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Bisphosphonates Derived from Fatty Acids are Potent Growth Inhibitors of *Trypanosoma cruzi*

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Abstract—We have investigated the effect of a series of bisphosphonates derived from fatty acids against *Trypanosoma cruzi* proliferation in in vitro assays. Some of these drugs proved to be potent inhibitors against the intracellular form of the parasite exhibiting IC_{50} values at the low micromolar level. As bisphosphonates are FDA clinically approved for treatment of bone resorption, their potential innocuousness makes them good candidates to control tropical diseases.

American trypanosomiasis (Chagas' disease) caused by the hemoflagellate protozoon *Trypanosoma cruzi* is a major health problem widespread in Latin America.[1,2] In rural areas, the parasite is transmitted to humans and other vertebrate hosts by reduviid bugs as a result of their blood sucking activity.[3] Similarly to other kinetoplastid parasites, *T. cruzi* has a complex life cycle. It multiplies in the insect gastrointestinal track as a non-infective epimastigote form, which differentiates to the nondividing highly infecting metacyclic trypomastigotes. This form is released within the insect feces and the circulatory system is attained through wounds produced by the blood-sucking activity of the vector. Once in the mammalian host, *T. cruzi* invades different kinds of tissues with preference for the cardiac muscle and the gastrointestinal track. After invasion the parasite differentiates to the amastigote form, which proliferates intracellularly. Finally, this form redifferentiates to the nondividing trypomastigote form, which is liberated to the bloodstream.[3–5] The bloodstream trypomastigote form can either invade other tissues or can infect the respective Chagas' disease vectors closing the cycle. In large urban centers and countries where this illness is not endemic, the main way of transmission is via the placenta and by blood transfusion.[6–8]

Chemotherapy against Chagas' disease is unsatisfactory.[9] It is based on old and fairly unspecific drugs like nifurtimox and benznidazole, which are able to cure at least 50% of recent infections. Nevertheless, these drugs suffer from serious drawbacks: (a) in acute infections, therapy has not been consistent among distinct geographical areas, presumably due to selective drug sensitivity on different *T. cruzi* strains;[10] (b) both drugs produce serious side effects; and (c) long term treatment is another disadvantage.[11] In addition, Gentian Violet is the only drug available to prevent infection of *T. cruzi* from blood transfusion. Unfortunately, the safety of this drug is in doubt because it is carcinogenic in animals.[12] These reasons emphasize the urgent need of having new chemotherapeutic agents that are effective against all strains of *T. cruzi* with minor or no side effects.

Recent studies have indicated that *T. cruzi* contains massive amounts of pyrophosphate and polyphosphates stored in an acidic organelle named the acidocalcisome[13,14] and that bisphosphonates inhibit parasite growth in vitro and in vivo with no toxicity for the host cells.[15] Bisphosphonates are analogues of pyrophosphate in which the oxygen bridge between the two phosphorus atoms is replaced by a hydroxyalkyl group. In fact, representative members of this family of compounds such as pamidronate (**1**),[16] alendronate (**2**), and risedronate (**3**) are able to impair proliferation of

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T. cruzi amastigotes in vitro (IC_{50} = 65 μM, 65 μM, and 300 μM, respectively).[15] Pamidronate is also able to reduce parasitemia in a murine model of acute Chagas' disease (Fig. 1).[15] Compounds **1**–**3** and other bisphosphonates are currently in use for treatment of bone resorption and other bone disorders.[17] As bisphosphonates are structurally related to pyrophosphate and polyphosphate, it can be postulated that these drugs are putative inhibitors of pyrophosphate-related metabolic pathways. Certainly, protein prenylation occurs in pathogenic trypanosomes.[18] This process is responsible for the attachment of farnesyl and geranylgeranyl groups to the C-terminal cysteine residues of a number of proteins of the cell, mainly small GTPases such as Ras, Rac, Rab, and Rho.[19] These attached prenyl groups play an important role in anchoring proteins to membranes. Three enzymes have been identified in eukariotic cells: protein farnesyl transferase (PFT) and protein geranylgeranyl transferase I and II (PGGT-I and II).[20] Selective inhibition of PFT impairs growth of human tumors due to farnesylation inhibition on oncogenic Ras.[21] This finding led to the development of many PFT inhibitors[21] to be used as potential anticancer agents; some of them resulted in being potent inhibitors of *T. brucei* and *T. cruzi* growth.[18] The target of aminobisphosphonates in osteoclasts,[22–24] plants,[25] and *D. discoideum*[26] is the farnesyl pyrophosphate synthase, which catalyzes the formation of the substrate for protein prenylation.[27] This is because nitrogen-containing bisphosphonates behave as carbocation transition state analogues for isoprenoid synthesis.[28] Since bisphosphonate derivatives are FDA-approved drugs for long term treatments of bone resorption, low toxicity might be anticipated for new compounds bearing the bisphosphonate moiety.

