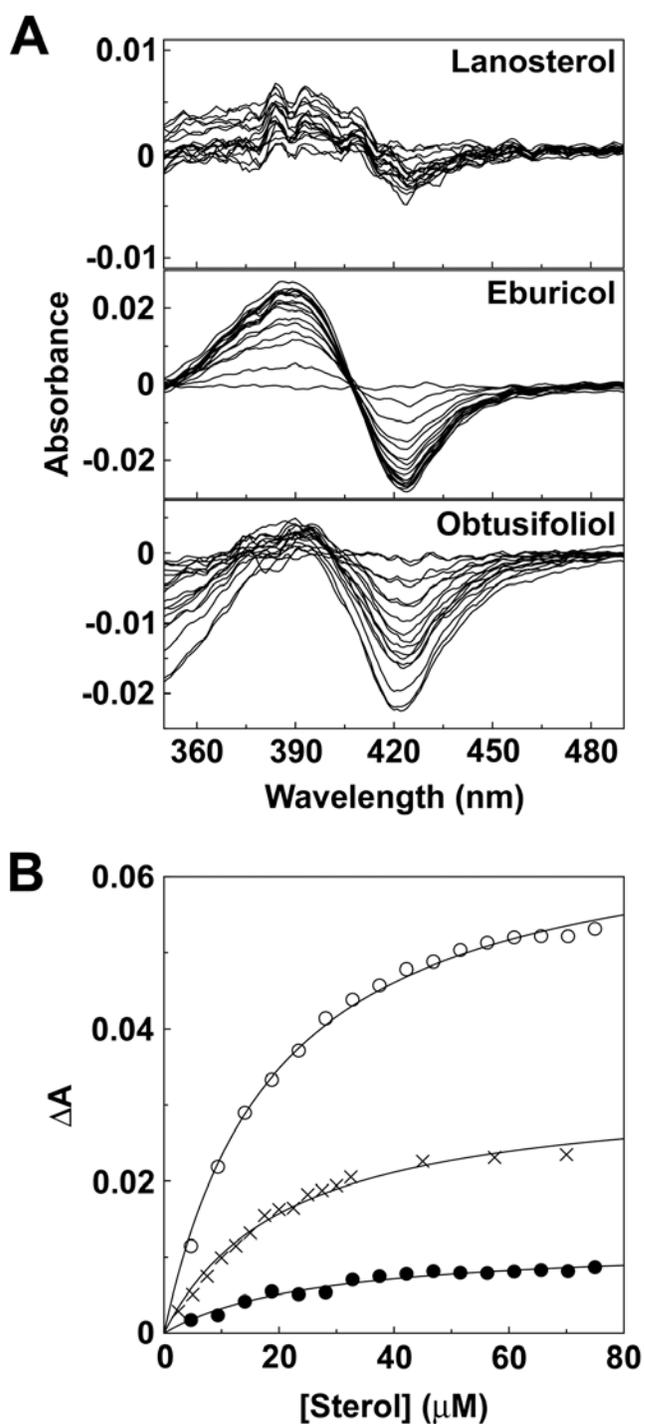


FIG. 2. Sterol binding properties of *Trub51*. Absorbance difference spectra (A) were measured during the progressive titration of 5  $\mu\text{M}$  *Trub51* with lanosterol, eburicol and obtusifoliol. Saturation curves (B) for lanosterol (filled circles), eburicol (hollow circles) and obtusifoliol (crosses) were constructed from the absorbance difference  $\Delta A_{388-421}$  of the type I difference spectra observed. Sterol binding data were fitted using the Michaelis-Menten equation.

