
New administration model of trans-chalcone biodegradable polymers for the treatment of experimental leishmaniasis

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Abstract

The present study was designed to investigate a new administration model and the antileishmanial activity of a semi-synthetic chalcone, benzylideneacetophenone (trans-chalcone). The antileishmanial activity of this product was first tested in vitro against promastigotes of L. braziliensis, L. tropica, L. infantum and L. amazonensis. An in vivo experiment was carried out using subcutaneous administration of trans-chalcone and implants of synthetic biodegradable polymers, polylactic acid (PLA) and polyactic/glycolic acid (PLGA). This compound showed potent inhibitory effects on the growth of all Leishmania strains examined. Subcutaneous administration of trans-chalcone at a single dose of 4 mg/kg of body weight reduced lesion development in mice infected with L. amazonensis. A similar inhibition of the lesion growth in mice treated with trans-chalcone and pentamidine was observed. PLA and PGLA implants of trans-chalcone at 4 mg/kg were administered to mice infected with L. amazonensis. PLGA implants induced a highest reduction in the lesion size (31.25%) than PLA implants (10.75%). Treatment in vitro with trans-chalcone at IC50, completely inhibited the pathogenicity of this parasite in vivo. The development of this model provides a new practical technique for delivering drugs and can be useful for experimental leishmaniasis treatment.

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1. Introduction

Leishmaniasis is a parasitic disease with a wide range of clinical symptoms: cutaneous, macucutaneous and visceral, produced by various species of the protozoan parasite Leishmania. This ailment is considered by the World Health Organization (WHO) among the six most
important tropical diseases (Borst and Oullette, 1995; Gallagher et al., 1994).

Human leishmaniasis is distributed worldwide, but mainly in the tropics and subtropics, with a prevalence of 12 million cases and an approximate incidence of 0.5 million cases of visceral leishmaniasis and 1.5 million cases of cutaneous leishmaniasis in the 88 countries where the disease is endemic, and approximately 80% of the population earns less than $2 a day. It is also considered that there is a current population of 350 million of people under risk of infection (Hirst and Stapley, 2000).

In Europe, leishmaniasis is an opportunistic infectious disease affecting patients treated with immunosuppressive agents or with the human immunodeficiency virus (HIV) infection (Albrecht et al., 1996), occurring in 2–7% of AIDS patients (Montalban et al., 1990; Dereure et al., 1995; Rosenthal et al., 1995). The number of cases of visceral leishmaniasis/HIV co-infection reported to WHO up to December 1999 was 1627 (Desjeux, 2001).

The pentavalent antimonials, sodium stibogluconate and N-methylglucamine antimoniate are the drugs currently in use, while amphotericin and pentamidine are secondary chemotherapy agents. Treatments are not consistently effective because these agents display high liver and heart toxicities, develop clinical resistance after a few weeks of treatment and currently contribute to increase co-infections leishmaniasis–HIV in some countries (Faraut-Gambarelli et al., 1997). For all this, it is necessary the development of new, effective and safe drugs for the treatment of leishmaniasis.

Chalcones are a group of natural products characterised by the presence of a 1,3-diphenylprop-2-en-1-one skeleton. They present a broad spectrum of biological activities including antibacterial (Friis-Moller et al., 2002; Tsukiyama et al., 2002), antihelmintic and amoebicidal effects. Previous studies have shown that chalcones have strong antileishmanial (Chen et al., 1994; Hermoso et al., 2003; Liu et al., 2003) and antimalarial activities (Chen et al., 1997; Go et al., 2004). These drugs alter the structure of the parasite's mitochondria and inhibit their function (Zhai et al., 1995). More recently, the mechanism of action was elucidated showing that chalcones inhibited the respiration of the parasite and the activity of mitochondrial dehydrogenases (Zhai et al., 1999).

