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Effect of Antifungal Agents on Lipid Biosynthesis and Membrane Integrity in *Candida albicans*

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Eight antifungal agents were examined for effects on lipid biosynthesis and membrane integrity in *Candida albicans*. Lipids were labeled in vivo or in vitro with [^{14}C]acetate and analyzed by thin-layer and gas chromatography. Membrane integrity was measured by a recently developed [^{14}C]aminoisobutyric acid radiolabel release assay. The imidazole antifungal agents miconazole, econazole, clotrimazole, and ketoconazole, at concentrations inhibiting ergosterol biosynthesis (0.1 μM), decreased the ratio of unsaturated to saturated fatty acids in vivo but not in vitro. Similarly, naftifine, tolnaftate, and the azasterol A25822B, at concentrations inhibiting ergosterol biosynthesis (10, 100, and 1 μM, respectively), decreased the ratio of unsaturated to saturated fatty acids in vivo only. This suggests that the effect on fatty acids observed with ergosterol biosynthesis inhibitors may be secondary to the effect on ergosterol. With imidazoles, oleic acid antagonized inhibition of cell growth but not inhibition of ergosterol. This suggests that, with the C-14 demethylase inhibitors, decreased unsaturated fatty acids, rather than decreased ergosterol, are responsible for growth inhibition. Cerulenin, previously reported to be a potent inhibitor of both fatty acid and ergosterol biosynthesis, was found in the present study to inhibit the former (at 5 μM) but not the latter (up to 100 μM). Of the antifungal agents tested, econazole and miconazole (at 100 μM) produced complete release of [^{14}C]aminoisobutyric acid, which is consistent with membrane damage.

Candida albicans is a dimorphic yeast of increasing clinical importance as a human pathogen (28). Normally found in the alimentary tract as a commensal, it is a major cause of fungal infections in immunocompromised patients and topical infections in healthy individuals (21, 24, 53). Most antibiotics active against this organism act at the level of membrane lipids by inhibiting either their function (polyenes) or their biosynthesis (azoles, allylamines, azasterol, cerulenin) (2, 8, 14, 23). Thus, yeast lipids are of obvious interest as chemotherapeutic targets and have been studied extensively in recent years (19, 20, 31, 32, 45, 48).

The present report describes the effects of eight antifungal agents on lipid biosynthesis and membrane integrity in *C. albicans*. The antifungal agents studied were clotrimazole, econazole, miconazole, ketoconazole, naftifine, tolnaftate, 15-azasterol (A25822B), and cerulenin. Effects on lipids were examined in vivo and in vitro by labeling with [^{14}C]acetate and then analyzing lipids by thin-layer (TLC) and gas (GC) chromatography. Effects on membrane integrity were measured by a recently developed [^{14}C]aminoisobutyric acid radiolabel release assay and, conventionally, as release of intracellular potassium. For comparison purposes, the effects of antifungal agents on mammalian lipids were also examined by using a tissue culture system.

MATERIALS AND METHODS

Materials. Cerulenin, dithiothreitol, α-aminoisobutyric acid, glucose-1-phosphate, glutathione, coenzyme A, NAD, NADP, EDTA, *Helix pomatia* β-glucuronidase, and lipid standards were obtained from Sigma Chemical Co. (St. Louis, Mo.); [1,2-^{14}C]acetate (specific activity, 56 μCi/μmol), α-[1-^{14}C]aminoisobutyric acid (specific activity, 50 μCi/μmol), and L-[*methyl*-^{14}C]methionine (specific activity, 48 μCi/μmol) were from ICN Pharmaceuticals Inc. (Irvine, Calif.); DL-[2-^{14}C]mevalonic acid lactone (specific activity, 53 μCi/μmol) was from Amersham Corp. (Arlington Heights, Ill.); [7-^{14}C]benzoic acid (specific activity, 10 μCi/μmol) and Aquasol were from New England Nuclear Corp. (Boston, Mass.); E. Merck precoated TLC plates (silica gel 60-F254; 20 by 20 cm; 0.25 mm thick for analytical TLC, 0.5 mm thick for preparative TLC) were from American Scientific Products (McGaw Park, Ill.); glass beads (0.45 to 0.52 mm in diameter) were from Thomas Scientific (Philadelphia, Pa.); GFA-1 glass fiber filter cups were from Amicon Corp. (Lexington, Mass.). All other chemical reagents were analytical grade, purchased from Fisher Scientific Co. (Pittsburgh, Pa.).