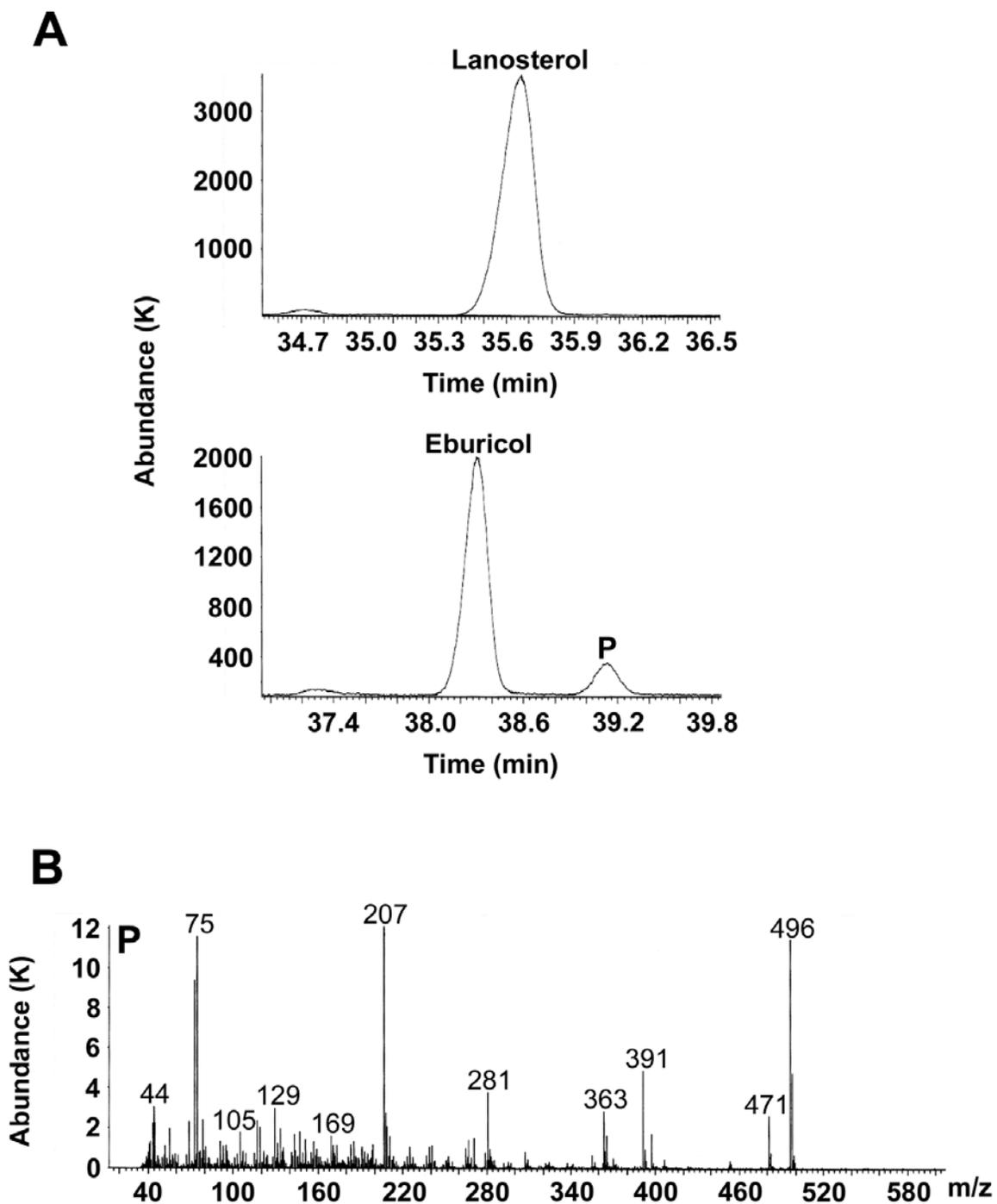


FIG. 3. GC/MS analysis of *Trub51* reconstitution assay metabolites. GC traces (A) for *Trub51* reconstitution assays (37°C, 15 min) using lanosterol and eburicol as substrates are shown. In addition the mass fragmentation pattern (B) for the TMS-derivatized C14-demethylated eburicol ( $M^+$  496 – 'P') product is shown. Abundance is expressed in units of one thousand (K).

