ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 2008, p. 3597–3603 Vol. 52, No. 10
0066-4804/08/$08.00+0 doi:10.1128/AAC.00517-08

Differential Azole Antifungal Efficacies Contrasted Using a *Saccharomyces cerevisiae* Strain Humanized for Sterol 14α-Demethylase at the Homologous Locus[▽]

J. E. Parker,[1] M. Merkamm,[2] N. J. Manning,[3] D. Pompon,[2] S. L. Kelly,[1] and D. E. Kelly[1]*

Institute of Life Science and School of Medicine, Swansea University, Swansea, Wales SA2 8PP, United Kingdom[1]; Laboratoire d'Ingénierie des Protéines Membranaires, CNRS-Centre de Génétique Moléculaire, 91198 Gif-sur-Yvette Cedex, France[2]; and Clinical Chemistry, Sheffield Children's Hospital, Sheffield S10 2TH, United Kingdom[3]

Received 22 April 2008/Returned for modification 29 May 2008/Accepted 28 July 2008

Inhibition of sterol-14α-demethylase, a cytochrome P450 (CYP51, Erg11p), is the mode of action of azole antifungal drugs, and with high frequencies of fungal infections new agents are required. New drugs that target fungal CYP51 should not inhibit human CYP51, although selective inhibitors of the human target are also of interest as anticholesterol agents. A strain of *Saccharomyces cerevisiae* that was humanized with respect to the amino acids encoded at the *CYP51* (*ERG11*) yeast locus (BY4741:huCYP51) was produced. The strain was validated with respect to gene expression, protein localization, growth characteristics, and sterol content. The MIC was determined and compared to that for the wild-type parental strain (BY4741), using clotrimazole, econazole, fluconazole, itraconazole, ketoconazole, miconazole, and voriconazole. The humanized strain showed up to >1,000-fold-reduced susceptibility to the orally active azole drugs, while the topical agents showed no difference. Data from growth kinetic measurements substantiated this finding but also revealed reduced effectiveness against the humanized strain for the topical drugs. Cellular sterol profiles reflected the decreased susceptibility of BY4741:huCYP51 and showed a smaller depletion of ergosterol and accumulation of 14α-methyl-ergosta-8, 24(28)-dien-3β-6α-diol than the parental strain under the same treatment conditions. This strain provides a useful tool for initial specificity testing for new drugs targeting CYP51 and clearly differentiates azole antifungals in a side-by-side comparison.

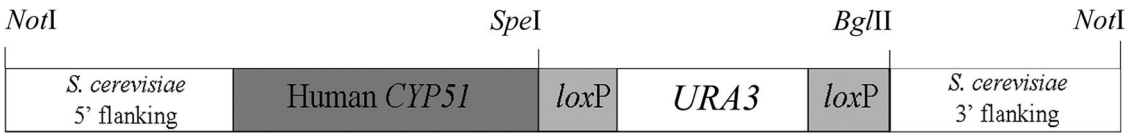
Azoles inhibit the synthesis of ergosterol in fungi through direct binding to the cytochrome P450 (CYP) sterol 14α-demethylase (CYP51, Erg11p), a mono-oxygenase that undertakes sequential reactions to remove the methyl group (26). A nitrogen of the azole ring binds to the iron atom in the heme prosthetic group of CYP51, located in the active site of the enzyme, while the N-1 substituent group interacts with amino acids in the folded enzyme structure above the heme (10). The inhibition of sterol 14α-demethylation results in a depletion of ergosterol and an accumulation of 14α-methylated sterols, including 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol, which inhibit fungal growth (17). Mutations which affect the binding of azoles to CYP51 are commonly found in azole-resistant fungal strains (15, 23), and new drugs are required to address this problem as well as the spectrum of fungal diseases.

One obvious potential side effect of new CYP51 inhibitors is the inhibition of human CYP51, which may result in a reduction in the synthesis of cholesterol and affect the endocrine system (29). Human and fungal CYP51 sequences are around 36% identical (human CYP51 and *Candida albicans* CYP51 are 35.9% identical; human CYP51 and *Saccharomyces cerevisiae* CYP51 are 35.8% identical) and catalyze the sterol 14α-demethylase reaction. Despite their relatively low sequence similarities, CYP51s are likely to be topologically similar in function and appear to contain structurally conserved regions (13). The similarities in the structures of human and fungal CYP51s have posed queries and problems for the treatment of fungal infections with azoles, in addition to the potential for drug-drug interactions due to the inhibition in humans of other CYPs that alter the half-lives of other agents that may be administered to patients (18). Azole antifungals are divided into the imidazoles (e.g., miconazole, clotrimazole, econazole, and ketoconazole) and the triazoles (e.g., itraconazole, fluconazole, and voriconazole). The earliest clinical imidazole-based azole antifungals (clotrimazole, econazole, and miconazole) were originally used as topical treatments. Ketoconazole was the first oral treatment for systemic fungal infections (7) but was limited by its toxicity/adverse effects (14, 21, 22). The triazoles were developed in an aim to produce more-specific, less-toxic, and more-potent antifungal drugs. Fluconazole and itraconazole have good antifungal activity and are less toxic than ketoconazole (16). However, the emergence of fluconazole resistance and absorption problems with itraconazole have led to the development of a second generation of triazoles, including voriconazole, which can be used to treat fluconazole-resistant strains (5) and aspergillosis (24). The adverse effects of azole drugs could be due to interactions with human CYPs, including CYP51. It is therefore important that antifungal drugs for systemic use are selective for fungal CYP51.

