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PRELIMINARY COMMUNICATION

EFFECT OF THE ALLYLAMINE ANTIFUNGAL DRUG SF 86-327* ON THE GROWTH AND STEROL SYNTHESIS OF LEISHMANIA MEXICANA MEXICANA PROMASTIGOTES

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In the search for drugs which are clinically effective against infections of the Leishmania species, attention has been directed recently towards the use of azole derivatives that have potent antifungal activity [1-10]. Thus, ketoconazole was shown [2-5] to inhibit the multiplication of L. mexicana mexicana promastigotes in culture and also to prevent the propagation of amastigotes of various Leishmania species in human monocyte-derived macrophages and in mouse peritoneal macrophages. Ketoconazole was also effective against cutaneous and mucocutaneous leishmaniasis in man [6-8] and against visceral leishmaniasis in the golden hamster when administered subcutaneously [9], but it was apparently ineffective against cutaneous leishmaniasis in BALB/C mice when given orally [10].

Ketoconazole and similar antifungal agents prevent the production of ergosterol in fungi by inhibiting the C-14 demethylation of lanosterol, and there is a consequent accumulation of 14α-methylsterols [11-13]. The C-14 demethylation reaction is cytochrome P-450 dependent [14], and it appears that the imidazole and triazole antimycotics lower the content of the microsomal cytochrome P-450 in yeasts [15].

The major sterol of several Leishmania species is ergosta-5,7,24(28)-trien-3β-ol [16-18], and it is apparently biosynthesized by a route which is similar to that producing sterol in fungi [18]. The inhibition of multiplication of L. mexicana mexicana promastigotes by ketoconazole is accompanied by alterations in the pattern of sterol biosynthesis [2]. This results in the accumulation of 14α-methylsterols [19] as previously observed with similarly treated fungi [11, 12].

The fact that an antimycotic drug which affects sterol synthesis is also effective against the pathogenic protozoan L. mexicana mexicana has prompted us to test the effect of another new class of antimycotic agents, the allylamines, on this organism. The allylamines (naftifine, SF 86-327) are very active towards a wide variety of filamentous fungi and yeasts [20-23]. They act at the molecular level by inhibiting squalene-2,3-epoxidase with a resultant accumulation of squalene and decline in the sterol content of the fungal cell [23-26]. This communication describes the effects of the allylamine SF 86-327 on the growth of L. mexicana mexicana and upon the incorporation of [2-^{14}C]mevalonic acid into squalene and sterols by this organism.

MATERIALS AND METHODS

Culture of promastigotes. L. mexicana mexicana Walter Reed 227 (obtained from Dr. J.D. Berman, Walter Reed Army Institute of Research) was grown in 1-liter batches of RE III medium of Steiger and Steiger [27] supplemented with additional hemin (5 mg/liter),

*SF 86-327 is (E)-N-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalenemethanamine hydrochloride.

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solvent extracted Bacto Peptone (1 g/liter), rabbit whole blood lysate (6.7 ml/liter) and heat-inactivated fetal calf serum (100 ml/liter). Medium and supplements were filter sterilized (Millipore, 0.22 μm); the initial pH was 7.4. Cultures were inoculated to give a starting population of about 3 x 10^6 promastigotes/ml. Cell counts were made using a hemocytometer.

Exposure of promastigotes to SF 86-327. Cultures of promastigotes in slanted plastic tissue culture tubes were incubated for 4 days with added SF 86-327 (0-10 μg/ml medium) supplied as a solution in dimethyl sulfoxide (DMSO, 0-5 μl/ml medium). After incubation, the numbers of elongate motile promastigotes were counted. The minimal inhibitory concentration (MIC) was the lowest concentration of SF 86-327 required to stop population growth.

Effect of SF 86-327 on the incorporation of [2-^{14}C]mevalonic acid into lipids of L. mexicana mexicana. Six cultures (each 1 liter) of L. mexicana mexicana in the supplemented RE III medium were grown for 3 days to give cell counts in the range of 30 to 38 x 10^6 cells/ml. [2-^{14}C]Mevalonic acid (NEN, 10 μCi, sp. act. 50 Ci/mole) in ethanol (1 ml) was added to each flask; two flasks were set aside as the controls. To two other flasks was added 5 ml DMSO while the final two flasks each received 5 ml DMSO containing 10 mg SF 86-327 to give a final concentration of 10 μg SF 86-327/ml. The cultures were then maintained for a further 20 hr before harvesting by centrifugation (4000 rpm for 15 min). The cells were extracted exhaustively with $CHCl_3$-MeOH [20] and the bulk of the solvent was removed by rotary evaporation. The mixture was then partitioned with petroleum ether to give a lipid sample containing squalene and sterols. The lipids were separated by chromatography on a 20 g column of alumina, Brockman grade III, eluted with 200 ml portions of petroleum ether (mainly hydrocarbons including squalene); petroleum ether-diethyl ether (P-E) 98:2 (steryl esters); P-E, 96:6 (4,4-dimethylsterols); P-E, 91:9 (4α-methylsterols); and P-E, 80:20 (4-desmethylsterols). The radioactivity in the samples was determined by liquid scintillation counting (Omnifluor, NEN).

