International Journal for Parasitology 42 (2012) 443–452

Contents lists available at SciVerse ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Giardia lamblia: A new target for miltefosine

Maha M. Eissa *, Eglal I. Amer

Department of Medical Parasitology, Faculty of Medicine, Alexandria University, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 22 November 2011
Received in revised form 7 February 2012
Accepted 9 February 2012
Available online 19 April 2012

Keywords:
Miltefosine
Giardia lamblia
In vivo
In vitro
Adherance assay
Electron microscopy
Histopathology

ABSTRACT

Giardia lamblia, the causative agent of giardiasis, is an intestinal infection with worldwide distribution and high rates of prevalence. Increased resistance of the parasite and the side effects of the reference drugs employed in the treatment of giardiasis make it necessary to seek new therapeutic agents. Therefore, the aim of this study was to examine the activity of hexadecylphosphocholine (miltefosine), a membrane active alkylphospholipid, that is licensed as an antileishmanial agent against giardiasis. The efficacy of miltefosine was evaluated both in vitro and in vivo in Swiss albino mice. Results of the in vitro testing revealed susceptibility of *G. lamblia* trophozoites to miltefosine with the following effective concentrations: EC_{50}s of between 20 and 40 μM, and EC_{90}s of between 20 and 80 μM. Immediate total lysis of the organisms was achieved by 100 μM. In vivo testing showed that oral administration of miltefosine, in a daily dose regimen course of 20 mg/kg for three successive days, to infected mice resulted in total elimination of the parasite from the intestine and amelioration of intestinal pathology. Scanning and transmission electron microscopy studies revealed that miltefosine induced severe morphological alterations to *G. lamblia* trophozoites, mainly at the level of cell membrane and adhesive disc. In conclusion, we believe that this is the first study highlighting *G. lamblia* as a possible new target for miltefosine.

1. Introduction

Giardia lamblia is an intestinal flagellated protozoan parasite that may give rise to diarrhoea with or without malabsorption. It has been identified as the etiological agent in numerous waterborne outbreaks of diarrhoeal disease (Adam, 2001). This parasite is endemic throughout the world and it is one of the most frequently found intestinal parasites in children living in developing countries. According to the World Health Organization, the worldwide incidence is approximately 500,000 new cases per year (WHO, 1998).

Although various drugs have been available for several decades to treat this infection, such as metronidazole, tinidazole, furazolidone, paramomycin and nitazoxanide (Adagu et al., 2002; Ávila et al., 2002; Morrone et al., 2004), none of those is entirely satisfactory due to the high incidence of undesirable side-effects, frequent relapses, activity against normal intestinal flora, possible carcinogenicity and parasite resistance (Ellis et al., 1993; Upcroft et al., 1999; Petri, 2003). Resistance to metronidazole and/or the nitroimidazole-related compounds, secnidazole, tinidazole and ornidazole (Townson et al., 1994), is a potential problem (Upcroft et al., 1999; Petri, 2003). In such cases, albendazole has been proposed as an alternative to metronidazole but it is not always effective (Brasseur and Favennec, 1995). To date, there is no known highly effective alternative to metronidazole available, therefore, there is an obvious need for alternative antigiardial agents.

Alkylphosphocholines are phospholipid-like substances that exhibit antineoplastic activity in vitro as well as in vivo (Unger et al., 1989; Eibl and Unger, 1990). The model compound, hexadecylphosphocholine (miltefosine), is licensed for the topical treatment of breast cancer skin metastases and is the first oral drug licensed for the treatment of leishmaniasis (Ganguly, 2002). Miltefosine is being investigated by researchers seeking treatments for infections which have become resistant to existing drugs. In vitro and animal studies have shown that miltefosine possesses broad anti-protozoal activity. More data has become available on its activity against other Kinetoplastidae: *Trypanosoma cruzi* and *Trypanosoma brucei* (Croft et al., 1996), other protozoan parasites, metronidazole-resistant variants of *Trichomonas vaginalis* (Blaha et al., 2006), *Entamoeba histolytica* (Seifert et al., 2001) and several free-living amoebas (Walochnik et al., 2009; Polat et al., 2011). Indeed, in 2005 miltefosine was designed as an orphan medicinal product for the treatment of *Acanthamoeba* keratitis by the European Medicines Agency (http://www.emea.eu.int/pdfs/human/comp/20357405en.pdf).

Apart from its antiprotozoal effect, miltefosine also has been demonstrated to have antifungal (Widmer et al., 2006), antibacterial (Llull et al., 2007) and most recently, antihelminthic and

* Corresponding author. Tel.: +20 1 223160355; fax: +20 3 4831498.
E-mail address: mahaeissa19@yahoo.com (M.M. Eissa).

0020-7519/$36.00
http://dx.doi.org/10.1016/j.ijpara.2012.02.015

molluscicidal properties against *Schistosoma* spp. (Eissa et al., 2011a,b).

To the best of our knowledge, there are no previous reports on the antigiardial effect of miltefosine. Therefore, given its promising broad antiprotozoal activity and with the aim of contributing to the search for new therapeutic alternatives for treatment of giardiasis, the current study was carried out to evaluate the in vitro and in vivo efficacies of miltefosine against *G. lamblia.*

2. Materials and methods

2.1. Parasite collection and preservation

Diarrhoeic stool samples were collected from patients attending the Paediatric Department, El-Shatby Hospital, Alexandria, Egypt, and the Tropical Medicine Department, Alexandria Main University Hospital, Alexandria, Egypt. Informed consent was obtained from the patients. All specimens were microscopically screened by conventional diagnostic methods using a direct wet saline smear (Garcia and Bruckner, 1997) An iodine smear was prepared for the detection of *Giardia* cysts. Positive samples were emulsified in saline solution and filtered through two layers of gauze to remove coarse particles. The suspension was then centrifuged at 400*g* for 10 min. The sediment was stored in an equal volume of 2.5% potassium dichromate solution at 4 °C until required (Khademi et al., 2006).

