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Antifungal Activity of the Allylamine Derivative Terbinafine In Vitro

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Terbinafine, an allylamine derivative, represents the most effective of this new chemical class of antimycotic compounds. Under in vitro conditions, terbinafine proved to be highly active against dermatophytes (MIC range, 0.001 to 0.01 μg/ml), aspergilli (MIC range, 0.05 to 1.56 μg/ml), and *Sporothrix schenckii* (MIC range, 0.1 to 0.4 μg/ml) and also exerted good activity against yeasts (MIC range, 0.1 to >100 μg/ml). The growth of *Malassezia furfur* was inhibited also (MIC range, 0.2 to 0.8 μg/ml). Terbinafine displays a primary fungicidal action against dermatophytes, other filamentous fungi, and *S. schenckii*. The type of action against yeasts is species dependent and can be primarily fungicidal (*Candida parapsilosis*) or fungistatic (*Candida albicans*). The in vitro activity of terbinafine is pH dependent and rises with increasing pH value.

The discovery of the antifungal activity of naftifine (Exoderil), which has been shown in vitro and in vivo against a variety of medically important species of fungi (3, 6), led to intensive chemical and biological investigations of the structure-activity relationships of allylamine derivatives at the Sandoz Forschungsinstitut, Vienna, Austria (12, 13). The antifungal activities of these compounds are based on the inhibition of fungal ergosterol biosynthesis at the point of squalene epoxidation (5, 8–10).

The most active derivative of this new chemical class of antimycotics found so far is terbinafine (7, 14; G. Petranyi, J. G. Meingassner, and H. Mieth, 9th Int. Congr. Int. Soc. Hum. Anim. Mycol. (ISHAM), abstr. no. P1-27, 1985). In this report we describe the antifungal activity of this compound in vitro.

(These data were presented in part at the 13th International Congress of Chemotherapy, 28 August to 2 September 1983, Vienna, Austria [Petranyi et al., session 116, p. 9–12].)

MATERIALS AND METHODS

Antimicrobial agents. Terbinafine (SF 86-327; lot 80 901) was synthesized at Sandoz Forschungsinstitut, Vienna, Austria. The following reference compounds were kindly made available by the manufacturing companies: clotrimazole (lot Pt 0782 A; Bayer A.G., Wuppertal-Elberfeld, Federal Republic of Germany), miconazole (lot E 25/1; Janssen Pharmaceutica, Beerse, Belgium), econazole (lot 780766; Cilag-Chemie, Schaffhausen, Switzerland), ketoconazole (lot C 4701; Janssen Pharmaceutica), tolnaftate (lot EL-75; H. Lundbeck u. Co., Copenhagen, Denmark), amphotericin B (lot 6C448; Squibb, von Heyden, Munich, Federal Republic of Germany), and griseofulvin (lot 15819/9; Biochemie, Kundl, Austria). Solutions of the test compounds were prepared with 10% dimethyl sulfoxide for dilution tests or with ethanol for loading filter paper disks. Doubling dilutions of 0.2 or 0.1% stock solutions were performed in assay medium to obtain final concentrations of 0.0008 to 100 μg/ml.

Test organisms. The fungal strains used either were obtained from the American Type Culture Collection, Rockville, Md., and Centraalbureau voor Schimmelcultures, Baarn, The Netherlands, or were clinical isolates from the Hygiene-Institut, Würzburg, Federal Republic of Germany; II. Universitäts-Hautklinik, Vienna, Austria; II. Universitäts-Frauenklinik, Vienna, Austria; Universitäts-Hautklinik, Munich, Federal Republic of Germany; and Veterinärmedizinische Universität, Vienna, Austria.

Fungal stock cultures. With the exception of *Malassezia furfur*, yeast blastospores were obtained from approximately 30-h-old shaken cultures incubated at 37°C in yeast nitrogen base (Difco Laboratories, Detroit, Mich.). Filamentous fungi were harvested with a spatula from 21-day-old cultures grown on Kimmig agar (E. Merck AG, Darmstadt, Federal Republic of Germany) at 30°C. This macerate was then finely suspended in Sabouraud dextrose 2% broth (E. Merck), using a glass homogenizer. Dimethyl sulfoxide was added to the fungal suspensions as a cryoprotective agent in final concentrations of 5%, which were then stored as 1.5-ml portions in liquid nitrogen (2). The CFU of the stock cultures were determined after freezing. Appropriate dilutions were made with saline or broth before use.

