ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2004, p. 2124–2131 Vol. 48, No. 6
0066-4804/04/$08.00+0 DOI: 10.1128/AAC.48.6.2124–2131.2004

Application of Real-Time Quantitative PCR to Molecular Analysis of *Candida albicans* Strains Exhibiting Reduced Susceptibility to Azoles

Andrew S. Chau, Cara A. Mendrick, Frank J. Sabatelli, David Loebenberg, and Paul M. McNicholas*

Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Received 27 October 2003/Returned for modification 9 December 2003/Accepted 12 February 2004

Real-time quantitative PCR was used to measure expression levels of genes encoding efflux pumps, *ERG11* and two control genes, *ACT1* and *PMA1*, in a collection of 14 fluconazole-susceptible *Candida albicans* isolates. For each gene, average expression levels and variations within the population were determined. These values were then used as reference points to make predictions about the molecular basis of resistance in 38 clinical isolates (the majority of which were resistant to fluconazole) obtained from 18 patients treated with posaconazole for refractory oropharyngeal candidiasis. For each of the 38 isolates, the expression levels of genes encoding efflux pumps, *ERG11* and the control genes, were measured as above. Comparison of the two data sets revealed that expression of *ACT1* and *PMA1* did not vary significantly between the two sets of isolates. In contrast, *MDR1, ERG11*, *CDR1*, and *CDR2* were overexpressed in 3, 4, 14, and 35, respectively, of the isolates from patients treated with azoles. In addition to these changes, the patient isolates all had at least one and often multiple missense mutations in *ERG11*. Select *ERG11* alleles were expressed in *Saccharomyces cerevisiae*; all of the alleles tested conferred reduced susceptibility to fluconazole. Despite both the increases in pump expression and the *ERG11* mutations, only one of the patient isolates exhibited a large decrease in posaconazole susceptibility.

Nearly 90% of individuals who are infected with human immunodeficiency virus or have AIDS experience at least one episode of oropharyngeal candidiasis during the course of their disease (7). Azoles have been the drugs of choice for the treatment of oropharyngeal candidiasis. Azoles inhibit the enzyme lanosterol 14α-demethylase (encoded by *ERG11*), which catalyzes an important step in the synthesis of ergosterol. The resulting depletion of ergosterol from the membrane, in combination with the accumulation of methylated sterols, is proposed to adversely affect membrane integrity as well as the function of some membrane-associated proteins (11, 18).

To combat the development of resistance to azoles in yeasts, in particular to fluconazole, and to expand the spectrum of susceptible pathogens, new azoles have been developed. One such agent is posaconazole, a broad-spectrum triazole in phase III trials. Posaconazole is more active than fluconazole against *Candida* spp. and, unlike fluconazole, is active against *Aspergillus* spp. (1, 14). In addition, posaconazole appears to be less affected by mutations in the gene encoding the azole target site than either voriconazole or fluconazole (D. Sanglard, F. Ischer, and J. Bille, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. M-221, 2002). Furthermore, although posaconazole is a substrate for the ATP-dependent efflux pumps encoded by *CDR1* and *CDR2*, posaconazole is not a substrate for the major facilitator pumps encoded by *MDR1* and *FLU1* (Sanglard et al., 42nd ICAAC).

Azoles are fungistatic against yeasts, and patients often require long-term therapy; this has promoted the development of resistance. The past decade has seen numerous publications describing the molecular mechanisms of azole resistance in yeasts (most recently reviewed in references 2 and 17). The two most prevalent mechanisms of resistance are mutations in the *ERG11* gene, resulting in reduced drug binding to the target enzyme and decreased intracellular drug accumulation. The latter is most often caused by increased expression of efflux pump genes, the best characterized of which are *MDR1*, which encodes a major facilitator pump, and *CDR1* and *CDR2*, which encode distinct but related ATP-dependent pumps. To identify changes in gene expression, researchers have focused on sequential isolates from a single patient. In such collections, changes in gene expression are readily identified by comparing expression levels in the resistant isolates with those measured in an isogenic, azole-susceptible baseline strain. However, such analyses become problematic when a susceptible baseline isolate is not available.

To circumvent this problem, we used quantitative real-time PCR (RT-PCR) to determine the normal range of expression levels of genes known to confer azole resistance in a collection of azole-susceptible isolates. These ranges were then used as reference points to make predictions about the molecular basis of resistance in isolates from patients receiving posaconazole therapy for treatment of refractory oropharyngeal candidiasis.

(Part of this work was presented at the 103rd Annual Meeting of the American Society for Microbiology, Washington, D.C., May 2003.)

* Corresponding author: Mailing address: Schering-Plough Research Institute, 2015 Galloping Hill Rd., K15.4-4700, Kenilworth, NJ 07033. Phone: (908) 740 7644. Fax: (908) 740 3918. E-mail: paul.mcnicholas@spcorp.com.

MATERIALS AND METHODS

Fungal strains. The identity of the clinical isolates was confirmed with the Vitek Identification System with the Yeast Biochemical Card (bioMérieux Inc., Hazelwood, Mo.).

TABLE 1. Sequences of RT-PCR primer-probe sets

Gene	Probe and primers	Sequence
ACT1	Probe	TTGACCTTGAGATACCCAATTGAACACGGTA
	Forward primer	TTGGTGATGAAGCCCAATCC
	Reverse primer	CATATCGTCCCAGTTGGAAACA
PMA1	Probe	AGATGTCCACGAAAACTACCAAAACACCGTT
	Forward primer	TTGAAGATGACCACCCAATCC
	Reverse primer	GAAACCTCTGGAAGCAAATTCG
ERG11	Probe	TGCCTGACCCTGATTATAGTTCAATGGTGG
	Forward primer	AACTACTTTTGTTTATAATTTAAGATGGACTATTGA
	Reverse primer	AATGATTTCTGCTGGTTCAGTAGGT
MDR1	Probe	TCGCAAGGCTAAAAGATTGAGAGCCATCA
	Forward primer	TTACCTGAAACTTTTGGCAAAACA
	Reverse primer	ACTTGTGATTCTGTCGTTACCG
CDR1	Probe	TAACCCATATGTCAGAAGTGCCCGGG
	Forward primer	TTTAGCCAGAACTTTCACTCATGATT
	Reverse primer	TATTTATTTCTTCATGTTCATATGGATTGA
CDR2	Probe	TCCCGGGTTTTGGATTTTCATGTACAGA
	Forward primer	GGTATTGGCTGGTCCTAATGTGA
	Reverse primer	GCTTGAATCAAATAAGTGAATGGATTAC

