Amiodarone Destabilizes Intracellular Ca\(^{2+}\) Homeostasis and Biosynthesis of Sterols in *Leishmania mexicana*

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Leishmaniasis represents a serious public health problem worldwide. The first line of treatment is based on glucantime and pentostan, which generate toxic effects in treated patients. We have recently shown that amiodarone, frequently used as an antiarrhythmic, possesses activity against *Trypanosoma cruzi* through the disruption of mitochondrial Ca\(^{2+}\) homeostasis and the inhibition of parasite ergosterol biosynthesis, specifically at the level of oxidosqualene cyclase activity (G. Benaim, J. Sanders, Y. Garcia-Marchan, C. Colina, R. Lira, A. Caldera, G. Payares, C. Sanoja, J. Burgos, A. Leon-Rossell, J. Concepcion, A. Schijman, M. Levin, E. Oldfield, and J. Urbina, J. Med. Chem. 49:892–899, 2006). Here we show that at therapeutic concentrations, amiodarone has a profound effect on the viability of *Leishmania mexicana* promastigotes. Additionally, its effect on the viability of the parasite was greater against intracellular amastigotes than against promastigotes, and it did not affect the host cell. Using fluorimetric and confocal microscopy techniques, we also demonstrated that the mechanism of action of amiodarone was related to the disruption of intracellular Ca\(^{2+}\) homeostasis through a direct action not only on the mitochondria but also on the acidocalcisomes. On the other hand, analysis of the free sterols in promastigotes incubated with amiodarone showed that this drug also affected the biosynthesis of 5-dehydroepisterol, which results in squalene accumulation, thus suggesting that amiodarone inhibits the squalene epoxidase activity of the parasite. Taken together, the results obtained in the present work point to a more general effect of amiodarone in trypanosomatids, opening potential therapeutic possibilities for this infectious disease.

Chemotherapy is the most effective treatment against leishmaniasis, due to the lack of an effective vaccine (14, 20). The recommended first-line therapy for different forms of leishmaniasis includes pentavalent antimonials like glucantime and pentostan (8, 25). However, these drugs have secondary effects on the renal, cardiac, and hepatic systems (25). Resistance to pentavalent antimonial drugs is also an important factor that limits the treatments available in several countries (1, 13). Another disadvantage of antimonial therapy is the requirement for long-term parenteral administration in order to obtain optimal results (12, 21). At present, miltefosine appears to represent a major advance in the treatment of visceral and cutaneous leishmaniasis, despite its teratogenic characteristics, which limits its use during pregnancy (11).

Amiodarone (Fig. 1), an antiarrhythmic class III drug commonly used to treat several cardiomypathies, has been the subject of recent studies as an anticytocidal and parasitocidal agent, since this drug possesses excellent pharmacokinetic properties and a relative low cost. Thus, this drug has been shown to have potent effects against the yeast *Saccharomyces cerevisiae* as well as *Candida albicans* and other fungi (9). The reported mechanism of action of amiodarone against *Saccharomyces cerevisiae* (19) and *Trypanosoma cruzi* (7) is, at least in part, the disruption of Ca\(^{2+}\) homeostasis. The crucial role of Ca\(^{2+}\) in the regulation of many important processes for cellular viability has been demonstrated in different trypanosomatids (6, 23, 24). At the plasma membrane level, it has been reported that a Ca\(^{2+}\)-ATPase is stimulated by calmodulin in *T. cruzi* (3, 5), *Trypanosoma brucei* (4), and different species of *Leishmania* (2, 22). In all of these parasites, the regulation of cytoplasmic Ca\(^{2+}\) is carried out by three major organelles: the endoplasmic reticulum; the unique mitochondrion that is present in these parasites; and the acidocalcisome, a special compartment that is devoted to the accumulation of polyphosphates and that is also probably involved in the regulation of Ca\(^{2+}\) (17). The parasite mitochondrion possess a Ca\(^{2+}\)-electrophoretic uniporter in its internal membrane which utilizes the difference in the proton electrochemical potential between the intramitochondrial space and the cytoplasm as the driving force for the accumulation of this cation (2, 15). This mitochondrial Ca\(^{2+}\) transport system is characterized by a low affinity for Ca\(^{2+}\) but a high capacity for Ca\(^{2+}\) accumulation. All these attributes are very similar to the mitochondrial Ca\(^{2+}\) transport system in mammals (2, 15). The acidocalcisomes, however, are acidic organelles with high levels of pyrophosphates and polyphosphates which are capable of accumulating large amounts of Ca\(^{2+}\) (16, 17). All these systems, which operate in a concerted fashion, contribute to the maintenance of intracellular Ca\(^{2+}\) homeostasis, keeping
the intracellular (cytoplasmic) Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) under 100 nM, well below the extracellular Ca\(^{2+}\) concentration (which is on the order of mM), thus allowing this cation to perform its essential function as an internal signaling messenger (6).