Characterization of radioactive squalene. (i) Carrier squalene (50 μg) was added to the labelled hydrocarbon fraction. The mixture was then subjected to TLC on silica gel developed with cyclohexane-diethyl ether (98:2) and an autoradiogram was prepared (Kodak X-Omat AR film). (ii) Carrier squalene (50 or 100 mg) was added to portions of the hydrocarbon fractions from the control and SF 86-327 incubations; the squalene was then purified via the thiourea clathrate [28].

Quantitation of squalene and sterols. These compounds were estimated by GLC on a 30 m x 0.32 mm SPB-5 capillary column [19]; n-docosane was used as the internal standard for squalene estimation and 5α-cholestane was employed for the sterol quantitations.

RESULTS AND DISCUSSION

SF 86-327 effectively retarded L. mexicana mexicana growth over a range of concentrations from 0.5 to 10 μg/ml (Table 1). The minimal inhibitory concentration (MIC) value of 10 μg/ml compares with the value reported [22] for some fungal species (Aspergillus, Candida) but is considerably higher than that found for others (Trichophyton, Microsporum) [22, 29].

When cells were cultured with [2-^{14}C]mevalonic acid, radioactivity was incorporated significantly into squalene and 4-desmethylsterols with some labelling also in the steryl esters, 4,4-dimethylsterols, and 4α-methylsterols (Table 2). In the presence of 0.5% DMSO, these compounds were also labelled but the proportion of radioactivity in squalene increased somewhat and there was a corresponding decline in the radioactivity associated with the 4-desmethylsterols. DMSO has been reported previously to stimulate the incorporation of [2-^{14}C]mevalonic acid into squalene by rat liver slices [30]. When the cells were cultured in

Table 1. Effect of SF 86-327 on the growth of L. mexicana mexicana

SF 86-327 (μg/ml)	Growth of promastigotes (% of control)
0.1	100 (105-95)
0.5	88 (84-92)
1.0	71 (68-77)
5.0	23 (18-27)
10.0	1 (0-3)

Data represent population growth of promastigotes in experimental groups expressed as a percentage of growth of promastigotes in controls cultivated at the same time (mean and range of three experiments). The number of organisms in control cultures was 33-49 x 10^6/ml after 4 days of incubation in supplemented RE III medium.

the presence of SF 86-327, there was a very striking increase in the labelling of the squalene while the proportion of radioactivity incorporated into the sterols fell sharply. The identity of the labelled squalene was confirmed by TLC-radioautography which showed radioactivity to co-chromatograph with the carrier squalene (R_f 0.5). Moreover, radioactivity was retained by the squalene after purification via the thiourea clathrate (control: initial sp. act. 136 dpm/mg, purified 146 dpm/mg; SF 86-327: initial 145 dpm/mg, purified 134 dpm/mg). The increased incorporation of radioactivity into squalene was accompanied by an approximately 4-fold increase in the quantity of squalene in the cells (Table 2). It can therefore be concluded that SF 86-327 is probably effective against L. mexicana mexicana in a manner similar to that in fungi, namely by inhibiting squalene epoxidation and causing the accumulation of excessive amounts of squalene which possibly may be disruptive to membrane structure [24-26].

Table 2. Effects of SF 86-327 and DMSO on the incorporation of [2-^{14}C]mevalonic acid into squalene and sterols of L. mexicana mexicana promastigotes

Compound	Control (-DMSO)	+DMSO (0.5%)	+DMSO (0.5%) and SF 86-327 (10 μg/ml)
Petroleum ether soluble lipid	43,550*	37,370	34,450
Squalene	7,910 (20.4)†	9,210 (27.4)	17,950 (55.7)
Steryl esters ‡	4,280 (11.0)	2,870 (8.5)	1,520 (4.7)
4,4-Dimethylsterols §	2,060 (5.3)	2,170 (6.5)	1,849 (5.7)
4α-Methylsterols ‖	5,150 (13.3)	6,300 (18.7)	2,060 (6.4)
4-Desmethylsterols ¶	19,050 (49.2)	12,890 (38.3)	8,540 (26.5)
Squalene content (ng/10^6 cells)	2.50	2.15	9.50
Squalene sp. act. (dpm/μmole)	16.8 x 10^3	17.3 x 10^3	8.2 x 10^3

* Dpm incorporated. † Values in parentheses are percentage distribution of radioactivity. ‡ Not characterized and may include squalene-2,3-oxide. § Mainly lanosterol. ‖ Mainly 4α,14α-dimethylcholesta-8,24-dien-3β-ol. ¶ Consists mainly of cholesterol (35-40%) derived from the medium (assumed to be unlabelled) and ergosta-5,7,24(28)-trien-3β-ol (45-60%) biosynthesized by the organism (see refs. 18 and 19).

The exact manner in which the allylmine drugs inhibit squalene epoxidation is not yet understood. The epoxidase enzyme is located in the microsomal membrane and it is a multicomponent system with both catalytic and non-catalytic protein moieties [31-33]. There is some evidence for the presence of two pools of squalene in rat liver cells, one having a slow rate of turnover [34]. In the present work, the sp. act. of the squalene accumulated in the presence of SF 86-327 was about 50% of that produced in the absence of the drug (Table 2). Pools of squalene of varying metabolic activity may also exist in Leishmania cells and the immobilization of these pools is perhaps a significant factor of allylamine action.

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