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The purine transferase from *Trypanosoma cruzi* as a potential target for bisphosphonate-based chemotherapeutic compounds

Daniel Fernández,[a] Mary Anne Wenck,[b] Sydney P. Craig, III[c] and José M. Delfino[a,*]

[a]*Departamento de Química Biológica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina*
[b]*Department of Epidemiology, University of North Carolina School of Public Health, Chapel Hill, NC 27599-7435, USA*
[c]*University of Connecticut, Storrs, CT 06269-3125, USA*

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Abstract—We identified and tested bisphosphonates as inhibitors of a protozoan molecular target. Computational modeling studies demonstrated that these compounds are mimics of the natural substrate of the enzyme. The most potent bisphosphonates in vitro are **pamidronate** and **risedronate**, which inhibit the purine transferase from *Trypanosoma cruzi* in the micromolar range.

Purine phosphoribosyltransferase proteins (PRTs)[1,2] catalyze the transfer of the ribose–phosphate moiety of phosphoribosylpyrophosphate (PRPP) to a free purine base yielding the corresponding nucleotide and pyrophosphate. The substrate PRPP binds to PRT as the dimagnesium complex,[3] and the metal cations appear to play an essential role in substrate binding and activation.[2] The reaction recycles preformed purine rings so that free hypoxanthine and guanine are salvaged by the hypoxanthine-guanine PRT (HPRT).[4] The precise chemistry of the HPRT catalyzed reaction is a current matter of debate.[1,5] Purine salvage processes provide a key metabolic function to protozoan parasites due to their lack of de novo synthesis of purine rings.[4,6] Although the salvage pathways utilized by the parasites are in general similar to those used in the host, there are metabolic differences between them that could lead to potential intervention against the parasites.[7–9] For hemoflagellated parasites of the genera Leishmania and Trypanosoma, one could take advantage of this fact to attack the intracellular forms that are pathogenic in man.[10] *Trypanosoma cruzi* is the causative agent of Chagas' disease (American trypanosomiasis), which is a major public health problem in Latin America, where it constitutes one of the largest parasitic disease burdens.[11] Currently, millions of people are infected with *T. cruzi*, and the available medications to treat Chagas' disease are relatively ineffective or toxic.[11,12]

The aim of our investigation is to develop novel chemotherapeutic compounds by using HPRT from *T. cruzi* (TcHPRT) as the target molecule. PRTs have been proposed as targets for the development of antiparasitic drugs.[6,9] Target validation of HPRT has been demonstrated through the reversal of the biological effect of selective inhibitors by an excess amount of hypoxanthine.[13,14] TcHPRT inhibitors developed prior to our work include the analogues of the purine base (the most potent inhibitor of this type showing an IC_{50} of 12 μM).[15] More recently, new compounds were identified through an automated flexible docking of a 2D database onto the TcHPRT closed conformation structure.[13,16] The resulting compounds show little similarity to the known HPRT ligands—the most potent of these inhibit TcHPRT with a K_i of 0.5 μM[13]—but were demonstrated to be selective against the parasite HPRT. However, this group includes chrysene-containing compounds, which are chemically undesirable and belong to a class of known carcinogens with a high hydrophobic character.[17]

The use of the PRPP structure to create a TcHPRT inhibitor has so far remained unexplored. Thus we searched the small-molecule 3D Cambridge Structural

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* Corresponding author. Tel.: +54-11-4962-5506/4964-8289-90-91x116; fax: +54-11-4962-5457; e-mail: delfino@qb.ffyb.uba.ar

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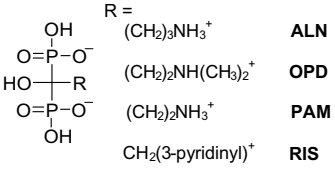
Database (CSD)[18] for molecules having the structural and electrical features of this substrate. Among the resulting hits we chose compounds from the most populated group of structures for further investigation. In order to gain insights on their ability to interact with the enzyme, selected structures were evaluated by molecular mechanics methods implemented in MacroModel/BatchMin software.[19] On the other hand, selected compounds were assayed in vitro to determine their potency as inhibitors of TcHPRT. In the future, the structure determination of complexes of TcHPRT with these inhibitors will help to understand in detail the basis of this interaction and permit the rational optimization of lead compounds.

