The Tetrazole VT-1161 Is a Potent Inhibitor of *Trichophyton rubrum* through its 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 μM), but displayed substrate specificity insofar as only eburicol was demethylated in CYP51 reconstitution assays (turnover number 1.55 min^{-1}, K_m value 2 μM). The investigational agent VT-1161 bound tightly to *T. rubrum* CYP51 (K_d = 242 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 nM). IC_{50} determinations using 0.5 μM CYP51 showed VT-1161 was a tight-binding inhibitor of *T. rubrum* CYP51 activity yielding an IC_{50} value of 0.14 μM compared to 0.26, 0.4 and 0.6 μM for itraconazole, fluconazole and ketoconazole, respectively. When tested against 34 clinical isolates, VT-1161 was a potent inhibitor of *T. rubrum* growth with MIC_{50}, MIC_{90}, and geometric mean MIC values of ≤0.03, 0.06, and 0.033 μg 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 corporus 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 (±80) nmoles per liter culture as determined by carbon monoxide difference spectroscopy (20). Purification by Ni^{2+}-NTA agarose chromatography resulted in an 84% recovery of native Trub51 protein yielding a stock 48 µM solution after dialysis. SDS polyacrylamide gel electrophoresis confirmed the purity of the Ni^{2+}-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 α, β, Soret (γ) and δ 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 ±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

(ΔA_{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 ±1.2 μM, 22.7 ±3.6 μM and 20.9 ±0.3 μM were obtained for eburicol, lanosterol and obtusifoliol, respectively, indicating all three sterols bound with similar affinity.

CYP51 reconstitution assays. Trub51 did not catalyze the 14α-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 14α-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 μM 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 μM and 225 μM, respectively. The maximum eburicol turnover number was 1.55 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 (±29) nM whilst clotrimazole, fluconazole, voriconazole, ketoconazole and VT-1161 all apparently bound less tightly to Trub51 with similar K_d values of 179 (±83), 173 (±53), 304 (±64), 312 (±36) and 242 (±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 μM), followed by itraconazole (IC_{50} 0.26 μM), then fluconazole and ketoconazole (IC_{50} values 0.4 and 0.6 μM). Given the concentration of CYP51 used in this assay was 0.5 μM, the expected IC_{50} value for an extremely tight-binding azole antifungal would be 0.25 μM. 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 μg ml^{-1}) to the highest MIC values of 0.06 μg ml^{-1}. VT-1161's MIC_{50}, MIC_{90} and geometric mean values of ≤0.03, 0.06, and 0.033 μg ml^{-1} were slightly less than those for itraconazole (0.06, 0.06, and 0.052 μg ml^{-1}, respectively), and both of these CYP51 inhibitors were significantly more potent that fluconazole (2, 16, and 2.3 μg 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 μM were comparable with CYP51 enzymes from *Candida albicans* (11 to 28 μM) (32), *Mycosphaerella graminicola* (11 to 13 μM) (33), *Aspergillus fumigatus* CYP51B (9 to 23 μM) (29), *Cryptococcus neoformans* (12 to 21 μM) (30) and *Malassezia globosa* (23 to 32 μM) (31). However, the K_d values for lanosterol and eburicol with *Trypanosoma cruzei* CYP51 were lower at 1.9 and 1.2 μM (34), and the K_d values for lanosterol with *H. sapiens* and *Mycobacterium tuberculosis* CYP51s were lower than Trub51 at 0.5 to 6 μM (28, 35) and 1 μM (21), respectively. However, Trub51 only catalyzed the 14α-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 μM was comparable to the substrate K_m values previously obtained for CYP51

enzymes from *C. albicans* and *Saccharomyces cerivisiae* (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 ≤0.03 to 0.06 μg/ml, slightly more potent than for itraconazole (≤0.03 to 0.12 μg/ml) and significantly more potent than for fluconazole (0.5 to >64 μg/ml). This MIC potency range for VT-1161 compares favorably to published MIC values for *T. rubrum* of 0.03 to 256 μg ml^{-1} for fluconazole, 0.008 to 0.25 μg ml^{-1} for itraconazole, 0.06 to 2 μg ml^{-1} for ketoconazole and 0.06 to 1 μg 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).

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_{50} 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$^+$: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$^+$ 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$^+$ expression vector. Gene integrity was confirmed by DNA sequencing.

