



Swansea University  
Prifysgol Abertawe



## Cronfa - Swansea University Open Access Repository

---

This is an author produced version of a paper published in:  
*Antimicrobial Agents and Chemotherapy*

Cronfa URL for this paper:

<http://cronfa.swan.ac.uk/Record/cronfa29154>

---

### Paper:

Warrilow, A., Parker, J., Price, C., Nes, W., Garvey, E., Hoekstra, W., Schotzinger, R., Kelly, D. & Kelly, S. (2016). The Investigational Drug VT-1129 Is a Highly Potent Inhibitor of Cryptococcus Species CYP51 but Only Weakly Inhibits the Human Enzyme. *Antimicrobial Agents and Chemotherapy*, 60(8), 4530-4538.  
<http://dx.doi.org/10.1128/AAC.00349-16>

---

This item is brought to you by Swansea University. Any person downloading material is agreeing to abide by the terms of the repository licence. Copies of full text items may be used or reproduced in any format or medium, without prior permission for personal research or study, educational or non-commercial purposes only. The copyright for any work remains with the original author unless otherwise specified. The full-text must not be sold in any format or medium without the formal permission of the copyright holder.

Permission for multiple reproductions should be obtained from the original author.

Authors are personally responsible for adhering to copyright and publisher restrictions when uploading content to the repository.

<http://www.swansea.ac.uk/iss/researchsupport/cronfa-support/>

1 **The Investigational Drug VT-1129 is a Highly Potent Inhibitor**  
2 **of *Cryptococcus* species CYP51 but only Weakly Inhibits the**  
3 **Human Enzyme.**

4  
5 Andrew G.S. Warrillow<sup>a</sup>, Josie E. Parker<sup>a</sup>, Claire L. Price<sup>a</sup>, W. David Nes<sup>b</sup>, Edward P.  
6 Garvey<sup>c</sup>, William J. Hoekstra<sup>c</sup>, Robert J. Schotzinger<sup>c</sup>, Diane E. Kelly<sup>a</sup> and Steven L.  
7 Kelly<sup>a\*</sup>

8  
9 Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University  
10 Medical School, Swansea, Wales SA2 8PP, United Kingdom<sup>a</sup>; Center for Chemical  
11 Biology, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock,  
12 Texas 79409-1061, USA<sup>b</sup>; Viamet Pharmaceuticals, Inc., Durham, NC 27703, USA<sup>c</sup>

13  
14 **Running title:** VT-1129 and cryptococcal CYP51s.

15 **Keywords:** CYP51, VT-1129, *Cryptococcus*, azole antifungal.

16  
17 \*Corresponding author.

18 Mailing address: Institute of Life Science, Swansea University Medical School,  
19 Swansea, Wales SA2 8PP, United Kingdom. Phone: +44 1792 292207 Fax: +44 1792  
20 503430 Email: [s.l.kelly@swansea.ac.uk](mailto:s.l.kelly@swansea.ac.uk)

21

22

23 **Cryptococcosis is a life-threatening disease often associated with HIV infection.**  
24 **Three *Cryptococcus* species CYP51 enzymes were purified and catalyzed the 14 $\alpha$ -**  
25 **demethylation of lanosterol, eburicol and obtusifoliol. The investigational agent**  
26 **VT-1129 bound tightly to all three CYP51 proteins (dissociation constant [ $K_d$ ]**  
27 **range, 14 to 25 nM) with affinities similar to those of fluconazole, voriconazole,**  
28 **itraconazole, clotrimazole, and ketoconazole ( $K_d$  range, 4 to 52 nM), whereas VT-**  
29 **1129 bound weakly to human CYP51 ( $K_d$ , 4.53  $\mu$ M). VT-1129 was as effective as**  
30 **conventional triazole antifungal drugs at inhibiting cryptococcal CYP51 activity**  
31 **(50% inhibitory concentration [ $IC_{50}$ ] range, 0.14 to 0.20  $\mu$ M), while it only weakly**  
32 **inhibited human CYP51 activity ( $IC_{50}$ , ~600  $\mu$ M). Furthermore, VT-1129 weakly**  
33 **inhibited human CYP2C9, CYP2C19, and CYP3A4, suggesting a low drug-drug**  
34 **interaction potential. Finally, the cellular mode of action for VT-1129 was**  
35 **confirmed to be CYP51 inhibition, resulting in the depletion of ergosterol and**  
36 **ergosta-7-enol and the accumulation of eburicol, obtusifolione and**  
37 **lanosterol/obtusifoliol in the cell membranes.**

38

39

40 Cryptococcosis is the most common systemic fungal infection in HIV/AIDS  
41 immunocompromised patients and is caused by the opportunistic basidiomycete yeast  
42 pathogen *Cryptococcus neoformans* (1) leading to infections of the lungs and brain.  
43 Meningoencephalitis is the most lethal manifestation of cryptococcosis with a life  
44 expectation of less than a month if untreated (2). Pathogenic *Cryptococcus* species  
45 cause disease in almost one million people annually with over 620,000 deaths and a  
46 third of all HIV/AIDS deaths are attributable to *Cryptococcus* species infection (1).  
47 Current treatment options are limited to a handful of drugs, namely initial induction  
48 therapy with a combination of amphotericin B and flucytosine followed by a maintenance  
49 regime of fluconazole (2). Even after administering the recommended treatment, three-  
50 month mortality rates of 10 to 20% are common (3, 4). In addition, adopting such  
51 treatment is costly and often impractical (with amphotericin B requiring intravenous  
52 administration), especially in developing countries where mortality rates can approach  
53 100% (5, 6).

54 Three main *C. neoformans* varieties are observed in clinical infections. *C.*  
55 *neoformans* var. *grubii* (primarily serotype A), ubiquitous in the environment especially in  
56 soil, is globally distributed and is responsible for almost all cryptococcal infections in  
57 HIV/AIDS patients (6-8). *C. neoformans* var. *neoformans* (primarily serotype D) is less  
58 likely to cause severe infection and is more commonly found in Europe (4). *C.*  
59 *neoformans* var. *gattii* (primarily serotypes B and C), a tree-dwelling basidiomycete  
60 yeast primarily located in the tropics and sub-tropics with localized outbreaks in  
61 northeast America, is now considered a separate species (*C. gattii*) and is  
62 predominantly a primary pathogen infecting healthy (immunocompetent) individuals but  
63 will also infect immunocompromised patients if opportunity arises (9). Most

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

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

81 In this study we compared the novel tetrazole antifungal VT-1129 (18, 19) (Fig. 1)  
82 with clinical azole antifungal drugs in terms of its potency and selectivity of binding to  
83 and inhibition of three recombinant cryptococcal CYP51 enzymes compared to human  
84 CYP51, and also to human CYPs that are critical xenobiotic-metabolizing enzymes. In  
85 addition, the *in vivo* mode of action for VT-1129 was demonstrated through sterol profile  
86 analysis.

87

88 **MATERIALS AND METHODS**

89 **Construction of pCWori<sup>+</sup>:CneoCYP51, pCWori<sup>+</sup>:CgruCYP51 and**  
90 **pCWori<sup>+</sup>:CgatCYP51 expression vectors.** The *C. neoformans* var. *neoformans* CYP51  
91 gene (CneoCYP51 - UniProtKB accession number Q5KQ65), the *C. neoformans* var.  
92 *grubii* CYP51 gene (CgruCYP51 - Q09GQ2) and the *C. gattii* CYP51 gene (CgatCYP51  
93 - E6QZS1) were synthesized by Eurofins MWG Operon (Ebersberg, Germany)  
94 incorporating an *Nde*I restriction site at the 5' end and a *Hind*III restriction site at the 3'  
95 end of the genes cloned into the pBSISK<sup>+</sup> plasmid. In addition the first eight amino  
96 acids were changed to 'MALLAVF' (20) and a four-histidine extension  
97 (CATCACCATCAC) was inserted immediately before the stop codon. The cryptococcal  
98 CYP51 genes were excised by *Nde*I / *Hind*III restriction digestion followed by cloning  
99 into the pCWori<sup>+</sup> expression vector. Gene integrities were confirmed by DNA  
100 sequencing.

101 **Heterologous expression and purification of recombinant cryptococcal**  
102 **CYP51 proteins.** The pCWori<sup>+</sup>:CYP51 constructs were transformed into competent  
103 DH5α *E. coli* cells and expressed as previously described (21). Recombinant CYP51  
104 proteins were isolated according to the method of Arase *et al* (22) except that 2%  
105 (wt/vol) sodium cholate was used in the sonication buffer and Tween-20 was omitted.  
106 The solubilized CYP51 proteins were purified by affinity chromatography using Ni<sup>2+</sup>-NTA  
107 agarose as previously described (23, 21) prior to characterization. Human CYP51 with a  
108 deletion of 60 amino acids from the N-terminus (Δ60 truncated human CYP51) was  
109 expressed and purified as previously described (24) and was shown to be comparable  
110 to the full-length human CYP51 in terms of binding azole antifungal drugs. Protein  
111 purities were assessed by SDS polyacrylamide gel electrophoresis.

112           **Cytochrome P450 protein determinations.** Reduced carbon monoxide  
113 difference spectroscopy was performed (25) with carbon monoxide being passed  
114 through the cytochrome P450 solution prior to addition of sodium dithionite to the  
115 sample cuvette (light-path 10 mm). An extinction coefficient of  $91 \text{ mM}^{-1} \text{ cm}^{-1}$  (26) was  
116 used to calculate cytochrome P450 concentrations from the absorbance difference  
117 between 447 and 490 nm. Absolute spectra were determined between 700 and 300 nm  
118 (light-path 10 mm). All spectral determinations were made using a Hitachi U-3310  
119 UV/VIS spectrophotometer (San Jose, California).

120           **Ligand binding studies.** Stock 2.5 mM solutions of lanosterol, eburicol and  
121 obtusifoliol were prepared in 40% (wt/vol) (2-hydroxypropyl)- $\beta$ -cyclodextrin (HPCD)  
122 using an ultrasonic bath. Sterol was progressively titrated against 5  $\mu\text{M}$  CYP51 protein in  
123 a quartz semi-micro cuvette (light-path 4.5 mm) with equivalent amounts of 40% (wt/vol)  
124 HPCD added to the reference cuvette which also contained 5  $\mu\text{M}$  CYP51. The difference  
125 in the spectrum between the absorbance at 500 and that at 350 nm was determined  
126 after each incremental addition of sterol (up to 75  $\mu\text{M}$ ). The sterol saturation curves were  
127 constructed from the difference spectra (difference in the  $A_{390}$  and  $A_{425}$ ). The substrate  
128 dissociation constants ( $K_{ds}$ ) were determined by non-linear regression (Levenberg-  
129 Marquardt algorithm) using the Michaelis-Menten equation.

