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SYNERGISM *IN VITRO* OF LOVASTATIN AND MICONAZOLE AS ANTI-LEISHMANIAL AGENTS

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Abstract—The antifungal drug miconazole and the cholesterol-lowering agent lovastatin (mevinolin) were used in combination to assess their potency as anti-leishmanial agents. The drug combination was synergistic, being more potent in terms of inhibition of promastigote proliferation, macrophage infection and amastigote numbers. In promastigote cultures the effect was more marked in *Leishmania amazonensis* than *L. donovani*. Analysis of the sterol compositions of both promastigote and amastigote cultures revealed the inhibition of sterol 14α-demethylation by miconazole and showed some apparent evidence of inhibition of sterol biosynthesis by lovastatin.

Inhibitors of sterol biosynthesis are widely used as antifungal agents. The imidazole antifungal drugs such as miconazole and ketoconazole inhibit the action of the cytochrome P450-dependent 14α-methylsterol 14α-demethylase enzyme and consequently reduce production of ergosta-5,7,22-trien-3β-ol (ergosterol), the major fungal sterol. These inhibitors have been investigated as potential anti-leishmanial agents because the sterol biosynthesis pathway in *Leishmania* is very similar to that in the fungi. *Leishmania* species biosynthesize sterols such as ergosta-5,7,24(24[1])-trien-3β-ol‡ and ergosta-5,7,22-trien-3β-ol, and biosynthesis is thought to proceed via lanosterol as in fungi, rather than involving cycloartenol as occurs in photosynthetic plants [1–3].

Ketoconazole and other azoles inhibit sterol 14α-demethylase in *Leishmania* promastigotes [4–6], amastigotes [7, 8] and other closely related protozoans such as the epimastigote form of *Trypanosoma cruzi* [9, 10]. This is accompanied by an accumulation of 14α-methylsterols, a decrease in the usual parasite sterols and inhibition of amastigote proliferation in mouse peritoneal macrophages [11–13] which is assumed to be a result of the disruption of sterol biosynthesis by analogy with the conclusions drawn from fungal studies [14, 15]. Importantly, ketoconazole has been reported to be effective in the treatment of human cutaneous leishmaniasis [16, 17].

A major drawback to the use of the azole drugs is their ability to inhibit other cytochrome P450-dependent enzyme systems. The conversion of androgens to form oestrogens is affected by ketoconazole in pigs, as are the cholesterol side chain cleavage enzymes in bovine adrenal cortex [14]. The steroid 17, 20-lyase and 17-hydroxylase are even more strongly affected. In humans, ketoconazole and, to a lesser extent, miconazole inhibit steroid 17α-hydroxylase, 17,20-lyase and 11β-hydroxylase, all important enzymes in the biosynthesis of the adrenal and testicular steroids [18]. Although mammalian cytochrome P450-dependent enzymes have been shown to be 30–70 times less sensitive than yeast systems to the azole drugs [15], inhibition of the enzymes involved in steroid hormone production is still an important consideration when using these drugs. Other undesirable side-effects are known and about a quarter of patients treated with ketoconazole suffer severe gastric discomfort [19]. It is therefore preferable to limit the drug dose administered and this may possibly be achieved through combination therapy of two dissimilar drugs which exhibit synergism in their actions. In this respect, ketoconazole has been used previously in combination with inhibitors of a number of other steps in sterol biosynthesis in fungi [20], and it has been shown that the allylamine SF-86-327 and ketoconazole exhibit antiproliferative synergism with epimastigotes and amastigotes of *T. cruzi* [21].

In this paper we describe the use of the sterol 14α-demethylase inhibitor miconazole in combination with lovastatin. Lovastatin is an inhibitor of HMG-CoA reductase, a rate-limiting and regulatory enzyme in sterol biosynthesis in humans [22] and fungi [23]. It is used as an anticholesterolemic agent in humans, acting by lowering serum cholesterol [24, 25]. Lovastatin also inhibits sterol biosynthesis and cell proliferation in epimastigotes of *T. cruzi* with strong retardation of parasite growth at a concentration in the order of 50 μg/mL [26]. This dose is below the toxic level for mammals, even that for a strain of abnormally lovastatin-sensitive rabbits [27]. A combination of lovastatin and miconazole has been used previously against *Saccharomyces cerevisiae*: treatment with lovastatin at 10 μg/mL reduced the minimal

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‡ IUPAC-IUB recently recommended some changes in sterol nomenclature [IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN), The nomenclature of steroids. *Eur J Biochem* **186**: 429–458, 1989], notably a change in the numbering of the side chain such that a 24-alkyl substituent, previously assigned as C-28, now becomes C-24[1]. This new numbering system has been adopted in this report.

inhibitory concentration of miconazole 32-fold compared to 10-fold and 6-fold for ketoconazole and clotrimazole, respectively [28]. As the biosynthesis of sterols in *Leishmania* is so similar to that in fungi the potential of this combination as an anti-leishmanial measure has been investigated.