We have previously shown that amiodarone affects the viability of \textit{T. cruzi} by disrupting mitochondrial Ca\(^{2+}\) homeostasis in the parasite (7). In the present study, we demonstrate that amiodarone also affects the viability of \textit{Leishmania mexicana} by destabilizing Ca\(^{2+}\) homeostasis in both the mitochondrion and the acidocalcisome.

Similar to \textit{T. cruzi}, however, \textit{L. mexicana} contains ergosterol instead of cholesterol as the main sterol component on its membranes. This property constitutes a notable difference between trypanosomatids and humans and validates the use of the sterol biosynthetic pathway as a potential target for the development of new drugs (31). We have also demonstrated that amiodarone blocks \textit{T. cruzi} ergosterol biosynthesis at the level of the oxidosqualene cyclase (7). In this context, in this work we also show that amiodarone inhibits the \textit{L. mexicana} sterol pathway at the level of the squalene epoxidase, which is essential for the synthesis of 5-dehydrosterol.

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**MATERIALS AND METHODS**

**Chemicals.** Amiodarone [(2-butyl)-3-benzofuranyl]-[4-[2-(diethylamino)ethyl]-3,5-diiodophenyl] methane hydrochloride), EGTA, digitonin, fluoro-carboxylic anhydride P-(trifluoromethoxy) phenylhydrazone (FCFP), bafloyn, A. nigerin, lectin from the coral tree (\textit{Erythrina cristagalli}), and \textit{t}-polylysine were from Sigma (St. Louis, MO). Fura 2-acetoxymethyl ester (FURA 2-AM), rhodamine 123, rhod 2-AM, and acridine orange were from Molecular Probes (Eugene, OR).

**Culture of promastigotes and determination of cellular proliferation.** Promastigotes of \textit{L. mexicana} were cultured in RPMI 1640 medium (Gibco) supplemented with 10% inactivated fetal bovine serum (Gibco) under continuous agitation in both the mitochondrial and the acidocalcisome.

**Confocal microscopy determinations.** Promastigotes of \textit{L. mexicana} were immobilized on coverslips on four- to eight-well plates coated with lectin from the coral tree (\textit{Erythrina cristagalli}), which interacts with glycoproteins on the parasite surface membrane that contain oligosaccharides with galactosyl (\(\beta\)-1,4)-N-acetylglucosamine, as described by Rohloff et al. (28). This lectin was demonstrated to be able to immobilize \textit{T. cruzi} (28) and also \textit{L. mexicana} (this work) in confocal microscopy experiments while maintaining their viability. The lectin was dissolved in Dulbecco's phosphate-buffered saline (PBS) at a concentration of 1 mg/ml. The coverslips with parasites were loaded with 10 \(\mu\)g/ml of rhodamine 123 for 30 min at 25°C. All measurements were performed in a Hitachi 2000 spectrofluorimeter at 29°C and under continuous agitation; the spectrofluorimeter was coupled to a computer with an appropriate data acquisition system.

