In vitro activity of C20-diterpenoid alkaloid derivatives in promastigotes and intracellular amastigotes of Leishmania infantum

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Abstract

The in vitro anti-proliferative effects are described of several atisine-type diterpenoid alkaloids against the protozoan parasite Leishmania infantum, which causes human visceral leishmaniasis and canine leishmaniasis in the Mediterranean basin, as well as human cutaneous leishmaniasis throughout the Mediterranean region. From a total of 43 compounds tested, including C19- and C20-diterpene alkaloids from several chemical classes, only 15, 22-O-diacetyl-19-oxo-dihydroatisine, azitine and isoazitine were highly active against cultures of the parasite (promastigote form) with IC50 values within the range of the reference drug pentamidine-isothionate (7.39–12.80 mg/L for the test compounds, 11.32 mg/L for the positive control). These compounds were not toxic to the host cell. When treated with a dosage of 5 μg/mL of the active compounds (half of their IC50), the promastigote forms lost 80% of their infection capacity and the multiplication of extracellular forms of L. infantum was severely affected. The study showed that atisine-type C20-diterpenoid alkaloids exhibited promising anti-leishmanial properties with strong molecular selectivity. These might have implications for other intracellular pathogens- or phylogenetically related parasites, such as Trypanosoma spp.

1. Introduction

Among the major worldwide health problems, especially in developing countries, are protozoan diseases of different types. Leishmaniasis, caused by several species of flagellated protozoa belonging to genus Leishmania, which are transmitted exclusively by the bite of female phlebotomine sandfly, affects some 12 million people. About 350 million are exposed to the risk of infection, with over 400 000–2 000 000 new cases annually [1]. Over the last few years, regions not formerly considered endemic to Leishmania, registered increases in recorded cases. In Mediterranean Europe, visceral leishmaniasis is recognized as a re-emerging disease [2]. The classic forms of leishmaniasis (e.g. visceral and cutaneous leishmaniasis) still impose specific difficulties in terms of diagnosis and treatment. Drug treatment today is restricted to a limited number of clinically useful drugs, such as pentamidine or amphotericin B. However, both drugs have serious side effects. Furthermore, especially improved formulations, such as liposome-encapsulated amphotericin B, though effective, have made general treatment unaffordable for many afflicted countries, revealing an urgent need for new, safer and cheaper drugs [3].

In Central and South America, where there is a high prevalence of these diseases, the drug of choice, commonly used increases in recorded cases.
in the industrialized world, is rarely available, and moreover, most affected patients belong to the poorer classes, who cannot afford these expensive medicines. Instead, following traditional medicine, these countries use plants to treat most diseases, including leishmaniasis [4]. For all these reasons, one strategy to discover new drug leads is to investigate natural products from traditional medicinal plants. Plant species of the genera *Aconitum*, *Delphinium* and *Consolida* are known sources of C19-norditerpene and C20-diterpene alkaloids (NDAs and DAs, respectively) of pharmacological and economic importance [5,6]. NDAs act as potent nicotinic cholinergic receptor (nAChR) agonists and antagonists in invertebrates, including insects and in vertebrates [7]. The insecticidal and anti-feedant activity of NDAs [8–10], suggest a defensive role played by these compounds. However, only a few DAs have been studied for their plant defensive and pharmacological properties, including their effects on *Trypanosoma cruzi* epimastigote forms [9,11–15] and their neurotoxic effects are unknown.

Here, for the first time, we report the inhibitory effects of DAs on the extracellular promastigote and the intracellular amastigote stages of *Leishmania infantum* in comparison with their direct effects on macrophage host cells. These data suggest that DAs are a class of compounds with potential for further development for anti-protozoal therapy.

2. Materials and methods

2.1. Parasite strain

*L. infantum* strain UCM10 (zimodeme MON1) was isolated in Madrid (Spain) and kindly supplied by Professor Alunda JM of the Faculty of Veterinary Medicine Madrid (Spain).

