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### Paper:

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1 Isavuconazole and voriconazole inhibition of sterol 14 $\alpha$ -  
2 demethylases (CYP51) from *Aspergillus fumigatus* and  
3 *Homo sapiens*

4  
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18  
19 Running title: Isavuconazole & voriconazole inhibition of CYP51

20  
21 Keywords: Isavuconazole, CYP51, *Aspergillus fumigatus*

22

22 **Highlights**

23 ● First evaluation of the molecular mechanism for isavuconazole inhibition of

24 CYP51s

25 ● Isavuconazole inhibits CYP51 through direct coordination with the heme

26 ferric ion

27 ● Isavuconazole as effective as voriconazole at inhibiting *A. fumigatus*

28 CYP51A & CYP51B

29 ● Isavuconazole is a strong inhibitor of AfCYP51A:G54W and

30 AfCYP51A:M220K enzymes

31 ● Isavuconazole is a potent inhibitor of cellular CYP51 activity in *A.*

32 *fumigatus* Af293

33

34

34 ABSTRACT

35 We report here the first evaluation of isavuconazole for inhibition of *A. fumigatus*  
36 CYP51 and of sterol biosynthesis in the fungus. Voriconazole and isavuconazole  
37 both bound tightly to recombinant AfCYP51A and AfCYP51B isolated in *E. coli*  
38 membranes. CYP51 reconstitution assays confirmed AfCYP51A and AfCYP51B  
39 in addition to three AfCYP51A mutants (G54W, L98H and M220K) were strongly  
40 inhibited by both triazoles. Voriconazole bound relatively weakly to purified  
41 HsCYP51 unlike isavuconazole, which bound tightly. However, isavuconazole  
42 was a relatively poor inhibitor of HsCYP51 activity with an IC<sub>50</sub> value of 25 µM  
43 which was 55- to 120-fold greater than those observed for the *A. fumigatus*  
44 CYP51 enzymes, albeit not as poor an inhibitor of HsCYP51 as voriconazole  
45 which gave an IC<sub>50</sub> value of 112 µM. Sterol analysis of triazole-treated *A.*  
46 *fumigatus* Af293 cells confirmed isavuconazole and voriconazole both inhibited  
47 cellular CYP51 activity with the accumulation of 14-methylated sterol substrates  
48 and depletion of ergosterol levels. Isavuconazole elicited a stronger perturbation  
49 of the sterol composition in Af293 than voriconazole at 0.0125 µg ml<sup>-1</sup> indicating  
50 increased potency. However, complementation studies in *Saccharomyces*  
51 *cerevisiae* using strains containing AfCYP51A and AfCYP51B indicated  
52 isavuconazole to be equally as effective at inhibiting CYP51 activity as  
53 voriconazole. These *in vitro* studies suggest isavuconazole is an effective  
54 alternative to voriconazole as an antifungal agent against the target CYP51 in  
55 *Aspergillus fumigatus*.

56

57

57 **1. Introduction**

58 Mortality associated with invasive fungal disease has increased over the  
59 past four decades, primarily through increasing numbers of cancer patients  
60 undergoing chemotherapy and patients undergoing organ transplantation (1, 2,  
61 reviewed in 3). The majority of the invasive fungal infections observed are  
62 caused by *Candida* and *Aspergillus* species with mortality rates being high,  
63 particularly for aspergillosis, reaching up to 90%. In addition, increased incidence  
64 of invasive infections by *Cryptococcus* species, *Fusarium* species, *Trichosporon*  
65 species, *Scedosporium* species and *Mucorales* (4, 5) requires antifungal drugs  
66 with broader spectra of activity to combat these infections and to overcome  
67 increasing resistance observed against triazole antifungals in some *Candida* and  
68 *Aspergillus* strains.

69 Currently available antifungal agents include polyenes, echinocandins and  
70 azoles. The polyene amphotericin B is a broad spectrum antifungal but is limited  
71 by intra-venous administration and nephrotoxicity. Echinocandins, such as  
72 caspofungin, have good safety profiles but lack oral formulations, have a  
73 relatively narrow spectrum of activity against *Candida* and *Aspergillus* species  
74 and there is increasing resistance to echinocandins amongst certain *Candida*  
75 species. Triazole antifungals have good safety profiles and remain the most  
76 commonly prescribed antifungal agents to treat fungal infections in the clinic and  
77 amongst outpatients (6, 7). Fluconazole has excellent oral bioavailability and is  
78 primarily effective against yeasts and dimorphic fungi but lacks potency against  
79 filamentous fungi, however, incidence of fluconazole resistance amongst

80 *Candida* species is increasing. More recent azoles, including voriconazole (Fig  
81 1), itraconazole and posaconazole, have a broader spectrum of activity to include  
82 filamentous fungi such as *Aspergillus* species, with posaconazole extending  
83 activity further against *Mucorales*. These second generation triazoles, however,  
84 exhibit significant drug interactions and interactions with host liver cytochrome  
85 P450 monooxygenases.

86 Isavuconazole (Fig 1) is a new broad-spectrum triazole antifungal with  
87 activity against yeasts, dimorphic fungi, *Aspergillus* species, molds and  
88 *Mucorales* (8-11). Isavuconazole has a good safety profile and excellent  
89 pharmacokinetic properties making this triazole particularly effective in treating  
90 invasive fungal infections and is currently recommended for the treatment of  
91 invasive aspergillosis and invasive mucormycosis (12). Isavuconazole is  
92 administered as a water-soluble prodrug isavuconazonium, which is rapidly  
93 cleaved by plasma esterases to release the active drug isavuconazole *in situ* (13,  
94 14).

95 Isavuconazole's mode of action is assumed to be similar to other triazole  
96 antifungals, causing inhibition of sterol 14 $\alpha$ -demethylase (CYP51) which is  
97 essential for ergosterol biosynthesis in fungi. However no previous publications  
98 have investigated this in detail. Ergosterol is responsible for the regulation of  
99 membrane integrity, fluidity and permeability. Inhibition of CYP51 leads to the  
100 accumulation of 14 $\alpha$ -methylated sterols, which pack more loosely in lipid bilayers  
101 leading to 'leaky' and unstable membranes causing arrested cell growth and  
102 division (15). Isavuconazole appears to be as effective as voriconazole in the

103 treatment of invasive aspergillosis but with the advantages of a broader spectrum  
104 of activity, more linear pharmacokinetics, less inter-patient variability, increased  
105 water solubility and fewer CYP enzyme-mediated drug-drug interactions than  
106 voriconazole (8, 9, 16). Isavuconazole displayed similar efficacy against  
107 mucormycosis as amphotericin B (9), supporting the use of isavuconazole as  
108 both a front-line and a salvage treatment for mucormycosis.

