

Effect of Itraconazole and Terbinafine on *Leishmania Major* Promastigotes

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ABSTRACT. The antiproliferative effect induced *in vitro* by two antifungal compounds, the azole itraconazole and the allylamine terbinafine on *Leishmania major* promastigotes are reported. Treatment of promastigote cultures with itraconazole or with terbinafine induced growth arrest. Concentrations of 0.75 μ /l or more of itraconazole induced cell lysis after 72 hours. However, even relatively large concentrations of terbinafine (2.0 μ /l) did not induce cell lysis. The IC 50 for itraconazole and terbinafine were 0.31 μ /l and 3.3 μ /l respectively.

Keywords: *Leishmania major*, Promastigotes, Itraconazole, Terbinafine

Introduction

Leishmania parasites cause a wide range of human diseases from localized self-healing cutaneous lesions to fatal visceral disease. It is a major global public health problem since 2 -3 million people are affected by the disease and 350 million people in 80 countries are at risk^[1]. Pentavalent antimonials are the most widely used antileishmanial agents since the 1940s. This drug is not always effective and requires long term administration that is associated with drug toxicity and severe kidney, heart, and liver disorders^[2-4]. Many investigators are therefore currently involved in seeking alternatives with improved activity and safety. Although there are several compounds under various stages of development, no drug has yet been discovered which is definitively effective in achieving a complete cure of the infections^[1].

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Since the discovery that some antifungal agents had a potent leishmanicidal activity, the chemotherapy of *Leishmania* appeared to stagnate^[5]. Several studies examined the effect of antifungal agents on visceral leishmaniasis^[6-12]. However, only few studies have been reported on the treatment of cutaneous leishmaniasis with antifungal agents^[13-15]. The azole ketoconazole and allylamine terbinafine, when tested against *Leishmania* promastigotes and amastigotes *in vitro*, were found to have a suppressive effect on cell growth. Furthermore, both compounds were found to induce ultrastructural changes by producing large multivesicular bodies in both amastigotes and promastigotes^[16]. To the best of the authors' knowledge, there is no particular study that has been run to study the effect of itraconazole and terbinafine on *L. major* *in vitro*. However, a pilot clinical trial that has tested the effect of terbinafine on 27 patients and the overall clinical response in 10 patients, who continued the study, was estimated to be 71.5%^[15].

The aim of this study was to investigate the effect of itraconazole and terbinafine on cultured promastigotes of *L. major*, which is one of the major causes of cutaneous leishmaniasis in Saudi Arabia^[17].

Materials and Methods

The parasite: Freshly isolated promastigotes of *L. major* clone FV-1 (MHOM/IL/80/Friedlin) were maintained in Schneider's *Drosophila* medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS) pH 7.0 at 26° C in 25 cm² tissue culture flasks using air as the gas phase. These promastigotes were used to start new cultures at a density of 5×10^5 cell/ml.

The drug: Itraconazole (cis-4-[4-[4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazole-1-yl)methyl]-1,3-dioxolan-4-yl]methoxy]-phenyl]-1-piperazinyl]phenyl)-2,4-dihydro-2-(1-methylpropyl)-3h-1,2,4-triazol-3-one) was kindly provided by Dr. Marcel Jansen of the Janssen Research Foundation, Belgium. The drug was added as an aqueous solution titrated to pH 2.4 with HCl and sterilized by filtration. The initial stock solution concentration was 0.5 mg/ml.

Terbinafine: ((E)-(6,6-dimethyl-2-hepten-4-ynyl)-N-methyl-1-naphthalenemethanamine) was kindly provided by Dr. Barbara Willi of Novartis Pharma AG, Basel, Switzerland. It was added as dimethyl sulfoxide (DMSO). The final concentration of DMSO never exceeded 1% (vol/vol) (and it had no effect by itself on the growth and multiplication of promastigotes.) The initial stock solution concentration was 0.5 mg/ml.