Previous experimental studies on the specificities of azoles with respect to human and fungal enzymes have relied upon the expression of recombinant proteins, assaying their activities in reconstituted systems and determining the 50% inhibi-

* Corresponding author. Mailing address: Institute of Life Science and School of Medicine, Swansea University, Swansea, Wales SA2 8PP, United Kingdom. Phone: 44 1792 602184. Fax: 44 1792 602280. E-mail: D.Kelly@swansea.ac.uk.

▽ Published ahead of print on 11 August 2008.




```
1    GCGGCCGCTT TCCCTTTTCG TTATTGGTAA ATTATTTATA AAAAGATTGA TAAGCAGTAT CGTTCAGCGT GTGTATTTTC GATAGGAAGT AATAATTTTT
101  CATTATATTA ATTAATAGTT TCCAGAAAAA ATTTTTTTTC CTTCACAATT GCAGCAGGCT TGAATAGAAA CAGAACAAAC GAGTAATAGG ATCCAAAatg
201  gctgctgctg ctggtatgtt gttgttgggt ttgttgcaag ctggtggttc tgttttgggt caagctatgg aaaaagttac tggtggtaat ttgttgtcta
301  tgttgttgat tgcttgtgct tttactttgt ctttggttta tttgattaga ttggctgctg gtcatttggt tcaattgcca gctggtgtta aatctccacc
401  atatattttt tctccaattc cattttggg tcatgctatt gcttttggta aatctccaat tgaatttttg gaaaatgctt atgaaaaata tggtccagtt
501  ttttctttta ctatggttgg taaaactttt acttatttgt tgggttctga tgctgctgct ttgttgttta attctaaaaa tgaagatttg aatgctgaag
601  atgtttattc tagattgact actccagttt ttggtaaagg tgttgcttat gatgttccaa atccagtttt tttggaacaa aaaaaaatgt tgaaatctgg
701  tttgaatatt gctcatttta aacaacatgt ttctattatt gaaaaagaaa ctaaagaata ttttgaatct tggggtgaat ctggtgaaaa aaatgttttt
801  gaagctttgt ctgaattgat tattttgact gcttctcatt gtttgcatgg taaagaaatt aggtctcaat tgaatgaaaa agttgctcaa ttgtatgctg
901  atttggatgg tggtttttct catgctgctt ggttgttgcc aggttggttg ccattgccat cttttagaag aagagataga gctcatagag aaattaaaga
1001 tatttttat aaagctattc aaaaaagaag acaatctcaa gaaaaaattg atgatatttt gcaaactttg ttggatgcta cttataaaga tggtagacca
1101 ttgactgatg atgaagttgc tggtatgttg attggtttgt tgttggctgg tcaacatact tcttctacta cttctgcttg gatgggtttt tttttggcta
1201 gagataaaac tttgcaaaaa aaatgttatt tggaacaaaa aactgtttgt ggtgaaaatt tgccaccatt gacttacgat caattgaaag atttgaattt
1301 gttggatata tgtattaaag aaactttgag attgagacca ccaattatga ttatgatgag aatggctaga actccacaaa ctgttgctgg ttatactatt
1401 ccaccaggtc atcaagtttg tgtttctcca actgttaatc aaagattgaa agattcttgg gttgaaagat tggattttaa tccagatага tatttgcaag
1501 ataatccagc ttctggtgaa aaatttgctt atgttccatt tggtgctggt agacatagat gtattggtga aaattttgct tatgttcaaa ttaaaactat
1601 ttggtctact atgttgagat tgtatgaatt tgatttgatt gatggttatt ttccaactgt taattatact actatgattc atactccaga aaatccagtt
1701 attagatata aaagaagatc caaataaTCA GGTGCTCATC ACCATCACCA TCACTAAGAA TTCTAAATAA AACTAGTATC TTAGATCTGG TTTGTAACTG
1801 AGAAAAAAAA AAAAAACAGA AAAAGAGAGA AGTTGTACAT TCACGTGGGC CTTATCGCCA CACTCATCCA TCTCCAATGA TTTATTTTAT TTGCCAGTAC
1901 GTTTGATATG ACATCGAGTT ATGTTCTTTA GTTACTTTCA AACTTGGACA ACCATCCCGC ATTATTTATG AGTGGTATGG TTTGCGGCCG C
```

FIG. 1. Diagram and sequences of the hu*CYP51* replacement cassette. The upstream and downstream flanking regions of *S. cerevisiae CYP51* are shown in uppercase. The hu*CYP51* sequence with *S. cerevisiae* codon bias (codon adaptation index = 0.571 [25]) and conflicting restriction enzyme sites removed is shown in lowercase. NotI sites for excision from the pUC57 vector are shown underlined. BglII and SpeI sites for insertion of the *URA3* marker are shown in boldface. Transcription initiation (AAA) and transcription termination (polyadenylation, TAAATAAA) sequences are in boldface and underlined. The sequence between the stop codon (taa) and the polyadenylation site contains the sequence for a histidine tag not utilized in this study.

tory concentrations (IC_{50}s) of drugs (3, 11, 12, 27). Eukaryotic CYP51 is a membrane-bound protein, and although recombinant proteins enable a direct comparison of drug binding and protein activity in a cell-free system, functional CYP51 is extremely difficult and time consuming to express, purify, and reconstitute with a highly lipophilic substrate and reductase partner. Therefore, these techniques do not present a convenient test for the specificity of new CYP51 inhibitors. In this study, we report on a strain of *S. cerevisiae* containing human *CYP51* (hu*CYP51*) targeted to replace the native *S. cerevisiae CYP51* (Sc*CYP51*) at the chromosomal locus and under the control of the yeast *CYP51* promoter, which may be used as a tool for testing the specificity of azoles and for general chemical screen technology based on the assessment of growth.