2.2. Reagents compounds

The compounds tested here were isolated from *Aconitum*, *Delphinium* and *Consolida* spp. [10,15] (Fig. 1).

2.3. Promastigote assay

The promastigote forms used for the chemotherapy assays were cultured at 28 °C in RPMI 1640 medium (Flow Laboratories, Irvine, UK) in Roux flasks (Corning, USA) of 75 cm² in surface area, supplemented with 10% inactivated

![Fig. 1. Structure of the three arsine-type diterpine alkaloids (ADs) used.](image-url)
calf serum. At the exponential-phase flagellate growth, the liquid medium was centrifuged at 1500 rpm for 10 min and the number of parasites were counted and distributed in 96 multiwell plates (Becton Dickinson, USA) to 2 × 10^6 parasites/mL.

The compounds were dissolved in dimethyl sulfoxide (DMSO, Panreac, Barcelona, Spain) at a concentration of 0.1%, after this had been assayed as non-toxic and without inhibitory effects on parasite growth, as previously demonstrated [16]. The compounds were dissolved in the culture medium, and the dosages used were: 100, 50, 25, 10 and 1 μg/mL. The effect of each compound at these concentrations against promastigote forms, were evaluated at 24, 48 and 72 h, using a Neubauer haemocytometric chamber. The leishmanicidal effects were expressed as IC_{50}, that is, the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations used.

2.4.2. Experimental model no. 1 (M and 95% air)

Two experimental procedures were designed for this study, in all cases beginning with macrophages J774A.1, which were diluted to 10^6 cells/mL in RPMI medium plus 10% IFCS, plated in 24-well tissue-culture chamber slides and allowed to adhere for 24 h at 37 °C in a mixture of 5% CO_2 and 95% air.

2.4.2.1. Amastigote-macrophage assay

Adherent macrophages were infected in vitro at a ratio of 10:1 with L. infantum promastigotes in the exponential growth phase. The drugs (5 μg/mL concentrations) were added at the same time and the trays were incubated for 12 h at 37 °C in 5% CO_2 in air. Afterwards, non-phagocyotised parasites and the drugs were removed by washing, and infected cultures were grown for 8 days in fresh RPMI medium. The medium was renewed every 48 h.

2.4.2.3. Experimental model no. 2 (Mb + Li + drug)

Adherent macrophages were infected with exponential-phase promastigotes of L. infantum at a ratio of 10:1 and maintained for 12 h at 37 °C in 5% CO_2 in air. Non-phagocytosed parasites were removed by washing, and the infected cultures were incubated with the drugs (5 μg/mL concentrations) for another 12 h and afterwards washed and cultured for 8 days in fresh RPMI medium. The culture medium was renewed every 48 h.

In all cases, the drug activity was determined from the percentage of infected cells and number of amastigotes per infected macrophage in treated and untreated cultures in methanol-fixed and Giemsa-stained preparations. Values are means of four separate determinations.

2.5. Ultrastructural alterations

The parasites, at a density of 5 × 10^6 cells/mL, were cultured in their corresponding medium, containing the three drugs at 5 μg/mL. After 72 h, the cultures were centrifuged at 1500 × g for 10 min, and the pellets washed in PBS and then fixed with 2% (v/v) p-formaldehyde-glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) for 2 h at 4 °C. Pellets were prepared for transmission-electron microscopy following the technique of Luque et al. [16].