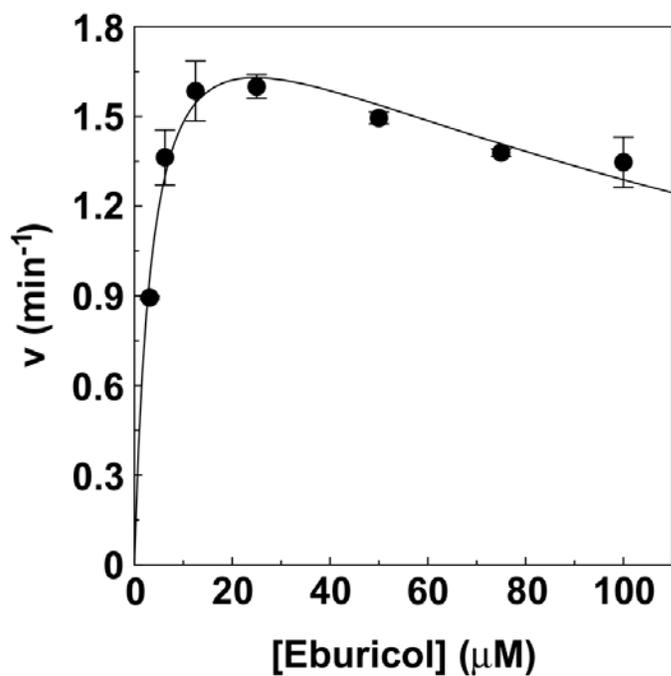


FIG. 4.  $K_m$  determination for eburicol. A velocity curve was constructed for eburicol with Trub51 using the CYP51 reconstitution assay (34, 55). The single substrate inhibition equation [ $v = (V_{max} \cdot [S]) / \{K_m + [S] \cdot (1 + [S]/K_i)\}$ ] (57) was used to fit the velocity curve. Mean values from three replicates and the associated standard deviation bars are shown.