Heterologous expression and purification of recombinant Trub51 protein. The pCWori$^+$:*Trub51* construct was transformed into competent DH5α *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^{2+}-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 mM^{-1} 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 mg ml^{-1} solutions of lanosterol, obtusifoliol and eburicol were prepared in 40% (wt/vol) (2-hydroxypropyl)-β-cyclodextrin (HPCD). Sterol was progressively titrated against 5 μM 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 μM Trub51. The absorbance difference spectrum between 500 and 350 nm was determined after each incremental addition of sterol (up to 75 μM). 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 mg ml^{-1} solutions of the azoles were prepared in dimethylsulfoxide and progressively titrated against 2 μM of Trub51 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 $\Delta A_{peak\text{-}trough}$ 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 μM Trub51, 1 μM *Aspergillus fumigatus* cytochrome P450 reductase isoenzyme 1 (AfCPR1 - UniProt accession number Q4WM67), 50 μM sterol substrate, 50 μM dilaurylphosphatidylcholine, 4% (wt/vol) HPCD, 0.4 mg ml^{-1} isocitrate dehydrogenase, 25 mM trisodium isocitrate, 50 mM NaCl, 5 mM $MgCl_2$ and 40 mM MOPS (pH ~7.2). Assay mixtures were incubated at 37°C prior to initiation with 4 mM β-$NADPHNa_4$ followed by shaking at 37°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°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 μM whilst maintaining a constant HPCD concentration of 4% (wt/vol). The single substrate inhibition equation [$v = (V_{max}.[S])/\{K_m + [S].(1+[S]/K_i)\}$] (57) was used to fit the data and to determine K_m and K_i values.

Azole IC_{50} determinations. IC_{50} 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-$NADPHNa_4$. The IC_{50} assays contained 25 µM eburicol, 0.5 µM Trub51, 1 µM AfCPR1 and 4 mM β-$NADPHNa_4$.

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 10^4, 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^{-1}, and for fluconazole from 0.125-64 µg ml^{-1}. *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 $\log_2$ 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^{-1}, 128 μg ml^{-1} 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 μg ml^{-1}, 0.03 μg 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-aminolevulenic acid were obtained from Foremedium Ltd (Hunstanton, UK). Ni^{2+}-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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Table 1. MICs of VT-1161, itraconazole, and fluconazole against 34 clinical isolates of *T. rubrum*.

	MIC (µg ml^{-1})			
Drug	50%	90%	Geometric mean	Range
VT-1161	≤0.03	0.06	0.033	≤0.03 – 0.06
Itraconazole	0.06	0.06	0.052	≤0.03 – 0.12
Fluconazole	2	16	2.3	0.5 – >64

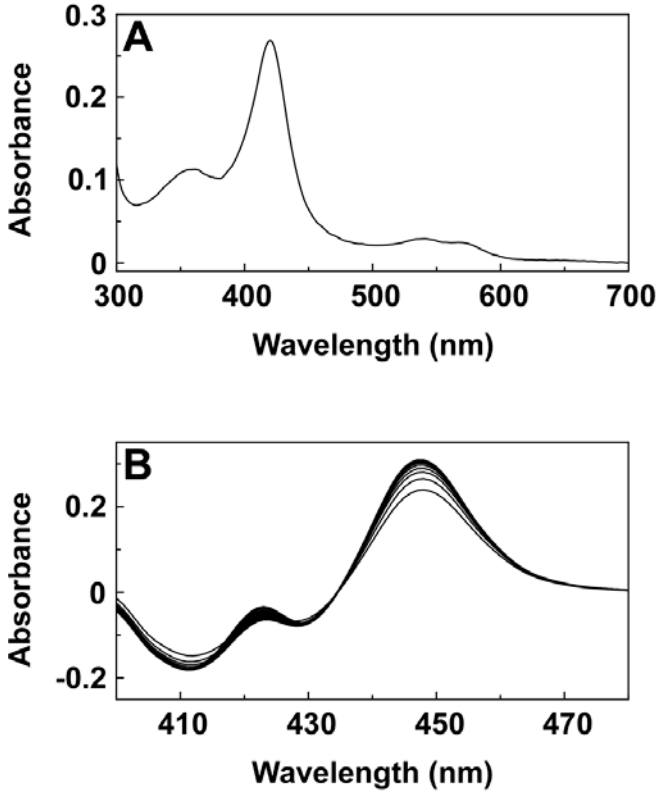

FIG. 1. *Spectral characteristics of Trub51.* Absolute spectra (A) were determined using 3 μM purified Trub51 in the oxidised resting state. Reduced carbon monoxide difference spectra (B) were determined using 3 μM purified Trub51 with sequential measurements made every 45 seconds.