3. Results and discussion

From a total of 43 diterpene alkaloids (x NDAs and y DAs from several chemical classes) tested at 100 μg/mL, only three aistine-type ADs markedly inhibited the growth of the in vitro forms of L. infantum promastigotes (data not shown). The in vitro leishmanicidal activity of the active compounds (1, 2 and 3, Fig. 1) against promastigote L. infantum are shown in Table 1. Pentamidine-isothionate was included here as the anti-leishmanial reference drug. Compound 3 exhibited the highest toxicity to the extracellular L. infantum parasites and the drugs were removed by washing, and infected cultures were grown for 8 days in fresh RPMI medium. The medium was renewed every 48 h.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC_{50} (mg/L)</th>
<th>Toxicity IC_{50} (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td>Pentostam</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1</td>
<td>24.58</td>
<td>15.74</td>
</tr>
<tr>
<td>2</td>
<td>26.30</td>
<td>15.35</td>
</tr>
<tr>
<td>3</td>
<td>13.38</td>
<td>9.70</td>
</tr>
</tbody>
</table>

IC_{50}, the concentration required to give 50% inhibition, calculated by linear regression analysis from the Kc values at the concentrations tested (1, 10, 25, 50 and 100 μg/mL). Note: average of four separate determinations.

On J774.2 macrophages at 72 h of culture.
parasites (IC₅₀ of 13.38, 9.70 and 7.39 mg/L at 24, 48 and 72h of culture, respectively) with IC₅₀ values lower than those obtained for the reference drug (IC₅₀ of 11.32 mg/L at 72h of culture). Alkaloids 2 and 3 also showed greater effects against promastigotes (IC₅₀ of 10.12 and 12.80 mg/L, respectively, at 72h of culture). This leishmanicidal activity was associated with a lack of toxicity to murine macrophages by compound 3 (at 5 mg/L) on infected macrophages (MT) may be seen. Table 2 shows the effect of compounds 1-3 (at 5 mg/L) on the infection rate of *L. infantum* macrophages and the average number of *Leishmania infantum* amastigotes per infected macrophage during 8 days of culture, under different conditions.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>(%)/Mₐ³</th>
<th>IC₅₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>48 h</td>
<td>96 h</td>
<td>144 h</td>
</tr>
<tr>
<td>None (control)</td>
<td>78.4 ± 0.6</td>
<td>79.2 ± 1.1</td>
</tr>
<tr>
<td>Mₐ – Li¹</td>
<td>41.0 ± 4.2</td>
<td>64.0 ± 6.0</td>
</tr>
<tr>
<td>Mₐ – Li²</td>
<td>46.4 ± 4.2</td>
<td>74.4 ± 6.0</td>
</tr>
<tr>
<td>Mₐ – Li³</td>
<td>46.1 ± 4.2</td>
<td>38.8 ± 6.0</td>
</tr>
<tr>
<td>Mₐ – Li + E²</td>
<td>75.2 ± 4.2</td>
<td>78.4 ± 6.0</td>
</tr>
<tr>
<td>Mₐ – Li + F³</td>
<td>16.0 ± 4.2</td>
<td>14.4 ± 6.0</td>
</tr>
<tr>
<td>Mₐ – Li + G³</td>
<td>27.2 ± 4.2</td>
<td>33.2 ± 6.0</td>
</tr>
</tbody>
</table>

- a Percentage macrophage parasitism. Values are mean ± standard deviations of four separate determinations.
- b Number of amastigotes per macrophage infected. Values are mean ± standard deviations of four separate determinations.
- c Details are given in Section 2.

Table 2 shows the effect of compounds 1-3 on the infection rate of *L. infantum* macrophages and the average number of amastigotes per infected macrophage at 8 days of culture. Two experimental models were used to discriminate the effects of the test compounds on the growth of the extracellular forms or the infection capacity and growth of the intracellular forms of *L. infantum*. The first experimental model (Mₐ + Li + drug) consisted of adherent macrophages infected with drug-treated promastigotes in exponential growth phase (MT) may be seen.

The results of this experimental model confirm that: (1) alkaloids 2 and 3, to a lesser extent 1, are toxic to promastigote forms of *L. infantum*, (2) these compounds lower the infective capacity of these forms of the parasite and (3) the three compounds assayed are very active against the extracellular forms of the parasite.