MATERIALS AND METHODS

Promastigote cultures. Leishmania strains used in this study were *L. donovani* (MHOM/BL/67/ITMAP263 and *L. amazonensis* MPRO/BR/73/M1845; LV78. *L. donovani* and *L. amazonensis* promastigotes were grown at 27° in the semi-defined HO minimum essential medium supplemented with 15% heat-inactivated foetal calf serum. Cultures (15 mL) were initiated at 2×10^6 cells/mL and allowed to grow for 48 hr before harvesting by centrifugation. Cells were then washed twice with Locke's solution before extraction of sterols. Cell populations were determined using a Coulter counter.

Macrophage and amastigote cultures. Mouse peritoneal macrophages were obtained as described previously [5] after i.p. injection of 2 mL starch solution. Cells were cultured in RPMI supplemented with 15% heat-inactivated foetal calf serum [30] at a density of $20 \times 10^6/75\,cm^2$ flask (40×10^6 total cells) and were allowed to spread at 37° for 24 hr before addition of *L. amazonensis* (LV78) promastigotes (20 promastigotes/macrophage). Cells were incubated at 35° and left for 24 hr to allow an infection to become established. The medium was changed and cells incubated for a further 24 hr before drug treatment.

Drug treatments. All drugs were added as solutions in dimethyl sulphoxide. Treatment was for 24 hr before harvest with amastigote cultures and 48 hr from culture initiation until harvest with promastigotes.

Isolation of amastigotes. Infected macrophages were washed with warm Locke's solution and detached from the flask in 5 mL fresh Locke's solution using a rubber policeman. A portion of the flask was left unscraped and stained with Geimsa for counting. Cells were pelleted, resuspended in 2 mL phosphate-buffered saline containing 2 mM EDTA and lysed, releasing the amastigotes, by 20 passes in a Potter–Elvejhem homogenizer. The resulting material was pelleted and resuspended in 7.5 mL 45% percoll in 0.25 M NaCl. This was layered onto a 2.5 mL cushion of 90% percoll in 0.25 M NaCl and centrifuged at 4000 *g* for 30 min in a swing-out rotor. The released amastigotes were isolated from the 90%:45% interface, which was diluted 10 times with Locke's solution before recovery of the parasites by centrifugation.

Extraction of sterols. Harvested cells were frozen at −20° until required. After defrosting and resuspension in 1 mL distilled water, 5α-cholestane was added as an internal standard (1 μg to the amastigote culture, 10 μg to the promastigote culture). Samples were stirred in 10 mL $CHCl_3$:MeOH (2:1) for 3 hr at room temperature. After this time, the volume of the extract was reduced under N_2 until cloudy. It was then extracted with 2×10 mL redistilled petroleum ether (40–60° b.p.), dried over Na_2SO_4 and then taken to dryness under N_2. A portion was removed and derivatized to form the sterol trimethylsilyl ethers for analysis by GC-MS as described previously [2]. All GC-MS identifications were in agreement with data presented in our previous publications [2, 4–7].

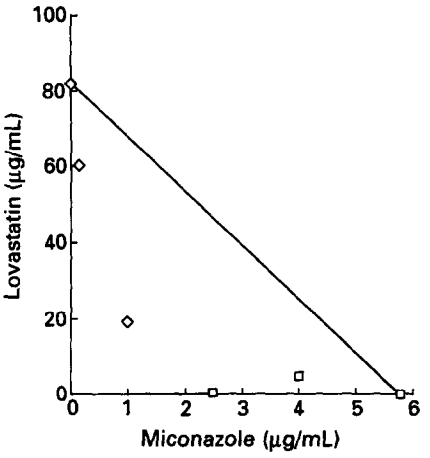

Fig. 1. Isobologram analysis of culture population of *L. donovani* promastigotes treated with combinations of lovastatin and miconazole. The points show the IC_{50} for miconazole (□) either alone or with varying concentrations of lovastatin, and the IC_{50} for lovastatin (◇) used alone or with varying concentrations of miconazole.

