Efficacy of topical risedronate and risedronate - Eudragit E complex in a model of cutaneous leishmaniasis induced by *Leishmania (Leishmania) amazonensis*

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**ARTICLE INFO**

Keywords:
- Cutaneous leishmaniasis
- Risedronate
- Eudragit
- Topical treatment
- *Leishmania amazonensis*

**ABSTRACT**

An efficacious topical treatment for cutaneous leishmaniasis (CL) is highly desirable but still an ongoing challenge. Systemic risedronate (Ris) has been reported to have anti-leishmanial properties and Eudragit EPO (EuE) has shown *in vitro* activity against *L. (L.) amazonensis*. The aim of this work was to investigate the *in vivo* efficacy of topical Ris and EuE-Ris complexes on CL. Surface charge and Ris release kinetics from the different dispersions were analyzed. BALB/c mice were infected intradermally with promastigotes of *L. (L.) amazonensis*. Ulcers were treated with Ris or EuE-Ris hydrogels. All the lesions that received topical Ris or EuE-Ris showed an improvement with respect to control: reduction of ulcer average size, cicatrization, flattened edges and no signs of necrosis. In addition, a marked parasitic inhibition of 69.5 and 73.7% was observed in the groups treated with Ris and EuE-Ris, respectively, with the IgG2a levels indicating a tendency towards cure. The results are promising and the system should now be enhanced to achieve total parasite elimination.

1. Introduction

Leishmaniasis is, according to the World Health Organization, one of the most important tropical orphan diseases. This is due to inadequate treatments, especially in the case of rural populations [1]. The cutaneous form is the most prevalent, with an annual incidence of 1–1.5 million cases, and is endemic in 98 countries [2]. Its lesions are characterized by nodules, which sometimes progress to ulcers. Changes in the skin reflect the immune response to the infection, resulting in hyperplasia, epidermal thickening, and commonly a strong inflammation with destruction of the epidermis resulting in an ulcer [3].

The current first line pharmacotherapy involves the use of pentavalent antimonials, which have a high toxicity and significant side-effects, administered parentally or orally [4, 5]. A standard therapy involves daily intramuscular injections for 20–30 days, which is distressing and requires specialized professionals. An efficacious topical treatment would improve patient compliance due to ease of use, diminished hurt, decreased side effects and the possibility of use in a rural context with low medical infrastructure. Depending on the size or number of the lesions, topical paromomycin ointment may be recommended, with several topical formulations of paromomycin having been developed for the treatment of CL with variable results [6, 7, 8]. Nevertheless, there is still no commercial formulation available.

Macrophage-like cells, or osteoclasts, are targets of bisphosphonate antiresorptive drugs, and it has been observed that risedronate (Ris) have considerable activity against leishmania parasites. The literature shows Ris anti-leishmanial efficacy *in vitro* in amastigotes strain of *L. (L.) donovani*, and low toxicity indices in macrophages [9]; efficacy was also demonstrated in promastigotes strain of *L. infantum* [10]. The *in vivo* efficacy of Ris was demonstrated in a visceral leishmaniasis (*L. (L.) donovani*) model of mice after its intra-peritoneal administration [11], with more than 85 % suppression of the amastigotes load in the liver.

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https://doi.org/10.1016/j.heliyon.2021.e07136
Received 29 June 2020; Received in revised form 17 December 2020; Accepted 19 May 2021
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without apparent toxicity. **In vivo** efficacy of other bisphosphonates such as pamidronate after intra-peritoneal administration has also been described in a cutaneous leishmaniasis model (L. mexicana amazonensis), with the disappearance of the lesions and more than 99 % of the amastigotes at the site of the lesion [12]. However, Ris topical efficacy in cutaneous leishmaniasis (CL) has not yet been tested.

Eudragit EPO (EuE, a basic polyelectrolyte) has shown good in vitro activity in an L. (L.) amazonensis model with a high selectivity index [13]. In vitro studies have shown that the complexation of Ris with EuE gives rise to a new material, referred to as EuE-Ris, which has advantageous properties. These include high loading capacity and high affinity for the phosphonate groups of the drug, leading to a controlled Ris release [14]. This material has also shown a reduction in the topical irritation potential in a gastric model.