We first queried the CSD for candidate compounds by searching the substructure P–X–P, where X represents any atom different from a metal and P is always bound to three oxygen atoms. The output (1114 hits) was then filtered to retrieve compounds that were able to coordinate mono and divalent metal cations. More than half the hits (243 out of 462) showed the P–C–P connectivity, compounds, which are collectively known as bisphosphonates (BPs). Remarkably, BPs are chemically and biologically stable analogues of the naturally occurring inorganic pyrophosphate (PPi).[20] In addition, BPs are medications prescribed for the treatment of skeletal disorders, for example, alendronate (Fosamax) is used for osteoporosis and pamidronate (Aredia) is used for hypercalcemia.[21] Much research has been conducted with BPs as enzyme inhibitors, the common feature being the mimicry of pyrophosphate or phosphate-containing metabolites. BPs have been proposed as antiresorptive agents by their ability to inhibit human farnesyl pyrophosphate synthase, a key enzyme in the isoprenoid pathway (K_i 3–200 nM).[22] In connection with novel therapeutic targets for *T. cruzi*, BPs have also been shown to inhibit farnesyl pyrophosphate synthase from this parasite (K_i 32–2020 nM),[23] as well as to arrest the proliferation of *T. cruzi* in an in vivo assay.[24] Here we propose to demonstrate that BPs are able to inhibit the reaction catalyzed by TcHPRT. This idea prompted us to investigate in depth the PRT–BP interaction.

We used an optimized Monte Carlo procedure[25] to explore the conformational space available to selected BPs. These included **alendronate** (4-amino-1-hydroxy butylidene-1,1-bisphosphonic acid; **ALN**), **olpadronate** (*N*,*N*-dimethyl-3-amino-1-hydroxypropylidene-1,1-bis phosphonic acid; **OPD**), **pamidronate** (3-amino-1-hydroxy propylidene-1,1-bisphosphonic acid; **PAM**), and **risedronate** (1-hydroxy-2-(3-pyridinyl)ethylidene-1,1-bisphosphonic acid; **RIS**). At the end of the conformational search, all nonenantiomeric conformers within 20 kJ/mol above the global minimum were tabulated.



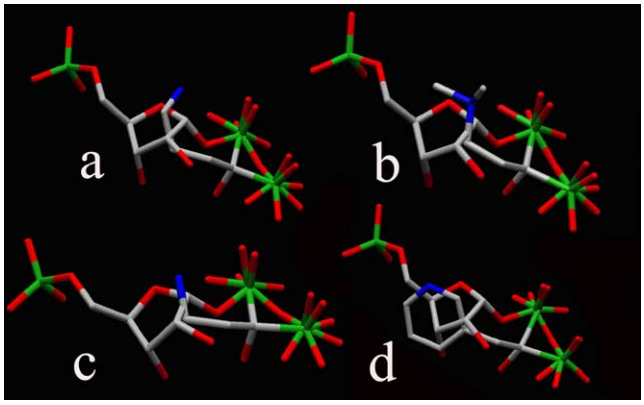


Figure 1. Structural comparison of BPs and PRPP. (a) **ALN**, (b) **OPD**, (c) **PAM**, and (d) **RIS**.

The structures of the conformers thus obtained were then rigidly superimposed onto the PRPP structure as found in the TcHPRT complex.[16] For all four compounds, the P–O group of each moiety of the BPs superimposes well on the corresponding group in PRPP (RMS deviations under 0.2 Å). Examples of superimposed structures are shown in Figure 1. It becomes evident that the size of each BP molecule is roughly one half that of PRPP. Moreover, for all BPs assayed the N atom locates close to the position of the C1–O bond of the ribose ring in PRPP. Once this comparison is included in the superimpositions, by defining an 'average atom' midway along the C1–O bond, RMS deviations below 0.4 Å for linear BPs (Fig. 1a–c) and below 0.6 Å for that containing the heterocycle (Fig. 1d) were found.

The BPs were then docked into the closed conformation of TcHPRT (PDB code: 1TC2), which is considered to be a structure that approximates the transition state.[16] The flexible loop II is closed over the active site, and the invariant residue Y104 plays an important role by preventing the access of external solvent to the catalytic site. Here exist two metal centers: Mg^{2+} in site I is coordinated by PRPP with indirect participation of the protein via the carboxylate of E133 through a water bridge, while in site II the coordination sphere of Mg^{2+} is built by bidentate PPi oxygens, three water molecules and the carboxylate of the invariant D193 residue. We favored the preservation of the interactions present in the latter, by anchoring the P–C–P moiety of BPs to this site. As regards Mg^{2+} in site I, we envisaged two possibilities: to leave it out of the simulations, because the bonds provided by PRPP will be missing (option 1), or to include it, due to a possible bond to a third phosphonic O atom and water molecules (option 2).[25] In the first case, the positively charged N atom of **PAM** (Fig. 2a), **RIS** (Fig. 2c) and **ALN** (not shown) locates close to the position of site I, presumably interacting strongly with the carboxylate of E133. This suggests a loose mimicry of the metal cation. In the second case, expectedly the N atom of these compounds (Fig. 2b and d) moves away from site I, and suggestively lies close to the position of the endocyclic O atom of the ribose in PRPP. This situation would not interfere with the closure of the aromatic ring of Y104 upon this site. Here,