Antifungal agents and susceptibility testing. Posaconazole was prepared at the Schering-Plough Research Institute (SPRI, Kenilworth, N.J.) as a micronized powder. Itraconazole and amphotericin B powders were obtained from Janssen Pharmaceutica Inc. (Beerse, Belgium) and Sigma Chemical Co. (St. Louis, Mo.), respectively. Voriconazole and fluconazole were obtained from Pfizer Inc. (New York, N.Y.). All drugs except voriconazole (which was dissolved in water) were dissolved in dimethyl sulfoxide, and serial dilutions were made in RPMI 1640 medium (BioWhittaker, Walkersville, Md.). MICs were determined by the procedures of the National Committee for Clinical Laboratory Standards (NCCLS) (10). When assaying the effects of expressing *ERG11* alleles in *Saccharomyces cerevisiae*, MICs were determined in yeast nitrogen base (Qbiogene, Carlsbad, Calif.) broth supplemented with 2% raffinose and 2% galactose.

DNA typing techniques for strain identification. Repetitive-element PCR was performed with a DiversiLab Candida kit and run on a Caliper 1000 analyzer as described by the manufacturer (Bacterial BarCodes, Houston, Tex.). The resultant data were analyzed with DiversiLab System software.

Measurement of transcript levels. Strains were grown overnight at 35°C in YPD liquid medium (Qbiogene). Overnight cultures were diluted into fresh medium, adjusted to an optical density at 530 nm of 0.1, and grown for 3 h. Total RNA was extracted with the RNeasy minikit (Qiagen Inc., Valencia, Calif.). RNA quality (i.e., the presence of discreet 18S and 28S rRNA peaks) and quantity were measured with an Agilent Technologies (Palo Alto, Calif.) RNA 6000 Nano Assay kit run on an Agilent Technologies 2100 bioanalyzer. Multiplex RT-PCRs were run in duplicate with an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif.). The 6-carboxyfluorescein-labeled test probe sets were designed in-house with Primer Express software (Applied Biosystems) and purchased from Applied Biosystems (Table 1). The 6-carboxyrhodamine 6G-labeled 18S TaqMan rRNA control reagents kit for the detection of endogenous 18S rRNA was also purchased from Applied Biosystems.

Expression levels as measured by RT-PCR are expressed in C_T units; this is the first cycle in which the signal above a preset threshold (known as the threshold cycle) is detected. Consequently, the C_T value is inversely proportional to the amount of transcript detected (i.e., the C_T value for an abundant transcript will be numerically lower than the value measured for a less abundant transcript). Furthermore, a decrease in the C_T value from x to $x - 1$ corresponds to a twofold increase in the amount of transcript detected. To control for variations in the amount of input RNA, multiplex reactions were run with the 18S rRNA probe as an internal control. Relative gene expression levels (ΔC_T) were calculated as ΔC_T[test gene] = C_T[test gene] − C_T[18S]. All probe sets were tested, alone and in a multiplex mode, with serial dilutions of cDNA. The signal from all probe sets was proportional to the amount of input cDNA over a 1,000-fold concentration range, irrespective of the presence of the 18S rRNA probe.

PCR amplification, sequencing, and cloning of *ERG11* alleles. *ERG11* was PCR amplified in overlapping 600-bp segments from total genomic DNA. Both strands were sequenced by MWG-Biotech Inc, (High Point, N.C.) and compared to the sequence published in GenBank (accession number X13296). Select *ERG11* alleles were PCR amplified from chromosomal DNA with PFU Turbo (Stratagene, La Jolla, Calif.) with the following oligonucleotides to generate fragments with flanking SalI and BamHI restriction sites, 5′-ACGCGTCGACA ATATGGCTATTGTTGAAACTGTC-3′ and 5′-GCGGATCCTTAAAACAT ACAAGTTTCTCTTTT-3′. To engineer the mutation encoding the A61V substitution, the 5′ and 3′ regions of *ERG11* were separately PCR amplified with the above oligonucleotides in combination with mutagenic oligonucleotides (5′-TT GGTTTGGTTCTGTAGCTTCATATGGT-3′ and 5′-ACCATATGAAGCTAC AGAACCAAACCAA-3′). The resulting PCR fragments were fused together by PCR with the same oligonucleotides used for cloning *ERG11*. The amplified *ERG11* genes were cloned into the multicopy plasmid YEp51 under control of the *GAL10* promoter, sequenced to confirm accurate amplification, and transformed into *S. cerevisiae* strain YKKB-13 (plasmid YEp51 and *S. cerevisiae* strain YKKB-13 were kindly supplied by D. Sanglard, University of Vaudois, Vaudois, Switzerland) (16).

RESULTS

Clinical isolates and azole susceptibilities. Fourteen *C. albicans* clinical isolates with fluconazole MICs of ≤0.5 μg/ml were randomly chosen from the SPRI culture collection (Table 2). All 14 isolates were susceptible to posaconazole, itraconazole, voriconazole, and amphotericin B. This collection of isolates is referred to below as the SPRI azole-susceptible population.