In the present study, our aim was to investigate the efficacy of topical administration of Ris and EuE-Ris complexes in an animal model of CL.

2. Materials and methods

2.1. Preparation of EuE-Ris and Ris hydrogels

A Ris (risedronate monosodium monohydrate, kindly provided by IVAX Argentina S.A.) aqueous solution was first prepared. Then, in order to facilitate its topical application, hydroxypropyl methylcellulose (HPMC, Parafarm®, Argentina) was added to the Ris solution, producing a hydrogel. A final concentration of 2 % HPMC and 20 mg/mL of Ris was obtained. This concentration was selected according to doses used in the literature for experimental leishmaniasis i.p. formulations [9, 11].

The EuE-Ris complex was prepared as previously described in Guzman et al. [14]. HPMC was added to an aqueous dispersion of this complex, to give an hydrogel with a final concentration of 20 mg/mL of Ris, 40 mg/mL of EuE and 2 % of HPMC. Both hydrogels are thus named Ris\textsubscript{HPMC} and EuE-Ris\textsubscript{HPMC} respectively.

2.2. EuE-Ris\textsubscript{HPMC} and Ris\textsubscript{HPMC} hydrogels physicochemical characterisation

The pH was determined in all the systems obtained, using a Mettler Toledo Seven Multi pH meter equipped with a combined Ag/AgCl electrode (Mettler Toledo DG 115-SC). The surface charge was analyzed using a Zetasizer NanoSerie DTS 1060 (Malvern Instruments S.A., Worcestershire, UK). The hydrogels were diluted (1:10) in deionized water, and zeta potential (ZP) was determined at 25 °C in triplicate.

Bicompartimental Franz cells were used to study the release behavior of the hydrogels obtained. One (1) mL of EuE-Ris\textsubscript{HPMC} (pH = 5.5) and Ris\textsubscript{HPMC} (pH = 5.2) containing 5 mg of Ris was used. An aqueous Ris solution (pH = 4.8) and EuE-Ris (pH = 4.7) dispersion containing equivalent Ris concentration were used as references. Ris concentration for the assay was selected in order to reach sink conditions. For that, the volume of the receptor compartment, sampling frequency and concentration of Ris in the donor compartment were considered. The experiments were performed as described in Guzman et al [14] with the receptor compartment filled with milli Q water or physiological solution (NaCl 0.9 %). Ris concentrations were determined by UV spectroscopy at \( \lambda = 262 \) nm. For comparison, Ris release slopes from Ris solutions were taken in terms of severity. At the beginning of the experiment, all lesions were ulcers. Flattened edges were considered to be a sign of cure of the lesion.

Leishmania related lesions are initially popular. Gradually they take on a darker reddish tone, while becoming infiltrated and bigger. The lesions then progress into nodular lesions, or a deeply infiltrated plaque, at the center of which a seropurulent exudate begins to sprout, whose desiccation may give rise to a tightly adhering scab. In this phase, the ulcerative lesion may have a variable dimension; the skin that covers it has a wine-violaceous red tone and is often surrounded by an edematous and indurated area. Dermal necrosis may also be seen [17].

The stages of the lesions, which are related to their severity, were classified as follows:

a) Necrotic ulcer: dry and regular rounded edges with lesions having a dark red/black color in the center.
b) Ulcer: hollow injury covered with furfuraceous scales, at the center of which sprouts a seropurulent exudate, with thick high borders.

c) Partially flattened edges: ulcerated lesion that begins to heal and flatten, but still having an ulcerated area that is less than 50 % of the initial area.

d) Flattened edges: where the ulcer has disappeared and the edges are flattened in the whole perimeter of the lesion.

2.3.6. Immunoglobulin levels
At the end of treatment, blood was extracted in order to measure immunoglobulin levels. Parasite-specific IgG1 and IgG2a from serum were measured by ELISA as described in a previous work [15].