**Free sterol content determinations.** Free sterol content were determined by high-resolution gas-liquid chromatography coupled with mass spectrometry, as described previously (33). Briefly, for the extraction and the separation of neutral lipids, \textit{L. mexicana} was cultured in the presence of amiodarone, as described above (see Table 2). Lipids were extracted with chloroform-methanol (2:1, vol/vol). The extract was dried and suspended in a minimum volume of chloroform. The chloroform suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with 5 column volumes of chloroform to separate the neutral lipids from the chloroform. The chloroform suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with 5 column volumes of chloroform to separate the neutral lipids from the chloroform. The chloroform suspension was applied to a silicic acid column (1.5 by 4 cm) and washed with 5 column volumes of chloroform to separate the neutral lipids from the chloroform. The chloroform suspension was applied to a si...
RESULTS

Susceptibility of *L. mexicana* promastigotes to amiodarone. *L. mexicana* promastigotes were exposed to different concentrations of amiodarone, and their viability was determined for 5 days. As can be seen in Fig. 2, amiodarone produced a dose-dependent inhibition of growth. Inhibition of 100% was obtained with a concentration of 5 μM amiodarone, and the 50% inhibitory concentration (EC_{50}) was 900 nM (Fig. 2). This result indicates that amiodarone is able to affect the viability of these parasites with an even greater efficacy than it affects the viability of *Trypanosoma cruzi* epimastigotes, for which the drug concentration needed for 100% inhibition was 1 order of magnitude greater (EC_{50}, 9 μM) (7).

**Effect of amiodarone on *L. mexicana* intracellular amastigotes.** Macrophages were infected with promastigotes in order to evaluate the effects of different concentrations of amiodarone on the number of infected macrophages. Figure 3A shows that as the amiodarone concentration increased, a concomitant decrease in the proportion of infected macrophages was observed. This effect was more pronounced than the inhibition of the promastigotes that was observed, since the EC_{50} was 8 nM. This fact was also reflected in the MIC of 20 nM obtained under these conditions. Figure 3B also shows that amiodarone did not affect the viability of macrophages in culture, as expected.

**Effect of amiodarone on the [Ca^{2+}]_i of promastigote from of *L. mexicana*.** The promastigotes were initially loaded with the Ca^{2+} indicator Fura 2. Figure 4 and Table 1 show that 5 μM amiodarone induced an increase in the [Ca^{2+}]_i in promastigote populations when an extracellular medium containing 2 mM Ca^{2+} was employed. In order to determine if the observed increase in the [Ca^{2+}]_i was due to the entrance of this cation from the extracellular milieu through the opening of a Ca^{2+} channel in the plasma membrane or, instead, the cytoplasmic Ca^{2+} concentration was elevated as a result of the release of the cation from intracellular compartments, we performed the same experiment but in the absence of extracellular Ca^{2+} (and in the presence of EGTA). Since a similar response was obtained (Fig. 4B and Table 1), the results demonstrate that the increase in the parasite [Ca^{2+}]_i is produced by the Ca^{2+} released from intracellular organelles.

**Effect of amiodarone on the mitochondrial electrochemical potential of *L. mexicana*.** Promastigotes were loaded with rhodamine 123, a reagent that senses the electrochemical potential of the mitochondria. Figure 5A shows that amiodarone affected the mitochondrial electrochemical potential in the promastigotes. This result was corroborated when the parasites were exposed to FCCP, a classical protonophore uncoupler that dissipates the mitochondrial H^+ gradient. Accordingly, FCCP generated the same effect as amiodarone (Fig. 5B). These results suggest that, similar to its effect in *T. cruzi*, amiodarone affects the parasite’s mitochondria.

**Effect of amiodarone on acidocalcisomes from *L. mexicana*.** When the promastigotes were loaded with acidine orange (Fig. 6), it was observed that amiodarone generated a rapid alkalinization of the parasite acidocalcisomes. This effect was similar to the effect generated by the vacuolar H^-ATPase inhibitor bafilomycin A (Fig. 6A). It was seen that only a partial alkalinization was generated by amiodarone and/or bafilomycin A, since nigericin, an electroneutral K^+-H^+ ex-
changer known to alkalinize the acidocalcisomes from these parasites (17), produced a second alkalinization response of a greater magnitude (Fig. 6A and 6B). These results suggest that the acidocalcisomes also participate in the increase in the parasite’s [Ca\(^{2+}\)](i) produced by amiodarone.