A second collection comprised 38 *C. albicans* clinical isolates cultured from 18 patients who were receiving posaconazole for treatment of refractory oropharyngeal candidiasis (Table 3). We focused on isolates that exhibited significant reductions in susceptibility to fluconazole, voriconazole, itraconazole, and posaconazole. None of the isolates showed any changes in

TABLE 2. MIC and RT-PCR data for SPRI azole-susceptible *C. albicans* isolates

Isolate	MIC[a] (μg/mL)				ΔC_T						Substitution(s) in Erg11p
	POS	ITZ	FLZ	VOR	*ACT1*	*PMA1*	*ERG11*	*MDR1*	*CDR1*	*CDR2*	
C34	0.016	0.06	0.25	<0.004	8.1	7.1	10.3	11.1	11.3	13.5	None
C40	0.016	0.06	0.25	<0.004	7.2	6.2	10.6	11.9	9.6	14.1	D116E, K128T[b]
C43	<0.03	0.06	0.25	0.03	7.4	6.3	10.3	15.1	10.2	14.4	ND[c]
C60	0.016	0.06	<0.12	<0.004	7.6	6.3	8.6	10.9	11.8	15.9	None
C72	0.03	0.06	0.06	<0.008	7.9	6.7	8.7	12.0	10.4	12.5	D116E
C84	0.008	0.06	<0.12	<0.004	7.3	6.4	9.3	14.4	10.2	13.8	D116E
C128	0.008	0.03	<0.12	<0.004	7.5	6.1	11.1	14.4	10.5	14.6	D116E, K128T,[b] D496
C132	0.016	0.06	0.25	<0.004	7.5	5.8	11.3	13.2	10.0	14.5	D116E, K128T[b]
C294	0.008	0.016	0.25	<0.008	8.9	8.3	10.9	12.8	12.3	16.0	None
C392	<0.004	0.03	0.5	<0.008	7.9	6.7	11.1	14.0	11.2	14.6	None
C393	<0.004	0.016	0.25	<0.008	7.8	6.7	11.3	13.0	10.7	14.7	ND
C394	<0.004	0.016	0.25	<0.008	7.9	6.3	10.9	16.6	10.6	13.9	ND
C395	<0.004	0.016	0.25	<0.008	7.9	6.8	11.5	12.4	10.7	15.6	V437I
C548	<0.004	<0.008	<0.125	<0.008	7.6	6.3	10.0	14.2	12.3	14.4	V437I
Avg ΔC_T					7.8	6.6	10.4	13.6	10.8	14.5	
SD					0.4	0.6	0.9	2	0.8	0.9	
3-SD range					6.6–9.0	4.8–8.4	7.7–13.1	7.6–19.6	8.4–13.2	11.8–17.2	

[a] POS, posaconazole; ITZ, itraconazole; FLZ, fluconazole; VOR, voriconazole.
[b] Mutation in only one copy of *ERG11.*
[c] ND, not done.

susceptibility to amphotericin B. Relatedness among isolates from individual patients was tested by repetitive-element PCR and also inferred from the conservation of both silent and missense mutations in *ERG11*. The majority of the patients appeared to be colonized by a single isolate (data not shown). The baseline isolates from the 18 patients were classified according to NCCLS criteria as follows: 10 were fluconazole resistant (MIC, ≥64 μg/ml); six were fluconazole susceptible, dose dependent (S-DD) (MIC, 16 to 32 μg/ml); and two were fluconazole sensitive (MIC, ≤8 μg/ml). Of the 38 isolates analyzed, 24 were fluconazole resistant, 10 were fluconazole S-DD, and 4 were fluconazole sensitive. Of the 24 fluconazole-resistant isolates, 17 had a voriconazole MIC of >1 μg/ml, one had a posaconazole MIC of >1 μg/ml, and eight were itraconazole resistant by NCCLS criteria (MIC of >1 μg/ml).

RT-PCR measurement of gene expression in the SPRI azole-susceptible population. Total RNA was isolated from exponentially growing cultures and analyzed by RT-PCR. The average C_T value for the 18S rRNA probe was 14.9; the standard deviation (SD) was 0.4, which corresponded to a fluctuation of ±1.3-fold. The *ACT1* and *PMA1* genes, encoding actin and a plasma membrane ATPase, respectively, have been reported to be constitutively expressed in *C. albicans* (9; D. Perlin, personal communication). The average ΔC_T values for *ACT1* and *PMA1* were 7.8 (SD, 0.4) and 6.6 (SD, 0.6), respectively. Similarly, the SDs associated with the ΔC_T values for *CDR1*, *CDR2*, and *ERG11* were all <1, which corresponds to a <2-fold fluctuation in expression levels. Expression of *MDR1* exhibited the most fluctuation; the SD was 2, which corresponds to a ±4-fold range of expression levels.

To determine the intrastrain variability in gene expression as well as the experimental error associated with the procedure, we repeated the analysis with RNA extracted from a single isolate (C72) grown on three separate occasions. The SDs for the three measurements ranged from a high of 0.6 for *CDR2* to a low of 0.3 for *MDR1*; the values for *PMA1*, *ACT1*, *CDR1*, and *ERG11* all fell within this range (data not shown).

RT-PCR measurement of *ACT1* and *PMA1* gene expression in *C. albicans* isolates from oropharyngeal candidiasis patients. The 38 *C. albicans* isolates exhibiting various levels of azole resistance were analyzed as above (Table 3). The average C_T value for the 18S rRNA probe was 14.8 (SD, 0.4). The average ΔC_T values for the control genes *ACT1* and *PMA1* were 7.5 (SD, 0.5) and 6.7 (SD, 0.7), respectively. These values are very similar to those measured in the 14 SPRI azole-susceptible isolates. Employing a two-tailed Student's *t* test, we confirmed that the expression levels of both *ACT1* and *PMA1* did not differ significantly ($P > 0.05$) between the two populations of isolates. These data suggest that the experimental protocol (i.e., the growth conditions and extraction procedures) did not discriminate against either population.

Assignment of overexpression cutoffs for the analysis of *C. albicans* isolates from oropharyngeal candidiasis patients. As shown above, the ΔC_T values for test genes in the SPRI azole-susceptible population exhibited relatively minor differences in expression levels, as reflected in the SD values (the exception was *MDR1*). For a given test gene, the variation in ΔC_T was a function of both the strain-to-strain variability in expression levels and the experimental error associated with the RT-PCR measurements. For the majority of the test genes (the exception was *MDR1*) in the SPRI azole-susceptible population, the variations in the ΔC_T values were normally distributed. Therefore, a ΔC_T value that differed from the average value by more than 3 SDs would be considered statistically different (with a confidence interval of >99%). These cutoff values (Table 2) were applied to the analysis of gene expression in the isolates from the oropharyngeal candidiasis patients; genes with a measured ΔC_T value that fell outside the 3-SD range measured in SPRI azole-susceptible population (Table 3) were designated as being over- or underexpressed.