109 In this study the biochemical mechanism of isavuconazole inhibition of  
110 *Aspergillus fumigatus* CYP51 isoenzymes A and B (AfCYP51A and AfCYP51B)  
111 was demonstrated for the first time using a combination of ligand binding and  
112 CYP51 inhibition studies with recombinant enzymes and modulation of the sterol  
113 profile of *A. fumigatus* Af293 at inhibitory concentrations of isavuconazole. In  
114 addition, the *in vitro* effectiveness of isavuconazole as a sterol 14 $\alpha$ -demethylase  
115 inhibitor was compared against voriconazole using recombinant *Homo sapiens*  
116 CYP51 (HsCYP51), AfCYP51B and AfCYP51A enzymes, including three  
117 prevalent AfCYP51A amino acid substitutions (G54W, L98H and M220) known to  
118 confer azole resistance (17-19).

119

120

120 **2. Materials and methods**

121 *2.1. Heterologous expression, isolation and purification of recombinant A.*  
122 *fumigatus and H. sapiens CYP51 proteins.*

123 The pCWori<sup>+</sup>: $\Delta$ 60HsCYP51 ( $\Delta$ 60-truncation of UniProtKB accession  
124 number Q16850), pCWori<sup>+</sup>:AfCYP51A (Q4WNT5), pCWori<sup>+</sup>:AfCYP51A:G54W,  
125 pCWori<sup>+</sup>:AfCYP51A:L98H, pCWori<sup>+</sup>:AfCYP51A:M220K and pCWori<sup>+</sup>:AfCYP51B  
126 (Q96W81) expression constructs were created as previously described (20, 21).  
127 The pCWori<sup>+</sup>:CYP51 constructs were transformed into competent DH5 $\alpha$  *E. coli*  
128 cells and transformants selected using 0.1 mg/ml ampicillin. Growth and  
129 expression conditions, protein isolation and purification were identical to those  
130 previously reported (20, 21). Previously,  $\Delta$ 60HsCYP51 was shown to have the  
131 same ligand binding properties as the full-length HsCYP51 enzyme (20) and is  
132 therefore referred to as HsCYP51 in this manuscript.

133

134 *2.2. Recombinant CYP51 protein characterization.*

135 The binding properties of isavuconazole and voriconazole (Fig 1) to *A.*  
136 *fumigatus* CYP51s A and B and *H. sapiens* CYP51 were determined  
137 spectrophotometrically as previously described (20) using quartz split-cuvettes  
138 (light path 4.5 mm). Azole antifungals were progressively titrated against 4  $\mu$ M  
139 HsCYP51, AfCYP51A and AfCYP51B purified proteins and 1  $\mu$ M AfCYP51A and  
140 AfCYP51B suspensions in *E. coli* membranes isolated from the expression  
141 clones diluted with 0.1 M Tris-HCl (pH 8.1) and 20% (wt/vol) glycerol at 22°C.



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142 Azole saturation curves were constructed from  $\Delta A_{\text{peak-trough}}$  of the resultant  
143 difference spectra versus azole concentration.

144 The triazole concentrations that cause 50% inhibition of CYP51 activity  
145 ( $IC_{50}$ ) were determined using the CYP51 reconstitution assay system previously  
146 described (21). *H. sapiens* CYP51 assays contained 0.5  $\mu\text{M}$  HsCYP51 and 2  $\mu\text{M}$   
147 *H. sapiens* cytochrome P450 reductase (UniProt accession number P16435)  
148 using lanosterol as substrate. *A. fumigatus* CYP51 assays used 50  $\mu\text{l}$  *E. coli*  
149 membrane preparations containing 0.5  $\mu\text{M}$  AfCYP51A, G54W:AfCYP51A,  
150 L98H:AfCYP51A, M220K:AfCYP51A or AfCYP51B with 1  $\mu\text{M}$  *A. fumigatus*  
151 cytochrome P450 reductase (UniProt accession number Q4WM67) and eburicol  
152 as substrate. Azole antifungal agents were added in 2.5  $\mu\text{l}$  DMSO followed by 10  
153 minutes incubation at 37°C prior to assay initiation with 4 mM  $\beta\text{-NADPH-Na}_4$ .  
154 Incubation times were 4 minutes for HsCYP51 and 15 minutes for AfCYP51A and  
155 AfCYP51B at 37°C. Sterol metabolites were recovered by ethyl acetate  
156 extraction and analyzed by gas chromatography mass spectrometry (section  
157 2.3.).

158

### 159 2.3. Sterol composition analysis.

160 Spore suspensions of *Aspergillus fumigatus* Af293 (ATCC MYA-4609,  
161 CBS 101355) were prepared in Tween 80 saline, containing 0.025% (wt/vol)  
162 Tween 80 and 0.8% (wt/vol) NaCl. Spores were used to inoculate Sabouraud  
163 media (final concentration of  $1 \times 10^4$  cells/ml) in the absence (DMSO control, 1%  
164 vol/vol) or presence of azole. Voriconazole and isavuconazole stocks were

165 prepared in DMSO and added to the media to give a final concentration of 0.125  
166 µg/ml azole and 1% (vol/vol) DMSO. Cultures were incubated at 37°C, 250 rpm  
167 for 48 hours. Mycelia were harvested and non-saponifiable lipids were extracted  
168 as previously described (22). Sterols were derivatized using 0.1ml BSTFA:TMCS  
169 (99:1) and 0.3 ml anhydrous pyridine with heating at 80°C for 2 hours (23). TMS-  
170 derivatized sterols were analysed by GC/MS using a Thermo 1300 GC coupled  
171 to a Thermo ISQ mass spectrometer (Thermo Scientific, Loughborough, UK) and  
172 identified with reference to relative retention times, mass ions and fragmentation  
173 spectra. GC/MS data files were analyzed using Xcalibur software (Thermo  
174 Scientific).

175

#### 176 2.4. *Complementation studies in Saccharomyces cerevisiae.*

177 YUG37-*pcyp51A* and YUG37-*pcyp51B* constructs in *Saccharomyces*  
178 *cerevisiae* (24) were used to assess the relative azole sensitivities of wild-type  
179 AfCYP51A and AfCYP51B towards isavuconazole, voriconazole and  
180 itraconazole. YUG37-*pcyp51A* and YUG37-*pcyp51B* cells were grown in 1.34%  
181 yeast nitrogen base without amino acids (Difco), 2% galactose, 2% raffinose,  
182 leucine and tryptophan (both at 100 mg/l) and doxycyclin (5 µg/ml) at 30°C for 72  
183 h as previously described (24). MIC determinations were performed in triplicate  
184 according to the CLSI M27-A2 broth dilution method, except for the use of  
185 doxycyclin induction media to grow the cells used for the  $2.5 \times 10^3$  cells/ml  
186 inoculums in the microtiter plates. Azole concentrations of 0.001 to 2 µg/ml were  
187 assessed and MIC plates were read visually after 72 h at 30°C. MIC here is

188 defined as the minimum drug concentration that causes at least 80% inhibition of  
189 growth.