Clotrimazole and tolnaftate were gifts from Schering-Plough Corp. (Kenilworth, N.J.); ketoconazole was from Janssen Life Sciences Products (Piscataway, N.J.); miconazole was from Johnson and Johnson (New Brunswick, N.J.); naftifine was from Sandoz Pharmaceuticals (East Hanover, N.J.); 15-azasterol (A25822B) was from Eli Lilly & Co. (Indianapolis, Ind.); and brefeldin A was from G. Tamura of the University of Tokyo (Tokyo, Japan).

Organism and growth conditions. *C. albicans* SC 5314 was from the Squibb Culture Collection and, on the basis of cell morphology, biochemical properties, and antifungal susceptibility profile, was a typical strain (Georgopapadakou and Smith, unpublished data). The organism was grown with shaking in YECD medium (0.5% yeast extract, 0.5% Casitone, 0.5% glucose) at 37°C.

In vivo lipid biosynthesis. *C. albicans* was grown at 37°C in 10 ml of YECD broth supplemented with 0.01 mM [^{14}C]acetate (1 μCi) and the appropriate antifungal agent until late-log phase (optical density at 660 nm, 1.3). Cells were harvested by centrifugation, washed once with cold 5% trichloroacetic acid, and extracted once with 1.5 ml of methanol followed by 1 ml of a 1:1 mixture of methanol-

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benzene (42). Lipid extracts (~200,000 cpm) were spotted on silica gel TLC plates and developed to half the length of the plate with chloroform-acetic acid-methanol-acetone-water (50:10:10:20:5 by volume; CAM system) (16). The CAM system resolved phospholipids into individual species, while neutral lipids migrated with the solvent front. Plates were thoroughly dried and then developed to the full length of the plate with heptane-acetic acid-isopropyl ether (60:4:40 by volume; HAP system) (4). The HAP system resolved neutral lipids into individual classes. Alternatively, lipid extracts were spotted on two plates which were then developed in parallel to the full length of each plate with the HAP and CAM solvent systems. Lipid bands were routinely localized by overnight exposure on Kodak XAR-5 film. They were identified originally by comparison with authentic samples which were visualized by UV illumination, reaction with iodine vapor (passing air through a glass wool-plugged Pasteur pipet containing iodine crystals), or spraying with ninhydrin or antimony trichloride in chloroform (Carr-Price reagent) (15) (see Table 1). For quantitation, individual lipid bands were scraped into scintillation vials, mixed with 5 ml of Aquasol, and counted for radioactivity. The numbers obtained were converted to percentages of total lipids, averaged from three replicate experiments (variability, <10%), rounded to the nearest percent, and tabulated.

The R_f values of the major lipids (see Table 1) were as follows: phosphatidylinositol, 0.12; phosphatidylserine, 0.18; phosphatidylcholine, 0.23; and phosphatidylethanolamine, 0.45 (CAM solvent system); and ergosterol, 0.19; lanosterol, 0.25; free fatty acids, 0.44; triglycerides, 0.69; and squalene and sterol esters, 0.89 (HAP solvent system). Squalene and sterol esters could be differentiated by saponification (30% aqueous sodium hydroxide–100% ethanol [1:1 by volume]) at 55°C for 1 h.

