In Vivo and in Vitro Investigation on Anti-Leishmanial Efficacy of Artemisinin on Iranian Strain of Leishmania major

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Abstract

Background: Meglomin antimonate (glucantime) is the first-line drug for the treatment of cutaneous leishmaniasis, but it is usually toxic, leading to some side effects. Artemisinin, is an established anti-malarial compound that has indicated some anti-leishmanial activity.

Objectives: The objective of this study was to investigate the anti-leishmanial effects of artemisinin in comparison with glucantime against Iranian strain of Leishmania major in vitro and in vivo.

Materials and Methods: L. major was cultured in the RPMI1640 medium and was exposed to different concentrations of artemisinin and glucantime. The inhibitory effect, as the IC₅₀, was calculated by linear regression analysis. For in vivo assay, the base of the tail of Balb/c mice was injected intradermally with 2×10⁶ promastigotes. Four groups of mice (8/group) were treated by intra-peritoneal injection of artemisinin, glucantime, and respected drug vehicles, following the occurrence of lesions. The anti-leishmanial effects were assessed by
the lesion size, proliferation of amastigotes inside macrophage and parasite visceralisation in the liver and spleen.

**Results:** The IC\textsubscript{50} for artemisinin was determined 283 µM and glucantime was less than 62.5 µM in 24 hours. The proliferation of amastigotes inside macrophage in the lesion smear, represented significant decrease in artemisinin-treated mice ($P<0.05$) and artemisinin as well as glucantime, inhibited 87.5% and 100% of parasite visceralisation in liver and spleen, respectively.

**Conclusions:** Although, artemisinin has inhibitory effect on the Iranian strain of *L. major* and could be suggested as a substitute for glucantime in the treatment of cutaneous leishmaniasis, more studies are required to complete this concept.

**Keywords:** *Leishmania major*, Artemisinin, Glucantime

### 1. Background

Leishmaniasis caused by the genus *Leishmania*, is a major worldwide health problem, with high endemicity in developing countries including Iran. The disease is transmitted by Phlebotomine sandflies. According to the World Health Organization, approximately 350 million people in 88 countries are affected by leishmaniasis [1, 2]. There are three main clinical manifestations of leishmaniasis including; cutaneous (CL), mucocutaneous and visceral leishmaniasis [3]. CL usually produces ulcers on the exposed parts of the body, such as the face, arms and legs. In the absence of an effective vaccine, chemotherapy remains the sole weapon against leishmaniasis. The pentavalent antimonial drugs, such as meglumin antimonate (glucantime), are currently the first-line drugs for the treatment of leishmaniasis [4]. However, these drugs have serious side effects and progressive antimonial resistance [5]. Clinical reports indicate that a large number of cases are becoming unresponsive to chemotherapy. Variable efficacy in endemic areas, toxicity, requirement of long courses of parenteral administration, or combinations of these factors, has been reported [6].

Artemisinin is isolated from a Chinese herb, *Artemisia annua*. Its derivatives have anti-malarial activities and are associated with low toxicity to animals [7]. Artemisinin is a powerful anti-malarial drug having significant activity against strains of the parasite which are resistant to chloroquine [8]. Artemisinin has demonstrated some activities against other parasites e.g. *Schistosoma japonicum* [9]. The anti-leishmanial activity of an ethanolic extract of *A. indica* has been demonstrated against some leishmania species in animal models of CL [10]. There are several reports about anti-leishmanial activities of herbal extract of *A. aucheri*, *A. kulbadica*, *A. santolina*, *A. ciniformis* and *A. herba alba* against CL in different regions of Iran [10-14].

The objective of this study was to investigate the anti-leishmanial effects of artemisinin in comparison with glucantime against endemic Iranian strain of *L. major* (MRHO/IR/75/ER) *in vitro* and *in vivo* on experimental model of CL.
2. Materials and Methods

Cultivation of *L. major*. The parasite used in this study was the standard Iranian strain of *L. major*, MRHO/IR/75/ER. The infectivity of the parasite was maintained by regular passage in susceptible Balb/c mice. The parasite was cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 µg/ml L-glutamine and 4.5 mg/ml glucose (all supplied by Sigma). Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days as determined previously [15].

*In vitro* assessment of anti-promastigote activity of drugs. Anti-promastigote effects of artemisinin (Sigma, Aldrich) and glucantime (Aventis, Pharma, Brazil) were evaluated using a direct counting assay based on growth inhibition. Two hundred and fifty µl of RPMI medium containing $2 \times 10^6$ promastigote/ml in the early log phase was added to the microplate wells and then allowed to multiply for 72 hours in presence of 0 µl of different concentrations of drugs (61.5, 125, 250, 500 µM). The culture plate was incubated at 26 °C and the parasites were counted daily by a Neubauer chamber with a light microscope. The results were compared with control wells (only medium and promastigotes). Each assay was performed in triplicate and whole series of experiments were repeated twice. The IC$_{50}$, which is the concentration required to inhibit 50% growth of promastigotes after 24 hours, was calculated by linear regression analysis [16, 17].

