ORIGINAL ARTICLE

In vitro evaluation of the efficacy of deferoxamine against *Leishmania major* life cycle stages

Saad M. Bin Dajem

Biology Department, College of Science, King Khalid University, Kahmis Mushyit 61961, P.O. Box 1148, Abha, Saudi Arabia

Abstract Many regions of the world, including Saudi Arabia, are medically vulnerable by the spread of leishmaniasis. Although the effectiveness, the current available drugs suffer from the appearance of resistant strains in epidemic areas and their toxic side-effects. We report the use of the iron chelator deferoxamine (DFRX) that can be used as antileishmanial agents. Our experimental procedure was to follow up the growth of *Leishmania major* stages (extracellular promastigotes, exinic extracellular amastigotes and intracellular amastigotes) in presence of the tested compound in the culture media in different concentrations (10 μg/ml, 25 μg/ml, 50 μg/ml and no treatment) and comparing it to the untreated control. The compound showed antileishmanial activities as it showed great activity against the growth of the three *Leishmania* stages in a time and dose dependent manner. Finally, this compound shows a promising potential activity to be a possible antileishmanial compound alternatively or in combination with the currently used drugs.

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1. Introduction

Leishmaniasis is a parasitic protozoan disease that is caused by obligate intra-macrophage parasite of genus *Leishmania* that is transmitted by sandflies of genus *Phlebotomus*. It is endemic in large areas of the tropics, subtropics and the Mediterranean area (WHO, 2007). It consists of three main clinical forms: cutaneous leishmaniasis (CL); muco-cutaneous leishmaniasis

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(MCL) and visceral leishmaniasis (VL). The species responsible for CL are mainly *Leishmania major* and *Leishmania tropica*. Scars, psychological and social disgrace are the main consequences of the CL. It is affecting approximately 12.5 million people worldwide (WHO, 2007). *Leishmania donovani* and *Leishmania infantum* are responsible for VL. It produces a life-threatening disease with 500,000 new cases and around 50,000 deaths annually (WHO, 2007). It is lethal if left untreated (François et al., 2007; Arevalo et al., 2007).

Leishmaniasis manifests a significant health hazard in Saudi Arabia. Cutaneous leishmaniasis (CL) occurs almost in all parts of the country except the empty quarter. *L. major* and *L. tropica* are the causative agents of CL in the country while *L. donovani* and *L. infantaum* are responsible for VL (Peters and Al-Zaharni, 1987; Ibrahim et al., 1994).

Only few drugs are available for the treatment of different forms of leishmaniasis. Currently, it includes pentavalent

antimonials, amphotericin B and miltefosine. Pentavalent antimonials are of high cost and has severe side-effects (Melby, 2002). The development of resistance is of particular concern (Sundar, 2001; Sundar et al., 2007). Amphotericin B has been the alternative of antimonials as the first-line treatment for VL in some endemic areas (Berman, 2005). Milefosine has been introduced as relatively safe and highly effective drug but it has some side-effects (Bhattacharya et al., 2007). Due to these problems associated with these drugs, researchers have utilized many new compounds, inhibitors and drugs to overcome the disadvantages appeared to the available leishmaniasis drugs.

Deferoxamine (DFRX), an iron chelator, and the similar xenobiotics (e.g., cycloheximide, and pyrimethamine) have been used as antiparasite drugs. Since it was introduced into the market in 1960s, DFRX has been used as antiprotozoal drug and its efficacy has been investigated against several protozoan parasites. These include: *Plasmodium* spp. (Pradines et al., 2006), *Toxoplasma gondii*, and *Trypanosoma brucei* (Tanja et al., 2002). The efficacy of DFRX has been investigated against several protozoan parasites including *Plasmodium* spp. (Pradines et al., 2006), *T. gondii*, and *T. brucei* (Tanja et al., 2002). It exhibit moderate efficacy against *Babesia* (Matsuu et al., 2008). Also, it was used to treat *Perkinsus olseni* the protozoan parasite of bivalve and showed *in vitro* inhibition of the parasite proliferation (Laurence et al., 2005). Desferrioxamine, a derivative of deferoxamine, was used as liposome-entrapped compound and found to be inactive against of promastigotes of *L. donovani* parasite and showed a moderate efficacy against amastigotes stages (Segovia et al., 1989; Ketty et al., 1995).