RESULTS

Effects on promastigote proliferation

Isobologram analysis of the populations of *L. donovani* promastigote cultures treated with a range of concentrations of miconazole and lovastatin is shown in Fig. 1. The IC_{50} values for miconazole and lovastatin were 6 and 82 μg/mL, respectively. Combinations of the two drugs show clear potentiation as all data points representing these treatments are found close to the axes on the isobologram. The reduction of promastigote cell numbers by a combination of the two drugs thus exceeded the additive effect of the two drugs alone.

A similar experiment was performed on *L. amazonensis* promastigotes (Fig. 2). The potentiation effect of the combination of the two drugs on cell proliferation was similar to that observed with *L. donovani*, except that the apparent IC_{50} values were 3 and 20 μg/mL for miconazole and lovastatin, respectively. *L. amazonensis* was found to be considerably more sensitive to lovastatin than *L. donovani*.

Effects on promastigote sterols

The sterol combinations of the above *L. donovani* cultures were examined (Table 1). Untreated cultures contained primarily cholesterol, which is derived by uptake from the culture medium [2, 5], ergosta-5,7,22-trien-3β-ol and ergosta-5,7,24(24[1])-trien-3β-

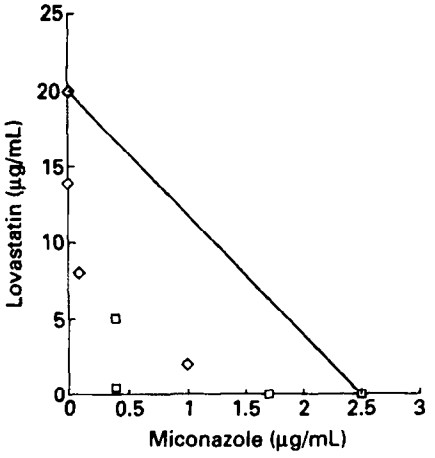

Fig. 2. Isobologram analysis of culture population of *L. amazonensis* promastigotes treated with combinations of lovastatin and miconazole. The points show the IC_{50} for miconazole (□) either alone or with varying concentrations of lovastatin, and the IC_{50} for lovastatin (◇) used alone or with varying concentrations of miconazole.

ol. There was also a significant amount of cholesta-5,7,22,24-tetraen-3β-ol together with traces of ergostadienols, stimasta-5,7,22-trien-3β-ol and stigmasta-7,22-dien-3β-ol.

Treatment with 1.0 μg/mL miconazole resulted in the accumulation of 14α-methylsterols, particularly 14α-methylcholesta-8,24-dien-3β-ol, 14α-methylergosta-8,22,24(24[1])-trien-3β-ol, 14α-methylergosta-8,24(24[1])-dien-3β-ol and 4α-14α-dimethylcholesta-8,24-dien-3β-ol. The accumulation of these sterols demonstrates that inhibition of the 14α-demethylase enzyme and consequent retention of the 14α-methyl group does not preclude the elimination of the second 4α-methyl group, the introduction of the Δ^{22}-double bond or the *S*-adenosylmethionine-dependent sterol C-24 transmethylation. It is important to note that the accumulation of apparently large amounts of these abnormal 14α-methylsterols was accompanied, at this concentration of miconazole, by no significant effect on cell proliferation. The amount of total sterol (μg sterol per 10^8 cells) was only slightly reduced when compared to the control value. The increase in 14α-methylsterols was balanced by the decline in the cholesta- and ergostatetraenols and the ergostadienols, whilst the proportion of ergosta-5,7,22-trienol-3β-ol remained relatively unchanged. Only cholesterol and ergosta-5,7,22-trien-3β-ol were detected in the cellular material recovered from the culture exposed to 10 μg/mL miconazole which causes severe inhibition of growth.

