EVALUATION OF MILTEFOSINE AGAINST LEISHMANIA MAJOR (MRHO/IR/75/ER): IN VITRO AND IN VIVO STUDIES

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Abstract- Cutaneous leishmaniasis is endemic in 88 different countries. There are an estimated 1.5 million new cases each year, with over 90% occurring in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria (Old World) and in Brazil and Peru (New World). Miltefosine is effective in vitro and in vivo against Leishmania species and it was demonstrated efficacy in animals via the oral route. This study is the first one for evaluating the effect of miltefosine on cutaneous leishmaniasis of L. major (MRHO/IR/75/ER) by in vivo and in vitro studies in the BALB/c mouse model. As it was shown, miltefosine has a better effect on reduction of size of lesion compared to Glucantime®, also it was not significant by statistical analysis. The results of this study show that miltefosine has a good activity against the proliferation of amastigotes of L. major. The results suggest that oral miltefosine might be a promising approach for developing new anti-Leishmanial drugs.

INTRODUCTION

Cutaneous leishmaniasis is endemic in 88 different countries. There are an estimated 1.5 million new cases each year, with over 90% occurring in Afghanistan, Algeria, Iran, Iraq, Saudi Arabia, Syria (Old World) and in Brazil and Peru (New World) (1). Both cutaneous and visceral forms of leishmaniasis are endemic in different parts of Iran. Zoonotic cutaneous leishmaniasis (CL) caused by Leishmania major is common in many rural areas of Iran (2). Antimonial compounds particularly meglumine antimoniate (Glucantime®) are the first line drugs for the treatment of all forms of leishmaniasis in Iran (3). Based on a few studies that have been carried out in recent years, about 10 to 15% of CL has not desirable response to meglumine antimoniate (Mohebali, unpublished data, 4). Recent circumstantial evidences are suggesting that an increasing number of Iranian patients with cutaneous leishmaniasis are unresponsive to meglumine antimoniate (Glucantime®) (4).

Miltefosine (hexadecyl-phosphocholine, Impavido®) interacts with cell signal transduction pathways and inhibits phospholipids and sterol biosynthesis (5). It was originally developed as an anticancer agent. Miltefosine is effective in vitro and in vivo against Leishmania species (6, 7); it was demonstrated efficacy in animals via the oral route (6). The first clinical test of miltefosine used dosages that ranged from 50 mg given every other day up to 250 mg/day in Indian patients with kala azar in 2002 (8). In a later large phase II trial, treatment with 100–150 mg for 28 days cured 86 (96%) of 90 viscerally infected Indian patients (9). Miltefosine is now registered to
Miltefosine against leishmaniasis in Germany and India, as well as cutaneous leishmaniasis in Colombia (10). For the first time, effectiveness and tolerability of Miltefosine were evaluated for the treatment of 63 Iranian zoonotic cutaneous leishmaniasis caused by \textit{L. major} comparing to meglumine antimoniate (11).

In this study, we aimed to clarify assessing the effectiveness of Miltefosine against \textit{L. major} (Iranian strain) comparing with Glucantime® \textit{in vitro} and \textit{in vivo} conditions.

**MATERIALS AND METHODS**

**Materials**

Miltefosine was a gift from Zentaris GmbH (Zentaris, GmbH, Frankfurt, Germany) provided by Dr. M. R. Goli (Arya Daro Co.). Glucantime® (Rorer Rhone-Poulenc Specia, Paris, France) received from the Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences.

**Parasites culture**

\textit{Leishmania major} promastigotes, MHROM/IR/75/ER, were grown in Schneider’s medium supplemented with 10\% heat-inactivated FBS, 100 g/ml streptomycin and 100 IU/ml penicillin G at 23-25°C as previously described (12). The parasites were kept in a virulent state by regular passage in susceptible BALB/c mice. Stationary phase promastigotes were harvested and centrifuged at 3,000 rpm for 10 min at 4\°C. The pellet was washed 3 times in PBS (8 mM Na₂HPO₄, 1.75 mM KH₂PO₄, 0.25 mM KCl, 0.137 mM NaCl).