Animals. Female inbred Balb/c mice were supplied by the Laboratory Animal Unit (Pasteur Institute of Iran) and used in this study. The initial body weight were $18.2 \pm 1.3$ g (mean±SE) and mice were housed at room temperature (20–23 °C) on a 12 hours light and 12 hours dark cycle, with unlimited access to food and tap water. Experiments with animals were done according to the ethical standards formulated in the Declaration of Helsinki, and measures taken to protect animals from pain or discomfort. It has been approved by institutional ethical review board (*Ethical Committee of the Pasteur Institute of Iran*), in which the work was done. [18]

Infection of Balb/c mice with *L. major*. Promastigotes of *L. major* harvested from culture media, were counted and used to infect Balb/c mice. The base of the tail was injected intradermally with inoculums of $2 \times 10^6$ promastigotes in the stationary phase of parasite growth which was obtained in 6 days of culture [20]. The lesions appeared at the eight week after inoculation. The mice were randomly divided into five groups (8 mice/group) following the occurrence of lesions.

Treatment of mice. Two groups of mice were treated by 300 µl interaperitoneal injection of glucantime (20 mg/kg/day) and artemisinin (10 mg/kg/day), two other groups received 300 µl saline and methanol 0.5% as drug vehicles for glucantime and artemisinin, daily up to 4 weeks. The fifth group of mice received no treatment as a control.
In vivo assessment of the anti-leishmanial effects of drugs. The anti-leishmanial effects of drugs were evaluated by considering lesion size, proliferation of amastigotes inside macrophages (MQ) and visceralisation of parasite in the target organs (liver and spleen). The lesion size was measured every week after treatment by a digital caliper (Chuan Brand, China) in two diameters ($D$ and $d$) at right angles to each other, and the size (mm) was determined according to the formula: $S = (D + d) / 2$ [19].

At the end of the experimental period, lesions were cleaned with ethanol and punctured at the margin with a sterile lancet and exudation material was smeared. The smears were stained with giemsa for detection of amastigotes by light microscopy. Five MQ per smear were selected randomly; contained amastigotes were counted and mean number was calculated as indicators for the degree of proliferation of parasites inside the MQ. Also, to evaluate the rate of visceralisation, impression smears were prepared from liver and spleen of mice and stained with giemsa for detection of amastigotes [20, 21].

Statistical analysis. Values for drug’s IC$_{50}$ were enumerated by linear regression graph (Microsoft office Excel 2007) and values for lesion size were presented as the mean ± SE. The significance of differences between groups was determined by analysis of variances (ANOVA) using Graph Pad Prism Software (Graph Pad, San Diego, USA).

3. Results

The inhibitory effect of drugs on promastigotes in vitro. Growth inhibition of promastigotes following exposure to different concentrations of artemisinin and glucantime are presented in Table 1. The resultant IC$_{50}$ for artemisinin was determined 283 µM in 24 hrs and 126 µM in 48 hrs. The IC$_{50}$ of glucantime was less than 62.5 µM in 24 hrs.

Anti-leishmanial effects of artemisinin and glucantime in vivo. The anti-leishmanial effects of drugs were evaluated by considering lesion sizes, proliferation of amastigotes inside MQ in the lesions and target organs (liver and spleen).

The mean values of lesion size (mm) at the end of experiment were 5.3 ± 1.4 for untreated group, 3.8±1.2 for artemisinin and 4.6±1.4 for glucantime-treated mice. There were no significant differences between the experiment groups. Unexpectedly, there is a decline on lesion size (3.5±1.1) in methanol group as well as artemisinin group (Fig. 1).
Table 1. Growth inhibition of promastigotes following exposure to different concentrations of artemisinin and glucantime after 24, 48 and 72 hrs.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Percent of killed promastigotes following exposure to the drugs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisinin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>250</td>
<td>125</td>
<td>62.5</td>
<td>35</td>
</tr>
<tr>
<td>250</td>
<td>47</td>
<td>62</td>
<td>50</td>
<td>43</td>
</tr>
<tr>
<td>125</td>
<td>35</td>
<td>50</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>62.5</td>
<td>28</td>
<td>43</td>
<td>43</td>
<td>43</td>
</tr>
<tr>
<td>Glucantime</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>77</td>
<td>89</td>
<td>50</td>
<td>89</td>
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<tr>
<td>250</td>
<td>68</td>
<td>79</td>
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<td>62</td>
</tr>
<tr>
<td>62.5</td>
<td>67</td>
<td>74</td>
<td>74</td>
<td>67</td>
</tr>
</tbody>
</table>

The proliferation of amastigotes inside MQ in lesion smears, represented a significant decrease in artemisinin-treated mice (P<0.05). The mean number of amastigotes inside MQ was 19.0±0.8 for control, and 14.3±1.6 for artemisinin-treated mice. The result showed ability of artemisinin in parasite reduction inside MQ in lesion smears (Fig. 2). Rate of positive smears for artemisinin-treated mice, were 12.5% for the liver and 0 for the spleen. In other words, artemisinin as well as glucantime, inhibited 87.5% and 100% of parasite visceralisation in liver and spleen, respectively (Table 2).

