Isavuconazole and voriconazole inhibition of sterol 14α-demethylases (CYP51) from *Aspergillus fumigatus* and *Homo sapiens*

Andrew G.S. Warrilow[a], Josie E. Parker[a], Claire L. Price[a], Nicola J. Rolley[a], W. David Nes[b], Diane E. Kelly[a], and Steven L. Kelly[a*]

Author affiliations: Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University Medical School, Swansea, Wales, UK[a]; Center for Chemical Biology, Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas, USA[b].

[*]Corresponding author: Professor Steven Kelly
Centre for Cytochrome P450 Biodiversity, Institute of Life Science, Swansea University Medical School, Swansea, SA2 8PP, Wales, UK.
E-mail address: s.l.kelly@swansea.ac.uk
Phone: +44 (0)1792 292207; Fax: +44 (0)1792 503430

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Highlights

- First evaluation of the molecular mechanism for isavuconazole inhibition of CYP51s
- Isavuconazole inhibits CYP51 through direct coordination with the heme ferric ion
- Isavuconazole as effective as voriconazole at inhibiting *A. fumigatus* CYP51A & CYP51B
- Isavuconazole is a strong inhibitor of AfCYP51A:G54W and AfCYP51A:M220K enzymes
- Isavuconazole is a potent inhibitor of cellular CYP51 activity in *A. fumigatus* Af293

ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

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

The pCWori^{+}:*Δ60HsCYP51* (Δ60-truncation of UniProtKB accession number Q16850), pCWori^{+}:*AfCYP51A* (Q4WNT5), pCWori^{+}:*AfCYP51A:G54W*, pCWori^{+}:*AfCYP51A:L98H*, pCWori^{+}:*AfCYP51A:M220K* and pCWori^{+}:*AfCYP51B* (Q96W81) expression constructs were created as previously described (20, 21). The pCWori^{+}:*CYP51* constructs were transformed into competent DH5α *E. coli* cells and transformants selected using 0.1 mg/ml ampicillin. Growth and expression conditions, protein isolation and purification were identical to those previously reported (20, 21). Previously, Δ60HsCYP51 was shown to have the same ligand binding properties as the full-length HsCYP51 enzyme (20) and is therefore referred to as HsCYP51 in this manuscript.

2.2. Recombinant CYP51 protein characterization.

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

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

The triazole concentrations that cause 50% inhibition of CYP51 activity (IC_{50}) were determined using the CYP51 reconstitution assay system previously described (21). *H. sapiens* CYP51 assays contained 0.5 µM HsCYP51 and 2 µM *H. sapiens* cytochrome P450 reductase (UniProt accession number P16435) using lanosterol as substrate. *A. fumigatus* CYP51 assays used 50 µl *E. coli* membrane preparations containing 0.5 µM AfCYP51A, G54W:AfCYP51A, L98H:AfCYP51A, M220K:AfCYP51A or AfCYP51B with 1 µM *A. fumigatus* cytochrome P450 reductase (UniProt accession number Q4WM67) and eburicol as substrate. Azole antifungal agents were added in 2.5 µl DMSO followed by 10 minutes incubation at 37°C prior to assay initiation with 4 mM β-NADPH-Na_4. Incubation times were 4 minutes for HsCYP51 and 15 minutes for AfCYP51A and AfCYP51B at 37°C. Sterol metabolites were recovered by ethyl acetate extraction and analyzed by gas chromatography mass spectrometry (section 2.3.).

2.3. Sterol composition analysis.

Spore suspensions of *Aspergillus fumigatus* Af293 (ATCC MYA-4609, CBS 101355) were prepared in Tween 80 saline, containing 0.025% (wt/vol) Tween 80 and 0.8% (wt/vol) NaCl. Spores were used to inoculate Sabouraud media (final concentration of $1x10^4$ cells/ml) in the absence (DMSO control, 1% vol/vol) or presence of azole. Voriconazole and isavuconazole stocks were

