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Naturally Azole-Resistant *Leishmania braziliensis* Promastigotes Are Rendered Susceptible in the Presence of Terbinafine: Comparative Study with Azole-Susceptible *Leishmania mexicana* Promastigotes

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Leishmania braziliensis **(isolate 2903) was naturally resistant to ketoconazole or the bis-triazole D0870, inhibitors of sterol C-14 demethylase, which produced only moderate effects on the proliferation of promastigotes at 10 μM. In contrast, *Leishmania mexicana* (isolate NR) was extremely susceptible to the azoles, as complete growth arrest and cell lysis were induced by incubation of the parasites with 0.05 μM concentrations of the drugs for 72 h. The opposite response was observed with terbinafine, an inhibitor of squalene epoxidase: *L. braziliensis* 2903 was three times more susceptible to the drug than *L. mexicana* NR (MICs of 5 and 15 μM, respectively). However, when the *L. braziliensis* stock was grown in the presence of 1 μM terbinafine, which by itself produced only marginal (<10%) effects on growth, it became highly susceptible to the azoles, with an MIC of 0.03 μM. Analysis of cellular free sterols by high-resolution capillary gas chromatography coupled to mass spectrometry showed that 14-methyl sterols can support normal growth of *L. braziliensis* 2903 but not of *L. mexicana* NR. On the other hand, the higher susceptibility of the *L. braziliensis* isolate to terbinafine was correlated with a massive accumulation of squalene in the presence of the allylamine while no significant effects on *L. mexicana* sterol composition were observed at drug concentrations up to 1 μM. Thus, the >300-fold increase in the susceptibility of *L. braziliensis* promastigotes to azoles in the presence of terbinafine was attributed to the combined effect of squalene and the methylated sterol precursors on the physical properties of the cell's membranes, leading to the loss of cell viability. Combination therapy with azoles and terbinafine in the treatment of human *L. braziliensis* infections deserves further study.**

Despite the considerable progress made in recent years in the study of the biochemistry, physiology, and molecular biology of parasitic organisms of the *Leishmania* genus, the treatment of the varied human infections caused by these organisms is still mostly based on pentavalent antimonials (Sb^V, sodium stibogluconate, and meglumine antimoniate), an empirical treatment developed more than 50 years ago (12, 13, 27, 36). Pentavalent antimonials require parenteral administration and prolonged treatments, which commonly have toxic side effects, including cardiotoxicity; although visceral and cutaneous leishmaniasis usually responds to these treatments, severe mucosal disease does not (27). Moreover, resistance of both visceral and mucocutaneous leishmaniasis to pentavalent antimonials has been increasing in all areas of endemicity (36). Second-line drugs such as amphotericin B and pentamidine are less effective and even more toxic; recent new formulations of the same drugs, such as lipid-associated amphotericin B, are less toxic and significantly more active against susceptible organisms, but they are still very expensive and have to be administered intravenously (12, 13, 36). Among potential orally active drugs for the treatment of these complex diseases, sterol biosynthesis inhibitors offer an attractive possibility, as *Leishmania* parasites synthesize de novo specific sterols which seem to be essential for cell proliferation and viability (6–8, 20–22, 24, 25). However, although the proliferation of many species of *Leishmania* has been shown to be inhibited in vitro by azole antifungals such as ketoconazole and itraconazole, which block sterol synthesis at the level of the sterol C-14α demethylase (6, 8, 22, 51, 52), the effects of these compounds in the treatment of human infections have been equivocal, ranging from high efficacy against *Leishmania mexicana* and *Leishmania major* infections to little or no activity against *Leishmania braziliensis* and *Leishmania donovani* infections (1, 10, 15, 16, 27, 30, 33, 35, 36, 41, 49, 55, 56). Although some of this variability could be related to the pharmacokinetic profile of the drugs, a placebo-controlled study carried out in Guatemala to compare the relative efficacy of sodium stibogluconate (Pentostam) and ketoconazole in the treatment of cutaneous leishmaniasis showed that *L. braziliensis* infections were responsive to stibogluconate but much less so to ketoconazole while for *L. mexicana* infections the reverse was true (33). These results strongly suggested different intrinsic susceptibilities of the etiological agents to the drugs, but no information on the cellular or biochemical basis for these differences exists.