130           Studies evaluating the binding of clotrimazole, fluconazole, voriconazole,  
131 itraconazole, ketoconazole and VT-1129 to the cryptococcal CYP51 proteins were  
132 performed as previously described (27, 21) using split-cuvettes with a 4.5-mm light path.  
133 Stock  $0.1\text{-mg}\cdot\text{ml}^{-1}$  solutions of the azole antifungal drugs were prepared in dimethyl  
134 sulfoxide (DMSO) and progressively titrated against 2  $\mu\text{M}$  CYP51 in 0.1 M Tris-HCl (pH

135 8.1) and 25% (wt/vol) glycerol. The difference spectra between 500 and 350 nm were  
136 determined after each incremental addition of azole and binding saturation curves were  
137 constructed from the difference in the absorption at the peak and the absorption at the  
138 trough ( $\Delta A_{\text{peak-trough}}$ ) against the azole concentration. The properties of VT-1129 binding  
139 with 5  $\mu\text{M}$  recombinant human CYP51 was also determined (24). The  $K_{\text{d}}$ s of the  
140 enzyme-azole complex were determined by non-linear regression (Levenberg-  
141 Marquardt algorithm) using a rearrangement of the Morrison equation for tight ligand  
142 binding (28, 29). Tight binding occurs where the  $K_{\text{d}}$  for a ligand is similar or lower than  
143 the concentration of the enzyme present (30).

144 **CYP51 reconstitution assays.** Cryptococcal CYP51 reconstitution assays (31,  
145 32) contained 0.5  $\mu\text{M}$  CYP51, 1  $\mu\text{M}$  *Aspergillus fumigatus* cytochrome P450 reductase  
146 (AfCPR1 - UniProtKB accession number Q4WM67), 50  $\mu\text{M}$  C-14 methylated sterol  
147 substrate (lanosterol, eburicol, obtusifolol), 50  $\mu\text{M}$  dilaurylphosphatidylcholine, 4%  
148 (wt/vol) (2-hydroxypropyl)- $\beta$ -cyclodextrin (HPCD), 0.4  $\text{mg ml}^{-1}$  isocitrate dehydrogenase,  
149 25 mM trisodium isocitrate, 50 mM NaCl, 5 mM  $\text{MgCl}_2$  and 40 mM MOPS  
150 (morpholinepropanesulfonic acid; pH  $\sim$ 7.2). Assay mixtures were incubated at 37°C prior  
151 to initiation with 4 mM  $\beta$ -NADPHNa<sub>4</sub> followed by shaking at 37°C for 15 minutes. Human  
152 CYP51 reconstitution assays were performed as above except 0.5  $\mu\text{M}$  soluble human  
153 CYP51 (24) and 2  $\mu\text{M}$  human cytochrome P450 reductase (UniProtKB accession  
154 number P16435) were used and the reaction time reduced to 5 minutes at 37°C. Sterol  
155 metabolites were recovered by extraction with ethyl acetate followed by derivatization  
156 with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and tetramethylsilane (TMCS)  
157 prior to analysis by gas chromatography (GC)-mass spectrometry (MS) (33).



158 Determinations of the 50% inhibitory concentration (IC<sub>50</sub>s) were performed using  
159 50 µM lanosterol as substrate in which various fluconazole, itraconazole, voriconazole  
160 and VT-1129 concentrations in 2.5 µl DMSO were added prior to incubation at 37°C and  
161 addition of β-NADPHNa<sub>4</sub>.

162 **Cryptococcus sterol analysis.** *C. neoformans* var. *neoformans* (strain ATCC  
163 MYA-565), *C. neoformans* var. *grubii* (strain ATCC 208821), and *C. gattii* (strain ATCC  
164 MYA-4071) were grown in MOPS buffered RPMI (0.165 M MOPS), pH 7.0, at 37°C and  
165 200 rpm. MOPS buffered RPMI, pH 7.0, in the absence (1% vol/vol, DMSO control) or  
166 with fluconazole or VT-1129 was inoculated at a final concentration of 2.5 x 10<sup>4</sup> cells ml<sup>-1</sup>.  
167 <sup>1</sup>. *C. neoformans* var. *neoformans* was grown in the presence of 0.2 µg ml<sup>-1</sup> fluconazole  
168 or 0.0039 µg ml<sup>-1</sup> VT-1129, *C. neoformans* var. *grubii* was grown in the presence of 0.4  
169 µg ml<sup>-1</sup> fluconazole or 0.0039 µg ml<sup>-1</sup> VT-1129, and *C. gattii* was grown in the presence  
170 of 0.4 µg ml<sup>-1</sup> fluconazole or 0.0078 µg ml<sup>-1</sup> VT-1129. The cultures were grown for 2  
171 days at 37°C, 200 rpm and nonsaponifiable lipids were extracted as previously reported  
172 (34).

173 Sterones were derivatized with methoxyamine-HCl by the addition of 200 µl of  
174 methoxyamine-HCl (2%, wt/vol, in anhydrous pyridine) and incubated for 30 min at  
175 70°C. Samples were mixed with 2 ml of saturated NaCl, and the lipids extracted in three  
176 sequential 2-ml volumes of ethyl acetate. The combined ethyl acetate fractions were  
177 washed with 2-ml volumes of NaCl-saturated 0.1 M HCl, saturated NaCl, NaCl-saturated  
178 5% (wt/vol) sodium bicarbonate solution and saturated NaCl. The samples were then  
179 dried over anhydrous magnesium sulphate and evaporated using a vacuum centrifuge.  
180 Sterols in the dried extracts were derivatized with 0.1 ml BSTFA-TMCS (99:1) and 0.3  
181 ml anhydrous pyridine (2 h at 80°C) prior to analysis by GC-MS (33). Individual sterols

182 and sterones were identified by reference to the retention times, mass ions, and  
183 fragmentation patterns of sterol and sterone standards. Sterol composition was  
184 calculated using peak areas.

185       **Inhibition of human liver CYP enzymes.** *In vitro* studies determined the IC<sub>50</sub>s of  
186 the test compounds for CYP2C9, CYP2C19, and CYP3A4 (with either midazolam or  
187 testosterone as substrates) in intact human liver microsomes. A separate series of  
188 incubation mixtures was prepared with each test compound at final concentration in  
189 reaction ranging from 0.0128 to 200 μM. Each incubation mixture contained pooled  
190 human liver microsomes at an assay concentration of 1 mg ml<sup>-1</sup> microsomal protein (Life  
191 Technologies, Grand Island, NY) and metabolic substrates of isozymes for CYP2C9,  
192 CYP2C19, and CYP3A4 (diclofenac, omeprazole, and midazolam or testosterone,  
193 respectively) at their experimentally determined *K<sub>m</sub>* concentrations. Active control wells  
194 contained microsomes, a substrate(s), and the test-compound diluent (i.e. DMSO-  
195 acetonitrile-phosphate buffer, 5:5:190) substituted for test compound solutions. The  
196 reaction was initiated by addition of an enzyme cofactor source (NADPH-regenerating  
197 solution; BD Biosciences, San Jose, CA) and the mixtures were incubated at 37°C. After  
198 10 min, incubation mixtures were quenched with acetonitrile, mixed, and centrifuged.  
199 The supernatant was analyzed by high-performance liquid chromatography-tandem MS  
200 for the hydroxy metabolite of the substrates. Each product peak area was normalized to  
201 be represented as a percentage of the enzyme control average. The IC<sub>50</sub> of each test  
202 compound was determined by fitting a 4-parameter logistical fit to the dose-response  
203 data and graphically determining the inhibitor concentration at 50% of the maximal  
204 enzymatic response.

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

209       **Chemicals.** VT-1129 was provided by Viamet Pharmaceuticals, Inc. (Durham,  
210 USA). All other chemicals were obtained from Sigma Chemical Company (Poole, UK).  
211 Growth media, sodium ampicillin, IPTG (isopropyl- $\beta$ -D-thiogalactopyranoside), and 5-  
212 aminolevulinic acid were obtained from Foremedium Ltd (Hunstanton, UK). Ni<sup>2+</sup>-NTA  
213 agarose affinity chromatography matrix was obtained from Qiagen (Crawley, UK).

214

## 215 **RESULTS**

216       **Expression and purification of cryptococcal CYP51 proteins.** Following  
217 heterologous expression in *E. coli*, CneoCYP51, CgruCYP51 and CgatCYP51 were  
218 extracted by sonication with 2% (wt/vol) sodium cholate (22), which yielded 240 ( $\pm$ 90),  
219 160 ( $\pm$ 50) and 290 ( $\pm$ 80) nmoles per liter culture, as determined by carbon monoxide  
220 difference spectroscopy (25). Purification by chromatography on Ni<sup>2+</sup>-NTA agarose  
221 resulted in 70%, 54% and 45% recoveries of native CneoCYP51, CgruCYP51 and  
222 CgatCYP51 proteins, respectively. SDS polyacrylamide gel electrophoresis confirmed  
223 the purity of the cryptococcal CYP51 proteins eluted on Ni<sup>2+</sup>-NTA agarose to be greater  
224 than 90% when assessed by staining intensity, with apparent molecular weights being  
225 55,000 to 58,000; the predicted molecular weights when the N-terminal modifications  
226 and the 4His C-terminal extensions are included were 62,708 for *C. neoformans* var.  
227 *neoformans*, 62,310 for *C. neoformans* var. *grubii*, and 62,689 for *C. gattii*.

228           **Spectral properties of cryptococcal CYP51 proteins.** The absolute spectra of  
229 the resting oxidized forms of all three CYP51 proteins (Fig. 2A) were typical for a low-  
230 spin ferric cytochrome P450 enzyme (23, 35) with  $\alpha$ ,  $\beta$ , Soret ( $\gamma$ ) and  $\delta$  spectral bands at  
231 566, 536, 418 and 360 nm, respectively. Reduced carbon monoxide difference spectra  
232 (Fig. 2B) gave the red-shifted heme Soret peak at 447 nm, characteristic of P450  
233 enzymes, indicating that all three CYP51 proteins were expressed in the native form.

