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

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ABSTRACT

Here we report the first evaluation of isavuconazole inhibition of *Aspergillus fumigatus* CYP51 and thus sterol biosynthesis in the fungus. Voriconazole and isavuconazole both bound tightly to recombinant *A. fumigatus* CYP51 isoenzymes A and B (AfCYP51A and AfCYP51B) isolated in *Escherichia coli* membranes. CYP51 reconstitution assays confirmed that AfCYP51A and AfCYP51B as well as three AfCYP51A mutants known to confer azole resistance (G54W, L98H and M220K) were strongly inhibited by both triazoles. Voriconazole bound relatively weakly to purified *Homo sapiens* CYP51 (HsCYP51), unlike isavuconazole that bound tightly. However, isavuconazole was a relatively poor inhibitor of HsCYP51 activity, with an IC_{50} value (half-maximal inhibitory concentration) 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 with an IC_{50} value of 112 μM. Sterol analysis of triazole-treated *A. fumigatus* Af293 cells confirmed that 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 *A. fumigatus* Af293 than voriconazole at 0.0125 μg/mL, indicating increased potency. However, complementation studies in *Saccharomyces cerevisiae* using strains containing AfCYP51A and AfCYP51B showed isavuconazole to be equally as effective at inhibiting CYP51 activity as voriconazole. These in vitro studies suggest that isavuconazole is an effective alternative to voriconazole as an antifungal agent against the target CYP51 in *A. fumigatus*.

1. Introduction

Mortality associated with invasive fungal diseases has increased over the past four decades, primarily due to increasing numbers of cancer patients undergoing chemotherapy and patients undergoing organ transplantation ([1,2], reviewed in [3]). The majority of invasive fungal infections are caused by *Candida* and *Aspergillus* spp., with high mortality rates, particularly for aspergillosis, reaching up to 90%. In addition, an increased incidence of invasive fungal infections caused by *Cryptococcus* spp., *Fusarium* spp., *Trichosporon* spp., *Scedosporium* spp. 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 the polyenes, echinocandins and azoles. The polyene amphotericin B is a broad-spectrum antifungal but is limited by the requirement for intravenous 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 *Aspergillus* spp., and there is increasing resistance to echinocandins amongst certain *Candida* spp. 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, the incidence of fluconazole resistance among *Candida* spp. 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* spp., with posaconazole extending activity further against Mucorales. These second-generation triazoles, however, exhibit significant drug–drug interactions as well as interactions with host liver cytochrome P450 (CYP) monooxygenases.

Isavuconazole (Fig. 1) is a new broad-spectrum triazole antifungal with activity against yeasts, dimorphic fungi, *Aspergillus* spp.,

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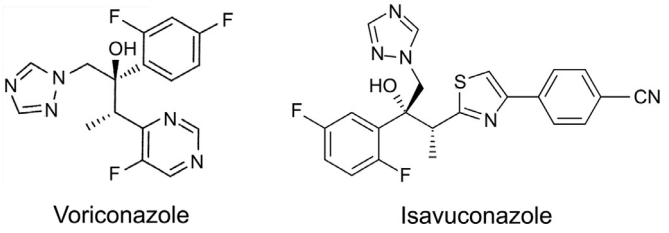

Fig. 1. Chemical structure of voriconazole (molar mass 349 g/mol) and isavuconazole (molar mass 437 g/mol).

moulds 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 it is currently recommended for the treatment of invasive aspergillosis and invasive mucormycosis [12]. Isavuconazole is administered as the water-soluble prodrug isavuconazonium, which is rapidly cleaved by plasma esterases to release the active drug isavuconazole in situ [13,14].

The mode of action of isavuconazole is assumed to be similar to other triazole antifungals, causing inhibition of sterol 14α-demethylase (CYP51) that is essential for ergosterol biosynthesis in fungi. However, no previous publications have investigated this in detail. Ergosterol is responsible for 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 interpatient variability, increased water solubility, and fewer CYP enzyme-mediated drug–drug interactions than voriconazole [8,9,16]. Isavuconazole displayed similar efficacy to amphotericin B against mucormycosis [9], supporting the use of isavuconazole both as a front-line and 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 as well as 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 with voriconazole using recombinant *Homo sapiens* CYP51 (HsCYP51), AfCYP51B and AfCYP51A enzymes, including three prevalent AfCYP51A amino acid substitutions (G54W, L98H and M220K) known to confer azole resistance [17–19].