MATERIALS AND METHODS

Strains and growth conditions. We used *S. cerevisiae* BY4741 (ATCC 201388) MAT**a** *his3*Δ1; *leu2*Δ0; *met15*Δ0 *ura3*Δ0 (EUROSCARF, Frankfurt, Germany). Unless stated otherwise, both the wild-type and humanized strains were grown on yeast minimal media (YM), pH 7.0, containing a 0.67% (wt/vol) yeast nitrogen base without amino acids (Difco, Detroit, Michigan) and 2% (wt/vol) glucose (Fisher, Loughborough, United Kingdom), with 50 μg/ml of histidine and 100 μg/ml each of leucine, methionine, and uracil added (Sigma, Poole, United Kingdom).

Escherichia coli DH5α (Stratagene, La Jolla, CA) was used for plasmid construction and DNA amplification.

Construction of strain BY4741:huCYP51. The human *CYP51A1* cDNA sequence (accession number Q16850) was obtained from Swiss-Prot (http://expasy.org/sprot/). The 5′-upstream- and 3′-downstream-flanking sequences of the *S. cerevisiae CYP51* open reading frame (accession number YHR007C) were obtained from the *Saccharomyces* genome database (http://www.yeastgenome.org/). A DNA sequence consisting of the 5′-flanking sequence from *S. cerevisiae*, the human *CYP51* sequence, and the 3′-flanking sequence from *S. cerevisiae* was designed. Restriction enzyme sites were added to facilitate cloning and insertion of the *URA3* marker gene, flanked by *loxP* sites, for selection in yeast. The nucleotide sequence was optimized for expression in *S. cerevisiae* (codon adaptation index of 0.571, compared to a codon adaptation index of 0.0859 for the unoptimized human *CYP51* sequence [25]; synthesized by GeneCust [Evry, France]) (Fig. 1). The synthesized gene was cloned into a NotI site in vector pUC57 (pUC57:hu*CYP51*). The *URA3-loxP* marker was excised from pUG72 (accession number P30117; EUROSCARF), using SpeI and BglII, and was ligated (T4 ligase; Promega, Madison, WI) into the pUC57:hu*CYP51* vector cut with SpeI and BglII (Promega). The replacement hu*CYP51* cassette was amplified in *E. coli* and excised from pUC57:hu*CYP51* by using NotI (Promega). *S. cerevisiae* strain BY4741 was transformed with the hu*CYP51* cassette by electroporation. A 10-ml culture containing 1×10^7 cells/ml grown in YPD medium (1% [wt/vol] yeast extract [Duchefa, Haarlem, The Netherlands], 2% [wt/vol] peptone [Duchefa], 2% [wt/vol] glucose [Fisher]) was harvested, washed twice with sterile water, washed sequentially with 1 M sorbitol (Fisher) and 1 M sorbitol–20 mM HEPES (Sigma), and resuspended in 80 μl of 1 M sorbitol–20 mM HEPES. Electroporation was performed in 2-mm gap cuvettes at 1.5 kV, 25 μF, and 200 Ω in a Gene Pulser II system (Bio-Rad). One milliliter of ice-cold YPD medium was added, and cells were incubated at room temperature for 1 h before being plated on YM–uracil containing 2% (wt/vol) agar (Duchefa). Replacement of the native Sc*CYP51* by hu*CYP51* at its chromosomal locus was confirmed by PCR and DNA sequencing (MWG, London, United Kingdom) (data not shown). Excision of the *URA3-loxP* marker was achieved by induction of Cre recombinase from BY4741:huCYP51:pSH62 transformants (EUROSCARF) (6).

Transcript analysis. Quantitative reverse transcriptase PCR (RT-PCR) was used to analyze the transcription levels of the native *CYP51* and the hu*CYP51* gene in the two strains. Total RNA was isolated from 3×10^8 cells from overnight cultures of BY4741 and BY4741:huCYP51 by hot phenol (4). RNA samples were adjusted to 100 ng/μl by using a NanoDrop spectrophotometer (Thermo Scientific), and cDNA was synthesized by using an Ambion RETROscript kit. An internal control, the *S. cerevisiae* actin gene (accession number YFL039C), was amplified by using primers actin F (5′-TCCCAGGTATTGCC GAAAGAAT-3′), actin R (5′-AGCCAAGATAGAACCACCAATC-3′; amplicon size, 125 bp), native Sc*CYP51*, Sc*CYP51*F (5′-CATTCACCGTTGGAACA AAG-3′) and Sc*CYP51*R (5′-GAGAGCTGACACCCTTAGA-3′; 96 bp), and hu*CYP51*, hu*CYP51*F (5′-GTGCTGGTAGACATAGATGTATTG-3′) and hu*CYP51*R (5′-CCATCAATCAAATCAAATTCATAC-3′; 103 bp). The PCR mixtures contained 10 μl of Sybr green master mix (Thermo Scientific), 2.5 μl of a 4-μM concentration of the forward and reverse primer, and 5 μl of the cDNA template (1 in 100 dilution) or RNA (1 in 200 dilution). The process was performed with negative controls containing water and no template, and a

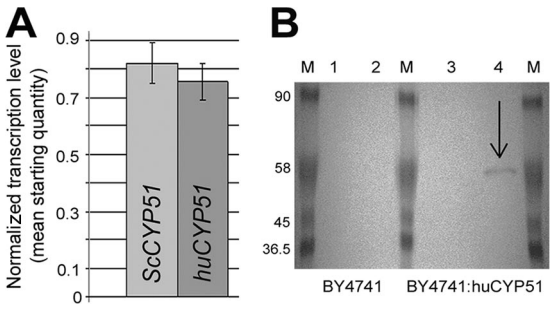


FIG. 2. (A) Transcription levels of native *ERG11* and hu*CYP51*. RT-PCR was performed, mean starting quantities were determined, and data were normalized to the values for yeast actin. Error bars show SEs for different quantities. (B) Western blot analysis of huCYP51p localization from cytosolic fractions (lanes 1 and 3) and microsomal fractions (lanes 2 and 4). Molecular mass markers (M) are shown in kilodaltons. An arrow indicates the band corresponding to huCYP51p (56.8 kDa), present in a microsomal fraction from the BY4741:huCYP51 strain.

four-step, 10-fold dilution series was used to calculate the efficiency of the primer sets (determined as ≥94%) and quantify the mean starting quantity of each transcript (according to Bio-Rad iCycler software, version IQ5 2.0).

