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Antiparasitic activity of risedronate in a murine model of acute Chagas' disease

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Abstract

We report the results of a study on the activity of the farnesyl-pyrophosphate synthase inhibitor risedronate (Ris) in a murine model of acute Chagas' disease. This compound displays rapid, cytocidal activity in vitro against *Trypanosoma cruzi*, but its in vivo activity had not been investigated previously. A murine model of acute Chagas' disease was used, in which experimental animals were infected with 10^3 trypomastigotes and intravenous treatment was started 24 h post-infection. In this model, Ris, at doses as low as 1 mg/kg per day given for 7 days, induced >90% reductions in parasitaemia and increased very significantly ($P = 0.001$) the survival of treated animals. Higher doses (up to 10 mg/kg per day) led to further reductions in parasitaemia and mortality, with no deleterious effects on weight gain and general physical condition of the treated animals. There was no relapse of parasitaemia after discontinuation of treatment, suggesting trypanocidal, rather than trypanostatic, activity. This interpretation was confirmed by the almost complete disappearance of amastigote nests in the hearts of treated animals. However, no parasitological cures were observed in infected animals that received the bisphosphonate, probably due to the short treatment period. Taken together, these results indicate that Ris could be a useful lead compound for the development of new drugs effective against Chagas' disease.

Keywords: Risedronate; *Mouse model*; Amastigote; Trypanocidal

1. Introduction

There is an unmet need for new, specific chemotherapeutic approaches for the treatment of Chagas' disease, the largest parasitic disease burden in Latin America [1,2], since currently available drugs have serious limitations due to limited efficacy, particularly in the prevalent chronic stage of the disease and frequent toxic side effects [3–5].

New rational approaches to aetiological treatment are being developed based on the growing knowledge of the basic biology of the causative agent of Chagas' disease, the trypanosomatid parasite *Trypanosoma cruzi* [3–6]. In recent work, we found that *T. cruzi* contain large stores of condensed phosphates, such as pyrophosphate [7–9] and triphosphate, together with novel, plant-like pyrophosphatase enzymes. This prompted us to investigate the possible utility of pyrophosphate analogues, bisphosphonates, as potential anti-parasitic agents. We found that these compounds, currently used in the treatment of bone resorption disorders [10], have potent activity against a variety of parasitic organisms, both in vitro and in vivo (see [4,5,7,11–16]). They are now thought to act by inhibiting the enzyme farnesyl pyrophosphate synthase (FFPS) [10,12,17–20], and parasite selectivity is believed to be due, at least in part, to selective uptake into the pyrophosphate storage vacuoles (acidocalcisomes) in the parasite cells. Risedronate

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(Ris, 2-(3-pyridyl)-1-hydroxy-ethane-1,1-bisphosphonate, Actonel®, Procter and Gamble) has been shown to be one of the most potent compounds against *T. cruzi* in vitro, where it displays rapid trypanocidal action [7,11,13], but there have been no reports of its in vivo activity against this parasite. In this work, we present the results of a series of studies on the activity of Ris in a murine model of Chagas' disease. We show that Ris has potent and selective activity in this model of acute disease, and we present evidence that it has trypanocidal activity in vivo.

2. Materials and methods

2.1. Parasites

The Y [21] strain of *T. cruzi* was used throughout this work. Handling of live *T. cruzi* was performed according to established guidelines [22].

2.2. Experimental chemotherapy

Groups of 10 outbred female Swiss albino mice, weighing 20–25 g, were inoculated intraperitoneally with 10^3 blood trypomastigotes of the Y strain; intravenous (i.v., through the tail vein) treatment with Ris, dissolved in phosphate buffered saline (PBS) was started 24 h p.i. and was given daily for a total of 7–14 doses. Control (untreated) animals received the same amount of PBS, i.v. Surviving animals were followed for 40 to 60 days p.i.

2.3. Parasitological tests

Parasitological tests were carried out as described previously [23,24]. Briefly, parasitaemia was measured daily during the first 3 weeks and weekly thereafter, in a haematocytometer using tail blood. Haemocultures were carried out by inoculating 5 ml of liver infusion medium with 0.2–0.4 ml of blood obtained from the orbital sinus of the experimental animals. Cultures were incubated without agitation at 28 °C and examined for the presence of proliferative epimastigote forms every 2 weeks, up to 60 days. A polymerase chain reaction (PCR)-based test of blood samples was carried out using the methods described previously [25].

2.4. Histological analysis

Thin (3 μm) sections of heart tissue from *T. cruzi* infected mice (14 days p.i.), treated or not treated with Ris (10 mg/kg per day), were formaldehyde-fixed, dehydrated and embedded in paraffin. Sections were stained by haematoxylin–eosin (HE) and analysed by light microscopy (400×). Fifty randomly selected microscopic fields were examined to quantify the number of amastigote nests, mononuclear cell infiltrates and fibrotic processes.