234           **Sterol binding properties of cryptococcal CYP51 proteins.** Progressive  
235 titration with lanosterol, eburicol and obtusifoliol gave characteristic type I difference  
236 spectra for all three CYP51 proteins with a peak at 390 nm and a trough at 425 nm (Fig.  
237 3). Type I binding spectra occur when the substrate or another molecule displaces the  
238 water molecule coordinated as the sixth ligand to the low-spin hexa-coordinated heme  
239 prosthetic group, causing the heme to adopt the high-spin penta-coordinated  
240 conformation (35). The cryptococcal CYP51 proteins had similar affinities for the three  
241 sterols (Table 1) with  $K_d$  values being 16 to 18  $\mu$ M for lanosterol, 12 to 16  $\mu$ M for  
242 eburicol and 12 to 21  $\mu$ M for obtusifoliol. This result suggests that all three 14 $\alpha$ -  
243 methylated sterols are potential substrates for the cryptococcal CYP51 proteins.

244           The sterol binding affinities of the three cryptococcal CYP51 proteins ( $K_d$  range,  
245 12 to 21  $\mu$ M) were similar to those reported for other CYP51 proteins. For example,  $K_d$   
246 values for lanosterol and eburicol were 11 to 16 and 25 to 28  $\mu$ M, respectively, with  
247 *Candida albicans* CYP51 (21), 11 and 13  $\mu$ M, respectively, with *Mycosphaerella*  
248 *graminicola* CYP51 (36); and the  $K_d$  values were 0.5 to 18  $\mu$ M for lanosterol with human  
249 CYP51 (24, 37, 38). However, the sterol  $K_d$  values obtained were 10- to 20-fold higher  
250 than those obtained for lanosterol with *Mycobacterium tuberculosis* CYP51 (1  $\mu$ M) (23)

251 and for lanosterol and eburicol with *Trypanosoma cruzi* CYP51 (1.9 and 1.2  $\mu\text{M}$ ,  
252 respectively) (32).

253 **CYP51 reconstitution assays.** CYP51 assays using 50  $\mu\text{M}$  sterol gave turnover  
254 numbers of 1.2 to 1.9  $\text{min}^{-1}$  for lanosterol, 3.7 to 7.6  $\text{min}^{-1}$  for eburicol and 3.5 to 4.5  $\text{min}^{-1}$   
255 for obtusifoliol (Table 1), confirming that all three cryptococcal CYP51 proteins readily  
256 catalyzed the  $14\alpha$ -demethylation of these three sterols. Both CneoCYP51 and  
257 CgruCYP51 displayed a substrate preference for eburicol over obtusifoliol and  
258 lanosterol, whilst CgatCYP51 displayed a substrate preference for obtusifoliol over  
259 eburicol and lanosterol. The ability of CgatCYP51, in particular, to readily demethylate  
260 obtusifoliol indicates a preference for a C-24-methylated sterol substrate.

261 **Azole binding properties of CYP51 proteins.** All five medical azole antifungal  
262 agents and the agent being investigated, VT-1129, bound tightly to all three cryptococcal  
263 CYP51 proteins, producing type II binding spectra. The binding spectra and saturation  
264 curves obtained for fluconazole and itraconazole (Fig. 4) and for VT-1129 (Fig. 5) are  
265 shown with a peak at  $\sim 429$  nm and a trough at  $\sim 412$  nm. Type II binding spectra are  
266 caused by the triazole ring N-4 (fluconazole, itraconazole, and voriconazole) or the  
267 imadazole ring N-3 (clotrimazole, ketoconazole) coordinating as the sixth ligand with the  
268 heme iron (39) to form the low-spin CYP51-azole complex, resulting in a 'red-shift' of the  
269 heme Soret peak. The interaction of VT-1129 with the heme ferric ion is through a  
270 terminal (N-3 or N-4) tetrazole nitrogen atom. CneoCYP51 bound the azole antifungal  
271 agents the strongest, with apparent  $K_d$  values of 4 to 11 nM (Table 1), followed by  
272 CgatCYP51 with apparent  $K_d$  values of 5 to 24 nM, and CgruCYP51 bound the azole  
273 antifungal agents the weakest, with apparent  $K_d$  values of 14 to 52 nM. None of the  
274 cryptococcal CYP51 enzymes appeared to be inherently resistant to azole antifungal

275 agents, as the range of  $K_d$  values observed (4 to 52 nM) was similar to those observed  
276 with *C. albicans* CYP51 (10 to 56 nM) (24), whereas *Aspegillus fumigatus* CYP51A  
277 appeared to be inherently resistant to fluconazole with an apparent  $K_d$  value of 11.9  $\mu$ M  
278 (40). The affinity of VT-1129 binding to all three cryptococcal CYP51 proteins was strong  
279 ( $K_d$  range, 11 to 25 nM) and similar to that of the other five clinical azole antifungal  
280 agents examined, suggesting VT-1129 would be effective as a therapeutic agent against  
281 *Cryptococcus* species infections. The similar azole binding properties of the three  
282 cryptococcal CYP51 proteins agree with their close sequence homology with  
283 CneoCYP51 sharing 98% and 96% sequence identity with CgruCYP51 and CgatCYP51,  
284 respectively.

285 In contrast, VT-1129 bound relatively weakly to human CYP51 (Fig. 5) with an  
286 apparent  $K_d$  of 4.53  $\mu$ M (Table 1). The interaction of VT-1129 with human CYP51 was  
287 atypical, as it gave rise to a red-shifted type I difference spectrum (peak at 410 nm and  
288 trough at 426 nm) rather than the expected type II difference spectrum normally  
289 observed for the interaction of azole antifungal agents with CYP51 proteins. This  
290 suggests that the mode of interaction of VT-1129 with the human CYP51 was different  
291 from that observed with the three cryptococcal CYP51 proteins. VT-1129 still perturbs  
292 the heme environment of human CYP51, as a difference spectrum was observed,  
293 though it was not through the azole nitrogen directly coordinating with the heme ferric  
294 ion. This altered interaction of VT-1129 with human CYP51 resulted in very weak  
295 inhibition of CYP51 activity in the CYP51 reconstitution assay (see below). The  $K_d$   
296 values obtained for VT-1129 with the cryptococcal CYP51 enzymes were 180- to 410-  
297 fold lower than the  $K_d$  value obtained with the human homolog, confirming the high  
298 selectivity of VT-1129 for the fungal target enzyme. This compared favorably with the

299 findings for fluconazole and voriconazole, which gave  $K_d$  values that were 370- to 1,300-  
300 fold and 120- to 570-fold lower, respectively, for cryptococcal CYP51 enzymes than  
301 human CYP51. VT-1129 exhibited far greater selectivity than clotrimazole,  
302 ketoconazole, and itraconazole toward cryptococcal CYP51 enzymes than toward the  
303 human homolog, with clotrimazole, ketoconazole, and itraconazole exhibiting  $K_d$  values  
304 that were only 1.3- to 15-fold lower for the fungal CYP51 than for the human CYP51.

305 **Azole  $IC_{50}$  determinations.**  $IC_{50}$  determinations (Fig. 6) confirmed that all three  
306 cryptococcal CYP51 proteins tightly bound fluconazole, itraconazole voriconazole and  
307 VT-1129, giving rise to strong inhibition of the CYP51 demethylation of lanosterol.  $IC_{50}$ s  
308 of 0.14 to 0.20  $\mu$ M (Table 1), which were obtained which were close to half the CYP51  
309 concentration present in the assay system, were obtained. VT-1129 proved equally as  
310 effective at inhibiting cryptococcal CYP51 activity as the three other azole antifungal  
311 drugs, suggesting VT-1129 would be effective at combating *Cryptococcus* infections. In  
312 contrast, VT-1129 only weakly inhibited human CYP51 activity ( $IC_{50}$ , ~600  $\mu$ M) (Fig. 7),  
313 in agreement with the weak perturbation of the heme environment of human CYP51  
314 observed with VT-1129 (Fig. 5), whereas clotrimazole severely inhibited human CYP51  
315 activity ( $IC_{50}$ , 1.9  $\mu$ M). The  $IC_{50}$ s of VT-1129 observed for the cryptococcal CYP51  
316 enzymes were 3,300- to 4,000-fold lower than that obtained with the human homolog  
317 (Table 1), again confirming high selectivity for the fungal target enzyme. This was  
318 comparable to the findings for fluconazole, where the  $IC_{50}$ s for the fungal CYP51  
319 enzymes were 6,500- to 9,000-fold lower than those for human CYP51 and with the  
320 selectivity observed with fluconazole being significantly better than that observed with  
321 voriconazole and itraconazole (Table 1). The  $IC_{50}$ s of VT-1129 were more potent than  
322 the  $K_d$  values for binding to cryptococcal CYP51 enzymes, suggesting that the  $K_d$  values

323 calculated by the Morrison equation were an overestimate, in part due to the relatively  
324 high CYP51 protein concentrations required for *in vitro* binding studies.

325 **Cryptococcus sterol content.** The treatment of *Cryptococcus* spp. with 0.2 to  
326 0.4  $\mu\text{g ml}^{-1}$  fluconazole and 0.0039 to 0.0078  $\mu\text{g ml}^{-1}$  VT-1129 resulted in the  
327 accumulation of eburicol (Table 2), obtusifolione and lanosterol/obtusifoliol. The  
328 accumulation of CYP51 substrates is indicative of direct CYP51 inhibition in treated  
329 cells. Both azole treatments resulted in the depletion of the post-CYP51 sterol  
330 metabolites ergosta-7,22-dienol and ergosta-7-enol and the partial depletion of  
331 ergosterol levels (Table 2), showing CYP51 inhibition. In these cellular experiments, VT-  
332 1129 was significantly more potent than fluconazole, as VT-1129 caused greater  
333 inhibition of cryptococcal CYP51 activity at a 50-fold lower concentration than  
334 fluconazole (relative to the results observed with the DMSO control, VT-1129 caused  
335 greater reductions in ergosterol levels than fluconazole at a 50-fold higher concentration,  
336 and in all cases, the accumulation of the 14-methylated product showed that CYP51  
337 was inhibited in cells; Table 2).

