New Insights into Molecular Recognition of 1,1-Bisphosphonic Acids by Farnesyl Diphosphate Synthase

Mariana Ferrer-Casal, Catherine L, Melina Galizz, Carlos A. Stortz, Sergio H. Szajnman, Roberto Docampo, Silvia N. J. Moreno, and Juan B. Rodriguez

Abstract

As part of our project pointed at the search of new antiparasitic agents against American trypanosomiasis (Chagas disease) and toxoplasmosis a series of 2-alkylaminoethyl-1-hydroxy-1,1-bisphosphonic acids has been designed, synthesized and biologically evaluated against the etiologic agents of these parasitic diseases, *Trypanosoma cruzi* and *Toxoplasma gondii*, respectively, and also towards their target enzymes, *T. cruzi* and *T. gondii* farnesyl pyrophosphate synthase (FPPS), respectively. Surprisingly, while most pharmacologically active bisphosphonates have a hydroxyl group at the C-1 position, the additional presence of an amino group at C-3 resulted in decreased activity towards either *T. cruzi* cells or *Tc*FPPS. Density functional theory calculations justify this unexpected behavior. Although these compounds were devoid of activity against *T. cruzi* cells and *Tc*FPPS, they were efficient growth inhibitors of tachyzoites of *T. gondii*. This activity was associated with a potent inhibition of the enzymatic activity of *Tg*FPPS. Compound 28 arises as a main example of this family of compounds exhibiting an ED$_{50}$ value of 4.7 μM against tachyzoites of *T. gondii* and an IC$_{50}$ of 0.051 μM against *Tg*FPPS.

Introduction

Farnesyl pyrophosphate synthase (FPPS) can be considered as a valid target not only for bone related disorders, but also for different parasitic diseases.$^1$ FPPS catalyzes the consecutive condensation of IPP with DMAPP and with geranyl diphosphate (GPP) to produce farnesyl diphosphate (FPP). FPP is the substrate for enzymes catalyzing the first committed step for the biosynthesis of sterols, ubiquinones, dolichols, heme a, and prenylated proteins. FPP could be condensed with an additional molecule of IPP by the
geranylgeranyl pyrophosphate synthase (GGPPS) to form the 20-carbon isoprenoid GGPP.
In trypanosomatids, isoprenoid biosynthesis occurs via the classical mevalonate pathway
(Scheme 1). The FPPS gene has been cloned from T. cruzi and T. brucei. Both of these
genes are single copy. RNA interference showed that the T. brucei FPPS gene is essential.

In Apicomplexan parasites such as Toxoplasma gondii isoprenoids are biosynthesized
through the DOXP/MEP pathway as illustrated in Scheme 2. In addition, T. gondii
possesses a bifunctional FPPS/GGPPS (TgFPPS) that is able to catalyze the formation of
both FPP and GGPP. Comparison of the amino acid sequence of FPPSs from different organisms (bacteria to
eukaryotes) shows the presence of seven conserved regions including two aspartate-rich
domains that are very important for the catalytic action and most likely act as the
binding sites for IPP and the allylic substrates. All the FPPSs that have been characterized
are homodimeric enzymes, and require divalent cations such as Mg$^{2+}$ or Mn$^{2+}$ for activity.

On the other hand, bisphosphonates (2) are pyrophosphate (1) analogues in which a
methylene group replaces the oxygen atom bridge between the two phosphorus atoms of the
pyrophosphate moiety. The substitution of carbon with different side chains has generated a
large family of compounds. Several bisphosphonates are potent inhibitors of bone resorption
and are in clinical use for the treatment of different bone disorders. Acidocalciosomes are
in bone mineral, facilitates their antiparasitic action. Aminobisphosphonates such as pamidronate (3), alendronate (4), and risedronate (5), were first found to be effective in the inhibition of T. cruzi in vitro and in vivo without toxicity to the host cells (Figure 1). In addition, some bisphosphonates were growth inhibitors of T. gondii, T. brucei rhodesiense, Leishmania donovani and Plasmodium falciparum. In vivo testing in mice has shown that risedronate can significantly increase the survival of mice infected by T. cruzi. All these results indicate that bisphosphonates are promising
candidate drugs to treat infections by T. cruzi and other pathogenic parasites. In fact, they
are extremely potent inhibitors of the enzymatic activity of T. cruzi FPPS, but they are extremely potent inhibitors of the
enzymatic activity of T. gondii FPPS exhibiting IC$\_50$ values of 35 nM and 60 nM, respectively. The high selectivity observed by these fluorine-containing bisphosphonates against TgFPPS versus TcFPPS can be rationalized by the evidence that the amino acid
sequences of these enzymes have less than 50% identity. (Figure 2). As mentioned before,
are promising anti- T. cruzi agents. For example, exhibit an ED$\_50$ value of 0.84 µM
against T. cruzi (amastigotes), which is fifteen times more potent than the well-known
antiparasitic agent WC-9 under the same assays conditions. In addition, is an extremely
potent inhibitor of the enzymatic activity of TcFPPS (IC$\_50$ = 0.058 µM) and of TgFPPS
(IC$_{50}$ = 0.095 μM), whereas the long chain length derivative 14 is an effective growth inhibitors of intracellular T. cruzi proliferation (ED$_{50}$ = 0.67 μM) compared to benznidazole (ED$_{50}$ = 2.77 μM). Sulfur-containing bisphosphonates also presents good prospective as putative lead drugs. For example, 15 and 16 are potent anti-Toxoplasma agents and, to a lesser extent, efficient anti-T. cruzi agents. For example, 15 has a potent cellular activity against tachyzoites of T. gondii (ED$_{50}$ = 1.8 μM), which was associated with a potent inhibition of the enzymatic activity of TgFPPS (IC$_{50}$ = 0.21 μM). Compound 15 is also effective towards TcFPPS (IC$_{50}$ = 0.097 μM). Moreover, 16 exhibits an extremely potent inhibitory action against TgFPPS with an IC$_{50}$ value as low as 0.009 μM. (Figure 2). It is worth mentioning that similar activity have also been reported for some lipophilic bisphosphonates, including 2-alkylaminoethyl compounds lacking the hydroxyl group at C-1, against another Apicomplexan parasite, P. falciparum.