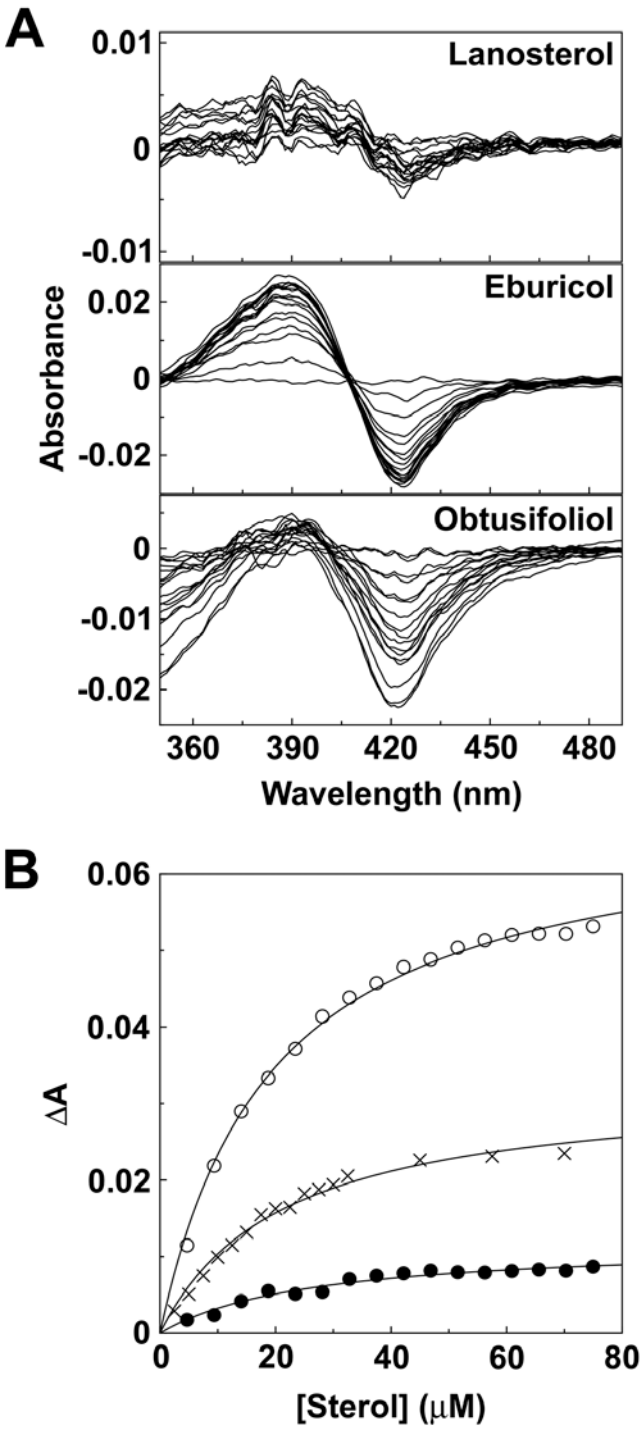

FIG. 2. *Sterol binding properties of Trub51.* Absorbance difference spectra (A) were measured during the progressive titration of 5 μM Trub51 with lanosterol, eburicol and obtusifoliol. Saturation curves (B) for lanosterol (filled circles), eburicol (hollow circles) and obtusifoliol (crosses) were constructed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