Malassezia strains were cultivated at 37°C in broth media by the method of Weary and Graham (15) by serial transfers.

Assessment of antifungal activity. Serial dilution tests were used to determine the MICs of the test compounds for dermatophytes, molds, biphasic fungi, and yeasts. The MIC for *Malassezia* strains was evaluated by an agar dilution test. Activity against dermatophytes was assessed by an agar diffusion test also. Since it was found in radiolabeling studies that terbinafine tends to stick to plasticware, the assays were performed in glass.

Serial dilution test. The MIC for filamentous fungi was determined in Sabouraud dextrose 2% broth (initial pH, 6.5). Malt extract broth (initial pH, 6.5; E. Merck) was used for yeasts.

Glass tubes (16 by 160 mm) were filled with 0.1 ml of drug solution, 1.8 ml of broth medium, and 0.1 ml of fungal suspension, yielding a final concentration of 10^3 CFU/ml. Control tubes contained 0.1 ml of drug vehicle instead of the test compound.

The tubes were incubated for 2 (yeasts), 3 (molds), or 7 days (*Sporothrix schenckii* and dermatophytes) at 30 or 37°C (yeasts) before being read. The MIC was defined as the lowest concentration at which neither turbidity nor sediment formation was detected. Vigorous fungal growth always occurred in the control tubes.

Each serial dilution test was performed in triplicate. The MICs reported represent the results of at least two replications.

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TABLE 1. MICs of terbinafine, clotrimazole, and econazole against clinical isolates of *C. albicans* and *C. parapsilosis* by serial dilution test

Species (no. of isolates)	Antifungal agent	MIC (μg/ml)		
		50%[a]	95%[a]	Range
C. albicans (78)	Terbinafine	25	50	6.25–100
	Clotrimazole	3.13	6.25	1.56–100
	Econazole	6.25	12.5	1.56–12.5
C. parapsilosis (20)	Terbinafine	0.283	2.919	0.1–3.13
	Clotrimazole	0.141	0.8	0.05–1.56
	Econazole	3.13	5.832	0.4–6.25

[a] Statistical evaluation according to Friedman (10a).

The influence of inoculation size and initial pH on MICs was determined analogously, using cell concentrations of 10^1 to 10^6 CFU/ml and a pH range of 3.5 to 7.5.

Agar dilution test. For *M. furfur*, the agar dilution test was performed with medium C (initial pH, 6.0), as described by Faergemann and Bernander (1).

Agar dilution was done in 9-cm-diameter petri dishes which had been filled with 20 ml of liquified assay medium and containing 2 ml of drug solution. One plate was used for each concentration. All plates were prepared the day before the experiment and stored at +4°C. On the following day the plates were inoculated with a Steer replicator (Production Products, Providence, R.I.), dipped into a diluted stock culture suspension of 85% turbidity (535 nm, 1-cm path length). Three stamps per test strain were used. Growth controls were made on plates containing the corresponding maximum quantity of solvent. The plates were read after an incubation period of 5 days at 37°C. The MIC was defined as the highest dilution step at which no fungal growth was seen at the sites of inoculation. Fungal growth was always visible in the control plates.

Agar diffusion test. Agar diffusion tests were done in 9-cm-diameter petri dishes on Kimmig agar, malt extract agar, yeast morphology agar, or Sabouraud dextrose 2% agar, using 9-mm-diameter paper disks (Schleicher & Schuell, Inc., Keene, N.H.). The dissolved test compound was loaded onto the disks at concentrations of 0.01 and 0.1 μg per disk. The assay was performed with *Trichophyton mentagrophytes* strain Δ158 with an inoculum of about 70 CFU/cm^2. Inhibition zone diameters were measured with sliding calipers after an incubation time of 7 days at room temperature (23°C).