Cell culture: Schneider's *Drosophila* medium supplemented with 10% FCS of different concentrations of itraconazole and terbinafine (0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 µl/l) were prepared and all culture flasks were inoculated with an original concentration of promastigotes of 5×10^5 . Cell densities were estimated daily using an improved Neubauer haemocytometer under phase contrast microscopy.

Data analysis: All results were entered in a PC. Plots were made using Harvey Graphics V.3.0) and analyzed with “dose effect analysis with microcomputers, Biosoft” software and the IC (Inhibition Concentration) 50 of each drug was estimated.

Results

Promastigotes of *L. Major* were cultured in Schneider’s *Drosophila* medium, supplemented with 10% FCS, containing different concentrations of itraconazole and terbinafine and cell densities were checked daily as described above. The logarithm of parasite cell densities vs. days in culture were plotted in Figures 1 and 2. Population doubling times were indicated in Tables 1 and 2.

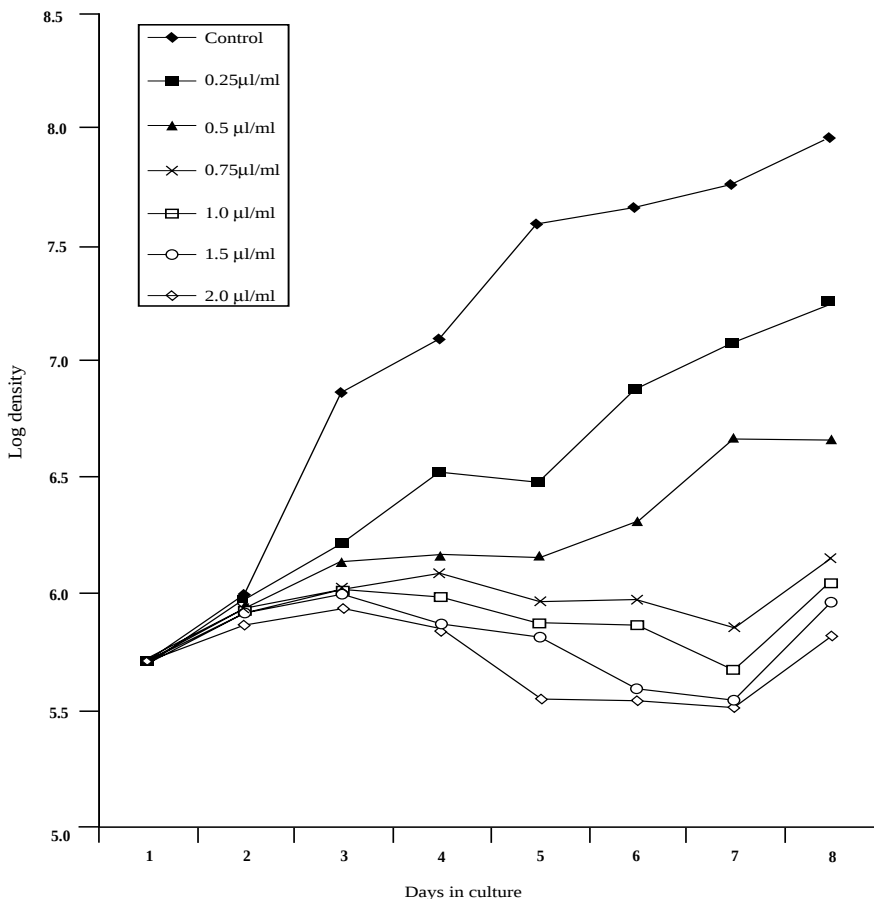


Fig. 1. Growth of *L. major* promastigotes in different concentrations of Itraconazole