In the present study we compared two *Leishmania* isolates, a naturally ketoconazole-resistant *L. braziliensis* stock (2903) and a highly susceptible *L. mexicana* isolate (NR), and found that the lack of activity of the azoles against the former organ-

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ism was the result of a fundamental biochemical difference which allows them to grow with 14α-methyl sterols as sole membrane sterols. However, we also found that azole-resistant promastigotes were rendered susceptible when treated with combinations of azoles and the allylamine terbinafine, which blocks sterol biosynthesis at the level of squalene epoxidase (40). These results confirm previous findings from our laboratory on the synergistic effects on *Trypanasoma cruzi* of combinations of sterol biosynthesis inhibitors acting at sequential steps of the pathway (32, 42, 44, 47, 48).

MATERIALS AND METHODS

Parasites. *L. braziliensis* 2903, a reference strain (MHOM/BR/75/M-2903), was obtained from the IBBA Laboratory, La Paz, Bolivia, through Shirley Kutner (26). Its promastigote form was maintained in Schneider's medium (29), which was also used for the experiments on drug susceptibility. *L. mexicana* NR was confirmed as such by the analysis of the ribosomal gene spacer (39); it was maintained in liver infusion-tryptose medium (17). In both cases the culture medium was supplemented with 10% fetal calf serum (GIBCO).

In vitro drug susceptibility tests. Parasites were cultivated in the media indicated above; cultures were initiated with a cell density of 10^6 promastigotes per ml, and drugs were added when the cell density reached 2×10^6 promastigotes per ml. Cell densities were measured by turbidimetry and with an electronic particle counter (model ZBI; Coulter Electronics Inc. Hialeah, Fla.). Cell viability was verified by light microscopy using trypan blue.

Studies on lipid composition and metabolism. Total lipids from control and drug-treated cells were extracted and fractionated by silicic acid column chromatography as described before (31, 43, 44). The neutral lipid fractions were first analyzed by thin-layer chromatography (on Merck 5721 silica gel plates with heptane-isopropyl ether-glacial acetic acid, 60:40:4, as the developing solvent) and conventional gas-liquid chromatography (isothermic separation in a 4-m glass column packed with 3% OV-1 on Chromosorb 100/200 mesh, with nitrogen as the carrier gas at 24 ml/min and flame ionization detection in a Varian 3700 gas chromatograph). For quantitative analysis and structural assignments the neutral lipids were separated in a capillary high-resolution column (HP-5 MS column, 30 m by 0.25 mm [inside diameter], 5% phenylmethyl silicone, 0.25-μm film thickness) in a Hewlett-Packard 5890 series II gas chromatograph equipped with an HP5971A mass-sensitive detector. The lipids were injected in ethyl-acetate, the column was kept at 50°C for 1 min, and then the temperature was increased to 250°C at a rate of 10°C per min and finally to 280°C at a rate of 1°C per min. The carrier gas (He) flow was kept constant at 1.1 ml/min. The injector temperature was 250°C, and the detector was kept at 280°C. The assignment of 5,7-diene structures was based on characteristic fragmentation patterns in mass spectrometry but also by chromatography in $AgNO_3$-impregnated Merck 5721 silica gel thin-layer plates and UV spectrophotometry, with a Hewlett-Packard 8452A UV–visible-spectrum spectrophotometer.