**Evaluation of effect of amiodarone on *L. mexicana* promastigotes and amastigotes by confocal microscopy.** The [Ca\(^{2+}\)](i) and the membrane electrochemical potentials of promastigotes (Fig. 7A) and of intracellular amastigotes from an infected macrophage (Fig. 7B) were also evaluated by confocal microscopy in order to identify the intracellular locus of action involved in the effect of amiodarone in these parasites.

The rhod 2 fluorescence level allowed the measurement of the amount of Ca\(^{2+}\) released from the organelles. The use of this indicator is based on its relatively low affinity for calcium, so that the fluorescence observed is visible, in principle, only in compartments with relatively high calcium concentrations. An increase in the fluorescence is obtained only when calcium is released from intracellular compartments and reaches a concentration high enough to be detected by the indicator, which is also present in the cytoplasm. On the other hand, rhodamine 123 measures energized mitochondria (Fig. 7A and B, top panels). The bottom panel of Fig. 7A shows that amiodarone

<table>
<thead>
<tr>
<th>Ca(^{2+}) concn in extracellular medium</th>
<th>Free cytoplasmic Ca(^{2+}) concn (nM)</th>
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<tbody>
<tr>
<td>Control</td>
<td>Amiodarone*</td>
</tr>
<tr>
<td>2 mM</td>
<td>98 ± 14 (n = 10)</td>
</tr>
<tr>
<td>0 mM</td>
<td>94 ± 18 (n = 8)</td>
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* Free cytoplasmic Ca\(^{2+}\) concentrations were determined with Fura 2, as described in Materials and Methods.

* Amiodarone concentration, 5 μM.

**FIG. 4.** Effect of amiodarone (AMIOD) on the [Ca\(^{2+}\)](i) of *L. mexicana* promastigotes. Populations of promastigotes (4 \times 10^6 cells in 1.5 ml) of *L. mexicana* were loaded with Fura 2-AM (6 μM) in a loading buffer (137 mM NaCl, 4 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 8.5 mM Na\(_2\)HPO\(_4\), 11 mM glucose, 1 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), and 20 mM HEPES-NaOH, pH 7.4, containing 2.4 mM probenecid), and the [Ca\(^{2+}\)](i) was calculated as described in Materials and Methods. (A) Effect of 5 μM amiodarone on the parasite [Ca\(^{2+}\)](i) in the presence of 2 mM Ca\(^{2+}\); (B) effect of 5 μM amiodarone on promastigotes loaded with Fura 2 in the absence of external Ca\(^{2+}\).

**FIG. 5.** Action of amiodarone (AMIOD) on the mitochondrial electrochemical potential of *L. mexicana* promastigotes. Parasites (4 \times 10^6 cells in 1.5 ml) were incubated in the presence of rhodamine 123 (10 μg/ml) for 30 min at room temperature in the same loading buffer (137 mM NaCl, 4 mM KCl, 1.5 mM KH\(_2\)PO\(_4\), 8.5 mM Na\(_2\)HPO\(_4\), 11 mM glucose, 1 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 20 mM HEPES-NaOH, pH 7.4). The cells were permeabilized with 1 μM digitonin (DIG). (A) Effect of amiodarone (5 μM), followed by the addition of FCCP (1 μM), on the mitochondrial electrochemical potential; (B) effect of FCCP (1 μM), followed by the addition of amiodarone (5 μM), on the mitochondrial electrochemical potential.