Treatment with lovastatin alone at 0.5 and 5.0 μg/mL resulted in a significant increase in the proportion of ergosta-5,7,22-trien-3β-ol; in cultures treated with 0.5 μg/mL lovastatin there was 37.2% of this sterol in the mixture compared with 15.3% in the control. The proportion of ergosta-5,7,24(24[1])-trien-3β-ol remained constant with increasing lovastatin concentration except in cultures treated with 50 μg/mL, in which it was not detected. Interestingly, the only sterols observed in cells treated with 50 μg/mL lovastatin were cholesterol, a diminished proportion of ergosta-5,7,22-trien-3β-ol, some cholesta-5,7-dien-3β-ol and traces of 14α-methylcholesta-8,24-dien-3β-ol.

Treatment of cultures with combinations of the drugs resulted in accumulation of 14α-methylsterols in a similar manner to that seen with miconazole alone, although as the concentration of lovastatin was increased, the percentage of these sterols declined, with the exception of 14α-methylergosta-8,22,24(24[1])-trien-3β-ol. A possible explanation for the continued presence of this sterol is that, in trying to preserve an essential metabolically active sterol in a certain cellular location, the organism inserts a Δ^{22}-bond into the 14α-methylsterol in order that it may, at least partially, fulfil this role. For any given concentration of lovastatin, the amount of sterol was considerably higher in the presence of 1.0 μg/mL miconazole than with lovastatin alone. The serum-derived sterol, cholesterol, became the predominant sterol whilst the proportions of leishmanial sterols were considerably reduced in cultures treated with drug combinations containing 50 μg/mL lovastatin or 10 μg/mL miconazole.

The occurrence of stigmast-5-en-3β-ol (sitosterol) in one incubation is notable. The previously described pathways of sterol biosynthesis in *Leishmania* promastigotes [2, 3] exclude this sterol as the parasite was believed to be unable to reduce the Δ^7-double bond in $\Delta^{5,7}$-sterols, a view supported by the absence of C_{28}-Δ^5-sterols such as ergost-5-en-3β-ol in promastigotes. The various $\Delta^{5,7}$-sterols found in the promastigotes were thus believed to be the final products of *de novo* synthesis in the organisms. A subsequent study (data not shown) has demonstrated that the foetal calf serum used to supplement the culture medium did not contain a detectable amount of stigmast-5-en-3β-ol and therefore it does not appear that the material detected in the promastigote culture could have originated by uptake from this source. The observation of stigmast-5-en-3β-ol in *Leishmania* requires further investigation to determine its origin and to see if its appearance is possibly correlated with stressing of cultures, for example by drug treatment as in the present case.

Effects on amastigote proliferation

Treatment of *L. amazonensis* amastigotes in mouse peritoneal macrophages showed that miconazole was more effective than lovastatin in inhibiting amastigote proliferation (Fig. 3). For example, treatment with 5.0 μg/mL miconazole reduced the mean number of amastigotes per infected macrophage from 34 to 20 and also reduced the percentage of infected macrophages from 95 to 58%. The IC_{50} for miconazole was found to be 8 μg/mL. Treatment with lovastatin up to a concentration of 10 μg/mL had little effect on the percentage of macrophages infected or the number of amastigotes in the macrophages (Fig. 3). Within the limitations of drug solubility it was not possible to obtain an IC_{50} for lovastatin, but it was considered to be well in excess of 10 μg/mL.

When miconazole and lovastatin were used in

Table 1. Percentage sterol composition of *L. donovani* promastigotes treated with miconazole and lovastatin