Rationale

The present study was motivated to get further insight into the molecular recognition processes of 2-alkyl(amino)ethyl-1,1-bisphosphonates taking compounds 12–14 as reference structures. We have recently demonstrated that TcFPPS inhibitors 12 and 13 bind to the allylic site of the enzyme with the phosphates group of the bisphosphonate unit coordinating three Mg$^{2+}$ atoms, which bridge the compounds to the enzyme in a similar way to that observed for the physiological substrates. Binding of either 12 or 13 is enthalpically unfavorable. The favorable entropy, which dominates the favorable free-energy, results from a delicate balance between two opposing effects: the unfavorable loss of conformational entropy, due to freezing of single bond rotations of the inhibitor (and binding site side chains), and the favorable increase of entropy associated with burial of the hydrophobic alkyl chains. The nitrogen atom at the C-3 position is very important to maintain a potent inhibition of the enzymatic activity of TcFPPS, but does not coordinate any Mg$^{2+}$ atom at the active site as we had been initially considered. It has a crucial role to drive the spatial alignment of the alkyl chains for better fitting.

We have envisioned that the introduction of a hydroxyl group at the C-1 position in compounds 12 and 13 is a relevant structural variation for a number of reasons: (a) it is known that the presence of an electron withdrawing group at C-1 would enhance the ability to coordinate Ca$^{2+}$ or Mg$^{2+}$ in a tridentate manner; (b) the presence of an electron withdrawing group at C-1 of 1,1-bisphosphonic acids would increase acidity in at least one order of magnitude compared to those where these groups are absent mimicking the pK$_a$ value of pyrophosphoric acid; (c) most of the bisphosphonates clinically in use for the treatment of bone disorders have a hydroxyl group bonded at C-1; (d) compound 6 impairs its efficiency as inhibitor of the enzymatic activity against TcFPPS when the hydroxyl group is absent as occurs with compound 8.

Bearing in mind the above statements, the role of the hydroxyl group at C-1 on biological activity in a variety of bisphosphonates is still uncertain. Experimental evidence indicates that that the hydroxyl group at C-1 does not interact with Mg$^{2+}$ at the active site of FPPS suggesting that the function of this group is circumvented to influence the ability of the adjacent bisphosphonic unit to coordinate Mg$^{2+}$ as well as to increase the pK$_a$ of the gem-phosphonate functionality.

Results and Discussion

As previously discussed, we selected 2-alkylaminoethyl-1-hydroxy-1,1-bisphosphonic acid derivatives 27–31 as the title compounds for the present study. These compounds were straightforwardly prepared starting from benzyl bromoacetate and the corresponding linear.

Bioorg Med Chem. Author manuscript; available in PMC 2015 January 01.
amine. Nucleophilic displacement reaction between each amine and benzyl bromoacetate in acetonitrile as a solvent, according to slightly modified published procedures, afforded the respective benzyl $n$-alkylaminoacetates 17–21 in yields ranging 29–84%. Benzyl groups were cleaved by catalytic hydrogenation employing palladium on charcoal as catalyst to give the respective free acids 22–26 in 46–98% yields. These 2-(n-alkylamino)acetic acids were the substrates to prepare the title compounds 27–31. Then, in independent experiments, on treatment with phosphorous acid and phosphorous trichloride employing benzenesulfonic acid as a solvent at 65 °C followed by hydrolysis, 22–26 were converted into 27–31 according to the widely employed method for the preparation of 1-hydroxy-1,1-bisphosphonic acids from carboxylic acids (Scheme 3). Biological evaluation of 2-alkylaminoethyl-1-hydroxy-1,1-bisphosphonates has lead to surprising results. Contrary to it was expected, all of these compounds were almost devoid of biological activity as inhibitors of $T$. cruzi proliferation and also as inhibitor of the enzymatic activity of $T$. cruzi FPPS confirming our previous finding in compound 29. However, some of these compounds showed an extremely potent inhibition of the enzymatic activity of $T$. cruzi FPPS. For example 28 and 30 are potent inhibitors of $T$. cruzi FPPS exhibiting IC$_{50}$ values of 0.051 μM and 0.039 μM, respectively. This enzymatic activity was associated with an efficient cellular activity showing ED$_{50}$ values of 4.7 μM and 2.0 μM, respectively. Risedronate was used as positive control (ED$_{50}$ = 2.4 μM). This selectively observed towards the target enzymes ($T$. cruzi FPPS versus $T$. cruzi FPPS) has previously been observed and it can be justified by the fact that sequences of these enzymes have less than 50% identity. Moreover, these results are more comprehensible taking into account that $T$. cruzi FPPS is a bifunctional enzyme, hence as $T$. cruzi FPPS also catalyzes formation of both FPP (C-15) and GGPP (C-20), it is reasonable to assume that its enzymatic activity could be inhibited by compounds of long chain length, which are structurally rather similar to GGPP in contrast to $T$. cruzi FPPS, the enzyme that catalyses formation of FPP as final product exclusively. The biological evaluation is presented in Table 1.