**TABLE 1.** Effect of amiodarone on free cytoplasmic Ca\(^{2+}\) concentration of *L. mexicana* promastigotes

- a Free cytoplasmic Ca\(^{2+}\) concentrations were determined with Fura 2, as described in Materials and Methods.
- b Amiodarone concentration, 5 μM.
Effect of amiodarone on synthesis of free sterols in *L. mexicana* promastigotes. After quantification of the neutral lipid fraction in a gas chromatograph equipped with a mass-sensitive detector, it was possible to determine that control promastigotes contained 67% 5-dehydroepisterol, similar to the values reported by others (27). On the other hand, we also demonstrated in this experiment that these parasites contain 19.2% squalene at the basal level. Interestingly, when promastigotes were exposed to 900 nM amiodarone, which corresponded to the EC_{50} of this drug, a drastic reduction (Table 2) in the amount of the main sterol, 5-dehydroepisterol, was seen and the accumulation of a significant amount of squalene (88.46%) was seen. These results strongly suggest that amiodarone affects the squalene epoxidase activity, which is essential for overall sterol biosynthesis (Table 2 and Fig. 8).

**DISCUSSION**

The results obtained in this work clearly demonstrate that amiodarone generates a dose-dependent effect on *L. mexicana* promastigote populations. It had an EC_{50} (900 nM) that was 1 order of magnitude lower than that which we found for *T. cruzi* epimastigotes (EC_{50}, 9 μM) (7) and that was also significantly less than the EC_{50}s of other drugs with inhibitory actions on *L. mexicana*, such as glibenclamide, an inhibitor of K^{+}-ATP[ε] channels (EC_{50}, 54.3 μM), as reported by our group (29). To evaluate the effect of this drug on the clinically relevant stage of the disease caused by *L. mexicana*, we performed experiments with macrophages infected with *L. mexicana* amastigotes. It was possible to observe that amiodarone was 2 orders of magnitude more potent against parasites at this stage (EC_{50}, 8 nM) than it was against free promastigotes. Interestingly, the effect of amiodarone on *L. mexicana* amastigotes was more than 2 orders of magnitude less than it was against cells infected with *T. cruzi* amastigotes (EC_{50}, 8 nM and 2.7 μM, respectively). It was also interesting to observe that the drug did not affect the viability of the host cells.

Similar to the effect on epimastigotes and amastigotes from *T. cruzi* (7), amiodarone increased the [Ca^{2+}], in the promastigotes and amastigotes of *L. mexicana* at least in part by the same mechanism that it does in *T. cruzi* epimastigotes and amastigotes, since we could demonstrate that this drug induces the release of Ca^{2+} from the unique mitochondrion present in these parasites. This was demonstrated in several ways. First, we showed that amiodarone can simulate the effect of the protonophore FCCP on the release of rhodamine 123 from the mitochondrial internal membrane, which indicates the collapse of the electrochemical potential. This was first performed with cell populations (Fig. 5). Second, this was demonstrated with individual cells by the use of confocal microscopy with isolated promastigotes and amastigotes present in infected macrophages. This mechanism of action of amiodarone in *L. mexicana* (and similar to that in *T. cruzi*) is different from that in fungi reported by Gupta et al. (19) and Courchesne and Ozturk (10), since those authors observed that even though in these organisms the drug also acted by disrupting Ca^{2+} homeostasis, the increase in the [Ca^{2+}], was mediated by an MD-1 caffeine-sensitive Ca^{2+} channel in the plasma membrane.

Remarkably, in this study we also demonstrated that amio-
Amiodarone is able to affect another important organelle characteristic of all trypanosomatids, namely, the acidocalcisomes. Our results clearly demonstrated that, besides the mitochondrion, these organelles are also targeted by amiodarone, since the drug induced a rapid alkalinization very similar to that induced by bafilomycin A, a specific inhibitor of the vacuolar H\(^+\) ATPase responsible for acidification and subsequent Ca\(^{2+}\) accumulation characteristic of these organelles (16, 17). We did not test for this possibility in our previous study with T. cruzi (7). Taken together, these results strongly support the idea that both mitochondria and acidocalcisomes are involved in the increment of the [Ca\(^{2+}\)]\(_i\) induced by amiodarone in L. mexi-

![Image](image-url)

**FIG. 7.** Determination of the intracellular site of action of amiodarone in promastigotes and amastigote-infected macrophages from *L. mexicana* by confocal microscopy. (A) *L. mexicana* promastigotes were loaded with rhod 2 (red) and rhodamine 123 (green), as described in Materials and Methods. The top row shows control cells not exposed to amiodarone, while the bottom row shows cells treated with 5 μM amiodarone for 20 min. (B) Amastigotes inside an infected macrophage loaded with rhod 2 (red) and rhodamine 123 (green), as described in Materials and Methods. The top row shows a control cell not exposed to amiodarone, while the bottom row shows the same cell treated with 20 nM amiodarone after 20 min. Ψ, electrochemical potential.
cana, and this could be an argument for the potent effect of amiodarone on the viability of L. mexicana.