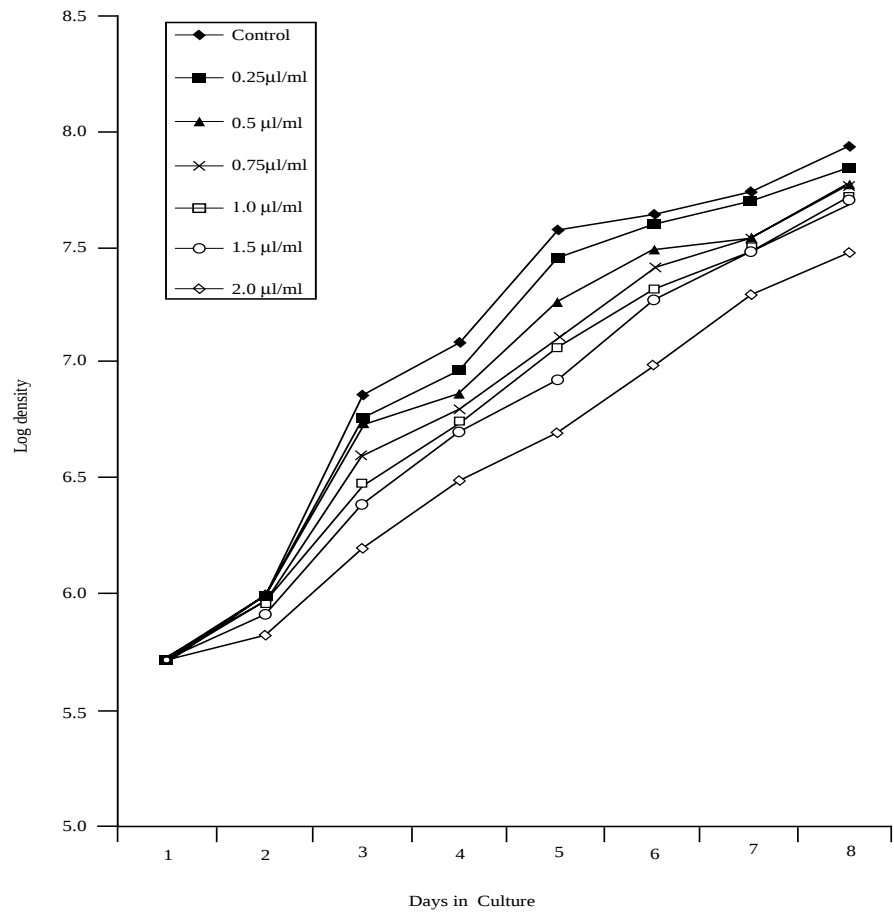


Fig. 2. Growth of *L. major* promastigotes in different concentrations of Terbinafine

TABLE 1. Population doubling time of *L. major* promastigotes cultured in Schneider's *Drosophila* medium supplemented with 10% FCS in different concentrations of itraconazole.

Concentration (µl/l) of Itraconazole	Population Doubling Time (hr.)
Control	15.6
0.25	26.8
0.50	48.4
0.75	57.8
1.00	80.3
1.50	138.9
2.00	173.4

TABLE 2. Population doubling time of *L. major* promastigotes cultured in Schneider's *Drosophila* medium supplemented with 10% FCS in different concentrations of terbinafine.

Concentration ($\mu\text{l/l}$) of Terbinafine	Population Doubling Time (hr.)
Control	15.6
0.25	17.2
0.50	18.7
0.75	19.7
1.00	20.8
1.50	21.9
2.00	27.9

Itraconazole: Incubation of promastigotes in cell cultures containing different concentrations of itraconazole produced growth arrest. This was judged by the decrease in cell densities as the culture progressed. Furthermore, cell lysis was observed with concentrations of itraconazole reaching 0.75 $\mu\text{l/l}$ or more. The IC 50 was estimated to be 0.3 $\mu\text{l/l}$ and less than 0.5 $\mu\text{l/l}$ concentrations. They were found enough to double the population doubling time.

Terbinafine: Incubation of promastigotes in cultures with different concentrations of terbinafine produced growth arrest as judged by the decrease in cell densities as the culture progressed. However, unlike itraconazole, even high concentrations of terbinafine (2 $\mu\text{l/l}$) did not induce cell lysis and were not found enough to double the population doubling time. The IC 50 was found to be 3.3 $\mu\text{l/l}$.