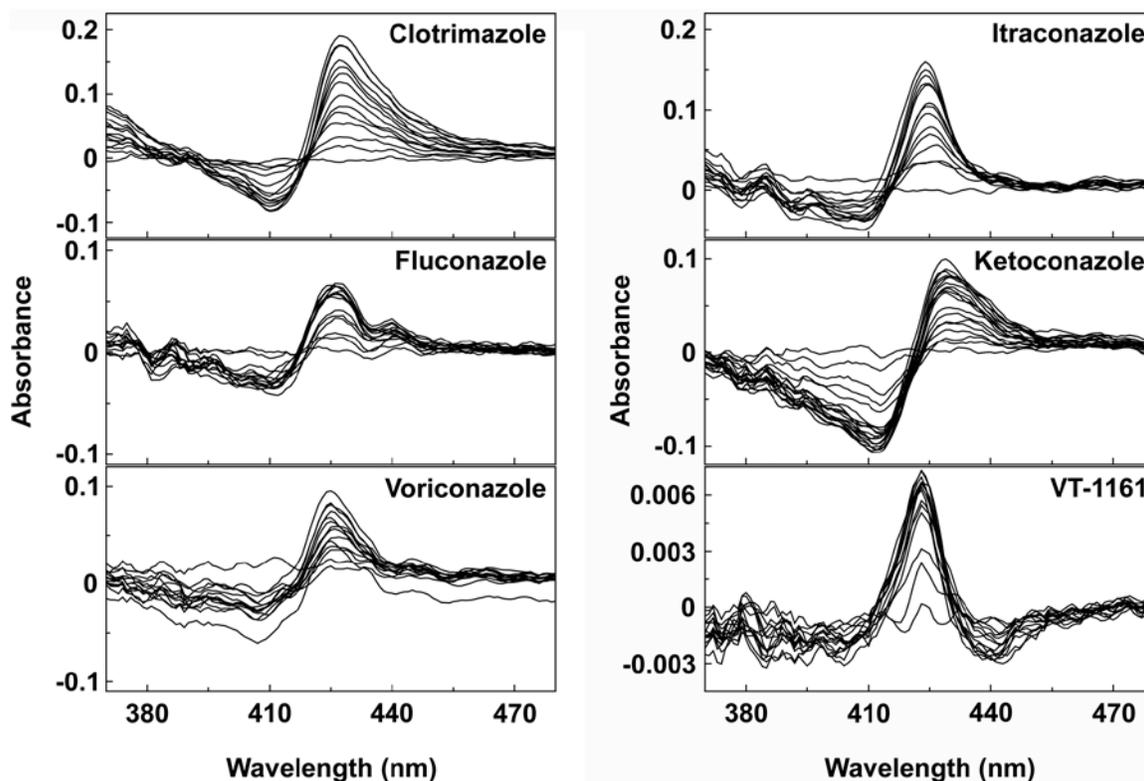


FIG. 5. Type II azole binding spectra for *Trub51*. Clotrimazole, fluconazole, voriconazole, itraconazole, ketoconazole and VT-1161 were progressively titrated against 2  $\mu$ M CYP51 protein with the difference spectra determined after each addition of azole. The resultant type II difference spectra obtained for each azole are shown. Each experiment was performed in triplicate although only one replicate is shown.

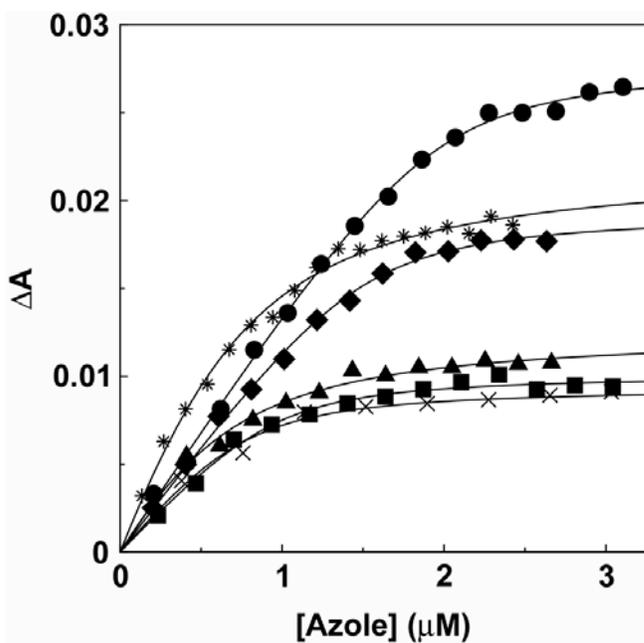


FIG. 6. Azole binding saturation curves for *Trub51*. Saturation curves were constructed from the absorbance difference  $\Delta A_{\text{peak-trough}}$  of the type II difference spectra (Fig. 5) for clotrimazole (circles), fluconazole (squares), voriconazole (triangles), itraconazole (diamonds), ketoconazole (asterisks) and VT-1161 (crosses). A rearrangement of the Morrison equation (25) was used to fit the tight ligand binding observed. Each experiment was performed in triplicate although only one replicate is shown.

