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Original Article

Cytotoxicity of Miltefosine against *Leishmania major* Promastigotes

Hayder Z. Ali
Department of Biology, College of Science, University of Baghdad
Email: h.z.ali@scbaghdad.edu.iq

ABSTRACT

Leishmaniasis has poor vaccination trials and the available drugs have side effects on patients. Therefore, development of new chemicals is important for public health. Miltefosine is the first drug used for oral treatment of visceral leishmaniasis. Previous studies approved that miltefosine is an effective treatment against visceral leishmaniasis, the visceral form of Leishmania, and also on L. major, the cutaneous form of Leishmania. Here, we investigated the in vitro cytotoxicity of miltefosine on the procyclic promastigotes forms of L. major. Different concentrations of miltefosine were investigated against the flagellated form of L. major, and results showed that miltefosine has inhibited the in vitro proliferation of the parasite, approving the cytotoxic effect of the compound on Leishmania, EC50 was determined after 24 hours exposure for the drug and it was 1.54 µM. Our findings indicate that miltefosine has a cytotoxic effect on the insect stage of the old world L. major and can be a new anti-Leishmanial drug.

INTRODUCTION

Leishmania species is eukaryote parasites, transmitted by the bite of female insect belongs to the family *Phlebotominae; Leishmania* generates two forms during its life cycle which required an invertebrate and invertebrate host to be completed; flagellated insect stage form called promastigotes and vertebrate non-flagellated form called amastigote [1]. Leishmaniasis is considered one of the neglected tropical health problems in which about 350 people living at risk of infection, worldwide [2,3]. Distribution area of the leishmaniasis has been broadly subdivided into the "New world" or the American and the "Old world" (Africa, Asia and Europe); *Leishmania* species are generally associated with one or other of the two subdivisions [4]. Old world leishmaniasis, *L. major,* is usually causes a small lesions but parasites replication is progressively controlled and the lesion heals slowly [5], while New World leishmaniasis, *L. mexicana* complex, when compared to *L. major* in mouse model, it has been proved that *L. mexicana* infection induces a chronic infection with non-healing lesions [6]. Growing interest in leishmaniasis has occurred in recent years due to the increasing number of overseas travelers, U.S. Gulf War veterans, and acquired immuno-deficiency syndrome (AIDS) patients who have developed the disease, also the economic development, environmental changes, and an increased number of worldwide travelers have led to the increased incidence of the disease [7,8,9]. *Leishmania* usually reside within the macrophage of the vertebrate host; to enter the macrophage, *Leishmania* utilizes a variety of cellular receptors to mediate endocytosis. Once inside the macrophage, *Leishmania* is protected from phagolysosome degradation by a variety of adaptations to inhibit cellular defense mechanisms [10,11]. *Leishmania* is a genus of several species and the prognosis of the disease varies with the species, therefore, the treatment is usually depends on the causative *Leishmania* [12]. The specific identification of *Leishmania* species in laboratory cultures is usually fastidious and time consuming that's why in many countries the physicians tend to treat patients with leishmaniasis depending on the clinical features rather than species specific identification [13]. The proposed choice of local or systemic treatment of cutaneous leishmaniasis is guided by the risk of developing mucosal disease. Widely available treatments for leishmaniasis are pentavalent antimonial which is usually used by (85 mg Sb/mL) for intramuscular administration and sodium stibogluconate (100 mg Sb/mL) for intravenous and intramuscular administration. The biochemical basis for their effectiveness is unknown, but may involve inhibition of ATP synthesis. A

possible disadvantage of this drug is the short course treatment which it could contribute to the appearance of resistance [14]. Another chemical which is pentamidine,: aromatic diamidine, its mechanism of action has not been established yet but it is considered the first line treatment for cutaneous leishmaniasis in Latin America also known to have side effect [15, 16]. One of the new drugs against leishmaniasis, which is still under investigations, is miltefosine (hexadecyl-phosphocholine, HePC), a phosphocholine analogue which was originally developed as anti-cancer agent and intended for breast cancer and other solid tumors. It showed high *in vitro* activity against *Leishmania* [17]. Miltefosine does interact with cell signal transduction pathways and inhibits phospholipids and sterols biosynthesis (18). First clinical miltefosine trial was tested on Indian patients with Kala-azar in 2002 and it was given by 250 mg/day [19]. Another trial was determined for Indian visceral leishmaniasis treatment with 100-150 mg for 28 days which cured 96% of patients [20]. In this study, we have investigated the cytotoxicity of miltefosine on the *in vitro* promastigotes-culture of *L. major,* the causative agent of old world leishmaniasis. Different concentrations of Miltefosine were used against the *in vitro* culture of the parasite to determine the cytotoxicity of this drug against *L. major*, using Alamr Blue® assay.