For fatty acid analysis, whole-lipid extracts or individual lipid components extracted from the silica gel with chloroform-methanol (2:1 by volume) after preparative TLC were saponified as described above. Fatty acids were isolated by preparative TLC, extracted with chloroform-methanol, dried under nitrogen, redissolved in 0.2 ml of methanol (13 by 100 mm tubes), and esterified, after addition of 0.2 ml of methanol-sulfuric acid (80:20 by volume), for 20 h at 22°C (6). The solution was neutralized with saturated $NaHCO_3$ (~2 ml per tube) and extracted twice with 0.8-ml portions of ethyl ether. Extracts were dried under nitrogen and, if not analyzed immediately, were stored at −20°C. In preliminary experiments, conventional esterification with diazomethane in dry ether at 0°C (6) produced side products, at least with linolenic acid, and was subsequently abandoned.

Fatty acid methyl esters were identified by comparison with authentic samples by gas chromatography (GC), selected ion mass spectrometry, and positive chemical ionization. The quadrupole mass spectrometer was built with Extranuclear rods, electronics with a Simulscan source, and 700- and 300-liter/min diffusion pumps on the source and analyzer, respectively. A Shimadzu GC 4B was interfaced by means of 1.6-mm outside diameter glass-lined stainless steel tubing entering the source at an angle of 90° to the ion beam. The mass spectrometer was controlled by a Teknivent 69 KD computer system operating in the selected ion-monitoring mode.

The GC was equipped with a column (0.8 m by 2 mm) packed with 3% OV-101 on 80/100 Supelcoport. Conditions were as follows: injector temperature, 240°C; column temperature, 145°C isothermal; interface line, 190°C; helium flow rate, 30 ml/min. Samples were dissolved in 30 μl of methylene chloride, and 0.5 to 1.0 μl of solution was injected into the chromatograph for each run.

The mass spectrometer source was kept at 180°C with the electron energy at 300 V and a current of 1,000 μA. The reagent gas (H_2O) was introduced directly opposite the GC line by means of a 0.25-in. (1 in. = 2.54 cm) outside diameter stainless steel line connected to a metering valve and a glass bulb containing the outgassed water. The pressure of the water was adjusted to make the ion at 55^+ [$(H_2O)_3H^+$] a maximum (approximately 1.6 torr [1 torr = 133.322 Pa] total pressure).

Comparison of the retention time and molecular ion plus hydrogen (MH^+) of authentic compounds and test samples was made under identical conditions. The relative amount of each ester was determined by summing the area of each gas chromatographic peak and dividing each by the sum. The major fatty acid species were palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), and γ-linolenic (18:3) acids (see Table 2).

In vitro lipid biosynthesis. *C. albicans* was grown at 37°C in 250 ml of YECD broth until late-log phase. Cells were harvested by centrifugation and ruptured with glass beads (1 g of cells, 2 g of glass beads, 3 to 4 ml of 50 mM Tris hydrochloride [pH 7.5]) (10) in a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) (setting 7; three times for 2 min each with 3-min cooling intervals). The suspension was briefly centrifuged to remove the glass beads, and the supernatant was assayed immediately. The assay mixture contained, in a total volume of 0.5 ml (13 by 100 mm tubes): 200 mM potassium phosphate buffer (pH 7.0), 10 mM $MgCl_2$, 1 μmol of ATP, 6 μmol of glutathione, 2.5 μmol of glucose-1-phosphate, 50 μmol of coenzyme A, 0.3 μmol of NAD, 0.4 μmol of NADP, 0.5 μmol of [^{14}C]acetate (500,000 cpm) or 0.05 μmol of [^{14}C]mevalonate (50,000 cpm), and cell homogenate (300 to 600 μg of protein determined as described by Lowry et al. [18] but with the addition of 1% sodium dodecyl sulfate) (9). After 60 min of incubation at 37°C, 5 nCi of [^{14}C]benzoic acid was added as an internal standard, followed by 0.75 ml of 15% potassium hydroxide in 95% ethanol to saponify lipids. The tubes were covered with steel caps and incubated at 75°C for 75 min. The mixture was neutralized with 0.4 ml of 6 N HCl, and saponified lipids were extracted with chloroform-methanol and separated on silica gel TLC (HAP solvent system). The mobility of benzoic acid was 1.2 relative to lanosterol. Fatty acids were extracted from the silica gel with chloroform-methanol, dried under nitrogen, redissolved in methanol, and esterified with methanol-sulfuric acid as described above.