Amplification was performed by using an iCycler (Bio-Rad) with the following conditions: 15 min at 95°C, 50 cycles of 15 s at 94°C, 30 s at 56°C, and 30 s at 72°C. Melt curve analysis and gel electrophoresis verified the specificity of each amplicon. All reactions were performed in triplicate on each plate, and each plate was duplicated. Starting quantities of the target genes (hu*CYP51*, Sc*CYP51*) in the RNA samples were subtracted from the cDNA samples and were normalized against actin levels.

Immunoblotting and protein localization. An antibody against the huCYP51 peptide, W_{80}AKSPPESENKEQLE$_{94}$C (GeneCust), was made. Yeast cytosolic and microsomal protein fractions were separated by sodium dodecyl sulfate from both BY4741 and BY4741:huCYP51 and were transferred to Hybond-P membranes (Amersham). A primary antibody against huCYP51p (anti-huCYP51) was diluted 1:1,000 in blocking buffer (50 g of low fat milk powder in 0.1 M potassium phosphate [pH 7.5] containing 0.05% [vol/vol] Tween 20 and 0.15 M NaCl). Binding of the antibody to antigens was detected with alkaline phosphatase-conjugated rabbit immunoglobulin G (Sigma) and detected by using a Sigmafast BCIP (5-bromo-4-chloro-3-indolylphosphate)/Nitro Blue Tetrazolium alkaline phosphatase substrate tablet (Sigma).

Identification of sterols. Both BY4741 and BY4741:huCYP51 were grown in 20 ml of YM (pH 7.0) with an initial inoculum of 2.25×10^3 cells/ml for 48 h at 30°C at 200 rpm. All azole antifungals were dissolved in dimethyl sulfoxide (adjusted to 1% [vol/vol] for azole solutions and negative controls). Cells were harvested and washed twice with sterile water. Nonsaponifiable lipids were extracted as reported previously (9). Samples were dried in a vacuum centrifuge (Heto). Sterol samples were derivatized by using bistrimethylsilyltrifluoroacetamide; Sigma), and gas chromatography mass spectrometry was performed by using a VG12-250 mass spectrometer (VG Biotech) with a split ratio of 20:1. Individual sterols were identified by reference to relative retention times, mass ions, and fragmentation patterns.

Susceptibility testing. The susceptibilities of BY4741 and BY4741:huCYP51 to clotrimazole (0 to 16 μg/ml), econazole (0 to 16 μg/ml), fluconazole (0 to 128 μg/ml), itraconazole (0 to 128 μg/ml), ketoconazole (0 to 16 μg/ml), miconazole (0 to 16 μg/ml), and voriconazole (0 to 128 μg/ml) were determined by an adaptation of the CLSI (formerly NCCLS) M-27A broth microdilution method (19) for use with *S. cerevisiae* strains, using microtiter plates in triplicate. Overnight cultures were diluted in YM, pH 7.0, to give an inoculum density of 2.5×10^3 cells/ml in a final volume of 200 μl per well with 20 μl of drug diluted in YM. Cells were grown at 30°C for a period of 48 h. MICs were determined as the concentrations of the drugs causing an 80% inhibition of growth.

Growth rates of azole-treated cells. The growth of BY4741 and BY4741:huCYP51 was monitored by using a Bioscreen C (Oy Growth Curves Ab Ltd.). Three hundred microliters from overnight cultures was diluted to 1×10^5 cells/ml in YM, pH 7.0. Azoles were dissolved in dimethyl sulfoxide, and controls were added as before to each well of a honeycomb 2 plate to final concentrations ranging from 0.0625 μg/ml to 16 μg/ml. Cultures were incubated at 30°C, and the optical density at 600 nanometers (OD_{600}) was measured every 15 min for 48 h. All strain/drug combinations were replicated on a second plate and were repeated on each plate, giving a total of four measurements for each. Maximum growth rates for each strain/drug treatment were determined from all four measurements, using the Excel (Microsoft) trend line function. The maximum OD was determined by calculating the mean maximum OD reached by each strain/drug combination.

RESULTS

Characterization of the BY4741:huCYP51 strain. *CYP51* (*ERG11*) gene deletion in *S. cerevisiae* is lethal, producing an obligate anaerobic ergosterol auxotroph (8), and functional complementation by huCYP51 is evidenced by growth under aerobic conditions without the requirement for exogenous ergosterol. For comparative studies and to avoid overexpression of huCYP51, we placed the gene under the control of the yeast *CYP51* (Sc*CYP51*) promoter. Expression was monitored by RT-PCR and Western blot analysis. RT-PCR showed that the yeast and hu*CYP51* transcripts were equally abundant (Fig. 2A). Western blot analysis of cytosolic and microsomal extracts from both BY4741 and BY4741:huCYP51 with anti-huCYP51 showed the human enzyme to be correctly targeted to the microsomal membranes (Fig. 2B, lane 4).