2. Materials and methods

2.1. Heterologous expression, isolation and purification of recombinant Aspergillus fumigatus and Homo sapiens CYP51 proteins

The pCWori$^+$:Δ*60HsCYP51* (Δ60-truncation of UniProtKB accession no. **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 *Escherichia coli* cells and transformants were 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 characterisation

The binding properties of isavuconazole and voriconazole (Fig. 1) to AfCYP51A, AfCYP51B and HsCYP51 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% (w/v) glycerol at 22 °C. Azole saturation curves were constructed from $\Delta A_{peak-trough}$ of the resultant difference spectra versus azole concentration.

The triazole concentration causing 50% inhibition of CYP51 activity (IC_{50}) was determined using the CYP51 reconstitution assay system previously described [21]. *Homo sapiens* CYP51 assays contained 0.5 µM HsCYP51 and 2 µM *H. sapiens* cytochrome P450 reductase (HsCPR) (UniProt accession no. **P16435**) using lanosterol as substrate. *Aspergillus fumigatus* CYP51 assays used 50 µL of *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 (AfCPR) (UniProt accession no. **Q4WM67**) and eburicol as substrate. Azole antifungal agents were added in 2.5 µL of dimethyl sulfoxide (DMSO) followed by 10 min of incubation at 37 °C prior to assay initiation with 4 mM β-NADPH-Na_4. Incubation times were 4 min for HsCYP51 and 15 min for AfCYP51A and AfCYP51B at 37 °C. Sterol metabolites were recovered by ethyl acetate extraction and were analysed by gas chromatography–mass spectrometry (GC-MS) (Section 2.3).

2.3. Sterol composition analysis

Spore suspensions of *A. fumigatus* Af293 (ATCC MYA-4609, CBS 101355) were prepared in Tween 80 saline containing 0.025% (w/v) Tween 80 and 0.8% (w/v) NaCl. Spores were used to inoculate Sabouraud medium (final concentration 1×10^4 cells/mL) in the absence (DMSO control, 1% v/v) or presence of azoles. Voriconazole and isavuconazole stocks were prepared in DMSO and were added to the medium to give a final concentration of 0.125 µg/mL azole and 1% (v/v) DMSO. Cultures were incubated at 37 °C at 250 rpm for 48 h. Mycelia were harvested and non-saponifiable lipids were extracted as previously described [22]. Sterols were derivatised using 0.1 mL of BSTFA:TMCS (99:1) and 0.3 mL of anhydrous pyridine with heating at 80 °C for 2 h [23]. Trimethylsilyl (TMS)-derivatised sterols were analysed by GC-MS using a TRACE^{TM} 1300 gas chromatograph coupled to an ISQ^{TM} mass spectrometer (Thermo Scientific, Loughborough, UK) and were identified with reference to relative retention times, mass ions and fragmentation spectra. GC-MS data files were analysed using Xcalibur^{TM} software (Thermo Scientific).

2.4. Complementation studies in Saccharomyces cerevisiae

YUG37-p*cyp51A* and YUG37-p*cyp51B* constructs in *S. cerevisiae* [24] were used to assess the relative azole sensitivities of wild-type AfCYP51A and AfCYP51B to isavuconazole, voriconazole and itraconazole. YUG37-p*cyp51A* and YUG37-p*cyp51B* 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 doxycycline (5 µg/mL) at 30 °C for 72 h as previously described [24]. Minimum inhibitory concentration (MIC) determinations were performed in triplicate by the broth dilution method according to

Clinical and Laboratory Standards Institute (CLSI) M27-A2 guidelines, except for the use of doxycycline induction medium to grow the cells used for the 2.5×10^3 cells/mL inocula in the microtitre plates. Azole concentrations of 0.001–2 μg/mL were assessed and MIC plates were read visually after 72 h at 30 °C. In this study, the MIC was defined as the minimum drug concentration that caused ≥80% growth inhibition.

2.5. Data analysis

Spectral determinations were made using quartz semi-micro cuvettes with a Hitachi U-3310 UV/Vis spectrophotometer (Hitachi, San Jose, CA, USA). Curve fitting of ligand binding data was performed using a rearrangement of the Morrison equation [25] with the computer program QuantumSoft Pro Fit v.6.2.11 (non-linear regression Levenberg–Marquardt algorithm) to determine K_d values of the azole–CYP51 complexes. Ligand titration was performed in triplicate and the mean ± standard deviation (S.D.) K_d value was calculated.

