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Effects of Squalene Epoxidase Inhibitors on *Candida albicans*

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The relationship between sterol biosynthesis inhibition, membrane integrity, and cell growth inhibition in *Candida albicans* was examined for five squalene epoxidase inhibitors. The compounds were the thiocarbamates tolnaftate and tolciclate and the allylamines naftifine, terbinafine, and SDZ 87-469. All compounds inhibited sterol biosynthesis, with the concentrations that caused a 50% decrease in the total sterol-to-squalene ratio ranging from ≤0.01 μM for terbinafine and SDZ 87-469 to 500 μM for tolnaftate. At 100 μM, the compounds also caused up to a 30% release of intracellular [^{14}C]aminoisobutyric acid. With terbinafine and SD2 87-469, aminoisobutyric acid release further increased in cells grown at concentrations that inhibited ergosterol biosynthesis. It is suggested that inhibition of ergosterol synthesis may render the *C. albicans* membrane susceptible to further damage, including direct damage from squalene epoxidase inhibitors.

Allylamines and the structurally related thiocarbamates are synthetic antifungal agents which inhibit ergosterol biosynthesis at the level of squalene epoxidase, causing the accumulation of squalene (4, 13, 18, 19, 22, 23, 26, 27). The compounds are highly fungicidal against dermatophytes and are used clinically in the treatment of dermatomycoses and onychomycoses (10, 14). They are also moderately active against *Candida albicans* in vitro, although they are not clinically useful against candidoses. This is in contrast to the azole antifungal agents, which inhibit ergosterol biosynthesis at the level of C-14 demethylase and are clinically useful as broad-spectrum antifungal agents (3, 7).

Ergosterol biosynthesis has been an attractive target ever since azoles were found to inhibit it (6, 29). However, early enthusiasm has been tempered by the realization that ergosterol synthesis inhibitors are mostly fungistatic over a wide concentration range (21). Large variations in MICs have been reported. These variations have been attributed to the morphological state of the organism, inoculum size, growth medium, duration of incubation, and end points (16). Conventional susceptibility testing is thus not a very reliable predictor of the in vivo activities of these compounds (9, 18, 20).

In the present study we examined in *C. albicans* the effects of the following five squalene epoxidase inhibitors: the thiocarbamates tolnaftate and tolciclate and the allylamines naftifine, terbinafine, and SDZ 87-469. The relationship between inhibition of sterol synthesis in growing cells, membrane integrity, and cell growth and viability in *C. albicans* for the five compounds was compared with the relationship between these factors for the more extensively studied C-14 demethylase inhibitors. All five compounds inhibited sterol synthesis, as expected, and at higher concentrations, they stimulated some release of intracellular material. In the case of terbinafine and SD2 87-469, the latter effects were more pronounced after inhibition of ergosterol synthesis, suggesting an increased fragility of the ergosterol-depleted plasma membrane.

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Tolnaftate and α-aminoisobutyric acid were purchased from Sigma Chemical Co. (St. Louis, Mo.); [1,2-^{14}C]acetate (specific activity, 56 μCi/μmol) was from ICN Pharmaceuticals Inc. (Irvine, Calif.); precoated thin-layer chromatography (TLC) plates (silica gel 60-F254; 20 by 20 cm; 0.25 mm thick; E. Merck) were from American Scientific Products (McGaw Park, Ill.). Naftifine, terbinafine, and SDZ 87-469 were gifts from Sandoz Forschungsinstitut (Vienna, Austria); tolciclate was from Farmitalia Carlo Erba (Milan, Italy); econazole and ketoconazole were from Jannsen Pharmaceutica (New Brunswick, N.J.); fluconazole was from Pfizer Central Research (Sandwich, United Kingdom); and amphotericin B was from Bristol-Myers Squibb Inc. (Princeton, N.J.).

C. albicans SC 5314 was from the Squibb culture collection (12). Cells were grown (5% inoculum from a culture with an optical density at 660 nm [OD_{660}] of 0.7, ca. 10^6 CFU/ml) in casitone-yeast extract-glucose (CYG) medium (5 g of each component per liter; Difco Laboratories, Detroit, Mich.) at 30°C or in RPMI 1640 (GIBCO Life Technologies, Inc., Grand Island, N.Y.)–0.165 M MOPS (morpholinepropanesulfonic acid; pH 7.0; USB Corp., Cleveland, Ohio) medium at 35°C (28).