Determination of type of activity. Fungicidal or fungistatic activities were tested by using unshaken Sabouraud dextrose 2% broth (pH 6.5) inoculated with 10^3 blastospores of *Candida albicans* and *Candida parapsilosis* or 10^3 CFU of *Aspergillus fumigatus*, *Scopulariopsis brevicaulis*, *S. schenckii*, *T. mentagrophytes*, and *Microsporum canis* per ml. Terbinafine was added at concentrations one and five times the MIC for the test strains. After organisms and compounds had been added to the flasks, subcultivation of *C. albicans* and *C. parapsilosis* was made on Sabouraud dextrose 2% agar after 4, 8, 24, 30, and 48 h, and subcultivation of *A. fumigatus*, *Scopulariopsis brevicaulis*, and *S. schenckii* was done after 24 h and once daily for 7 consecutive days. Subcultivation of *T. mentagrophytes* and *Microsporum canis* was made on Kimmig agar after 24 h and once daily for 7 consecutive days after organisms and compounds had been added to the flasks. All of the plate cultures were incubated at 30°C and 60% relative humidity.

TABLE 2. MICs of terbinafine, clotrimazole, and ketoconazole for *M. furfur* by agar dilution test

Strain	MIC (μg/ml)		
	Terbinafine	Clotrimazole	Ketoconazole
Δ22	0.8	12.5	0.1
Δ23	0.2	1.6	0.05
Δ24	0.8	12.5	0.1
Δ25	0.2	1.6	0.05
Δ46	0.8	6.3	0.1
Δ47	0.8	6.3	0.1

RESULTS AND DISCUSSION

The test results obtained in the serial dilution test for the various genera, species, and strains of fungi are presented in Tables 1 to 4. These findings demonstrate that terbinafine possesses excellent activity against dermatophytes, *A. fumigatus*, and *S. schenckii*.

In general, the most striking activity is displayed against dermatophytes of the genera *Trichophyton*, *Microsporum*, and *Epidermophyton* (MIC range, 0.0015 to 0.01 μg/ml) followed by *A. fumigatus* (MIC range, 0.05 to 1.56 μg/ml) and *S. schenckii* (MIC range, 0.1 to 0.4 μg/ml). This high activity of terbinafine against dermatophytes, molds, and biphasic fungi in vitro has also been shown by Clayton (Y. M. Clayton, Proc. 13th Int. Congr. Chemother., p. 13–14, section 116, 1983; Y. M. Clayton, 9th Int. Congr. ISHAM, abstr. no. P2–41, 1985), Shadomy et al. (11), and Goudard et al. (4; M. Goudard, P. Regli, Y. Buffard, and B. Gabriel, Pathol. Biol., in press).

The susceptibility of the yeasts to terbinafine was examined by determining the MICs for freshly isolated medically important yeasts such as *C. albicans* and *C. parapsilosis* for which MICs for 50% of the strains (MIC_{50}s) proved to be 25 and 0.28 μg/ml, respectively (Table 1). However, both azoles, clotrimazole and econazole, with MIC_{50}s of 3.13 and 6.25 μg/ml, respectively, were more active than terbinafine against *C. albicans*.

The growth of *M. furfur* was inhibited by terbinafine also (Table 2). The activity of terbinafine was inferior to that of ketoconazole but superior to that of clotrimazole in the assay used.

Terbinafine was effective against *A. fumigatus*, with a calculated MIC_{50} of 0.8 μg/ml, and *S. schenckii*, with a calculated MIC_{50} of 0.4 μg/ml (Table 3). These values are comparable or superior to the results obtained with the standards clotrimazole, miconazole, econazole, ketoconazole, and amphotericin B (Table 3).

