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In Vitro Activities of Terbinafine against Cutaneous Isolates of *Candida albicans* and Other Pathogenic Yeasts

NEIL S. RYDER,* SONJA WAGNER, AND INGRID LEITNER

Novartis Research Institute, A-1235 Vienna, Austria

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Terbinafine is active in vitro against a wide range of pathogenic fungi, including dermatophytes, molds, dimorphic fungi, and some yeasts, but earlier studies indicated that the drug had little activity against *Candida albicans*. In contrast, clinical studies have shown topical and oral terbinafine to be active in cutaneous candidiasis and *Candida* nail infections. In order to define the anti-*Candida* activity of terbinafine, we tested the drug against 350 fresh clinical isolates and additional strains by using a broth dilution assay standardized according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) M27-A assay. Terbinafine was found to have an MIC of 1 μg/ml for reference *C. albicans* strains. For 259 clinical isolates, the MIC at which 50% of the isolates are inhibited (MIC_{50}) of terbinafine was 1 μg/ml (fluconazole, 0.5 μg/ml), and the MIC_{90} was 4 μg/ml (fluconazole, 1 μg/ml). Terbinafine was highly active against *Candida parapsilosis* (MIC_{90}, 0.125 μg/ml) and showed potentially interesting activity against isolates of *Candida dubliniensis*, *Candida guilliermondii*, *Candida humicola*, and *Candida lusitaniae*. It was not active against the *Candida glabrata*, *Candida krusei*, and *Candida tropicalis* isolates in this assay. *Cryptococcus laurentii* and *Cryptococcus neoformans* were highly susceptible to terbinafine, with MICs of 0.06 to 0.25 μg/ml. The NCCLS macrodilution assay provides reproducible in vitro data for terbinafine against *Candida* and other yeasts. The MICs for *C. albicans* and *C. parapsilosis* are compatible with the known clinical efficacy of terbinafine in cutaneous infections, while the clinical relevance of its activities against the other species has yet to be determined.

The allylamine antimycotic terbinafine is employed both orally and topically in the therapy of fungal infections of the skin, nails, and hair. Numerous earlier reports have documented the activity of terbinafine against a wide range of pathogenic fungi in vitro, as reviewed previously (2, 25, 26). The mechanism of action of terbinafine involves the specific inhibition of fungal squalene epoxidase, resulting in ergosterol deficiency and accumulation of intracellular squalene, and appears to be identical in dermatophytes, molds, and yeasts (23). Terbinafine has extremely potent in vitro activity against dermatophytes (25), correlating well with its established clinical efficacy against these organisms (4). In the case of dermatophytes and a number of other filamentous fungi, in vitro testing of terbinafine has proved to be fairly straightforward, and consistent results have been reported by investigators using a variety of methods (25). This situation probably reflects the primary fungicidal action of terbinafine against these organisms (3, 19), which results in clear zero-growth end points in conventional determinations of MICs. In contrast, widely varying MICs have been reported for *Candida* species, and terbinafine has generally been considered to have little or no activity against *Candida albicans* yeasts in vitro, although the filamentous form is susceptible (31). The activity of the drug against *C. albicans* is primarily fungistatic (19).

Despite the unpromising in vitro data, terbinafine has proven clinical efficacy against cutaneous candidiasis with either topical or oral therapy, (8–10, 34, 37) and against *Candida* nail infections (17, 32). There is thus a discrepancy between clinical efficacy and apparent poor in vitro activity against the responsible pathogen. Earlier tests were performed with a variety of different assay media and conditions which delivered correspondingly varied results, and similar problems of compatibility with clinical data were encountered with other drugs, such as fluconazole. More recently, the National Committee for Clinical Laboratory Standards (NCCLS) has established guidelines for standardized susceptibility testing of yeasts with azoles, amphotericin B, and flucytosine in the form of the M27-A broth dilution assay (16). In order to clarify the question of the in vitro activity of terbinafine, we have investigated the applicability of the M27 assay to testing this drug against *Candida* and other yeasts. We report here the activity of terbinafine against six reference strains of *Candida* in comparison with standard drugs and the results of testing over 350 clinical isolates and other strains in comparison with fluconazole.

