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In Vitro Analysis of the Ability of *Trichophyton rubrum* To Become Resistant to Terbinafine

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In this study, we have investigated in vitro the resistance frequency and development of resistance to terbinafine of *Trichophyton rubrum*. Results demonstrated that naturally occurring mutants are rare and that *T. rubrum* appears to have little capacity to develop resistance to terbinafine even after prolonged exposure.

Dermatophytosis is a common disease which can affect a large proportion of the population (12). The main causative agent of skin and especially nail infections is *Trichophyton rubrum* (3, 8). Terbinafine is highly effective in treating fungal infections (6), but despite extensive use of the drug, reports of *T. rubrum* isolates resistant to terbinafine are rare (9). In order to better understand the reasons for this rarity, we have investigated in vitro how frequently spontaneous terbinafine-resistant *T. rubrum* mutants occur and to what extent this dermatophyte is able to develop resistance to increasing concentrations of terbinafine during extended periods of exposure.

Strains tested were from the Novartis Fungal Index (NFI) collection. To prepare stock inocula, cultures were grown on potato dextrose agar (PDA) (Merck, Whitehouse Station, N.J.) at 30°C for 1 to 5 weeks. The conidia and mycelia were then harvested, dispersed in Sabouraud 2% dextrose broth (Merck), and stored at −80°C after the addition of 5% (vol/vol) dimethyl sulfoxide as cryoprotectant. The numbers of CFU in these stock inocula were then determined, after rapid thawing, by spreading 50 μl from 10-fold serial dilutions in a physiological saline solution onto PDA plates and counting the colonies after incubation for 1 week at 30°C.

The minimum fungicidal concentrations (MFCs) of terbinafine were ≤0.06 μg/ml for the tested *T. rubrum* strains, and resistance frequencies were determined on PDA plates containing terbinafine HCl (Novartis, Basel, Switzerland) at this MFC level. A total of about 10^9 CFU of each *T. rubrum* strain was plated and incubated at 30°C for 3 weeks, and colonies were then counted. The resistance frequency was calculated by dividing the number of colonies grown on PDA medium containing 0.06 μg of terbinafine/ml by the total number of CFU spread on these plates. To estimate the level of resistance, each colony grown at a terbinafine concentration of 0.06 μg/ml was transferred to a PDA plate containing 0.5 μg of terbinafine/ml. Growth was checked after incubation for 1 week at 30°C. For the seven strains tested, resistance frequencies did not exceed 5×10^{-9} (Table 1). To our knowledge, this experiment is the first of this type with dermatophytes, although some similar studies have been carried out with *Aspergillus* and yeasts. For example, *Aspergillus fumigatus* was shown to have a resistance frequency of 10^{-7} when cultured on plates containing 32 μg of miconazole/ml (7). *Candida glabrata* exposed to high concentrations of fluconazole and miconazole developed resistance at a frequency of about 3×10^{-4} and 3.3×10^{-5}, respectively (2, 11). In contrast, *Candida albicans* starting with 10^7 cells failed to produce resistant mutants to miconazole (2). The frequency of resistance found in *T. rubrum* to terbinafine compares favorably with these few published values, and this low frequency is compatible with resistance based on a single genetic mutation. Several antifungal drugs were then tested to determine their MICs for the isolated resistant colonies (Table 2). The MICs of a range of antifungal drugs were determined with 96-well flat-bottom assay plates with a slight modification of NCCLS microdilution procedure M38-A (4, 10). In addition to terbinafine, other drugs tested were naftifine and itraconazole (Novartis, Basel, Switzerland), amorolfine (Roche Pharmaceuticals, Basel, Switzerland), tolciclate (Montedison, Milan, Italy), and tolnaftate and griseofulvin (Sigma Chemical Co., St. Louis, Mo.). Fluconazole was also tested after extraction and purification from commercial tablets of Diflucan (Pfizer) at Novartis, Vienna, Austria. The method of this extraction had previously been validated. All drugs were dissolved in dimethyl sulfoxide at a final concentration of 100-fold. The final con-

TABLE 1. Resistance frequency of seven *T. rubrum* strains to terbinafine

Strain	Background	Total CFU plated	No. of resistant colonies	Resistance frequency
NFI 1895	Clinical strain[a]	4.8×10^8	1	2.1×10^{-9}
NFI 5132	Onychomycosis	4.0×10^8	2	5.0×10^{-9}
NFI 5139	Tinea pedis	4.5×10^8	1	2.2×10^{-9}
NFI 5140	Tinea pedis	5.8×10^8	1	1.7×10^{-9}
NFI 5141	Tinea pedis	2.3×10^9	2	8.8×10^{-10}
NFI 5143	Onychomycosis	4.0×10^8	1	2.5×10^{-9}
NFI 5182[b]	Dermatophytosis	9.2×10^8	4	4.3×10^{-9}

[a] Exact infection type unknown. All strains were isolated from different patients; strains NFI 5139, NFI 5140, and NFI 5141 were isolated before patients were treated. The exact clinical background of the patients from which the other strains were isolated is unknown.

[b] NFI 5182 corresponds to the ATCC 18759.