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

2.4. Complementation studies in Saccharomyces cerevisae.

YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs in *Saccharomyces cerevisiae* (24) were used to assess the relative azole sensitivities of wild-type AfCYP51A and AfCYP51B towards isavuconazole, voriconazole and itraconazole. YUG37-pcyp51A and YUG37-pcyp51B cells were grown in 1.34% yeast nitrogen base without amino acids (Difco), 2% galactose, 2% raffinose, leucine and tryptophan (both at 100 mg/l) and doxycyclin (5 µg/ml) at 30°C for 72 h as previously described (24). MIC determinations were performed in triplicate according to the CLSI M27-A2 broth dilution method, except for the use of doxycyclin induction media to grow the cells used for the 2.5 x 10^3 cells/ml inoculums in the microtiter plates. Azole concentrations of 0.001 to 2 µg/ml were 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

211 Chemicals (Bournemouth, UK), Isavuconazole from BOC Sciences (Shirley, New
212 York) and Growth media, sodium ampicillin, IPTG and 5-aminolevulenic 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 μM) 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 μM).

234

235 ## *3.2. Azole inhibition of CYP51 sterol 14α-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

238 AfCYP51A mutations (G54W, L98H and M220K) associated with azole
239 resistance in *A. fumigatus*, yielding IC_{50} 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_{50} curves dipping below those for voriconazole, however, the difference in IC_{50}
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_{50} 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_{50} 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

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

3.4 Complementation studies in Saccharomyces cerevisiae.

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

284 0.016, 0.004, 0.125 and 0.063 μg/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 μg/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 μg/ml for voriconazole, itraconazole and isavuconazole, respectively,
294 compared with 0.001, 0.0313 and 0.001 μg/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

4. Discussion

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

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

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

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

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

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

The two-fold increase in IC_{50} values for AfCYP51A:L98H over the wild-type enzyme indicates L98H on its own does not confer the full azole resistance

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

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

The prevalence of the AfCYP51A:TR34/L98H genotype is increasing both numerically and geographically amongst azole resistant *A. fumigatus* clinical isolates (29, 30) and other tandem repeat linked AfCYP51A mutations are emerging, such as TR46/Y121F/T289A (31), TR34/L98H/S297T/F495I (32) and TR46/Y121F/M172I/T289A/G448S (28). The emergence of these mutations suggest *A. fumigatus* is undergoing a similar process previously observed in *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. cerevisae*, 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

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

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

5. Conclusions

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

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

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

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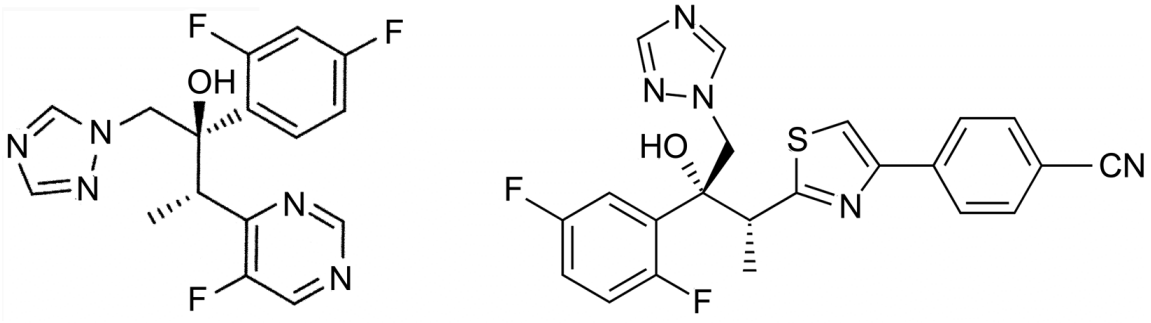


613

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

615 isavuconazole [MW 437].