Some studies against L. amazonensis with methoxychalcone isolated from Piper aduncum extract showed significant in vitro and in vivo activity (Torres-Santos et al., 1999a,b). These studies showed that inhibitory action on amastigotes is apparently a direct effect on the parasites by disorganization of the mitochondria of promastigotes.

In recent years, alternative therapies using new formulation and drug delivery systems may reduce the toxicity and improve the activity of antileishmanial compounds. Implantable drug delivery systems have meant significant progress in developing novel therapeutic methods. Their advantage includes decreased overall dose and administration frequency, reduction of possible side effects and enhanced treatment efficiency.

Among the various biodegradable polymers, PLGA [poly(lactic-co-glycolide)] was particularly suitable to be used for the drug delivery application. This polymers are already approved by the US Food and Drug Administration (FDA), such as biodegradable structures, implantable screws, pins, drug delivery devices and tissue engineering (Lee et al., 2001; Eliaz and Kost, 2000).

The present study was designed to investigate a new administration model and antileishmanial activity of a semi-synthetic chalcone: benzylideneacetophenone (trans-chalcone). A previous study has show that this drug exhibited promising leishmanicidal and trypanocidal activities (Lunardi et al., 2003).

The in vivo experiment was carried out using subcutaneous implants of synthetic biodegradable polymers. The development of this model provides a new local drug delivery system that could be useful for experimental leishmaniasis treatment.

2. Material and methods

2.1. Drugs

The tested chalcone (Fig. 1) was obtained from Sigma–Aldrich Chemical Co. (Madrid, Spain) and solubilized in dimethyl sulphoxide (DMSO; Sigma) to prepare a working solution of 10 mg/ml. Later on it was diluted in RPMI 1640 medium to the final highest concentration of DMSO on 1.5%, which was not toxic to the parasites.

2.2. Implants preparation

Biodegradable poly(lactic acid (PLA, weight-average molecular weight 40,000) and poly(lactic/glycolic) acid (PLGA, weight-average molecular weight 47,000 and

![Fig. 1. Chemical structure of trans-chalcone.](image)
LA/GA) containing 40% (w/v) of drug were prepared by a solvent casting technique (Kwong et al., 1986). Briefly, the drug was first dissolved in a 30% (w/v) polymer-methylene dichloride solution. The films were prepared by casting this solution into petri disher. The methylene dichloride was allowed to evaporate slowly at 2–8°C for 48 h and the films were vacuum-dried in a dessicator at room temperature for 12 h to remove the residual solvent. The resulting simple monolithic film was cut into small discs of a 3 mm diameter and each one was weighed. The final weight and thickness of the discs, thus obtained was 1.97 ± 0.127 mg and 0.148 ± 0.028 mm, respectively.

2.3. Parasite culture

In this study we used L. braziliensis (MHOM/PE/95/LQ8), which was isolated in the province of La Convención, Cuzco, Perú, L. tropica (MON 58/LEM 2578) and L. infantum (MON 183/LEM 2592). Promastigotes were adapted for culture in RPMI 1640 liquid medium (Gibco-BRL) supplemented with 20% heat inactivated fetal bovine serum, vitamins and amino acids, at 22°C. Logarithm phase cultures of promastigotes were used for experimental purposes. We also used one strain of L. amazonensis (MHOM/BR/77/LTB0016). Promastigotes, obtained from amastigotes forms isolated from mouse lesions, were maintained at 26°C in Schneider’s medium (Schneider’s Insect Medium; Sigma Cell Culture, St. Louis, MO, USA) containing a 10% heat inactivated fetal calf serum, 100 U of penicillin/ml and 100 μg of streptomycin/ml. Subcultures were made in the late-log phase of growth and parasites were used no later than at the fifth passage.

2.4. Animals

Male BALB/c mouse, with a body weight of approximately 20 g, were used in this study. The infection experiments were conducted in accordance with the Oswaldo Cruz Foundation guidelines for experimentation with animals.