IC_{50} enzyme velocities were calculated from gas chromatogram peak areas for product and substrate. Velocities were standardised against those observed in the absence of azole antifungal. IC_{50} experiments were performed in duplicate and the mean ± S.D. IC_{50} value was calculated.

The sterol composition of *A. fumigatus* Af293 was calculated using gas chromatogram peak areas with mass fragmentation patterns confirming sterol identification. The mean ± S.D. percentage composition for each sterol was calculated from three replicate experiments.

2.6. Chemicals

All chemicals, unless otherwise stated, were obtained from Sigma Chemical Company (Poole, UK). Voriconazole was obtained from Discovery Fine Chemicals (Bournemouth, UK), isavuconazole was from BOC Sciences (Shirley, NY, USA), and growth media, sodium ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-aminolevulinic acid were from Formedium Ltd. (Hunstanton, UK).

3. Results

3.1. Azole ligand binding studies

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

Azole saturation curves (Fig. 3) confirmed that isavuconazole and voriconazole bound tightly to AfCYP51A and AfCYP51B when isolated in the *E. coli* membrane fraction from the expression clones, with K_d values of 20–60 nM (Table 1). In contrast, voriconazole and isavuconazole binding to purified AfCYP51A and AfCYP51B was less tight (Table 1). Voriconazole bound to both purified *A. fumigatus* CYP51 isoenzymes with similar affinity (K_d value ~1 μM), whilst isavuconazole bound more tightly to AfCYP51B than AfCYP51A reflected in the 10-fold lower K_d value for AfCYP51B (Table 1). Isavuconazole bound tightly to HsCYP51 (K_d = 68 nM), whereas voriconazole bound less tightly (K_d value ~2.3 μM).

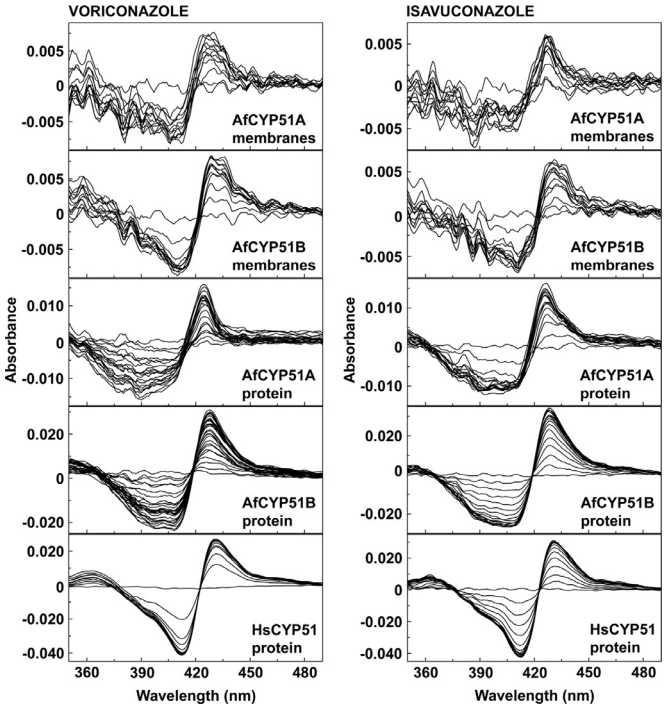

Fig. 2. Type II binding spectra. Type II difference spectra were obtained by progressive titration of voriconazole and isavuconazole against 4 μM purified HsCYP51, AfCYP51A and AfCYP51B proteins and *Escherichia coli* membrane suspensions containing 1 μM AfCYP51A and AfCYP51B. All spectral determinations were performed in triplicate, although only one replicate is shown. HsCYP51, *Homo sapiens* CYP51; AfCYP51A and AfCYP51B, *A. fumigatus* CYP51 isoenzymes A and B.

Table 1
K_d values for voriconazole and isavuconazole

CYP51[a]	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

[a] Hs, *Homo sapiens*; Af, *Aspergillus fumigatus*.