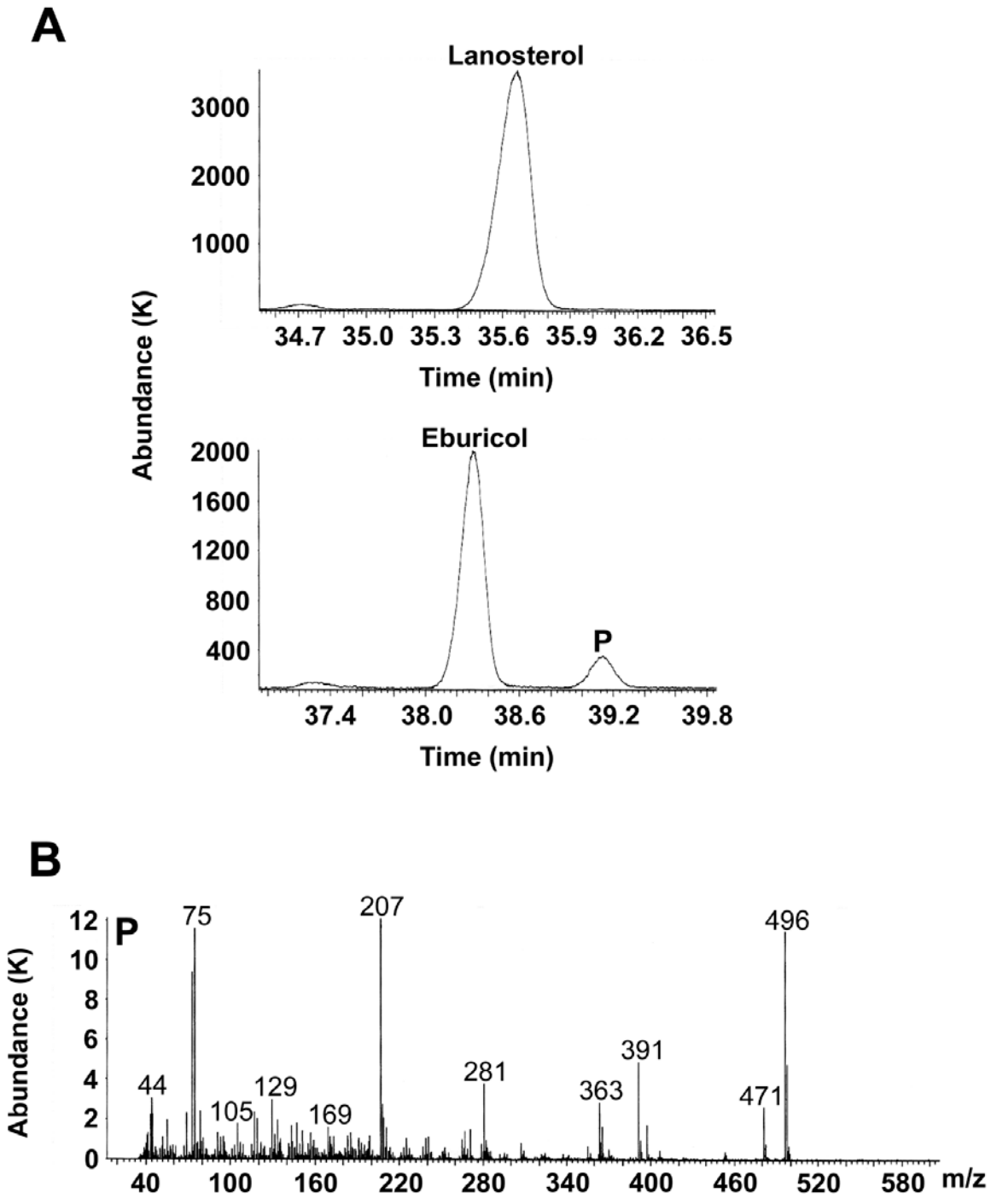

FIG. 3. *GC/MS analysis of Trub51 reconstitution assay metabolites.* GC traces (A) for Trub51 reconstitution assays (37°C, 15 min) using lanosterol and eburicol as substrates are shown. In addition the mass fragmentation pattern (B) for the TMS-derivatized C14-demethylated eburicol (M^+ 496 – 'P') product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

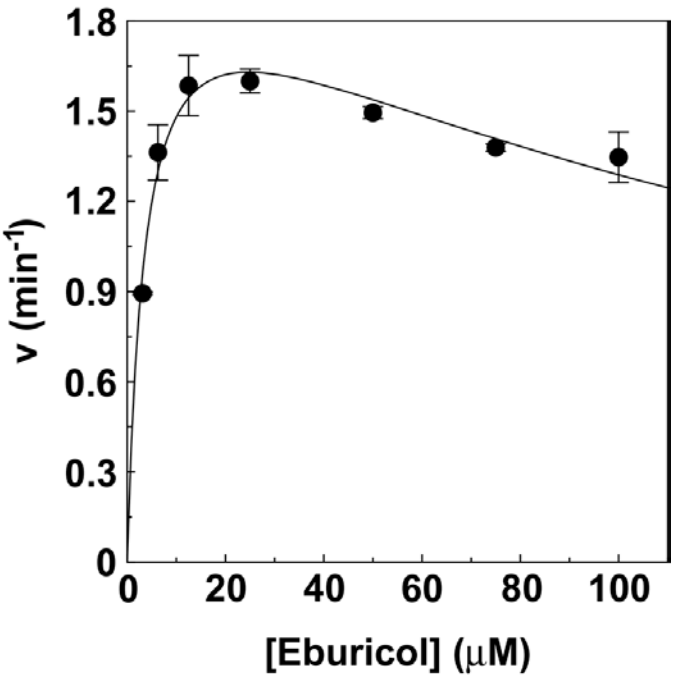

FIG. 4. *K_m determination for eburicol.* A velocity curve was constructed for eburicol with Trub51 using the CYP51 reconstitution assay (34, 55). The single substrate inhibition equation [v = (V_{max}.[S])/{K_m + [S].(1+[S]/K_i)}] (57) was used to fit the velocity curve. Mean values from three replicates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

