EVALUATION OF MILTEFOSINE ACTIVITY AGAINST VIABILITY AND DNA INTEGRITY OF LEISHMANIA DONOVANI PROMASTIGOTES IN VITRO

Braa A. Alshakir* and Khawla H. Zghair**

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

*Author for Correspondence: Braa A. Alshakir
Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.

ABSTRACT
Visceral leishmaniasis is the most severe disease from all leishmaniasis forms and can be fatal without treatment. Miltefosine was the first oral drug approved in several countries for treatment of visceral and cutaneous leishmaniasis. Regional variations in susceptibility of Leishmania donovani clinical isolates have been recorded to sodium stibogluconate but not other antileishmanial drugs. This study investigates the viability of Leishmania donovani promastigotes using the colorimetric MTT assay and DNA integrity in vitro after exposed to different concentrations of miltefosine in comparison to untreated parasites. The results were expressed as inhibitory concentration IC50. The IC50 of miltefosine drug after 24, 48 and 72 hr was 13.32 µM, 11.73µM and 10.63µM against L. donovani promastigotes respectively. Miltefosine induce apoptosis like death in L. donovani promastigotes. Degradation of DNA was revealed at 11.04, 12.26, 14.72 and 17.17µM after 24 hr, and the clearly degradation was noticed for these concentrations of miltefosine drug after 48 hr ,while the highest concentration of miltefosine (19.62µM) caused completely lysis of DNA after 24hr. Miltefosine in lower concentrations affect L. donovani viability and induce apoptosis like death, thus it might be a promising approach for developing new oral anti- visceral Leishmanial drug.

KEYWORDS: Leishmania donovani, Miltefosine, promastigotes.

INTRODUCTION
Leishmaniasis is a clinically heterogeneous group of diseases, caused by protozoa of the genus Leishmania. Disease can range from a solitary, spontaneous healing ulcer (Cutaneous leishmaniasis), to generalized involvement with visceral leishmaniasis (Neuber,2008).Visceral Leishmaniasis (VL) also known as kala azar, is caused by Leishmania donovani (LD) in the Indian subcontinent, Africa and Asia (Herwaldt, 2005), L. infantum in Mediterranean regions, and L. chagasi in the New World (Berman, 2008).

The classical treatment of leishmaniasis requires the administration of toxic and poorly tolerated drugs. The pentavalent antimonials- meglumine antimoniate (Glucantime) and sodium stibogluconate (Pentostam) are the first-line compounds used to treat leishmaniasis. Other drugs that may be used include pentamidine and amphotericin B (Croft et al., 2005; Natera et al., 2007). Miltefosine is the first effective orally active drug against leishmaniasis.

Miltefosine is effective in vitro against both promastigotes and amastigotes of different species of Leishmania (Croft et al., 1996; Escobar et al., 2002), Kinetoplastidae (Trypanosoma cruzi, T. brucei) and other protozoan parasites (Acanthamoeba, Entamoeba histolytica) (Walochnik et al., 2002).

This study aimed to assess the activity of miltefosine different concentrations against the viability and DNA integrity of local L. donovani promastigotes in comparison to pentostam activity in vitro.

MATERIALS AND METHODS
Growth of L. donovani promastigote in vitro
To obtain large amount of parasites in promastigote stage in vitro, inoculums of one ml was transferred from culture contain growth to screw tube vials contain five ml of media (m 199) with 10% FCS and then incubated at 26°C.

Drug concentrations
Miltefosine (10g, molecular weight 407.57 and purity 99%) was manufactured by (Xian Wango Biopharm Co., Ltd. China), and Pentostam (An injectable ampoules 100mg/ml) was manufactured by (Glaxo Operations UK Limited Castle, Member of the Glaxo Smith Kline Group companies) were used in this study.
A stock solution of each of them was used to prepare different concentrations (1.22, 2.45, 4.90, 9.81, 11.04, 12.26, 14.72, 17.17 and 19.62µM) immediately before exposed to *L. donovani* promastigotes. They were performed in triplicate, incubated at 25°C for 24, 48 and 72hr in vitro.

Testing the viability of the parasites

MTT [3-(4, 5-dimethylthiazol-2-y)-2, 5-diphenyltetrazolium bromide; thiazoly blue] is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes (Terry *et al*., 2004). Formazan can be solubilized using Dimethyl sulfoxide (DMSO), and the dissolved material is measured spectrophotometrically yielding absorbance as a function of concentration of converted dye (Mosmann, 1983).

The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.5 mg/ml and incubated for 4 hour (Terry *et al*., 2004). The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 490 nm using a microplate reader (Tanaka *et al*., 2007). Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula.

\[
\text{Viable cells} = \frac{(AT-AB)}{(AC-AB)} \times 100
\]

Where AC is the absorbance of the untreated samples, AT is the absorbance of the treated samples, and AB is the absorbance of the blank (Verma and Dey, 2004).