MATERIALS AND METHODS

Fungal isolates. Fresh isolates of *C. albicans* and other yeasts were obtained directly from clinical centers performing trials of topical formulations of terbinafine against cutaneous candidiasis. The countries of origin included the United States, Dominican Republic, Ecuador, Guatemala, Honduras, and Panama. Isolates were plated onto Sabouraud dextrose agar, and cultures were established from a single colony. The identification of the cultures was confirmed by using the API 20C kit (Biomerieux, Marcy l'Etoile, France) according to the maker's instructions. In addition, the species-selective chromogenic media CHROMagar (Chromagar Company, Paris, France) and Albicans ID Agar (Biomerieux) were used, as well as routine morphological examination. In certain cases, the identity of *C. albicans* isolates was additionally confirmed by observation of chlamydospore formation on rice agar. Cultures were grown up in liquid shake culture (Sabouraud dextrose broth, pH 6.5) for 30 h at 30°C and then stored at −80°C as cell suspensions in ampoules with 5% (vol/vol) dimethyl sulfoxide (DMSO) as cryoprotectant before testing. Reference strains (for validation of the assay) and additional test strains were purchased from the American Type Culture Collection, Rockville, Md. (ATCC numbers), and the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands (CBS numbers), or were from the Novartis collection (NFI numbers).

Antifungal drugs. Terbinafine, itraconazole, and ketoconazole were synthesized at Novartis. Fluconazole was extracted from commercial tablets of Diflucan (Pfizer). Terbinafine was used as the standard hydrochloride salt (weight correction factor, 1.12 with respect to the pure base), while the azoles were analytically

* Corresponding author. Mailing address: Novartis Research Institute, Brunner Strasse 59, A-1235 Vienna, Austria. Phone: (431) 86634-324. Fax: (431) 86634-354. E-mail: neil.ryder@pharma.novartis.com.

TABLE 1. MICs of terbinafine and comparison drugs against reference strains of *Candida* species

Organism and reference no.	MIC (μg/ml)[a]					
	Terb	Flu	Itra	Keto	AmB	5FC
C. albicans ATCC 90028	1	0.25	0.125	0.06	0.25	0.5
C. albicans ATCC 24433	1	0.5	0.06	0.06	0.125	2
C. krusei ATCC 6258	>128	32	0.06	0.125	0.5	8
C. parapsilosis ATCC 22019	0.03	2	0.06	0.03	0.5	0.25
C. parapsilosis ATCC 90018	0.03	0.5	0.03	0.01	0.25	0.06
C. tropicalis ATCC 750	>128	2	0.125	0.06	0.25	0.125

[a] Results are from three separate experiments each in duplicate (5FC from two experiments) in which the MIC did not differ by more than one dilution step. Terb, terbinafine; Flu, fluconazole; Itra, itraconazole; Keto, ketoconazole; AmB, amphotericin B.

pure. Amphotericin B, formulated as Fungizone (weight correction factor, 1.82), was purchased from Bristol-Myers Squibb GmbH, Munich, Germany. 5-Flucytosine (5FC) was obtained from Sigma Chemical Corp. (catalog #F-7129).

Assay medium. Assays were performed in RPMI 1640 medium without $NaHCO_3$ but with L-glutamine (GIBCO BRL, Paisley, Scotland) buffered with 0.165 M 3-[*N*-morpholino]propanesulfonic acid (MOPS) (Sigma M-8899). The medium was adjusted to be at pH 7.0 at 35°C, sterile filtered, aliquoted into 160- by 16-mm glass tubes (1.8 ml/tube), and stored at 4°C until used.