616

617

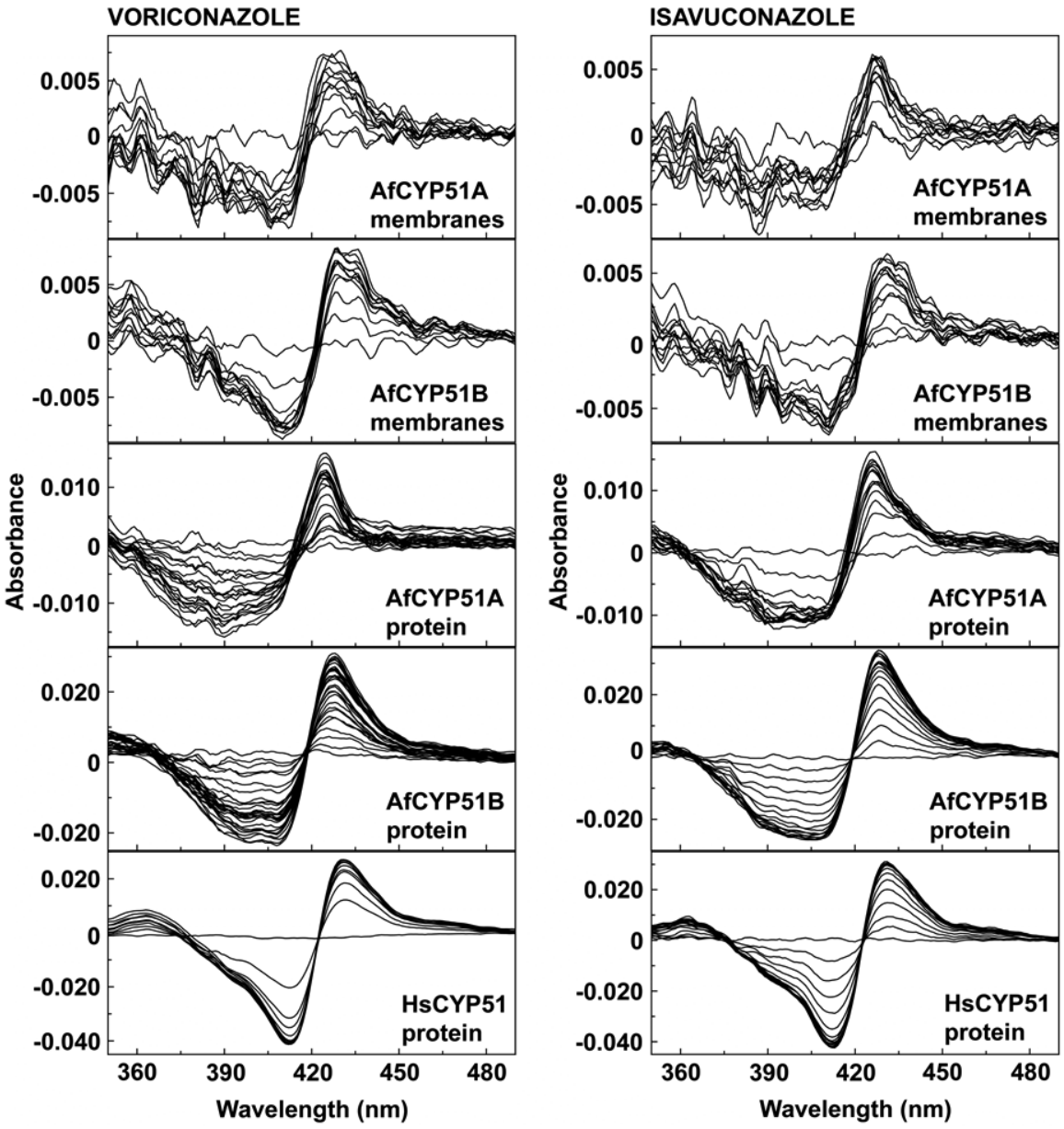