2.5. Statistical analysis

Mean value comparisons were performed by using Student's *t*-test or ANOVA and Krunskal–Wallis tests. Survival analysis was carried out by using the Kaplan–Meier non-parametric method to estimate the survival functions of the different experimental groups. Rank tests (log-rank, which gives equal weight to all observations, and the Peto–Peto–Wilcoxon test, which uses an estimate of the survival functions for its weightings) were used to analyze the results obtained. These analyses were performed by using the Survival Tools package for StatView 4.5 (Survival Functions for StatView, Abacus Concepts Inc., Berkeley, CA) on a Power Macintosh G4 Cube computer. In all cases, *P*-values below 0.05 were considered significant.

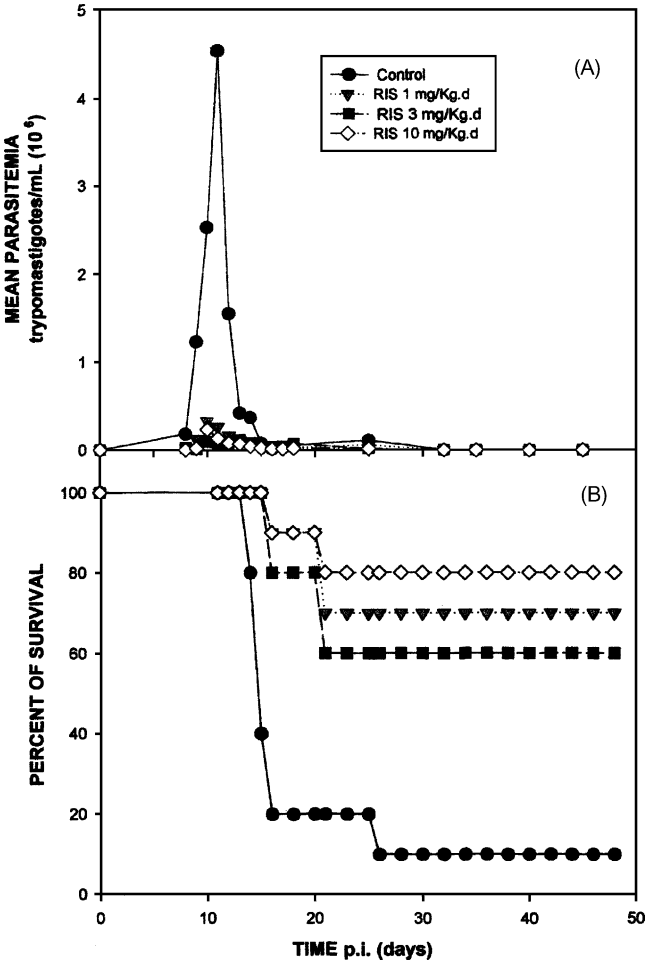

Fig. 1. Effects of risedronate (Ris) on parasitaemia (A) and survival (B) in a murine model of acute Chagas' disease. Female Swiss albino mice were inoculated with 10^3 blood trypomastigotes of the Y strain and drug treatment started 24 h later. Ris was given daily intravenously at the indicated doses, for 7 days; controls received only vehicle (phosphate-buffered saline). Statistical analysis using both the log-rank (Mantel–Cox) and Peto–Peto–Wilcoxon tests indicated a very significant ($P = 0.004$) difference between the control (untreated) animals and all those that received drug treatments. For details, see Section 2.

2.6. Drug

Risedronate (2-(3-pyridyl)-1-hydroxy-ethane-1,1-bisphosphonate, monosodium salt) was synthesised as described previously [25]. Elemental analysis and ^{1}H, ^{13}C and ^{31}P NMR spectra indicated that the compound was 98.8% pure. Stock solutions were prepared in phosphate buffered saline (PBS) (pH adjusted to 7.4) and sterilised by using a 0.2 μm filter (Millipore).