The outstanding efficacy of terbinafine against derma-

TABLE 3. MIC_{50}[a] of terbinafine based on MICs obtained for *A. fumigatus* and *S. schenckii* (six strains each) and compared with standard antimycotic agents

Compound	MIC_{50}, μg/ml (range) for:	
	A. fumigatus	*S. schenckii*
Terbinafine	0.8 (0.05–1.56)	0.4 (0.1–0.4)
Clotrimazole	1.11 (0.1–1.56)	>100
		>100
Miconazole	1.56 (0.4–3.13)	0.28 (0.2–0.8)
Econazole	0.28 (0.1–0.8)	0.56 (0.4–0.8)
Ketoconazole	17.6 (0.8–25.0)	1.56 (0.4–3.13)
Amphotericin B	1.56 (0.8–1.56)	50.0 (12.5–>100)

[a] Statistical evaluation according to Friedman.

TABLE 4. Calculated MIC_{50}s[a] and MIC_{95}s[a] of terbinafine and reference compounds based on MICs obtained for 112 clinical isolates of dermatophytes

Compound	Concn (μg/ml) for:				
	T. rubrum (24)[b]	*T. mentagrophytes* (24)	*T. verrucosum* (16)	*E. floccosum* (24)	*Microsporum canis* (24)
Terbinafine					
MIC_{50}	0.006	0.003	0.003	0.003	0.006
MIC_{95}	0.006	0.006	0.006	0.006	0.01
Range	0.0015–0.006	0.003–0.006	0.0015–0.006	0.0015–0.006	0.006–0.01
Tolnaftate					
MIC_{50}	0.05	0.1	0.006	0.05	0.05
MIC_{95}	0.1	0.283	0.02	0.141	0.1
Range	0.01–0.1	0.01–0.8	0.003–0.02	0.02–0.2	0.02–0.1
Clotrimazole					
MIC_{50}	0.4	0.141	0.4	0.2	0.1
MIC_{95}	0.8	0.8	0.4	0.4	0.2
Range	0.1–1.56	0.05–1.56	0.2–0.4	0.1–0.4	0.1–0.4
Econazole					
MIC_{50}	0.05	0.141	0.2	0.02	0.1
MIC_{95}	0.2	0.566	0.4	0.071	0.1
Range	0.01–0.4	0.01–0.8	0.1–0.4	0.01–0.1	0.05–0.2
Griseofulvin					
MIC_{50}	6.25	6.25	3.13	3.13	3.13
MIC_{95}	6.25	12.5	15.39	6.25	6.25
Range	3.13–12.5	3.13–12.5	0.8–25	0.8–6.25	3.13–6.25
Ketoconazole					
MIC_{50}	1.56	6.25	6.25	0.2	6.25
MIC_{95}	4.423	17.678	7.695	0.8	12.5
Range	0.4–6.25	0.05–25	3.13–12.5	0.2–0.8	3.13–12.5

[a] Statistical evaluation according to Friedman.
[b] The number of strains is shown in parentheses.

tophytes was confirmed by determining the MICs for 112 clinical isolates of the following species: *Trichophyton rubrum*, *T. mentagrophytes*, *Trichophyton verrucosum*, *Epidermophyton floccosum*, and *Microsporum canis* (Table 4). A comparison carried out on the basis of a calculated MIC_{50} revealed that terbinafine is significantly more active in vitro than the reference compounds tolnaftate, clotrimazole, econazole, griseofulvin, and ketoconazole.

The activity of terbinafine was also confirmed in an agar diffusion assay, using various media (Table 5). With the test organism used (*T. mentagrophytes*, which was susceptible to terbinafine), at a MIC of 0.006 μg/ml determined in the serial dilution test, terbinafine produced the largest inhibition zones on Sabouraud dextrose agar. On this medium terbinafine at 0.01 and 0.1 μg/ml caused inhibition zones of 16 to 40 mm in diameter, whereas on malt extract, Kimmig, and yeast morphology agars, the same concentrations led to inhibition zones of 11 to 35 mm, 13 to 31 mm, and 14 to 34 mm in diameter, respectively.

The uniformly high activity of terbinafine against dermatophytes indicates a high specificity, which is underlined by the primary fungicidal activity against these organisms (Fig. 1). The compound is also primarily fungicidal against molds such as *A. fumigatus* and *Scopulariopsis brevicaulis* as well as the biphasic fungus *S. schenckii*. The compound is also fungicidal for *Histoplasma capsulatum* and *Blastomyces dermatitidis*, as shown by Shadomy et al. (11).