190

#### 191 2.5. *Data analysis.*

192 Spectral determinations were made using quartz semi-micro cuvettes with  
193 a Hitachi U-3310 UV/VIS spectrophotometer (San Jose, California). Curve-fitting  
194 of ligand binding data was performed using a rearrangement of the Morrison  
195 equation (25) with the computer program QuantumSoft ProFit (version 6.2.11)  
196 (non-linear regression Levenberg-Marquardt algorithm) to determine  $K_d$  values of  
197 the azole-CYP51 complexes. Ligand titrations were performed in triplicate and  
198 mean  $K_d$  values with standard deviations calculated.

199  $IC_{50}$  enzyme velocities were calculated from gas chromatogram peak  
200 areas for product and substrate. Velocities were standardized against those  
201 observed in the absence of azole antifungal.  $IC_{50}$  experiments were performed in  
202 duplicate and mean  $IC_{50}$  values and standard deviations calculated.

203 Sterol composition of *A. fumigatus* Af293 was calculated using gas  
204 chromatogram peak areas with mass fragmentation patterns confirming sterol  
205 identification. Mean percentage compositions with standard deviations for each  
206 sterol were calculated from three replicate experiments.

207

#### 208 2.6. *Chemicals.*

209 All chemicals, unless otherwise stated, were obtained from Sigma  
210 Chemical Company (Poole, UK). Voriconazole was obtained from Discovery Fine

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211 Chemicals (Bournemouth, UK), Isavuconazole from BOC Sciences (Shirley, New  
212 York) and Growth media, sodium ampicillin, IPTG and 5-aminolevulinic acid from  
213 Foremedium Ltd (Hunstanton, UK).

214

215

215 **3. Results**

216 3.1. *Azole ligand binding studies.*

217 Type II binding spectra were observed between all three CYP51 proteins  
218 and both isavuconazole and voriconazole (Fig 2), yielding a peak at ~428 nm and  
219 a trough at ~412 nm, and indicative of the triazole N-4 nitrogen coordinating as  
220 the sixth ligand with the heme iron (26) to form the low-spin CYP51-azole  
221 complex resulting in a 'red-shift' of the heme Soret peak. Similar spectra were  
222 also observed with *E. coli* membrane suspensions of AfCYP51A and AfCYP51B,  
223 although the spectra were more ragged, in part due to the increased turbidity  
224 caused by the membrane suspensions.

225 Azole saturation curves (Fig 3) confirmed isavuconazole and voriconazole  
226 bound tightly to AfCYP51A and AfCYP51B when isolated in the *E. coli* membrane  
227 fraction from the expression clones with  $K_d$  values of 20 to 60 nM (Table 1). In  
228 contrast, voriconazole and isavuconazole binding to purified AfCYP51A and  
229 AfCYP51B was less tight (Table 1). Voriconazole bound to both purified *A.*  
230 *fumigatus* CYP51 isoenzymes with similar affinity ( $K_d$  ~1  $\mu$ M) whilst  
231 isavuconazole bound more tightly to AfCYP51B than AfCYP51A reflected in the  
232 10-fold lower  $K_d$  value with AfCYP51B (Table 1). Isavuconazole bound tightly to  
233 HsCYP51 ( $K_d$  68 nM) whereas voriconazole bound less tightly ( $K_d$  ~2.3  $\mu$ M).

234

235 3.2. *Azole inhibition of CYP51 sterol 14 $\alpha$ -demethylase activity.*

236  $IC_{50}$  determinations for voriconazole and isavuconazole (Fig 4) indicated  
237 both were equally effective at inhibiting the enzyme activity of the three

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238 AfCYP51A mutations (G54W, L98H and M220K) associated with azole  
239 resistance in *A. fumigatus*, yielding IC<sub>50</sub> values of 0.4 to 0.8 μM, with the only  
240 noticeable difference being slightly higher residual CYP51 activities observed at  
241 high isavuconazole concentrations with the G54W and L98H mutants compared  
242 to voriconazole. Isavuconazole was marginally more effective at inhibiting wild-  
243 type AfCYP51A and AfCYP51B than voriconazole (Fig 4), with the isavuconazole  
244 IC<sub>50</sub> curves dipping below those for voriconazole, however, the difference in IC<sub>50</sub>  
245 values were less than two-fold (Table 2). Both voriconazole and isavuconazole  
246 were weak inhibitors of HsCYP51 activity *in vitro* with 32 μM voriconazole  
247 causing 25% inhibition of CYP51 activity compared to 57% inhibition in the  
248 presence of 32 μM isavuconazole (Fig 4). The 4.5-fold difference in IC<sub>50</sub> values  
249 obtained with HsCYP51 reflected the stronger inhibition exhibited by  
250 isavuconazole (Table 2). The apparent selectivity for *A. fumigatus* CYP51s over  
251 human CYP51 based on IC<sub>50</sub> values were 290- to 340-fold and 110- to 120-fold  
252 for voriconazole and isavuconazole, respectively.

253

#### 254 3.3 Sterol composition analysis.

255 *Aspergillus fumigatus* Af293 was grown from spores in the presence of  
256 0.0125 μg/ml (0.0358 μM) voriconazole and 0.0125 μg/ml (0.0286 μM)  
257 isavuconazole and in the absence of azole antifungals (DMSO control) and the  
258 sterol content of the cells extracted and then analyzed. The predominant sterol in  
259 the control sample was ergosterol, comprising nearly 91% of the total sterol  
260 content (Table 3) with only 0.6% eburicol present. Treatment with 0.0125 μg/ml

261 voriconazole and isavuconazole both resulted in sharp rises in the relative  
262 abundance of the 14-methylated sterols eburicol and lanosterol, indicative of  
263 CYP51 inhibition in the cells (Table 3). The increased 14-methylated sterol  
264 content was more pronounced in the isavuconazole-treated sample, reaching  
265 34% eburicol and 9% lanosterol, than the voriconazole-treated sample that  
266 contained 20% eburicol and 6% lanosterol. Therefore, isavuconazole appeared  
267 to be a more potent inhibitor of cellular CYP51 activity in strain Af293 than  
268 voriconazole, especially bearing in mind the molar isavuconazole concentration  
269 used was 20% lower than that for voriconazole. Levels of toxic 14-methyl-  
270 ergosta-8,24(28)-dien-3,6-diol (22) remained low when cells were grown in  
271 0.0125 µg/ml triazole, comprising just 0.7% and 2.6% of the sterol composition  
272 for isavuconazole- and voriconazole-treated cells, respectively. Cellular  
273 ergosterol depletion, another indicator of CYP51 inhibition, was also evident in  
274 the triazole-treated cells falling from 91% of the sterol composition in the control  
275 cells to 55% and 65% in isavuconazole- and voriconazole-treated cells,  
276 respectively.