To confirm that complementation was based on in vivo sterol 14α-demethylation, we analyzed the sterol content for both strains (Table 1). Similar percentages of ergosterol were observed in BY4741:huCYP51 and BY4741: 54% (± 0.82 standard error [SE]) versus 57% (± 1.99 SE), respectively. However, the huCYP51 gene product may be slightly less effective than native ScCYP51, since a greater percentage of the total

TABLE 1. The composition of major sterols in both untreated and azole-treated BY4741 and BY4741:huCYP51

Strain	Sterol[b]	% (± SE) Total sterols with indicated treatment:[a]				
		Untreated	16 μg/ml of fluconazole	8 μg/ml of itraconazole	4 μg/ml of ketoconazole	0.25 μg/ml of voriconazole
BY4741	Erg	57 ± 1.00	ND[c]	6 ± 1.63	12 ± 3.89	10 ± 1.41
	Lan	17 ± 1.22	42 ± 3.19	42 ± 5.66	35 ± 0.41	35 ± 1.08
	3,6 diol	ND[c]	29 ± 3.54	24 ± 7.07	19 ± 5.66	24 ± 0.41
BY4741:huCYP51	Erg	54 ± 0.82	46 ± 1.78	52 ± 1.41	51 ± 1.41	49 ± 1.47
	Lan	23 ± 1.08	24 ± 1.08	24 ± 2.12	27 ± 4.60	27 ± 0.41
	3,6 diol	ND[c]	6 ± 0.4	5 ± 1.08	7 ± 3.19	4 ± 0.82

[a] Values shown are the means of two replicate samples.
[b] Erg, ergosterol; Lan, lanosterol; 3,6 diol, 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol.
[c] ND, not detected.

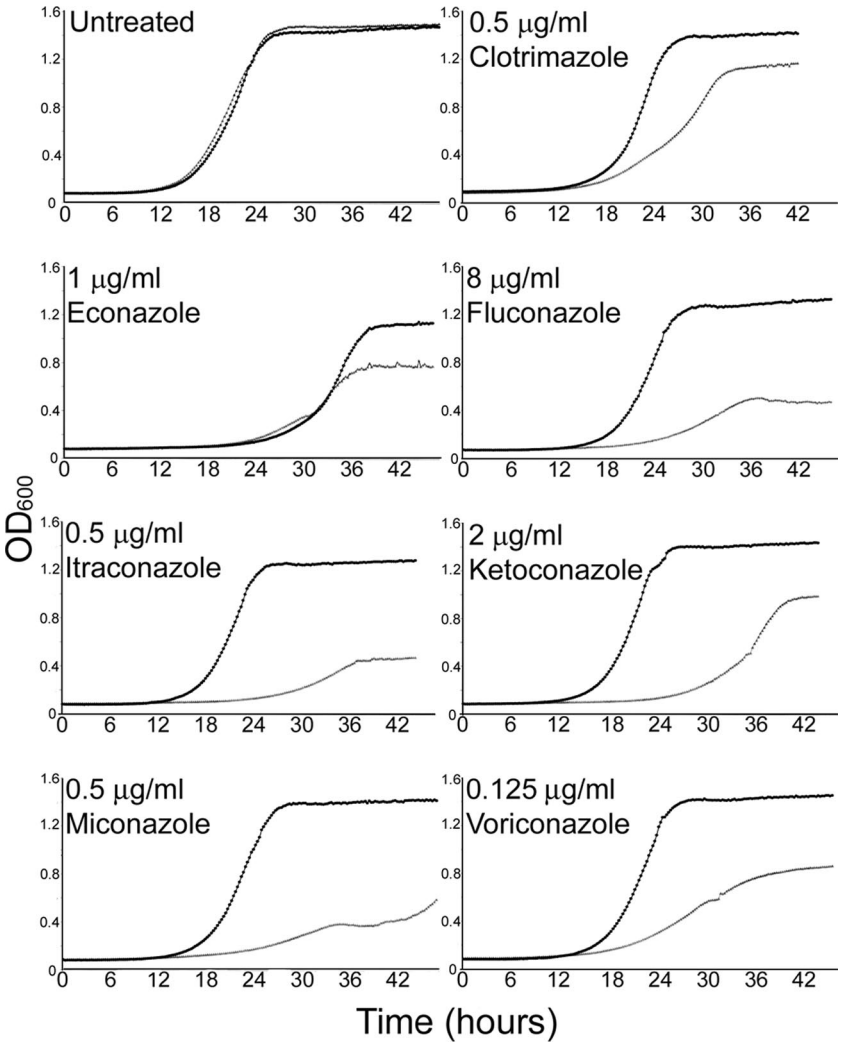


FIG. 3. Growth curves of BY4741 (gray lines) and BY4741:huCYP51 (black lines). The growth curves of untreated cultures and the lowest concentration of a treatment that resulted in significantly slower growth of a strain are shown.

sterol in repeat measurements was lanosterol (23% [± 1.08 SE] versus 17% [± 1.22 SE] in BY4741).

Prior to the drug treatments, it was important to determine whether the growth of the humanized strain was impaired or altered in comparison to that of the wild-type strain. Figure 3 shows the growth curves obtained for the untreated BY4741 and BY4741:huCYP51 strains. Both strains grew to a similar final OD_{600} of around 1.4, and the maximal growth rates were of the same order (Table 2); in fact, the humanized strain appeared to grow slightly faster (an increase of an OD of 0.157 per hour) than the wild-type strain (an increase of an OD of 0.144 per hour).

Susceptibility testing. The susceptibility of both strains to azole antifungal drugs was tested. Of the drugs tested, the triazoles (fluconazole, itraconazole, and voriconazole) and ketoconazole (an imidazole used for oral treatment) showed specificity in their effects on the strains, with much-reduced efficacy for inhibiting the humanized strain. The earlier drugs used topically exhibited no specificity in this comparison (Table 3).