RT-PCR measurement of efflux pump and *ERG11* expression levels in previously characterized *C. albicans* isolates. Three sequential *C. albicans* isolates were previously analyzed by Sanglard and coworkers (16). The first two isolates in the

TABLE 3. MIC and RT-PCR data for *C. albicans* isolates from candidiasis patients

Patient no.	Isolate no.	MIC[a] (μg/ml)				ΔC_T[b]						Substitution(s) in Erg11p
		POS	ITZ	FLZ	VOR	*ACT1*	*PMA1*	*ERG11*	*MDR1*	*CDR1*	*CDR2*	
1	C482	0.25	0.5	16	0.5	7.9	7.5	10.4	13.7	9.2	***9.4***	A107T
	C486	0.25	1	16	0.25	8.0	8.1	9.8	12.3	11.2	***10.1***	A107T
2	C491	0.12	0.25	2	0.03	7.4	7.2	8.9	12.9	10.1	***10.0***	D116E
	C504	0.25	0.25	16	0.25	7.3	6.2	7.9	15.5	***8.2***	***7.7***	F449S
3	C498	0.008	0.06	1	0.008	7.4	5.4	9.6	12.6	***8.2***	***10.8***	F145L, E266D, V488I
	C502	0.008	0.06	1	0.008	7.2	5.9	***7.4***	11.7	10.6	***10.8***	F145L, E266D, V488I
	C513	0.25	0.25	16	0.12	6.7	6.4	***7.5***	10.6	***7.6***	***5.8***	F145L, E266D, V488I
4	C527	0.12	0.5	256	4	7.5	6.6	9.1	12.6	8.9	***8.6***	Y132H, G450E
	C528	0.12	0.5	256	4	7.3	5.9	9.0	11.7	***7.7***	***7.7***	Y132H, G450E
	C530	0.12	0.25	256	0.5	7.0	6.3	8.5	12.0	8.8	***9.9***	G450E, G464S
	C534	0.12	0.5	256	4	7.3	6.4	8.1	11.0	***8.3***	***8.8***	Y132H, G450E
	C546	0.12	0.25	128	2	8.1	7.5	9.6	11.9	9.4	***9.4***	Y132H, G450E
	C536	0.25	0.5	256	4	8.2	7.7	9.8	11.7	9.2	***9.4***	Y132H, G450E
	C572	0.25	0.25	>256	8	8.0	7.4	9.5	12.1	9.5	***9.2***	Y132H, G450E
5	C481	0.25	0.5	32	0.5	7.9	7.2	9.9	15.7	9.4	***9.0***	F128T, F145L
	C483	0.25	0.5	8	0.12	8.2	7.3	11.0	14.8	10.0	***9.5***	F145L
6	C535	0.5	2	256	8	7.7	7.1	9.8	9.0	9.6	***10.9***	Y132H, G448V
	C539	0.25	2	128	8	7.2	6.8	11.0	8.3	***6.6***	***7.1***	Y132H, G448V
7	C516	0.12	0.25	32	0.5	7.3	7.1	9.8	14.0	9.7	***9.7***	K128T, Y132F, F145L
8	C470	0.5	0.5	32	0.25	7.3	7.0	9.0	13.5	8.7	***8.5***	S405F
	C478	0.5	0.5	64	0.5	7.6	6.9	8.6	12.6	9.1	***8.5***	S405F
9	C490	0.5	1	32	0.25	8.3	7.8	10.6	9.4	9.0	***9.2***	V452A
	C497	0.5	2	64	0.5	8.4	7.7	9.1	12.5	10.0	***9.3***	K128T, V452A
10	C526	0.5	1	32	0.5	7.8	7.1	9.6	13.6	9.2	***9.2***	V509M
	C533	0.5	0.5	32	1	7.9	7.4	9.8	13.6	9.3	***9.9***	V509M
11	C594	0.5	2	128	16	7.2	6.5	9.0	11.3	***8.2***	***7.5***	Y132H, S405F
	C600	1	4	>256	16	7.7	7.1	8.5	12.3	9.4	***8.2***	Y132H, S405F
12	C577	0.06	0.06	128	0.5	***6.5***	5.0	9.0	***5.3***	***8.1***	12.9	G464S
	C583	0.25	0.25	256	1	6.8	5.5	9.4	***5.7***	***6.9***	***10.7***	G464S
	C587	0.5	0.5	256	4	7.7	7.3	9.2	8.2	10.2	***9.6***	G464S
13	C480	0.03	0.12	128	8	8.3	7.4	9.3	9.2	12.2	15.1	K128T, G464S, R467I
14	C438	0.5	0.5	128	2	6.6	6.0	7.9	13.3	***7.1***	***7.5***	Y257H, G464S
	C439	1	4	>256	16	7.1	6.2	***7.6***	13.5	***7.7***	***7.5***	Y257H, G464S, G307S
	C440	4	8	>256	>16	7.3	6.4	8.0	13.5	***7.9***	***7.7***	Y257H, G464S, G307S, A61V
15	C444	0.5	0.5	256	0.5	7.0	5.8	***7.4***	13.2	***7.8***	***7.8***	K143R
16	C489	0.25	0.25	128	1	7.2	6.1	8.8	12.8	10.1	***9.5***	Y132H, Y257H, E266D
17	C477	1	0.25	256	16	7.6	6.1	9.3	***7.3***	10.5	15.3	K128T, G464S, R467I
18	C507	1	2	64	8	***6.1***	7.2	8.6	10.5	***8.3***	***8.4***	Y132H, H283R, G464S

[a] See Table 2, footnote *a*.
[b] Values that fall outside the 3-SD range measured in the azole-susceptible isolates (see text for details) are shown in boldface italic.

series were fluconazole susceptible; Northern blot analysis confirmed that there were no significant increases in expression of *ERG11*, *MDR1*, or *CDR* (the probe was unable to discriminate between *CDR1* and *CDR2*) in these isolates. The last isolate in the series was fluconazole S-DD, and Northern blot analysis revealed an eightfold increase in *CDR* expression. RT-PCR analysis of the same isolates confirmed these findings. In the first two isolates, the expression levels of *CDR1*, *CDR2*, *MDR1*, and *ERG11* fell within the ranges measured in the SPRI azole-susceptible isolates (data not shown). In contrast, in the last isolate, expression of *CDR2* was 16-fold higher than the average value measured in the SPRI azole-susceptible isolates. In

addition, in a recent study we described an analysis of a series of *C. albicans* isolates exhibiting reduced susceptibility to posaconazole (3). As part of this analysis, we measured gene expression by both Northern blotting and RT-PCR (with the same probe sets employed in this analysis); the data from both techniques were in complete agreement.

