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In vivo activity of perifosine against *Leishmania amazonensis*

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ABSTRACT

Miltefosine has been established as the first oral administration drug against cutaneous and visceral leishmaniasis. Other alkyl-phospholipids such as edelfosine have been tested against *Leishmania* showing an *in vitro* antiparasitic activity. Perifosine *in vitro* activity has been previously demonstrated against different *Leishmania* species including *Leishmania amazonensis*. In this study edelfosine and perifosine were orally administered to *BALB/c* mice at doses of 1 and 2.5 mg/kg/day during 28 days and 5 mg/kg/day during 14 days, starting the treatment 2 weeks after the first treatment scheme. Lesion sizes and parasitic burden as well as viability were determined in order to establish the treatment effectiveness. An assay to compare miltefosine at standard dose of 2.5 mg/kg/day during 28 days to an *in vivo* treatment with perifosine at the most effective treatment scheme observed in this study 5 mg/kg/day during 14 days, was also developed. Perifosine showed the higher activity in the *in vivo* assay and is showing as a new possibility within the alkyl-phospholipids group for the treatment against cutaneous leishmaniasis caused by *L. amazonensis*.

1. Introduction

Parasitic tropical diseases affect hundreds of millions of people worldwide but have been largely neglected for drug development because they mainly affect poor people in poor regions of the world. Most of the current used drugs are decades old and have many limitations, including the emergence of drug resistance. It is estimated that the annual incidence of cutaneous leishmaniasis (CL) is about 2 million persons. In the New World, leishmaniasis is presented in almost the whole American continent and is caused by a multitude of *Leishmania* species, members of *L. vianna* subgenus and members of the *L. mexicana* complex such as *L. m. amazonensis* and *L. m. mexicana* (Soto and Berman, 2006). In this pathology, the primary lesion spontaneously ulcerates after a few days and then the ulcer grows in surface, deep becoming a massive ulcer. After a few months, the lesion could reach various centimetres of length and often the parasites could invade, at this stage, the lymphatic nodes and mucosal tissues and even reach visceralization (Palacios et al., 2001; Peters and Sacks, 2006; Soto and Berman, 2006).

CL should be treated to accelerate cure in order to prevent dissemination to the mucosa, pentavalent antimonials such as, the meglumine antimoniate (Glucantime®) are currently the first election treatment (Croft et al., 2005; Murray et al., 2005; Soto and Berman, 2006). In 2005, miltefosine (Impavido®, Æterna Zentaris, 2002) was approved for the treatment of CL in Colombia due to the previous good results (Berman et al., 2006; Soto and Berman, 2006). In the clinical trials carried out against American cutaneous leishmaniasis, the standard posology by oral administration is 2.5 mg/kg/day for 28 days (Soto et al., 2004).

Other alkyl-phospholipids such as edelfosine have been tested against *Leishmania* showing a lower *in vitro* antiparasitic activity than miltefosine (Croft and Yardley, 2002; Escobar et al., 2002; Santa-Rita et al., 2004; Azzouz et al., 2005). Perifosine is another alkyl-phospholipid which is being used against cancer (Posadas et al., 2005; Knowling et al., 2006). In 2007, it was demonstrated the *in vitro* activity of perifosine against different *Leishmania* species from the New and Old World, being three of them potential agents of CL o MCL (Cabrera-Serra et al., 2007). In this study the *in vivo* activity of edelfosine, perifosine and miltefosine was evaluated in a *BALB/c* murine model which was infected with *Leishmania amazonensis*.

2. Material and methods

2.1. Drugs

Miltefosine was obtained from Zentaris GmbH (Frankfurt am Main, Germany). Edelfosine and perifosine were purchased from

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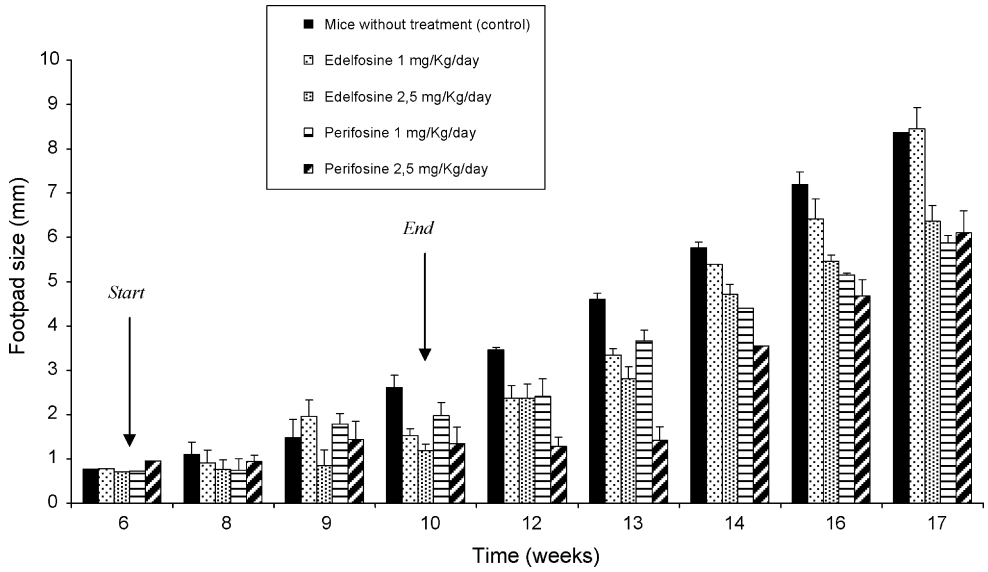