We have recently developed aryloxyethyl thiocyanate derivatives as a new class of extremely potent inhibitors against *T. cruzi* growth, in which compounds **4** and **5** arise as the main members of this family (Fig. 2).[29,30] The mode of action of these drugs is the blockade of the ergosterol biosynthetic pathway.

As bisphosphonates and thiocyanate derivatives have different molecular targets, it was of interest to replace the thiocyanate group by a bisphosphonates, keeping the nonpolar skeleton of **4** and **5**, giving rise to compounds **7**, **11**, and **15**. Therefore, a potential intramolecular synergistic effect can be anticipated. In addition, in order to study the influence of the nitrogen atom on biological activity, it was decided to prepare bisphosphonates derived from fatty acids, in which the nitrogen atom is not present. Compound **7** was easily prepared from phenylacetic acid (**6**) by a slightly modified Kiecykowski and Jobson procedure in an extremely careful anhydrous condition.[31] On treatment with phosphorous acid and phosphorous trichloride in the presence of benzenesulfonic acid at 65 °C followed by hydrolysis, **6** was converted into 2-phenoxy-1-hydroxyethane-1,1-bisphosphonate (**7**) as a mono sodium salt in almost quantitative yield.[32] NMR analysis of **7** exhibited the typical signals for this bisphosphonate-containing family of compounds. For example, the corresponding ^{31}P NMR spectrum showed a peak centered at 18.13 ppm, while the ^{13}C NMR spectrum presented a triplet centered at 75.1 ppm with a coupling constant of 133 Hz. The preparation of **11** and **15** was carried out starting from **8** and **12**, respectively. Therefore, **8** was treated with ethyl bromoacetate and potassium hydroxide in methyl sulfoxide to give the corresponding ethyl ester **9**, which after treatment with potassium carbonate afforded the free acid **10**. As depicted for **7**, carboxylic acid **10** was transformed into 2-(2,4-dichlorophenoxy)-1-hydroxyethane-1,1-bisphosphonate (**11**) in very good yield. In a similar approach, **12** gave rise to 2-(4-phenoxyphenoxy)-1-hydroxyethane-1,1-bisphosphonate (**15**) in good overall yield (Scheme 1).

The 1-hydroxyalkyl-1,1-bisphosphonates **17**, **19**, **21**, **23**, **25**, **27**, and **29** were prepared according to the general procedure from commercially available fatty acids (Scheme 2).

The biological results on *T. cruzi* were very encouraging.[33] Some bisphosphonate drugs derived from short chain fatty acids proved to be very potent inhibitors against the intracellular form of *T. cruzi*, while all of these compounds (aromatic and aliphatic bisphosphonates) were devoid of antiparasitic activity against epimastigotes. The results are presented in Table 1. Thus, aromatic bisphosphonate drugs were moderately active agents against amastigotes of the parasite, which is the clinically more relevant form of *T. cruzi*. The 4-phenoxyphenoxy derivative **11** presented 37% of inhibition at 50 μM. On the other hand, the aliphatic bisphophonates proved to be potent inhibitors against

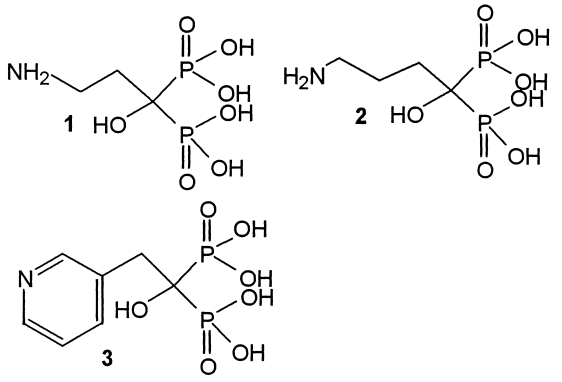


Figure 1. Chemical structures of representative FDA-approved bisphosphonates employed clinically for bone resorption.