2.5. Antipromastigote activity

The inhibition of promastigotes growth in vitro was assessed as previously described (Piñeiro et al., 2002). Parasites were added to sterilized microtitre plates with 24 wells (Corning) at a concentration of 5 × 10⁴/well (500 μl/well), exposed to drugs for 48 h and counted on a Coulter Counter model Z1. The 50% inhibitory concentration (IC₅₀) was determined by linear regression analysis with 95% confidence limits. Tests were performed in at least triplicate on three different days in order to verify the results.

2.6. In vivo drug assay

The in vivo activity was carried out in BALB/c mouse infected with L. amazonensis, in the same conditions as previously described (Alves et al., 2003). On day 0, mice were injected subcutaneously with 0.05 ml of stationary-phase promastigotes suspension containing 1 × 10⁷ parasites, in the footpad. After 5 weeks, lesions of measurable size developed. Mice were randomly sorted into group of five and were treated in different schemes, subcutaneously with 4 mg/kg of trans-chalcone and 4 mg/kg of pentamidine, which is a concentration equivalent to those used in the treatment of leishmaniasis. The lesion size was measured every week in a period of 2 months.

In another experiment, groups of five mice each received an ear implant with 4 mg/kg body weight of trans-chalcone, made in two different polymers, PLA and PGLA. The lesion size was measured every 15 days during 2.5 months.

2.7. Statistical analysis

A paired two-tailed t-test was used for analysis of the data. Values of P<0.05 were considered significant.

2.8. In vivo infectivity assay

This experiment was carried out in BALB/c mouse infected with parasite treated previously with trans-chalcone at the IC₅₀ concentration. The lesions size was measured weekly in a period of 2 months after lesion development.

2.9. Histopathological analysis

At the end of the experiment, the mice used in the in vivo drugs assay, control, trans-chalcone and pentamidine treatment, were killed under ether inhalation. The liver, skin, brain, lung, heart, spleen and kidney were removed from the mouse, fixed with 10% formol and embedded in paraffin. The tissues were cut into small pieces and stained by hematoxylin-eosin, PAS, silver-metenamin and Giemsa.

3. Results

3.1. Leishmanicidal activity

The antileishmanial activity of trans-chalcone was first tested in vitro against promastigotes of L. braziliensis.
Table 1
Leishmanicidal activity of trans-chalcone in vitro on promastigote forms of L. braziliensis, L. tropica, L. infantum and L. amazonensis, respectively

<table>
<thead>
<tr>
<th>Chalcone</th>
<th>Leishmanicidal activity (µM)</th>
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<tr>
<td></td>
<td>L. braziliensis</td>
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<tr>
<td>Trans-chalcone</td>
<td>1.58 ± 0.76</td>
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</tbody>
</table>

This compound showed potent inhibitory effects on the growth of all Leishmania strains examined (Table 1). Among the four parasite species, L. braziliensis proved to be the most susceptible, while drug activity was 2-6 times lower against the other strains. This compound clearly showed a concentration-dependent inhibitory effect on the in vitro growth of parasites.

3.2. Effects of trans-chalcone treatment

As shown in Fig. 2, the subcutaneous administration of trans-chalcone at doses of 4 mg/kg in mice infected with L. amazonensis produced, 8 weeks after treatment, a significant (P < 0.05) reduction of the average lesion size (21%) compared with untreated mice. During the last 3 weeks of the experiment, the lesion size increased rapidly in no treated mice, while in treated mice it increased more gradually. Mice treated with pentamidine and trans-chalcone developed similar lesion sizes during the first 3 weeks. In the last 2 weeks of the experiment, a similar inhibition of the lesion growth in mice treated with trans-chalcone and pentamidine was observed (P > 0.05).

The results of the effect of ear implants administration of trans-chalcone are shown in Fig. 3. PGLA implants of trans-chalcone at 4 mg/kg reduced lesion size (31.25%) in the mice infected with L. amazonensis compared with those untreated animals (P < 0.05). In mice with PLA implants lesion size reduction was less than the one obtained in those with PLGA implants. During the first 2 months of the experiment lesion size in mice treated with PLA implants, with or without drugs, was similar. In the last 2 weeks mice with PLA implants with drug showed significantly smaller lesions than those having PLA implants without drug.