338 **Inhibition of human liver drug-metabolizing CYPs.** The inhibition of three  
339 critical xenobiotic-metabolizing CYPs by the four approved azole drugs and VT-1129 is  
340 shown in Table 3. The  $\text{IC}_{50}\text{s}$  of the marketed agents available in the literature (41-43)  
341 agree well with those measured in this study. The imidazole-containing agent  
342 clotrimazole was the most potent CYP inhibitor, inhibiting the activities of all CYPs at  
343 sub- or low-micromolar concentrations. The three triazole-containing agents had  
344 variable inhibitory potencies, with itraconazole potently inhibiting CYP3A4 with either  
345 substrate ( $\text{IC}_{50}\text{s}$ , 0.08 and 0.13  $\mu\text{M}$ ), voriconazole inhibiting the activities of all CYPs with



346 a relatively tight range of potencies (IC<sub>50</sub>s, 4 to 13 μM), and fluconazole showing a  
347 slightly broader range (IC<sub>50</sub>s, 6 to 34 μM). In contrast, VT-1129 weakly inhibited the  
348 activities of each of these enzymes (IC<sub>50</sub>s, 79 to 178 μM).

349

## 350 **DISCUSSION**

351 Sionov *et al* (14) demonstrated that *C. neoformans* strains are heteroresistant to  
352 fluconazole, with each strain yielding a sub-population that can survive in the presence  
353 of fluconazole concentrations well above the MIC values through disomy of  
354 chromosome 1, which duplicates the CYP51 and AFR1 transporter genes. The disomy  
355 of chromosome 1 coupled with reported G484S and Y145F CYP51 mutations (12, 13)  
356 increased CYP51 and AFR1 expression levels (14), and the genome plasticity post  
357 infection (15) may explain the divergent range of MIC values of fluconazole of 0.5 to 64  
358 μg ml<sup>-1</sup> reported for *Cryptococcus* spp. (44-47). The MIC values reported for  
359 voriconazole (0.008 to 0.5 μg ml<sup>-1</sup>), itraconazole (0.015 to 0.5 μg ml<sup>-1</sup>), and  
360 posaconazole (0.008 to 0.5 μg ml<sup>-1</sup>) were lower and less variable than those reported for  
361 fluconazole (44-47), indicating the therapeutic efficacy of these triazole antifungals and  
362 their potential for use should fluconazole tolerance become problematic. However, as  
363 previously observed with *Candida* spp. and *Aspergillus* spp., it can be anticipated that  
364 tolerance against current triazole therapeutics will emerge in *Cryptococcus* spp.

365 New antifungal drug candidates for the treatment of systemic *Cryptococcus*  
366 infection which target CYP51 should ideally have high potency against the intended  
367 cryptococcal CYP51 target enzymes and minimal interaction with human CYP51 and  
368 other critical CYP enzymes, such as those that metabolize xenobiotics. VT-1129 meets

369 both these criteria by binding tightly to cryptococcal CYP51 enzymes ( $K_d$  range, 11 to 25  
370 nM) with a high affinity similar to that of other pharmaceutical azole antifungal agents ( $K_d$   
371 range, 4 to 52 nM) while binding weakly to the CYP51 of the human host *in vitro* ( $K_d$ ,  
372 4.53  $\mu$ M). Binding studies (Fig. 4 and 5) provide useful preliminary information on a  
373 cyclized nitrogen-containing antifungal drug candidate's likely effectiveness at inhibiting  
374 CYP51 activity. However, only  $IC_{50}$  determinations using a CYP51 reconstitution assay  
375 system can determine the functional activity of each compound as a CYP51 inhibitor.  
376  $IC_{50}$  determinations confirmed that VT-1129 is a strong inhibitor of cryptococcal CYP51  
377 activity, consistent with tight binding inhibition, but only weakly inhibits human CYP51  
378 (13% inhibition at 150  $\mu$ M VT-1129). The selectivity of VT-1129 for the cryptococcal  
379 CYP51 protein over the human homolog was ~3,300-fold in terms of inhibiting CYP51  
380 catalysis, and VT-1129 was as effective as conventional triazole antifungal drugs at  
381 inhibiting cryptococcal CYP51 activity. VT-1129's selectivity for inhibiting cryptococcal  
382 CYP51 was similarly high compared to its selectivity for inhibiting key human xenobiotic-  
383 metabolizing CYPs, suggesting a low potential for clinical drug-drug interactions.

384 Sterol profile analysis confirmed that VT-1129 inhibited cryptococcal CYP51  
385 activity in whole cells, resulting in the depletion of ergosterol and ergosta-7-enol from the  
386 cell membranes and the accumulation of the 14-methylated compounds eburicol and  
387 lanosterol/obtusifoliol and obtusifolione. In a separate study measuring a large number  
388 of *Cryptococcus* species isolates and using 50% inhibition as the endpoint, the  $MIC_{90}$  of  
389 VT-1129 was 0.060  $\mu$ g ml<sup>-1</sup> for 180 isolates of *C. neoformans* and 0.25  $\mu$ g ml<sup>-1</sup> for 321  
390 isolates of *C. gattii* (19), confirming that VT-1129 is a potent inhibitor of *Cryptococcus*  
391 growth. In both studies, VT-1129 was a more potent inhibitor of *Cryptococcus* CYP51  
392 than fluconazole. In addition, VT-1129 retains all or most of its antifungal potency

393 against 50 Ugandan clinical isolates of *C. neoformans* with elevated fluconazole MIC  
394 values (48). This potency coupled with its excellent selectivity for fungal rather than  
395 human CYP enzymes shown here supports VT-1129 as a good candidate for the  
396 treatment of systemic *Cryptococcus* infections. Given the unmet need for more potent  
397 drugs for the treatment of cryptococcosis, especially in sub-Saharan Africa, further  
398 assessments in clinical trials are warranted, with VT-1129 Phase 1 studies with healthy  
399 volunteers now being underway.

400

#### 401 **ACKNOWLEDGMENT**

402 We are grateful to the Engineering and Physical Sciences Research Council National  
403 Mass Spectrometry Service Centre at Swansea University and Marcus Hull for  
404 assistance with GC-MS analyses.

405 This work was supported in part by the European Regional Development  
406 Fund/Welsh Government funded BEACON research program (Swansea University), the  
407 National Science Foundation of the United States (grant NSF-MCB-09020212 awarded  
408 to W. David Nes, Texas Tech University), and by Viamet Pharmaceuticals, Inc.  
409 (Durham, NC 27703, USA).

410

#### 411 **FUNDING INFORMATION**

412 This work, including the efforts of W. David Nes, was funded by NSF (NSF-MCB-  
413 09020212).

414

415

416

417 **REFERENCES**

- 418 1. **Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller**  
419 **TM.** 2009. Estimation of the current global burden of cryptococcal meningitis  
420 among persons living with HIV/AIDS. *Aids.* **23**:525-530.
- 421 2. **Bicanic T, Harrison TS.** 2004. Cryptococcal meningitis. *Br. Med. Bull.* **72**:99-  
422 118.
- 423 3. **Lortholary O, Poizat G, Zeller V, Neuville S, Boibieux A, Alvarez M,**  
424 **Dellamonica P, Botterel F, Dromer F, Chene G, and the French**  
425 **Cryptococcosis study group.** 2006. Long-term outcome of AIDS-associated  
426 cryptococcosis in the era of combination antiretroviral therapy. *Aids.* **20**:2183-  
427 2191.
- 428 4. **Dromer F, Mathoulin-Pelissier S, Launay O, Lortholary O.** 2007. Determinants  
429 of disease presentation and outcome during cryptococcosis: the Crypto A/D  
430 study. *PLoS Med.* **4**:e21.
- 431 5. **Mwaba P, Mwansa J, Chintu C, Pobee J, Scarborough M, Portsmouth S,**  
432 **Zumla A.** 2001. Clinical presentation, natural history, and cumulative death rates  
433 of 230 adults with primary cryptococcal meningitis in Zambian AIDS patients  
434 treated under local conditions. *Postgrad. Med. J.* **77**:769-773.
- 435 6. **French N, Gray K, Watera C, Nakiyingi J, Lugada E, Moore M, Lalloo D,**  
436 **Whitworth JAG, Gilks CF.** 2002. Cryptococcal infection in a cohort of HIV-1-  
437 infected Ugandan adults. *Aids.* **16**:1031-1038.
- 438 7. **Canteros CE, Brundy M, Rodero L, Perrotta D, Davel G.** 2002. Distribution of  
439 *Cryptococcus neoformans* serotypes associated with human infections in  
440 Argentina. *Rev. Argent. Microbiol.* **34**:213-218.

- 441 8. **Banerjee U, Datta K, Casadevall A.** 2004. Serotype distribution of *Cryptococcus*  
442 *neoformans* in patients in a tertiary care center in India. *Med. Mycol.* **242**:181-  
443 186.
- 444 9. **Byrnes EJ, Bartlett KH, Perfect JR, Heitman J.** 2011. *Cryptococcus gattii*: an  
445 emerging fungal pathogen infecting humans and animals. *Microbes Infect.*  
446 **13**:895-907.
- 447 10. **Hagen F, Khayhan K, Theelen B, Kolecka A, Polacheck I, Sionev E, Falk R,**  
448 **Parnmen S, Lumbsch HT, Boekhout T.** 2015. Recognition of seven species in  
449 the *Cryptococcus gattii* / *Cryptococcus neoformans* species complex. *Fungal*  
450 *Genet. Biol.* **78**:16-48.
- 451 11. **Perfect JR, Cox GM.** Drug resistance in *Cryptococcus neoformans*. 1999. *Drug*  
452 *Resist. Update.* **2**:259-269.
- 453 12. **Rodero L, Mellado E, Rodriguez AC, Salve A, Guelfand L, Cahn P, Cuenca-**  
454 **Estrella M, Davel G, Rodriguez-Tudela JL.** 2003. G484S amino acid  
455 substitution in lanosterol 14- $\alpha$  demethylase (ERG11) is related to fluconazole  
456 resistance in a recurrent *Cryptococcus neoformans* clinical isolate. *Antimicrob*  
457 *Agents Chemother* **47**:3653-3656.
- 458 13. **Sionov E, Chang YC, Garraffo HM, Dolan MA, Ghannoum MA, Kwon-Chung**  
459 **KJ.** 2012. Identification of a *Cryptococcus neoformans* cytochrome P450  
460 lanosterol 14 $\alpha$ -demethylase (Erg11) residue critical for differential susceptibility  
461 between fluconazole/voriconazole and itraconazole/posaconazole. *Antimicrob.*  
462 *Agents Chemother.* **56**:1162-1169.