Sterol	Control	Miconazole			Lovastatin			Lovastatin with increasing miconazole concentrations							
	A*	B	C	D	E	F	G	H	I	J	K	L	M	N	O
1†	17.0	19.2	16.5	85.5	21.2	17.1	77.0	25.3	19.0	19.1	11.3	62.7	90.4	83.4	82.8
2a	8.7	1.3	—	—	1.3	1.4	6.6	1.6	0.8	—	—	—	—	4.3	—
3	—‡	—	11.4	—	3.4	5.9	0.8	—	—	10.9	6.9	—	1.7	5.4	2.2
4	—	—	10.5	—	—	—	—	—	—	12.6	10.9	10.3	—	—	—
5	12.5	1.6	—	—	4.1	2.1	—	2.4	2.1	—	—	—	—	—	—
6	15.3	34.9	12.7	14.5	37.2	34.1	9.4	33.5	36.8	13.8	28.5	23.6	4.9	6.9	11.5
7	—	—	15.5	—	—	—	—	—	—	14.6	12.0	3.4	—	—	—
8	4.6	—	—	—	1.3	3.8	—	0.7	—	—	—	—	2.9	—	2.3
9	4.2	—	—	—	—	—	—	—	—	—	—	—	—	—	—
10	—	3.0	9.0	—	—	—	—	2.7	3.1	10.1	8.1	—	—	—	—
11	27.9	30.0	17.5	—	27.6	31.5	—	21.5	28.2	10.4	10.3	—	—	—	—
12	1.8	—	3.0	—	—	—	—	—	—	3.8	1.7	—	—	—	—
13	3.0	5.5	1.5	—	3.8	3.8	—	11.4	5.3	2.2	1.7	—	—	—	—
14	tr	—	2.5	—	—	—	—	—	—	2.5	0.9	—	—	—	—
15	5.0	—	—	—	—	—	—	—	—	—	1.4	—	—	—	—
16a	—	—	—	—	—	—	—	—	—	—	5.3	—	—	—	—
17a	—	—	—	—	—	—	—	—	—	—	1.1	—	—	—	—
TC	2.25	2.37	2.29	0.56	2.36	2.50	1.59	2.33	2.18	1.68	1.24	1.05	0.39	0.49	0.58
SC	12.53	5.56	9.38	4.07	4.23	7.95	2.39	5.26	21.88	8.91	34.02	4.51	2.46	5.02	4.81

* Treatment with miconazole (μg/mL)/lovastatin(μg/mL): (A) 0.0/0.0; (B) 0.1/0.0; (C) 1.0/0.0; (D) 10/0.0; (E) 0.0/0.5; (F) 0.0/5.0; (G) 0.0/50; (H) 0.1/0.5; (I) 0.1/5.0; (J) 1.0/0.5; (K) 1.0/5.0; (L) 1.0/50; (M) 10/0.5; (N) 10/5.0; (O) 10/50.

† Sterol identifications: (1) cholest-5-en-3β-ol; (2a) cholesta-5,7-dien-3β-ol; (3) 14α-methylcholesta-8,24-dien-3β-ol; (4) 14α-methylergosta-8,22,24(24^1)-trien-3β-ol; (5) cholesta-5,7,22,24-tetraen-3β-ol; (6) ergosta-5,7,22-trien-3β-ol; (7) 14α-methylergosta-8,24(24^1)-dien-3β-ol; (8) ergosta-5,7,22,24(24^1)-tetraen-3β-ol; (9) ergosta-7,22-dien-3β-ol; (10) 4α,14α-dimethylcholesta-8,24-dien-3β-ol; (11) ergosta-5,7,24(24^1)-trien-3β-ol; (12) ergosta-5,7-dien-3β-ol; (13) ergosta-7,24(24^1)-dien-3β-ol; (14) stigmasta-5,7,22-trien-3β-ol; (15) 4α,14α-dimethylergosta-8,24(24^1)-dien-ol; (16a) stigmast-5-en-3β-ol; (17a) stigmasta-7,22-dien-3β-ol; (TC) total cells $\times$ 10^8; (SC) sterol (μg) per 10^8 cells.

‡—, not detected.

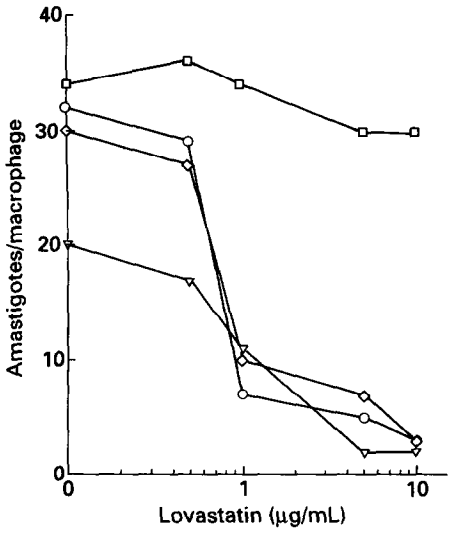

Fig. 3. Treatment of *L. amazonensis* amastigotes with combinations of lovastatin and miconazole. Amastigotes were grown in mouse peritoneal macrophages and were treated for 18 hr before harvesting. Amastigote population is expressed as number of amastigotes per infected macrophage. (□) Control; (◇) 0.5 μg/mL miconazole; (○) 1.0 μg/mL miconazole; (▽) 5.0 μg/mL miconazole.