277

#### 278 3.4 *Complementation studies in Saccharomyces cerevisiae.*

279 Previously both *A. fumigatus* CYP51 isoenzymes A and B were found to  
280 complement *S. cerevisiae* sterol 14 $\alpha$ -demethylase function (24) using the  
281 YUG37-*pcyp51A* and YUG37-*pcyp51B* constructs. MIC values for fluconazole,  
282 clotrimazole, voriconazole, itraconazole and posaconazole with YUG37-*pcyp51A*  
283 were 8, 0.016, 0.004, 0.125 and 0.063 µg/ml, respectively, compared to 0.5,

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284 0.016, 0.004, 0.125 and 0.063  $\mu\text{g/ml}$  for YUG37-pcyp51B (24). The control  
285 construct YUG37-pCTRL gave MIC values of 0.25, 0.016, 0.004, 0.031 and  
286 0.063  $\mu\text{g/ml}$  against fluconazole, clotrimazole, voriconazole, itraconazole and  
287 posaconazole, respectively (24). Therefore AfCYP51A conferred tolerance  
288 towards fluconazole, whilst AfCYP51A and AfCYP51B were equally susceptible  
289 to inhibition by clotrimazole, voriconazole, itraconazole and posaconazole.

290 In this study, MIC determinations with voriconazole and itraconazole were  
291 repeated along with MIC determinations for the new triazole antifungal  
292 isavuconazole. MIC values obtained with YUG37-pcyp51A were 0.002, 0.0625  
293 and 0.002  $\mu\text{g/ml}$  for voriconazole, itraconazole and isavuconazole, respectively,  
294 compared with 0.001, 0.0313 and 0.001  $\mu\text{g/ml}$  for YUG37-pcyp51B. Therefore  
295 isavuconazole was equally effective at inhibiting both AfCYP51A and AfCYP51B  
296 as voriconazole and was 300-fold more effective than itraconazole.

297

298



298 **4. Discussion**

299 The type II binding spectra observed between voriconazole and  
300 isavuconazole and the three CYP51 proteins (Fig 2) indicated that the mode of  
301 interaction was the same for both triazoles, namely through the triazole N-4  
302 nitrogen coordinating as the sixth ligand with the heme iron (26). Both triazoles  
303 bound tighter to AfCYP51A and AfCYP51B isolated in the *E. coli* membrane  
304 fraction from the expression clones than to the purified proteins. The fold-  
305 difference in the calculated  $K_d$  values between purified and membrane-isolated  
306 proteins with voriconazole were 19- and 25-fold for AfCYP51A and AfCYP51B,  
307 respectively, compared to 39- and 11-fold with isavuconazole (Table 1). This  
308 suggests the enzyme conformation adopted by AfCYP51A and AfCYP51B in free  
309 solution was subtly different to that in a lipid bilayer membrane and is supported  
310 by the observation that CYP51 catalysis was ten-fold higher for *A. fumigatus*  
311 CYP51 proteins isolated in *E. coli* membranes (21). The tight triazole binding  
312 observed with the membrane *A. fumigatus* CYP51 proteins suggested AfCYP51A  
313 and AfCYP51B would be strongly inhibited by both voriconazole and  
314 isavuconazole. This was confirmed by the low  $IC_{50}$  values obtained which were  
315 approximately half the CYP51 concentration and indicative of tight binding  
316 inhibitors (Table 2).

317 The  $K_d$  value for isavuconazole with HsCYP51 was 34-fold lower than that  
318 obtained for voriconazole, suggesting that isavuconazole would be a stronger  
319 inhibitor of HsCYP51 activity. This was confirmed by the  $IC_{50}$  values obtained  
320 with HsCYP51 (Table 2), however, the degree of inhibition caused by

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321 isavuconazole was less than expected considering the low  $K_d$  value of 68 nM.  
322 AfCYP51A in *E. coli* membranes had a similar  $K_d$  for isavuconazole (61 nM) and  
323 yet the  $IC_{50}$  for isavuconazole was 0.21  $\mu$ M compared to 25  $\mu$ M obtained with  
324 HsCYP51 (Table 2). This suggests suspension of HsCYP51 in a lipid bilayer in  
325 the presence of substrate and CPR redox partner weakens *in situ* isavuconazole  
326 binding. This requires further investigation to ascertain the biophysical and  
327 biochemical mechanisms involved. Therefore initial concerns about the relatively  
328 poor selectivity of isavuconazole for the *A. fumigatus* CYP51s over the human  
329 homolog based on ligand binding data (1.1- to 3.2-fold differences in  $K_d$ ) were not  
330 observed when  $IC_{50}$  values were measured (108- to 119-fold differences). For  
331 voriconazole the selectivity for the *A. fumigatus* CYP51s was 45- to 60-fold based  
332 on  $K_d$  and 290- to 340-fold based on  $IC_{50}$  (Table 2), indicating voriconazole was  
333 more selective for *A. fumigatus* CYP51s over the human homolog than  
334 isavuconazole, albeit with isavuconazole still being a strong inhibitor of *A.*  
335 *fumigatus* CYP51 activity *in vitro*. Azole ligand binding studies provide a useful  
336 preliminary screen for new potential CYP51-inhibitory compounds that contain an  
337 azole functional group, including mechanistic information on the mode of  
338 interaction, but confirmatory CYP51 reconstitution assays are required to  
339 determine *in vitro*  $IC_{50}$  values for each compound.

340  $IC_{50}$  values obtained for voriconazole and isavuconazole against the  
341 G54W, L98H and M220K proteins were only two-fold greater than the wild-type  
342 AfCYP51A indicating both triazoles strongly inhibited CYP51 activity of all three  
343 mutants, with isavuconazole proving marginally more potent than voriconazole,

344 albeit at the expense of slightly increased residual activities at high  
345 isavuconazole concentrations (Fig 4). Therefore, isavuconazole is as effective as  
346 voriconazole in terms of inhibiting AfCYP51A and AfCYP51B activity and is a  
347 strong inhibitor of the CYP51 activity of the AfCYP51A mutants G54W, L98H and  
348 M220K which are often associated with resistance / tolerance to itraconazole and  
349 posaconazole.