2.2. Purification of Giardia cysts

Giardia cysts were separated from faecal debris using 1 M of sucrose as follows; in a conical glass centrifuge tube, 2 ml of 2.5% potassium dichromate containing *Giardia* cysts were layered on the top of 3 ml of sucrose solution. The tube was centrifuged at 800*g* for 5 min and the cysts that had concentrated at the emulsion–sucrose interface were removed and transferred to a clean tube. Distilled water (1:10) was added to each tube and the cysts were sedimented by centrifugation at 800*g* for 10 min. The cysts were then washed twice in distilled water. After the supernatant was discarded, the pellet was resuspended in a sterile tube containing 3% sodium hypochlorite to kill other microorganisms. Tubes containing *Giardia* cysts in 3% sodium hypochlorite solution were chilled in an ice bath for 10 min and then washed three times with PBS that was free of calcium and magnesium. The process of purification was performed using an aseptic technique (Buraud et al., 1991).

2.3. Drugs

Milteforan® 2% veterinary oral solution (Miltefosine, Virbac, Italy) was used and kindly supplied by Dr. Paolo Bianciardi, Virbac, Italy.

Metronidazole (flagyl®, Amriya Pharmaceutical Industries, Alexandria, Egypt), intravenous infusion sterile solution 500 mg/ml, was purchased from a local pharmacy.

2.4. Experimental animals

Animal experimentation was approved by the Ethics Committee of the Faculty of Medicine, Alexandria University, Egypt. All animal experiments comply with the Egyptian national regulations for animal experimentation.

Experiments were performed on 100 Swiss albino mice of both sexes, 4–6 weeks old and weighing 20–25 g at the onset. They were purchased from the Animal House Medical Parasitology Department, Faculty of Medicine, Alexandria University, Alexandria, Egypt. Mice were housed in separate cages in air conditioned rooms. Animals were fed wheat, milk and bread on alternate days.

2.4.1. In vitro assay

Ten laboratory bred Swiss albino mice, 4–6 weeks old of both sexes, were inoculated orally with 2×10^5 *G. lamblia* cysts. On day 6 p.i., the mice were sacrificed and the entire small intestine was removed, cut longitudinally and placed in tubes containing 3 ml of cold sterile saline solution for at least 10 min. Tubes were vortexed to ensure complete detachment of parasites (Leméea et al., 2000). *Giardia* trophozoites were separated from the mucosa by gauze filtration, and filtrates were cultured on TYI-S-33 medium supplemented with bile salts, 10% heat inactivated bovine serum, 10 μg/ml of ampicillin and 10 μg/ml of gentamicin sulphate (Keister, 1983; Cedillo et al., 1991). Stock cultures of trophozoites were grown in screw-cap glass tubes at 37 °C. Organisms were subcultured every 72 h by chilling the culture tube in an ice water bath for 5 min. The trophozoites were dislodged from the glass by inverting the chilled tube vigorously. The number of organisms per ml was determined and 10^4 organisms from a logarithmically growing culture were transferred into fresh medium. For susceptibility tests, a 2 mM miltefosine stock solution was prepared by dissolving miltefosine in 5% (w/v) ethanol. Miltefosine and metronidazole were used in a range of concentrations: 10, 20, 40, 80 and 160 μM/ml. Three separate experiments were performed for each concentration with the incubation performed at 37 °C. The effect on the morphology of the trophozoites was recorded after 30 min, 1 h and 24 h using light microscopy. Living and dead cells, as revealed by 0.3% Trypan Blue staining, were counted on a haemocytometer (Neubauer Improved, Germany) and 50% effective concentrations (EC_{50}) and 90% effective concentrations (EC_{90}) were calculated. One hundred percent eradication was shown by inoculation of the respective cell pellets into fresh culture medium without the addition of miltefosine. The susceptibility assays were performed in triplicate, in two independent experiments. For each test a control series was included in the experiments (Blaha et al., 2006).

For the adherence inhibition assay, 10^4 trophozoites were inoculated into culture media and various concentrations of each drug were dispensed into culture tubes and incubated at 37 °C for 2 h. The effluents were collected in disposable test tubes. For enumeration of adhered trophozoites to borosilicate culture tubes, the tubes were chilled on ice for 5 min, then trophozoite concentrations quantitated using a haemocytometer. Experiments were performed in triplicate and the results were calculated as follows:

$$\% \text{ adherence} = 100 - [C_e/C_o \times 100]$$

where Ce is the concentration of *Giardia* in the effluent and Co is the concentration in the original suspension (Khademi et al., 2006).

2.4.2. In vivo assay

One hundred and eight Swiss albino mice were used. Their stools were examined for three successive days by direct wet saline smear, iodine and Sheather's sugar flotation method to exclude the presence of parasites (Garcia and Bruckner, 1997). Mice were inoculated orally with 2×10^5 cysts and allocated equally into three groups.

Group I: control group (infected untreated mice).

Group II: therapeutic control group (metronidazole-treated mice) with metronidazole given orally on the sixth day p.i. in a daily dose of 50 mg/kg for 1 day (group IIa), three successive days (group IIb), and five successive days (group IIc), with 12 mice in each subgroup (Leméea et al., 2000).

Group III: miltefosine-treated mice with miltefosine given orally on the sixth day p.i. in a daily dose of 20 mg/kg for 1 day (group IIIa), three successive days (group IIIb), and five successive days