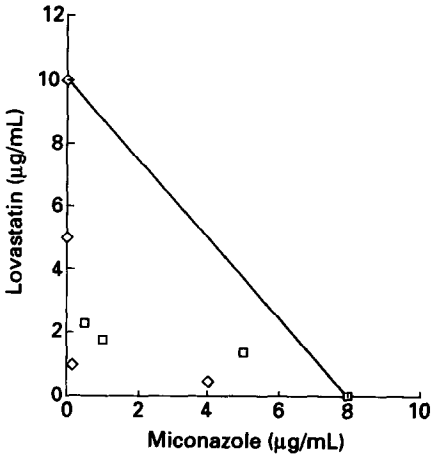

Fig. 4. Isobologram analysis of populations of *L. amazonensis* amastigotes per infected macrophage treated with combinations of lovastatin and miconazole. The points show the IC_{50} for miconazole (□) either alone or with varying concentrations of lovastatin, and the IC_{50} for lovastatin (◇) used alone or with varying concentrations of miconazole. Within the constraints of this experiment it was impossible to obtain an IC_{50} value for lovastatin. For this analysis the IC_{50} has been taken to be 10 μg/mL, although the real value is well in excess of this concentration.

combination, a similar synergistic effect was observed to that seen with the promastigotes. For example, 1.0 μg/mL miconazole reduced the IC_{50} of lovastatin to 1.8 μg/mL. Similarly, 0.5 μg/mL lovastatin reduced the IC_{50} of miconazole to 0.13 μg/mL. Thus, the effect of the two drugs in combination appears to be synergistic as the reduction in infection can not be explained by a simple addition of the effects of the two inhibitors. When the numbers of amastigotes per infected macrophage in drug-treated cultures are subjected to isobologram analysis (Fig. 4) the potentiation between these two drugs becomes clear.

Effects on amastigote sterols

Analyses of the sterols of amastigote cultures treated with miconazole and lovastatin combinations are shown in Table 2. The composition of control cultures was similar to that reported previously [7, 8]. The amastigotes contained cholesterol, presumably derived from the macrophage, ergosta-5,7,24(24^1)-trien-3β-ol and ergosta-7,24(24^1)-dien-3β-ol, together with the C_{29}-sterols stigmasta-5,7-dien-3β-ol, stigmast-5,7,24(24^1)-trien-3β-ol and stigmasta-7,24(24^1)-dien-3β-ol. Considerable amounts of the C_{29}-sterols have been noted previously in amastigotes compared to the rather low amounts of these particular sterols present in the promastigote form of the organism [2, 7]. In several of these amastigote cultures there were also significant quantities of stigmast-5-en-3β-ol. The presence of ergosta-5,24(24^1)-dien-3β-ol (24-methylenecholesterol) in the control amastigotes indicates the possible conversion by the parasite of cholesta-5,24-dien-3β-ol (desmosterol) derived from the host macrophage cells as reported previously [31, 32] (see also Discussion). The *L. amazonensis* strain used for the present work is apparently unable to biosynthesize ergost,5,7,22-trien-3β-ol in any quantity, presumably because the Δ^{22}-desaturase enzyme activity is limiting. This had been observed previously in promastigotes and amastigotes of *L. mexicana* [2, 4, 7].

Treatment with 1.0 μg/mL miconazole caused accumulation of 14α-methylergosta-8,24(24^1)-dien-3β-ol, 4α, 14α-dimethylcholesta-8,24-dien-3β-ol and 4α, 14α-dimethylergosta-8,24(24^1)-dien-3β-ol. At the higher concentrations of lovastatin, which caused some reduction in amastigote numbers, there was an increase in the proportion of ergosta-5,7,24(24^1)-trien-3β-ol present in the sterol mixture. It is possible that the cell tries to maintain the concentration of this sterol for a specific function. Treatment with miconazole and lovastatin together had similar effects on the sterol composition of amastigotes as on that of promastigotes. The presence of miconazole caused the accumulation of 14α-methylsterols and the percentage of ergosta-5,7,24(24^1)-trien-3β-ol was maintained at the expense of the ergostadienols. It is striking that in the amastigotes the proportion of stigmasta compounds was also maintained at a relatively high level after all drug treatments which is suggestive of some particular needs for these C_{29}-sterols by the amastigote form compared to the promastigotes which generally have a rather low content of these sterols.