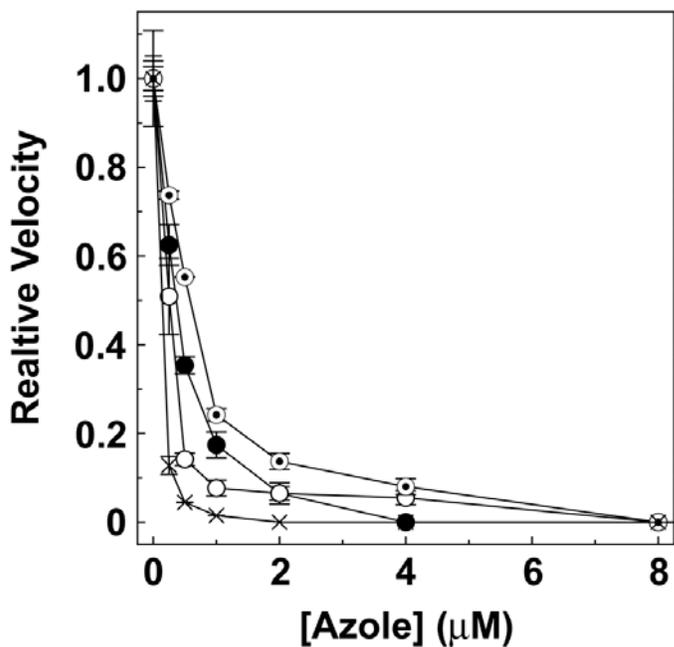


FIG. 7.  $IC_{50}$  determinations for antifungal agents. CYP51 reconstitution assays were performed using a CYP51:AfCPR ratio of 1:2 for 0.5  $\mu$ M Trub51 with 25  $\mu$ M eburicol as substrate at varying fluconazole (filled circles) itraconazole (hollow circles), ketoconazole (bullets) and VT-1161 (crosses) concentrations from 0 and 8  $\mu$ M. Mean relative velocity values are shown along with the associated standard deviation values. Relative velocities of 1.00 were equivalent to velocities of 1.55  $\text{min}^{-1}$ .