The type of activity against yeasts depends on the species,

TABLE 5. Agar diffusion tests with terbinafine in different nutritive media (*T. mentagrophytes*)

Test substance (μg per disk)	Inhibition zone (mm)			
	Sabouraud dextrose agar (pH 5.5)	Malt extract agar (pH 6.5)	Kimmig agar (pH 6.5)	Yeast morphology agar (pH 6.5)
0.01	16	11	13	14
0.1	40	35	31	34

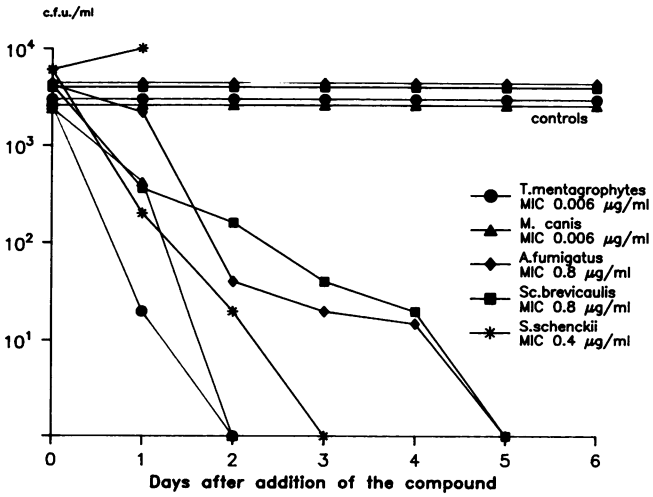

FIG. 1. Fungicidal activity of terbinafine against *T. mentagrophytes*, *Microsporum canis*, *A. fumigatus*, *Scopulariopsis brevicaulis*, and *S. schenckii*.

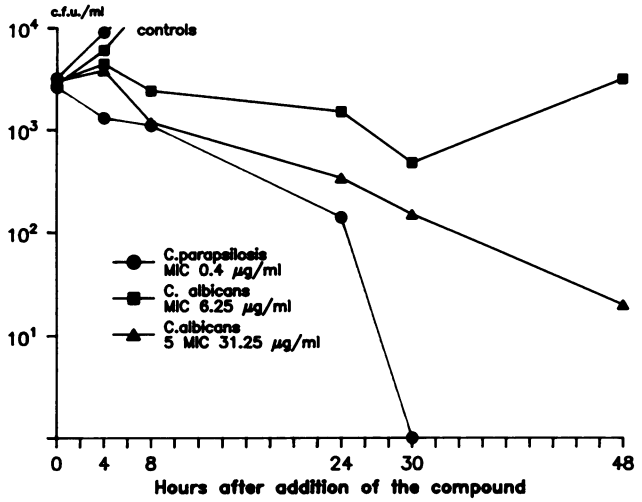

FIG. 2. Fungistatic and fungicidal activity of terbinafine against *C. albicans* and *C. parapsilosis*, respectively.

being, for example, fungicidal against *C. parapsilosis* and fungistatic against *C. albicans* (Fig. 2).

The in vitro activity of terbinafine is pH dependent and increases with initial pH. Experiments with *T. mentagrophytes* and *Microsporum canis* revealed, at pH 4.5, MICs of 0.05 and 0.012 μg/ml, respectively; at pH 6.5, 0.006 and 0.003 μg/ml, respectively; and at pH 7.0, an MIC of 0.003 μg/ml.

Determination of the MIC at cell densities ranging from 10^1 to 10^5 CFU of *T. mentagrophytes* and *Microsporum canis* per ml showed an increase in the MICs from 0.0015 (*T. mentagrophytes*) and 0.006 (*Microsporum canis*) μg/ml, respectively, at 10^1 CFU/ml to 0.012 μg/ml in both strains at 10^5 CFU/ml.

The in vitro data presented for terbinafine in this study indicate a very interesting profile of activity. The broad antifungal spectrum and its primary fungicidal action, particularly against filamentous fungi, stimulated further investigations with this new allylamine derivative in vivo, in experimental animals, and subsequently in the clinic.

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