2.3.7. Smears
The presence of parasites in the lesions was examined after sacrificing the animals. Skin tissue samples were aseptically excised with scissors, smeared in glass slides and dyed using DiffQuick (Biopur Diagnostics) to enable recognition of the nucleus and cytoplasm of the macrophages and parasites. By this technique, the parasites in the lesions could be observed and identified by the kinetoplast joined to the nucleus. Photographs were taken in a Zeiss optical microscope at 40X.

2.3.8. Parasitic inhibition in the lesion
The number of parasites (amastigotes) remaining in the lesions after the end of treatment was determined from macerates of the skin lesion at the inoculation site. To obtain the amastigotes from the tissue, a protocol described in a previous work [18] was followed. Briefly, 5 skin tissue samples were homogenized and the parasites were separated through mechanical tissue disruption using a glass grinder. The percentage of parasite inhibition with regard to controls was calculated as: (Total number of amastigotes disruption using a glass grinder. The percentage of parasite inhibition et al.

2.3.9. Statistical analysis
The results of lesion sizes and immunoglobulin levels were analyzed using the unpaired Mann-Whitney test, with p < 0.05 denoting significant differences. GraphPad Prism (v.6) software was used for this analysis. The results are presented as means and standard error. The macroscopic aspect of the lesions at the end of the treatments was plotted as a percentage of case frequency for each experimental group.

3. Results and discussion
3.1. EuE-Ris-HPMC and Ris-HPMC hydrogels physicochemical characterization
The preparation method produced transudic semisolid homogeneous hydrogels of EuE-Ris-HPMC and Ris-HPMC without tendency for phase separation, with an adequate viscosity that allowed easy expelling through the syringe and manual administration. EuE-Ris-HPMC hydrogels presented a pH value of 6.0 ± 0.5, Ris-HPMC hydrogel pH was 5 ± 0.4. Zeta potential of EuE-Ris-HPMC was high and positive (20.6 ± 0.2) and Ris-HPMC zeta potential was 5.5 ± 0.9.

3.2. In vivo anti-leishmanial efficacy of hydrogels
As shown in Figure 2C, in this model of CL the mice develop a single lesion, at the inoculation site. Topical Ris-HPMC and EuE-Ris-HPMC treatment resulted in a reduction of the size of the lesions compared to untreated mice. In the group treated with EuE-Ris-HPMC, the lesions borders started to partially flatten and the ulcer to show cicatrization from the first week of treatment. From day 19, lesions had completely flattened borders and no ulcers were visible in this group. We described this behavior as 0 mm² lesion area at days 19 and 22 in Figure 2A.

In the group treated with Ris-HPMC, the lesions started to flatten, show cicatrization and diminish size at day 19. All the lesions treated with either Ris-HPMC or EuE-Ris-HPMC showed an improvement in lesion appearance, with flattened edges either in a part or in the complete perimeter of the lesion, along with a recovery of the epidermis in the previously ulcerated central region. At the end of treatment 100 % and 50 % of the lesions treated with EuE-Ris-HPMC and Ris-HPMC, respectively, showed flattened edges in the complete perimeter of the lesion and cicatrization of the ulcer (Figure 2B). Even in animals treated with Ris-HPMC showing only a partially flattened edge, the ulcer showed cicatrization (Figure 2B and C). While 60 % of untreated control mice developed necrosis, this was never observed in the groups treated with Ris-HPMC or EuE-Ris-HPMC. A marked parasitic inhibition in the lesions at the end of treatment was found in the groups treated with Ris-HPMC or EuE-Ris-HPMC (69.5 % and 73.7 % respectively, Table 1). No animals were kept alive after treatment completion to evaluate recidivism, since we found approx. 2 × 10⁷ parasites in each lesion at the end of treatment. Since this amount of parasites is enough to produce a recidiva of the lesion, the animals cannot be considered cured.

Smears from the control group lesions revealed a higher amount of amastigotes per field than those of Ris-HPMC and EuE-Ris-HPMC groups. Both Ris-HPMC and EuE-Ris-HPMC groups showed development of promastigotes when the recovered lesions were cultured in USMARU, indicating the presence of viable remaining parasites at the end of treatment. However, the amastigotes found in the lesions treated with EuE-Ris-HPMC showed vacuoles and a less compact nucleus (Figure 3A). The cytoplasmatic vacuolization in the parasites treated with EuE-Ris-HPMC suggest that autophagy is the mechanism underlying leishmaniasis death [24]. However, specific experiments should be performed to confirm if EuE itself is the responsible for this mechanism or it is potentiating Ris anti-leishmanial activity.