DISCUSSION

The sterol biosynthesis-inhibiting drugs lovastatin

Table 2. Percentage sterol composition of *L. amazonensis* amastigotes treated with miconazole and lovastatin

Sterol	A*	B	C	D	E	F	G	H
1†	11.7	18.1	20.7	11.2	37.3	11.8	14.5	8.7
2b	1.3	1.2	—	—	—	—	—	—
7	1.5	8.8	4.3	3.0	0.9	1.0	—	1.0
10	—‡	13.5	8.5	8.5	10.1	4.5	—	—
11	18.3	17.0	11.1	11.1	6.6	9.9	34.5	24.3
13	12.7	8.2	7.6	3.9	13.5	3.3	18.6	8.5
15	—	4.7	3.7	—	2.3	—	2.6	1.6
16a	24.4	1.8	15.9	33.3	—	37.7	—	26.0
16b	11.8	—	8.3	15.6	—	19.8	2.4	14.5
17b	11.4	18.8	13.3	10.2	16.2	7.9	20.0	11.2
17c	6.8	7.9	6.6	3.4	13.0	4.0	7.4	4.1
SA	13.44	3.41	5.15	13.69	3.46	9.90	7.80	13.69

* Treatment with miconazole (μg/mL)/lovastatin (μg/mL): (A) 0.0/0.0; (B) 1.0/0.0; (C) 1.0/1.0; (D) 5.0/1.0; (E) 1.0/10; (F) 0.0/1.0; (G) 0.0/5.0; (H) 0.0/10.

† Sterol identifications: (a) cholest-5-en-3β-ol; (2b) ergosta-5,24(24[1])-dien-3β-ol (24-methylenecholesterol); (7) 14α-methylergosta-8,24(24[1])-dien-3β-ol; (10) 4α,14α-dimethylcholesta-8,24-dien-3β-ol; (11) ergosta-5,7,24(24[1])-trien-3β-ol; (13) ergosta-7,24(24[1])-dien-3β-ol; (15) 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol; (16a) stigmast-5-en-3β-ol; (16b) stigmasta-5,7-dien-3β-ol; (17b) stigmasta-5,7,24(24[1])-trien-3β-ol; (17c) stigmasta-7,24(24[1])-dien-3β-ol; (SA) sterol (μg) per culture.

‡ —, not detected.

and miconazole inhibit proliferation of *L. donovani* promastigotes *in vitro* having IC_{50} values of 82 and 6 μg/mL, respectively. When used in combination these values were reduced considerably, for example, to 19 μg/mL for lovastatin with 1.0 μg/mL miconazole and to 3 μg/mL for miconazole with 0.5 μg/mL lovastatin. Similar effects were observed with *L. amazonensis* amastigotes. Isobologram analysis revealed a significant degree of potentiation between the two drugs in the systems tested. The concentrations of the drugs needed in combination treatment were considerably lower than those required for elimination of *Leishmania* promastigotes [6] or amastigotes [7, 8] and also of *T. cruzi* epimastigotes [9, 21, 33] by azoles alone, and for elimination of *T. cruzi* epimastigotes by lovastatin alone [5]. They are also lower than the concentrations found to be effective in combination against *S. cerevisiae* [28].

The antimycotic drug miconazole inhibits the 14α-methylsterol 14α-demethylase enzyme of sterol biosynthesis in both *Leishmania* promastigotes and amastigotes. This results in the accumulation of 14α-methylsterols, such as 4α,14α-dimethylcholesta-8,24-dien-3β-ol, 4α,14α-dimethylergosta-8,24(24[1])-dien-3β-ol and 14α-methylergosta-8,24(24[1])-dien-3β-ol. 14α-Methylergosta-8,22,24(24[1])-tetraen-3β-ol was also observed in the promastigotes. Similar results have already been reported following treatment of *Leishmania* with other azole drugs such as ketoconazole [5, 6]. The occurrence of the 14α-methylsterols indicates that the action of the 4α-demethylase, the Δ^{22}-desaturase and the *S*-adenosylmethionine-dependent sterol C-24 transmethylase are relatively unaffected by the inhibition of 14α-demethylase and consequent presence of a 14α-methyl group in the sterol substrate(s). It is also interesting to note that, despite such an apparently large accumulation of 14α-methylsterol, there was no appreciable effect on growth unless a high concentration of drug was administered.