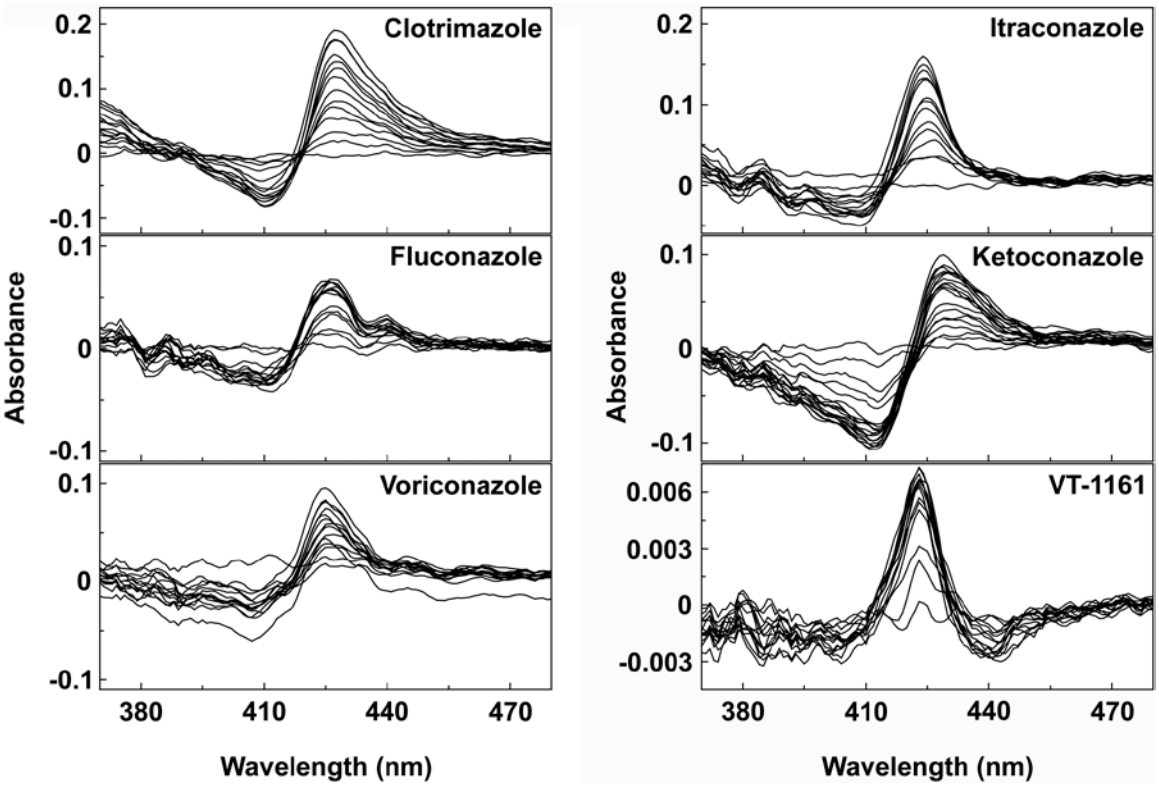

FIG. 5. *Type II azole binding spectra for Trub51.* Clotrimazole, fluconazole, voriconazole, itraconazole, ketoconazole and VT-1161 were progressively titrated against 2 μM CYP51 protein with the difference spectra determined after each addition of azole. The resultant type II difference spectra obtained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

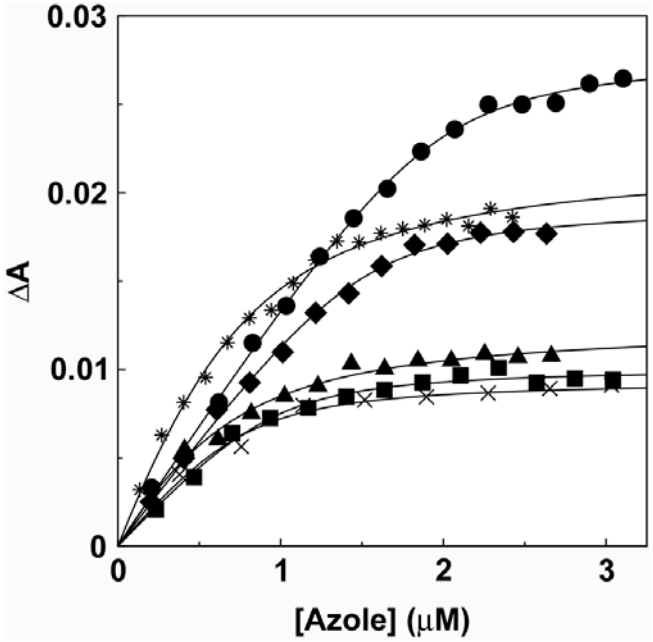

FIG. 6. *Azole binding saturation curves for Trub51.* Saturation curves were constructed from the absorbance difference $\Delta A_{peak\text{-}trough}$ of the type II difference spectra (Fig. 5) for clotrimazole (circles), fluconazole (squares), voriconazole (triangles), itraconazole (diamonds), ketoconazole (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.