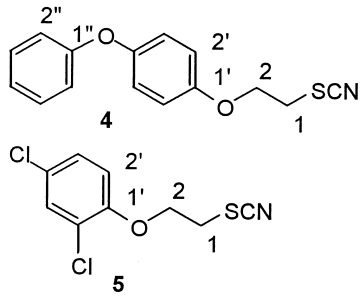
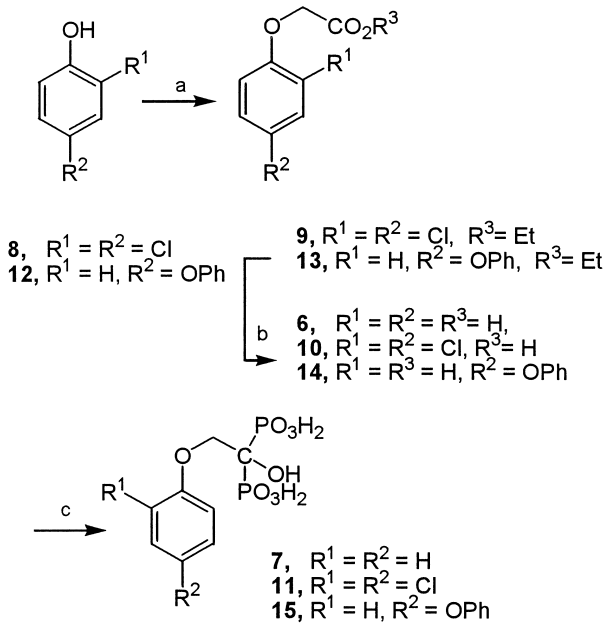
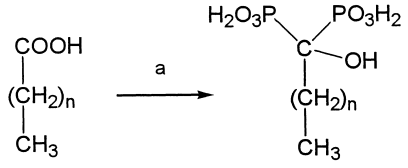


Figure 2. Chemical structures of two representative aryloxyethyl thiocyanates that block the ergosterol biosynthetic pathway.



Scheme 1. Reagents: (a) KOH, $BrCH_2CO_2Et$, DMSO, rt, 16 h (78% for **9**, 86% for **13**); (b) K_2CO_3, MeOH–H_2O, rt, 3 h (95% for **10**, 92% for **14**); (c) (i) H_3PO_3, PCl_3, $PhSO_3H$, 65 °C, 16 h, (ii) H_2O, 100 °C, 5 h (90% for **7**, 78% for **11**, 86% for **15**).



16, n = 2
18, n = 4
20, n = 5
22, n = 6
24, n = 7
26, n = 8
28, n = 12

17, n = 2
19, n = 4
21, n = 5
23, n = 6
25, n = 7
27, n = 8
29, n = 12

Scheme 2. Reagents: (a) (i) H_3PO_3, PCl_3, $PhSO_3H$, 65°C, 16 h, (ii) H_2O, 100 °C, 5 h (99% for **17**, 92% for **19**, 84% for **21**, 82% for **23**, 77% for **25**, 74% for **27**, 67% for **29**).

Table 1. Growth inhibition against *T. cruzi* (epimastigotes and amastigotes) for aromatic and aliphatic bisphosphonates

Compound	Epimastigotes IC_{50} (μM)	Amastigotes IC_{50} (μM)
4	2.2[30]	16.0[29]
7	nt[b]	>70.0 (16%)[a]
11	nt	>50.0 (37%)[a]
15	nt	>50.0 (23%)[a]
17	>70.0	21.4
19	>70.0	70.0 (34%)[a]
21	>70.0	18.1
23	>70.0	>70.0 (41%)[a]
25	>70.0	65.8
27	>70.0	>70.0 (10%)[a]
29	nt	53.5

[a]Maximum inhibition values obtained at the indicated concentrations (ca. 70.0 or 50.0 μM) are given in parentheses.
[b]nt, Not tested.