Antifungal testing in vitro. MICs were determined in broth macrodilution assays according to a modification of the NCCLS M27-A protocol (16). Terbinafine was first dissolved at 100-fold highest final concentration in DMSO containing 5% Tween 80, after which sequential twofold dilutions were made in DMSO followed by fivefold dilutions of each solution in RPMI medium. Dilution procedures for the other drugs were as described for the reference method (16). Inocula for assays were prepared from stocks frozen at −80°C by dilution in growth medium to give a final viable cell count of 2.5×10^3 CFU/ml. Each assay was performed with a duplicate series of drug dilutions. Drug solution (0.1 ml) and fungal inoculum (0.1 ml) were added to each tube prefilled with 1.8 ml of medium to give a total volume of 2 ml. Tubes were capped with loose-fitting stainless-steel caps, vortexed, and then incubated for 48 h (72 h for *Cryptococcus*) at 35°C in air. Two end points were recorded: the MIC of amphotericin B was defined as the lowest drug concentration causing 100% inhibition of fungal growth, while those of terbinafine, fluconazole, and other drugs were defined as the lowest drug concentrations causing at least 80% inhibition. A solvent control was included in each set of assays; the DMSO diluent at maximum final concentration of 1% had no effect on fungal growth. Each set of assays was validated by testing the reference strain ATCC 24433 in parallel and ensuring that the MIC of the standard drug fluconazole was within the NCCLS-recommended range.

Statistical analysis. Primary data were stored in Excel 5.0 tables for statistical analysis. For subsequent calculations, MICs of >128 were set to the next higher value of 256. The MICs were then converted to their log (base 2) for calculation of geometric means, and comparisons between test drugs were made by using the Student *t* test (two-tailed) with paired data.

RESULTS

Application of the NCCLS assay to terbinafine. The macrodilution assay described above was initially validated with standard drugs by using the NCCLS-recommended reference strains of *Candida*, after which terbinafine was also tested against these strains (Table 1). Values obtained with the standard drugs were in agreement with the recommended ranges (16). Using 80% inhibition of growth as the assay end point, clear and reproducible MICs were obtained of terbinafine for the *C. albicans* and *C. parapsilosis* strains, while the *C. krusei* and *C. tropicalis* strains appeared to be resistant in this assay (Table 1). Using complete growth inhibition as the end point, MICs could be obtained only for *C. parapsilosis*, consistent with the previously established fungicidal action of terbinafine against this species. Neither terbinafine nor fluconazole achieved complete growth inhibition of the other *Candida* species, consistent with a fungistatic action. The results obtained were highly reproducible between experiments. Using *C. albicans* ATCC 24433 as a reference in a series of over 60 separate sets of assays performed during an 18-month period, consistent results were obtained with MICs of terbinafine of 1 or 2 μg/ml and of fluconazole of 0.5 μg/ml.

Since our method of inoculation of the assays (using stocks maintained at −80°C) differed from that of the NCCLS reference standard (which uses freshly grown cells), a direct comparison was made of the two inocula using the six reference *Candida* strains shown in Table 1. At equivalent viable cell counts, use of fresh or frozen inocula had no effect on the MICs of terbinafine, fluconazole, and amphotericin B, the values obtained being identical to those given in Table 1. In *C. albicans*, a 10-fold variation in the viable cell count (from 0.5×10^3 to 5×10^3 CFU/ml) did cause minor variation in the MICs of terbinafine and fluconazole with both fresh and frozen inocula, as expected. The maximum observed variation in MIC was two dilution steps, and no MIC with either fresh or frozen inocula varied by more than one dilution step from the values in Table 1. A fixed inoculum of 2.5×10^3 CFU/ml was used in all other assays reported here.