Drugs. Ketoconazole (51) was provided by Ingrid Straziota, Janssen Pharmaceutica, Caracas, Venezuela. It was added to the culture medium as an aqueous solution titrated to pH 2.4 with HCl and sterilized by filtration through membrane filters (mean pore size, 0.22 μm; Millipore Corp., Bedford, Mass.). Terbinafine (SF-86-327) (40) was provided by A. Lindenmann and H. Stahelin, Sandoz, Ltd., through Luis Rodriguez, Sandoz de Venezuela, S.A.; D0870 [(R)-2-(2,4-difluorophenyl)-1-(3-[(Z)-4-(2,2,3,3-tetrafluoropropoxy)stiryl]-1,2,4-triazol-1-yl)-propan-2-ol] (13, 43) was kindly provided by Zeneca Pharmaceuticals, Macclesfield, United Kingdom, through William Bastain. The last two drugs were added to the culture medium as dimethyl sulfoxide solutions; the final concentration of dimethyl sulfoxide in the medium never exceeded 1% (vol/vol) and had no effect on growth or morphology of the promastigotes.

RESULTS

Different susceptibilities of *L. braziliensis* and *L. mexicana* to antimycotic azoles. Figure 1A shows that growth of *L. braziliensis* 2903 promastigotes was affected by the sterol C-14 demethylase inhibitor ketoconazole only at 10 μM concentrations of the drug. At these relatively high concentrations azoles are known to produce nonspecific effects on lipid membranes (51–53). By contrast, the *L. mexicana* NR isolate was extremely susceptible to ketoconazole; incubation with 0.03 to 0.06 μM concentrations was able to induce complete growth arrest and cell lysis in 72 h (Fig. 2A). Similar results were obtained with both isolates with the bis-triazol derivative D0870 (results not shown). When we investigated the susceptibility of the two isolates to terbinafine, a sterol synthesis inhibitor which acts at the level of squalene epoxidase (40, 51, 54), the inverse situation was observed: as can be seen in Fig. 3 and 4, *L. braziliensis* 2903 was threefold more susceptible than *L. mexicana* NR to this drug (MICs of 5 and 15 μM, respectively). However, when we incubated the *L. braziliensis* promastigotes with 1 μM terbinafine, which by itself produced a very modest (<10%) effect on growth rate, these organisms became highly susceptible to ketoconazole: as can be seen in Fig. 1B, the MIC of the azole in combination with the allylamine was 0.03 μM, i.e., >300-fold lower than when the azole acted alone, indicating strong synergism. On the other hand, combined treatment of *L. mexicana* promastigotes with ketoconazole and 1 μM terbinafine did not significantly alter the high susceptibility of this strain to the azole (Fig. 2B). Again, essentially identical results were obtained for combinations of terbinafine and D0870.

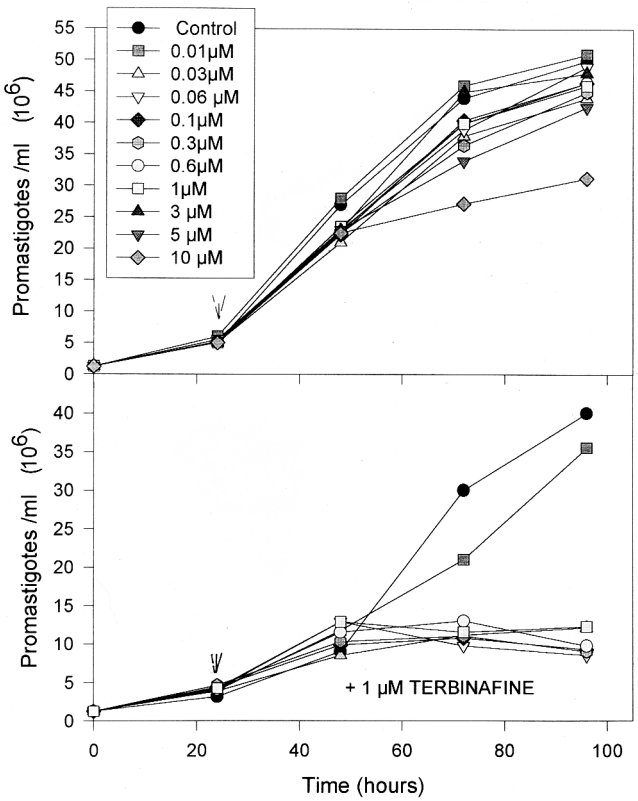


FIG. 1. Effects of ketoconazole on *L. braziliensis* 2903 promastigotes in the absence (top panel) or presence (bottom panel) of 1 μM terbinafine. The cells were cultivated in Schneider's medium (29) with the indicated concentrations of the azole. Arrows indicate the time of addition of the drug(s). Each experimental point is the mean value of three independent cultures; the standard deviations were equal to or less than 10% of the means.