VT-1161 inhibition of *T. rubrum* CYP51

	10	20	30	40	50	60
Af51B	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....
Trub51	-----MG	LIAFILDGIC	KHCSTQSTWV	LVGIGLLSIL	AVSVIINVLO	QLLFFK--NPH
Mgram51	-----MG	LLADIVSRFC	ENCSTLSTAA	LVASAISAFI	VLSIVINVLO	QLLFRG--DPT
Af51A	-----	LLQEVLQAQFD	AQFGQTSLWK	LVGLGFLAFS	TLAILLNVLS	QLLFRG-KLS
Cneo51	MSAIIPQVQO	LLGQVAQFFP	PWFAALPTSL	KVAIAVVGIP	ALIIGLNVFQ	QLCLPR-RKD
Mglob51	-----	MLQEIG-AWP	VWQQALT---	----FLVGGL	ALIVGINVLV	QVLVPR-NKS
Calb51	-----MA	IVETVIDGIN	YFLSLSVT--	---QQISILL	GVPFVYNLVW	QYLYSL-RKD
ClustalX					. ** *	
	70	80	90	100	110	120
Af51B	..... .....	..... .....	..... .....	..... .....	..... .....	..... .....
Trub51	EPVVVFWHWP	FIGSTISYGI	DPYKFFDCCR	AKYGDIFTFI	LLGKKTIVYL	GTKGNDFILN
Mgram51	KPPVVFWHWP	IIGSTISYGI	DPYKFFDDCK	EKYGDIFTFI	LLGKKTIVFL	GTKGNDFILN
Af51A	DPPLVFWHWP	FIGSTITYGI	DPYKFFFCR	EKYGDVFTFI	LLGKKTIVCL	GTKGNDFILN
Cneo51	EPMPVFWHWP	YLGSTISYGI	DPYKFFACR	EKYGDIFTFI	LLGQKTIVYL	GVQNEFILN
Mglob51	LPPVVPHYIP	WFGSAAYYGE	DPYKFLFECR	DKYGDIFTFI	LMGRRITVAL	GPKGNLNLG
Calb51	LPPMVFWHWP	VVGSAITYGM	DPYRFFNCR	EKYGDVFTFK	LFGRNVIVAL	GPKGSNLVFN
ClustalX	.*:*.:.*	.*: **	:**.*: *	:* **	****:*:*	*:*: ** * * : * : : .
	130	140	150	160	170	180
		<u>SRS-1</u>				
Af51B	GKLRDVCAAE	VYSPLTTPVF	GRHVYDPCN	AKLMEQKKFV	KYGLTSDALR	SYVPLITDEV
Trub51	GKLRDVCAED	VYSPLTTPVF	GRHVYDPCN	SKLMEQKKFV	KFGLTSEALR	SYVTLITKEV
Mgram51	GKLRDVNAEE	IYSPLTTPVF	GKDVVYDPCN	SKLMEQKKFV	KYGLTTSALQ	SYVTLIAAET
Af51A	GKLRDVNAEE	VYSPLTTPVF	GSDVVYDPCN	SKLMEQKKFI	KYGLTQSALE	SHVPLIEKEV
Cneo51	GKISQVSAEE	AYTHLTPVF	GKGVVYDPCN	EMLMQKKFI	KSGLTTESLQ	SYPPMITSEC
Mglob51	GRLTQVSAEE	AYTSLTTPVF	GKGVVYDPCN	AVLMEQKRFV	KSGLSMENFR	MYVTQIESEV
Calb51	AKLSDVSAED	AYKHLTPVF	GKGVVYDPCN	SRLMEQKKFA	KFALTTDSFK	RYVPKIREEI
ClustalX	::: * **:	*. *****	* *:* **	**:*:*	*.*: . : .	: . * *
	190	200	210	220	230	240
						<u>SRS-2</u>
Af51B	ESFVKNS---	PAFQG--HKG	VFDVCKTIAE	ITIIYTASRSL	QGKEVRSKFD	STFAELYHNL
Trub51	EQFFESS---	PIFKG--DSG	VFNVSVMMAE	ITIIYTASRSL	QGKEVRGKFD	SSFALYSDL
Mgram51	RQFFDRNNPH	KKFAS--TSG	TIDLPPALAE	LTIYTASRSL	QGKEVREGFD	SSFADLYHYL
Af51A	LDYLRDS---	PNFQG--SSG	RVDISAAMAE	ITIFTAARAL	QGQEVRSKLT	AEFADLYHDL
Cneo51	EDFFTKEVGI	SPQKP---SA	TLDLLKAMSE	LIILTASRTL	QGKEVRESLN	GQFAKYEDL
Mglob51	KDFINNDAAF	LPLQKGATSV	TVDIFNVFSE	ITILTASRTL	QGKEVRESLD	KTFAKLYHDL
Calb51	LNPFVTDSEF	KLKEK--THG	VANVMKTQPE	ITIFTASRSL	FGDEMRRIFD	RSFAQLYSDL
ClustalX	.. .		:: . *	: * **:*:*	*.*: :	** * *
	250	260	270	280	290	300
		<u>SRS-3</u>				
Af51B	DMGFAPINFM	LPWAPLPHNR	KRDAAQRKLT	ETymeIIKAR	RQAGSKKDSE	-DMVWNLMSC
Trub51	DMGFAAINFM	FPWFPPHNR	KRDAAQRKMA	QVYTDIIRQR	RAAGGEKDSE	-DMVWNLMS
Mgram51	DMGFPTINFM	LPWAPLPQNR	RRDYAQKKMS	ETyMSIIQKR	RESKTGEHEE	-DMIHNLMQC
Af51A	DKGFPTINFM	LPWAPLPHNK	KRDAAHARMR	SIYVDIITQR	RLDGEKDSQK	SDMIWNLMC
Cneo51	DGGFTPLNFM	FPNLPLPSYK	RRDEAQKAMS	DFYLKIMENR	RK-GESD-HE	HDMIENLQSC
Mglob51	DSGFPTINFM	IPNLPLPNNF	RRDRAQRLMS	DFYLGIIKKR	RE-GNTEGTE	HDMISALMEQ
Calb51	DKGFPTINFM	FPNLPLPHYW	RRDAAQKKIS	ATYMKEIKSR	RERGDIDPNR	DLIDSLLIHS
ClustalX	* **:.***:	* *:*	:** *:	: * **:*:*	*.*: :	: *