amastigotes. Drugs **17** and **21** exhibited IC_{50} values close to 20 μM each, and were more potent drugs as antiparasitic agents than bisphoshosphonates employed clinically for bone resorption, such as pamidronate and risedronate with IC_{50} values of 65 μM.[15] The rest of the designed drugs were moderately active against amastigotes. Drug **4** had previously exhibited a similar potency on amastigotes under the same assay conditions.[29] The more potent effect of bisphosphonates on amastigotes than on epimastigotes could be explained by their preferential uptake by tissue culture cells and intracellular amastigotes that could be potentially more capable of fluid-phase pinocytosis than epimastigotes.[36] Fluid-phase pinocytosis is the likely route of cellular uptake of bisphosphonates.[37] In conclusion, we have designed, prepared and evaluated new bisphosphonates that behaved as potent inhibitors of intracellular *T. cruzi*, which are now lead structures for the design of new drugs with the advantage of the potential lack of toxicity presented by the current clinically in use chemoprophylactic agents.

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32. **General procedure for the preparation of bisphosphonates (mono sodium salt).** To a flame dried 100 mL three neck flask having an addition funnel and a reflux condenser through which was circulated water at 0 °C was added the carboxylic acid (38 mmol), PO_3H_3 acid (3.2 g, 38 mmol), and anhydrous benzenesulfonic acid (15 g) under argon atmosphere. The reaction mixture was heated to 65 °C, then PCl_3 (7 mL, 80 mmol) was added dropwise with vigorous stirring. The reaction was stirred at 65 °C for 16 h. The reaction was allowed to cool to rt. Cold water (60 mL) was added and the reaction was stirred at 100 °C for 5 h. The reaction was cooled to rt and the pH was adjust to 4.3 with a 50% aqueous NaOH solution. Ethanol (20 mL) was added, and the resulting mixture was cooled to 0 °C for 24 h. The product was filtered and crystallized with water–ethanol. The products were characterized by ^1H, ^{13}C, and ^{31}P NMR spectrometry and mass spectra. The purity obtained was >99% in all cases.
33. **Drug screening.** Biological assays on epimastigotes were performed as previously described.[34] *T. cruzi* epimastigotes (Y strain) were grown in 20 mL screw-cap tubes at 28 °C in a liquid medium containing brain–heart infusion (37 g/L), hemin chlorohydrate (20 mg/L) (dissolved in 50% triethanolamine) and 10% newborn calf serum. The initial inoculum contained $2–3\times10^6$ cells/mL (as determined by counting in a Neubauer chamber) in a final volume of 1 mL. The concentration of cells was determined by measuring the absorbance of the culture medium containing parasites at 600 nm against a blank with culture medium alone. Each drug was tested at four different concentrations (1, 5, 10, and 20 μg/mL) each one in quadruplicate. Drugs were dissolved in ethanol. A control without drug was done with each group that was tested.

To calculate percent inhibition, the following formula was used: $100-(\Delta A_d\times100)/\Delta A_c$ = percent inhibition, where ΔA_c and ΔA_d are the differences in the absorbance of control cultures and drug-treated cultures, respectively, at the beginning and at the end of the experiment. The maximum amount of solvent used (1% ethanol) did not have any significant effect on the epimastigotes growth. The values of IC_{50} were estimated by linear and polynomial regression.

Experiments on the intracellular form of the parasite were conducted on *T. cruzi*-infected L_6E_9 myoblasts (Y strain) as described before.[35] L_6E_9 myoblasts were exposed to 2000 rads of gamma radiation and plated on 75 cm^2 flasks at a density of 1.2×10^7 cells/flask in DMEM containing 20% fetal calf serum in a total volume of 10 mL. After 24 h of incubation at 35 °C, the cells were exposed to a suspension of 5×10^7 trypomastigotes/flask in DMEM containing 20% fetal calf serum for 2 h, then cultures were washed twice with Dulbecco' PBS and the culture medium was replaced. Different concentration of drugs were added to the cultures that were labeled with 1.0 μCi of [5-6-^{3}H]uracil and incubated for an additional 72 h. Incorporation of [^{3}H]uracil was measured, the percent inhibition of [^{3}H]uracil incorporation (parasite proliferation) was calculated employing the following formula: inhibition percent = $[(A-B/A]\times100$, where A and B are the mean counts per minute of infected control-treated myoblasts and infected drug-treated myoblasts, respectively.
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