In parallel to the determination of MICs, sensitivities to the drugs, as assessed by their effects on growth rates and maximum ODs, were also measured, using a Bioscreen C instrument (Fig. 3 and Table 2). The data showed the same trend with regard to the specificities of the drugs, with a higher affinity for the yeast CYP51 than for the human CYP51 observed for all drugs tested. This extended to clotrimazole, econazole, and miconazole, with which there was clearly reduced growth in the humanized strain compared to the treated parent strain, although the MIC was not altered in the humanized strain.

Voriconazole displayed the greatest specificity of the drugs tested, and the growth data match the MICs determined in this study. A significant reduction in growth rate and the restriction of maximum OD_{600} to <1 was seen with BY4741 at 0.125 μg/ml of voriconazole, while no significant reduction in growth was seen at 16 μg/ml with BY4741:huCYP51. The MIC of 0.125 μg/ml of voriconazole determined for BY4741 versus >128 μg/ml for BY4741:huCYP51 is in agreement with these data. Fluconazole significantly reduced the growth rate of the wild-type strain and reduced the maximum OD_{600} to less than

TABLE 2. Maximum growth rates and ODs of BY4741 and BY4741:huCYP51 treated with azoles

Treatment and drug amt (μg/ml)	BY4741		BY4741:huCYP51	
	Mean maximum growth rate	Mean maximum OD	Mean maximum growth rate	Mean maximum OD
Untreated	0.144	1.46	0.157	1.39
Clotrimazole				
0.5	**0.110**	1.16	0.162	1.43
1	**0.123**	1.29	0.173	1.41
2	**0.088**	1.08	0.169	1.33
Econazole				
1	**0.084**	**0.87**	0.157	1.22
Fluconazole				
8	**0.036**	**0.47**	0.160	1.34
16	**0.030**	**0.55**	0.171	1.42
Itraconazole				
0.5	**0.042**	**0.55**	0.166	1.40
1	**0.042**	**0.55**	0.176	1.42
2	**0.044**	**0.56**	0.170	1.36
4	**0.039**	**0.56**	0.164	1.33
8	**0.036**	**0.52**	**0.136**	1.28
16	**0.035**	**0.54**	**0.136**	1.25
Ketoconazole				
2	**0.088**	**0.98**	0.188	1.35
4	**0.060**	**0.73**	0.204	1.18
8	**0.039**	**0.62**	**0.119**	1.19
Miconazole				
0.5	**0.024**	**0.76**	0.174	1.41
1	**0.034**	**0.72**	0.141	1.25
2	**0.023**	**0.30**	0.167	1.34
Voriconazole				
0.125	**0.057**	**0.89**	0.157	1.45
0.25	**0.046**	**0.75**	0.159	1.44
0.5	**0.046**	**0.66**	0.153	1.45
1	**0.039**	**0.54**	0.156	1.42
2	**0.051**	**0.62**	0.154	1.35
4	**0.043**	**0.62**	0.154	1.36
8	**0.043**	**0.67**	0.168	1.43
16	**0.039**	**0.67**	0.159	1.33

[a] All drugs were tested between 0.0625 μg/ml and 16 μg/ml. Values are the means of four replicate samples. The SEs of growth rate measurements do not exceed ±10 % of the means except the measurements for BY4741 treated with 0.125 μg/ml of voriconazole, 2 and 4 μg/ml of ketoconazole, 0.5 μg/ml of clotrimazole, and 0.5 μg/ml of miconazole, which did not exceed 20%. The significance of the difference between the values for untreated growth and treated growth was measured by Student's *t* test, using a confidence interval of 99%. Growth rates that are significantly less than those for the untreated strains and OD maximums below 1.0 are shown in boldface. Only treatments resulting in significantly slower growth of at least one of the strains are shown; treatments resulting in no growth are not shown.

TABLE 3. MICs of seven azole drugs for BY4741 and BY4741:huCYP51

Drug	MIC (μg/ml) for indicated strain:	
	BY4741	BY4741:huCYP51
Clotrimazole	8	8
Econazole	8	8
Fluconazole	8	>128
Itraconazole	1	>128
Ketoconazole	1	8
Miconazole	8	8
Voriconazole	0.125	>128

[a] Results were consistent for three experiments.

1 at 8 μg/ml, whereas growth of the humanized CYP51 strain was unaffected at 16 μg/ml. The growth rate measurements obtained with fluconazole agree with our results obtained in tests for MICs. Itraconazole significantly reduced the growth rate and restricted the maximum OD_{600} to <1 at 0.5 μg/ml for the wild-type strain, and although a significantly slower growth rate was observed for the humanized CYP51 strain at 8 and 16 μg/ml, the maximum OD_{600} was not greatly affected. These results concur with results for MICs, although the concentration required to inhibit the growth of the wild-type strain is lower when tested in this way. Treatment with ketoconazole resulted in a significantly lower growth rate at 2 μg/ml, higher than the MIC of 1 μg/ml. However, growth of the humanized strain with ketoconazole was observed at 8 μg/ml, which is also above the MIC we estimated under different conditions. In the cases of clotrimazole, econazole, and miconazole, the concentrations required to inhibit the wild-type strain were lower (0.5, 1, and 0.5 μg/ml, respectively), and indeed, no growth could be detected at all with concentrations above, 2, 1, or 2 μg/ml, respectively.

Sterol composition of azole-treated strains. The sterol composition of strains treated with azoles for 48 h was determined. Azole drugs inhibit CYP51 (sterol 14α-demethylase); therefore, it was expected that an increase in the concentration of the substrate, lanosterol, would be observed upon treatment with azole. An increase in the sterol, 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol, was also associated with growth arrest and the mode of action in previous studies (17).