Fatty acid esters were separated on a Hewlett-Packard (Palo Alto, Calif.) gas chromatograph (model 5750) equipped with a thermal conductivity detector and a Supelco 10% SP-2330 column (6 ft. [1 ft. = 30.48 cm] by 0.125 in). Conditions were as follows: injector temperature, 310°C; column temperature, 180 to 220°C at 2°C/min; helium flow rate, 20 ml/min. Approximately 5 μl containing 10 μg of fatty acid methyl esters was injected into the chromatograph for each run. Fatty acid methyl esters were identified by comparison with the retention times of reference compounds chromatographed under identical conditions (total elution time, 25 min). Emerging peaks were collected in polyethylene tubing (Intramedic; 1.14-mm inside diameter, 1.57-mm outside diameter, 140 mm long), flushed with 0.2 ml of toluene into 5 ml of Aquasol, and counted for radioactivity.

Assays for membrane integrity. *C. albicans* was grown at 37°C in 50 ml of YECD broth until the optical density at 660 nm was 0.5. Cells were harvested by centrifugation, sus-

pended in one-tenth of the original volume of 50 mM sodium phosphate (pH 7.0)–2% glucose, and incubated at 37°C for 30 min.

For the radiolabel release assay, [^{14}C]aminoisobutyric acid was added to a final concentration of 1 mM (1.5 μCi/ml of cell suspension), and the mixture was incubated at 37°C for an additional 30 min. Cells were harvested by centrifugation, washed twice with 50 mM sodium phosphate (pH 7.0)–2% glucose, and suspended in one-tenth of the original culture volume of sodium phosphate-glucose (optical density at 660 nm, 1.5 to 2.0). Samples of 0.1 ml were removed and incubated with the appropriate antifungal agent (added as a 100-fold concentrated solution) at 37°C for 60 min. Samples were transferred to glass fiber filter cups, filtered, washed twice with 2 ml of cold water, dried, and counted in Aquasol. Radiolabel release was calculated as the percent difference in cell-associated radioactivity between test samples and untreated controls. Typically, cell-associated radioactivity in untreated controls was 10,000 to 12,000 cpm, representing approximately 10% of the radioactivity initially added.

For the potassium efflux assay (7, 56), 1-ml samples of the cell suspension were incubated with the appropriate antifungal agent at 37°C for 60 min. Cells were removed by centrifugation, and potassium was measured in the supernatant with a flame photometer (model 443; Instrumentation Laboratory, Inc., Lexington, Mass.). Potassium efflux was calculated as a percentage of total intracellular potassium, the latter being the amount released in a boiled (5 min) control. Typically, the potassium released to the supernatant by boiled cells was 0.5 to 0.7 mM.

For the dye exclusion assay, 1-ml samples of the cell suspension were incubated with the appropriate antifungal agent at 37°C for 60 min. A 50-μl sample was added to 5 μl of an aqueous solution of 0.04% methylene blue, and 20 μl of the resulting suspension was placed in a hemacytometer. Total cells and stained cells were counted, and the percentage of stained cells was calculated.

RESULTS AND DISCUSSION

The lipid composition of *C. albicans* SC5314 is shown in Tables 1 and 2. It was similar to that reported for *C. albicans* (19, 32, 45) and *Saccharomyces cerevisiae* (13). The major phospholipids were phosphatidylcholine and phosphatidylethanolamine, with 16- and 18-carbon fatty acid chains. The major sterol was ergosterol, predominantly in the free, unesterified form. Sterol esters accumulated in stationary-phase cells (Georgopapadakou and Smith, unpublished data). Surprisingly, nonradioactive ergosterol caused darkening of the X-ray film during autoradiography of the TLC plates. Therefore, radioactivity in lipids was not determined densitometrically.

