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Ergosterol Biosynthesis Inhibition by the Thiocarbamate Antifungal Agents Tolnaftate and Tolciclate

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The thiocarbamate antimycotics tolnaftate and tolciclate blocked sterol biosynthesis in fungal cells and cell extracts, with accumulation of squalene. This point of action was confirmed by the direct inhibition of microsomal squalene epoxidase from *Candida albicans*. There was no inhibition of other steps in ergosterol biosynthesis. In whole *Candida* cells, ergosterol biosynthesis inhibition was not complete at drug concentrations up to 100 mg/liter, whereas full inhibition occurred in a cell-free test system. Rat liver cell-free cholesterol biosynthesis was much less sensitive to the drugs. The biochemical action of tolnaftate and tolciclate is thus similar to that of the allylamine antimycotics naftifine and terbinafine.

Many antifungal compounds have been shown to act as ergosterol biosynthesis inhibitors, including the azoles which inhibit lanosterol 14-demethylation (13, 14) and the allylamines which inhibit squalene epoxidase (5, 6, 10). The thiocarbamate drugs tolnaftate (4) and tolciclate (3, 3a) are selectively active against dermatophytes (2, 15) and are used clinically as a topical treatment for such infections. Their mode of action has not been reported, but ultrastructural studies suggested that it involves a disturbance of cell wall biosynthesis (1, 16). While testing a range of compounds as potential ergosterol biosynthesis inhibitors, we discovered that tolnaftate and tolciclate act on this pathway. In the present report we describe the inhibitory effects of these compounds in comparison with the allylamine antimycotic terbinafine (SF 86-327) which we have previously characterized (7, 10) as a squalene epoxidase inhibitor.

MATERIALS AND METHODS

Inhibitors. Tolnaftate (*O*-2-naphthyl-*m,N*-dimethylthiocarbanilate) was a gift from H. Lundbeck Co., Copenhagen, Denmark. Tolciclate [*O*-(1,2,3,4-tetrahydro-1,4-methanonaphthalen-6-yl)-*m,N*-dimethylthiocarbanilate] was donated by Montedison Pharmaceutical, Milan, Italy. Terbinafine [(*E*)-*N*(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalenemethanamine] was synthesized at Sandoz Forschungsinstitut by A. Stütz (12).

Fungal cultures. The fungal strains used were *Candida albicans* 63 (a clinical isolate), *Candida parapsilosis* ATCC 46589, and *Trichophyton mentagrophytes* CBS 56066. Cultures were grown and maintained as described previously (7, 11).

Measurement of sterol biosynthesis. Ergosterol biosynthesis in fungal cells was measured as described previously (7, 11). Briefly, cells were incubated for 2 h with [U-^{14}C]acetate at pH 6.5, the nonsaponifiable lipids were extracted and separated by thin-layer chromatography, and the fractions were counted for radioactivity. Sterol biosynthesis was also measured in whole cells by incorporation of radioactivity from L-[*methyl*-^{14}C]methionine into the sterol side chain as described previously (8). Sterol biosynthesis in cell extracts of *C. albicans* was measured by incorporation of [2-^{14}C]mevalonate (7, 11). A similar system was used to measure cholesterol biosynthesis in rat liver cell extracts (7). Squalene epoxidase (EC 1.14.99.7) was assayed with microsomal preparations from *C. albicans* in the presence of soluble cytoplasm as described by Ryder and Dupont (9, 10). All experiments were performed with triplicate incubations (with standard deviations routinely less than 10% of the mean) and were repeated to obtain consistent results.

RESULTS AND DISCUSSION

In preliminary tests, tolnaftate inhibited sterol biosynthesis in *C. albicans* cell extracts. Autoradiography of the labeled nonsaponifiable lipids showed that radioactivity accumulated in squalene, a pattern previously observed with the allylamines naftifine (11) and terbinafine (7). In *T. mentagrophytes* cells, tolnaftate caused a dose-dependent inhibition of [^{14}C]acetate incorporation into sterols and an accumulation of labeled squalene (Table 1). The lack of accumulation of radioactivity in the 4-methylsterols in treated cells (Table 1) tends to rule out inhibition of steps subsequent to squalene epoxidation, at least at drug concentrations up to 0.1 mg/liter, at which point essentially complete blockade of squalene epoxidation occurred. There was no evidence for inhibition of earlier steps in the sterol pathway; indeed, tolnaftate caused some stimulation of acetate incorporation into the total nonsaponifiable lipids (Table 1). This effect was also reported in the case of the allylamines which, however, caused no direct stimulation of earlier enzymes in the pathway (7). Results with tolciclate were very similar to those described for tolnaftate.