The lack of biological activity of this family of compounds against $T$. cruzi is quite unexpected and cannot be attributable simply to the presence of a hydroxyl group at C-1. In fact, either compound 6 or risedronate (5), both bearing a hydroxyl group at C-1, are effective inhibitors of $T$. cruzi FPPS. Why our title compounds are devoid of antiparasitic activity against $T$. cruzi? In risedronate the nitrogen atom is bonded one position further than is bonded in our compounds. Evidently, the position of this nitrogen atom has a strong influence in the observed biological activity. Removal of the hydroxyl group keeping the nitrogen atom at C-3 results in extremely potent 2-alkylaminoethyl bisphosphonates, such as 12–14. Therefore, these two groups did not produce the expected synergistic effect. On the contrary, the combination of these two groups reduced their activity. We have attempted to rationalize this behavior using density functional theory (DFT) calculations. Analogs of 12–14 (12a) and 27–31 (27a) carrying, for the sake of simplicity, $N$-methylamino groups instead of longer alkylamino moieties were submitted to energy minimizations at the B3LYP/6-311+G(d,p) level, simulating the presence of water as solvent with the polarizable continuum method (PCM). In order to reproduce better the possible conformation of these compounds in the biological environment, the molecules 12a and 27a were considered to have the acidic hydrogen atoms expected to appear at physiological pH ($\approx$ 6.5), i.e. two hydrogen atoms for 12a, and only one for the more acidic 27a. One magnesium atom was added to complete each molecule (Figure 3).

Results show that the minimization always leads to the formation of a six-membered ring containing C-1, both P atoms, two O atoms and the Mg atom, being the distance between each O and the Mg of 1.91–1.96 Å. However, the “exocyclic” atoms generate different hydrogen bond patterns (even in the simulated water environment) with distinct geometries.
and energies. It is known that the strength of the hydrogen bonds depends, under the geometric criteria, on acceptor-hydrogen distances as short as possible (for very strong bonds, the distance can be even shorter than 2 Å), and donor-hydrogen-acceptor angles as close to 180° as possible.\textsuperscript{54,55} Table 1 shows the results for the main conformers of each analog. For 12a, the most stable geometry shows a strong “1,3-diaxial” hydrogen bond between an axial O-H and an axial O bonded to different P atoms ($d_{\text{H-O}} = 1.82$ Å), and a very strong hydrogen bond between the other acidic hydrogen (equatorial) and the nearby nitrogen atom ($d_{\text{H-N}} = 1.68$ Å), which fixes the conformation of the side chain (Figure 4). Another conformer, with similar energy, has the same hydrogen bond features but a different conformation of the carbon chain. On the other hand, compound 27a does not have two acidic hydrogens. Thus, the only acidic hydrogen should be involved in either the 1,3-diaxial H-bond interaction or the interaction with the N. The most stable geometry (Figure 4, Table 1) shows the strong diaxial interaction ($d_{\text{H-O}} = 1.62$ Å, $\theta = 161°$), and two weaker interactions (Table 1), as deduced from directional factors ($\theta = 124–134°$). Other conformations, with higher energies show other bonding patterns: conformer 4 shows a similar pattern, whereas conformers 2 and 3 show a strong hydrogen bond between the acidic hydrogen and the nitrogen atom, as occurred with 12a (Table 1). However, these conformers have energies surpassing in about 2 kcal/mol that of the most stable conformer. The strength of the bond in conformer 3 of 27a is equivalent to that observed for the main conformer of 12a, but the hydrogen bond in conformer 2 is slightly weaker, as deduced from the distance and angles (Table 1).

These results might be a clue that in compounds like 12–14, having two acidic hydrogen atoms, the flexibility of the carbon chain is strongly reduced, to the point of giving an almost “fixed” conformation carrying the nitrogen atom in a favorable arrangement for biological action. On the other hand, compounds like 27–31, which carry both a hydroxyl group at C-1 and a N atom on C-2 display a higher flexibility of the carbon chain, thus generating a manifold of conformations, for which only some (less stable) display the arrangement needed for an optimal molecular recognition.

NMR analyses support the above statement. Compound 32 is an interesting example of an isosteric analog of compounds 12–14.\textsuperscript{56} It has been demonstrated that 32 forms a rather stable six-membered ring via a hydrogen bond based on the chemical shift of the phosphorus atom at the C-3 position as illustrated in Figure 5.\textsuperscript{56} In addition, proton NMR data of 28 and the $n$-propyl derivative of 12–14 (compound 33), performed in anhydrous deuterated DMSO, indicated the occurrence of this conformational restriction on these compounds. Two signals of protons bonded to heteroatoms were observed in the $^1$H NMR spectrum of 33 at 7.70 ppm and 8.95 ppm, respectively. The first signal moved upfield (5.40 ppm) when one equivalent of Mg$^{2+}$ was added. A similar behavior was observed from the proton NMR spectrum of 28. Once again, two signals that appeared at 6.69 ppm and 9.11 ppm. The first one moved upfield (4.41 ppm) when one equivalent of Mg$^{2+}$ was added.

In summary, it can be concluded that the activity against \textit{T. cruzi} of compounds of type 12–14 can be attributed due to these compounds adapt a restricted conformation having no tension of torsion that would be benefit for molecular recognition, whereas the 1-hydroxy derivatives 27–31 present a manifold of conformations that would not lead to a successful interaction with the target enzyme. Nevertheless, these bisphophonate derivatives exhibited a selective and potent inhibitory action towards TgFPPS. Efforts in optimizing lead structures 12–14 are currently being pursued in our laboratory.
Experimental Section

General

The glassware used in air- and/or moisture-sensitive reactions was flame-dried and reactions were carried out under an argon atmosphere. Unless otherwise noted, chemicals were commercially available and used without further purification. Solvents were distilled before use. Acetonitrile was distilled from phosphorus pentoxide.