Activity of terbinafine against cutaneous *C. albicans* isolates. Having established the reproducibility of the NCCLS assay with terbinafine, we used this method to test susceptibility of fresh *Candida* isolates obtained during clinical trials of topical terbinafine formulations in cutaneous candidiasis. As expected, *C. albicans* was the predominant species isolated. Fluconazole was chosen as comparator drug as there are extensive data available on the use of the NCCLS assay with this drug and its relationship to clinical efficacy. Terbinafine and fluconazole both had MICs over the full test range of 0.03 to >128 μg/ml for 259 *C. albicans* isolates, with geometric mean values of 1.4 and 0.6 μg/ml, respectively (Table 2). However, analysis of the data (Table 3) demonstrates that over 90% of MICs of both drugs were within a much narrower range of 0.25 to 4 μg/ml. For each drug, there was a small subpopulation of isolates which displayed higher MICs. In the case of fluconazole, 242 of 259 isolates had MICs of 4 μg/ml or lower, while the remaining 17 isolates had MICs of 128 μg/ml or higher. These groups of isolates are classified respectively as susceptible and fully resistant according to the NCCLS resistance breakpoints of ≤8 and >64 μg/ml (16). Clinically relevant breakpoints are currently not available for terbinafine, but a distinct group of 16 isolates with higher MICs (>8 μg/ml) was also observed (Table 3). By the same criteria, six isolates (about 2% of the total) were found to be resistant to both drugs.

Activity of terbinafine against other yeasts. Testing of the non-*C. albicans* clinical isolates (Table 2) confirmed the potent activity of terbinafine against *Candida parapsilosis*, with a MIC at which 90% of the isolates are inhibited (MIC_{90}) of 0.125 μg/ml. In contrast to the other species tested, 100% growth inhibition end points were also attained in *C. parapsilosis* by using terbinafine, with a MIC_{50} of 4 μg/ml and a range of 0.5 to >128 μg/ml ($n = 11$). In order to gain a more complete picture of the spectrum of terbinafine, additional isolates for testing were obtained from culture collections, as listed in Table 2. Terbinafine had potentially interesting activity against

TABLE 2. MICs of terbinafine and fluconazole against cutaneous fungal isolates[a]

Organism	*n*	MIC of terbinafine			MIC of fluconazole			*P*
		MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	
C. albicans[b]	259	1	4	0.03–>128	0.5	1	0.06–>128	<0.001
C. parapsilosis[b]	11	0.06	0.125	0.03–0.25	1	4	0.5–16	<0.001
C. dubliniensis[c]	2			0.125–1			0.25–32	
C. glabrata[b]	15	>128	>128	>128	32	>128	2–>128	<0.001
C. guilliermondii[b]	8	2		0.5–>128	4		0.25–>128	0.6
C. humicola[b]	1			1			4	
C. krusei[c]	4	>128		>128	32		32–64	
C. lusitaniae[c]	3	0.5		0.125–0.5	1		0.25–1	
C. tropicalis[b]	26	>128	>128	>128	>128	>128	1–>128	0.004
C. albicans var. *stellatoidea*[c]	3	0.5		0.25–>128	0.5		0.5–>64	
B. capitatus[c]	4	2		0.5–>64	16		4–>64	
C. laurentii[b]	7	0.25		0.125–0.25	2		1–4	<0.001
C. neoformans[c]	4	0.125		0.06–0.125	4		2–8	
T. beigelii[b]	5	2		0.5–>128	2		1–8	0.3

[a] Values are for *n* isolates, with *P* indicating the statistical significance of the difference between the two drugs (only calculated when $n = 5$ or more).
[b] Fresh clinical isolates from cutaneous lesions.
[c] Isolates obtained from culture collections.

isolates of several other *Candida* species, including *Candida guilliermondii*, *Candida dubliniensis*, *Candida humicola*, and *Candida lusitaniae* but showed no activity in this assay against *Candida glabrata*, *Candida krusei*, and *Candida tropicalis*. Terbinafine was highly active against both *Cryptococcus* species tested and significantly superior to fluconazole. It was also effective against some isolates of *Blastoschizomyces capitatus* and *Trichosporon beigelii.*