Correlating changes in expression profiles and/or mutations in *ERG11* with azole resistance in the oropharyngeal candidiasis isolates. Three of the isolates from the azole-treated patients overexpressed *MDR1*; all three isolates were fluconazole resistant. *CDR1* was upregulated in 14 patient-derived isolates, one of which was fluconazole susceptible. Similarly, of the 35 isolates overexpressing *CDR2*, four were fluconazole susceptible. *ERG11* was overexpressed in four isolates, one of which was fluconazole susceptible. However, for all four isolates, the ΔC_T values for *ERG11* were only marginally greater than the cutoff values given in Table 2.

Previous work demonstrated that azole resistance in *Candida* spp. frequently resulted from multiple molecular mechanisms of resistance (12, 13, 17). It is clear from Table 3 that the majority of the oropharyngeal candidiasis isolates not only exhibited significant changes in efflux pump expression levels but had also acquired mutations in *ERG11*. In the following analysis, we focused on collections of isolates in which changes in resistance profiles appeared to result from a single molecular mechanism.

Mutations in *ERG11* resulting in cross-resistance to fluconazole and voriconazole. Seventeen of the 38 isolates analyzed exhibited cross-resistance to fluconazole (MIC, $\geq$64 μg/ml) and voriconazole (in the absence of established breakpoints, we labeled an isolate resistant to voriconazole if the MIC was $>$1 μg/ml). Sixteen of the 17 isolates (the exception was C587) exhibited the same pattern of mutations in *ERG11*; a substitution close to the N terminus of the protein (K128T, Y132H, or Y257H) together with a substitution towards the C terminus of the protein (G405F, G448V, G450E, or G464S). All but one of these isolates remained susceptible to posaconazole (see below). To confirm that the substitutions were responsible for the changes in susceptibility, select *ERG11* alleles were cloned into a multicopy plasmid and expressed in *S. cerevisiae*. All of the *ERG11* alleles tested conferred significant reductions in susceptibility to fluconazole, and most also conferred reduced susceptibility to voriconazole (see Table 5).

Mutations in *ERG11* resulting in changes in fluconazole susceptibility. Sixteen clinical isolates were either fluconazole S-DD or fluconazole resistant but remained susceptible to voriconazole and posaconazole (Table 3). With the exception of isolate C513 (see below), we were unable to associate a pattern of overexpression of efflux pumps or *ERG11* with the changes in azole susceptibility in these isolates. However, eight of these isolates (C504, C516, C470, C478, C577, C583, C444, and C489) also had substitutions in Erg11p that were previously shown to cause a reduction in fluconazole susceptibility (12, 13, 15). The remaining seven isolates (C482, C486, C481, C490, C497, C526, and C533) also had substitutions in Erg11p; it remains to be determined if the substitutions are responsible for the change in fluconazole susceptibility.

Overexpression of *CDR2* and azole resistance. In the three isolates from patient 3, the fluconazole MIC increased from a baseline level of 1 μg/ml in isolates C498 and C502 to 16 μg/ml in C513; the MICs of posaconazole, itraconazole, and voriconazole increased similarly (Table 3). The increase in the MIC was accompanied by a 30-fold increase in expression of *CDR2*. Other minor variations in gene expression included a slight increase in *ERG11* expression in isolates C502 and C513 and an increase in *CDR1* expression in isolates C498 and C513. All three isolates had the same three missense mutations in *ERG11*.

Mutations in *ERG11* resulting in reduced susceptibility to posaconazole. Isolates C438, C439, and C440 were cultured from patient 14 (Table 3). The baseline isolate was resistant to fluconazole and voriconazole. Isolate C439 was resistant to itraconazole, and the final isolate exhibited reduced susceptibility to all azoles, including posaconazole. There were no significant increases in the expression levels of either *ERG11* or any of the pumps in these three isolates. Sequence analysis of *ERG11* revealed an ordered acquisition of mutations: isolate C438 carried two substitutions (Y257H and G464S), C439 acquired an additional substitution (G307S), and isolate C440 had acquired a fourth substitution (A61V). When the three *ERG11* alleles were expressed in *S. cerevisiae*, they conferred the same pattern of resistance seen in the clinical *C. albicans* isolates (Table 4). The mutation encoding the A61V substitution was introduced into a wild type *ERG11* allele and expressed in *S. cerevisiae*. The A61V substitution alone did not confer a significant reduction in susceptibility to posaconazole (Table 5).

DISCUSSION

Azole resistance in *C. albicans* frequently results from overexpression of genes encoding efflux pumps or from mutations in or overexpression of *ERG11*. Northern blot analysis has proved useful for identifying significant changes in gene expression, particularly when comparing isogenic azole-susceptible and -resistant isolates side by side on the same gel. However, such analyses are more problematic, if not impossible, if a susceptible baseline strain is unavailable. The goals of this study were twofold. First, we used RT-PCR to determine what constitutes a "normal" level of gene expression for a collection of genes that encode proteins known to confer azole resistance in azole-susceptible *C. albicans* isolates. The second goal was to use these population averages to identify changes in expression in a collection of *C. albicans* isolates exhibiting various levels of azole resistance.