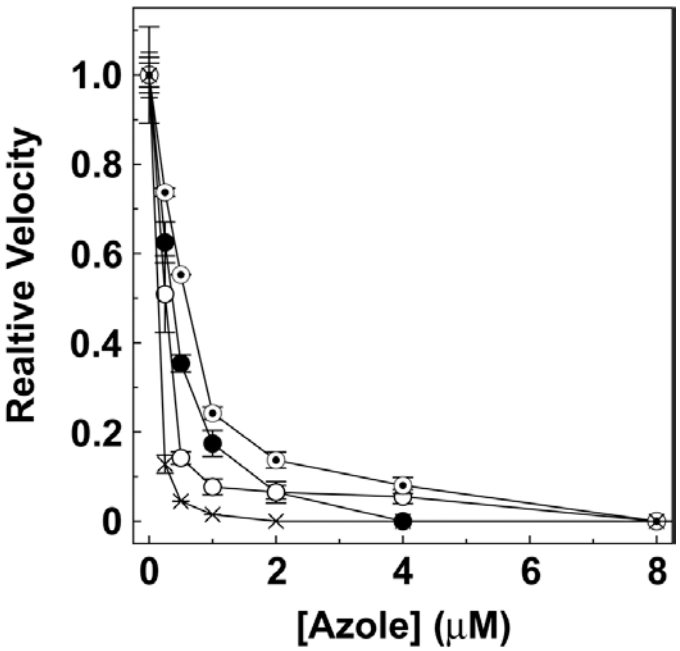

FIG. 7. *IC_{50} determinations for antifungal agents.* CYP51 reconstitution assays were performed using a CYP51:AfCPR ratio of 1:2 for 0.5 μM Trub51 with 25 μM eburicol as substrate at varying fluconazole (filled circles) itraconazole (hollow circles), ketoconazole (bullets) and VT-1161 (crosses) concentrations from 0 and 8 μM. 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 min^{-1}.

```
                10         20         30         40         50         60
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Af51B   --------MG LIAFILDGIC KHCSTQSTWV LVGIGLLSIL AVSVIINVLQ QLLFK--NPH
Trub51  --------MG LLADIVSRFC ENCSTLSTAA LVASAISAFI VLSIVINVLQ QLLFK--DPT
Mgram51 --------MG LLQEVLAQFD AQFGQTSLWK LVGLGFLAFS TLAILLNVLS QLLFRG-KLS
Af51A   ---------- ---------- ----MVPMLW LTAYMAVAVL T-AILLNVVY QLFFRLWNRT
Cneo51  MSAIIPQVQQ 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   EPPVVFHWFP FIGSTISYGI DPYKFFFDCR AKYGDIFTFI LLGKKTTVYL GTKGNDFILN
Trub51  KPPVVFHWFP IIGSTISYGI DPYKFFDDCK EKYGDIFTFI LLGKKTTVFL GTKGNDFILN
Mgram51 DPPLVFHWVP FIGSTITYGI DPYKFFFSCR EKYGDVFTFI LLGKKTTVCL GTKGNDFILN
Af51A   EPPMVFHWVP YLGSTISYGI DPYKFFFACR EKYGDIFTFI LLGQKTTVYL GVQGNEFILN
Cneo51  LPPVVFHYIP WFGSAAYYGE DPYKFLFECR DKYGDLFTFI LMGRRITVAL GPKGNNLSLG
Mglob51 LPPMVFHWVP VVGSAITYGM DPYRFFFNCR EKYGDVFTFK LFGRNVTVAL GPKGSNLVFN
Calb51  RAPLVFYWIP WFGSAASYGQ QPYEFFESCR QKYGDVFSFM LLGKIMTVYL GPKGHEFVFN
ClustalX .*:**::.*  .**:  **  :**.*:  *:  ****:*:*  *:*:  ** * * :* :: :.

                130        140        150        160        170        180
        ....|....SRS-1_____| ....|....| ....|....| ....|....| ....|....|
Af51B   GKLRDVCAEE VYSPLTTPVF GRHVVYDCPN AKLMEQKKFV KYGLTSDALR SYVPLITDEV
Trub51  GKLKDVCAED VYSPLTTPVF GRHVVYDCPN SKLMEQKKFV KFGLTSEALR SYVTLITKEV
Mgram51 GKLKDVNAEE IYSPLTTPVF GKDVVYDCPN SKLMEQKKFV KYGLTTSALQ SYVTLIAAET
Af51A   GKLKDVNAEE VYSPLTTPVF GSDVVYDCPN SKLMEQKKFI KYGLTQSALE SHVPLIEKEV
Cneo51  GKISQVSAEE AYTHLTTPVF GKGVVYDCPN EMLMQQKKFI KSGLTTESLQ SYPPMITSEC
Mglob51 GRLTQVSAEE AYTSLTTPVF GKGVVYDVPN AVLMEQKRFV KSGLSMENFR MYVTQIESEV
Calb51  AKLSDVSAED AYKHLTTPVF GKGVIYDCPN SRLMEQKKFA KFALTTDSFK RYVPKIREEI
ClustalX .:: :* **:  *. ****** *  *:** **   **:**:*  * .*: . :.  : . *  *

                190        200        210        220        230        240
        ....|....| ....|....| ....|....| ....|....| ....|....| ....|SRS-2