**In vivo studies**

Male BALB/c mice (6-8 weeks old) were obtained from the Animal Breeding Stock Facility of Razi Institute of Iran, Karaj, Iran. Male BALB/c mice were infected with subcutaneously 2 x 10⁵ \textit{L. major} promastigotes (MHROM/IR/75/ER) at the base of tail. The weight and diameter of lesions were measured before treatment. Impression smears were prepared from lesions; methanol fixed, and stained with 10\% Giemsa stain in water. The mice were randomly divided to four groups, that three (15 mice per group) and one group (5 mice) for animal Lab control. The groups included:

- Group 1: Control mice non-infected and non-treated
- Group 2: Infected but non-treated
- Group 3: Infected treated with miltefosine 2.5 mg/kg by daily gavage for 28 days.
- Group 4: Infected treated with Glucantime® 60 mg/kg salt injected IP daily for 28 days.

The diameters of lesions one, two and 8 weeks after the beginning the treatment were measured. Also before treatment and one, two and 8 weeks after the beginning the treatment, slides were prepared and methanol fixed, Giemsa stained and examined by light microscopy (× 1000). Drugs efficacy were determined by comparing the diameters of lesions and the number of mice which slides contained amastigotes, between treated and untreated groups.

**In vitro studies**

**Mouse peritoneal macrophages**

The macrophages of peritoneal fluid of male BALB/c mice were collected and resuspended at 5 x 10⁷/ml in RPMI 1640 supplemented with 15\% FCS, as described by others (13). Cells were plated in eight-chamber LabTek tissue-culture slides, and adherent macrophages were infected with late-logarithmic promastigote parasites at a parasites-to-macrophage ratio of 5:1. After 2 h of incubation at 34 °C, extracellular parasites were removed by washing, and fresh medium containing the different fixed-ratio solutions miltefosine and glucantime® 1.25, 2.5, 5, 10, 20 \(\mu\)M or no drug was added. Each point was tested in triplicate. Each 5-ml ampoule of glucantime® contained 1.5 g meglumine antimoniate corresponding to 0.405 g of pentavalent antimony. The tissue-culture slides were incubated for 3 days, fresh Glucantime and miltefosine was added, and the slides were incubated for an additional 72 h. The slides were fixed and stained with Giemsa. Three slides were used for each concentration. The percentage of infected macrophages and the number of parasites per infected cell were evaluated by microscopic examination of at least 100 macrophages. The ED50 is defined in this study as
the effective dose of miltefosine and glucantime that reduces the survival of *Leishmania* parasites by 50% ED50 values were determined by linear regression analysis.

**Statistical analysis**

Statistical significance between groups was analyzed by Student’s t test using SPSS version 10. Values of *P* < 0.05 were considered statistically significant.

**RESULTS**

**In vivo studies**

**Effect on size of lesions**

The mean sizes of the lesions in three infected groups were measured before treatment and after one, two and eight weeks of treatment. The results are shown in Table 1. As shown in Table 1, both miltefosine (2.5 mg/kg by gavage for 28 days) and glucantime (60 mg/kg salt injected IP) produced a suppression and reduction effect in the size of lesions compared with control group in leishmania-infected mice. However, as shown in Fig 1, there is no significant difference between miltefosine and glucantime in reduction of lesion size.

**Parasitology**

The results of monitoring the slides for the presence of amastigotes are shown in Table 2. The positive cases are ones which slides contained *L. major* amastigotes and negative cases are ones without any *L. major* amastigotes.

The chi-square analysis of results shows a significant difference between the treated groups 3 (miltefosine) and 4 (glucantime) with control group (*P* < 0.05).

**In vitro studies**

*In vitro* EC50 for *L. major* amastigotes was determined after 3 days exposure to different concentration of miltefosine and Glucantime. The data represent the means ± standard deviations (SDs) of three independent experiments. The ED50 of miltefosine was 2.20 µM according to the linear regression was shown in Fig 2. More than 85% of *L. major* amastigotes-infected macrophages damaged when treated with 10 and 20 µM of miltefosine and was cytotoxic for both parasite and macrophage.

The ED50 of Glucantime was 7.2 µM according to the linear regression was shown in Figure 2.