VT-1161 inhibition of *T. rubrum* CYP51

	310	320	330	340	350	360
	.... ....	..	<u>SRS-4</u>	.... ....	.... ....	.... ....
Af51B	VYKNGTPVPD	EEIAHMMIAL	LMAGQHSSSS	TASWIVLRLA	TRPDIMEELY	QEQRVVLG-S
Trub51	VYKNGTPIPD	IEVAHMMIAL	LMAGQHSSSS	TGSWIVLRLA	SRPDILEELY	EEQKRVLG-E
Mgram51	KYKDGNAIPD	KEIAHMMIAL	LMAGQHSSSA	TESWITLRLA	SRPDIQDELL	QEQRKMLG-V
Af51A	TYKNGQQVPD	KEIAHMMITL	LMAGQHSSSS	ISAWIMLRLA	SQPKVLEELY	QEQLANLPGA
Cneo51	KYRNGVPLSD	RDIAHIMIAL	LMAGQHTSSA	TSSWTLLHLA	DRPDVVEALY	QEQRKQLG--
Mglob51	SYKNGRNIND	REIAHMMIAL	LMAGQHTSSA	TGSWAMLRLA	SRPEIIEELY	EEQKRIVYS--
Calb51	TYKDGVKMTD	QEIANLLIGI	LMGGQHTSAS	TSAWFLHLHG	EKPHLQDVIY	QEVVELLK--
ClustalX	*** :	* : : : : *	** .*** : :	* ** :	* : : :	* :
	370	380	390	400	410	420
	.... ....	.... ....	.... ....	..	<u>SRS-5</u>	.... ....
Af51B	DLP---PLT	YDNLQ-KLDL	HAKVIKETLR	LHAPIHSIIR	AVKNPMAVDG	-----
Trub51	DLP---PLT	YEALQ-KLDL	HNNVIKETLR	LHAPIHSILR	AVKSPMPVEG	-----
Mgram51	NADGSIKELT	YANLS-KLTL	LNQVVKETLR	IHAPIHSILR	KVKSPMPIEG	-----
Af51A	GPDGSLPPLQ	YKDLQ-KLPF	HQHVIRETLR	IHSSHSIMR	KVKSPMPVEG	-----
Cneo51	NPDGTFRDYR	YEDLK-ELPI	MDSIIRETLR	MHAPIHSIYR	KVLSDIPVPP	SLSAP----
Mglob51	DGTGGFAPLD	YDIQSSVPV	LDAVIRETLR	LHPPHSIMR	KVKSDMVVPP	TLAAPISSKG
Calb51	EKGGDLNDLT	YEDLQ-KLPS	VNNTIKETLR	MHMLPHSIFR	KVTNPLRIPE	-----
ClustalX	*	. :	: : : : *	* . : : *	* . : :	:
	430	440	450	460	470	480
	.... ....	.... ....	.... ....	.... ....	.... ....	.... ....
Af51B	---TSYVIPT	SHNVLSSPGV	TARSEEHPFN	PLEWNPWRWD	EN-----	-IAASAEDD-
Trub51	---TNYVVPT	SHNLLAAPGV	PSRDPQYFPD	PLVWNPWRWE	NN-----	-VGVTVVEAS
Mgram51	---TAYVIPT	THTLLAAPGT	TSRMEHPFD	CLHWEPWRWD	ESPSEKYKHL	SPTTALGSIA
Af51A	---TPYMIPP	GRVLLASPGV	TALSDEHPFN	AGCWDPWRWE	NQ-----	---ATKEQEN
Cneo51	SENGQYIIPK	GHYVIAAPGV	SQMDPRIWQD	AKVWNPWRWH	DEKG-----	F AAAAMVQYTK
Mglob51	SRDETYVIPK	GHYVIAAPGV	SQVDPKIWED	ASRFDPHRWL	GDK-----	--ANVMNQTD
Calb51	---TNYIVPK	GHYVLVSPGY	AHTSERYFDN	PEDFDPTRWD	TAAA-----	-KANSVSFNS
ClustalX	*** :	: : : *	. : : :	: : : *	* : : *	* : : :
	490	500	510	520	530	540
	.... ....	.... ....	<u>Heme-Cys</u>	.... ....	.... ....	.... ....
Af51B	-EKVDYGYGL	VSKGTNSPYL	PFGAGRHRCI	GEQFAYLQLG	TITAVLVRLF	RFRNLPG-V
Trub51	EEKTDYGYGL	VSKGANSPYL	PFGSGRHRCI	GEQFAYVQLG	TVTATLARLM	RWKQVEGTKD
Mgram51	EEKEDYGYGL	VSKGAASPYL	PFGAGRHRCI	GEQFAYVQLQ	TITATMVRDF	KFYVNDG-SD
Af51A	DKVVDYGYGA	VSKGTSSPYL	PFGAGRHRCI	GEKFAYVNLG	VILATIVRHL	RLFNVDG-KK
Cneo51	AEQVDYGFSG	VSKGTESPYQ	PFGAGRHRCV	GEQFAYTQLS	TIFTYVVRNF	TLKLAVP---
Mglob51	DAQEDFGWGM	VSTGANSPYL	PFGAGRHRCI	GEQFAYLQLG	TIISTFVRAF	DWRLET----
Calb51	SDEVYDYGFK	VSKGVSSPYL	PFGGRHRCI	GEQFAYVQLG	TILTTFVYNL	RWTIDGY---
ClustalX	*** :	** . * : *	*** .*** : *	** : ** :	* : : . :	:
	550	560	570			
	....	<u>SRS-6</u>	.... ....	...		
Af51B	GIPD	DYSSL FSKPL	GRSFV	EFEKRESATK	A--	
Trub51	VVPP	DYSSL FSKPF	GPNMV	SWEKRKQASQ	K--	
Mgram51	NVVG	DYSSL FSRPL	SPAVV	KWERREEKEE	KN-	
Af51A	GVPET	DYSSL FSGPM	KPSII	GWEKRKNTS	K--	
Cneo51	KFPET	NYRTM IVQPN	NPL-V	TFTLRNAEVK	QEV	
Mglob51	KLPAP	DYTSM VVLPT	QPANL	VFTPRKNKA-	---	
Calb51	KVPDP	DYSSM VVLPT	EPAEI	IWEKRETCMF	---	
ClustalX	.	: * : :	. * :	: * :	.	