With regard to the first goal, in a collection of 14 azole-susceptible isolates, five of the six test genes exhibited minor fluctuations in expression (as reflected in SD values of $<$1). The exception was *MDR1*, the SD value for which was 2, which corresponds to a $\pm$4-fold fluctuation in expression. To estimate how much of these variations were due to experimental error versus differences between individual strains, we made replicate measurements with a single strain. Expression of *MDR1* varied the least; the range of expression levels of the other test genes were similar to those measured in the SPRI azole-susceptible population. These data suggest that the fluctuations in *MDR1* expression seen in our survey resulted from strain-to-strain variations rather than from inconsistencies in the experimental methodology. Lyons and White also reported that *MDR1* expression varied considerably, which they ascribed to a

TABLE 4. Subpopulation of candidiasis patients colonized by *C. albicans* isolates exhibiting cross-resistance to fluconazole and voriconazole[a]

Patient no.	Isolate no.	MIC (μg/ml)				ΔC_T						Substitution(s) in Erg11p
		POS	ITZ	FLZ	VOR	*ACT1*	*PMA1*	*ERG11*	*MDR1*	*CDR1*	*CDR2*	
4	C527	0.12	0.5	256	4	7.5	6.6	9.1	12.6	8.9	***8.6***	Y132H, G450E
	C528	0.12	0.5	256	4	7.3	5.9	9.0	11.7	***7.7***	***7.7***	Y132H, F155L, G450E
	C534	0.12	0.5	256	4	7.3	6.4	8.1	11.0	***8.3***	***8.8***	Y132H, G450E
	C546	0.12	0.25	128	2	8.1	7.5	9.6	11.9	9.4	***9.4***	Y132H, G450E
	C536	0.25	0.5	256	4	8.2	7.7	9.8	11.7	9.2	***9.4***	Y132H, G450E
	C572	0.25	0.25	>256	8	8.0	7.4	9.5	12.1	9.5	***9.2***	Y132H, G450E
6	C535	0.5	2	256	8	7.7	7.1	9.8	9.0	9.6	***10.9***	Y132H, G448V
	C539	0.25	2	128	8	7.2	6.8	11.0	***8.3***	***6.6***	***7.1***	Y132H, G448V
11	C594	0.5	2	128	16	7.2	6.5	9.0	11.3	***8.2***	***7.5***	Y132H, S405F
	C600	1	4	>256	16	7.7	7.1	8.5	12.3	9.4	***8.2***	Y132H, S405F
12	C587	0.5	0.5	256	4	7.7	7.3	9.2	***8.2***	10.2	***9.6***	G464S
13	C480	0.03	0.12	128	8	8.3	7.4	9.3	9.2	12.2	15.1	K128T, G464S, R467I
14	C438	0.5	0.5	128	2	6.6	6.0	7.9	13.3	***7.1***	***7.5***	Y257H, G464S
	C439	1	4	>256	16	7.1	6.2	***7.6***	13.5	***7.7***	***7.5***	Y257H, G464S, G307S
	C440	4	8	>256	>16	7.3	6.4	8.0	13.5	***7.9***	***7.7***	Y257H, G464S, G307S, A61V
17	C477	1	0.25	256	16	7.6	6.1	9.3	***7.3***	10.5	15.3	K128T, G464S, R467I
18	C507	1	2	64	8	6.1	7.2	8.6	10.5	***8.3***	***8.4***	Y132H, H283R, G464S

[a] See Table 2, footnote *a*, and Table 3, footnote *b*.

rapid turnover of the *MDR1* transcript (4). In summary, we determined that by standardizing growth conditions to avoid the previously described growth phase-dependent variations in gene expression (4), it was possible to assign an average expression level across a collection of strains.

With regard to the second goal, 38 clinical isolates from 18 patients were analyzed. The expression levels of the control genes, *PMA1* and *ACT1*, did not vary significantly between the two populations of isolates, suggesting that the growth conditions and RNA extraction techniques were reproducible. To identify changes in expression, we designated a gene as being overexpressed if the ΔC_T value measured in the patient isolate differed from the average value calculated for the susceptible isolates by more than 3 SDs. Based on these criteria, *CDR2* was classified as being overexpressed in the majority of the patient isolates, including four fluconazole-susceptible isolates.

To determine if the changes in *CDR2* expression were stable, select isolates were passaged for a week in liquid medium with or without fluconazole. Contrary to the findings of a previous study (6), passaging cells in the absence of fluconazole did not lead to a reversal of the changes in *CDR2* expression (data not shown). Another unusual finding was that in contrast to previous reports (8, 19), *CDR1* and *CDR2* did not appear to be coregulated; the reason for this discrepancy is unknown. Few patient isolates exhibited overexpression of either *MDR1* or *ERG11*; for those isolates that did, there was no clear association between azole susceptibility and changes in expression of *MDR1* or *ERG11*. The only isolates for which there appeared

TABLE 5. Changes in azole susceptibility resulting from expressing *C. albicans ERG11* alleles in *S. cerevisiae*

Original *C. albicans* isolate	Substitution(s) in expressed *C. albicans* Erg11p	MIC[a] (μg/ml)			
		POS	ITZ	FLZ	VOR
None	None (vector alone)	0.06	0.125	4	0.016
C43	None (wild type)	0.06	0.5	16	0.125
C441	K143R	0.25	1	>256	1
C587	G464S	0.25	1	>256	1
C477	K128T, G464S, R467I	0.125	0.5	32	0.25
C530	G450E, G464S	1	1	128	1
C600	Y132H, S405F	0.5	>8	>256	16
C535	Y132H, G448V	0.25	1	>256	0.03
C572	Y132H, G450E	0.5	2	>256	8
C438	Y257H, G464S	0.5	1	128	4
C439	Y257H, G464S, G307S	1	1	>256	8
C440	Y257H, G464S, G307S, A61V	4	8	>256	16
NA[b]	A61V	0.25	0.5	16	0.125

[a] See Table 2, footnote *a*.
[b] NA, not applicable; mutation was engineered into a wild-type *ERG11* gene.