Concerning the anti-leishmanial serum immunoglobulin levels, the difference of IgG2a/IgG1 ratios was not significant between groups although the ratios tended to increase in the treated groups after...
treatments (Figure 3B). In the BALB/c - L. (L.) amazonensis model, the progression of the disease seven weeks after infection normally shows greatly increased IgG1 levels and decreased IgG2a levels [25]. This was observed in the control untreated group. After treatment with Ris-HPMC or EuE-RiS-HPMC, the IgG2a/IgG1 ratio continued to be lower than 1, which is typical since normalization of the immune profile after treatment requires a long time [26], and we measured the IgG levels immediately at the end of treatment. However, the IgG2a/IgG1 ratio tended to increase with the treatments, implying a tendency to a Th1 response. This is crucial because the activation of infected macrophages produces leishmanicidal products such as nitric oxide radicals, which are toxic for the parasite [27, 28].

Other topical drugs, including imiquimod and amphotericin B, have been tested in clinical trials, but results are equivocal and no major breakthroughs have yet been achieved [29, 30]. Very recently, three manuscripts have reported the good efficacy of topical Miltefosine or Miltefosine/AnfotericineB dispersions in CL animal models produced by different species of leishmania [31, 32, 33], including our own work (M.F. Peralta, et al 2021) [15]. Leishmania is however a parasite that is notable for showing a highly variable response to pharmacological therapy, depending not only on species but even also on geographical region. Consequently, the formulation of an effective pharmacotherapy containing a different kind of drug such as Risedronate is extremely important in the fight against the disease.

By flattening the borders of the lesions and eliminating ulcers, topical Ris-HPMC was effective against L. (L.) amazonensis. We did not observe significant differences between Ris-HPMC and EuE-RiS-HPMC regarding parasitic burden decrease, but a clearly higher efficacy in healing the lesions by EuE-RiS-HPMC was observed. This is especially important since untreated lesions produce unsightly anthropic scars [34]. EuE may have enhanced by itself the anti-leishmanial activity of Ris-HPMC in the complex [13]. In order to verify this, in vitro studies should be performed. Unfortunately, we were unsuccessful in performing in vitro studies due to the insolubility of Ris and EuE-Ris in culture media since aminobisphosphonates form insoluble complexes with calcium or other divalent cations [14, 35] and EuE precipitates at pH values above 5 [36].

Although the release of Ris from EuE-RiS-HPMC was slower than from Ris-HPMC, we observed a higher efficacy as anti-leishmanial, which may be related to the complementary action of EuE. This increased activity might be explained by the mode of action of individual components. Recently, Gadilha et al. proposed a mechanism of action for Ris in Leishmania infantum. Leishmania synthesizes ergosterol and Ris strongly inhibits farnesyl diphosphate synthase (FPPS) enzyme, a key intermediate for ergosterol biosynthesis. Moreover, they observed that promastigotes treated in vitro with Ris showed small vesicles in the Golgi region near the kinetoplast, mitochondrial swelling, blebbing of the plasma membrane, as well as nuclear pyknosis and chromatin condensation [10]. They also reported phosphatidylserine (PS) exposure on the membrane of the parasites, which indicates early apoptosis. In addition, bisphosphonates have been shown to accumulate in tissues susceptible to infection by some of these parasites and to possess immunomodulatory effects [37].

In line with that observation, our in vivo results showed that lesions treated with EuE-RiS-HPMC present vacuoles near the kinetoplast and...
amastigotes morphology. B) Serum anti-leishmanial antibodies at the end of treatment. Ratio of IgG2a/IgG1 (Absorbance) levels found in the serum of animals at the end of treatments. Measurements were obtained by ELISA. N = 5.