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TABLE 2. Comparison of MICs of several antimycotics for *T. rubrum* wild-type strains and terbinafine-resistant mutants derived from them

Strain[b]	MIC (μg/ml)[a]							
	Ter	Naf	Toln	Tolc	Flu	Itra	Amo	Gri
NFI 5132 wt	0.002	0.03	0.002	0.016	1	0.25	0.004	0.5
NFI 5132 r	0.5	4	0.13	0.25	4	0.5	0.016	1
NFI 5140 wt	0.004	0.016	0.002	0.03	4	0.25	0.004	0.5
NFI 5140 r	16	8	4	>128	8	0.5	0.004	0.5
NFI 5141 wt	0.004	0.03	0.03	0.016	8	0.5	0.008	0.25
NFI 5141 r	4	16	2	0.5	4	0.13	0.004	0.25
NFI 5143 wt	0.004	0.016	0.002	0.03	1	0.25	0.004	0.5
NFI 5143 r	4	64	0.5	1	1	0.5	0.008	0.5
NFI 5182 wt	0.002	0.016	0.001	0.008	1	0.5	0.002	0.5
NFI 5182 r1	4	16	0.13	0.13	1	0.25	0.008	1
NFI 5182 r2	2	32	0.13	0.13	1	0.25	0.008	1

[a] Ter, terbinafine; Naf, naftifine; Toln, tolnaftate; Tolc, tolciclate; Flu, fluconazole; Itra, itraconazole; Amo, amorolfine; Gri, griseofulvin.
[b] wt, wild-type; r, terbinafine-resistant; 1 and 2 refer to different isolated colonies.

centration of CFU/ml in each assay was 5×10^3. The MIC was defined as the lowest drug concentration that caused about 75% inhibition of fungal growth by visual inspection (score of 1 on a scale of 0 to 4). Interestingly, all terbinafine-resistant mutants able to grow at a concentration of 0.5 μg/ml were also cross-resistant to the other squalene epoxidase inhibitors tested (naftifine, tolciclate, and tolnaftate) but were normally susceptible to antifungals with a different mode of action, the lanosterol 14α-demethylase inhibitors (itraconazole and fluconazole), the inhibitor of sterol Δ^{14}-reductase and sterol Δ^7-Δ^8-isomerase amorolfine, and griseofulvin, which interferes with microtubule polymerization. The same phenomenon was observed with clinical terbinafine-resistant isolates (9). These results suggest that the resistance phenotype of all these mutants is due to alterations of squalene epoxidase, and currently work is ongoing to confirm this hypothesis.

TABLE 3. Decrease in susceptibility to terbinafine of four *T. rubrum* strains during their passaging in liquid or agar medium in the presence of increasing drug concentrations

NFI strain no.	Growth in liquid RPMI 1640 medium			Growth on PDA medium		
	Passage no.	Terbinafine concn (μg/ml)	Cumulative incubation time (wk)	Passage no.	Terbinafine concn (μg/ml)	Cumulative incubation time (wk)
1895	1	0.002	5	1	0.002	5
	2	0.004	14	2	0.008	8
	3	0.008	22	3	0.016	15
	4	0.016	28	4	0.016	20
	5	0.03	32	5	0.03	25
5139	1	0.002	6	1	0.002	2
	2	0.008	11	2	0.008	6
	3	0.03	19	3	0.008	13
	4	0.03	29	4	0.016	18
	5	0.03	40	5	0.016	23
				6	0.016	29
5143	1	0.002	2	1	0.002	2
	2	0.004	6	2	0.008	6
	3	0.008	11	3	0.008	13
	4	0.008	21	4	0.016	18
	5	0.016	29	5	0.016	28
5182	1	0.002	6	1	0.002	6
	2	0.004	11	2	0.004	8
	3	0.008	19	3	0.016	15
	4	0.008	29	4	0.016	20
	5	0.008	40	5	0.016	25

The potential for induction of acquired resistance in *T. rubrum* by culture in subfungicidal concentrations of terbinafine was investigated in four strains. Resistance development was investigated both in liquid and on agar cultures. RPMI 1640 medium (Invitrogen), buffered at pH 7.0 with 0.165 M 3-[*N*-morpholino] propanesulfonic acid (Sigma) and containing 0.002 μg of terbinafine/ml, was inoculated with 5×10^3 CFU of *T. rubrum* per ml and incubated at 30°C. About 5×10^2 CFU of *T. rubrum* per ml was spread onto PDA plates, also containing 0.002 μg of terbinafine/ml and incubated at 30°C. Parallel experiments investigating other culture conditions on PDA plates showed extremely poor growth at 35°C with or without 10% CO_2. When growth was well established, the mycelium was split and transferred in duplicate to the same medium containing (i) the same concentration of terbinafine, (ii) twofold the amount of the initial concentration, and (iii) fourfold the amount of the initial concentration of terbinafine. The passaging procedure was repeated several times by systematically continuing from the highest concentration of terbinafine in which the mycelium grew. As shown in Table 3, prolonged incubation times were necessary to observe some growth at low concentrations of terbinafine, and development of reduced susceptibility was also very slow and weak, indicating that *T. rubrum* cannot easily adapt to terbinafine. This might explain the nondetection of acquired resistance by *T. rubrum* in vivo in response to treatment with terbinafine (1, 5).

Our results support the conclusions drawn from previous clinical studies and indicate that spontaneous *T. rubrum* mutants resistant to terbinafine are very rare and that prolonged exposure of the organism to terbinafine does not lead to significant loss of susceptibility.

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