Biochemical studies. We investigated the biochemical basis for these findings. As can be seen in Tables 1 and 2, the sterol compositions of control (untreated) cells of both types were very similar, with episterol [ergosta-7,24(24^1)-dien-3β-ol] and 5-dehydro-episterol accounting for >60% of the total free sterols; squalene and cholesterol (this last compound most probably derived from the growth medium) were also present but at much lower levels. With the *L. braziliensis* isolate, incubation with 0.05 μM ketoconazole or D0870 (data not shown) for 72 h markedly reduced the proportion of 4-desmethyl sterols (from ca. 65% in control cells to ca. 38% in treated cells) and produced a concomitant increase in the levels of 14- and 4-methyl sterols (14-methyl-fecosterol, 14-methyl-5-dehydro-fecosterol, and 4,14-dimethyl-fecosterol), while with 1 μM con-

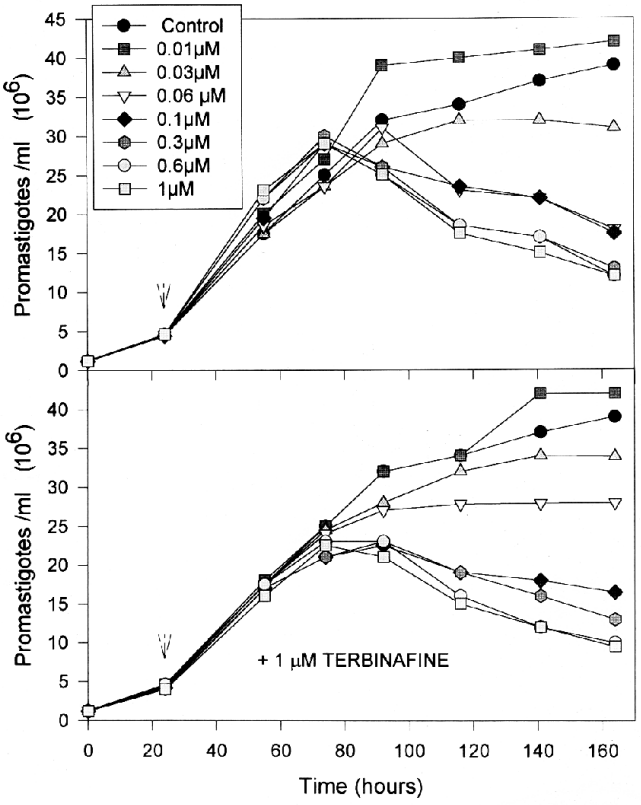


FIG. 2. Effects of ketoconazole on *L. mexicana* NR promastigotes in the absence (top panel) or presence (bottom panel) of 1 μM terbinafine. The cells were cultivated in liver infusion-tryptose medium (17) with the indicated concentrations of the azole. Arrows indicate the time of addition of the drug(s). Each experimental point is the mean value of three independent cultures; the standard deviations were equal to or less than 10% of the means.

centrations of any of the drugs only 14-methyl sterols were present, although no significant effects on growth rate could be detected (Fig. 1A). In contrast, *L. mexicana* NR promastigotes incubated for 72 h with 0.05 μM ketoconazole or D0870 (which corresponded to the MIC for this organism [Fig. 2A]) had no 4-desmethyl sterols; they were completely replaced by 14-methyl- and 4,14-dimethyl-sterols. Incubation of *L. braziliensis*