Af51B   ESFVKNS--- PAFQG--HKG VFDVCKTIAE ITIYTASRSL QGKEVRSKFD STFAELYHNL
Trub51  EQFFESS--- PIFKG--DSG VFNVSKVMAE ITIYTASRSL QGKEVRGKFD SSFAELYSDL
Mgram51 RQFFDRNNPH KKFAS--TSG TIDLPPALAE LTIYTASRSL QGKEVREGFD SSFADLYHYL
Af51A   LDYLRDS--- PNFQG--SSG RVDISAAMAE ITIFTAARAL QGQEVRSKLT AEFADLYHDL
Cneo51  EDFFTKEVGI SPQKP---SA TLDLLKAMSE LIILTASRTL QGKEVRESLN GQFAKYYEDL
Mglob51 KDFINNDAAF LPLQKGATSV TVDIFNVFSE ITILTASRTL QGKEVRESLD KTFAKLYHDL
Calb51  LNYFVTDESF KLKEK--THG VANVMKTQPE ITIFTASRSL FGDEMRRIFD RSFAQLYSDL
ClustalX  .:.  .                ::  . .* : * **:*:*  *.*:*  :    **. *  *

                250        260        270        280        290        300
        ___.|....| ....|...SRS-3___| ....|....| ....|....| ....|....|
Af51B   DMGFAPINFM LPWAPLPHNR KRDAAQRKLT ETYMEIIKAR RQAGSKKDSE -DMVWNLMSC
Trub51  DMGFAAINFM FPWFPFPHNR KRDRAQRKMA QVYTDIIRQR RAAGGEKDSE -DMVWNLMSS
Mgram51 DMGFTPINFM LPWAPLPQNR RRDYAQKKMS ETYMSIIQKR RESKTGEHEE -DMIHNLMQC
Af51A   DKGFTPINFM LPWAPLPHNK KRDAAHARMR SIYVDIITQR RLDGEKDSQK SDMIWNLMNC
Cneo51  DGGFTPLNFM FPNLPLPSYK RRDEAQKAMS DFYLKIMENR RK-GESD-HE HDMIENLQSC
Mglob51 DSGFTPINFV IPNLPLPNNF RRDRAQRLMS DFYLGIIKKR RE-GNTEGTE HDMISALMEQ
Calb51  DKGFTPINFV FPNLPLPHYW RRDAAQKKIS ATYMKEIKSR RERGDIDPNR DLIDSLLIHS
ClustalX * **:.:**: :*  *:*    :** *:  :    *   :  * *     .  .   :   *
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                310        320        330        340        350        360
          ....|....| ..______SRS-4_____ ....|....| ....|....| ....|....|
Af51B     VYKNGTPVPD EEIAHMMIAL LMAGQHSSSS TASWIVLRLA TRPDIMEELY QEQIRVLG-S
Trub51    VYKNGTPIPD IEVAHMMIAL LMAGQHSSSS TGSWIVLRLA SRPDILEELY EEQKRVLG-E
Mgram51   KYKDGNAIPD KEIAHMMIAL LMAGQHSSSA TESWITLRLA SRPDIQDELL QEQKDMLG-V
Af51A     TYKNGQQVPD KEIAHMMITL LMAGQHSSSS ISAWIMLRLA SQPKVLEELY QEQLANLGPA
Cneo51    KYRNGVPLSD RDIAHIMIAL LMAGQHTSSA TSSWTLLHLA DRPDVVEALY QEQKQKLG--
Mglob51   SYKNGRNIND REIAHMMIAL LMAGQHTSSA TGSWAMLRLA SRPEIIEELY EEQKRVYS--
Calb51    TYKDGVKMTD QEIANLLIGI LMGGQHTSAS TSAWFLLHLG EKPHLQDVIY QEVVELLK--
ClustalX   *::*  : *  ::*:::* : **.***:*::   :*  *:*.  :*.: : :  :*

                370        380        390        400        410        420
          ....|....| ....|....| ....|....| ..__SRS-5_ ....|....| ....|....|
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 YKDLD-KLPF HQHVIRETLR IHSSIHSIMR KVKSPLPVPG ----------