Nuclear magnetic resonance spectra were recorded using a AM-500 MHz spectrometer. Chemical shifts are reported in parts per million ($\delta$) relative to tetramethylsilane. Coupling constants are reported in Hertz. $^{13}$C NMR spectra were fully decoupled. $^{31}$P NMR spectra are referenced with respect to the peak of 85% $\text{H}_3\text{PO}_4$ as external reference. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quartet.

High-resolution mass spectra were obtained using a hybrid quadrupole time of flight mass spectrometer with MS/MS capability.

Melting points are uncorrected. Analytical TLC was performed on commercial 0.2 mm aluminum-coated silica gel plates (F$_{254}$) and visualized by 254 nm UV or immersion in an aqueous solution of (NH$_4$)$_6$Mo$_7$O$_{24}$•4H$_2$O (0.04 M), Ce(SO$_4$)$_2$ (0.003 M) in concentrated H$_2$SO$_4$ (10%). Elemental analyses were conducted by UMYMFOR (CONICET-FCEyN). The results were within ±0.4% of the theoretical values.

Synthesis of 2-(n-Alkylamino)acetic acids

General Procedure—To a solution of the corresponding $n$-alkylamine (1.00 g, 10 mmol) in anhydrous acetonitrile (15 mL) cooled at 0 °C was added dropwise benzyl bromoacetate (2.29 g, 10 mmol). Then, triethylamine (2.7 mL, 19.4 mmol) was added and the reaction mixture was stirred overnight. The solvent was evaporated and the residue was purified by column chromatography (silica gel) eluting with a mixture of hexane–EtOAc (19:1) to afford the corresponding benzyl esters 17–21 as colorless oils. Then, a solution of respective benzyl ester (8.0 mmol) in ethyl acetate (50 mL) in the presence of palladium on charcoal (50 mg) was treated with hydrogen at 3 atm in a Parr apparatus. The reaction mixture was shaken for 6 h and the mixture was filtered through a fritted glass funnel. The solvent was evaporated to yield the corresponding free 2-(n-alkylamino) acetic acids that were used in the next step without further purification.

1-[(n-Alkylamo)ethyl]-1-hydroxy-1,1-bisphosphonic acids

General Procedure—To a flame dried 100 mL three neck flask having an addition funnel and a reflux condenser through which water at 0 °C was circulated the corresponding 2-(n-alkylamino) acetic acid (2.9 mmol), $\text{H}_3\text{PO}_3$ acid (273 mg, 2.9 mmol), and anhydrous benzenesulfonic acid (1.0 g, 6.3 mmol) under argon atmosphere. The reaction mixture was heated to 65 ºC, then PCl$_3$ (500 $\mu$L, 5.8 mmol) was added dropwise with vigorous stirring. The reaction was stirred at 65 ºC for 16 h, and then allowed to cool to room temperature. Then, cold water (60 mL) was added and the reaction was stirred at 100 ºC for an additional 5 h. The reaction was cooled to room temperature and the pH was adjust to 4.3 with a 50% aqueous NaOH solution. Acetone (20 mL) was added, and the resulting mixture was cooled to 0 ºC for 24 h. The product was filtrated and crystallized from water–ethanol.

2-(n-Pentylamino)acetic acid (22): White solid; 46% yield; mp 185–190 ºC (desc.); $^1$H NMR (500.13 MHz, CD$_3$OD) $\delta$ 0.94 (t, $J=6.8$ Hz, 3H), 1.37 (m, 4H), 1.68 (p, $J=7.5$ Hz, 2H), 2.97 (dist t, $J=7.9$ Hz, 2H), 3.47 (s, 2H); $^{13}$C NMR (125.77 MHz, CD$_3$OD) $\delta$ 14.1, 27.2, 31.3, 61.4, 71.4.
23.2, 26.9, 29.7, 48.7, 50.6, 170.9. HRMS (ESI) calcd for C$_7$H$_{10}$O$_2$N$_2$Na [M+Na]$^+$ 168.1000; found: 168.1010.

1-[(n-Pentylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (27): White solid; 31% yield; mp 186–190 °C; $^1$H NMR (500.13 MHz, D$_2$O) $\delta$ 0.79 (t, J = 7.1 Hz, 3H), 1.26 (m, 4H), 1.62 (p, J = 7.4 Hz, 2H), 3.02 (t, J = 7.5 Hz, 2H), 3.40 (t, J = 11.7 Hz, 2H); $^{13}$C NMR (125.77 MHz, D$_2$O) $\delta$ 13.0, 21.4, 25.0, 27.7, 48.2, 49.9, 70.3 (t, J = 137.5 Hz); $^{31}$P NMR (202.46 MHz, D$_2$O) $\delta$ 14.82. HRMS (ESI) calcd for C$_7$H$_{10}$O$_2$NP$_2$Na [M+Na]$^+$ 314.0534; found: 314.0527.

2-(Hexylamino)acetic acid (23): White solid; 91% yield; mp 196–199 °C (desc.); $^1$H NMR (500.13 MHz, CD$_3$OD) $\delta$ 0.92 (t, J = 6.9 Hz, 3H), 1.35 (m, 4H), 1.39 (p, J = 7.8 Hz, 2H), 1.67 (p, J = 7.6 Hz, 2H), 2.97 (dist t, J = 7.9 Hz, 2H), 3.46 (s, 2H); $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 14.3, 23.5, 27.2, 27.3, 32.4, 48.7, 50.6, 170.9. HRMS (ESI) calcd for C$_8$H$_{21}$O$_2$N $[M+H]^+$ 160.1338; found: 160.1343.