DNA fragmentation assay

An apoptotic DNA ladder kit (Promega, USA) was used to extract DNA from treated and untreated parasites, and then loaded on agarose gel electrophoresis.

**Statistical analysis**

Results were expressed as the concentration that inhibited parasite growth by 50% (IC50) after 24, 48 and 72hr.

SAS (2012) program was used to analysis of data to effect of difference factors in study parameters. Least significant different -LSD test was used to significant compare between means of the present results.

**RESULTS AND DISCUSSION**

The present study tried to determine the effectiveness of miltefosine drug on the viability of *L. donovani* promastigotes in vitro, in comparison to pentostam standard drug by using cytotoxicity (MTT) assay and Oligonucleosomal-DNA fragmentation assay.

The viability of *L. donovani* promastigotes exposed to different concentrations of miltefosine and pentostam (1.22, 2.45, 4.90, 9.81, 11.04, 12.26, 14.72, 17.17, and 19.62µM) were determined by colorimetric assay (MTT) after 24, 48 and 72 hr.

Promastigotes exposed to different concentrations of pentostam revealed high viability rate after 24, 48 and 72 hr in comparison to promastigotes treated with miltefosine drug.

Table (1) revealed that all used concentrations of miltefosine showed significant (p< 0.05) differences in the percentages of viable cells, thus the lowest one (1.22µM) of the drug recorded (94 ± 3.4), (88.3 ± 3.5) and (75.1 ± 2.6) percentage of viability, and the highest concentration (19.62 µM) of the drug recorded (31.3 ± 1.8), (18.1 ± 0.81) and (5.8 ± 0.62) percentage of viable cells after 24, 48 and 72 hr, respectively.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>24 (µg/ml)</th>
<th>48 (µg/ml)</th>
<th>72 (µg/ml)</th>
<th>L.S.D value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.22</td>
<td>94.3 ± 3.4</td>
<td>88.3 ± 3.5</td>
<td>75.1 ± 2.6</td>
<td>9.31 *</td>
</tr>
<tr>
<td>2.45</td>
<td>91.9 ± 2.9</td>
<td>82.1 ± 3.1</td>
<td>68.8 ± 2.9</td>
<td>11.68 *</td>
</tr>
<tr>
<td>4.90</td>
<td>88.4 ± 3.6</td>
<td>80.9 ± 4.4</td>
<td>68.6 ± 3.0</td>
<td>9.43 *</td>
</tr>
<tr>
<td>9.81</td>
<td>83.6 ± 3.1</td>
<td>79.2 ± 3.6</td>
<td>62.6 ± 2.6</td>
<td>10.76 *</td>
</tr>
<tr>
<td>11.04</td>
<td>76.6 ± 2.5</td>
<td>56.1 ± 2.5</td>
<td>25.6 ± 1.9</td>
<td>12.67 *</td>
</tr>
<tr>
<td>12.26</td>
<td>52.3 ± 1.6</td>
<td>35.5 ± 1.9</td>
<td>18.1 ± 1.02</td>
<td>10.35 *</td>
</tr>
<tr>
<td>14.72</td>
<td>40.9 ± 2.4</td>
<td>21.3 ± 1.2</td>
<td>10.0 ± 0.89</td>
<td>8.46 *</td>
</tr>
<tr>
<td>17.17</td>
<td>37.1 ± 1.4</td>
<td>19.8 ± 0.94</td>
<td>8.2 ± 0.77</td>
<td>8.94 *</td>
</tr>
<tr>
<td>19.62</td>
<td>31.3 ± 1.8</td>
<td>18.1 ± 0.81</td>
<td>5.8 ± 0.62</td>
<td>6.85 *</td>
</tr>
<tr>
<td>L.S.D value</td>
<td>14.573 *</td>
<td>15.772 *</td>
<td>12.558 *</td>
<td>----</td>
</tr>
</tbody>
</table>