MICs were determined by broth microdilution (96-well plates, 100 μl [10^4 or 10^6 CFU/ml] per well) in CYG medium. The MIC was the lowest concentration which inhibited visible growth after 18 h of incubation at 35°C. Growth inhibition was also determined after 24 h of incubation (OD_{660} of 0.7 for a culture diluted to the desired CFU per milliliter) at 35°C with the test compound in RPMI 1640–MOPS medium. The 50% inhibitory concentration (IC_{50}) for growth was the concentration which produced a 50% decrease in cell turbidity (OD_{630}).

For determinations of sterol biosynthesis in intact cells, *C. albicans* was grown for 7 h in CYG medium supplemented with [^{14}C]acetate (10 μM, 0.2 μCi/ml) and the appropriate antifungal agent. Lipids were extracted and separated on TLC plates (heptane-acetic acid-isopropyl ether; 60:4:40, by volume), and the radioactivities of the neutral lipid bands were measured as described previously (12).

Membrane integrity was measured as the release of intracellular material. *C. albicans* was grown to an OD_{660} of 0.5, concentrated to 1/10th of the original volume, loaded with [^{14}C]aminoisobutyric acid when indicated, and then incubated with the appropriate antifungal agent at 37°C for 1 h (10). Potassium release was measured by atomic absorption,

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TABLE 1. Effects of squalene epoxidase inhibitors and standard compounds on *C. albicans* SC 5314 sterols and cell growth

Antifungal agent	IC_{50} (μM) for sterol synthesis		IC_{50} (μg/ml) for cell growth with inoculum of:		MIC (μg/ml) with inoculum of:	
	Erg/DMS + TMS[a]	Sterol/squalene[b]	10^4 CFU/ml	10^6 CFU/ml	10^4 CFU/ml	10^6 CFU/ml
Tolnaftate	ND[c]	100	>128	>128	>128	>128
Tolciclate	ND	0.3	>128	>128	>128	>128
Naftifine	ND	1.0	32	32	64	>128
Terbinafine	ND	0.005	1	4	8	>128
SDZ 87-469	ND	0.03	ND	ND	4	>128
Ketoconazole	0.001	ND	<0.125	8	16	64
Econazole	0.005	ND	ND	ND	ND	ND
Fluconazole	0.1	ND	ND	ND	ND	ND
Amphotericin B			<0.125	<0.125	<0.125	0.25

[a] Erg/DMS + TMS, ergosterol:(dimethylsterol plus trimethylsterol) ratio. The ratio was ~20 in untreated cells (ergosterol was 12% and dimethylsterol plus trimethylsterol was <1% of total lipids).
[b] The sterols ergosterol, dimethylsterol, and trimethylsterol were measured.
[c] ND, not determined.

and [^{14}C]aminoisobutyric acid release was measured by liquid scintillation counting (12).

As reported previously (19, 22, 26, 27), all five compounds inhibited ergosterol biosynthesis in growing *C. albicans* cells and caused the accumulation of squalene (Table 1). Inhibition was defined as a decrease in the sterol-to-squalene ratio, which was typically 20 in control cultures. IC_{50}s ranged from 0.005 μM for terbinafine to 500 μM for tolnaftate (Table 1). The azoles, which were included in the study for direct comparison, also inhibited ergosterol synthesis and caused the accumulation of methylated sterols (12, 29). Inhibition in this case was defined as the decrease in the ergosterol-to-methylated sterol ratio and the IC_{50} was defined as the concentration in which the ratio decreased to 50% of that of the control (8). Typically, the ergosterol-to-methylated sterol ratio of the controls was 18. The IC_{50} for terbinafine was similar to that for ketoconazole, suggesting that permeability is not a problem, as it is with tolnaftate (1). It should be noted that the TLC method used for sterol analysis is not useful with other ergosterol inhibitors, such as azasterol and the morpholine antifungal agents, in which different sterols accumulate.