350         These observations were consistent with the azole phenotypes of G54W  
351 and M220K in which G54W was found to confer resistance to itraconazole (MIC  
352 >16 µg/ml) and posaconazole (MIC >8 µg/ml) but not to voriconazole (MIC 0.25  
353 µg/ml) (17) and M220K to confer resistance to itraconazole (MIC >8 µg/ml),  
354 elevated MIC to posaconazole (MIC 1 to 2 µg/ml compared to 0.06 µg/ml for wild-  
355 type) but little increase in resistance to voriconazole (MIC 1 µg/ml) (18). Previous  
356 investigations utilizing recombinant G54W and M220K AfCYP51A proteins have  
357 shown these mutations confer resistance against CYP51 inhibition by  
358 itraconazole and posaconazole and limited tolerance to voriconazole (21). MIC  
359 values for isavuconazole with *A. fumigatus* strains containing the CYP51A G54W  
360 and M220K substitutions were 0.125 to 0.25 µg/ml and 1 to 4 µg/ml, respectively  
361 (27). As isavuconazole was equally effective at inhibiting the AfCYP51A:G54W  
362 and AfCYP51A:L98H proteins, the observed variability in the isavuconazole MICs  
363 for the AfCYP51A:M220K-containing strains suggest additional resistance  
364 mechanisms were also present.

365         The two-fold increase in IC<sub>50</sub> values for AfCYP51A:L98H over the wild-  
366 type enzyme indicates L98H on its own does not confer the full azole resistance

367 phenotype observed with AfCYP51A:TR34/L98H-containing strains. This is in  
368 agreement with previous studies using recombinant AfCYP51A:L98H protein (21)  
369 and with *A. fumigatus* transformation studies (19) in which both the tandem  
370 repeat and the L98H mutation are required to confer itraconazole resistance (MIC  
371 >16 µg/ml) and elevated MIC against voriconazole (2 µg/ml). MIC values for  
372 isavuconazole with AfCYP51A:TR34/L98H-containing strains are variable at 4 to  
373 >16 µg/ml (27), suggesting other azole resistance mechanisms are also present  
374 in some of these strains.

375         The relatively high residual CYP51 activities observed for AfCYP51A:L98H  
376 at 8, 16 and 32 µM voriconazole or isavuconazole suggests the L98H mutation  
377 may confer azole tolerance in a clinical setting by facilitating slow *A. fumigatus*  
378 growth under prolonged triazole treatment regimens, rather than arresting growth  
379 in strains that possess a wild-type AfCYP51A enzyme. In addition, when the  
380 L98H substitution is coupled to a 5-fold increase in AfCYP51A expression levels  
381 associated with TR34 over the wild-type form (28), this could explain the azole  
382 resistance phenotype observed for TR34/L98H combination.

383         The prevalence of the AfCYP51A:TR34/L98H genotype is increasing both  
384 numerically and geographically amongst azole resistant *A. fumigatus* clinical  
385 isolates (29, 30) and other tandem repeat linked AfCYP51A mutations are  
386 emerging, such as TR46/Y121F/T289A (31), TR34/L98H/S297T/F495I (32) and  
387 TR46/Y121F/M172I/T289A/G448S (28). The emergence of these mutations  
388 suggest *A. fumigatus* is undergoing a similar process previously observed in  
389 *Mycosphaerella graminicola* CYP51 in which complex genotypes with multiple

390 substitutions have been selected during the changing regimes of azole fungicides  
391 deployed over recent decades with the wild-type CYP51 alleles not seen in some  
392 countries (33).

393 Less frequently encountered AfCYP51A mutations that confer azole  
394 resistance include G138C, G138S, Y431C, G434C and G448S. Clinical strains  
395 containing AfCYP51A:G138C/S are resistant to isavuconazole, voriconazole and  
396 itraconazole (MIC 8 to >16 µg/ml) but display variable resistance towards  
397 posaconazole (MIC 1 to >16 µg/ml) (34-36). Similarly, clinical *A. fumigatus*  
398 strains containing the AfCYP51 substitutions Y431C, G434C and G448S are  
399 resistant against isavuconazole, voriconazole, itraconazole and posaconazole  
400 (34-36). Albarrag et al (34) confirmed that G138C and Y431C conferred  
401 resistance against voriconazole, itraconazole and posaconazole using  
402 complementation studies in *S. cerevisiae*, however, unexpectedly the  
403 AfCYP51A:G434C transformant caused hypersensitivity to azole antifungals. The  
404 molecular basis for azole resistance conferred by the AfCYP51A amino acid  
405 substitutions G138C, G138S, Y431C, G434C and G448S would be of interest for  
406 a future study, especially as these substitutions appear to confer the greatest  
407 resistance towards isavuconazole.

408 Sterol composition studies confirmed isavuconazole and voriconazole at  
409 0.0125 µg/ml both inhibited cellular CYP51 activity in *A. fumigatus* Af293,  
410 characterized by the accumulation of 14-methylated sterols and the depletion of  
411 ergosterol, demonstrating the *in situ* mode of action of both azoles.  
412 Isavuconazole elicited a stronger response than voriconazole even though the

413 molar concentration of isavuconazole was 20% lower, confirming isavuconazole  
414 as a more potent inhibitor of cellular CYP51 activity in this strain. Further *A.*  
415 *fumigatus* strains (azole sensitive and azole resistant) will need to be evaluated  
416 to establish whether this observation is strain specific or more general.

417 Isavuconazole was generally found to be as effective as voriconazole at  
418 inhibiting the growth of *Candida* spp. (37-39), as well as *Cryptococcus* spp. (37,  
419 38), *Coccidioides* spp. (38), *Fusarium* spp. (38), and *Aspergillus* spp. (37, 39) but  
420 less effective than voriconazole at inhibiting *Scedosporium* spp. growth (38). The  
421 FDA currently licenses isavuconazole for the treatment of invasive aspergillosis  
422 and invasive mucormycosis with a recent clinical study showing isavuconazole to  
423 be non-inferior to voriconazole for the primary treatment of invasive mould  
424 disease along with isavuconazole being well tolerated compared to voriconazole  
425 and with fewer drug-related side effects (8). Isavuconazole exhibits moderate  
426 activity towards *Mucorales*, whereas few *Mucorales* isolates could be classified  
427 as susceptible to voriconazole (40). However, direct comparisons of MIC values  
428 across compounds are not readily correlated to clinical effectiveness as factors  
429 such as *in vivo* bioavailability and pharmacokinetic interactions and stability also  
430 contribute to clinical effectiveness.

431

## 432 **5. Conclusions**

433 The biochemical mode of action of isavuconazole has been demonstrated  
434 for the first time both *in vitro* using recombinant CYP51 enzymes, where  
435 isavuconazole inhibits CYP51 activity through direct coordination of the triazole

436 nitrogen atom as the sixth axial ligand to the heme ferric ion, and at a cellular  
437 level by analysis of *A. fumigatus* sterol composition where isavuconazole inhibits  
438 CYP51 activity resulting in an accumulation of 14-methylated sterols and the  
439 depletion of ergosterol. The molecular mode of action of isavuconazole is  
440 confirmed to be the same as other triazole antifungals.