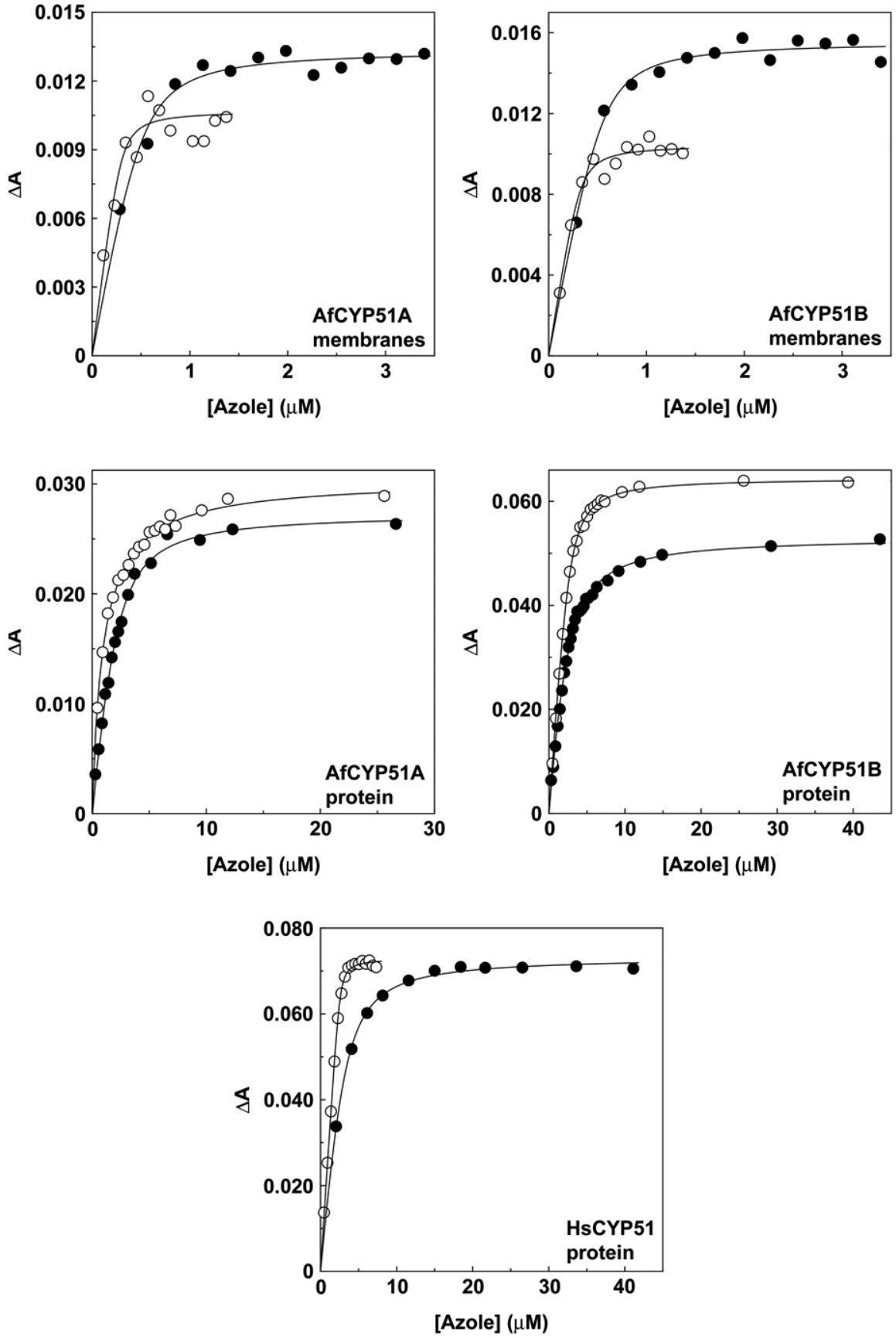
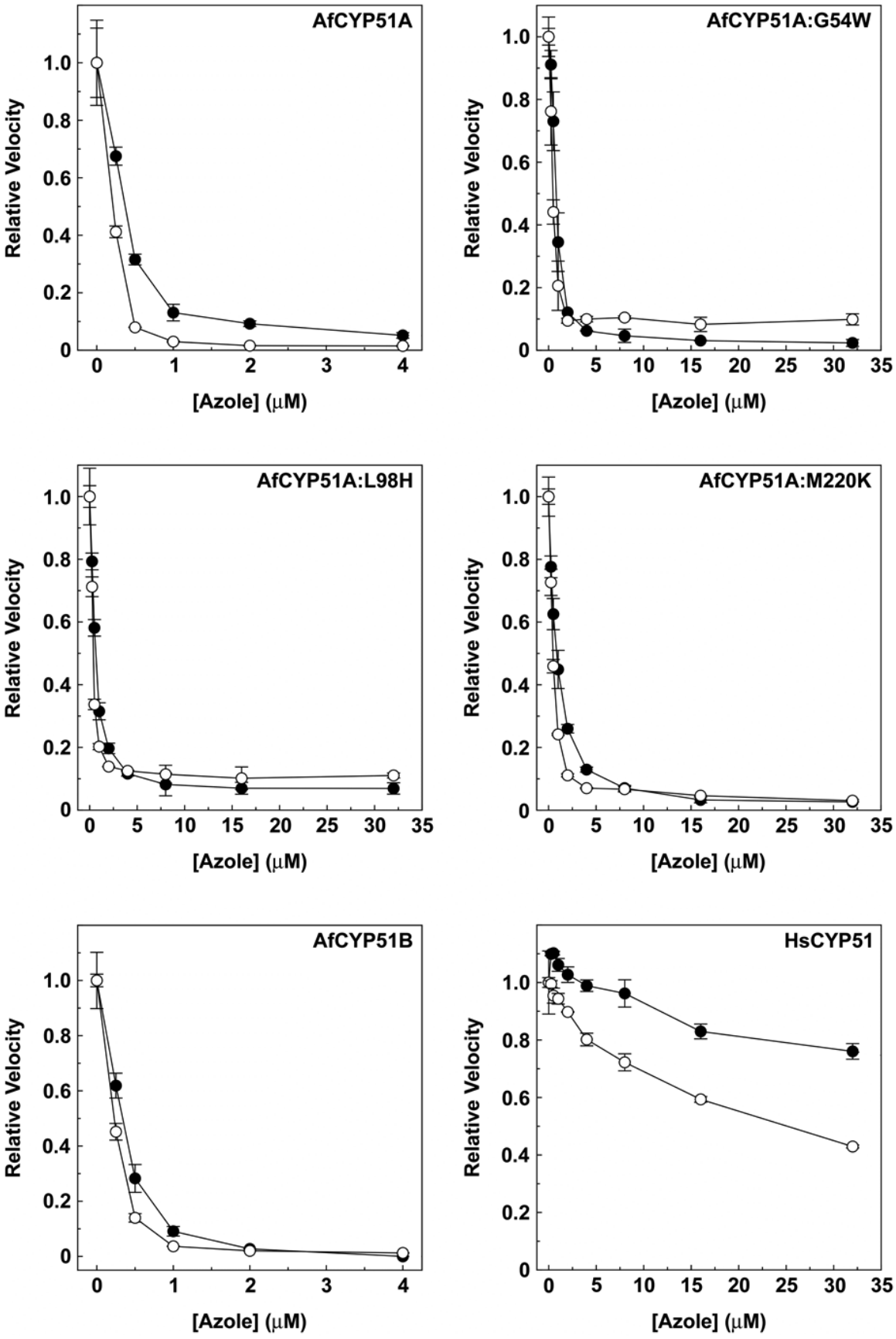