TABLE 1. Lipid composition of *C. albicans*

Component	% of total	Detection method
Ergosterol (free)	12	Long-wavelength UV, I_2, $SbCl_3$ (green)
Ergosterol (esters)	0.4	Long-wavelength UV, I_2, $SbCl_3$ (light green)
Lanosterol	0.6	Long-wavelength UV, I_2, $SbCl_3$ (brown-purple)
Free fatty acids	2.8	Short-wavelength UV, I_2, $SbCl_3$ (pink)
Triglycerides	6.2	I_2, $SbCl_3$ (pink)
Phosphatidylcholine	32	I_2, $SbCl_3$ (pink)
Phosphatidylethanolamine	21	I_2, ninhydrin, $SbCl_3$ (pink)
Phosphatidylserine	3	I_2, ninhydrin
Phosphatidylinositol	4	I_2

TABLE 2. Fatty acid composition of *C. albicans* lipids

Lipid	Fatty acid (% of total)					
	16:0	16:1	18:0	18:1	18:2	18:3
Sterol esters	38	12	9	29	6	4
Free fatty acids	35	20	8	20	12	4
Triglycerides	20	23	4	33	13	6
Phosphatidylcholine	20	23	10	22	20	6
Phosphatidylethanolamine	26	23	8	27	9	7

Phosphatidylcholine and ergosterol could be labeled specifically with [^{14}C]methionine, and lanosterol and ergosterol were labeled with [^{14}C]mevalonic acid. In the latter case, incorporation was 2 orders of magnitude lower than that for [^{14}C]acetate, suggesting that mevalonic acid does not readily enter *C. albicans* cells. Consistent with this, inclusion of 10 mM mevalonic acid in the culture medium did not decrease incorporation of [^{14}C]acetate into ergosterol relative to other lipid species or stimulate incorporation of [^{14}C]methionine (data not shown).

Lipid biosynthesis in vivo. As reported in the literature (5, 20, 51), clotrimazole, econazole, ketoconazole, and miconazole inhibited ergosterol biosynthesis by approximately 80% at 0.1 μM and caused accumulation of methylated sterols (R_fs, 0.23 and 0.25 in the HAP TLC system). Typically, the ergosterol/methylated sterol ratio changed from 20 to 0.2. They also decreased the unsaturated-to-saturated fatty acid ratio from 2.3 to 1.1 (52; Table 3). Naftifine and tolnaftate, at 10 and 100 μM, respectively, inhibited sterol biosynthesis by 70% and caused a 10-fold accumulation of squalene. In the case of naftifine, methylated sterols also increased, though to a lesser extent than with squalene. Squalene has been recently reported to potentiate the antifungal activity of amphotericin (22), suggesting that naftifine and tolnaftate might also synergize with amphotericin. Naftifine is known to inhibit squalene epoxidase (30, 39), whereas tolnaftate has been very recently suggested to act by a similar mechanism (26, 40). Both compounds also decreased the unsaturated-to-saturated fatty acid ratio from 2.3 to 1.4. The azasterol A25822B, at 1 μM, inhibited ergosterol biosynthesis, consistent with its reported effect on Δ14 reductase (12, 25, 54). It also decreased the unsaturated-to-saturated fatty acid ratio from 2.3 to 1.3. All of the above ergosterol inhibitors caused a shift in fatty acid length from C_{18} to C_{16} (Table 3). Some also inhibited phospholipid biosynthesis, though this effect was variable and less conclusive.