1-[(n-Hexylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (28): White solid; 38% yield; mp 189–190 °C (desc.); $^1$H NMR (500.13 MHz, D$_2$O) $\delta$ 0.77 (t, J = 7.2 Hz, 3H), 1.25 (m, 4H), 1.29 (p, J = 6.6 Hz, 2H), 1.61 (p, J = 7.5 Hz, 2H), 3.02 (t, J = 7.5 Hz, 2H), 3.39 (t, J = 11.6 Hz, 2H); $^{13}$C NMR (125.77 MHz, D$_2$O) $\delta$ 13.2, 21.6, 25.2, 25.3, 30.4, 48.2, 50.0, 70.4 (t, J = 138.4 Hz); $^{31}$P NMR (202.46 MHz, D$_2$O) $\delta$ 14.69. HRMS (ESI) calcd for C$_8$H$_{21}$O$_2$NP$_2$Na [M+Na]$^+$ 328.0691; found: 328.0684.

2-(n-Heptylamino)acetic acid (24): White solid; 67% yield; mp 187–191 °C; $^1$H NMR (500.13 MHz, CD$_3$OD) $\delta$ 0.91 (t, J = 7.0 Hz, 3H), 1.32 (m, 4H), 1.37 (m, 4H), 1.67 (p, J = 7.5 Hz, 2H), 2.97 (m, 2H), 3.46 (s, 2H); $^{13}$C NMR (125.77 MHz, D$_2$O) $\delta$ 13.6, 22.2, 28.5, 28.7, 29.4, 31.3, 47.4, 49.2, 169.5. HRMS (ESI) calcd for C$_9$H$_{23}$O$_2$N $[M+H]^+$ 174.1494; found: 174.1510. Anal. Calcd. for (C$_9$H$_{19}$O$_2$N): C, 62.39; H, 11.05; N, 8.08. Found C, 62.05; H, 10.62; N, 7.74.

1-[(n-Heptylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (29): White solid; 10% yield; mp 155–159 °C; $^1$H NMR (500.13 MHz, CDCl$_3$) $\delta$ 0.90 (t, J = 7.0 Hz, 3H), 1.18 (m, 6H), 1.24 (m, 2H), 1.60 (p, J = 7.4 Hz, 2H), 3.01 (t, J = 7.6 Hz, 2H), 3.39 (t, J = 11.7 Hz, 2H); $^{13}$C NMR (125.77 MHz, D$_2$O) $\delta$ 13.3, 21.8, 25.3, 25.5, 27.8, 30.7, 48.3, 49.9, 70.3 (t, J = 137.7 Hz); $^{31}$P NMR (D$_2$O) $\delta$ 15.31. HRMS (ESI) calcd for C$_9$H$_{23}$O$_2$NP$_2$ [M+H]$^+$ 320.1030; found: 320.1037. Anal. Calcd. for (C$_9$H$_{23}$O$_2$NP$_2$1.5H$_2$O): C, 31.22; H, 7.57; N, 4.05. Found C, 31.53; H, 7.75; N, 4.36.

2-(Octylamino)acetic acid (25): White solid; 96% yield; $^1$H NMR (500.13 MHz, CD$_3$OD) $\delta$ 0.90 (t, J = 6.8 Hz, 3H), 1.33 (m, 10H), 1.68 (p, J = 7.5 Hz, 2H), 2.97 (dist t, J = 7.9 Hz, 2H), 3.46 (s, 2H); $^{13}$C NMR (125.77 MHz, CD$_3$OD) $\delta$ 14.4, 23.7, 27.2, 27.6, 30.17, 30.19, 32.9, 48.7, 50.6, 170.9. HRMS (ESI) calcd for C$_{10}$H$_{22}$O$_2$N $[M+H]^+$ 188.1651; found: 188.1669.

1-[(n-Octylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (30): White solid; 43% yield; $^1$H NMR (500.13 MHz, DMSO-d$_6$) $\delta$ 0.85 (t, J = 6.9 Hz, 3H), 1.25 (m, 10H), 1.54 (p, J = 7.1 Hz, 2H), 2.90 (dist t, J = 7.1 Hz, 2H), 3.22 (t, J = 10.7 Hz, 2H); $^{13}$C NMR (125.77 MHz, DMSO-d$_6$) $\delta$ 13.9, 22.1, 25.6, 25.9, 28.46, 28.48, 31.1, 47.4, 49.8, 69.4 (t, J = 132.3 Hz); $^{31}$P NMR (DMSO-d$_6$) $\delta$ 14.85. HRMS (ESI) calcd for C$_{10}$H$_{25}$O$_2$NP$_2$Na [M+Na]$^+$ 356.1004; found: 356.0994.

Bioorg Med Chem. Author manuscript; available in PMC 2015 January 01.
2-(Decylamino)acetic acid (26): White solid; mp 184–187 °C; $^1$H NMR (500.13 MHz, CD$_3$OD) $\delta$ 0.89 (t, $J = 6.9$ Hz, 3H), 1.24 (m, 14H), 1.67 (p, $J = 7.5$ Hz, 2H), 2.97 (dist t, $J = 8.9$ Hz, 2H), 3.46 (s, 2H); $^{13}$C NMR (125.77 MHz, CD$_3$OD) $\delta$ 14.4, 23.7, 27.2, 27.6, 30.2, 30.4, 30.5, 30.6, 33.0, 48.7, 50.6, 170.9. HRMS (ESI) calcd for C$_{12}$H$_{26}$O$_2$N $[M+H]^+$ 216.1964; found: 216.1995.