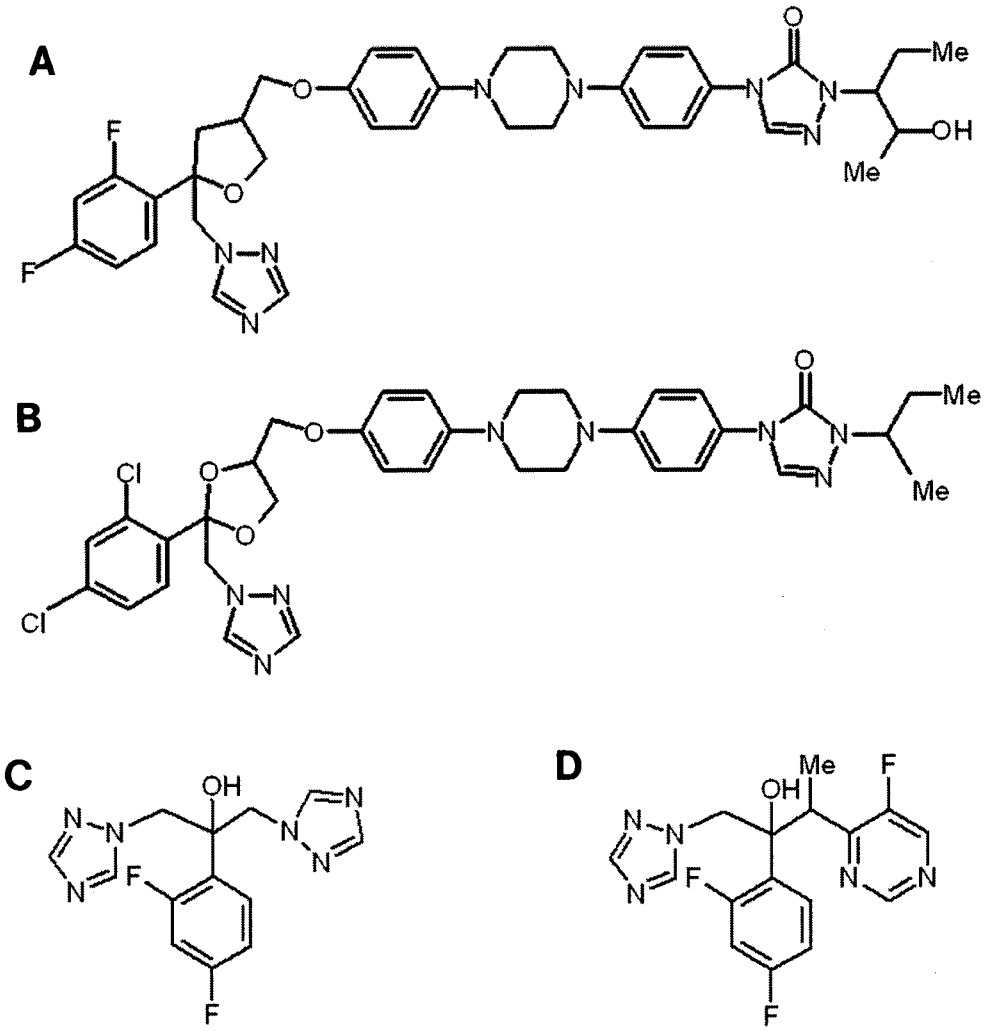

FIG. 1. Structures of posaconazole (A), itraconazole (B), voriconazole (C), and fluconazole (D).

to be a clear correlation between overexpression of *CDR2* and a change in susceptibility were from patient 3. However, there are a number of caveats to the approach taken here. For instance, since we measured gene expression in the absence of drug, we would not have identified isolates that had acquired mutations that enable them to respond more efficiently (i.e., a more rapid and possibly larger degree of overexpression) to a drug challenge. Similarly, we cannot rule out the possibility that the isolates have acquired mutations in the efflux pump genes that enable them to transport drugs more efficiently or have changed the substrate profile of the pumps.

In addition to the changes in pump expression detailed above, all of the isolates had acquired mutations in *ERG11*. The *ERG11* mutations in 28 of the 34 isolates that were either fluconazole resistant or fluconazole S-DD have all previously been associated with changes in azole susceptibility (5, 12, 13, 15). For the remaining six isolates, the mutations were clustered into two of the three hot-spot regions noted by Marichal and coworkers (5). We observed that a particular pattern of *ERG11* mutations was associated with cross-resistance to both fluconazole and voriconazole; 16 of the 17 fluconazole- or voriconazole-resistant isolates had a substitution close to the N terminus paired with a second substitution located towards the C terminus of the protein. A similar finding was reported recently (Sanglard et al., 42nd ICAAC). We confirmed that the mutations were responsible for the change in susceptibility by expressing alleles with these mutations in *S. cerevisiae*; all but one of the alleles tested conferred reduced susceptibility to fluconazole and voriconazole.

The high incidence of cross-resistance between fluconazole and voriconazole presumably results from their structural similarity (Fig. 1). Furthermore, the structural differences between the compact azoles (e.g., fluconazole and voriconazole) and those with long side chains (e.g., posaconazole and itraconazole) may explain why all but one of the 17 fluconazole- and voriconazole-resistant isolates remained susceptible to posaconazole (i.e., MIC, ≤ 1 μg/ml) and nine remained susceptible to itraconazole. To explain these differences, we constructed a three-dimensional model of Erg11p based on the X-ray structure of the Cyp51 orthologue from *Mycobacterium tuberculosis* (20). The long side chain of posaconazole and itraconazole, which is absent in fluconazole and voriconazole, is predicted to

make extensive hydrophobic interactions with Erg11p and may serve to stabilize binding of these drugs to some of the mutated forms of the protein. Consistent with our model, the A61V mutation in isolate C440, which specifically impacted the binding of posaconazole and itraconazole (as reflected in the MIC change), is predicted to interfere with binding of the long side chain (20). However, it is important to note that the A61V substitution alone does not confer significant levels of resistance to posaconazole; some or all of the additional substitutions seen in isolate C440 are required to cause the observed change in susceptibility to posaconazole (Table 5).

Interestingly, there was a marked difference in the distribution of silent mutations between the *ERG11* alleles from the sensitive and resistant populations. For example, seven isolates from the SPRI azole-susceptible population had silent mutations in the *ERG11* alleles; in six of these isolates the mutations were distributed heterogenously between the two *ERG11* alleles. In contrast, 23 of the isolates cultured from patients treated with azoles had at least two and frequently more than five silent mutations in *ERG11*. In all 23 isolates, the silent mutations were strictly conserved between the two *ERG11* alleles. We hypothesize that the absence of heterozygosity at the *ERG11* locus in the patient-derived isolates is a result of the selective pressures associated with azole treatment. More specifically, following the acquisition of an *ERG11* mutation that confers reduced susceptibility to azoles, there is presumably an additional growth advantage conferred on isolates that undergo a gene conversion event that results in introduction of the *ERG11* mutation, and thereby the linked silent mutations, into both copies of *ERG11*.