MATERIALS AND METHODS

Parasite culture

A- Procyclic promastigotes of *L. major* (MHOM/IL/81/Friedlin) was kindly provided by Dr Paul W Denny (Dept. of Chemistry, Durham University, UK).

B- Media preparation: Schneider's *Drosophila* medium (Sigma-Aldrich) was used for parasite culture and prepared as described by manufacturer's instruction (21).

C- Axenic culture of promastigotes was maintained at 26^0C, pH 7.0 in Schneider's *Drosophila* medium® supplemented with 15% heated inactivated foetal bovine serum (22).

Miltefosine

This drug was purchased from Cayman Chemical and prepared according to the manufacturer's procedure (23). In brief, crystalline solid miltefosine was dissolved in ethanol (1.25 mg/ml) and stored in -20.

Cytotoxicity screening [22]

A- Two of 96 well-plates (flat bottom) were set up for this experiment. Suspension of *L. major* promastigotes was used for 2-folded serial dilution to achieve triplicate of series concentration starting from $4\chi10^5$ parasite/ml and $1.25\ \chi10^5$ parasite/ml, each raw (100 µl /well).

B- Miltefosine was added into first plate starting from concentration of 50 µM and serial dilution was made to end up with 0.78 µM miltefosine. The 2^{nd} plate was prepared the same but with methanol (MeOH) added instead of miltefosine as positive control; blank media was used as negative control. Plates were incubated at 26^0C for 24 hours prior incubation with Alamar Blue® for 4 hours before assessing of cell viability which was determined by using fluorescent plate reader (Biotek; 650EX nm/600EM nm).

Alamar Blue® assay

Alamar Blue® is changing from blue to red as an indicator of cell viability. Alamar Blue® (Invitrogen) was added to the plates in a ratio of 1:10 and incubated for 4 hours at 26^0C prior result was detected by fluorescent plate reader (24, 25).

Statistical analysis

The t test was used to determine the significance of miltefosine effect and *EC50* was calculated as previously described by (22).

RESULTS AND DISCUSSION

Procyclic insect stage promastigotes of *L. major* was treated with different concentrations of miltefosine to detect the cytotoxicity of this drug on the parasite viability, *in vitro*. Results showed that there was a significance difference in the fluorescent absorption between test and control for the concentration of (50-0.78) µM miltefosine ($P < 0.05$) and there was no significance difference of the lowest concentration (0.78) µM miltefosine ($P \geq 0.05$) as shown in figure [1]. The viability percentage of the parasite was calculated based on the optical absorbance of treated parasites (T), untreated parasites (C) and blank media (B) using the formula: viable cells (%) = (T of each

replicate- mean of B)/(C of each replicate- mean of B) x 100. As shown in figure [2], it shows that the viability of the parasites was below 50% for the concentration of (0.78-50) µM miltefosine. Also the *EC50* was calculated after plotting cell viability (as percent of untreated control) against log miltefosine concentration (µM) as previously described [22]. The result showed that *EC50* is 0.19 which means that the *EC50* in this study is equal to 1.54 µM, figure [3]. A previous study [26,27] showed that after 48 hours incubation of *L. major* amastigotes and macrophages with 20 µM miltefosine, 80% of cells were killed and the *ED50* was 5.7 µM and they suggest that miltefosine may provoked DNA fragmentation in the parasites. Another studies [28, 29, 30] detected the leishmanicidal activity of miltefosine against the Iranian strain of *L. infantum* (causative agent of infant visceral leishmaniasis) and *L. donovani* and they suggested that miltefosine induces apoptosis death in *Leishmania* marked by nuclear condensation and DNA fragmentation and the *EC50* of miltefosine in the study was 7 µM while a previous study on *L. donovani* shows that the *EC50* of the same drug was 13 µM [31].The *EC50* which was calculated in our study is 1.54 µM, varying from previous work, such results show wide range of drug dosage and sensitivity of *Leishmania* spp. Miltefosine is effective *in vitro* against both promastigotes and amastigotes of various species of *Leishmania* or other protozoan parasites, although the actual mechanism of this drug is still partly known, most data refer to its ability of anti-tumor and anti leishmanial activity [32]. However, evidence of apoptosis-like death has been shown in *L. donovani* promastigotes treated with miltefosine, but how this family of compounds induces apoptosis in either mammalian cells or parasites is not entirely clear [31]. One of the hypotheses suggests that miltefosine does inhibit the phosphatidyl choline, the essential element in the synthesis of cellular membrane [33]. Also, a current hypothesis suggests that miltefosine moves across membranes via inward translocation, an energy-dependent process [26] as well as DNA fragmentation after miltefosine treated parasites [28, 29, 30]. In our study, we have used Alamar Blue® assay which has been known as non-toxic and it can be reduced by living cells and it is considered as quantitative and colorimetric assay when compared to MTT assay which is less sensitive when compared to Alamar Blue® [34].