1-[((n-Decylamino)ethyl]-1-hydroxy-1,1-bisphosphonic Acid (31): White solid; mp 188–190 °C; $^1$H NMR (500.13 MHz, DMSO-$_d_6$) $\delta$ 0.84 (t, $J = 6.7$ Hz, 3H), 1.25 (m, 14H), 1.54 (m, 2H), 2.90 (dist. t, $J = 7.0$ Hz, 2H), 3.22 (t, $J = 11.1$ Hz, 2H); $^{13}$C NMR (125.77 MHz, DMSO-$_d_6$) $\delta$ 13.9, 22.1, 25.6, 25.9, 28.5, 28.7, 28.8, 28.9, 31.3, 47.3, 50.0, 69.2 (t, $J = 130.0$ Hz); $^{31}$P NMR (DMSO-$_d_6$) $\delta$ 15.19. HRMS (ESI) calcd for C$_{12}$H$_{30}$O$_7$NP$_2$ $[M+H]^+$ 362.1498; found: 362.1493.

Drug Screening

*T. cruzi* amastigote assays—Gamma-irradiated (2,000 Rads) Vero cells (3.4 × 10$^4$ cells/well) were seeded in 96 well plates (black, clear bottom plates from Greiner Bio-One) in 100 μL RPMI media (Sigma) with 10% FBS. Plates were incubated overnight at 35 °C and 7% CO$_2$. After overnight incubation, Vero cells were challenged with 3.4 × 10$^5$ trypomastigotes/well (CL strain overexpressing a tdTomato red fluorescent protein) in 50 μL volume and incubated for 5 h at 35 °C and 7% CO$_2$. After infection, cells were washed once with Hanks solution (150 μL/well) to eliminate any extracellular parasites and compounds were added in serial dilutions in RPMI media in 150 μL volumes. Each dilution was tested in quadruplicate. Each plate also contained controls with host cells and no parasites (for background check), and controls with parasites and no drugs (positive control). Drugs were tested on *T. cruzi* at 1.56 μM, 3.125 μM, 6.25 μM, 12.5 μM, 25 μM. For each set of experiments, benznidazole was also used as a positive control 0.39 μM, 0.78 μM, 1.56 μM, 3.125 μM, and 6.25 μM. After drug addition, plates were incubated at 35 °C and 7% CO$_2$. At day 3 post-infection, plates were assayed for fluorescence. $^{57}$IC$_{50}$ values were determined by non-linear regression analysis using SigmaPlot. There was no evident cytotoxicity on the host cells (visual assay) with any of the drugs tested at concentrations as high as 25 μM.

*T. gondii* tachyzoites assays—Experiments on *T. gondii* tachyzoites were carried out as described previously$^{58}$ using *T. gondii* tachyzoites expressing red fluorescent protein.$^{59}$ Cells were routinely maintained in hTerT cells grown in High Glucose Dulbecco’s modified Eagle’s medium (DMEM-HG) supplemented with 1% fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, at 37 °C in a humid 5% CO$_2$ atmosphere. Confluent monolayers grown in 96-well black plates with optical bottoms (black, clear bottom plates from Greiner Bio-One) were used and drugs dissolved in the same medium and serially diluted in the plates. Freshly isolated tachyzoites were filtered through a 3 μm filter and passed through a 22 gauge needle, before use. The cultures were inoculated with 10$^4$ tachyzoites/well in the same media. The plates were incubated at 37 °C and read daily in a Molecular Devices fluorescence plate reader. To preserve sterility the plates were read with covered lids, and both excitation (510 nm) and emission (540 nm) were read from the bottom.$^{59}$ For the calculation of the EC$_{50}$, the percent of growth inhibition was plotted as a function of drug concentration by fitting the values to the function: $I = I_{max} C / (EC_{50} + C)$, where $I$ is the percent inhibition, $I_{max} = 100\%$ inhibition, C is the concentration of the inhibitor, and EC$_{50}$ is the concentration for 50% growth inhibition. There was no evident cytotoxicity on the host cells with any of the drugs tested (visual assay).
Computational methods

All the DFT calculations were performed using the Gaussian09 program, the B3LYP functional and the 6-311+G(d,p) basis set. Calculations were carried out with full geometry optimization, using standard termination conditions, and including in all cases the effect of the solvent (water) through the Tomasi’s polarized continuum model (PCM) as implemented in Gaussian09. Several different input geometries were used in order to find the most important conformers; the Mg atom always appeared coordinated to two oxygen atoms, and the hydrogen bonding usually drove the energy minimization processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the National Research Council of Argentina (PIP 1888 to J.B.R. and 55910 to C.A.S.), ANPCyT (PICT 2008 #1690 to J.B.R.), and the Universidad de Buenos Aires (20020100103801 to J.B.R. and W-759 to C.A.S.), the Bunge & Born Foundation to S.H.S, and the U.S. National Institutes of Health to R.D. (AI-082542) and S.N.J.M. (AI-102254).