- 463 14. **Sionov E, Lee H, Chang YC, Kwon-Chung KJ.** 2010. *Cryptococcus neoformans*  
464 overcomes stress of azole drugs by formation of disomy in specific multiple  
465 chromosomes. PLoS Pathogens. **6**:e1000848.
- 466 15. **Hu G, Wang J, Choi J, Jung WH, Liu I, Litvintseva AP, Bicanic T, Aurora R,**  
467 **Mitchell TG, Perfect JR, Kronstad JW.** 2011. Variation in chromosome copy  
468 number influences the virulence of *Cryptococcus neoformans* and occurs in  
469 isolates from AIDS patients. BMC Genomics. **12**:526.
- 470 16. **Sheng C, Miao Z, Ji H, Yao J, Wang W, Che X, Dong G, Lu J, Guo W, Zhang**  
471 **W.** 2009. Three-dimensional model of lanosterol 14 $\alpha$ -demethylase from  
472 *Cryptococcus neoformans*: active-site characterization and insights into azole  
473 binding. Antimicrob. Agents Chemother. **53**:3487-3495.
- 474 17. **Suzuki Y, Tokimatsu I, Sato Y, Kawasaki K, Sato Y, Goto T, Hashinaga K,**  
475 **Itoh H, Hiramatsu K, Kadota J.** 2013. Association of sustained high plasma  
476 trough concentration of voriconazole with the incidence of hepatotoxicity. Clin.  
477 Clim. Acta. **424**:119-122.
- 478 18. **Hoekstra WJ, Garvey EP, Moore WR, Rafferty SW, Yates CM, Schotzinger**  
479 **RJ.** 2014. Design and optimization of highly-selective fungal CYP51 inhibitors.  
480 Bioorg. Med. Chem. Lett. **24**:3455-3458.
- 481 19. **Lockhart SR, Fothergill AW, Iqbal N, Bolden CB, Grossman NT, Garvey EP,**  
482 **Brand SR, Hoekstra WJ, Schotzinger RJ, Ottinger E, Patterson TF,**  
483 **Wiederhold NP.** 2016. The investigational fungal Cyp51 inhibitor VT-1129  
484 demonstrates potent in vitro activity against *Cryptococcus neoformans* and  
485 *Cryptococcus gattii*. Antimicrob. Agents Chemother. In press AAC.02770-15.

- 486 20. **Barnes HJ, Arlotto MP, Waterman MR.** 1991. Expression and enzymatic activity  
487 of recombinant cytochrome P450 17 $\alpha$ -hydroxylase in *Escherichia coli*. Proc. Natl.  
488 Acad. Sci. USA. **88**:5597-5601.
- 489 21. **Warrilow AGS, Martel CM, Parker JE, Melo N, Lamb DC, Nes D, Kelly DE,**  
490 **Kelly SL.** 2010. Azole binding properties of *Candida albicans* sterol 14- $\alpha$   
491 demethylase (CaCYP51). Antimicrob. Agents Chemother. **54**:4235-4245.
- 492 22. **Arase M, Waterman MR, Kagawa N.** 2006. Purification and characterization of  
493 bovine steroid 21-hydroxylase (P450c21) efficiently expressed in *Escherichia coli*.  
494 Biochem. Biophys. Res. Com. **344**:400-405.
- 495 23. **Bellamine A, Mangla AT, Nes WD, Waterman MR.** 1999. Characterisation and  
496 catalytic properties of the sterol 14 $\alpha$ -demethylase from *Mycobacterium*  
497 *tuberculosis*. Proc. Natl. Acad. Sci. USA. **96**:8937-8942.
- 498 24. **Warrilow AGS, Parker JE, Kelly DE, Kelly SL.** 2013. Azole affinity of sterol 14 $\alpha$ -  
499 demethylase (CYP51) enzymes from *Candida albicans* and *Homo sapiens*.  
500 Antimicrob. Agents Chemother. **57**:1352-1360.
- 501 25. **Estabrook RW, Peterson JA, Baron J, Hildebrandt AG.** 1972. The  
502 spectrophotometric measurement of turbid suspensions of cytochromes  
503 associated with drug metabolism, p 303-350. In: Chignell CF (ed), Methods in  
504 Pharmacology, vol 2, Appleton-Century-Crofts, New York, NY.
- 505 26. **Omura T, Sato R.** 1964. The carbon monoxide-binding pigment of liver  
506 microsomes. J. Biol. Chem. **239**:2379-2385.
- 507 27. **Lamb DC, Kelly DE, Waterman MR, Stromstedt M, Rozman D, Kelly SL.** 1999.  
508 Characteristics of the heterologously expressed human lanosterol 14 $\alpha$ -

- 509 demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the  
510 purified human and *Candida albicans* CYP51 with azole antifungal agents. Yeast  
511 **15:755-763.**
- 512 28. **Lutz JD, Dixit V, Yeung CK, Dickmann LJ, Zelter A, Thatcher JA, Nelson WL,**  
513 **Isoherranen N.** 2009. Expression and functional characterization of cytochrome  
514 P450 26A1, a retinoic acid hydroxylase. *Biochem. Pharmacol.* **77:258-268.**
- 515 29. **Morrison JF.** 1969. Kinetics of the reversible inhibition of enzyme-catalysed  
516 reactions by tight-binding inhibitors. *Biochim. Biophys. Acta – Enzymol.* **185:269-**  
517 **286.**
- 518 30. **Copeland RA.** 2005. Evaluation of enzyme inhibitors in drug discovery: a guide  
519 for medicinal chemists and pharmacologists, p 178-213, Wiley-Interscience, New  
520 York, NY.
- 521 31. **Lepesheva GI, Ott RD, Hargrove TY, Kleshchenko YY, Schuster I, Nes WD,**  
522 **Hill GC, Villalta F, Waterman MR.** 2007. Sterol 14 $\alpha$ -demethylase as a potential  
523 target for antitrypanosomal therapy: enzyme inhibition and parasite cell growth.  
524 *Chem. Biol.* **14:1283–1293.**
- 525 32. **Lepesheva GI, Zaitseva NG, Nes WD, Zhou W, Arase M, Liu J, Hill GC,**  
526 **Waterman MR.** 2006. CYP51 from *Trypanosoma cruzi*: a phyla-specific residue  
527 in the B' helix defines substrate preferences of sterol 14 $\alpha$ -demethylase. *J. Biol.*  
528 *Chem.* **281:3577–3585.**
- 529 33. **Parker JE, Warrilow AGS, Cools HJ, Fraaije BA, Lucas JA, Rigdova K,**  
530 **Griffiths WJ, Kelly DE, Kelly SL.** 2011. Prothioconazole and prothioconazole-  
531 desthio activity against *Candida albicans* sterol 14 $\alpha$ -demethylase (CaCYP51).  
532 *Appl. Environ. Microbiol.* **79:1639-1645.**



- 533 34. **Kelly SL, Lamb DC, Corran AJ, Baldwin BC, Kelly DE.** 1995. Mode of action  
534 and resistance to azole antifungals associated with the formation of 14 $\alpha$ -  
535 methylergosta-8,24(28)-dien-3 $\beta$ ,6 $\alpha$ -diol. *Biochem. Biophys. Res. Comm.*  
536 **207**:910-915.
- 537 35. **Jefcoate CR.** 1978. Measurement of substrate and inhibitor binding to  
538 microsomal cytochrome P-450 by optical-difference spectroscopy. *Methods*  
539 *Enzymol.* **52**:258-279.
- 540 36. **Parker JE, Warrilow AGS, Cools HJ, Martel CM, Nes WD, Fraaije BA, Lucas JA, Kelly**  
541 **DE, Kelly SL.** 2011. Mechanism of binding of prothioconazole to *Mycosphaerella*  
542 *graminicola* CYP51 differs from that of other azole antifungals. *Appl. Environ.*  
543 *Microbiol.* **77**:1460-1465.
- 544 37. **Lepesheva GI, Nes WD, Zhou W, Hill GC, Waterman MR.** 2004. CYP51 from  
545 *Trypanosoma brucei* is obtusifoliol-specific. *Biochemistry* **43**:10789–10799.
- 546 38. **Strushkevich N, Usanov SA, Park HW.** 2010. Structural basis of human CYP51  
547 inhibition by antifungal azoles. *J. Mol. Biol.* **397**:1067-1078.
- 548 39. **Jefcoate CR, Gaylor JL, Calabrese RL.** 1969. Ligand interactions with  
549 cytochrome P450. I. Binding of primary amines. *Biochemistry* **8**:3455-3463.
- 550 40. **Warrilow AGS, Melo N, Martel CM, Parker JE, Nes D, Kelly DE, Kelly SL.**  
551 2010. Expression, purification, and characterization of *Aspergillus fumigatus*  
552 sterol 14- $\alpha$  demethylase (CYP51) isoenzymes A and B. *Antimicrob. Agents*  
553 *Chemother.* **54**:4225-4234.

- 554 41. **Niwa T, Shiraga T, Takagi A.** 2005. Effect of antifungal drugs on cytochrome  
555 P450 (CYP) 2C9, CYP2C19, and CYP3A4 activities in human liver microsomes.  
556 Biol. Pharm. Bull. **28**:1805-1808.
- 557 42. **Zhang S, Pillai VC, Mada SR, Strom S, Venkataramanan R.** 2012. Effect of  
558 voriconazole and other azole antifungal agents on CYP3A activity and  
559 metabolism of tacrolimus in human liver microsomes. Xenobiotica **42**:409-416.
- 560 43. **Zhang W, Ramamoorthy Y, Kilicarslan T, Nolte H, Tyndale RF, Sellers EM.**  
561 2002. Inhibition of cytochromes P450 by antifungal imidazole derivatives. Drug  
562 Met. Disp. **30**:314-318.
- 563 44. **Trilles L, Meyer W, Wanke B, Guarro J, Lazera M.** 2012. Correlation of  
564 antifungal susceptibility and molecular type within the *Cryptococcus neoformans* /  
565 *C. gattii* species complex. Med. Mycol. **50**:328-332.
- 566 45. **Pfaller MA, Castanheira M, Messer SA, Moet GJ, Jones RN.** 2011.  
567 Echinocandin and triazole antifungal susceptibility profiles for *Candida* spp.,  
568 *Cryptococcus neoformans*, and *Aspergillus fumigatus*: application of new CLSI  
569 clinical breakpoints and epidemiologic cutoff values to characterize resistance in  
570 the SENTRY antimicrobial surveillance program (2009). Diag. Microbiol. Infect.  
571 Dis. **69**:45-50.
- 572 46. **Bertout S, Drakulovski P, Kouanfack C, Krasteva D, Ngouana T, Dunyach-**  
573 **Remy C, Dongsta J, Aghokeng A, Delaporte E, Koulla-Shiro S, Reynes J,**  
574 **Mallie M.** 2012. Genotyping and antifungal susceptibility testing of *Cryptococcus*  
575 *neoformans* isolates from Cameroonian HIV-positive adult patients. Clin.  
576 Microbiol. Infect. **19**:763-769.