The concentrations of fluconazole (16 μg/ml), itraconazole (8 μg/ml), ketoconazole (4 μg/ml), and voriconazole (0.25 μg/ml) used were chosen by determining the highest concentrations which still resulted in sufficient growth (biomass) for sterol extraction. Table 1 shows the percentages of ergosterol, lanosterol, and 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol present in the strains. As expected, the wild-type strain (BY4741) showed a greater accumulation of lanosterol and 14α-methyl-ergosta-8,24(28)-dien-3β-6α-diol than the BY4741:huCYP51 strain when treated with azoles. Importantly, the percentage of ergosterol in BY4741 was greatly reduced by treatment, whereas in the BY4741:huCYP51 strain, a level of around 50%, which is typical of this strain when untreated, was maintained. For example, treatment of BY4741 with fluconazole (16 μg/ml) resulted in no detectable ergosterol, whereas the ergosterol level remained at 46% (±1.76 SE) in BY4741:huCYP51.

DISCUSSION

Azole antifungals target CYP51, causing an accumulation of 14 methyl sterol intermediates and a depletion of ergosterol. The effect of perturbed sterol content on the fungal membrane gives rise to growth inhibition (17, 28). CYP51 is also present in humans, and when designing new drugs to target fungal CYP51, the specificity of the new compounds must be considered. We have constructed and validated a strain that may be utilized for preliminary specificity screenings of new drugs. The yeast strain contains the human *CYP51* gene, but not the fungal *CYP51* gene, through integration at the chromosomal locus of yeast *CYP51*, and this strain has been shown to be less susceptible to some azole drugs than the wild-type yeast strain. The results obtained are complementary to those of previous

enzyme inhibition-based studies on the specificities of azole antifungals (3, 12, 27).

Experimental studies of the specificities of azole drugs have utilized recombinant protein to determine the K_m and V_{max} of the enzyme in reconstituted systems and the subsequent IC_{50}s (the inhibition of enzyme activity by drugs). Three studies have used these techniques to investigate the relative susceptibilities of *C. albicans* CYP51 and human CYP51 (3, 12, 27). These studies found the human enzyme to be less susceptible to fluconazole, itraconazole, and ketoconazole. Our study used MICs determined for an 80% inhibition of growth and resulted in an efficacy ratio comparing the parent strain to the humanized one of 16 for fluconazole, 128 for itraconazole, and 8 for ketoconazole, where the humanized strain was least inhibited in growth. Although our MIC data cannot be directly compared to in vitro enzyme inhibition data using *C. albicans* CYP51, our results are in reasonable agreement with those previously determined for ketoconazole (12, 27) and fluconazole (3) and with the ratio for itraconazole determined by Lamb et al. (12). Our MIC data show some correlation of trends with the results for IC_{90} determined by Barchiesi et al. (2) and Pfaller et al. for *S. cerevisiae* (20). Pfaller et al., however, found ketoconazole to be far less effective than indicated by the data presented here and by Barichiesi et al. The results from these different susceptibility tests vary greatly in their numerical values, which may be due to differences in methodology and/or the use of different strains of *S. cerevisiae*.

The data on the growth rates of drug-treated strains in this study agree with the MICs determined and enabled identification of growth inhibition at lower concentrations not detectable through MIC testing. One additional observation was that both strains exhibited significantly faster growth rates at some subinhibitory azole concentrations. At present we cannot explain this phenomenon. However, subinhibitory concentrations of azoles have been shown to affect the transcription levels of a large number of genes in *S. cerevisiae*, including genes involved in the control of the cell cycle (1), which could explain this increase in growth rate.

Importantly, the method we employed here gave reproducible results, which are required to detect significant differences in the sensitivities of the enzyme target. The humanized strain, BY4741:huCYP51, therefore provides a useful tool for the rapid and high-throughput initial specificity testing of new drugs designed to inhibit fungal CYP51. This is an in vivo system that allows direct comparisons of the specificities of the drugs and could also be used to further investigate inhibitors of CYP51 not only for antifungals but also for anticholesterol chemotherapies.

ACKNOWLEDGMENTS

This publication was generated in the context of the STEROLTALK project, funded under the 6th Framework Programme of the European Community (contract no. STREP-512096). The authors are solely responsible for its content, it does not represent the opinion of the European Community, and the Community is not responsible for any use that might be made of the information contained therein. Partial support arose from BBSRC and ERDF support for the Institute of Life Science.