3.3. Effects of trans-chalcone on L. amazonensis infectivity

To assess whether trans-chalcone affects infection capacity, an in vivo experiment was carried out. Stationary-phase promastigotes of L. amazonensis, treated with the drug at a concentration equivalent to the IC₅₀, and untreated parasites, were used to infect two groups of five male BALB/c mice.

As shown in Fig. 4, the BALB/c mice inoculated with treated parasites did not develop any lesion during the 8 weeks of experiment. This clearly indicates that promastigotes pre-treated with trans-chalcone lose all their infective capacity.

3.4. Histopathological analysis

The histopathological analysis realized in this work showed numerous parasites in skin samples with low inflammatory associated response. In the rest of tissues,

![Fig. 2. Effects of trans-chalcone and pentamidine (4 mg/kg body weight) subcutaneous administration on the lesion development in mice infected with L. amazonensis promastigotes. Results are means ± S.E.M. for five mice in each group.](image)

![Fig. 3. Effects of trans-chalcone PLA and PLGA ear implants administration on lesion development in mice infected with L. amazonensis promastigotes. Results are means ± S.E.M. for five mice in each group.](image)
only minimum unspecific lesion was found, such as a microscopic focus of tubulointerstitial mononuclear infiltrated in the kidney of an uninfected mouse, a pericardic calcification in a mouse treated with pentamidine and light signs of lobular mononuclear infiltrated in the liver of an infected mouse without treatment. The lung of some mice infected and treated with \textit{trans}-chalcone presented a light lymphocyte interstitial inflammatory infiltrate. Additionally, in all lung samples of pentamidine treated mice a mononuclear inflammatory infiltrate was observed.

4. Discussion

Previous studies have demonstrated that chalcones inhibits the in vitro and in vivo growth of extracellular promastigotes and intracellular amastigotes of several species of \textit{Leishmania} parasites. The leishmanicidal effect of several chalcones against \textit{L. major}, \textit{L. donovani} (Chen et al., 2001; Kayser and Kiderlen, 2001), \textit{L. infantum} and \textit{L. enrietti} (Kayser and Kiderlen, 2001) and \textit{L. braziliensis} (Nielsen et al., 1998) has been described.

Preliminary in vitro studies showed that the leishmanicidal activity of chalcones is determined by the number and nature of their oxygenated substituents. For highly active compounds, the ability to inhibit parasite growth apparently depends on the presence and ratio of a limited number of hydrophilic to lipophilic substituents at both aromatic rings (Kayser and Kiderlen, 2001; Langer, 1998). Our study demonstrated that leishmanicidal activity was not lost when chalcone common skeleton did not show any substituents. Previous studies (Nielsen et al., 1998) showed that the positions of the substituents seem to be important for the effectiveness of the antiprotosozial activity of the chalcones evaluated but, in this study \textit{trans}-chalcone also revealed pronounced leishmanicidal and trypanocidal activities. In this case, \textit{trans}-chalcone was about 2- to 13-fold more potent than the other substituted chalcones.

Our in vitro experiments against promastigote forms showed a dose-dependent inhibitory effect on the viability of parasite. Similar results were obtained in other studies (Torres-Santos et al., 1999a; Kayser and Kiderlen, 2001; Nielsen et al., 1998). The evaluation of the leishmanicidal activity in vivo on BALB/c mice infected with a New World strain of cutaneous leishmaniasis, showed the efficacy of \textit{trans}-chalcone.

Subcutaneous administration of \textit{trans}-chalcone at a single dose of 4 mg/kg reduced lesion development in mice infected with \textit{L. amazonensis}. Pentamidine and \textit{trans}-chalcone showed similar leishmanicidal activity against \textit{L. amazonensis} at same experimental conditions.