In this study, we also demonstrated that amiodarone inhibits the free sterol biosynthesis pathway in L. mexicana promastigotes. Indeed, we were able to show the accumulation of large amounts of squalene after amiodarone treatment, which suggests the inhibition of squalene epoxidase (Fig. 8), which is essential for the biosynthesis of sterols (31). Accordingly, the levels of 5-dehydroepisterol, the most abundant sterol present in this parasite (27), fell dramatically upon addition of the drug. This result is, in principle, different from that reported in our previous work with T. cruzi (7), in which we found that amiodarone inhibits oxidosqualene cyclase. However, it is perfectly conceivable that amiodarone simultaneously inhibits squalene epoxidase and oxidosqualene cyclase in L. mexicana, since the accumulation of squalene observed would prevent the later accumulation of squalene epoxide. Moreover, these parasites being different, it is possible that squalene epoxidase behaves differently in L. mexicana compared to T. cruzi. Moreover, these two enzymes are contiguous in the sterol biosynthetic pathway in both parasites. For this reason, both substrates, squalene and squalene epoxide, have very similar structures and sizes and differ only by the presence of an epoxide moiety in T. cruzi. Thus, we consider it plausible that amiodarone could inhibit both enzymes. However, by the experimental approach that we have used, it can only be suggested that the first enzyme is inhibited.

In any case, the inhibition of the sterol synthesis pathway

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<tr>
<th>Sterol</th>
<th>Retention time (min)</th>
<th>Mass % after treatment with:</th>
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<tr>
<td>Exogenous cholesterol</td>
<td>24.9</td>
<td>Control: 13.8, Amiodarone: 11.54</td>
</tr>
<tr>
<td>Endogenous 14-desmethyl ergosta-5,7,24(24')-trien-3β-ol (5-dehydroepisterol)</td>
<td>28.9</td>
<td>Control: 67.0, Amiodarone: ND</td>
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<tr>
<td>Ergosta-7,24(24'-dien-3β-ol (episterol)</td>
<td>29.2</td>
<td>Control: 12.2, Amiodarone: ND</td>
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<tr>
<td>Cholesta-8,24-dien-3β-ol (zymosterol)</td>
<td>25.9</td>
<td>Control: ND, Amiodarone: ND</td>
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<tr>
<td>Endogenous 14-methyl lanosterol</td>
<td>30.8</td>
<td>Control: 3.0, Amiodarone: ND</td>
</tr>
<tr>
<td>Squalene epoxide</td>
<td>22.6</td>
<td>Control: 4.0, Amiodarone: ND</td>
</tr>
<tr>
<td>Squalene</td>
<td>20.5</td>
<td>Control: 19.2, Amiodarone: 88.46</td>
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a ND, not detected.

FIG. 8. Part of the sterol biosynthetic pathway in Leishmania mexicana showing the possible site of action of amiodarone. Acetyl-CoA, acetyl coenzyme A; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.
could be a second argument that explains the effect of amiodarone on the viability of *L. mexicana*.

The results obtained in the present work point to a more general effect of amiodarone against trypanosomatids, which was indeed expected, since the mechanisms of action reported in *T. cruzi* imply that this family of parasites has targets that are common, such as the mitochondria, acidocalcisomes, and sterol biosynthesis. The possible therapeutic use of amiodarone for the treatment of leishmaniasis was addressed in a recently published case report (26). In that report, the authors postulated that a human patient with leishmaniasis had been cured by the use of amiodarone. These results also open the possibility that amiodarone may be used in combination with other drugs, such as miltefosine and/or inhibitors of sterol biosynthesis, and this possibility needs to be investigated.

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