441 Isavuconazole is a good alternative to voriconazole as an inhibitor of *A.*  
442 *fumigatus* CYP51 activity and *A. fumigatus* cellular growth and is an effective  
443 inhibitor of two AfCYP51A mutations (G54W and M220K) that confer tolerance  
444 towards itraconazole and posaconazole. Isavuconazole has the disadvantage of  
445 increased inhibition of human CYP51 activity compared to voriconazole.  
446 However, this is offset by increased bioavailability of isavuconazole, linear  
447 pharmacokinetics, fewer drug interactions and lower reported side effects  
448 compared to voriconazole.

449

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458

459

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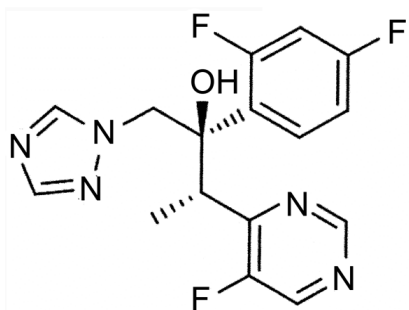
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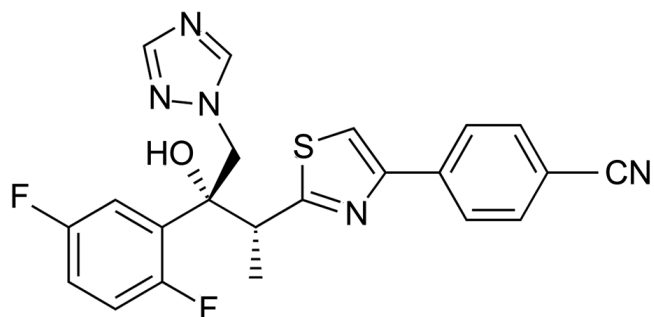
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Voriconazole



Isavuconazole

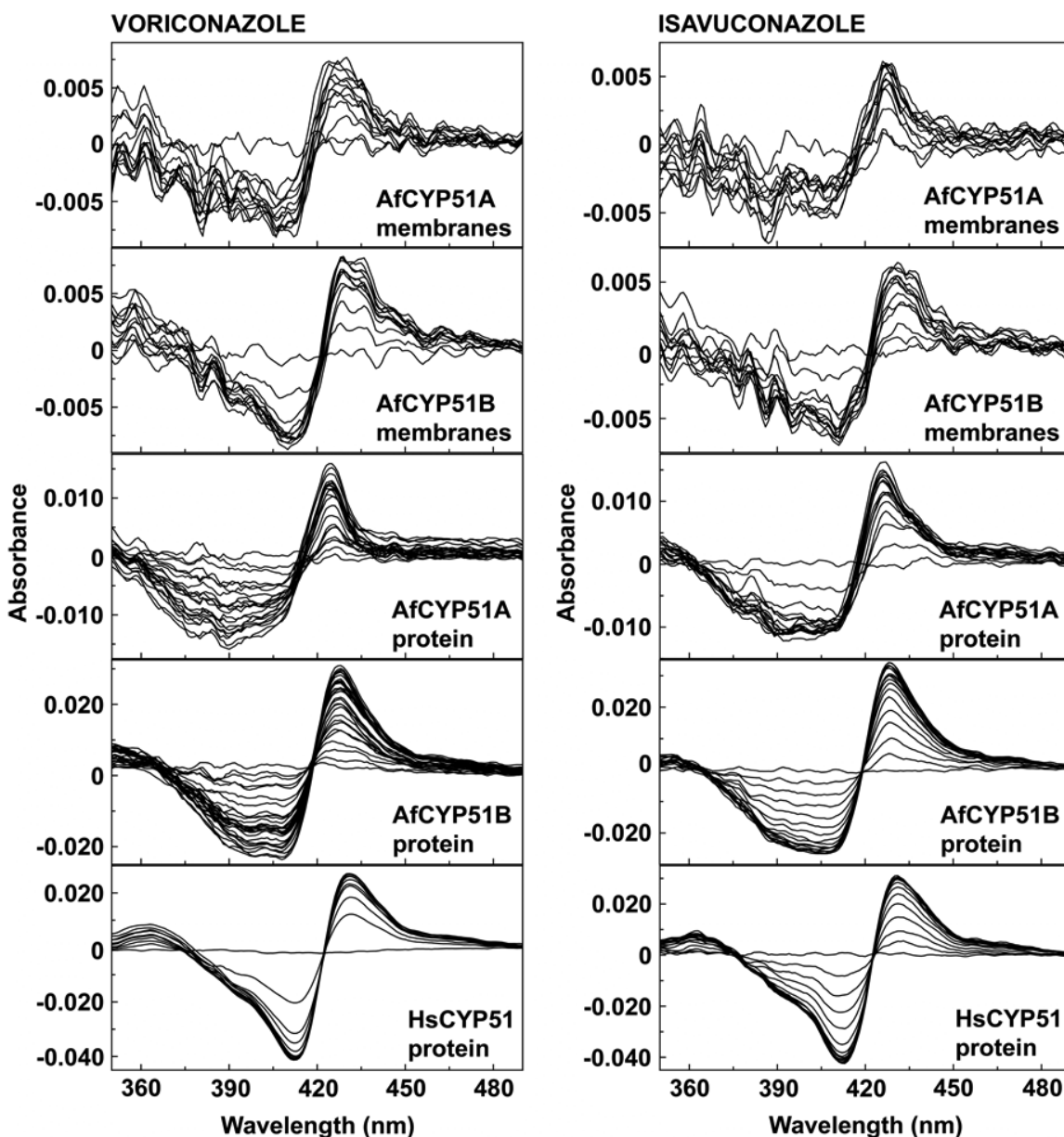
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614 **Fig. 1.** Chemical structures of voriconazole [molecular weight, MW 349] and

615 isavuconazole [MW 437].