Due to the problems associated with the drugs used against *Leishmania* and the limited progress in developing a vaccine against human leishmaniases, development of new chemotherapeutic agents is highly required. No research has been done to investigate the effect of this compound against *L. major* life stages (extracellular promastigotes, exinic extracellular amastigotes and intracellular amastigotes). In this study, we tested deferoxamine (DFRX) for its effectiveness as antileishmanial agents against the life cycle stages of *L. major*.

2. Materials and methods

2.1. Compounds and materials

All compounds and tissue culture chemicals used in this study (RPMI, sodium bicarbonate, streptomycin, penicillin, etc.) were mainly from Sigma Chemical Company (USA). Fetal calf serum (FCS) was purchased from HyClone, USA.

2.2. Parasite

L. major isolate was provided by Dr. Ahmed Alkahtani, King Fisal Specialist Hospital and research Center, Riyadh, Saudi Arabia. It was isolated from a Saudi patient from Riyadh, Saudi Arabia. This study was approved by the Ethics Committee at King Khalid University, Abha, Saudi Arabia.

2.3. Growth of Leishmania cells

Leishmania parasite was maintained in the laboratory in RPMI containing 10% fetal calf serum (FCS). Amastigotes

were obtained by incubating promastigotes in RPMI supplemented with 10% FCS and the pH of the media was adjusted to pH 5. Cells were incubated at 37 °C in 5% CO_2.

2.4. Growth and experimental infection of J774A.1 macrophages in vitro

J774A.1 macrophage cell line was maintained in RPMI-1640 medium with 10% FCS. Tissue culture flasks containing macrophages were placed in a CO2 incubator (5% CO_2) at 37 °C. The experiment was started by optimizing *in vitro* infection of macrophage with *Leishmania* promastigotes. Macrophages were inoculated with *L. major* in a 10:1 ratio (promastigote: macrophage) and the infection was monitored microscopically.

2.5. Drug administration to axenic amastigotes and promastigotes

Drug administration to both stages was carried out according to Al-Qahtani et al. (2009). Briefly, Parasites were grown in 24-well plates and seeded at 5×10^6 parasite/ml of culture media. DFRX was dissolved in a dimethylsulfoxide (DMSO) and added to the wells in triplicate in a final concentration of 10 μg/ml, 25 μg/ml or 50 μg/ml. The drug was added at day 1 and every 48 h. Control cells were treated with DMSO (0.4% final concentration) and were always included in all experiment. The cell counts were recorded using a Neubauer haemocytometer.

2.6. Drug administration to infected macrophages

Macrophages (10^6 cells) were growing in Lab-Tek Permanox® Chamber Slide (Electron Microscopy Sciences, Hatfield, PA, USA). Stationary phase promastigotes were added to each well in 10:1 ratio (promastigotes:macrophages) to each well. DFRX was added at different concentrations (10 μg/ml, 25 μg/ml or 50 μg/ml) to each well and the plates were incubated at 37 °C. Triplicate incubations in the presence or absence of DFRX were maintained in all experiments. DFRX has been used in concentrations that have been previously shown to be non-toxic to mammalian cells (Crichton and Ward, 1992). The drug was added at day 1 and then every 48 h during the course of the experiment. The cells and the intracellular amastigotes were counted directly under the microscope.

2.7. Statistical analysis

Differences in growth of the parasite in all experiments (treated with DFRX versus untreated control cells) were analyzed using one-way ANOVA test using SPSS software (version 11; SPSS Inc., Chicago, Illinois, USA). All p values < 0.05 were considered significant.

3. Results

3.1. In vitro infection of macrophage by L. major

The *in vitro* infection of macrophage by *Leishmania* was monitored microscopically. It was found that promastigotes were

Table 1 The effect of different doses of deferoxamine on the number of extracellular axenic promastigotes and axenic amastigotes.