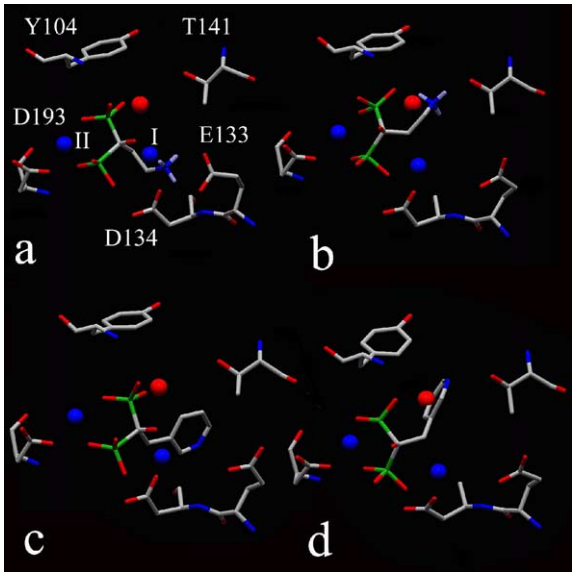


Figure 2. Docking of BPs into the active site of TcHPRT. BPs, metal centers (blue spheres labeled I and II in panel a) and selected residues are shown. Panels a and c illustrate results from the simulation of **PAM** and **RIS**, respectively, where the metal in site I is ignored (option 1 in main text). Panels b and d correspond to results of a similar simulation where both metal sites are considered (option 2). For the purpose of comparison, the position of the endocyclic O atom of the ribose in PRPP is also shown (red sphere).

rather than bonding to E133, the N atom would interact by hydrogen bonding to the OH of T141. By contrast, although **OPD** is endowed with a carbon chain of the same length as that of **PAM**, no hydrogen bond could ever be observed for its N atom, most likely due to the steric hindrance exerted by the methyl groups.

Overall, these results suggest that **PAM**, **RIS**, and **ALN**—but less so **OPD**—would bind well to the PRPP binding site. The common feature being the coordination of the P–C–P head group to the metal cation in site II, helped by ancillary stabilization expected from hydrogen bonding between the positively charged N atom and residues lining the active site. Noteworthy, from a steric viewpoint, BPs would partially fill the PRPP binding site, hence opening the possibility of taking advantage of empty spaces by adding suitable functional groups to enhance recognition.

The BPs **ALN**, **OPD**, **PAM**, and **RIS** were obtained as described,[20] and used in the kinetic studies.[29] In all cases, inhibition data were modeled consistently by a competitive inhibition mechanism versus the substrate PRPP (Table 1). The most potent inhibitors are **RIS** and **PAM**, which show values of K_i in the range of the K_m for the substrate PRPP. These results are fully consistent with predictions from molecular modeling, whereby simultaneous binding of BPs and PRPP is not possible. Significantly, when typical clinical doses of these compounds are administered intravenously, micromolar plasma concentrations are reached.[30]

Collectively, the work herein presented lends support to the view by which BPs could serve as lead compounds

Table 1. Inhibition of TcHPRT by bisphosphonates

Compounds	K_i, μM[a]
ALN	188.6(±11.8)[b]
OPD	126.0(±5.0)[b]
PAM	50.6(±3.7)[b]
RIS	23.2(±2.3)[b]

[a] Values are means of three experiments, standard deviation is given between parentheses.