TABLE 3. Effect of antifungal agents in vivo on *C. albicans* fatty acids

Antifungal agent	Concn (μM)	Fatty acid (% of total)					
		16:0	16:1	18:0	18:1	18:2	18:3
None		26	20	4	30	15	5
Clotrimazole	1	39	16	9	14	16	6
Econazole	1	40	15	10	14	16	6
Ketoconazole	1	42	13	8	14	18	6
Miconazole	1	38	16	10	12	19	6
Naftifine	10	37	18	5	22	14	4
Tolnaftate	100	36	20	6	20	15	3
Azasterol	1	33	20	6	21	16	4
Cerulenin	1	27	13	6	25	22	6

TABLE 4. Effect of antifungal agents in vitro on *C. albicans* ergosterol and total fatty acids

Antifungal agent	50% inhibitory concn (μM)	
	Ergosterol	Fatty acids
Clotrimazole	0.5	>10
Econazole	0.3	>10
Ketoconazole	0.5	>10
Miconazole	0.3	>10
Naftifine	5	>100
Tolnaftate	30	>100
Azasterol	1	>10
Cerulenin	>100	5

Cerulenin, contrary to a previous report (27), did not inhibit ergosterol biosynthesis up to 100 μM (Table 3). It inhibited fatty acids (phospholipids and triglycerides) in vivo by over 80% at 10 μM. The results are consistent with those from biochemical studies, in which 50% inhibitory concentrations for β-ketoacyl–acyl carrier protein synthase and 3-hydroxy-3-methylglutaryl coenzyme A synthase of 5 μM and 1 mM have been reported (29). The inhibitory effect of cerulenin on fatty acids is similar to that reported for other fungi (3, 27, 28). The reported inhibition of ergosterol biosynthesis was probably inhibition of sterol ester biosynthesis secondary to inhibition of fatty acid biosynthesis. Both free ergosterol and sterol esters are precipitable by digitonin, the method used to isolate ergosterol in that study, and therefore digitonin precipitation is not a reliable index of ergosterol concentration. Moreover, stationary-phase cells, which accumulate sterol esters, were used (27).

Lipid biosynthesis in vitro. To distinguish between primary and secondary effects on individual lipid components, an in vitro system was developed. Fatty acids were quantitated by liquid scintillation counting after GC, as described in Materials and Methods. The high background of nonradioactive fatty acids precluded quantitation by GC and mass spectrometry.

The imidazole antifungal agents naftifine, tolnaftate, and 15-azasterol inhibited [^{14}C]acetate incorporation into ergosterol (Table 4). Imidazoles caused fourfold accumulation of methylated sterols, whereas naftifine and tolnaftate caused threefold accumulation of squalene at their inhibitory concentrations. For naftifine, the 50% inhibitory concentration was 100-fold higher than that reported for the isolated enzyme system (40) and similar to that for cholesterol in human fibroblasts (data not shown). There was no significant effect on individual fatty acids (Table 5). Thus, the decrease in unsaturated fatty acids observed with these compounds in vivo was a secondary effect, probably the result of decreased Δ9 desaturase activity. Both phospholipid and fatty acyl coenzyme A Δ9 desaturases are microsomal enzymes (1, 35) and thus are likely to be affected by altered membrane fluidity.

The inhibitory effect of imidazoles on cell growth could be reversed by 1 mg of oleic acid per ml or by 1% (vol/vol) Tween 80, an oleic acid derivative. Ergosterol biosynthesis was still inhibited under these conditions, precluding a trivial explanation of direct interaction between imidazoles and fatty acids (e.g., formation of mixed micelles). The results are consistent with the findings of Yamaguchi (55) but not those of Van den Bossche et al. (50). Saturated fatty acids, such as stearic acid, did not reverse inhibition of cell growth by imidazoles.

It has been postulated that ergosterol has at least two types of function in membranes (37): a nonspecific, "bulk" function in the regulation of membrane fluidity and a specific, "sparking" function in the regulation of cell growth and proliferation (33, 36, 38). Thus, compounds which inhibit ergosterol biosynthesis substantially but not completely are likely to affect membrane fluidity only. The situation is presumably corrected by exogenous fatty acids but not ergosterol because of failure of the latter to be incorporated into the membrane (17, 47). The indirect effect of imidazoles on fungal fatty acids may explain why imidazoles are not active against bacteria unless they damage the membrane directly (44). Their effects on mitochondrial functions, such as respiration and ATPase activity (34, 46, 49), may be less critical to their mode of action.