Fig. 1. Treatment during 28 days, shown as number of weeks post-infection. Effects of edelfosine and perifosine on the lesion development of mice infected with *L. amazonensis* promastigotes. Results are shown as standard mean ± S.E.M. for the four mice included in each group.

Cayman chemical company (Tallinn, Estonia). Stock solutions were prepared in PBS 1× (Gibco) and stored at −20 °C.

2.2. Parasites and culture

The infection was carried out with *L. amazonensis*, MHOM/BR/77/LTB0016 promastigotes, obtained from amastigotes forms isolated from mice lesions. Subcultures were made in the late-log phase of growth and parasites were used no later than at the fifth passage.

2.3. Animals

Female and male *BALB/c* mice, with a body weight of approximately 20 g and 5 weeks of age, were used in this study. The infection experiments were conducted in accordance with the Spanish and European guidelines for experimentation with animals.

2.4. Antileishmanicidal in vivo activity

The *in vivo* assay was carried out in *BALB/c* mice infected with *L. amazonensis*. The infection was carried out in the same conditions as previously described (Piñero et al., 2006). Briefly, on day 0, mice were injected subcutaneously in the footpad with 0.05 ml of a suspension of promastigotes in stationary phase containing 1×10^7 parasites. After 6 weeks, lesions of measurable size were developed. Mice weight and gastrointestinal side effects were monitored during the whole infection process and the treatment scheme.

Mice were randomly sorted into groups of four and were treated orally with 1 and 2.5 mg/kg/day of edelfosine (E-1 and E-2.5) and perifosine (P-1 and P-2.5), during 28 days. Treatment was started on the 6th week post-infection.

In another experiment, groups of four mice were administered 5 mg/kg/day of edelfosine (E-5) and perifosine (P-5), during 14 days. Treatment was started on the 8 week post-infection. The lesion size was measured every week during a period of 4 months of infection (17 weeks).

Moreover, another assay was carried out with oral administration of miltefosine and perifosine. Groups of four mice each received 2.5 mg/kg/day of miltefosine during 28 days, and 5 mg/kg/day of perifosine during 14 days. This treatment scheme was started in the same day for the two alkyl-phospholipids in order to compare the best dose of perifosine referred to the standard dose of miltefosine.

2.5. Amastigote parasitic burden and viability evaluation in the tissues

The parasitic burden was determined 7 weeks after treatment (17th week of monitorization), thus tissues were homogenised (grinding) and amastigotes forms were released. After decanting for 5 min, amastigotes were separated from tissue and counted from supernatant on a Z1™ Series COULTER COUNTER® (Beckman Coulter). After counting the number of parasites in each sample, the obtained aliquots were centrifuged at room temperature during 5 min at 600 rpm. After this centrifugation, supernatant was discharged and aliquots were centrifuged again at 2000 rpm during 5 min. Obtained pellets were resuspended in 1 ml of RPMI medium without phenol red supplemented with a 10% of inactivated foetal bovine serum and 10 μl/ml of gentamicin (Roche). Pellets were then seeded in 96-well plates and parasite number was calculated using a COULTER™ Z1 cell counter as mentioned above. A working suspension of 1×10^6 parasites/ml was prepared and serial dilutions of this one were prepared in the assay plates at 2*x*, 4*x*, 8*x*, 16*x*, 32*x*, 64*x*, 128*xy* and 256*x* (each dilution was repeated 8 times in order to control homogeneity in the results). This method is a variation of a previously described method known as "*Limit Dilution*" method and it is used to determine the concentration, in ideal culture conditions, where amastigotes are able to differentiate into promastigotes, considering this ability as a viability parameter (Titus et al., 1985; Buffet et al., 1995; Lima et al., 1997; De Sá Oliveira et al., 2000; Teixeira et al., 2002). Briefly, the culture medium in these assays was RPMI medium without phenol red and a 10% of inactive foetal bovine serum at a pH of 7.2. Finally, a 10% of AlamarBlue™ assay was added to all wells and plates were incubated at 28 °C during 72 h. Absorbancies at 570 and 630 nm were measured using a Micro plate Reader Model 680 (BIO-RAD) (Mikus and Steverding, 2000; Cabrera-Serra et al., 2007). The growth rate percentages of each group were obtained in comparison to the non-treated mice group at 24, 48 and 72 h of incubation. Obtained data were processed with Excel software programme (Office 2003)