3.2. Azole inhibition of CYP51 sterol 14α-demethylase activity

IC_{50} determinations for voriconazole and isavuconazole (Fig. 4) indicated that both were equally effective at inhibiting the enzyme activity of the three AfCYP51A mutations (G54W, L98H and M220K) associated with azole resistance in *A. fumigatus*, yielding IC_{50} values of 0.4–0.8 μM, with the only noticeable difference being slightly higher residual CYP51 activities observed at high isavuconazole concentrations with the G54W and L98H mutants compared with voriconazole. Isavuconazole was marginally more effective at inhibiting wild-type AfCYP51A and AfCYP51B than voriconazole (Fig. 4), with the isavuconazole IC_{50} curves dipping below those for voriconazole, however the difference in IC_{50} values was less than two-fold (Table 2). Both voriconazole and isavuconazole were weak inhibitors of HsCYP51 activity in vitro, with 32 μM voriconazole causing 25% inhibition of CYP51 activity compared with 57% inhibition in the presence of 32 μM isavuconazole (Fig. 4). The 4.5-fold difference in IC_{50} values obtained with HsCYP51 reflected the stronger inhibition exhibited by isavuconazole (Table 2). The apparent selectivity for *A. fumigatus* CYP51s over human CYP51 based on IC_{50} values were 290–340-fold and 110–120-fold for voriconazole and isavuconazole, respectively.

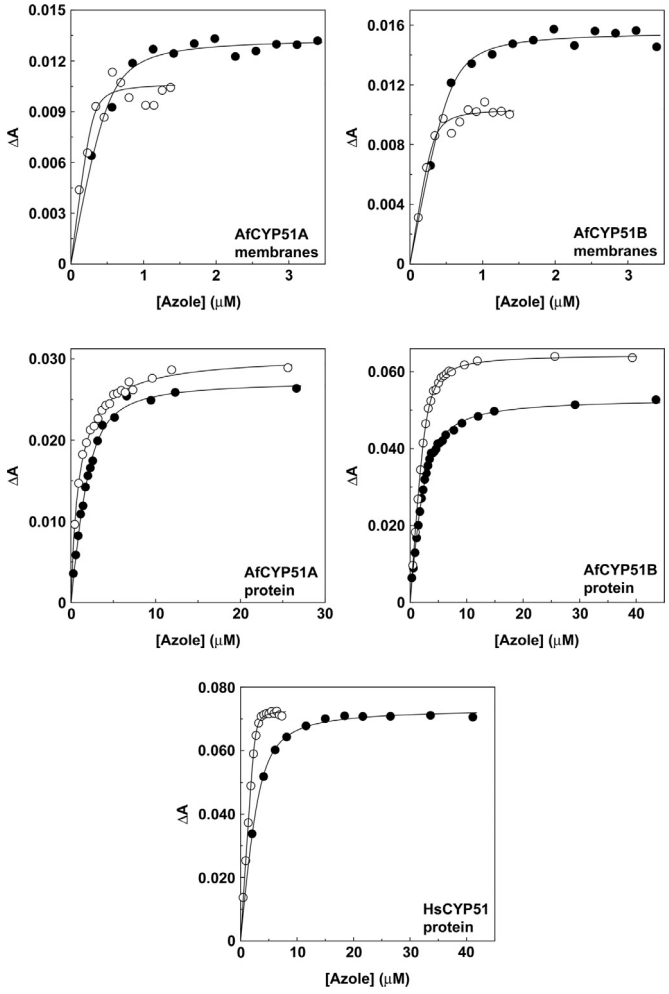

Fig. 3. Azole ligand saturation curves. Ligand saturation curves for voriconazole (•) and isavuconazole (○) were constructed from the type II difference spectra (Fig. 2) and were fitted using a rearrangement of the Morrison equation for tight ligand binding [25].