In summary, it is apparent that more than one mechanism of resistance may contribute to the overall resistance phenotype in many isolates. Other recent studies have made similar observations (11, 13, 19). It remains to be determined in what temporal order these individual mechanisms arise and to what degree each individual resistance mechanism contributes to the overall resistance phenotype. Such an analysis would require systematic elimination of each resistance mechanism by gene knockouts. At present, such manipulations are not feasible in clinical *C. albicans* isolates. The occurrence of multiple mechanisms of resistance presumably reflects both the long periods of drug exposure and the static mode of action of azoles against yeasts. From a therapeutic point of view, posaconazole appears to offer advantages over fluconazole and voriconazole, since posaconazole appears to be less affected by either mutations in *ERG11* or the overexpression of specific efflux pumps.

ACKNOWLEDGMENTS

We thank Todd Black (SPRI) for helpful comments, David Perlin and Steven Park (PHRI, Newark, N.J.) for sharing unpublished results, Michelle Treitel (SPRI) and Scott Walker (SPRI) for editing the manuscript, and Robyn Hawkinson (SPRI) for technical help.

REFERENCES

1. **Cacciapuoti, A., D. Loebenberg, E. Corcoran, F. Menzel, Jr., E. L. Moss, Jr., C. Norris, M. Michalski, K. Raynor, J. Halpern, C. Mendrick, B. Arnold, B. Antonacci, R. Parmegiani, T. Yarosh-Tomaine, G. H. Miller, and R. S. Hare.** 2000. In vitro and in vivo activities of SCH 56592 (posaconazole), a new triazole antifungal agent, against *Aspergillus* and *Candida*. Antimicrob. Agents Chemother. **44:**2017–2022.
2. **Canuto, M. M., and F. G. Rodero,** 2002. Antifungal drug resistance to azoles and polyenes. Lancet Infect. Dis. **2:**550–563.
3. **Li, X., N. Brown, A. S. Chau, J. L. López-Ribot, C. A. Mendrick, D. Loebenberg, R. S. Hare, B. J. DiDomenico, and P. M. McNicholas.** 2004. Changes in susceptibility to posaconazole (POS) in clinical isolates of *Candida albicans*. J. Antimicrob. Chemother. **53:**74–80.
4. **Lyons, C. N., and T. C. White,** 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. Antimicrob. Agents Chemother. **44:** 2296–2303.
5. **Marichal, P., L. Koymans, S. Willemsens, D. Bellens, P. Verhasselt, W. Luyten, M. Borgers, F. C. Ramaekers, F. C. Odds, and H. V. Bossche.** 1999b. Contribution of mutations in the cytochrome P450 14α-demethylase (Erg11p, Cyp51p) to azole resistance in *Candida albicans*. Microbiology **145:**2701–2713.
6. **Marr, K. A., C. N. Lyons, T. Rustad, R. A. Bowden, and T. C. White.** 1998. Rapid, transient fluconazole resistance in *Candida albicans* is associated with increased mRNA levels of *CDR*. Antimicrob. Agents Chemother. **42:**2584–2589.
7. **Meunier, F.** 1989. Candidiasis. Eur. J. Clin. Microbiol. Infect. Dis. **8:**438–447.
8. **Micheli, M., J. Bille, C. Schueller, and D. Sanglard.** 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. Mol. Microbiol. **43:**1197–1214.
9. **Morschhauser, J., S. Michel, and J. Hacker.** 1998. Expression of a chromosomally integrated, single-copy GFP gene in *Candida albicans*, and its use as a reporter of gene regulation. Mol. Gen. Genet. **257:**412–420.
10. **National Committee for Clinical Laboratory Standards.** 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. Document M27-A. National Committee for Clinical Laboratory Standards, Wayne, Pa.
11. **Nozawa, Y., and T. Morita.** 1986. Molecular mechanisms of antifungal agents associated with membrane ergosterol: dysfunction of membrane ergosterol and inhibition of ergosterol biosynthesis, p. 111–122. *In* K. Iwata and H. Vanden Bossche (ed.), In vitro and in vivo evaluation of antifungal agents. Elsevier Science Publishers, B. V., Amsterdam, The Netherlands.
12. **Perea, S., Lopez- J. L. Ribot, W. R. Kirkpatrick, R. K. McAtee, R. A. Santillan, M. Martinez, D. Calabrese, D. Sanglard, and T. F. Patterson.** 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob. Agents Chemother. **45:**2676–2684.
13. **Perea, S., J. L. Lopez-Ribot, B. L. Wickers, W. R. Kirkpatrick, R. K. McAtee, O. P. Dib, S. P. Bachmann, S. Keller, M. Martinez, and T. F. Patterson.** 2001. Molecular mechanisms of fluconazole resistance in *Candida dubliniensis* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob. Agents Chemother. **46:**1695–1703.
14. **Pfaller, M. A., S. A. Messer, R. J. Hollis, and R. N. Jones.** 2001. In vitro activities of posaconazole (SCH 56592) compared with those of itraconazole and fluconazole against 3685 clinical isolates of *Candida* spp. and *Cryptococcus neoformans*. Antimicrob. Agents Chemother. **45:**2862–2864.
15. **Sanglard, D., K. Kuchler, F. Ischer, J.-L. Pagani, M. Monod, and J. Bille.** 1998. Mechanisms of resistance to azole antifungal gents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agents Chemother. **39:**2378–2386.
16. **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.
17. **Sanglard, D., and F. C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect. Dis. **2:**73–85.
18. **Vanden Bossche, H., G. Willemsens, and P. Marichal.** 1987. Anti-Candida drugs-the biochemical basis for their activity. Crit. Rev. Microbiol. **15:**57–72.
19. **White, T. C., S. Holleman, F. Dy, F. D. Mirels, and D. A. Stevens.** 2002. Resistance mechanisms in clinical isolates of *Candida albicans*. Antimicrob. Agents Chemother. **46:**1704–1713.
20. **Xiao, L., V. Madison, A. S. Chau, D. Loebenberg, R. E. Palermo, and P. M. McNicholas.** Three dimensional models of the wild type and mutated forms of the cytochrome P450 14α-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into the binding of posaconazole. Antimicrob. Agents Chemother. **48:**568–574.