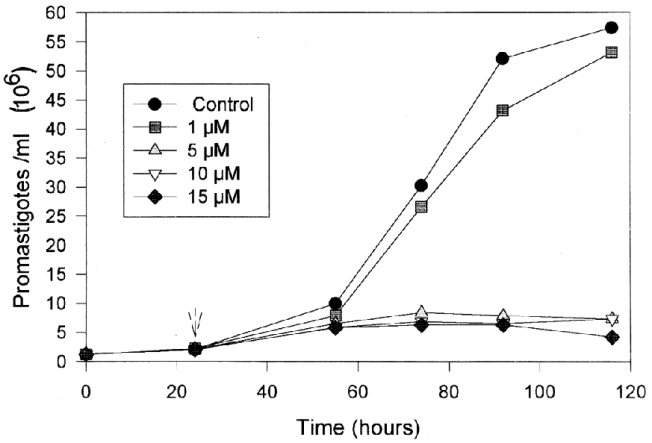


FIG. 3. Effects of terbinafine on *L. braziliensis* 2903 promastigotes. Details are as described for Fig. 1.

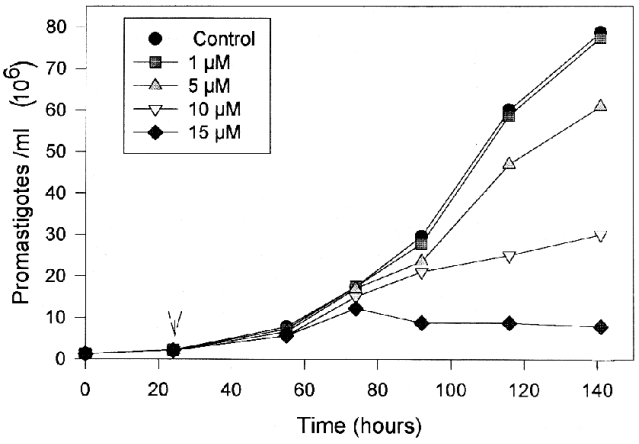


FIG. 4. Effects of terbinafine on *L. mexicana* NR promastigotes. Details are as described for Fig. 2.

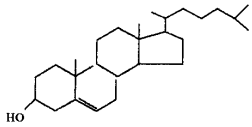
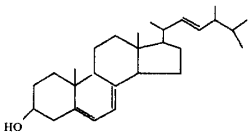
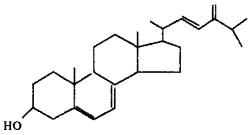
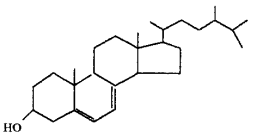
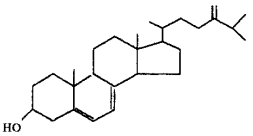
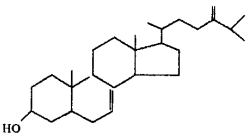
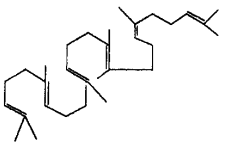
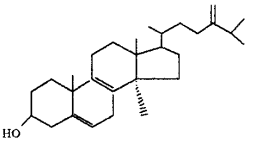
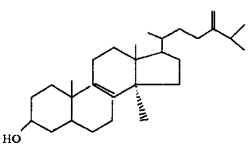
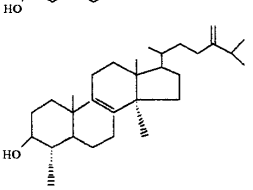
2903 with 1 μM terbinafine for 72 h produced a dramatic increase in the level of squalene (to ca. 70% [by weight] of the total mass of sterols and precursors) with a corresponding decrease in the level of 4-desmethyl sterols (Table 1); in contrast, no modification of the sterol composition of the *L. mexicana* NR promastigotes could be detected after an identical incubation period with the same concentration of the allylamine, and only at 10 μM was a significant accumulation of squalene detected (Table 2). For *L. braziliensis* 2903 promastigotes incubated with the combination of 0.03 μM ketoconazole or D0870 plus terbinafine, which produced complete growth arrest (Fig. 1B), squalene accounted for ca. 80% of the total mass of free sterols and precursors, the rest corresponding to cholesterol and 14-methyl-fecosterol; the sterol composition of *L. mexicana* incubated in the presence of 1 μM ketoconazole plus 1 μM terbinafine was indistinguishable from that of cells incubated with the azole alone.