REFERENCES

1. **Bammert, G. F., and J. M. Fostel.** 2000. Genome-wide expression patterns in *Saccharomyces cerevisiae*: comparison of drug treatments and genetic alterations affecting biosynthesis of ergosterol. Antimicrob. Agents Chemother. **44:**1255–1265.
2. **Barchiesi, F., D. Arzeni, A. W. Fothergill, L. F. Di Francesco, F. Caselli, M. G. Rinaldi, and G. Scalise.** 2000. In vitro activities of the new antifungal triazole SCH 56592 against common and emerging yeast pathogens. Antimicrob. Agents Chemother. **44:**226–229.
3. **Bellamine, A., G. I. Lepesheva, and M. R. Waterman.** 2004. Fluconazole binding and sterol demethylation in three CYP51 isoforms indicate differences in active site topology. J. Lipid Res. **45:**2000–2007.
4. **Collart, M. A., and S. Oliviero.** 1993. Preparation of yeast RNA, unit 13.12 ed., vol. 2. Wiley, New York, NY.
5. **Gothard, P., and T. R. Rogers.** 2004. Voriconazole for serious fungal infections. Int. J. Clin. Prac. **58:**74–80.
6. **Gueldener, U., J. Heinisch, G. J. Koehler, D. Voss, and J. H. Hegemann.** 2002. A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. **30:**e23.
7. **Heeres, J., L. J. Backx, J. H. Mostmans, and J. Van Cutsem.** 1979. Antimycotic imidazoles. Part 4. Synthesis and antifungal activity of ketoconazole, a new potent orally active broad-spectrum antifungal agent. J. Med. Chem. **22:**1003–1005.
8. **Kalb, V. F., C. W. Woods, T. G. Turi, C. R. Dey, T. R. Sutter, and J. C. Loper.** 1987. Primary structure of the P450 lanosterol demethylase gene from Saccharomyces cerevisiae. DNA **6:**529–537.
9. **Kelly, S. L., D. C. Lamb, A. J. Corran, B. C. Baldwin, and D. E. Kelly.** 1995. Mode of action and resistance to azole antifungals associated with the formation of 14α-methylergosta-8,24(28)-dien-3β,6α-diol. Biochem. Biophys. Res. Commun. **207:**910–915.
10. **Kelly, S. L., D. C. Lamb, C. J. Jackson, A. G. Warrilow, and D. E. Kelly.** 2003. The biodiversity of microbial cytochromes P450. Adv. Microb. Physiol. **47:**131–186.
11. **Lamb, D. C., D. E. Kelly, B. C. Baldwin, and S. L. Kelly.** 2000. Differential inhibition of human CYP3A4 and *Candida albicans* CYP51 with azole antifungal agents. Chem. Biol. Interact. **125:**165–175.
12. **Lamb, D. C., D. E. Kelly, M. R. Waterman, M. Stromstedt, D. Rozman, and S. L. Kelly.** 1999. Characteristics of the heterologously expressed human lanosterol 14α-demethylase (other names: P45014DM, CYP51, P45051) and inhibition of the purified human and *Candida albicans* CYP51 with azole antifungal agents. Yeast **15:**755–763.
13. **Lepesheva, G. I., and M. R. Waterman.** 2007. Sterol 14α-demethylase cytochrome P450 (CYP51), a P450 in all biological kingdoms. Biochim. Biophys. Acta **1770:**467–477.
14. **Lewis, J. H., H. J. Zimmerman, G. D. Benson, and K. G. Ishak.** 1984. Hepatic injury associated with ketoconazole therapy. Analysis of 33 cases. Gastroenterology **86:**503–513.
15. **Löffler, J., S. L. Kelly, H. Hebart, U. Schumacher, C. Lass-Flörl, and H. Einsele.** 1997. Molecular analysis of *cyp51* from fluconazole-resistant *Candida albicans* strains. FEMS Microbiol. Lett. **151:**263–268.
16. **Maertens, J. A.** 2004. History of the development of azole derivatives. Clin. Microbiol. Infect. **10** (Suppl. 1)**:**1–10.
17. **Marichal, P., J. Gorrens, L. Laurijssens, K. Vermuyten, C. Van Hove, L. Le Jeune, P. Verhasselt, D. Sanglard, M. Borgers, F. C. S. Ramaekers, F. Odds, and H. Vanden Bossche.** 1999. Accumulation of 3-ketosteroids induced by itraconazole in azole-resistant clinical *Candida albicans* isolates. Antimicrob. Agents Chemother. **43:**2663–2670.
18. **Masubuchi, Y., and T. Horie.** 2007. Toxicological significance of mechanism-based inactivation of cytochrome p450 enzymes by drugs. Crit. Rev. Toxicol. **37:**389–412.
19. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
20. **Pfaller, M. A., S. Messer, and R. N. Jones.** 1997. Activity of a new triazole, Sch 56592, compared with those of four other antifungal agents tested against clinical isolates of *Candida* spp. and *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **41:**233–235.
21. **Pont, A., P. L. Williams, S. Azhar, R. E. Reitz, C. Bochra, E. R. Smith, and D. A. Stevens.** 1982. Ketoconazole blocks testosterone synthesis. Arch. Intern. Med. **142:**2137–2140.
22. **Pont, A., P. L. Williams, D. S. Loose, D. Feldman, R. E. Reitz, C. Bochra, and D. A. Stevens.** 1982. Ketoconazole blocks adrenal steroid synthesis. Ann. Intern. Med. **97:**370–372.
23. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14α-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agents Chemother. **42:**241–253.
24. **Scott, L. J., and D. Simpson.** 2007. Voriconazole: a review of its use in the management of invasive fungal infections. Drugs **67:**269–298.
25. **Sharp, P. M., and W.-H. Li.** 1987. The Codon Adaptation Index—a measure of directional synonymous codon usage bias, and its potential applications. Nucleic Acids Res. **15:**1281–1295.
26. **Shyadehi, A. Z., D. C. Lamb, S. L. Kelly, D. E. Kelly, W. H. Schunck, J. N.**

Wright, D. Corina, and M. Akhtar. 1996. The mechanism of the acyl-carbon bond cleavage reaction catalyzed by recombinant sterol 14α-demethylase of *Candida albicans* (other names are: lanosterol 14α-demethylase, P-450$_{14DM}$, and CYP51). J. Biol. Chem. **271:**12445–12450.

27. **Trösken, E. R., M. Adamska, M. Arand, J. A. Zarn, C. Patten, W. Völkel, and W. K. Lutz.** 2006. Comparison of lanosterol-14α-demethylase (CYP51) of human and *Candida albicans* for inhibition by different antifungal azoles. Toxicology **228:**24–32.
28. **Watson, P. F., M. E. Rose, S. W. Ellis, H. England, and S. L. Kelly.** 1989. Defective sterol C5-6 desaturation and azole resistance: a new hypothesis for the mode of action of azole antifungals. Biochem. Biophys. Res. Commun. **164:**1170–1175.
29. **Zarn, J. A., B. J. Bruschweiler, and J. R. Schlatter.** 2003. Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14α-demethylase and aromatase. Environ. Health Perspect. **111:**255–261.