**Supplementary figure S1.** Sequence alignment of selected fungal CYP51 enzymes. This alignment shows the six substrate recognition sites (SRS) according to Strushkevich et al (1) and the twenty-three conserved CYP51 amino acid residues (2) in orange as well as the conserved heme-binding cysteine residue in red. The fungal CYP51 sequences aligned were *Aspergillus fumigatus* CYP51 isoenzyme A (Af51A – UniProt accession number Q4WNT5), *Aspergillus fumigatus* CYP51 isoenzyme B (Af51B – Q96W81), *Candida albicans* CYP51 (Calb51 – P10613), *Cryptococcus neoformans* CYP51 (Cneo51 – Q5KQ65), *Malassezia globosa* CYP51 (Mglob51 – A8Q3I7), *Mycosphaerella graminicola* CYP51 (Mgram51 – Q5XWE5) and *Trichophyton rubrum* CYP51 (Trub51 – F2SHH3). ClustalX consensus sequence indicates absolutely conserved residues (\*), conserved strong (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) groups (:), and conserved weaker (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY) groups (.) (<http://www.clustal.org>).

## REFERENCES

1. Strushkevich N, Usanov SA, Park HW. 2010. Structural basis of human CYP51 inhibition by antifungal azoles. *J Mol Biol* 397:1067-1078.
2. Lepesheva GI, Waterman MR. 2011. Structural basis for conservation in the CYP51 family. *Biochim Biophys Acta* 1814:88-93.