- 577 47. **Lockhart SR, Iqbal N, Bolden CB, DeBess EE, Marsden-Haug N, Worhle R,**  
578 **Thakur R, Harris JR.** 2012. Epidemiologic cutoff values for triazole drugs in  
579 *Cryptococcus gattii*: correlation of molecular type and in vitro susceptibility. *Diag.*  
580 *Microbiol. Infect. Dis.* **73**:144-148.
- 581 48. **Vedula P, Smith K, Boulware DR, Meya DB, Garvey EP, Hoekstra WJ,**  
582 **Schotzinger RJ, Nielsen K.** 2015. Activity of VT-1129 against *Cryptococcus*  
583 *neoformans* clinical isolates with high fluconazole MICs. 2015 Interscience  
584 Conference on Antimicrobial Agents and Chemotherapy, San Diego, CA. Poster  
585 F-763a.  
586

VT-1129 and cryptococcal CYP51s

587 **TABLE 1** Ligand binding affinities, azole IC<sub>50</sub>s, and turnover numbers for CYP51

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

588 <sup>a</sup> HsapCYP51, *Homo sapiens* CYP51.

589 <sup>b</sup> Values were taken from Warrilow *et al.* (24).

590 <sup>c</sup> Thirteen percent inhibition was observed in the presence of 150 μM VT-1129.

591

592

593

594 **TABLE 1** (Continued)

CYP51	IC <sub>50</sub> (μM)					Turnover no. (min <sup>-1</sup> )	
	Clotrimazole	Fluconazole	Itraconazole	Voriconazole	VT-1129	Lanosterol	Eburicol
CneoCYP51		0.17	0.17	0.17	0.16	1.4 ±0.2	6.1 ±0.2
CgruCYP51		0.2	0.19	0.2	0.18	1.9 ±0.3	7.6 ±0.3
CgatCYP51		0.14	0.16	0.16	0.15	1.2 ±0.2	3.7 ±0.2
HsapCYP51	1.9	~1,300 <sup>b</sup>	70 <sup>b</sup>	112	~600 <sup>c</sup>	22.7 ±4.8	

595

596

VT-1129 and cryptococcal CYP51s

597 **TABLE 2** Sterol profiles of *Cryptococcus* spp.

Sterols	Sterol composition (%) with the indicated treatment <sup>a</sup>						
	<i>C. neoformans</i> var. <i>neoformans</i>			<i>C. neoformans</i> var. <i>grubii</i>			<i>C. gattii</i>
	DMSO	+FLUC	+VT1129	DMSO	+FLUC	+VT1129	DMSO
Ergosta-5,7,22,24(28)-tetraenol	-	1.2 ±0.3	5.0 ±0.5	2.5 ±1.4	2.1 ±1.6	2.3 ±0.3	-
Ergosta-5,8,22-trienol	-	1.0 ±0.0	3.7 ±0.3	-	-	-	-
Ergosterol	60.6 ±2.5	42.2 ±0.7	11.5 ±3.9	43.9 ±4.3	34.1 ±3.6	18.7 ±0.6	49.3 ±9.7
Ergosta-7,22-dienol	7.4 ±0.4	-	-	9.3 ±1.1	-	-	10.8 ±6.0
Fecosterol (E8,24(28))	-	-	-	-	-	-	1.0 ±0.9
Ergosta-8-enol	-	1.6 ±0.4	-	-	-	-	-
Ergosta 5,7 dienol	-	-	-	-	-	-	3.0 ±0.6
Ergosta-7-enol	25.3 ±1.0	-	-	28.8 ±0.5	-	-	30.2 ±6.6
Eburicone	-	-	-	-	-	-	-
Lanosterol / Obtusifoliol	-	3.7 ±0.9	4.4 ±0.2	1.7 ±1.1	10.9 ±2.1	4.9 ±0.0	-
4-methyl fecosterol	-	-	-	2.5 ±0.4	-	-	-
Obtusifolione	-	35.9 ±1.8	17.1 ±1.0	-	22.1 ±1.5	24.5 ±0.6	-
Eburicol	1.5 ±0.8	12.8 ±0.7	55.8 ±5.7	6.8 ±2.0	30.0 ±2.1	49.1 ±1.1	3.1 ±2.5
4,4-dimethyl-ergosta-8,24(28)-dienol	-	-	-	4.0 ±0.8	-	-	-

598 <sup>a</sup> Mean values from three replicates ± standard deviations are shown. FLUC, fluconazole.

VT-1129 and cryptococcal CYP51s

600 **TABLE 3** Inhibition of human liver CYPs by fungal CYP51 inhibitors.

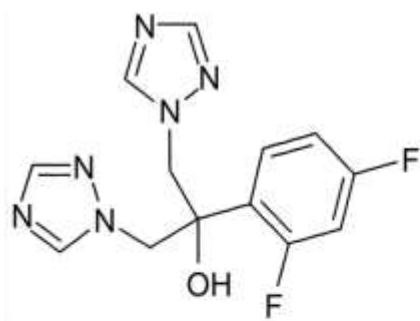
Inhibitor	IC <sub>50</sub> (μM) <sup>a</sup>			
	2C9	2C19	3A4 <sup>b</sup>	3A4 <sup>c</sup>
Clotrimazole	1.4 (0.1)	0.6 (0.2)	0.03 (0.01)	0.03 (0.01)
Fluconazole	34 (10)	13 (9)	32 (5)	32 (5)
Itraconazole	80 (28)	78 (31)	0.08 (0.02)	0.08 (0.02)
Voriconazole	10 (5)	10 (4)	13 (4)	13 (4)
VT-1129	87 (21)	110 (80)	79 (23)	79 (23)

601 <sup>a</sup> Values are averages of 2 to 4 separate determinations with standard deviations in parentheses.

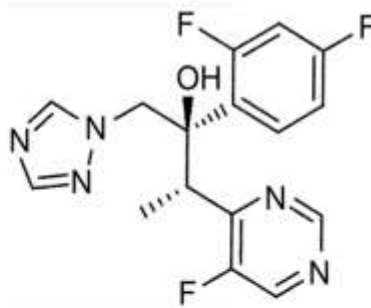
602 <sup>b</sup> Testosterone as substrate.

603 <sup>c</sup> Midazolam as substrate.

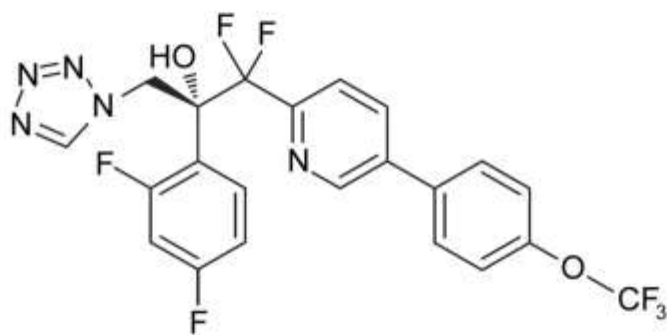
604



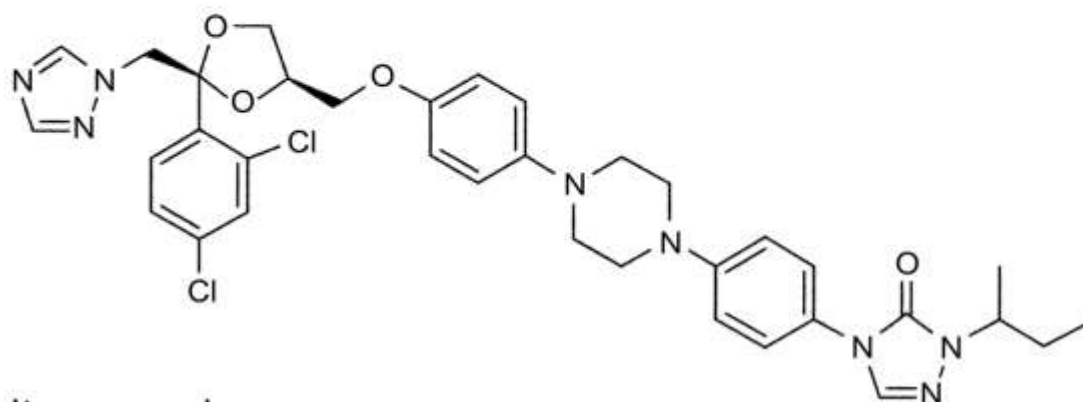
Fluconazole



Voriconazole



VT-1129



Itraconazole

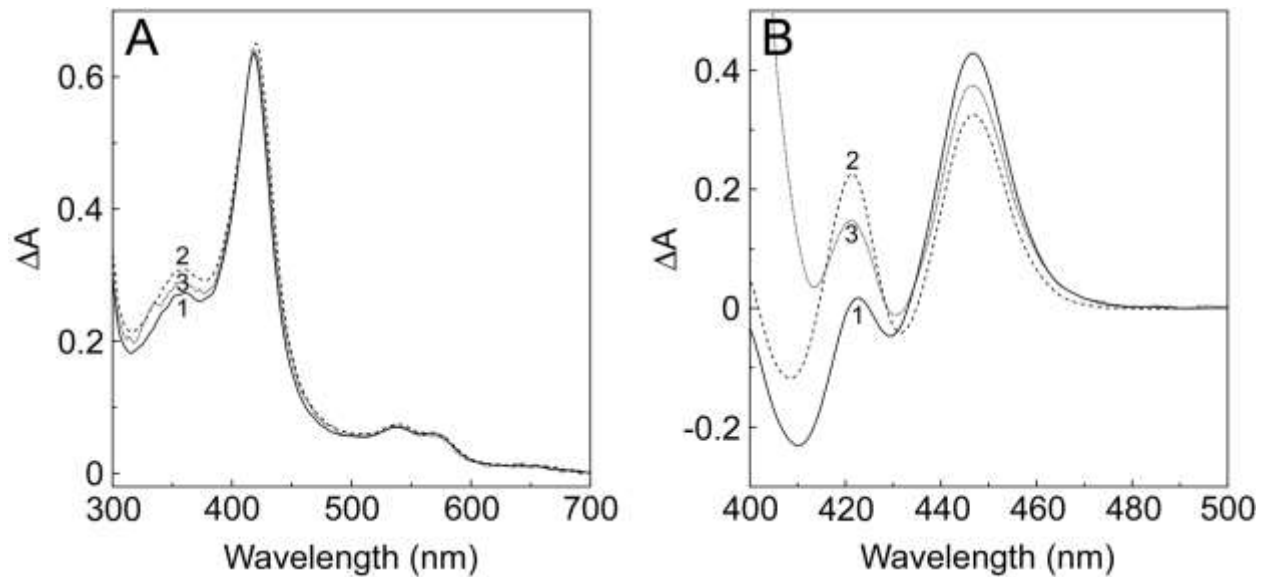
605 Itraconazole

606 **FIG 1** Chemical structures of the azole antifungals used for IC<sub>50</sub> studies. The chemical