[b] For **ALN** and **OPD**, values of K_i were obtained from Eq. $K_i = IC_{50}/3.5$, while for **PAM** and **RIS**, these values were obtained by a non-linear global fit of the data to a model of competitive inhibition versus PRPP.[29]

for the development of inhibitors of PRTs. Optimization will include (i) the design of more potent and parasite-specific derivatives of BPs, and (ii) the improvement of their oral bioavailability (prodrugs).[31] Among the BPs assayed, **PAM** might be the most useful candidate for modification given the combination of high potency as inhibitor, low molecular weight, and advantage from a synthetic standpoint. The availability of the primary amino function would allow diversity at this point of attachment. Notwithstanding this, valuable conclusions can also be learnt from **RIS**, because the presence of a nitrogen bearing heterocycle delocalizing a positive charge enhances binding to the enzyme. A specific inhibitor of TcHPRT should ideally avoid its interaction with the homologous human enzyme, for example, by taking advantage of the ability of PRTs to discriminate between different purine analogues.[15] Conceivably, such inhibitor (e.g., a bi-substrate analogue) might result from the covalent attachment of a suitable ring to the parent BP skeleton. In this regard, a desirable goal would consist in obtaining highly potent inhibitors of TcHPRT based on BP bearing transition state analogues. If the reaction follows a Sn2 mechanism, the positively charged N atom in the R group of a BP could enhance binding via interactions with polar side chains of active site residues, like those with E133 of TcHPRT herein predicted. Alternatively, if the reaction implies a Sn1 mechanism, the positive charge in the lateral chain could potentially mimic that predicted to develop on the ribose ring. In connection with this, the preservation of the positive charge can be important for improving the affinity of the inhibitor,[32] as illustrated by the inhibition of human and malarial PRTs by immucilins.[33] In this context, fundamental insights for rational drug design will come from the precise elucidation of the reaction mechanism, assisted by structure determination of complexes of the enzyme with BP derivatives.

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25. Molecular mechanics calculations were carried out with MacroModel/Batchmin[19] 7.0 running on a SGI O2 workstation (R10000) under the Irix 6.5 operating system. The conformational search was carried out with an optimized Monte Carlo method.[26] Starting geometries for BPs were generated from the atomic coordinates of the Ca^{2+} salt of **PAM** (CSD[18] code: XUGGEL).[27] We used the GB/SA solvation treatment and the AMBER* force field version implemented in MacroModel. For dynamics, we adopted a mixed Monte Carlo/stochastic dynamics (MC/SD) method.[28] These simulations were run at 300 K during 300 ps, after an equilibration time of 50 ps. A time step of 1.5 fs was used for the SD part. All atoms belonging to BPs, excepting the two oxygen atoms bound to the metal cation, and the side chains of Y104, E133, D134, T141, K165, and D193 were allowed to move freely. Crystallographic water molecules were eliminated. The original Mg^{2+} and Mn^{2+} metal cations were replaced by Ca^{2+}, due to requirements of the force field. The interaction of the N atom of the BPs with the enzyme was monitored. A hydrogen bond N–H···O is formed whenever the following criteria are met: a N···O distance of less than 3 Å, and an N–H···O bond angle larger than 120°. Apart from the exam of the average values for the population, we tabulated the occurrence of hydrogen bonding for each sampled structure with the command GEOM. This helped select representative structures as shown in Figure 2.
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29. TcHPRT was purified from an overexpression system, as described previously.[15] For enzyme kinetics we followed a standard procedure,[13] with minor modifications. Reactions were carried out at room temperature in 10 mM HEPES, 12 mM $MgCl_2$ buffer. The 50% inhibitory concentration (IC_{50}) of each compound was measured as the molar concentration, which reduces the enzymatic activity by 50% of that observed in its absence. These compounds were assayed in the range 4–500 μM in the presence of a fixed level of the substrates hypoxanthine and PRPP: each was kept at 2.5 times the value of the K_m for the corresponding substrate, that is 32.4 μM PRPP and 8.6 μM hypoxanthine. If the concentration of each substrate is held fixed, K_i is calculated as $K_i = IC_{50} * K_m/(K_m + [S])$, where $[S]$ is the concentration of the PRPP substrate. Alternatively, reactions were run where the concentration of PRPP varies (from 4 to 240 μM), the concentration of each inhibitor varies (between 4 and 250 μM), while that of hypoxanthine is held constant (at 2.5 times its K_m value). In this case, K_i is determined from a global nonlinear fit of the data to Eq $v/V_{max} = 1/[1 + (K_m/[S]) * (1 + [I]/K_i)]$, where $[S]$ and $[I]$ are the concentrations of PRPP substrate and inhibitor, respectively. Regression analysis was carried out with the program SigmaPlot (SPSS Inc., 233 S. Wacker Drive, Chicago, Illinois, IL, USA).
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