Table (2) revealed lower activity of pentostam drug used in the same concentrations. The lowest concentrations from 1.22µM to 12.26µM showed non-significant (p< 0.05) differences in the percentage of cell viability after 24, 48 and 72hr, while there were a significant (p<0.05) differences in the percentage of viable cells exposed to 14.72µM to 19.62µM of pentostam.
Table (2): The viability of *L. donovani* promastigotes exposed to Pentostam by MTT assay after 24, 48 and 72 hr.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Time (hr.)</th>
<th>24</th>
<th>48</th>
<th>72</th>
<th>L.S.D value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.22</td>
<td>98.9 ± 3.6</td>
<td>98.3 ± 4.6</td>
<td>97.0 ± 3.6</td>
<td>7.44 NS</td>
<td></td>
</tr>
<tr>
<td>2.45</td>
<td>98.0 ± 3.5</td>
<td>97.1 ± 3.8</td>
<td>95.0 ± 2.9</td>
<td>6.89 NS</td>
<td></td>
</tr>
<tr>
<td>4.90</td>
<td>97.3 ± 4.1</td>
<td>96.8 ± 3.6</td>
<td>95.0 ± 2.9</td>
<td>6.75 NS</td>
<td></td>
</tr>
<tr>
<td>9.81</td>
<td>96.6 ± 2.9</td>
<td>95.2 ± 3.6</td>
<td>94.0 ± 3.6</td>
<td>7.21 NS</td>
<td></td>
</tr>
<tr>
<td>11.04</td>
<td>96.1 ± 3.7</td>
<td>93.6 ± 4.1</td>
<td>93.0 ± 2.8</td>
<td>7.95 NS</td>
<td></td>
</tr>
<tr>
<td>12.26</td>
<td>94.2 ± 2.6</td>
<td>92.8 ± 3.5</td>
<td>90.0 ± 3.6</td>
<td>6.33 NS</td>
<td></td>
</tr>
<tr>
<td>14.72</td>
<td>93.7 ± 3.5</td>
<td>91.7 ± 3.8</td>
<td>83.0 ± 3.1</td>
<td>8.02 *</td>
<td></td>
</tr>
<tr>
<td>17.17</td>
<td>90.9 ± 4.1</td>
<td>86.8 ± 4.8</td>
<td>76.0 ± 2.9</td>
<td>9.54 *</td>
<td></td>
</tr>
<tr>
<td>19.62</td>
<td>87.7 ± 2.9</td>
<td>82.4 ± 3.1</td>
<td>71.0 ± 3.5</td>
<td>9.75 *</td>
<td></td>
</tr>
<tr>
<td>LSD value</td>
<td>9.331 *</td>
<td>11.841 *</td>
<td>9.052 *</td>
<td>----</td>
<td></td>
</tr>
</tbody>
</table>

* (P<0.05), NS: Non-significant.

The IC\(_{50}\) of miltefosine drug after 24, 48, and 72hr were 13.32µM, 11.73µM, and 10.63µM respectively, in comparison to pentostam drug which doesn’t revealed the IC\(_{50}\) in all used concentrations and periods.

This result was lower than the IC\(_{50}\) of *L. donovani* promastigotes treated with miltefosine obtained by Verma and Dey (2004) which was 25µM. While it was higher than the IC\(_{50}\) against *L. infantum* promastigotes obtained by Khademvatan et al., (2009) which was 7µM.

Also, Khademvatan et al., (2011) achieved the IC\(_{50}\) for miltefosine at 22 µM and 11µM for *L. major* and *L. tropica* promastigotes respectively, and the ED\(_{50}\) of *L. major* and *L. tropica* amastigotes at 5.7 µM and 4.2µM respectively.

These results revealed that different species and strains of *leishmania* promastigotes showed different responsiveness.

Promastigotes DNA were extracted and run on gel electrophoresis after 24, 48 and 72hr of exposure to different concentrations of miltefosine.

The lowest miltefosine concentrations (1.22, 2.45, 4.90 and 9.81µM) showed slightly promastigote DNA degradation after 24 and 48 hr, figure (1).

Obvious degradation of DNA was revealed at 11.04, 12.26, 14.72 and 17.17µM after 24 hr, and the clearly degradation was noticed for these concentrations of miltefosine drug after 48 hr, figure (2). The highest concentration of miltefosine (19.62µM) caused completely lysis of DNA after 24hr.

These results coincided and confirmed with other previous results which also showed a fragmentation of *Leishmania* DNA in different species under the effect of miltefosine drug but with different concentrations.
A study done by Paris et al. (2004) explain the effect of high concentrations of miltefosine drug on the DNA degradation of *L. donovani* promastigotes into oligonucleosomal fragments. They showed DNA analysis by agarose gel electrophoresis revealed DNA fragmentation into oligonucleosome-sized fragments (in multiples of 200 bp) in WT promastigotes treated with 40 µM miltefosine after 24 hr, whereas untreated cells did not show any DNA fragmentation.

Another study done by Verma and Dey (2004) explain the effect of miltefosine on *L. donovani* promastigotes and amastigotes DNA degradation into oligonucleosomal fragments at 25 µM concentration. Also, Khademvatan et al. (2009) showed the effect of miltefosine on DNA degradation into oligonucleosomal fragment (180-200 bp) of *L. major* promastigote after 24 hr at 32µM concentration and *L. infantum* promastigote undergo DNA degradation at 22µM concentration of miltefosine.

Miltefosine drug developed as an oral drug for leishmaniasis induces apoptosis-like death in *L. donovani* that show nuclear DNA condensation, DNA fragmentation with accompanying ladder formation (Verma and Dey, 2004). Cell death resembling metazoan apoptosis has been reported in several parasitic protozoans (Moreira et al., 1996; Welburn et al., 1999; Balanco et al., 2001; Das et al., 2001).

Induction of programmed cell death is one of the advantages of miltefosine against other currently used drugs, including antimony (Paris et al., 2004; Verma and Dey, 2004).

Our findings indicate that miltefosine in lower concentrations affect *L. donovani* viability and induce apoptosis like death.

REFERENCES