607 structures of fluconazole (molecular weight, 306), voriconazole (molecular weight, 349),

608 VT-1129 (molecular weight, 513), and itraconazole (molecular weight, 706) are shown.

609

610  
611

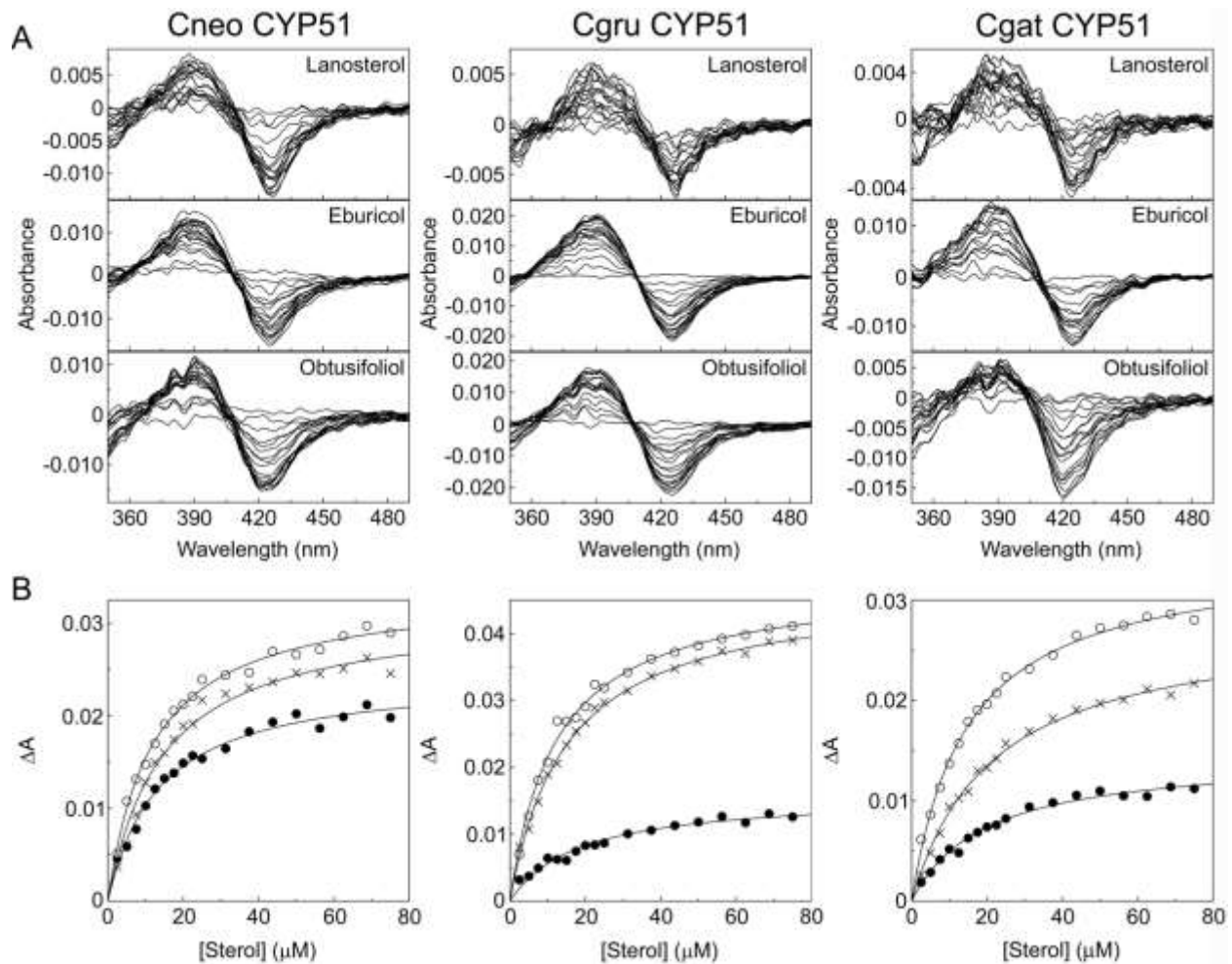
612  
613 **FIG 2** Absolute and reduced carbon monoxide spectra of cryptococcal CYP51 proteins.  
614 Absolute spectra in the oxidised resting state (A) and reduced carbon monoxide  
615 difference spectra (B) were determined using 5  $\mu$ M solutions of purified CneoCYP51  
616 (line 1), CgruCYP51 (line 2), and CgatCYP51 (line 3). Spectral determinations were  
617 made using quartz semimicrocuvettes with a path length of 10 mm.