References

1. Sun S, McKenna CE. Expert Opin Ther Patents. 2011; 21:1433–1451.
2. Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R. J Biol Chem. 2001; 276:33930–33937. [PubMed: 11435429]
3. Montalvetti A, Fernandez A, Sanders JM, Ghosh S, Van Brussel E, Oldfield E, Docampo R. J Biol Chem. 2003; 278:17075–17083. [PubMed: 12618430]
4. Moreno SNJ, Li Z-H. Expert Opin Ther Targets. 2008; 12:253–263. [PubMed: 18269336]
5. Ling Y, Li Z-H, Miranda K, Oldfield E, Moreno SN. J Biol Chem. 2007; 282:30804–30816. [PubMed: 17724033]
6. Li ZH, Cintrón R, Koon NA, Moreno SNJ. Biochemistry. 2012; 51:7533–7540. [PubMed: 22931372]
7. Blanchard L, Karst F. Gene. 1993; 125:185–189. [PubMed: 8096487]
8. Song L, Poulter CD. Proc Natl Acad Sci USA. 1994; 91:3044–3048. [PubMed: 8159703]
9. Ogura K, Koyama T. Chem Rev. 1998; 98:1263–1276. [PubMed: 11848932]
10. Rodan GA. Annu Rev Pharmacol Toxicol. 1998; 38:375–88. [PubMed: 9597160]
11. Docampo R, Moreno SNJ. Cell Calcium. 2011; 50:113–119. [PubMed: 21752464]
12. Urbina JA, Moreno B, Vierkotter S, Oldfield E, Payares G, Sanoja C, Bailey BN, Yan W, Scott DA, Moreno SNJ, Docampo R. J Biol Chem. 1999; 274:33609–33615. [PubMed: 10559249]
13. Martin MB, Grimley JS, Lewis JC, Heath HT III, Bailey BN, Kendrick H, Yardley V, Caldera A, Lira R, Urbina JA, Moreno SN, Docampo R, Croft SL, Oldfield E. J Med Chem. 2001; 44:909–916. [PubMed: 11300872]
14. Yardley V, Khan AA, Martin MB, Slifer TR, Araujo FG, Moreno SNJ, Docampo R, Croft SL, Oldfield E. Antimicrob Agents Chemother. 2002; 46:929–931. [PubMed: 11850291]
15. Martin MB, Sanders JM, Kendrick H, de Luca-Fradley K, Lewis JC, Grimley JS, Van Brussel EM, Olsen JR, Meints GA, Burzynska A, Kafarski P, Croft SL, Oldfield E. J Med Chem. 2002; 45:2904–2914. [PubMed: 12086478]
16. Rodriguez JB, Szajnman SH. Expert Opinion Ther Patents. 2012; 22:311–334.
17. Garzoni LR, Waghabi MC, Baptista MM, De Castro SL, de Meirelles MN, Britto CC, Docampo R, Oldfield E, Urbina JA. Int J Antimicrob Agents. 2004; 23:286–290. [PubMed: 15164970]
18. Bouzahzah B, Jelicks LA, Morris SA, Weiss LM, Tanowitz HB. Parasitol Res. 2005; 96:184–187. [PubMed: 15844009]
50. Zuliani V, Carmi C, Rivara M, Fantini M, Lodola A, Vacondio F, Bordi F, Plazzi PV, Cavazzoni A, Galetti M, Alfieri RR, Petronini PG, Mor M. Eur J Med Chem. 2009; 44:3471–3479. [PubMed: 19268405]

51. Kruijtzer JAW, Liskamp RMJ. Tetrahedron Lett. 1995;6969–6972.

52. Kieczykowski GR, Jobson RB, Melillo DG, Reinhold DF, Grenda VJ, Shinkai I. J Org Chem. 1995: 60:8310–8312.

53. Demoro B, Caruso F, Rossi M, Benitez D, Gonzalez M, Cerecetto H, Parajón-Costa B, Castiglioni J, Galizzi M, Docampo R, Otero L, Gambino D. J Inorg Biochem. 2010; 104:1252–1258. [PubMed: 20817265]

54. Pan Z, Chen J, Su G, Geng YZ, Zheng H, Ji Q. J Chem Phys. 2012; 136:164313. [PubMed: 22559488]

55. Swiatla-Wojcik D. Chem Phys. 2007; 342:260.

56. Delain-Biiton L, Turner A, Lejeune N, Villemin D, Hix GB, Jaffrès P-A. Tetrahedron. 2005; 61:6602–6609.

57. Canavaci AM, Bustamante JM, Padilla AM, Perez Brandon CM, Simpson LJ, Xu D, Boehlke CL, Tarleton RL. PLOS Negl Trop Dis. 2010; 4:e740. [PubMed: 20644616]

58. Gubbels MJ, Li C, Striepen B. Antimicrob Agents Chemother. 2003; 43:309–316. [PubMed: 12499207]

59. Agrawal S, van Dooren GG, Beatty WL, Striepen B. J Biol Chem. 2009; 284:33683–33691. [PubMed: 19808683]

60. Frisch, MJ.; Trucks, GW.; Schlegel, HB.; Scuseria, GE.; Robb, MA.; Cheeseman, JR.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, GA.; Nakatsuji, H.; Caricato, M.; Li, X.; Hratchian, HP.; Izmaylov, AF.; Bloino, J.; Zheng, G.; Sonnenberg, JL.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Vreven, T.; Montgomery, JA., Jr; Peralta, JE.; Ogliaro, F.; Bearpark, M.; Heyd, JJ.; Brothers, E.; Kudin, KN.; Staroverov, VN.; Kobayashi, R.; Normand, J.; Raghavachari, K.; Rendell, A.; Burant, JC.; Iyengar, SS.; Tomasi, J.; Cossi, M.; Rega, N.; Millam, JM.; Klene, M.; Knox, JE.; Cross, JB.; Bakken, V.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, RE.; Yazyev, O.; Austin, AJ.; Cammi, R.; Pomelli, C.; Ochterski, JW.; Martin, RL.; Morokuma, K.; Zakrzewski, VG.; Voth, GA.; Salvador, P.; Dannenberg, JJ.; Dapprich, S.;Daniels, AD.; Farkas, Ö.; Foresman, JB.; Ortiz, JV.; Cioslowski, J.; Fox, DJ. Gaussian 09, Revision C.01. Gaussian, Inc; Wallingford CT: 2009.

61. (a) Lee C, Yang W, Parr RG. Phys Rev B. 1988; 37:785–789. (b) Becke AD. J Chem Phys. 1993; 98:5648–5652.