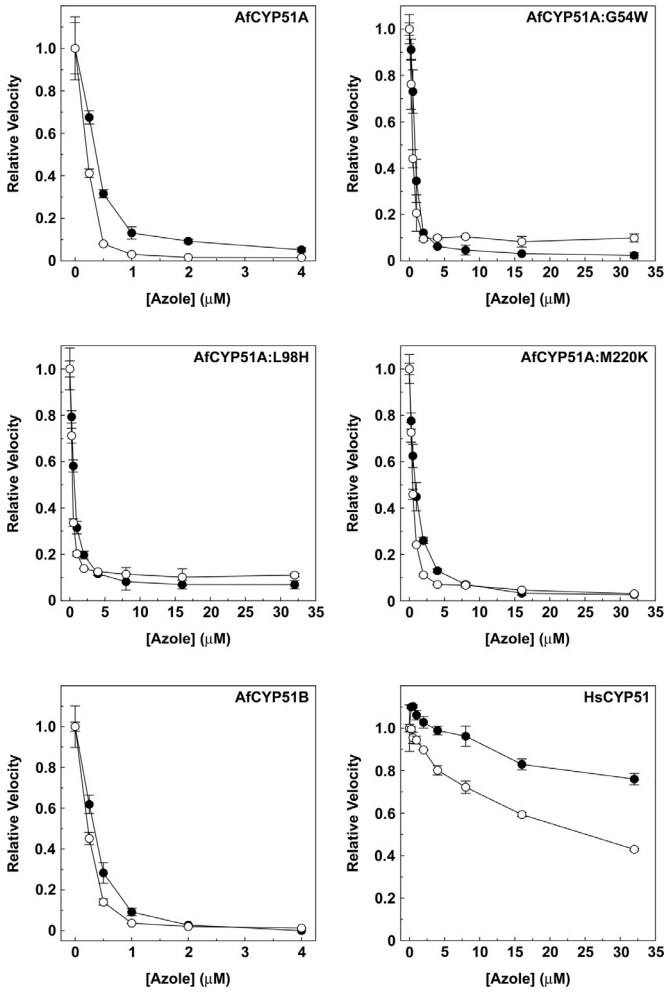

Fig. 4. Azole inhibition profiles. IC_{50} values for voriconazole (•) and isavuconazole (○) were determined using a CYP51 reconstitution assay system that contained either 0.5 μM HsCYP51, 2 μM HsCPR, 50 μM DLPC or 0.5 μM *A. fumigatus* CYP51 proteins isolated in *Escherichia coli* membrane fractions from the expression clones supplemented with 1 μM AfCPR. In addition, the relative velocities for HsCYP51 in the presence of 75 μM 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. IC_{50}, triazole concentration causing 50% inhibition of CYP51 activity; HsCYP51, *Homo sapiens* CYP51; HsCPR, *H. sapiens* cytochrome P450 reductase; AfCPR, *Aspergillus fumigatus* cytochrome P450 reductase; AfCYP51A and AfCYP51B, *A. fumigatus* CYP51 isoenzymes A and B.

Table 2
IC_{50} values for voriconazole and isavuconazole

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

IC_{50}, triazole concentration causing 50% inhibition of CYP51 activity.
[a] Hs, *Homo sapiens*; Af, *Aspergillus fumigatus*.
[b] As previously reported by Warrilow et al. [21].

3.3. Sterol composition analysis

Aspergillus fumigatus Af293 was grown from spores in the presence of 0.0125 μg/mL (0.0358 μM) voriconazole and 0.0125 μg/mL (0.0286 μM) isavuconazole and in the absence of azole antifungals (DMSO control) and the sterol content of the cells was then extracted and analysed. The predominant sterol in the control sample was ergosterol, comprising nearly 91% of the total sterol content (Table 3), with only 0.6% eburicol present. Treatment with 0.0125 μg/mL voriconazole and isavuconazole both resulted in a sharp rise 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, compared with 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 *A. fumigatus* Af293 than voriconazole, especially bearing in mind that the molar isavuconazole concentration used was 20% lower than that for voriconazole. Levels of toxic 14-methylergosta-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

Table 3
Sterol composition of control, voriconazole-treated and isavuconazole-treated *Aspergillus fumigatus* Af293

Sterol	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 ergostatrienol	4.8 ± 0.4	3.5 ± .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	

DMSO, dimethyl sulfoxide.

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-p*cyp51A* and YUG37-p*cyp51B* constructs. MICs for fluconazole, clotrimazole, voriconazole, itraconazole and posaconazole with YUG37-p*cyp51A* were 8, 0.016, 0.004, 0.125 and 0.063 μg/mL, respectively, compared with 0.5, 0.016, 0.004, 0.125 and 0.063 μg/mL for YUG37-p*cyp51B* [24]. The control construct YUG37-pCTRL gave MICs of 0.25, 0.016, 0.004, 0.031 and 0.063 μg/mL against fluconazole, clotrimazole, voriconazole, itraconazole and posaconazole, respectively [24]. Therefore, AfCYP51A conferred tolerance towards fluconazole, whilst AfCYP51A and AfCYP51B were equally susceptible to inhibition by clotrimazole, voriconazole, itraconazole and posaconazole.