Tolnaftate and tolciclate also inhibited ergosterol biosynthesis in *C. albicans* cells (Fig. 1). They caused slight stimulation of incorporation into nonsaponifiable lipids and accumulation of radioactivity in squalene only, in a fashion similar to that shown in Table 1. In *C. albicans*, the two thiocarbamates were considerably less active than terbinafine and gave only incomplete inhibition even at concentrations of 100 mg/liter (Fig. 1). Very similar results were obtained with *C. parapsilosis* cells.

We have previously characterized the microsomal squalene epoxidase from *C. albicans* (9) and shown that this

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TABLE 1. Effect of tolnaftate on incorporation of [^{14}C]acetate into sterols and squalene in *T. mentagrophytes* cells

Tolnaftate (mg/liter)	Total incorporation[a] (dpm)	% Total radioactivity incorporated by:			
		Ergosterol	4α-Methylsterol	4,4-Dimethylsterol	Squalene
0 (control)	148.8 ± 1.0	86.4	2.4	8.6	2.6
0.003	175.4 ± 15.1	47.2	1.0	4.0	47.8
0.01	202.8 ± 20.2	19.1	0.6	2.3	78.0
0.03	186.0 ± 8.8	7.0	0.4	1.4	91.3
0.10	179.2 ± 7.5	1.9	0.2	1.1	96.7

[a] Total incorporation into nonsaponifiable lipids given as mean ± standard deviation for triplicate incubations.

enzyme is the primary target of the allylamine antimycotics (10). Tolnaftate and tolciclate were both effective inhibitors of the epoxidase, although somewhat less active than terbinafine (Fig. 2). As with the allylamines (6, 7), tolnaftate and tolciclate up to 10 mg/liter had no detectable effect on rat liver cell-free cholesterol biosynthesis. In rat liver, at 100 mg/liter, tolnaftate and tolciclate caused 58.9 and 71.1% inhibition, respectively. This selectivity is further evidence for a significant difference between the fungal and mammalian squalene epoxidase enzymes, as previously indicated in the case of the allylamines (10).

Table 2 summarizes the quantitative results obtained with tolnaftate and tolciclate in comparison with terbinafine. In the case of the dermatophyte *T. mentagrophytes*, good correlation between inhibition of growth and inhibition of ergosterol biosynthesis has previously been demonstrated for terbinafine (7) and naftifine (6). Similarly, the high biochemical activity of the two thiocarbamates is consistent with their reported efficacy against dermatophyte fungi both in vitro and in vivo (1, 2, 15, 16). Furthermore, the potency of ergosterol biosynthesis inhibition correlates with the relative antifungal activities of the compounds, in the order terbinafine > tolciclate > tolnaftate. In *C. albicans*, tolnaftate and tolciclate were clearly less effective inhibitors of sterol biosynthesis in whole cells than in the cell-free test systems (Table 2), suggesting that they do not readily penetrate the *Candida* cell envelope. This may explain the lack of antifungal activity of these compounds against *C. albicans* and other yeasts (2, 15).

The effect on *C. albicans* cells was further investigated by measuring ergosterol biosynthesis toward the end of the pathway, using the sterol side chain methylation reaction (8). Cells treated with tolnaftate showed a considerable degree of residual sterol biosynthesis (Table 3) as previously found with the allylamines, a phenomenon caused by methylation of endogenous sterol precursors (15). The uniform activity at drug concentrations up to 100 mg/liter indicates the absence of any inhibition of steps distal to the primary block at squalene epoxidase. Parallel incubations with acetate as the substrate (Table 3) demonstrated the incomplete inhibition (also seen in Fig. 1). Measured by acetate incorporation, the compound was apparently less active at pH 4.5 than at pH 6.5. With methionine as the substrate this difference was not seen, presumably obscured by the high level of endogenous methylation, which is independent of external pH. Similar results were obtained with tolciclate. Tolciclate was reported to have some activity against *C. albicans* at low pH (MIC of 60 mg/liter at pH 4.33 [1]). This may indicate a different mode of action of the drug at high concentrations and low pH.

The good correlation between antifungal activity and ergosterol biosynthesis inhibition strongly suggests that this is the primary mode of action of the thiocarbamates. However, the existence of other mechanisms cannot be ruled out.