DISCUSSION

The NCCLS macrodilution assay appears to be suitable for testing terbinafine against *Candida* species. Because of the lipophilic nature of the drug, particular care is required in preparation of the sequential dilutions; the procedure described here provided clear drug solutions and consistent results. The MIC data for the standard fluconazole agreed well with published data, and results were highly reproducible, thus providing a basis for comprehensive testing of terbinafine against a wider range of isolates. Interestingly, the MICs of terbinafine against *C. albicans* were much lower than those obtained by earlier investigators (19, 26), who reported MIC_{50}s of around 25 μg/ml compared with 1 μg/ml in the present study. This is likely due to two characteristics of the NCCLS method. First, the readout is 80% growth inhibition, eliminating the uncertainty of trailing end points seen in *Candida* treated with dilution series of azoles or allylamines. Second, the NCCLS medium is buffered at neutral pH, while earlier methods used unbuffered media which are rapidly acidified by *Candida* species. Terbinafine is much less active at low pH (22), so that inclusion of a neutral buffer is an essential prerequisite for testing this and related drugs against yeasts. However, the new results confirm the potent activity of terbinafine against *C. parapsilosis* which had previously been noted by other investigators (19, 26).

The data for *C. albicans* provide an opportunity for analysis of antifungal resistance in "normal" isolates, which are unlikely to have had extensive previous exposure to the drugs, since AIDS or other immunocompromised patients were excluded from the clinical studies. As seen in Table 3, MICs of fluconazole formed two discrete clusters of susceptible (<8 μg/ml) or resistant (≥64 μg/ml) isolates, in accordance with NCCLS

TABLE 3. Analysis of MICs for terbinafine and fluconazole against 259 cutaneous isolates of *C. albicans*[a]

MIC of fluconazole (μg/ml)	MIC of terbinafine (μg/ml)												
	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	>32	Total
0.06	1	1		1									3
0.125				5	7	4							16
0.25			2	6	50	25	19	5				2	109
0.5				1	13	23	30	3	2			4	76
1					4	6	11	4	2	1		1	29
2						1	3	1	2			1	8
4												1	1
8													
16													
32													
64													
128						1		1			1		3
>128					1	2	3	2	1			5	14
Total	1	1	2	13	75	62	66	16	7	1	1	14	259

[a] Breakpoints for fluconazole resistance (16) are ≤8 μg/ml (susceptible), 16 to 32 μg/ml (susceptible-dose dependent), and ≥64 μg/ml (resistant). Breakpoints for terbinafine are not available, but for the purposes of discussion a breakpoint of >8 μg/ml is taken to indicate apparent in vitro resistance.

breakpoints (16), with no intermediate values. Although clinically relevant breakpoints are not available for terbinafine, a similar pattern is seen taking 8 μg/ml as a breakpoint for in vitro resistance. The two clusters of 17 fluconazole-resistant isolates and 16 terbinafine-resistant isolates partially overlap to give a group of six isolates resistant to both drugs. The nonoverlapping isolates show no correlation of MICs between the two drugs. Thus, at least two resistance mechanisms must be involved, specific for fluconazole and terbinafine, respectively, with the possibility of a third mechanism conferring resistance to both drugs. A number of mechanisms of azole resistance have been described, the most important apparently being mediated by multidrug resistance efflux transporters (1). Recently, Sanglard et al. have identified genes for several such transporters in *C. albicans* and shown that they could confer resistance to fluconazole, other azoles, and a variety of other compounds (27, 28). Three of these genes, *CDR1*, *CDR2*, and *BEN*^{r}, also conferred resistance to terbinafine, thus providing a potential mechanism for the partial fluconazole-terbinafine cross-resistance which we observed. However, since both terbinafine and azoles act on stages of the ergosterol biosynthesis pathway, other mechanisms may also be involved, and further studies are required to settle this question. Acquired resistance to terbinafine has never been reported, but the drug has been little used against *Candida* infections. Differential constitutive expression of multidrug resistance transporters may also explain the considerable variation in susceptibility of different *Candida* species to terbinafine as well as to fluconazole. The target enzyme of terbinafine, squalene epoxidase, was previously shown to differ in sensitivity to the drug by at most a factor of 10 between *C. albicans*, *C. parapsilosis*, and *C. glabrata* (22, 24), which does not explain the >1,000-fold differences in MICs for these species. Fluconazole is also inherently inactive against *C. krusei* (20) and frequently displays high MICs against *C. glabrata* and *C. tropicalis* (12, 21) as seen in the present study. Low intracellular accumulation of the drug has previously been implicated in the resistance of *C. krusei* to fluconazole (13). The critical role of efflux transporters in determining drug susceptibility in fungi as well as other pathogens has only recently become apparent (11), and additional studies are clearly needed to elucidate the mechanisms involved with respect to terbinafine and other commonly used antifungals.