DISCUSSION

The delayed lytic effect observed with ketoconazole in susceptible *L. mexicana* NR promastigotes is characteristic of sterol biosynthesis inhibitors and has also been found in our previous studies with *T. cruzi*, the causative agent of Chagas' disease (31, 44, 47, 48): the onset of growth arrest and cell lysis was associated with the complete replacement of the endogenous parasite sterols by metabolic precursors (Table 2). On the other hand, the almost complete insensitivity of *L. braziliensis* 2903 to sterol C-14 demethylase inhibitors (corroborated with two different azole derivatives) found in this study is at variance with the results of Beach et al. (6), who observed that their *L. braziliensis* isolates were among the most susceptible organisms of the seven species studied, their proliferation being >90% inhibited by 0.1-μg/ml (ca. 0.2 μM) concentrations of the drug in 5 days. This discrepancy illustrates the marked intra- as well interspecific differences in the susceptibility of the organisms of the *Leishmania* genus to this type of sterol biosynthesis inhibitor, as has been found with other drugs (12, 13). The resistant phenotype was not a result of the two different culture media used to grow the *L. braziliensis* and *L. mexicana* strains, as azole-susceptible *L. braziliensis* isolates also require Schneider's medium for growth.

Our biochemical results indicated that the basis for resistance of strain 2903 is not related to limited intracellular drug levels or to insensitivity or overproduction of the target en-

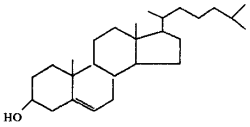
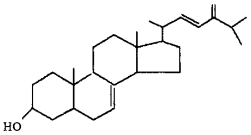
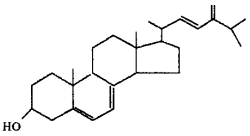
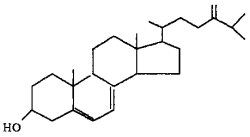
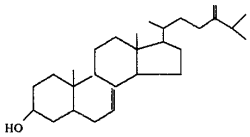
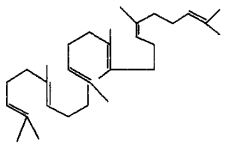
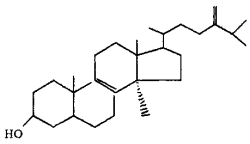
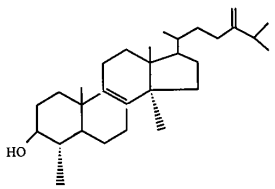
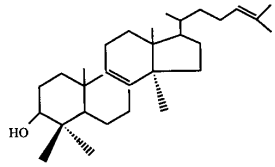
TABLE 1. Effects of ketoconazole and terbinafine on the free sterol composition of *L. braziliensis* 2903 promastigotes[a]

Compound	Molecular structure	Retention time (min)	Composition[b] in cells				
			Control	0.05 μM ketoconazole	1 μM ketoconazole	1 μM terbinafine	1 μM terbinafine +0.03 μM ketoconazole
Exogenous							
Cholesterol		24.2	7.1	11.6	5.3	3.2	6.2
Endogenous, 14-desmethyl							
Ergosterol		26.5	5.5	25.5	<1	11.2	6.6
Ergosta-5,7,22,24(24[1])-tetraen-3β-ol		26.7	3.4	<1	<1	<1	<1
Ergosta-5,7-dien-3β-ol		27.4	<1	<1	<1	3.1	<1
Ergosta-5,7,24(24[1])-trien-3β-ol (5-dehydroepisterol)		28.1	60.1	10.8	<1	9.2	<1
Ergosta-7,24(24[1])-dien-3β-ol (episterol)		28.5	3.9	1.8	<1	3.5	<1
Endogenous, 14-methyl							
Squalene		18.1	20.0	14.4	19.6	69.7	78.6
14-Methyl-ergosta-5,8,24(24[1])-trien-3β-ol		26.9	<1	9.4	14.7	<1	<1
14-Methyl-ergosta-8,24(24[1])-dien-3β-ol (14-methyl-fecosterol)		27.4	<1	24.0	52.5	<1	8.6
4,14-Dimethyl-ergosta-8,24(24[1])-dien-3β-ol (4,14-dimethyl-fecosterol)		29.0	<1	4.2	7.9	<1	<1