Allylamines, but not thiocarbamates, showed measurable growth-inhibitory activity against the organism under the assay conditions described here (Table 1). IC_{50}s for growth at 10^4 CFU/ml ranged from 1 μg/ml for SDZ 87-469 and terbinafine to >128 μg/ml for tolnaftate. MICs were higher than growth IC_{50}s, and both were increased when an inoculum of 10^6 CFU/ml was used. Terbinafine, which was examined in more detail together with the C-14 demethylase inhibitor ketoconazole, inhibited cell growth and viability at concentrations similar to those of ketoconazole (Fig. 1). As reported previously for azoles (12), the inhibitory effect of terbinafine on cell growth was antagonized by 1% (vol/vol) Tween 80, an oleic acid derivative (3a).

None of the five compounds induced leakage of intracellular potassium, while the polyene amphotericin B, which was included as a positive control, did (Table 2). However, all compounds caused some leakage of aminoisobutyric acid when they were used at a concentration of 100 μM, as did ketoconazole. Leakage increased when cells were grown with terbinafine, SDZ 87-469, or ketoconazole at concentrations that inhibited ergosterol synthesis. Some of the apparent isobutyric acid leakage may actually have been decreased uptake, because it also occurred in the absence of the compounds in the assay (Table 2). Decreased amino acid uptake following ergosterol inhibition by azoles has been reported recently (2, 24). Nevertheless, terbinafine and SDZ 87-469 may interact directly with membrane lipids in the manner postulated for ketoconazole and other azoles (5).

In conclusion, squalene epoxidase inhibitors, like C-14 demethylase inhibitors, are active against their target in intact *C. albicans* cells. Yet, in contrast to their activity against dermatophytes, they are only moderately active against *C. albicans*. While this may reflect different sterol requirements for physiological functions in different fungi (15, 17, 25), it may also reflect direct damage of ergosterol-depleted membranes by ergosterol inhibitors.

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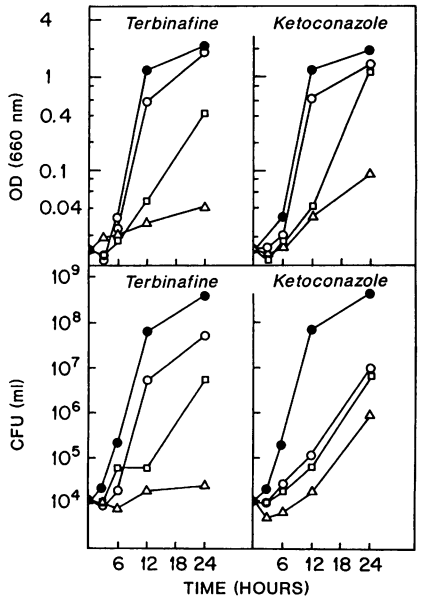

FIG. 1. Effect of terbinafine and ketoconazole on growth (OD_{660}) and viability (CFU per milliliter) of *C. albicans* SC 5314. Antibiotic concentrations were 0 (●), 0.16 (○), 1.6 (□), and 16 (△) μg/ml.

TABLE 2. Effects of terbinafine and ketoconazole on membrane integrity of *C. albicans* SC 5314

Antifungal agent	Concn (μM)		Release (% of total)	
	Growth medium[a]	Assay mixture	Potassium	Aminoisobutyric acid
None			<5	<5
Tolnaftate		100	<5	<5
Tolciclate		100	<5	25
Naftifine		100	<5	30
Terbinafine		100	<5	23
Terbinafine	0.1		<5	33
Terbinafine	0.1	100	<5	50
SDZ 87-469		100	<5	<5
SDZ 87-469	0.1		<5	44
SDZ 87-469	0.1	100	<5	71
Ketoconazole		100	<5	20
Ketoconazole	0.1		<5	48
Ketoconazole	0.1	100	<5	85
Amphotericin B		10	100	97

[a] Cells were grown in CYG medium at 30°C to an OD_{660} of 0.5, unless indicated otherwise.

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