Stage	Concentration Period							
	0 days		2 days		4 days		6 day	
	Extracellular promastigote	Extracellular amastigote	Extracellular promastigote	Extracellular amastigote	Extracellular promastigote	Extracellular amastigote	Extracellular promastigote	Extracellular amastigote
No treatment	$5 \times 10^6 \pm 0.0$	$5 \times 10^6 \pm 0.0$	$1 \times 10^7 \pm 352766.8$	$1.1 \times 10^7 \pm 577350.3$	$1.8 \times 10^7 \pm 881917.1$	$1.6 \times 10^6 \pm 346410.2$	$1.9 \times 10^6 \pm 230940.1$	$1.7 \times 10^6 \pm 240370.1$
10 µg/ml	$5 \times 10^6 \pm 0.0$	$5 \times 10^6 \pm 0.0$	68,66,667 ± 290593.3***	67,33,333 ± 240370.1***	$1.4 \times 10^5 \pm 721110.3$***	$1.2 \times 10^5 \pm 240370.1$***	$1.3 \times 10^5 \pm 240370.1$***	$1.5 \times 10^5 \pm 352766.8$***
25 µg/ml	$5 \times 10^6 \pm 0.0$	$5 \times 10^6 \pm 0.0$	51,33,333 ± 290593.3***b	35,33,333 ± 290593.3***c	$1 \times 10^7 \pm 176383.4$***c	$7.8 \times 10^5 \pm 230940.1$***c	96,66,667 ± 240370.1***c	95,33,333 ± 466666.7***c
50 µg/ml	$5 \times 10^6 \pm 0.0$	$5 \times 10^6 \pm 0.0$	30,66,667 ± 290593.3***c	25,33,333 ± 290593.3***c	46,00,500 ± 305505.1***c	40,66,667 ± 176383.4***c	46,66,667 ± 240370.1***c	29,33,333 ± 240370.1***c

All values are mean ± SE.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ in comparison with control group.
$p < 0.05$ a , $p < 0.01$ b , $p < 0.001$ c in comparison the dose of 10 µg/ml with the other treated groups in different days.

phagocytozed by macrophages, differentiated into amastigotes and multiplied within the host cells.

3.2. Effect of iron chelators on growth of Leishmania

Treatment *Leishmania* promastigotes with (DFXM) causes growth inhibition during the investigated intervals compared to non-treated cells. In Table 1, ANOVA analysis among different concentrations showed very high significant changes ($p < 0.001$) while the least significant differences test showed very highly significant decrease ($p < 0.001$) when 10 µg/ml was administered compared with 25 µg/ml and 50 µg/ml of (DFXM) was used. When axenically-grown amastigotes were incubated in the presence of (DFXM), multiplication of amastigotes was halted compared to vehicle-treated parasites. ANOVA analysis between different concentrations showed very high significant changes ($p < 0.001$) while the least significant differences test showed very highly significant decrease ($p < 0.001$) especially with 50 µg/ml dose (Table 1). To investigate the role of iron chelator on multiplication of *Leishmania* amastigotes within J774.1 macrophages, there was a great reduction in the number of intracellular parasites within host cells. Table 2 showed that ANOVA analysis between different concentrations were of very high significant changes ($p < 0.001$) while the least significant differences test showed very highly significant decrease especially with 50 µg/ml (Table 2).

4. Discussion

Owing to the worldwide distribution of leishmaniasis and the problems associated with the current drugs, development of new chemotherapeutic agents is essential. The success of the parasite to survive in the mammalian host depends largely on its ability to invade macrophages and to transform to amastigotes, the stage that is adopted to live in the tissue of the mammalian host (Bogdan et al., 1996). The organic pentavalent antimoniates, which are the clinical drugs most frequently employed against leishmaniasis, have been in use for several decades. However, antimoniates have been associated with considerable toxicity to humans. The spread of drug-resistant *Leishmania* strains in several endemic areas stresses the urgent need of identifying new chemotherapeutic means against leishmaniasis. Amphotericin B and miltefosine have been also used. Each of the above mentioned drugs has its own limitations (toxicity, price, efficacy and treatment schedules) (Santos et al., 2008; Davis and Kedzierski, 2005).