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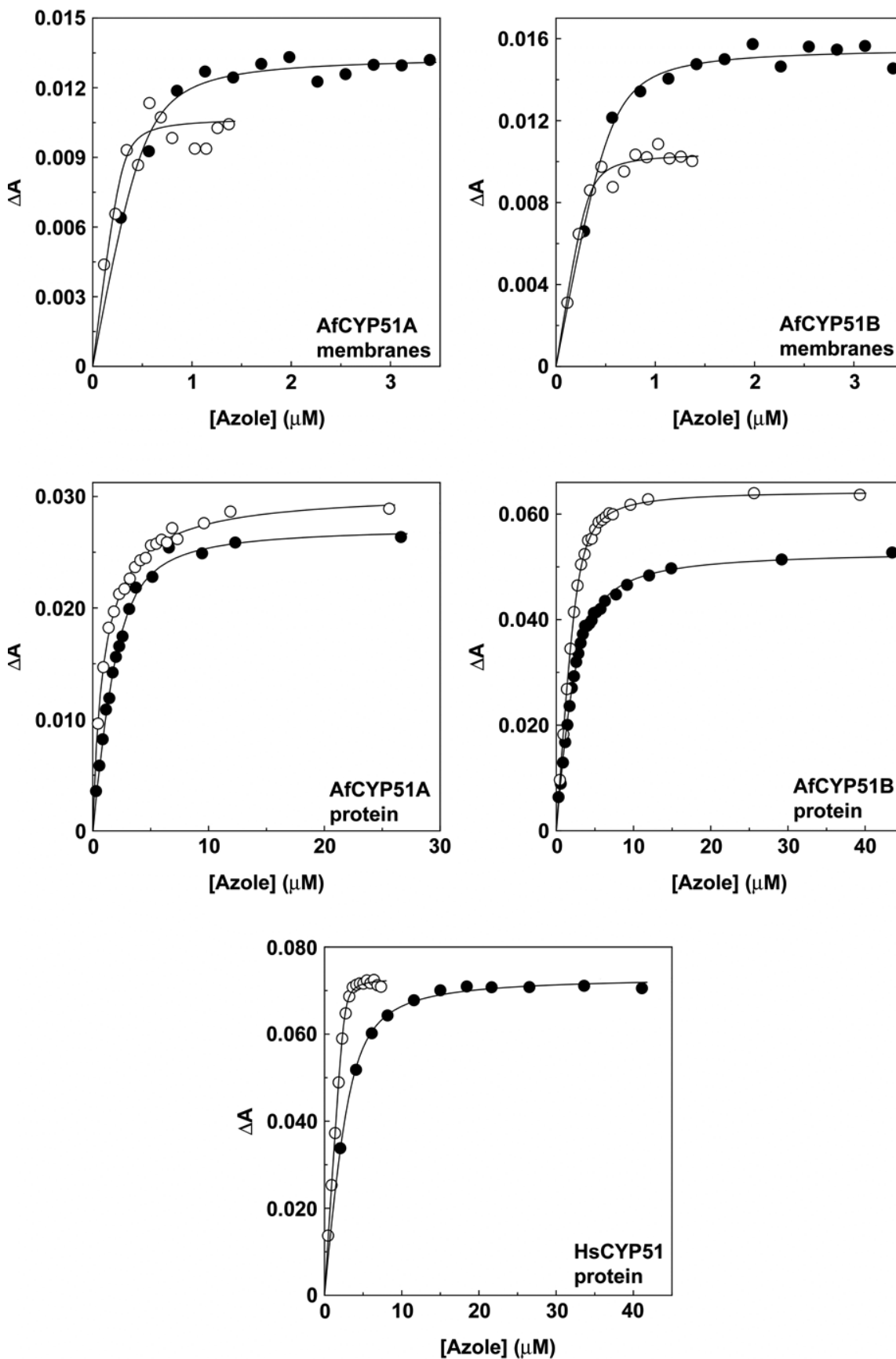
618 **Fig. 2.** Type II binding spectra. Type II difference spectra were obtained by the  
 619 progressive titration of voriconazole and isavuconazole against 4  $\mu$ M purified  
 620 HsCYP51, AfCYP51A and AfCYP51B proteins and *E. coli* membrane  
 621 suspensions containing 1  $\mu$ M AfCYP51A and AfCYP51B. All spectral  
 622 determinations were performed in triplicate, although only one replicate is shown.

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*Isavuconazole & voriconazole inhibition of CYP51*

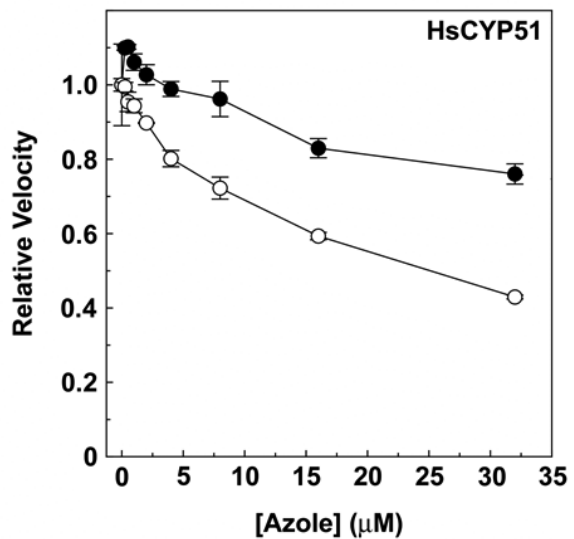
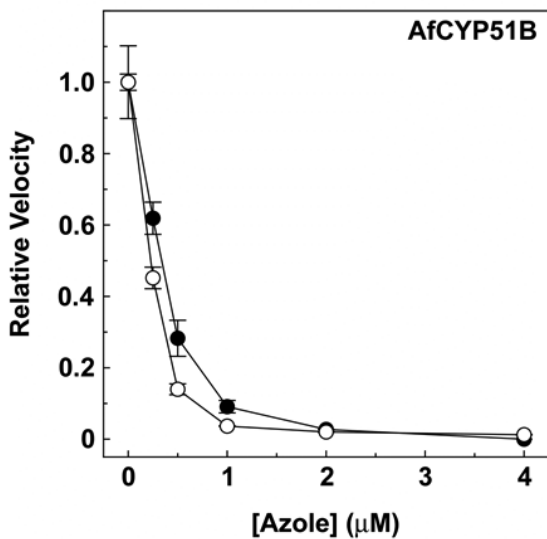
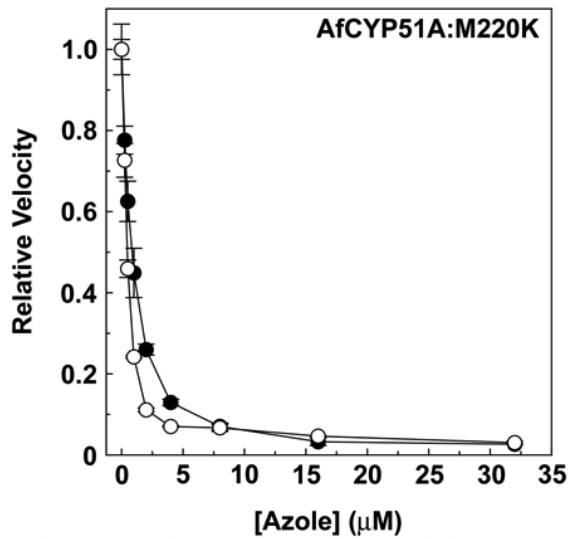
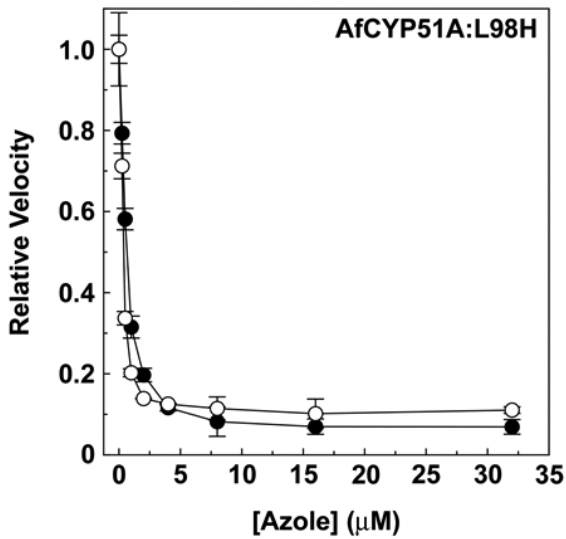
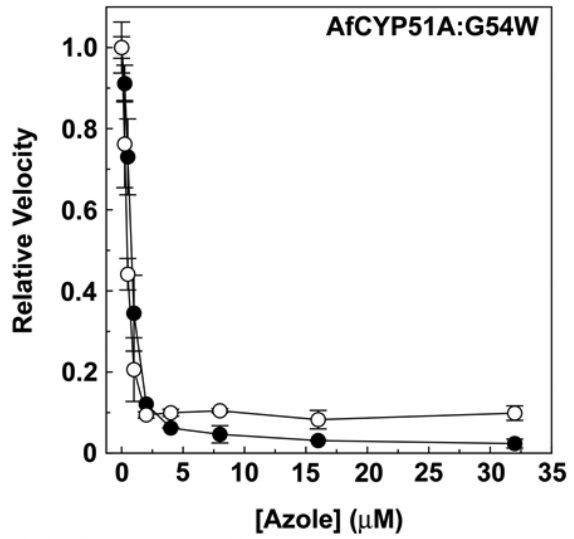
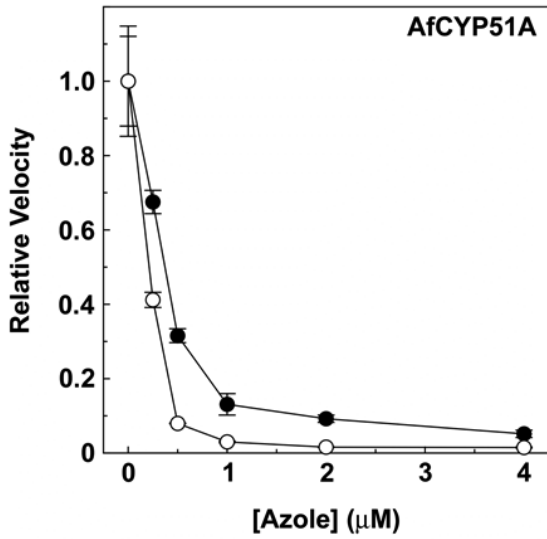