Previous studies showed that intraperitoneal administration of licochalcone A at doses of 2.5 and 5 mg/kg per day during 39 days completely prevented lesion development in BALB/c mice infected with \textit{L. major}. In contrast, intralesional administration of this product at doses of 1 and 2.5 mg/kg per day produced about 50% reduction in lesion size (Chen et al., 1994). In our study, one single administration of \textit{trans}-chalcone reduced about 21% the lesion size.

Drug incorporation in biocompatible polymers has an advantage over other systems due to easy preparation, longer shelf life, prolonged cession and greater stability in biological fluids (Langer, 1998). Nanoparticles prepared with biodegradable poly(lactide) have been proposed as a passive system of drug delivery to macrophages that would increase the therapeutic index of leishmanicidal drugs (Torres-Santos et al., 1999b; Rodrigues et al., 1994). Also, phospholipid microspheres and biodegradable nanoparticles prepared from PLGA were tested against experimental (Medda et al., 2003) and in vitro (Venier-Julienne et al., 1995) leishmaniasis. In this work PLA and PLGA \textit{trans}-chalcone implants were prepared to assess its effect on male BALB/c mice infected with \textit{L. amazonensis} promastigotes in their footpad. Each mouse received an ear implant, with or without drug and the treatment was initiated 5 weeks after inoculation, when the infection was well established and lesions were obvious.

The results showed that PLGA implants induced a higher reduction in the lesion size than PLA implants. The lesions sizes were reduced in 10.75% and 31.25% when animals received PLA and PLGA implants with drug (Fig. 3). In the first weeks mice treated with PLA implants without drug, presented lesions size smaller than mice treated with PLA implants with drug. It is possible that PLA have intrinsic anti-inflammatory activity and that cell types other than macrophages may
be activated by the PLA in vivo and indirectly induce parasite death in the lesions. Some polymers probably have direct activity against the parasites: isoalkyl cyanoacrylate have shown intrinsic activity against trypanosomes (Zherm et al., 1987) and L. donovani (Gaspar et al., 1992), stearylamine-bearing liposomes are cytotoxic toward T. cruzi (Yoshihara et al., 1987), T. brucei gambiense (Tachibana et al., 1988) and L. donovani (Dey et al., 2000). Although experiments carried out with chalone entrapment in PLA nanoparticles (Torres-Santos et al., 1999b) did not show a direct effect on L. amazonensis, our experiments using PLA implants showed direct activity against parasite.

Mice treated with PLGA implants with drug showed a significant reduction in lesion size, starting 45 days after initiation of treatment (Fig. 3).

On the other hand, treatment in vitro with trans-chalcone at IC50, completely inhibited the pathogenicity of this parasite in vivo. None of the mice inoculated with promastigote cultured with trans-chalcone developed the parasitic infection (Fig. 4).

Pathogenicity of Leishmania parasite is associated with two different groups of parasite molecules. One group consists of largely surface and secretory products, and the second group includes intracellular molecules, referred to as “pathoantigens”. The first group are invasive/evasive determinants, which are involved in the establishment of infection and survival. The second group is involved in pathology (Chang et al., 2003; Rivas et al., 2004).

In the present study we investigated the possible histological changes induced in mouse treated with free trans-chalcone and pentamidine. None of the organs examined presented visceral lesion. All the results obtained are considered lacking of pathological significance, indicating that trans-chalcone did not produce histological alterations.

In summary, the leishmanicidal activity of free or trans-chalcone implants was assessed in this study. Local delivery of chemotherapeutic agent by controlled-release polymers is a new strategy with the potential to maximize the leishmanicidal effect of a drug, we have demonstrated the effectiveness of using the biodegradable PLGA polymer to deliver trans-chalcone. Administration of drugs using biodegradable polymers provides a new way to study polymer-mediated therapy of experimental leishmaniasis.

Acknowledgments

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