As in vivo, cerulenin inhibited fatty acid biosynthesis in vitro with 50% inhibitory concentration of 5 μM. It did not inhibit ergosterol biosynthesis up to 100 μM.

Membrane integrity. The radiolabel release assay involves feeding cells a nonmetabolizable radioactive amino acid, treating cells with a test compound, and measuring the radioactivity remaining in the cells. The assay produced results similar to those obtained with the conventional potassium release assay (Table 6) but was more sensitive, accurate, convenient, and interference free. Potassium release, in turn, was more sensitive than methylene blue staining, most likely because of the smaller size of the reporter substance.

The polyenes amphotericin B and nystatin were positive in all three assays at 10 μM and thus served as a positive control. Of the eight antifungal agents studied, miconazole and econazole were positive, though at concentrations much higher than those affecting lipid biosynthesis or cell growth (43; Table 6). The direct membrane effects of imidazoles

TABLE 5. Effect of antifungal agents in vitro on individual fatty acids of *C. albicans*

Antifungal agent	Concn (μM)	Fatty acid (% of total)					
		16:0	16:1	18:0	18:1	18:2	18:3
None		40	11	12	28	8	3
Clotrimazole	1	45	7	16	26	5	2
Econazole	1	42	8	17	24	4	2
Ketoconazole	1	46	10	15	22	5	2
Miconazole	1	41	11	14	28	5	2
Naftifine	100	40	8	16	29	5	1
Tolnaftate	100	46	7	16	26	5	1
Azasterol	1	40	11	12	29	5	2

TABLE 6. Effects of antifungal agents on membrane integrity of *C. albicans*

Antifungal agent	Concn (μM)	% Total release of:		% of cells stained with methylene blue
		Potassium	Aminoisobutyric	
None		<5	<5	0
Amphotericin B	10	100	97	100
Nystatin	10	100	97	100
Clotrimazole	100	<5	20	0
Econazole	100	47	98	0
Ketoconazole	100	<5	20	0
Miconazole	100	66	94	60
Naftifine	100	<5	30	0
Tolnaftate	100	<5	<5	0
Azasterol	100	<5	<5	0
Cerulenin	100	<5	<5	0

have been reported to be both concentration and time dependent. For example, clotrimazole at 100 μM requires 2.5 h of incubation for 50% total intracellular potassium release (41). Interestingly, miconazole and econazole inhibited *Staphylococcus aureus* SC 2399 at 10 to 100 μM, as previously reported for a different strain (44). Tolnaftate produced cytopathic effects in human fibroblasts at 100 μM (Georgopapadakou and Dix, unpublished data) but no membrane damage in *C. albicans* under the assay conditions, raising the possibility that squalene accumulation is toxic to cells. This may be an important point in determining whether squalene epoxidase is a clinically useful target for antifungal agents or modulators of cholesterol biosynthesis.

The present studies were originally undertaken to establish convenient and unambiguous methods for determining the mode of action of novel antifungal agents. In the process, we found that brefeldin A, a C-16 fatty acid-derived cyclic antifungal agent (MIC for *C. albicans*, 5 μg/ml), caused membrane damage in *C. albicans* (50% release of [^{14}C]aminoisobutyric acid at 2 μg/ml). It also caused morphological changes (swelling, rounding, multiple budding in some cells) but, contrary to a previous report (11), did not inhibit phospholipid biosynthesis. We also found tolnaftate to act similarly to naftifine and cerulenin to act exclusively through inhibition of fatty acid biosynthesis. Interestingly, in human fibroblasts cerulenin inhibited cholesterol in addition to fatty acid synthesis (Georgopapadakou and Dix, unpublished data). The effects of imidazoles on fatty acids in vivo, though secondary to ergosterol inhibition and the inability of *C. albicans* to take up exogenous sterols, may be responsible for growth inhibition in *C. albicans*.

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