Fig. 2. Type II binding spectra. Type II difference spectra were obtained by the progressive titration of voriconazole and isavuconazole against 4 μM purified HsCYP51, AfCYP51A and AfCYP51B proteins and *E. coli* membrane suspensions containing 1 μM AfCYP51A and AfCYP51B. All spectral determinations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


AfCYP51A
membranes
AfCYP51B
membranes
AfCYP51A
protein
AfCYP51B
protein
HsCYP51
protein
ΔA
[Azole] (μM)

624

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


AfCYP51A
AfCYP51A:G54W
AfCYP51A:L98H
AfCYP51A:M220K
AfCYP51B
HsCYP51
Relative Velocity
[Azole] (μM)

630

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

644 **Table 1**

645 K_d values for voriconazole and isavuconazole.

	K_d (nM)			
	Proteins		Membranes	
CYP51	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_{50} values for voriconazole and isavuconazole.

	IC_{50} (μM)	
CYP51	Voriconazole	Isavuconazole
HsCYP51	112 ±27	25 ±2
AfCYP51A	0.38 ±0.05 [a]	0.21 ±0.03
AfCYP51A: G54W	0.80 ±0.09 [a]	0.45 ±0.08
AfCYP51A: L98H	0.65 ±0.13 [a]	0.39 ±0.05
AfCYP51A: M220K	0.84 ±0.08 [a]	0.46 ±0.04
AfCYP51B	0.33 ±0.07 [a]	0.23 ±0.03

649 [a] 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)	

654