[a] Sterols were extracted from cells exposed to the indicated drug concentrations for 72 h; they were separated from polar lipids by silicic acid column chromatography and analyzed by quantitative capillary gas-liquid chromatography and mass sp ectrometry as described in Materials and Methods.

[b] Expressed as mass percentage.

TABLE 2. Effects of ketoconazole and terbinafine on the free sterol composition of *L. mexicana* NR promastigotes[a]

Compound	Molecular structure	Retention time (min)	Composition[b] in cells					
			Control	0.05 μM ketoconazole	1 μM ketoconazole	1 μM terbinafine	10 μM terbinafine	1 μM terbinafine +1 μM ketoconazole
Exogenous								
Cholesterol		24.2	10.9	16.9	6.0	4.7	11.1	7.0
Endogenous, 14-desmethyl								
Ergosta-7,22,24(24[1])-trien-3β-ol		26.7	4.7	<1	<1	<1	<1	<1
Ergosta-5,7,22,24(24[1])-trien-3β-ol		27.1	5.2	<1	<1	<1	<1	<1
Ergosta-5,7,24(24[1])-trien-3β-ol (5-dehydroepisterol)		28.1	59.4	<1	6.5	63.1	44.2	<1
Ergosta-7,24(24[1])-dien-3β-ol (episterol)		28.5	7.5	<1	5.5	23.3	11.0	<1
Endogenous, 14-methyl								
Squalene		18.1	12.3	3.2	20.6	8.9	33.7	21.7
14-Methyl-ergosta-8,24(24[1])-dien-3β-ol (14-methyl-fecosterol)		27.4	<1	7.4	45.6	<1	<1	53.3
4,14-Dimethyl-ergosta-8,24(24[1])-dien-3β-ol (4,14-Dimethyl-fecosterol)		29.0	<1	65.4	13.1	<1	<1	15.3
Lanosterol		30.8	<1	7.1	2.7	<1	<1	2.7

[a] Sterols were extracted and analyzed as described in Table 1.
[b] Expressed as mass percentage.