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**Table 1. Effect of miltefosine and Glucantime® on the size of lesions (mm) in Balb/c mice infected by *L. major***

<table>
<thead>
<tr>
<th>Groups</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>8</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>74.03 ± 7.84</td>
<td>80.60 ± 8.01</td>
<td>82.60 ± 7.92</td>
<td>87.80 ± 8.00</td>
</tr>
<tr>
<td>3</td>
<td>64.90 ± 12.51</td>
<td>32.37 ± 6.78</td>
<td>22.48 ± 4.98</td>
<td>16.80 ± 4.15</td>
</tr>
<tr>
<td>4</td>
<td>66.20 ± 12.25</td>
<td>54.13 ± 9.70</td>
<td>39.40 ± 7.98</td>
<td>32.07 ± 7.79</td>
</tr>
</tbody>
</table>

Group 2: Infected but non-treated (Control group)
Group 3: Infected treated with miltefosine 2.5 mg/kg by gavage daily for 28 days
Group 4: Infected treated with glucantime (60 mg/kg salt injected IP) daily for 28 days

**Table 2. Parasitological results of the effect of miltefosine and Glucantime® in Balb/c mice infected by *L. major***

<table>
<thead>
<tr>
<th>Groups</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>8</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>P</td>
<td>N</td>
<td>P</td>
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<tr>
<td>2</td>
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<td>15</td>
<td>-</td>
<td>15</td>
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<tr>
<td>3</td>
<td>-</td>
<td>15</td>
<td>11*</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>15</td>
<td>8*</td>
<td>7</td>
</tr>
</tbody>
</table>

Abbreviations: N, Negative; P, Positive
Group 2: Infected but non-treated
Group 3: Infected treated with miltefosine 2.5 mg/kg by gavage for 28 days
Group 4: Infected treated with glucantime (60 mg/kg salt injected IP)
*: *P*<0.001 by χ² test
Miltefosine against leishmania

**Fig 1.** Effect of treatment with miltefosin and Glucantim® on size of lesions after one, two and eight weeks after the beginning of treatment. *: P<0.01. **: P<0.05. ***: P<0.001.

**Fig. 2.** Effect of different concentration of miltefosine on the proliferation of amastigotes of *L.major*.

**Parasit count = (72.95 ± 1.097) + [(-26.50 ± 1.34) * log concentration)]

**Fig. 3.** Effect of different concentration of Glucantime® on the proliferation of amastigotes of *L.major*

**DISCUSSION**

Leishmaniasis is a worldwide disease still treated with expensive compounds that present severe side effects, and are frequently ineffective, emphasizing the importance to search new compounds against this disease. The standard agents for leishmaniasis-pentavalent antimonials, pentamidine, and amphotericin B- have the disadvantages of repeated parenteral injection and of toxicity (14).

Oral medications have an obvious appeal with ease of administration, domiciliary treatment, no hospital costs, no limitation by bed capacity. Thus, the quest for an effective oral antileishmanial drug has been ongoing for a long period. Drugs such as allopurinol, ketoconazole, triazoles (fluconazole), atovaquone, have been tested either alone or in combination for the treatment of VL; however, they had either no effect or only a partial effect (15).

An earlier study in localized cutaneous leishmaniasis reported successful miltefosine treatment of patients with *Leishmania (viannia) panamensis* infections. Miltefosine has been used for up to 2 years in maintenance treatment in patients with HIV/visceral leishmaniasis, but we found no reports of long-term maintenance treatment of human beings with cutaneous forms of leishmaniasis.
Miltefosine is effective in vitro and in vivo against Leishmania species (6, 11), and Kuhlencord et al. demonstrated efficacy in animals via the oral route (6).

In previous studies, it was demonstrated that miltefosine can be applied orally without overt side effects. After a daily oral application of the miltefosine (10 mg/kg) to rats, a steady-state level of about 100 μM was obtained in serum (16), indicating that miltefosine is well absorbed from the gut. Furthermore, biodistribution studies of miltefosine in mice demonstrated an accumulation of the compound in spleen and liver (17).

An outstanding advantage of He-PC is its significant activity after oral administration, since few other antileishmanial drugs that are effective by oral administration are known. Ketocanazole, allopurinol, and allopurinol riboside are effective in vitro; however, clinical trials showed that cures could be achieved in only a few patients (18, 19).

The results of our study shows that miltefosine has a good suppression effects on the pro suggest that miltifosine oral might be a promising approach for developing new anti-Leishmanial drugs.

Conflict of interests
The authors declare that they have no competing interests.

REFERENCES