When the parasites were incubated with the macrophages for 12 h before the addition of the alkaloids (Section 2.4.3), the percentage of parasitism was not significantly affected. This result is not surprising since 12h incubation of the macrophages with the promastigotes in the absence of drugs is sufficient time to allow for maximum parasitism.

Nevertheless, the number of amastigotes was significantly reduced (71–49%) by the three products tested. This inhibitory effect increased slightly over the 8 experimental days, indicating that there was a direct action on the intracellular forms and their multiplication.

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Fig. 2A shows a control culture of *L. infantum* promastigotes with the characteristic structure of kinetoplastids. The typical kinetoplast (K) is formed by mass- and minicircles that appear as a proliferation of the mitochondria, the mitochondrial (M) have well-defined mitochondrial crypts and a number of glycosomes (G), a nucleus (N) with its nucleolus (Nu) normally centred and the characteristic microtubules (MT) may be seen. *L. infantum* promastigotes treated with compound 3 (Fig. 2B) showed major nuclear and cytoplasmic alterations. Although the cytoplasmic membrane appeared intact, we were able to detect small dense bodies (D) associated with the microtubular structure. The nucleus (N) appeared irregular and with little electrondensity, and a double nuclear membrane was not visible. The cytoplasm showed little electrondensity and contained a large number of empty vacuoles (V) through-out, and even the flagellar sack contained small vacuoles. The mitochondria (M) were very scarce.

Compound 3 (Fig. 2C) induced notable morphological changes in the parasites, which appeared with a rounded, extremely swollen appearance. The cytoplasm, with abundant vacuoles and little electrondensity, contained numerous glycosomes (G) and swollen mitochondria (M).

Similar alterations were provoked by 2 (Fig. 2D). The parasites appeared very swollen with enormous empty vacuoles (V) or vacuoles composed of granular material. In some of
these, a large vacuole occupied almost the entire cytoplasm. Also, the mitochondria in which the crypts could be distinguished, appeared swollen. The kinetoplast appeared disorganized, without visible maxi- or minicircles.

Compound 1 (Fig. 2E) proved to be the most harmful to *L. infantum* promastigotes. Most of the parasites were dead and many were fragmented, and thus, the culture was filled with cell fragments, and intact parasites were scarce. In some of these, the cytoplasmic membrane appeared disintegrated in some areas (arrow), leading to direct contact of the cytoplasmic organelles and the nucleus with the culture medium and resulting in fragmentation. This compound acts fundamentally at the level of the cytoplasmic membrane of the parasites, although alterations were detected also particularly in the mitochondria (M), which were extremely rare and lacked their normal morphology and appeared as uniform sacks without crypts. Compound 1 also led to the loss of the typical structure in maxi- and minicircles in the kinetoplast (K).

This is the first study that has been made on the leishmanicidal activity of DAs. Previous results have shown that *T. cruzi* mortality increased with ADs 13-oxo-cardiopetamine (inactive against *L. infantum*, data not shown) and 1, while 2 and 3 were inactive which suggests species-related selectivity for the anti-parasitic action of these compounds, with *L. infantum* being more sensitive to DAs than *T. cruzi*. Furthermore, none of 43 NDAs tested on *T. cruzi* affected its viability as shown here for *L. infantum* indicating a strong molecular selectivity for the trypanocidal and leishmanicidal effect of DAs.

Our results showed that compounds 1–3 were very active in vitro against both the extracellular as well as the intracellular forms of *L. infantum*. These compounds are not toxic for the host cells and are effective at concentrations similar to or lower than the reference drug used in the present study. The in vitro growth rate of *L. infantum* was reduced, its capacity to infect cells was negatively
affected and the multiplication of the amastigotes was greatly reduced.

In conclusion, our study provides data that DAs 1–3 has promising anti-leishmanial properties. These could have implications for other intracellular pathogens or phylogenetically related parasites as shown for Trypanosoma spp. The potent leishmanicidal activities of the alkaloids described here represent an exciting advance in the search for new antiprotozoal agents.

References