3. Results and discussion

We carried out three independent experiments to evaluate the in vivo activity of Ris, using a murine model of acute Chagas' disease. Female Swiss albino mice were inoculated intraperitoneally with 10^3 blood trypomastigotes of the Y strain, then i.v. treatment with Ris was started 24 h p.i. and given daily for a total of 7–14 doses. In the first experiment (summarised in Fig. 1 and Table 1), control animals developed a massive parasitaemia, which peaked at 11 days p.i., at which time animals began to die, reaching 90% mortality 26 days p.i. In contrast, animals which received Ris at just 1 mg/kg per day for 7 days had a 90.1% reduction in mean peak parasitaemia (Fig. 1A) and a very significant increase in survival (Fig. 1B), with only a 30% mortality at the end of the observation period (48 days; $P = 0.001$). Higher doses of Ris led to further reductions in peak parasitaemia (95.7% for 3 mg/kg per day and 94.8% for 10 mg/kg per day), but there were no significant differences in survival when compared with the group that received Ris at 1 mg/kg per day. These results were confirmed in two other, independent, experiments. In the second one (Table 1), animals received Ris at 10 mg/kg per day for 7 days, which led to a 96.6% reduction in peak parasitaemia with respect to control animals and a significant reduction of mortality: from 70% in control mice to 10% in treated animals, 40 days p.i. ($P = 0.004$). A third experiment was also carried out in which treatment with Ris at 10 mg/kg per day was extended to 14 days (Table 1). We observed, once again, a marked reduction in peak parasitaemia (92.8%) and mortality, at 60 days p.i., from 60% in untreated animals to 20% in those receiving Ris ($P = 0.05$), but these values were not significantly different from those obtained with the 7-day treatments (Table 1).

In all cases, there was no relapse of parasitaemia after the end of treatment (Fig. 1), suggesting trypanocidal, rather than trypanostatic, activity. This contrasts with the effects of pamidronate, another commercially available bisphosphonate (Aredia®), in a similar murine model of acute Chagas' disease, where although development of parasitaemia was very effectively suppressed during the treatment period, it resumed after the drug pressure was removed [7]. The idea that risedronate has trypanocidal activity in vivo, as seen previously in vitro [13], was supported by analysis of histological sections of heart tissue from control and treated animals. Fig. 2 shows that infected mice which received Ris at 10 mg/kg per day for 14 days (Table 1, experiment 3) exhibited normal heart tissue appearance (compare Fig. 2A, E and F) and a drastic reduction (91.4%, $P = 0.002$) in the number of *T. cruzi* amastigote nests, again when compared with non-treated animals (Fig. 2C and D). The reduction in the number of amastigote nests in the hearts of Ris-treated mice coincided very well with the decrease in mean peak parasitaemia in the same animals (Table 1, experiment 3); this fact strongly suggested that the two events were causally

Table 1
Effects of risedronate on parasitaemia and survival in a murine model of acute Chagas' disease[a]

Experiment	Treatment	Mean peak parasitaemia (trypomastigotes/ml × 10^4)	Survival
1	Control	458.2 ± 316.3	2/10[c]
	1 mg/kg per day Ris × 7 days	45.2 ± 33.8[b]	7/10[c,d]
	3 mg/kg per day Ris × 7 days	19.9 ± 15.0[b]	6/10[c,e]
	10 mg/kg per day Ris × 7 days	24.0 ± 14.1[b]	8/10[c,f]
2	Control	478.4 ± 209.5	3/10[g]
	10 mg/kg per day Ris × 7 days	16.4 ± 15.0[b]	9/10[g,h]
3	Control	253.2 ± 171.2	4/10[i]
	10 mg/kg per day Ris × 14 days	18.0 ± 11.4[b]	8/10[i,j]

[a] Female Swiss albino mice (20–25 g) were infected with 10^3 trypomastigotes/ml and i.v. treatment was started 24 h post-infection. Animals were followed for 40–60 days. Other details are described in Section 2.
[b] $P < 0.001$ for each group when compared with the corresponding control determined by the non-parametric Mann–Whitney or Kruskal–Wallis tests.
[c] 48 days.
[d] $P = 0.001$ with respect to control using log-rank and Peto–Peto–Wilcoxon tests.
[e] $P = 0.004$ with respect to control using log-rank and Peto–Peto–Wilcoxon tests.
[f] $P < 0.001$ with respect to control using log-rank and Peto–Peto–Wilcoxon tests.
[g] 40 days.
[h] P=0.004 with respect to control using log-rank and Peto–Peto–Wilcoxon tests.
[i] 48 days.
[j] $P = 0.05$ with respect to control using log-rank and Peto–Peto–Wilcoxon tests.