62. Tomasi J, Mennucci B, Cammi R. Chem Rev. 2005; 105:2999–3093. [PubMed: 16092826]
Figure 1.
Chemical structures of representative FDA-approved bisphosphonates clinically employed for different bone disorders.
Figure 2.
Chemical structures of representative members of bisphosphonic acids derived from fatty acids.
Figure 3.
Simplified models of 12–14 (12a) and 27–31 (27a) to carry out molecular modeling studies.
Figure 4.
Most stable geometries of simple models 12a (a) and 27a (b). Hydrogen bonds were labelled as strong (s) when $d_{\text{Acceptor-H}} < 2$ Å and $\theta_{\text{donor-H-Acceptor}} > 145^\circ$, or as weak (w) if they do not meet both of these criteria.
Figure 5.
Chemical structures of compound 32, exhibiting a typical six-membered hydrogen bond (see ref. 56), and its isosteric analogue 33.
Scheme 1.
Isoprenoid biosynthesis in trypanosomatids.
Scheme 2.
Isoprenoid biosynthesis in Apicomplexan parasites.
Scheme 3.
Synthetic approach for the preparation of modified alkylaminoethyl bisphosphonates.
Table 1

Biological activity of 2-alkylaminoethyl-1-hydroxy-1,1-bisphosphonic acids against TcFPPS, TgFPPS, T. cruzi (amastigotes), and tachyzoites of T. gondii.

| Compound | TcFPPS IC\textsubscript{50} (μM) | ED\textsubscript{50} T. cruzi amastigotes (μM) | TgFPPS IC\textsubscript{50} (μM) | ED\textsubscript{50} T. gondii tachyzoites (μM) |
|----------|-------------------------------|-----------------------------------------------|-------------------------------|-----------------------------------------------|
| 27       | > 10                          | > 20                                          | 0.067 ± 0.064                 | > 10                                          |
| 28       | > 10                          | > 20                                          | 0.051 ± 0.006                 | 4.68 ± 1.19                                   |
| 29       | > 10                          | > 20                                          | > 1.0                         | 0.00% at 10 μM                                |
| 30       | > 10                          | 46% at 10 μM cytotoxic at 10 μM               | 0.039 ± 0.033                 | 2.00 ± 0.95                                   |
| 31       | > 10                          | 42% at 10 μM                                 | 0.125 ± 0.023                 | 14.9% at 10 μM                                |
| Benzimidazole | 1.44 ± 0.97                   |                                               |                               |                                               |
| Risedronate | 0.027 ± 0.01\textsuperscript{53} | 55.0 ± 5.0\textsuperscript{53}               | 0.074 ± 0.017\textsuperscript{5} | 2.4 ± 0.7\textsuperscript{24}                 |

Bioorg Med Chem. Author manuscript; available in PMC 2015 January 01.
Table 2

Main conformers\(^a\) of \(12a\) and \(27a\), calculated by B3LYP/6-311+G(d,p) with PCM in water: energies and geometrical features of the hydrogen bonds.

| Conformer | Energy (kcal/mol) | Carbon chain\(^b\) | Hydrogen bonds Atoms involved | \(d_{\text{H-Acc}}\) (Å) | \(\theta_{\text{Don-H-Acc}}\) (°) |
|-----------|------------------|--------------------|-------------------------------|----------------|-----------------|
| 12a       |                  |                    |                               |                 |                 |
| 1         | 0.00             | equat.             | H(PO\text{ax}) -O\text{ax}(P') | 1.82            | 154             |
|           |                   |                    | H(PO\text{eq}) -N             | 1.68            | 157             |
| 2         | 0.59             | axial              | H(PO\text{ax}) -O\text{ax}(P') | 1.89            | 154             |
|           |                   |                    | H(PO\text{eq}) -N             | 1.73            | 155             |
| 3         | 6.50             | equat.             | H(PO\text{ax}) -O\text{ax}(P') | 1.86            | 154             |
|           |                   |                    | H(N)-O\text{eq}(P)           | 2.25            | 139             |
| 4         | 8.49             | equat.             | H(N)-O\text{eq}(P)           | 2.19            | 139             |
| 27a       |                  |                    |                               |                 |                 |
| 1         | 0.00             | equat.             | H(PO\text{ax}) -O\text{ax}(P') | 1.62            | 161             |
|           |                   |                    | H(PO\text{eq}) -N             | 2.01            | 124             |
|           |                   |                    | H(N)-O\text{eq}(P)           | 2.24            | 134             |
| 2         | 1.77             | equat.             | H(PO\text{ax}) -O\text{eq}(P) | 1.91            | 135             |
|           |                   |                    | H(PO\text{eq}) -N             | 1.78            | 153             |
| 3         | 2.17             | axial              | H(PO\text{ax}) -O\text{eq}(P) | 1.95            | 133             |
|           |                   |                    | H(PO\text{eq}) -N             | 1.67            | 157             |
| 4         | 3.75             | equat.             | H(PO\text{ax}) -O\text{ax}(P') | 1.62            | 162             |
|           |                   |                    | H(PO\text{eq}) -O\text{eq}(P) | 2.82            | 108             |
|           |                   |                    | H(PO\text{eq}) -O\text{eq}(P) | 2.14            | 142             |
| 5         | 4.86             | axial              | H(PO\text{ax}) -O\text{ax}(P') | 1.59            | 160             |
|           |                   |                    | H(PO\text{eq}) -O\text{eq}(P) | 2.25            | 127             |

\(^a\) For each of the nine H-bond/carbon chain arrangements, the conformer with the orientation of the \(N\)-methyl group with lower energy is shown.

\(^b\) The designation of substituents as axial or equatorial was made considering the presence of a six-membered cycle formed by the atoms C-1–P-1–O–Mg–O–P-2.