2-Alkylaminoethyl-1,1-Bisphosphonic Acids are Potent Inhibitors of the Enzymatic Activity of Trypanosoma cruzi Squalene Synthase

Carlos A. Rodríguez-Poveda,a Dolores González-Pacanowska,a Sergio H. Szajnman,b and Juan B. Rodríguezb

*a Instituto de Parasitología y Biomedicina López-Neyra, Consejo Superior de Investigaciones Científicas, Parque Tecnológico de Ciencias de la Salud, Avenida del Conocimiento, s/n, 18100 Armilla, Granada, Spain, and b Departamento de Química Orgánica and UMYMFOR (CONICET–FCEyN), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EHA, Buenos Aires, Argentina

Abstract As part of our efforts aimed at searching for new antiparasitic agents, the effect of representative 2-alkylaminoethyl-1,1-bisphosphonic acids on Trypanosoma cruzi squalene synthase (TcSQS) was investigated. These compounds had proven to be potent inhibitors against TcSQS. This cellular activity had been associated with an inhibition of the enzymatic activity of T. cruzi farnesyl diphasphate synthase. 2-Alkylaminoethyl-1,1-bisphosphonic acids appear to have a dual action since they also inhibit TcSQS at the nanomolar range.
Inhibitors of squalene synthase (SQS) have great potential not only as cholesterol lowering agents (23), but also as antiparasitic drugs (12,37). In trypanosomatids the enzyme catalyzes the first committed step in isoprenoid biosynthesis that leads to ergosterol instead of cholesterol as occurs in mammals (37,38). The reaction is a reductive coupling of two molecules of \((E,E)-farnesyl pyrophosphate\) \((1, \text{FPP})\) that takes place in two steps via the formation of a cyclopropane intermediate \((2)\) followed by a reductive rearrangement that gives rise to squalene \((3)\) as illustrated in FIG. 1. FPP is a branching point in isoprenoid biosynthesis. It can either be transformed into squalene or converted into other essential isoprenoids such as dolichol, coenzyme Q or prenylated proteins (21).

_Trypanosoma cruzi_ is the etiologic agent of American trypanosomiasis (Chagas disease) (40) and exhibits a complex life cycle involving blood-sucking Reduvid bugs and mammals (1). It multiplies in the insect gut as an epimastigote form and is spread as a non-dividing metacyclic trypomastigote from the insect feces by contamination of intact mucosa or wounds produced by the blood-sucking activity of the vector. In the mammalian host, the parasite proliferates intracellularly as the amastigote form that is released into the blood stream as a non-dividing trypomastigote (1). Distribution of Chagas disease could also take place via the placenta or by transfusion of infected blood (11,15).

Bisphosphonic acids \((4)\) are metabolically stable pyrophosphate \((5)\) analogues in which a methylene group replaces the oxygen atom bridge between the two phosphorus atoms of the pyrophosphate unit. Substitution at the bridge has produced a large number of compounds (27). Bisphosphonates such as pamidronate (6), alendronate (7), risendronate (8), and ibandronate (9) are in clinical use for the treatment different bone disorders (FIG. 2) (24,52,30). Bisphosphonic acids became relevant drugs since calcification studies done close to 40 years ago (8,9,10).

Besides their pharmacological properties on bone, aminobisphosphonic acids had proven to be potent inhibitors of _T. cruzi_ proliferation without toxicity to the host cells (20). Moreover, numerous bisphosphonic acids have shown to be effective growth inhibitors of parasites other than _T. cruzi_, such as _T. brucei rhodesiense_, _Leishmania spp_ and apicomplexan parasites such as _Toxoplasma gondii_ and _Plasmodium falciparum_ (17,22,29,32–36). As the acidocalcisomes are equivalent in composition to the bone mineral, gathering of bisphosphonic acids in these organelles facilitates their antiparasitic action (39). The mechanism of action of aminobisphosphonic acids has
been narrowed down to protein prenylation (26). Farnesyl pyrophosphate synthase (FPPS) constitutes the main target of bisphosphonic acids (3,6,13,14,28). FPPS catalyzes the two mandatory biosynthetic steps to form farnesyl pyrophosphate from dimethylallyl pyrophosphate. Inhibition of the enzymatic activity of FPPS blocks farnesyl pyrophosphate and geranylgeranyl pyrophosphate formation, which are required for the post-translational prenylation of small GTP-binding proteins within osteoclasts (4).

Of special interest are 1,1-bisphosphonic acids derived from fatty acids, particularly the 2-alkylaminoethyl-1,1-bisphosphonic acids derivatives, which were shown to be potent growth inhibitors of amastigotes T. cruzi, which is the clinically more relevant form of the parasite, exhibiting IC_{50} values at the nanomolar range (29,33). This class of bisphosphonic acids has proven to be more efficient than the parent drugs 1-hydroxy-, 1-amino-, and 1-alkyl-1,1-bisphosphonic acids as antiparasitic agents (33). Compound 12 arises as the main member of this class of bisphosphonic acids (15,29,32–36), with an IC_{50} value of 0.84 μM (33). In initial studies, this cellular activity had been exclusively associated with the inhibition of the enzymatic activity of TcFPPS (5), being a competitive inhibitor (35) with an IC_{50} value of 0.49 μM (33). 12 was also effective towards the enzymatic activity of T. gondii FPPS (IC_{50} = 0.14 μM) (33), and exhibited in vitro inhibitory action against tachyzoites of T. gondii (IC_{50} = 9.37 μM) (33) (FIG. 3).