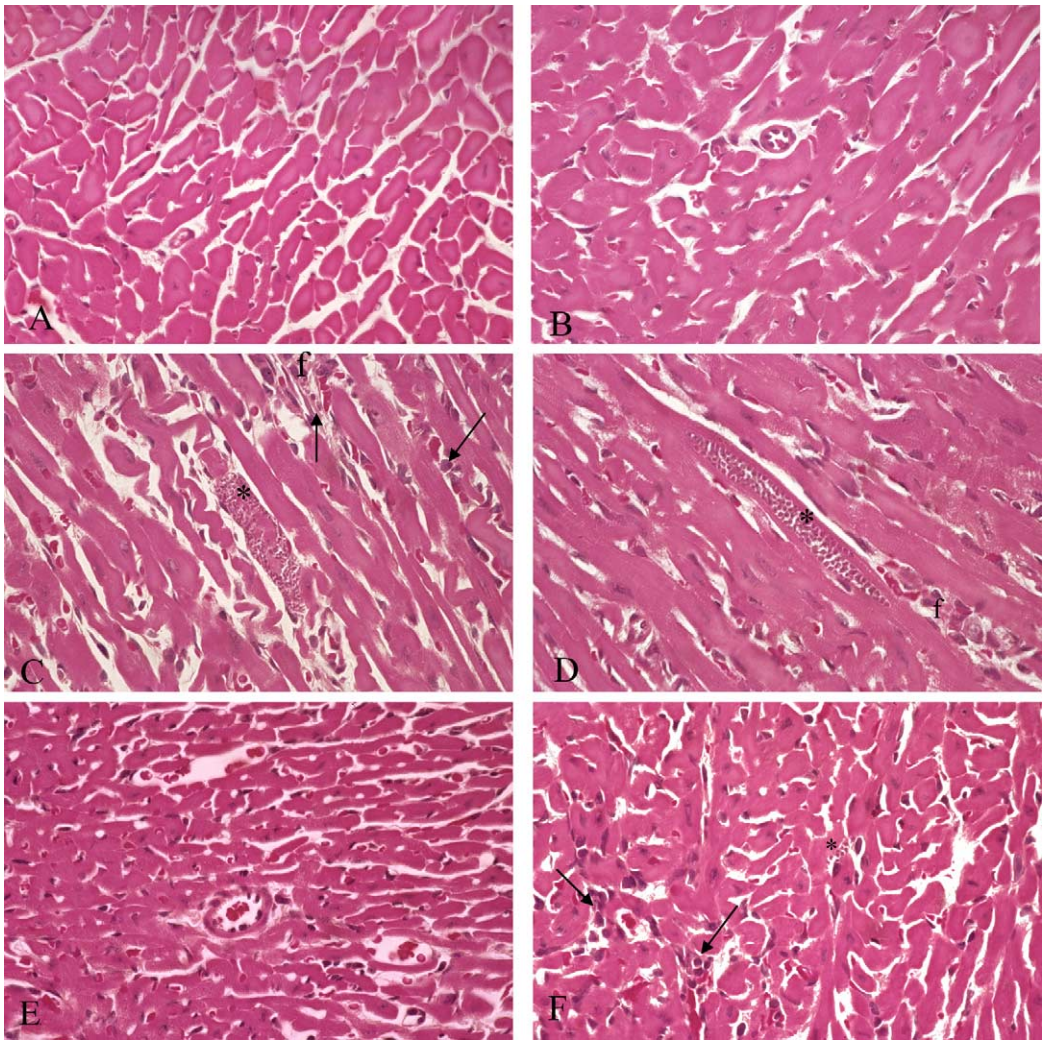

Fig. 2. Histological analysis of heart tissue from normal, *T. cruzi*-infected and infected, risedronate-treated mice. (A) Non-infected mice, (B) non-infected mice treated with risedronate (Ris) at 10 mg/kg per day for 14 days, showing no changes in tissue appearance. (C and D) Infected mice, 15 days p.i., presenting *T. cruzi* amastigote nests with large numbers of parasites (*) and an intense inflammatory process, with mononuclear cell infiltrates (arrow) and fibrosis (f). (E and F) infected but Ris-treated mice, the cardiac tissue appears normal, with very infrequent amastigote nests (*) and small mononuclear cell infiltrates (arrow). Optical magnification: 400×. For details, see Section 2.

related and that Ris has in vivo a cytocidal effect on the clinically relevant intracellular proliferative form of the parasite. The reduction in the number of both circulating and intracellular parasites induced by Ris in this murine model of acute Chagas' disease was very similar to that observed, with comparable doses and treatment times, on the *Leishmania donovani* amastigote burden of livers in a murine model of visceral leishmaniasis [15].

Despite the drastic reduction in parasite load in Ris-treated animals, no parasitological cures were obtained in any of the experimental protocols, as determined by using a combination of haemoculture and PCR-based blood assays ([25]; see Section 2). The incapacity of Ris (or pamidronate, which at 10 mg/kg per day can induce radical parasitological cures in a murine model of cutaneous leishmaniasis [16]) to induce a parasitological cure in this model of acute Chagas' disease, despite its potent in vitro and in vivo anti-*T. cruzi* activity, may be due to the disseminated nature of the infection and the short treatment periods used.

At all the doses and treatment lengths used in this work, Ris, given by the intravenous route was very well tolerated by the mice. There were no deleterious effects on weight gain and general physical condition of the treated animals and no histopathological effects on key organs, such as the heart (compare Fig. 2A and B).

In conclusion, the results we have presented above show that Ris has selective cytocidal activity against *T. cruzi* in a murine model of acute Chagas' disease and is well tolerated when administered intravenously. This and related compounds are therefore, interesting lead compounds for the development of new drugs against Chagas' disease.

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