An interesting observation with promastigote cultures, was that the highest and lowest concentrations of each drug reduced the total sterol content of the cells (Table 1) rather more than did the intermediary drug concentration. These effects could presumably be a consequence of cell death or diminished carbon flux through the pathway with high or low drug doses, respectively; the highest concentrations of lovastatin and miconazole may have caused cell death by rapid depletion of sterol whilst the sub-inhibitory concentrations merely lowered cell sterol content but only to a level which could still support some growth. Sterol accumulation was marked in two cultures (I and K in Table 1) where a high degree of drug synergism in antiprotozoal action was observed. After treatment of promastigotes with 1.0 μg/mL miconazole and 5.0 μg/mL lovastatin, the cell number was 55% of the control value but the cells contained 2.7 times as much sterol as the control cells.

An important consideration when discussing the above results is that the parasite cells have an additional source of sterol over and above that produced by their own biosynthetic pathways. The use of foetal calf serum to supplement the medium gives both the promastigote and macrophage cells access to a ready supply of cholesterol and also possibly to desmosterol. This exogenous sterol is then available to the amastigote, which also has a further cholesterol and desmosterol source from that biosynthesized *de novo* by the host macrophage. When sterol biosynthesis is restricted, for example with imidazole drugs, the amastigotes may be able to use macrophage-derived desmosterol and convert it to 24-methylenecholesterol (ergosta-5,24(24[1])-

dien-3β-ol). Further conversion to 24-methylcholesterol and 24-methylcholesta-5,22-dien-3β-ol is also possible in some *Leishmania* species which produce ergosta-5,7,22-trien-3β-ol as a major sterol component [31, 32]. In the case of the *L. amazonensis* strain used in this study these latter sterol interconversions would be unlikely to occur as the protozoan apparently has a limited capability to perform either $\Delta^{24(241)}$-bond reduction or introduction of the Δ^{22}-bond [2, 3].

Reports about organisms as diverse as plants [34], yeasts [35] and *Paramecium tetraurelia* [36] have suggested that sterols have two functions in normally growing cells. Firstly there is a "bulk" role as architectural molecules in membrane structure. The requirements for this role are rather unspecific with respect to sterol structure but relatively large quantities of sterol are needed. Secondly, there is the "sparking" function, also known as the "regulatory", "synergistic" or "metabolic" role. This is a requirement for a small quantity of sterol with specific structural features which is essential for cell proliferation. In relation to the sparking role of sterols, a recent study has examined the effects of sterol biosynthesis inhibitors on mitosis [37]. A proposed explanation for the growth inhibitory activity of the sterol 14α-demethylase inhibitors is that the accumulating 14α-methylsterols disrupt membrane structure by virtue of their axial 14α-methyl group which protrudes from the lower face of the otherwise planar sterol molecule. It is also considered possible that this effect may be secondary to the inhibition of production of the sparking sterols. The use of host sterol by the amastigote as a substrate for its C-24 transmethylase enzyme may be an attempt to produce a sterol sufficiently similar to the normal leishmanial sterols to act in the sparking role. Thus, depletion of any pool of sterol available to act directly, or indirectly after modification, in a possible sparking role appears to be attractive as an effective means of retardation of cell division. Recent work with yeast cultures also suggests that this might be the case [38].

24-Methylenecholesterol, which may be produced from the macrophage sterol demosterol, was present in control amastigote cultures and those treated with miconazole alone, but could not be detected after lovastatin treatment. It is possible that lovastatin, which is a drug with inhibitory action in mammalian cells, reduces macrophage sterol biosynthesis to such an extent that any desmosterol biosynthesized is required immediately by the macrophage itself for cholesterol biosynthesis and so is unavailable to the parasite.

The imidazole drugs such as miconazole are hydrophobic which may hinder absorption. It is possible that lovastatin, by reducing sterol production in *Leishmania*, may cause changes in membrane permeability and that this then allows greater uptake of the lipophilic molecules into the cell. The azoles may then act by causing more extensive disruption of sterol biosynthesis and adverse effects on growth by either increasing the 14α-methylsterols in the membranes or perhaps by direct incorporation of the drugs themselves into the membranes.

Whatever the reason for the synergistic effect observed with miconazole and lovastatin, their combined use appears to offer a promising approach for the treatment of leishmanial infections. The possibility of using drugs such as lovastatin, which has a low toxicity to man, to increase the efficacy of other triazole antifungal drugs with good pharmacological properties in order to develop an effective therapy for leishmaniasis warrants further investigation in an *in vivo* situation.

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