625 **Fig. 3.** Azole ligand saturation curves. Ligand saturation curves for voriconazole  
626 (filled circles) and isavuconazole (hollow circles) were constructed from the type  
627 II difference spectra (Fig 2) and were fitted using a rearrangement of the  
628 Morrison equation for tight ligand binding (25).

629

630

*Isavuconazole & voriconazole inhibition of CYP51*



631 **Fig. 4.** Azole inhibition profiles. IC<sub>50</sub> values for voriconazole (filled circles) and  
632 isavuconazole (hollow circles) were determined using a CYP51 reconstitution  
633 assay system that contained either 0.5 μM HsCYP51, 2 μM HsCPR, 50 μM  
634 DLPC or 0.5 μM *A. fumigatus* CYP51 proteins isolated in the *E. coli* membrane  
635 fractions from the expression clones supplemented with 1 μM AfCPR.  
636 Additionally, the relative velocities for HsCYP51 in the presence of 75 and 150  
637 μM voriconazole were 0.565 ±0.056 and 0.432 ±0.007. Relative turnover  
638 numbers of 1.00 equate to mean turnover numbers of 1.06, 1.13, 4.91, 2.47,  
639 1.11, and 11.72 min<sup>-1</sup> for AfCYP51A, AfCYP51A:G54W, AfCYP51A:L98H,  
640 AfCYP51A:M220K, AfCYP51B, and HsCYP51, respectively. IC<sub>50</sub> experiments  
641 were performed in duplicate with the mean values plotted and standard  
642 deviations presented as error bars.

643

644

*Isavuconazole & voriconazole inhibition of CYP51*

644 **Table 1**

645  $K_d$  values for voriconazole and isavuconazole.

CYP51	$K_d$ (nM)			
	Proteins		Membranes	
	Voriconazole	Isavuconazole	Voriconazole	Isavuconazole
HsCYP51	2290 ±120	68 ±23	-	-
AfCYP51A	980 ±239	2358 ±707	51 ±17	61 ±18
AfCYP51B	958 ±22	228 ±61	38 ±16	21 ±6

646

647

647 **Table 2**

648 IC<sub>50</sub> values for voriconazole and isavuconazole.

CYP51	IC <sub>50</sub> (μM)	
	Voriconazole	Isavuconazole
HsCYP51	112 ±27	25 ±2
AfCYP51A	0.38 ±0.05 <sup>a</sup>	0.21 ±0.03
AfCYP51A: G54W	0.80 ±0.09 <sup>a</sup>	0.45 ±0.08
AfCYP51A: L98H	0.65 ±0.13 <sup>a</sup>	0.39 ±0.05
AfCYP51A: M220K	0.84 ±0.08 <sup>a</sup>	0.46 ±0.04
AfCYP51B	0.33 ±0.07 <sup>a</sup>	0.23 ±0.03

649 <sup>a</sup> as previously reported by Warrilow et al (21).

650

651

651 **Table 3**

652 Sterol composition of control, voriconazole- and isavuconazole-treated *A.*  
 653 *fumigatus* Af293.

Sterols	Sterol composition (%)		
	DMSO (control)	Voriconazole (0.0125 µg/ml)	Isavuconazole (0.0125 µg/ml)
Ergosta-5,8,22-trienol	1.5 (±0.0)	1.0 (±0.0)	1.0 (±0.4)
Ergosterol	90.8 (±0.5)	64.5 (±0.9)	55.1 (±0.8)
Methylated ergosta- trienol	4.8 (±0.4)	3.5 (±0.3)	
14-methyl-ergosta- 8,24(28)-dien-3,6-diol		2.6 (±0.6)	0.7 (±0.1)
Lanosterol		6.4 (±0.3)	9.2 (±0.1)
Eburicol	0.6 (±0.1)	19.6 (±0.8)	34.0 (±0.7)
4,4 dimethyl-ergosta- 8,24-dienol	1.6 (±0.1)	1.0 (±0.2)	

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