In conclusion, the miltefosine has a valuable suppression effect, *in vitro,* on the promastigotes of *L. major* with *EC50* = 1.54 as we have defined in this study which is agreed with previous studies on different species of *Leishmania* and we can recommend future work on the direct effect of miltefosine on *Leishmania* which can be a promising new drug.

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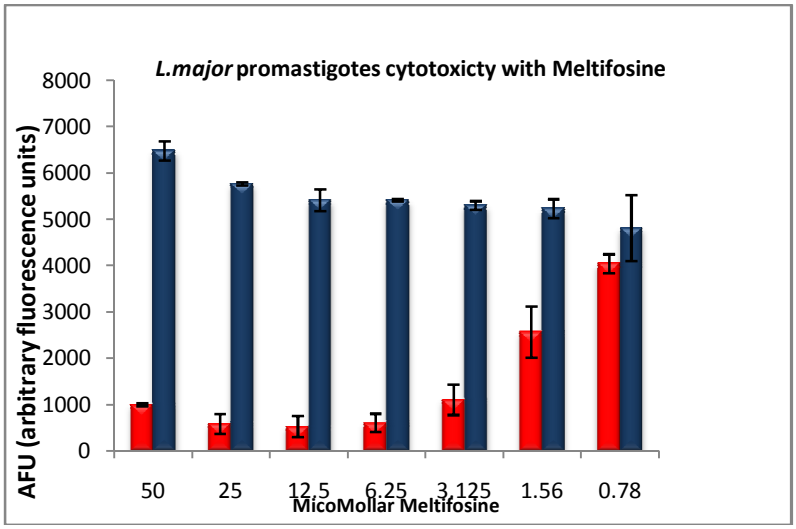

Figure-1: miltefosine cytotoxicity against *L. major* promastigotes, 24 hours incubation with the miltefosine, Alamar Blue® assay (red column=test, blue column=control).

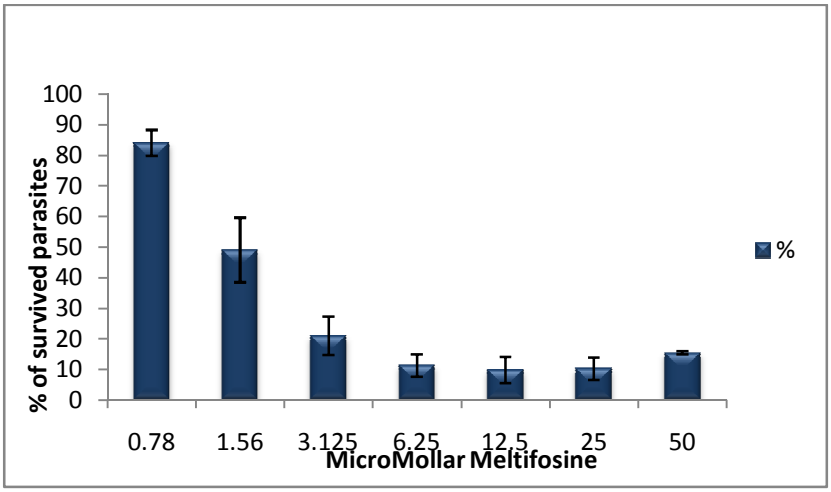

Figure-2: percentage viability of *L. major* promastigotes treated with miltefosine.

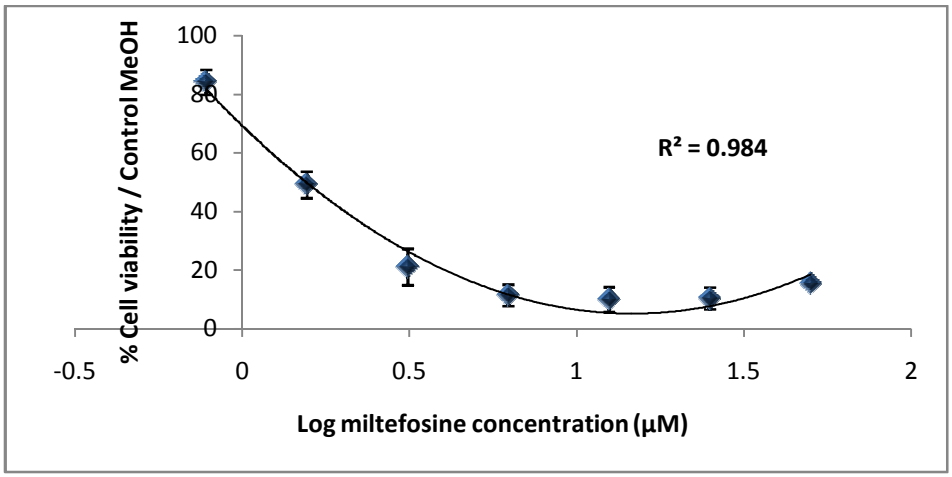

Figure-3: cell viability against log miltefosine concentration (µM), *EC50* = 1.54 µM.

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