618  
619  
620

621



622



623

624

625

626

627

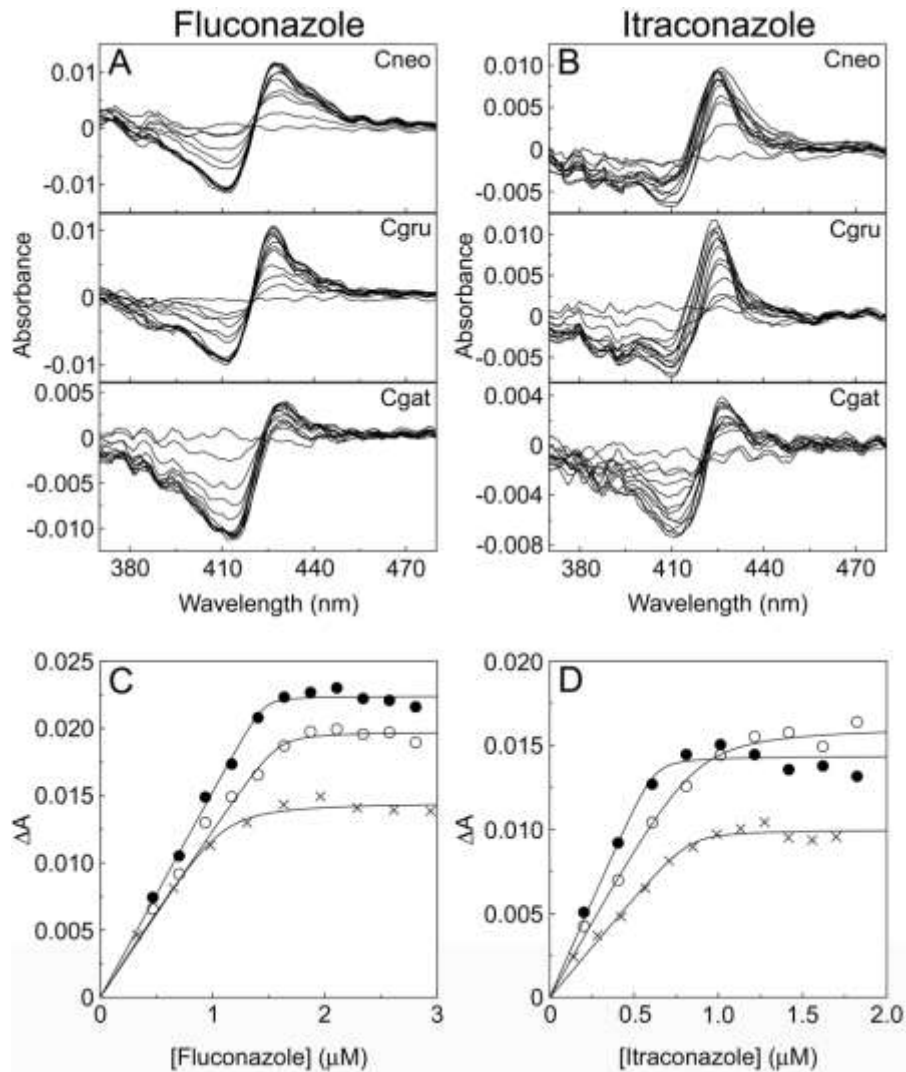
628

629

630

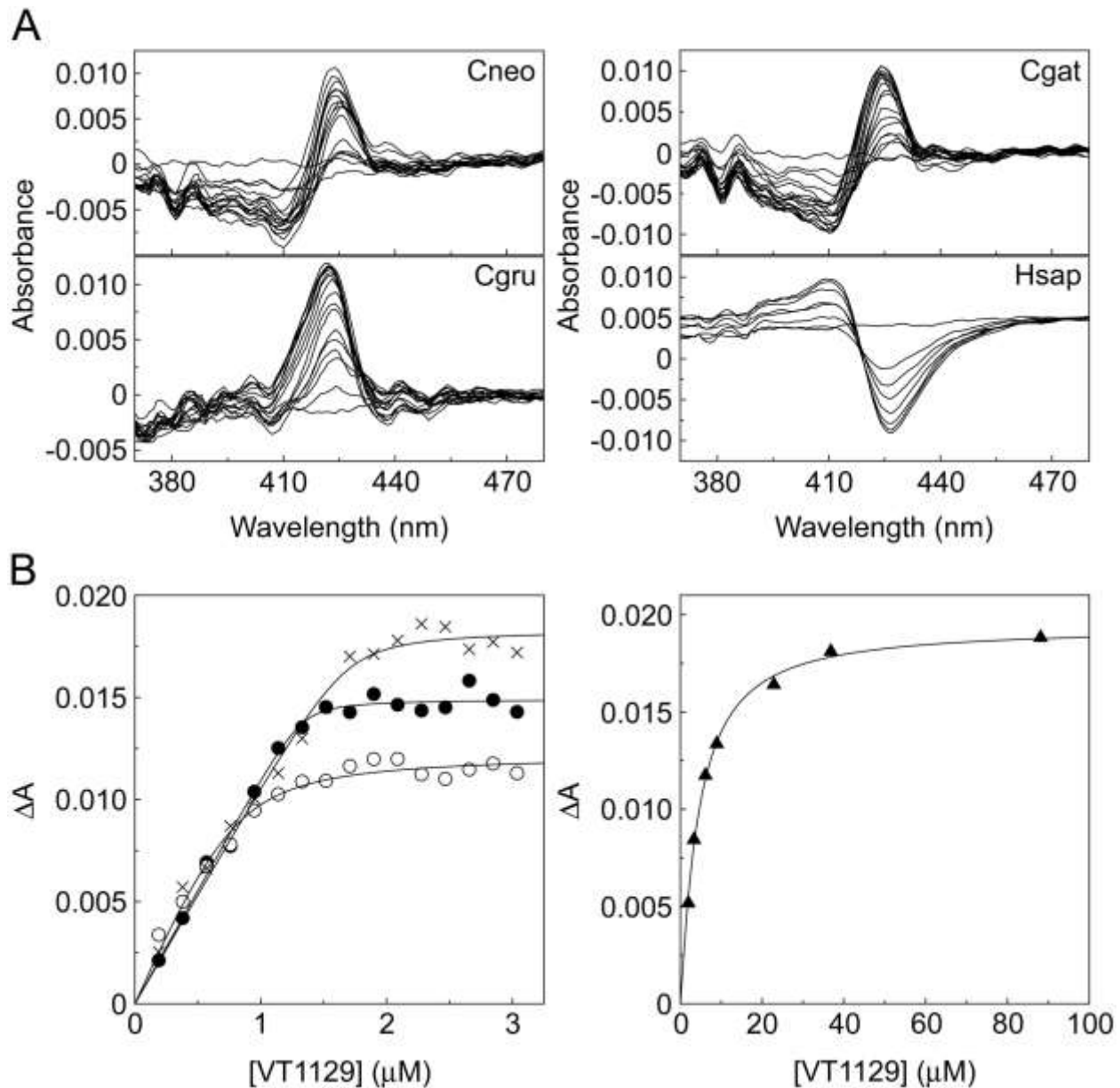
631

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



632  
 633  
 634 **FIG 4** Azole binding properties of cryptococcal CYP51 proteins. Fluconazole and  
 635 itraconazole were progressively titrated against 2  $\mu\text{M}$  CneoCYP51, CgruCYP51, and  
 636 CgatCYP51. (A and B) The resultant type II difference spectra obtained with fluconazole  
 637 (A) and itraconazole (B) are shown. (C and D) Fluconazole (C) and itraconazole (D)  
 638 saturation curves were constructed from the  $\Delta A_{\text{peak-trough}}$  of the type II binding spectra  
 639 observed for CneoCYP51 (solid circles), CgruCYP51 (hollow circles), and CgatCYP51  
 640 (crosses). A rearrangement of the Morrison equation was used to fit the tight ligand  
 641 binding observed. All experiments were performed in triplicate, although the results of  
 642 only one replicate are shown.

643



644

645

646

647

648

649

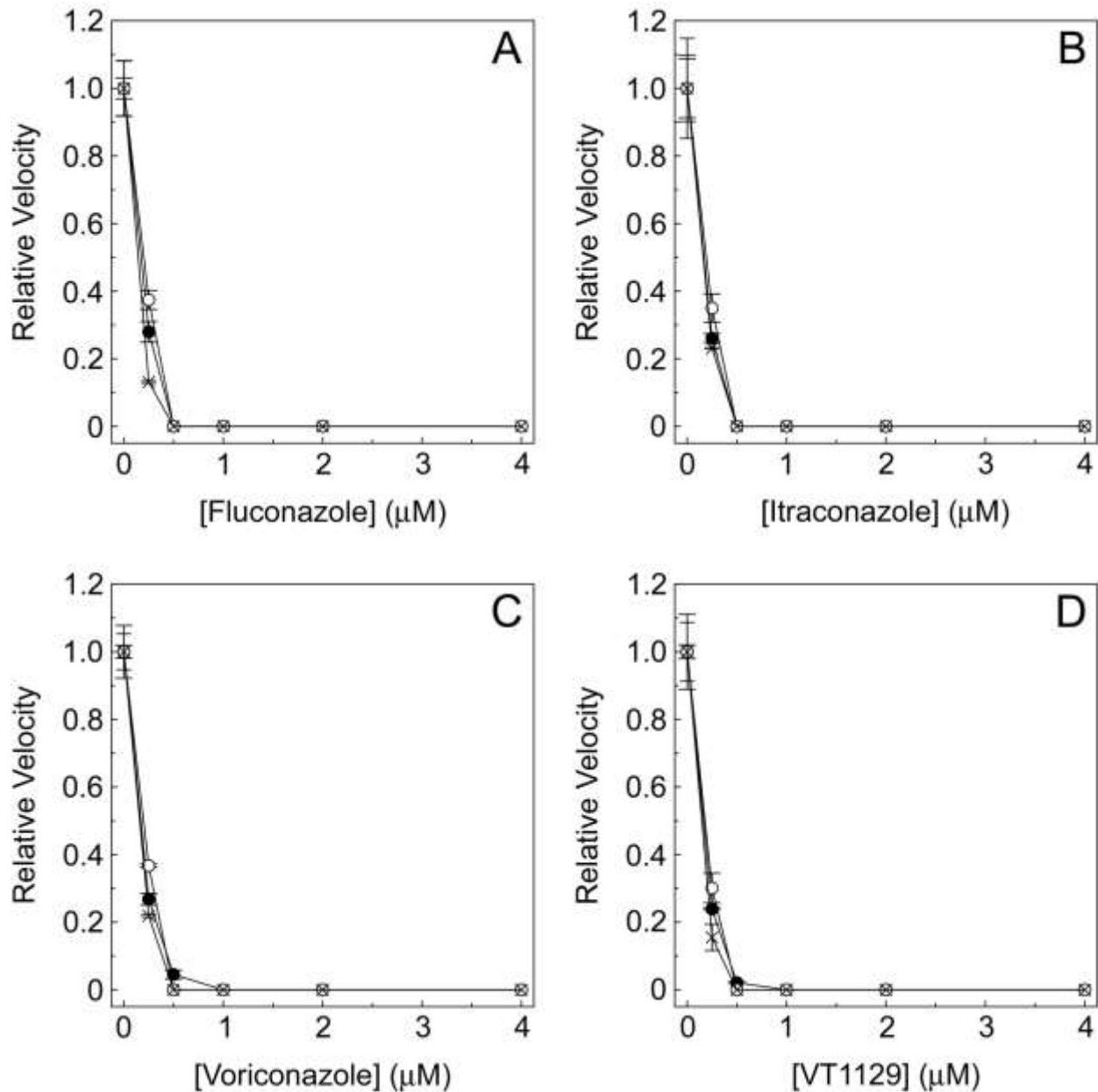
650

**FIG 5** VT-1129 binding properties of cryptococcal and human CYP51 proteins. VT-1129 was progressively titrated against 4  $\mu\text{M}$  CneoCYP51, CgruCYP51, and CgatCYP51 and 5  $\mu\text{M}$  human (*Homo sapiens*) CYP51 (Hsap). (A) The resultant type II difference spectra obtained with the three cryptococcal CYP51 proteins and the red-shifted type I difference spectrum with human CYP51 are shown. (B) Saturation curves were

*VT-1129 and cryptococcal CYP51s*

651 constructed from the  $\Delta A_{\text{peak-trough}}$  of the type II binding spectra observed for CneoCYP51  
652 (solid circles), CgruCYP51 (hollow circles), CgatCYP51 (crosses), and the red-shifted  
653 type I binding spectrum observed for human CYP51 (solid triangles). A rearrangement  
654 of the Morrison equation was used to fit the tight ligand binding observed. All  
655 experiments were performed in triplicate, although the results of only one replicate are  
656 shown.

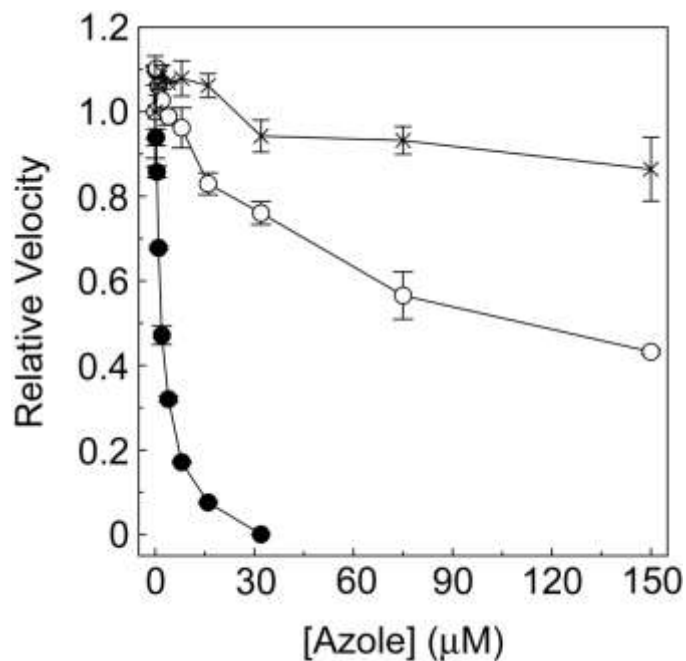
657

658  
659

660 **FIG 6** Azole  $\text{IC}_{50}$  determinations for cryptococcal CYP51 proteins. The  $\text{IC}_{50}$ s of  
 661 fluconazole (A), itraconazole (B), voriconazole (C) and VT-1129 (D) for 0.5  $\mu\text{M}$   
 662 CneoCYP51 (filled circles), CgruCYP51 (hollow circles) and CgatCYP51 (crosses) were  
 663 determined using the CYP51 reconstitution assay with 1  $\mu\text{M}$  AfCPR1 as the redox  
 664 partner.

665

666



667

668

669

670

671

672

673

674

**FIG 7** IC<sub>50</sub> determinations with human CYP51 for clotrimazole, voriconazole, and VT-1129. The CYP51 reconstitution assay contained 0.5 µM human CYP51 and 2 µM human cytochrome P450 reductase as the redox partner in the presence of clotrimazole (filled circles), voriconazole (hollow circles), and VT-1129 (crosses) at concentrations ranging from 0 to 150 µM.