It is worthy pointing out that 12 also has exhibited a modest inhibitory action (IC_{50} = 1.35 μM) against an important prenyltransferase in T. cruzi, a solanesyl diphosphate synthase (TcSPPS), which is involved in the synthesis of ubiquinone (7). This enzyme has been considered as another potential target for chemotherapy (7).

Certain bisphosphonate derivatives have in addition been reported to be potent inhibitors of mammalian squalene synthase. Such is the case of the isoprenoid derivatives 18–24 (18,19) and the closely structurally related compounds 25–27 (16) (FIG. 4). We therefore reasoned that, in a similar fashion, this could be the case of some of the compounds previously reported to be potent inhibitors of T. cruzi proliferation (compounds 10–17), which were straightforwardly prepared according to published procedures (33). Hence, here we tested a selection of bisphosphonic acids against recombinant TcSQS.
Truncated soluble *T. cruzi* SQS enzyme was expressed and purified as previously described (31). *Tc*SQS activity was based on measuring the conversion of $[^3H]FPP$ to $[^3H]squalene$. Final assay concentrations were 50 mM morpholinepropanesulfonic acid–NaOH (pH 7.4), 20 mM MgCl$_2$, 5 mM CHAPS ((cholamidopropyl)-dimethylammonio)-1-propanesulfonate), 1% Tween 80, 10 mM dithiothreitol, 0.025 mg/mL bovine serum albumin, 0.25 mM NADPH, and mg of purified recombinant *T. cruzi* SQS. The reaction was started with the addition of substrate ($[^3H]FPP$, 0.1 nmol, $2.22 \times 10^4$ dpm) and the final volume of the reaction was 200 μL. After incubation at 37 ºC for 5 min, 40 μL of 10 M NaOH were added to stop the reaction, followed by 10 μL of a (100:1) mixture of 98% EtOH and squalene. The resulting mixtures were mixed vigorously by vortexing, then 10 μL aliquots were applied to $2.5 \times 10^2$ cm channels of a silica gel thin layer chromatogram, and newly formed squalene was separated from unreacted substrate by chromatography in toluene–EtOAc (9:1). The region of the squalene band was scraped and immersed in Hydrofluor liquid scintillation fluid, and assayed for radioactivity. IC$_{50}$ values were calculated from the hyperbolic plot of percent of inhibition versus inhibitor concentration, using Sigma Plot® (31).

Biological evaluation of 2-(alkylamino)ethyl-1,1-bisphosphonic acids indicated that these compounds are potent inhibitors of the enzymatic activity of *T. cruzi* SQS. Particularly, compounds 11–13 arose as the most efficient members of this type of compounds. Interestingly, compound 11 exhibited an IC$_{50}$ value of 5.0 nM against *Tc*SQS and had previously shown a potent action as growth inhibitor of amastigotes of *T. cruzi* with an IC$_{50}$ value of 0.54 μM (33). However, compound 11 exhibited only a moderate inhibitory action towards *Tc*FPPS (IC$_{50}$ = 1.84 μM) (33). Compound 12 was another example of bisphosphonate that had previously exhibited potent activity against intracellular amastigotes (IC$_{50}$ = 0.84 μM) (33). Indeed this cellular activity had been attributed to inhibition of the enzymatic activity of *Tc*FPPS (IC$_{50}$ = 0.49 μM) (33) yet now we show that it is also an effective inhibitor of *Tc*SQS with an IC$_{50}$ value of 21 nM. Compound 13 was also a potent inhibitor of *Tc*SQS (IC$_{50}$ = 12 nM) while its cellular activity against *T. cruzi* amastigotes was moderate (IC$_{50}$ = 10.0 μM) (33). Thus, with the exception of compounds 15 and 16, all the tested compounds were potent inhibitors of *Tc*SQS with IC$_{50}$ in the low nanomolar range (TABLE 1). These results suggest that the primary target for some of these compounds may be *Tc*SQS instead of *Tc*FPPS as...
initially considered. This would imply that the interruption of carbon flow towards sterol intermediates is a major mechanism of action within the parasite. Work aimed at designing optimized bisphosphonate molecules targeting either \(Tc\)SQS or \(Tc\)FPPS is currently being pursued in our laboratory.

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REFERENCES


squalene synthase inhibitors: Chiral recognition in the interactions of an α-


FIGURE 1. Squalene formation catalyzed by squalene synthase (SQS).

FIGURE 2. General formula and chemical structure of representative FDA-approved bisphosphonic acids clinically employed for different bone disorders.

FIGURE 3. Representative members of 1-[(alkylamino)ethyl]-1,1-bisphosphonic acids.
FIGURE 4. Chemical structure of selected inhibitors of SQS activity bearing a bisphosphonate moiety.
<table>
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC$_{50}$ (nM)</th>
<th>IC$_{50}$ μM TcFPPS$^\dagger$</th>
<th>IC$_{50}$ μM (amastigotes)$^\dagger$</th>
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$^\dagger$ Data taken from reference (33).