Discussion

The effect of some antifungal compounds on *Leishmania* has been previously tested. In cases of visceral leishmaniasis, amphotericin B was shown to cure the diseases^[6-9]. Some antifungal agents such as lovastatin and miconazole have been shown to inhibit promastigote proliferation by inhibiting sterol biosynthesis^[10, 12], while others (*e.g.*, sinefungin) were found to have a limited effect on the sterol production and hence on inhibition of proliferation^[11].

In cases of cutaneous leishmaniasis, only few studies have been reported on the treatment with antifungal agents. Paromomycine sulphate was found to be effective against cultured promastigotes of *L. mexicana* at concentrations of 100 $\mu\text{g/ml}$ ^[13]. Clotrimazole and miconazole were also found effective in treating cutaneous leishmaniasis in the eastern province of Saudi Arabia, where *L. major* dominates^[17, 18]. However, only 16% of lesions treated with clotrimazole healed fully after 30 days treatment.

The only study that used Terbinafine in the treatment of cutaneous leishmaniasis was that of Bahamdan *et al*^[15]. Ten out of fourteen (10/14) attained complete or partial cure after 4 weeks of treatment with Terbinafine. However, the sample size was relatively small, the duration of the treatment course was short, and the *Leishmania* species was not identified.

In the present study, we found that both itraconazole and terbinafine had an inhibitory effect on the growth and proliferation of *L. major* promastigotes. Itraconazole had a more powerful leishmanicidal effect since only 0.75 µl/l concentrations induced cell lysis. With terbinafine, promastigote proliferation was suppressed to lesser extent and cell lysis was never observed.

There are several reasons that one can speculate on why itraconazole is more effective than terbinafine. One strong reason may be that different *Leishmania* species respond in different ways to different drugs. Also, the half life of itraconazole is known to be longer than that of terbinafine which would explain why large concentrations of terbinafine are required to induce growth arrest while, in contrast, only small concentrations of itraconazole are required to induce the same effect.

Combination of two drugs at the same time may show different results. One study showed that both terbinafine with ketoconazole had an inhibitory effect on growth and proliferation of *L. mexicana amazonensis* promastigotes. Combination of the two drugs had an additive effect on promastigote proliferation^[16].

It is well known that promastigotes (the culture form) and amastigotes (the mammalian form) of *Leishmania* react differently to different drugs^[19,20]. However, the ease in obtaining and culturing promastigotes makes it an ideal stage to start with when studying drug effects on such parasite before commencing a study on amastigotes *in vitro* or *in vivo*. Preliminary studies on the effect of itraconazole and terbinafine on *Leishmania* lesions in laboratory animals showed promising results (unpublished observations).

A continuation of the present study would be intended to examine the effect of both itraconazole and terbinafine together with lovastatin, a hypocholesterolaemia agent, on *Leishmania* amastigotes *in vitro* or *in vivo*. This is because *Leishmania* parasites have the ability to utilize some cholesterol from the host and not rely only on ergosterol. Besides miconazole was found to be more effective in inhibiting *Leishmania* parasites growth when used with lovastatin^[10, 21].

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تأثير العقاقير المضادة للفطريات على الطور السوطي لطفيل اليشمانيا العظيمة

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المستخلص. تم في هذه الدراسة اختبار تأثير مضادين للفطريات هما إتراكونازول و تيربينافين على الأطوار السوطية لطفيل اليشمانيا. و قد وجد أن وجود كمية ٧٥ , ٠ مايكروليتر في اللتر من مادة الإتراكونازول في مزرعة الأطوار السوطية تؤدي إلى موت الخلايا ثم تحللها. بينما وجود كمية كبيرة من مادة التيربينافين (٠ , ٢ مايكروليتر في اللتر) لم يؤد إلى تحلل الخلايا. وكان متوسط تركيز منع التكاث هو ٣١ , ٠ مايكروليتر في اللتر لمادة إتراكونازول و ٣ , ٣ مايكروليتر في اللتر لمادة تيربينافين.