Table 2. Anti-leishmanial effects of artemisinin and glucantime on parasite visceralisation in liver and spleen of Balb/c mice infected intradermally with inoculums of 2×10⁶ promastigotes

<table>
<thead>
<tr>
<th>8 mice/group (n=40)</th>
<th>Positive smear of organs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number (percent)</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Normal saline</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Glucantime</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Methanol</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Artemisinin</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Untreated</td>
<td>2 (25)</td>
</tr>
</tbody>
</table>
**Fig. 1. Lesion size variation in experimental groups**

Group 1 *(L. major + Normal saline, control 1)*, group 2 *(L. major + Glucantime, Test 1)*,
group 3 *(L. major + methanol 0.5%, control 2)*, group 4 *(L. major x Artemisinin, Test 2)* and
group 5 *(L. major + no treatment), (n=10 mice/group), *P<0.05, AxOVA*

4. Discussion

The classical treatment of CL is achieved by glucantime, however, it is usually toxic, and leading to widespread side effects [6]. Although artemisinin (qinghaosu) is widely used as anti-malarial agent, it has also demonstrated its anti-promastigote activities and inhibitory effect on leishmania proliferation [12, 22]. It has also demonstrated effectiveness in experimental models of schistosomiasis [23], toxoplasmosis [24] and clonorchiasis [25]. Analysis of data resulted from our *in vitro* experiment, indicated that artemisinin inhibited growth of *L. major* promastigotes. Its inhibitory activity against promastigotes showed the IC$_{50}$ values of 283 µM after 24 hrs (Table 1). In a report on *L. donovani*, the IC50 of artemisinin was 160 µM in promastigotes, which further decreased to 22 µM in amastigotes [10]. These results suggested that artemisinin possesses a proportional anti-leishmanial activity *in vitro* which may be associated with leishmania species and strains.

The *in vivo* results showed that the lesion size of artemisinin-treated mice was smaller than those receiving glucantime, also methanol as a solvent for artemisinin could decrease lesion size markedly (fig 1). This may have caused a bias in our experiment due to limitation of its application as a specific alcoholic solvent for artemisinin. This result leads us to an uncertainty about the exact role of artemisinin on the lesion size. Because the leishmanial lesions are often affected to secondary infection by aerobic bacteria [26], whether traces of metha-
nol (0.5% as a vehicle) and/or its metabolites could affect the parasite or bacteria is unclear.

This study showed a significant decline in proliferation of amastigotes inside the lesion's macrophages in the artemisinin-treated mice compared with the others ($P<0.05$). Macrophages when exposed to micro-organisms, exhibit a burst in oxygen consumption coincident with the generation of nitric oxide in huge amounts with H$_2$O$_2$, shows more potent leishmaniacidal effects [27]. It seems that artemisinin kills the leishmania amastigotes as well as activating macrophages to clear itself from the parasite [28]. It is known that reaction of artemisinin and its derivatives with a ferrous iron atom forms free radicals which contribute to their anti-malarial activities [29, 30]

Livers and spleen of mice were studied as target organs to detect amastigotes and to evaluate visceralisation of parasite. The data indicated that artemisinin as well as glucantime had anti-leishmanial activity by reduction of positive smears in liver and spleen. This could emphasize the role of artemisinin to inhibit visceralisation of *L. major* in target organs of infected susceptible Balb/c mice.

In conclusion, artemisinin has inhibitory effects on the Iranian strain of *L. major* promastigotes by decreasing the number of amastigots inside macrophages and inhibitory effects on parasite visceralisation in liver and spleen of Balb/c mice. Although, artemisinin could be suggested as a potent substitute for classical glucantime in the treatment of cutaneous leishmaniasis in animal model, more studies are required to complete this concept.

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**References**


**Fig. 2.** Anti-leishmanial effects of artemisinin and glucantime on the lesion size in Balb/c mice infected intradermally with inoculums of 2×10^6 promastigotes. Mice were treated daily by 300 µl interaperitoneal injection of glucantime (20 mg/kg), artemisinin (10 mg/kg), normal saline and methanol 0.5% as drug vehicles up to 4 weeks, following the occurrence of the lesions. (n=40, 8 mice/group).

**Fig. 3.** Anti-leishmanial effects of artemisinin (10 mg/kg) and glucantime (20 mg/kg) on proliferation of amastigotes inside macrophages in lesion smears in Balb/c mice infected intradermally with inoculums of 2×10^6 promastigotes. Mice were treated daily by 300 µl interaperitoneal injection of glucantime (20 mg/kg), artemisinin (10 mg/kg), normal saline and methanol 0.5% as drug vehicles up to 4 weeks, following the occurrence of the lesions. G=group (n=40, 8 mice/group). *P<0.05.

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