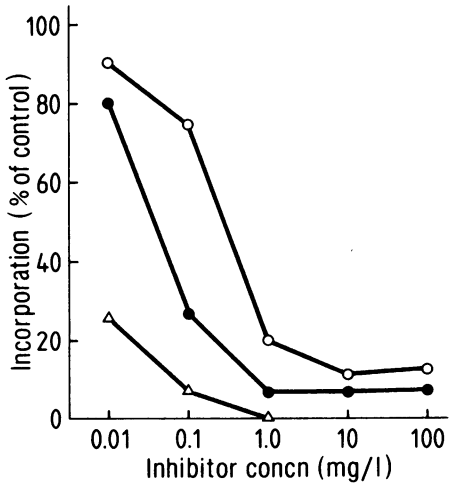

FIG. 1. Inhibition of ergosterol biosynthesis in *C. albicans* cells treated with tolnaftate (○), tolciclate (●), or terbinafine (△). Ergosterol biosynthesis was measured by incorporation of acetate as described in Materials and Methods.

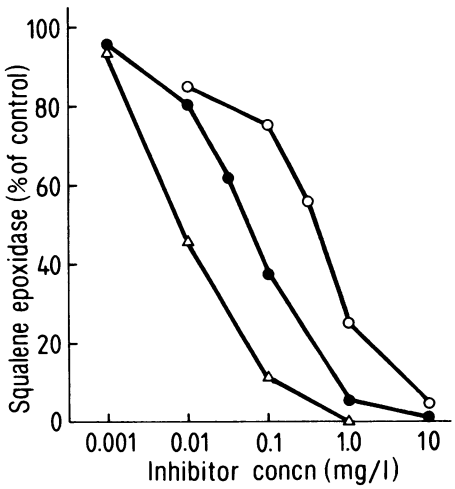

FIG. 2. Inhibition of squalene epoxidase by tolnaftate (○), tolciclate (●), and terbinafine (△). The microsomal enzyme preparations from *C. albicans* were assayed as described in Materials and Methods.

TABLE 2. Concentrations of tolnaftate, tolciclate, and terbinafine causing inhibition of ergosterol biosynthesis in fungal cells and cell extracts[a]

Test system	Inhibitory concn (mg/liter)					
	Tolnaftate		Tolciclate		Terbinafine	
	50%	95%	50%	95%	50%	95%
C. albicans						
Whole cells	0.25 ± 0.05	>100	0.04 ± 0.01	>100	0.008 ± 0.002	0.2 ± 0.1
Cell free	0.13 ± 0.03	10.0 ± 1.5	0.010 ± 0.004	1.0 ± 0.3	0.008 ± 0.002	0.3 ± 0.1
Squalene epoxidase	0.32 ± 0.12	12.5 ± 3.8	0.04 ± 0.01	1.0 ± 0.1	0.008 ± 0.002	0.7 ± 0.2
T. mentagrophytes (whole cells)	0.004 ± 0.0003	0.077 ± 0.003	0.0026 ± 0.0001	0.05 ± 0.02	0.0015 ± 0.0004	0.020 ± 0.002

[a] Values shown are concentrations of the drugs causing, respectively, 50 or 95% inhibition in the relevant assay. Ergosterol biosynthesis was measured in whole cells by incorporation of [^{14}C]acetate and in cell extracts by incorporation of [^{14}C]mevalonate and by assay for squalene epoxidase activity, all as described in Materials and Methods. Values are means ± error limits for two separate experiments (three in the case of squalene epoxidase) with triplicate incubations.

TABLE 3. Effect of tolnaftate on ergosterol biosynthesis in *C. albicans* cells at pH 4.5 and 6.5[a]

Tolnaftate (mg/liter)	Radioactivity incorporated (% of control)			
	Acetate		Methionine	
	pH 4.5	pH 6.5	pH 4.5	pH 6.5
1	23.8	19.8	45.3	52.2
10	16.7	9.7	45.9	48.2
100	18.8	8.6	51.8	50.4

[a] Sterol synthesis was measured by incorporation of either [U-^{14}C]acetate or [*methyl*-^{14}C]methionine into ergosterol as described in Materials and Methods. Results are the mean of triplicate incubations, with the standard deviation <10% of the mean in all cases. Control incorporations into ergosterol (mean ± standard deviation in units of 1,000 dpm) were: acetate, 63.2 ± 3.5 (pH 4.5) and 54.9 ± 0.9 (pH 6.5); methionine, 14.1 ± 0.6 (pH 4.5) and 11.8 ± 0.5 (pH 6.5).

ADDENDUM IN PROOF

Inhibition of ergosterol biosynthesis by tolnaftate was very recently reported by Morita and Nozawa (3a).

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