Iron is a required nutritional substance that is essential for eukaryotic cell growth and differentiation (Crichton and Ward, 1992). Pathogenic agents are dependent on iron for their growth, it has been postulated that iron-chelating compounds may be effective in eliminating or reducing infection. The discovery of transferrin receptor on *Leishmania* parasite suggests that sufficient availability of iron is essential for the life cycle (Ketty et al., 1995). In this study, deferoxamine showed great efficacy against *L. major* different stages. It has been also shown that the depletion of Iron results in impairment of DNA synthesis (Crichton and Ward, 1992) When treated with iron-chelating agent, It is assumed that pathogens inhibition is caused by inactivation of ribonucleotide reductase (RBRD), an iron-dependent rate-limiting enzyme in DNA synthesis (Hoffbrand et al.

Table 2 The effect of in different doses of Deferoxamine on the number of intracellular amastigotes.

Dose	Period								
	0 day	3 days	6 days	9 days	12 days	15 days	18 days	21 days	24 days
No treatment	0.0 ± 0.0	18 ± 0.6	21 ± 0.6	30 ± 0.6	35 ± 0.6	38 ± 0.6	47 ± 0.6	46 ± 0.6	46 ± 0.6
25 µg/ml	0.0 ± 0.0	18 ± 0.6	21 ± 0.6	16 ± 0.6***	26 ± 0.6***	31 ± 0.6***	35 ± 0.6***	35 ± 0.6***	32 ± 0.6***
50 µg/ml	0.0 ± 0.0	19 ± 0.6	18 ± 0.6***	18 ± 0.6***	20 ± 0.6***	23 ± 0.6***	15 ± 0.6***	15 ± 0.6***	15 ± 0.6***

All values are mean ± SE.
$*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in comparison with non-treated group.

1976; Jordan and Reichard, 1998). It seems reasonable to assume that same parameters could be applicable in both promastigotes and amastigotes of *Leishmania*. RBRD has identified in *Leishmania* with properties similar to that of its mammalian counterpart. However, inhibition of growth of intracellular amastigotes shown in this study may be due to several factors. In addition to the effects of iron chelator mentioned above, removal of iron has been shown to induce secretion of cytokines and to induce apoptosis in several cell lines. Both mechanisms could lead to reduction of growth of intracellular amastigotes. Lastly, one of the interesting findings of this research is the indirect evidence that *Leishmania* amastigotes possess RBRD activity since growth of these parasites was inhibited in the presence of DFXM. It has been also shown that both *Leishmania* amastigotes and promastigotes contain sizable quantities of iron stored in an organelle known as acidocalcisome (Al-Qahtani, 1990). Taken together, it is not surprising to find that iron depletion is detrimental to extracellular or intracellular growth of *Leishmania*.

This compound has been used as antiparasitic drug. When DFXM used against *T. brucei*, it has been found that it inhibits the proliferation of the parasites and was postulated that the effect of this compound on the activity of alternative oxidase of blood stages forms lower the rate of DNA synthesis (Cooper et al., 1996). *In vitro* and *in vivo* studies with *Trypanosoma cruzi, Plasmodium falciparum, Plasmodium vinckei,* and *Plasmodium berghei* in mice, rats and monkeys have all shown that this compound has the ability to inhibit the proliferation of these parasites. (Lalonde and Holbein, 1986; Fritsch et al., 1985; Gordeuk et al., 1993; Hershko and Peto, 1988; Pollack et al., 1987; Pradines et al., 2006) When used as antiparasite against *Babesia gibsoni in vitro* it exhibited intermediate effectiveness (Matsuu et al., 2008). In a study of bivalve clam parasite, *P. olseni*, it has been shown that, this iron chelator failed to eliminate light infections in the clam despite their *in vitro* antiprotozoal properties (Laurence et al., 2005). The present study suggested that iron depletion may be an effective against *Leishmania* infection. However, usage of iron chelators as a possible antileishmanial therapy must be taken with great care since they could create potential health disorders for host as has been noted with other infectious agents (Golenser et al., 2003)

In conclusion, the tested drug has shown to possess a promising antileishmanial activity *in vitro* system. Furthermore, their effect *in vivo* in experimental animals should be performed to draw a meaningful conclusion.

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