Figure 3. A) Optical microscopy images of smears from lesions. The intracellular amastigotes are identified by the rod-shaped kinetoplast and the circular nucleus in lesion smears of the control group without treatment (A1), Ris-HPMC group (A2) and EuE-Ris-HPMC group (A3). The amplified sections (4x digitally amplified from original images) show amastigotes morphology. B) Serum anti-leishmanial antibodies at the end of treatment. Ratio of IgG2a/IgG1 (Absorbance) levels found in the serum of animals at the end of treatments. Measurements were obtained by ELISA. N = 5.

pyknosis, and also that chromatin condensation occurred in the nucleus of the parasites (Figure 3 A3), suggesting that the combined therapy with EuE and Ris can improve anti-leishmanial efficacy. Considering that EuE is a polycation and EuE-Ris-HPMC presents a high and positive zeta potential, an increased capability of interaction with the negatively charged PS present in parasites membrane could be playing a role. In fact, once EuE-Ris-HPMC reaches the macrophages vacuoles, the interaction with PS can produce severe damage and parasite death [24,38]. However, more research is necessary to elucidate such mechanisms and to confirm if the combined therapy produces additive or synergistic effects.

Cutaneous wound healing in mammals is a complex multi-step process. It has also been suggested that microbes of lesions play significant roles in impaired wound healing, i.e., in complex chronic injury, microbiome have a detrimental effect on wound closure [39,40]. A moist wound environment is considered to promote the wound healing process, favoring the granulation step. In our case, Ris-HPMC and EuE-Ris-HPMC were dispersed in a hydrogel system containing polymers that could facilitate the re-epithelialization process by diminishing water loss [41]. Besides, by causing a reduction of the parasite load, EuE-Ris-HPMC presumably had complementary effects in healing.

A re-epithelialization process of the ulcers was observed, but with parasites still remaining in the dermis below the lesions, which may imply a persistence/recurrence of the disease. The persistence of leishmaniasis in skin or mucosal tissues was also reported in patients who achieved therapeutic cure of CL [42]. In fact, at present, the exact factors related to parasite persistence or elimination are unknown.

Being highly polar, Ris is a molecule with a very low permeability [43]. Thus, in order to reach dermal parasites it is probably necessary that Ris application takes place while there is still an ulcer, when there are no well-formed epidermal skin layers to penetrate. Thus, to improve parasite reduction, the dosage or frequency of administration could be increased, or combinations tried with other components of proven activity. Meanwhile, the repurposing of both Ris and EuE for topical treatment of CL is worth being explored.

This could be strategic since they are approved compounds, which can reduce time, cost and risk, and it is a particularly attractive approach for neglected tropical diseases where new medications are needed urgently to treat the poorest of people, making their translation to clinical use more feasible. In this sense, translational research, built on the solid foundation of existing and ongoing basic research, is a priority.

4. Conclusions

The development of novel chemotherapeutic approaches for CL is of great importance. The use of bisphosphonates is of interest as they have already been used for other clinical indications. Ris produced moderately encouraging results in the treatment of CL caused by L. (L.) amazonensis in an experimental animal model. The EuE-Ris-HPMC complex led to lesion cicatrization and a slightly higher reduction in the parasitic burden. Future work should be aimed at optimizing the concentration, dose frequency, drug or excipient combinations and system characteristics in order to attain total parasite elimination. To the best of our knowledge, this is the first report on the efficacy of bisphosphonates against CL as a topical treatment.

Declarations

Author contribution statement

Ma. Florencia Peralta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ma. Laura Guzman: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Ma. Eugenia Olivera: Contributed reagents, materials, analysis tools or data.

Ma. Estefanía Bracamonte: Performed the experiments.

J. Diego Marco: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ma. Eugenia Olivera: Contributed reagents, materials, analysis tools or data.

Dolores C. Carrer: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Paola A. Barroso: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Ma. Laura Guzman, Ma. Eugenia Olivera and Dolores C. Carrer were supported by Secretaría de Ciencia y Tecnología - Universidad Nacional de Córdoba; Dolores C Carrer was supported by Fundación Bunge y Born and by PUE - CONICET 2016-22920160100135CO; Paola A. Barroso was supported by FONCYT, Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación, Argentina.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.
Acknowledgements

Authors would like to thank Federico Ramos and Alejandro D. Uncos for their help with animal experiments.

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