In this study, MIC determinations with voriconazole and itraconazole were repeated along with MIC determinations for the new triazole antifungal isavuconazole. MICs obtained with YUG37-p*cyp51A* were 0.002, 0.0625 and 0.002 μg/mL for voriconazole, itraconazole and isavuconazole, respectively, compared with 0.001, 0.0313 and 0.001 μg/mL for YUG37-pcyp51B. Therefore, isavuconazole was equally effective at inhibiting both AfCYP51A and AfCYP51B as voriconazole and was 300-fold more effective than itraconazole.

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 co-ordinating as the sixth ligand with the heme iron [26]. Both triazoles bound more tightly 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 with 39- and 11-fold with isavuconazole (Table 1). This suggests that 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 10-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 that AfCYP51A and AfCYP51B would be strongly inhibited both by voriconazole and isavuconazole. This was confirmed by the low IC_{50} values obtained that were approximately one-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 with 25 μM obtained with HsCYP51 (Table 2). This suggests that 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 homologue 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 *A. fumigatus* CYP51s was 45- to 60-fold based on K_d value and 290- to 340-fold based on IC_{50} value (Table 2), indicating that voriconazole was more selective for *A. fumigatus* CYP51s over the human homologue 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, however 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 that 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 conferred resistance to itraconazole (MIC > 8 μg/mL), an elevated MIC to posaconazole (MIC of 1–2 μg/mL compared with 0.06 μg/mL for wild-type) but little increase in resistance to voriconazole (MIC 1 μg/mL) [18]. Previous investigations utilising recombinant G54W and M220K AfCYP51A proteins have shown that these mutations confer resistance against CYP51 inhibition by itraconazole and posaconazole and limited tolerance to voriconazole [21]. MICs for isavuconazole with *A. fumigatus* strains con-

taining the CYP51A G54W and M220K substitutions were 0.125–0.25 μg/mL and 1–4 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 suggests that additional resistance mechanisms were also present.

The two-fold increase in IC_{50} values for AfCYP51A:L98H over the wild-type enzyme indicates that 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 an elevated MIC against voriconazole (2 μg/mL). MICs for isavuconazole with AfCYP51A:TR34/L98H-containing strains are variable at 4 μg/mL to >16 μg/mL [27], suggesting that 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 that 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 the 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 suggests that *A. fumigatus* is undergoing a similar process previously observed in *Mycosphaerella graminicola* CYP51 in which complex genotypes with multiple substitutions have been selected during the changing regimens of azole fungicides deployed over recent decades, with the wild-type CYP51 alleles not seen in some countries [33].

Less frequently encountered AfCYP51A mutations that confer azole resistance include G138C, G138S, Y431C, G434C and G448S. Clinical strains containing AfCYP51A:G138C/S are resistant to isavuconazole, voriconazole and itraconazole (MICs of 8 μg/mL to >16 μg/mL) but display variable resistance to posaconazole (MICs of 1 μg/mL to >16 μg/mL) [34–36]. Similarly, clinical *A. fumigatus* strains containing the AfCYP51 substitutions Y431C, G434C and G448S are resistant to isavuconazole, voriconazole, itraconazole and posaconazole [34–36]. Albarrag et al. [34] confirmed that G138C and Y431C conferred resistance to voriconazole, itraconazole and posaconazole using complementation studies in *S. cerevisiae*; however, unexpectedly, the AfCYP51A:G434C transformant caused hypersensitivity to azole antifungals. The molecular basis for azole resistance conferred by the AfCYP51A amino acid substitutions G138C, G138S, Y431C, G434C and G448S would be of interest for a future study, especially as these substitutions appear to confer the greatest resistance towards isavuconazole.

Sterol composition studies confirmed that isavuconazole and voriconazole at 0.0125 μg/mL both inhibited cellular CYP51 activity in *A. fumigatus* Af293, characterised by the accumulation of 14-methylated sterols and depletion of ergosterol, demonstrating the in situ mode of action of both azoles. 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 -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 US Food and Drug Administration (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 with 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 comparison of MICs across compounds is not readily correlated with 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 co-ordination 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 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 with voriconazole. However, this is offset by increased bioavailability of isavuconazole, linear pharmacokinetics, fewer drug interactions and lower reported side effects compared with voriconazole.

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Declaration of Competing Interest

None declared.

Ethical approval

Not required.

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