zyme, as has been proposed for other organisms of this group (18). Figure 1 and Tables 1 and 2 show that, although the sterol C-14 demethylase of the 2903 isolate was significantly less sensitive in vivo than the corresponding enzyme of the NR cells, 2903 promastigotes in which the enzyme was fully inhibited and which consequently had all their natural sterols replaced by 14-methyl sterols (e.g., those exposed to 1 μM drug) still had a normal growth rate. This finding showed that in these cells 14-methyl sterols were able to perform both the bulk and the metabolic or "sparking" role of sterols (9, 11, 38, 50); in contrast, for the *L. mexicana* NR cells, growth arrest and loss of cell viability always correlated with the complete depletion of the normal (4-desmethyl) sterols, as has been found previously by us in the related organism *T. cruzi* (31, 44, 47, 48). The axial 14-α-methyl group has been frequently associated with the incapacity of lanosterol to support normal growth of several eukaryotic organisms (9, 37, 38, 50), a fact explained by the deleterious effects of this sterol on the packing of phospholipid molecules in bilayer membranes (14, 28, 46, 57). However, work with purified methyl sterols bearing the methyl substituent(s) at different positions of the steroid nucleus has shown that 14-methyl sterols can support normal growth of the *Saccharomyces cerevisiae* sterol auxotroph GL7 while lanosterol or sterols bearing a geminal dimethyl or a β-methyl group at C-4 do not (11, 34). This observation indicated that the inability of lanosterol to support growth in yeast cells and possibly in other cells is associated with its C-4 methyl substituents, which probably interfere with hydrogen bonding of the hydroxyl group at C-3 (34). These results suggest a fundamental limitation for C-14 demethylase inhibitors as antiproliferative agents against such organisms as yeasts, fungi, and all *Leishmania* species studied to date, in which the C-4 demethylase can act on its substrate even in the presence of a C-14 methyl group (5, 6). However, most yeasts and fungi are susceptible to azoles, but their currently accepted mechanism of action is the accumulation of toxic sterol intermediates and derivatives such as lanosterol or 24-methylene-dihydrolanosterol, 14α-methyl-ergosta-8,24(24^1)-3β,6α-diol, or obtusifolione (4, 51, 52). The absence of any such intermediates in azole-treated *Leishmania* cells (references 6–8, 20–23, 25, and 45 and this work) could provide an explanation for the resistance of the *L. braziliensis* 2903 isolate, while for azole-susceptible *Leishmania* isolates, including the *L. mexicana* NR stock studied here, we have to conclude, as we did previously in the case of *T. cruzi* (31, 44, 47, 48), that 14-methyl sterol cannot perform the metabolic roles of normal sterols. Knockout experiments with selected genes of the sterol biosynthesis pathway in different *Leishmania* and *T. cruzi* isolates could provide valuable insights concerning these fundamental questions (2–4, 19). Whatever the molecular explanation, our results demonstrate that basic biochemical differences exist among *Leishmania* organisms at the level of specific sterol requirements, which could account for the equivocal results obtained to date with azole antifungals as antileishmanial agents (1, 10, 15, 16, 27, 30, 33, 35, 36, 41, 49, 55, 56).

The differences in the sterol biosynthetic pathway and sterol requirements between *L. braziliensis* 2903 and *L. mexicana* NR were not restricted to sterol C-14 demethylase: as can be seen from Tables 1 and 2, there was a marked difference in susceptibility to terbinafine between the two types of cell in terms of the effects of the drug on both cell proliferation and the intracellular accumulation of squalene. Although these differences could be related to differential drug uptake, metabolism, extrusion, and/or intracellular levels or intrinsic susceptibility of the target enzyme, squalene epoxidase, the relatively high levels of squalene in control (untreated) cells of *L. braziliensis* 2903 and the dramatic rise of squalene levels in the presence of subinhibitory levels of terbinafine (Table 1) could indicate that in this organism the activity of the target enzyme was limiting. Moreover, our results also demonstrated large differences in the capacity of the two species to handle excess intracellular squalene: thus, despite the fact that *L. braziliensis* 2903 was more susceptible to terbinafine than *L. mexicana* NR (Fig. 3 and 4), it could handle very large intracellular levels (ca. 70% of the total mass of sterols and precursors) of the hydrocarbon without significant effects on cell proliferation (Table 1 and Fig. 3). In contrast, in *L. mexicana* NR cells, the accumulation of much lower levels of squalene was associated with sharply reduced growth rates (Table 2 and Fig. 4). These results again showed marked differences in the drug susceptibility of the sterol biosynthetic pathway and lipid requirements between organisms belonging to the *Leishmania* genus.

The marked synergism in the combined effects of ketoconazole and terbinafine on *L. braziliensis* 2903 (Fig. 1), probably resultant from the accumulation of sterols precursors (squalene and 14-methyl sterols) above a critical level to maintain membrane integrity (Table 1), resembles our previous results obtained with *Leishmania amazonensis* (54) and *T. cruzi* with combinations of sterol biosynthesis inhibitors acting at sequential steps of the pathway (32, 42, 44, 47, 48). Although the known insensitivity of murine models of cutaneous leishmaniasis to azoles (12) has limited our ability to test this drug combination in vivo, the fact that both types of drug have been found to be very effective in the treatment of human dermatomycoses suggests that combination therapies with azoles and allylamines, particularly terbinafine, in the treatment of human *L. braziliensis* infections deserve further study.

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