Cneo51    NPDGTFRDYR YEDLK-ELPI MDSIIRETLR MHAPIHSIYR KVLSDIPVPP SLSAP-----
Mglob51   DGTGGFAPLD YDIQKSSVPV LDAVIRETLR LHPPIHSIMR KVKSDMVVPP TLAAPISSKG
Calb51    EKGGDLNDLT YEDLQ-KLPS VNNTIKETLR MHMPLHSIFR KVTNPLRIPE ----------
ClustalX            *   . .:       ::**** :* .:*** *  * . : :

                430        440        450        460        470        480
          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
Af51B     ---TSYVIPT SHNVLSSPGV TARSEEHFPN PLEWNPHRWD EN-------- -IAASAEDD-
Trub51    ---TNYVVPT SHNLLAAPGV PSRDPQYFPD PLVWNPHRWE NN-------- -VGVTVVEAS
Mgram51   ---TAYVIPT THTLLAAPGT TSRMDEHFPD CLHWEPHRWD ESPSEKYKHL SPTTALGSIA
Af51A     ---TPYMIPP GRVLLASPGV TALSDEHFPN AGCWDPHRWE NQ-------- ---ATKEQEN
Cneo51    SENGQYIIPK GHYIMAAPGV SQMDPRIWQD AKVWNPARWH DEKG-----F AAAAMVQYTK
Mglob51   SRDETYVIPK GHYVIAAPGV SQVDPKIWED ASRFDPHRWL GDK------- --ANVMNQTD
Calb51    ---TNYIVPK GHYVLVSPGY AHTSERYFDN PEDFDPTRWD TAAA------ -KANSVSFNS
ClustalX      *::*   : :: :**  .    . : :    ::* **

                490        500        510        520        530        540
          ....|....| ....|....| ____Heme-Cys_____...| ....|....| ....|....|
Af51B     -EKVDYGYGL VSKGTNSPYL PFGAGRHRCI GEQFAYLQLG TITAVLVRLF RFRNLPG-VD
Trub51    EEKTDYGYGL VSKGANSPYL PFGSGRHRCI GEQFAYVQLG TVTATLARLM RWKQVEGTKD
Mgram51   EEKEDYGYGL VSKGAASPYL PFGAGRHRCI GEQFAYVQLQ TITATMVRDF KFYNVDG-SD
Af51A     DKVVDYGYGA VSKGTSSPYL PFGAGRHRCI GEKFAYVNLG VILATIVRHL RLFNVDG-KK
Cneo51    AEQVDYGFGS VSKGTESPYQ PFGAGRHRCV GEQFAYTQLS TIFTYVVRNF TLKLAVP---
Mglob51   DAQEDFGWGM VSTGANSPYL PFGAGRHRCI GEQFAYLQLG TIISTFVRAF DWRLET----
Calb51    SDEVDYGFGK VSKGVSSPYL PFGGGRHRCI GEQFAYVQLG TILTTFVYNL RWTIDGY---
ClustalX     *:*:*  **.*. ***  ***.*****: **:*** :*  .: : ..  :

                550        560        570
          ....|___SRS-6___....| ....|....| ...
Af51B     GIPDTDYSSL FSKPLGRSFV EFEKRESATK A--
Trub51    VVPPTDYSSL FSKPFGNPMV SWEKRKQASQ K--
Mgram51   NVVGTDYSSL FSRPLSPAVV KWERREEKEE KN-
Af51A     GVPETDYSSL FSGPMKPSII GWEKRSKNTS K--
Cneo51    KFPETNYRTM IVQPNNPL-V TFTLRNAEVK QEV
Mglob51   KLPAPDYTSM VVLPTQPANL VFTPRKNKA- ---
Calb51    KVPDPDYSSM VVLPTEPAEI IWEKRETCMF ---
ClustalX   .  .:* :: .  *     :  :  *.
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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/).

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