C. albicans is the predominant causative agent of candidiasis of either the skin or mucosal surfaces (18). However, *C. parapsilosis* was found to be the most frequently isolated yeast in the subungual space of the hand in healthy subjects (14) and has been associated with around 50% of *Candida* nail infections and mixed yeast-dermatophyte infections (17, 36). *C. parapsilosis* is also regarded as an important emerging nosocomial pathogen (6, 36). The in vitro activity of terbinafine against these two organisms is thus of interest with regard to potential clinical efficacy. Topically applied terbinafine was found to be highly effective in cutaneous candidiasis (8, 10, 37), but the high drug levels attained with topical therapy may render differences in MIC irrelevant. Oral terbinafine has also been reported to show efficacy in cutaneous candidiasis (9, 34) and in *Candida* nail infections (17, 32). After oral administration (250 mg/day), terbinafine attains peak levels of up to 12 μg/g in the stratum corneum (5), which is well above the MIC for most *C. albicans* isolates. In nails, peak levels are around 0.5 to 1.5 μg/g (5, 30), which covers the MIC of only 50% of *C. albicans* isolates but all of *C. parapsilosis*. Interestingly, in both of the studies cited above, nail infections due to *C. parapsilosis* responded significantly better than those involving *C. albicans*. This difference is presumably a reflection of the greater in vitro susceptibility of *C. parapsilosis* to terbinafine, as found in the present study.

The mean MICs obtained of terbinafine against clinical isolates of *C. albicans* (Table 2) were similar to those found by using the laboratory reference strains (Table 1), although the clinical isolates showed a broader range of values (Table 3). However, the results obtained with these cutaneous isolates are not necessarily predictive for isolates from other sites of infection, such as mucosal or systemic candidiasis. Oral terbinafine (250 mg/day) was not effective in a pilot study against AIDS-associated oral candidiasis (15), but it is not yet known whether this lack of efficacy is due to pharmacokinetic factors or to low susceptibility of the pathogens. On the other hand, a systemic *Candida* infection was reported to respond to treatment with higher doses of terbinafine (35).

Regarding the remaining *Candida* species tested, too little clinical experience is available to draw any conclusions with respect to correlation of in vitro and clinical data. The high MICs obtained for *C. glabrata* and *C. tropicalis* stand in contrast to the reported clinical efficacy of terbinafine in a small number of infections caused by these agents (32, 34). The significant activities against *C. dubliniensis*, *C. humicola*, and *C. lusitaniae* have not been previously reported for this drug. Terbinafine was found to be highly active against *Cryptococcus* species, confirming earlier reports using different assay conditions (7, 33), and suggesting that the drug might be clinically useful against these organisms, which are occasionally involved in skin disease as well as causing serious systemic infections. Oral terbinafine (250 mg/day) was recently reported to have cured a cutaneous *Cryptococcus* lesion which was resistant to treatment with fluconazole and itraconazole (29). The activities against isolates of *T. beigelii*, the agent of white piedra, and the opportunistic pathogen *B. capitatus* are also potentially of clinical interest.

In conclusion, the M27 macrodilution assay, which is widely used for testing of other antimycotics, has also been found to be suitable for application with terbinafine. For routine testing, a 96-well microdilution assay would be more convenient, and we are attempting to optimize this technique for agreement with the macro method when testing with terbinafine. By using the NCCLS assay, we obtained highly reproducible MICs for terbinafine, which were compatible with the known clinical efficacy of the drug against cutaneous *Candida* infections. Furthermore, the data indicated that terbinafine is active against a range of other pathogenic yeasts and may therefore have clinical applications against some of these organisms.

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