1. In Vitro and In Vivo Activity of Sulfur-Containing Linear Bisphosphonates against Apicomplexan Parasites

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ABSTRACT

We tested a series of sulfur-containing linear bisphosphonates against *Toxoplasma gondii*, the etiologic agent of toxoplasmosis. The most potent compound (**22**, 1-[(*n*-decylsulfonyl)ethyl]-1,1-biphosphonic acid) is a sulfone-containing compound, which had an EC_{50} of 0.11 ± 0.02 µM against intracellular tachyzoites. The compound showed low toxicity when tested in tissue culture with a selectivity index of >2,000. **22** also showed high activity *in vivo* in a toxoplasmosis mouse model. The compound inhibited the *Toxoplasma* farnesyl diphosphate synthase (*Tg*FPPS) but the concentration needed to inhibit 50% of the enzymatic activity (IC_{50}) was higher than the concentration that inhibited 50% of growth. We tested **22** against two other Apicomplexan parasites, *Plasmodium falciparum* (EC_{50} of 0.6 ± 0.01 µM), the agent of malaria, and *Cryptosporidium parvum* (EC_{50} of ~65 µM), the agent of cryptosporidiosis. Our results suggest that **22** is an excellent novel compound that could lead to the development of potent agents against Apicomplexan parasites.

INTRODUCTION

Human infections with *Toxoplasma gondii* are usually asymptomatic but this protozoan parasite is a major opportunistic pathogen of immune-deficient people, for example patients with AIDS (1) or patients immune-suppressed after organ transplantation or cancer chemotherapy (2). Infection of the fetus during pregnancy can cause severe disease (3), and severe ocular disease can also occur in immune-competent patients (4). Current drugs used against toxoplasmosis can produce toxic side effects, do not adequately reach the central nervous system, or are very expensive (5). There is an urgent need for safe and effective treatments for toxoplasmosis (6).

Cryptosporidium spp. can cause severe diarrheal disease in humans (7). The clinical disease can be debilitating, and life threatening in malnourished children and immune-compromised individuals. There is only one drug approved for treatment, nitazoxanide, which has modest efficacy and provides no benefit for AIDS patients infected with *Cryptosporidium*. Malaria is a parasitic disease caused by Apicomplexan parasites of the genus *Plasmodium*. These parasites infects nearly 250 million people and kills about 450,000 people every year (8). The emergence of drug resistant malaria parasites highlights the need for new treatments. The need for safe and effective treatments for toxoplasmosis, cryptosporidiosis and malaria is compelling.

Isoprenoids are essential compounds in all organisms due to their roles in a variety of biological processes and several enzymes of this pathway have been reported to be excellent molecular targets against pathogenic parasites (9). Despite their structural and functional diversity, all isoprenoids derive from common precursors: isopentenyl diphosphate (IPP), and its isomer, dimethylallyl diphosphate (DMAPP). In *T. gondii*, IPP and DMAPP are synthesized through the 1-deoxy-D-xylulose-5-phosphate (DOXP) pathway (10), which localizes to the apicoplast and is essential (11). To synthesize longer isoprenoids *T. gondii* possesses a bifunctional farnesyl diphosphate synthase/geranylgeranyl diphosphate synthase (FPPS/GGPPS; *Tg*FPPS) able to catalyze the formation of both FPP and GGPP (12, 13). In addition to its production of isoprenoids, *Toxoplasma* has the ability to salvage FPP and/or GGPP from the host, where they are produced through the mevalonate pathway (14).

Bisphosphonates are metabolically stable pyrophosphate analogues in which a methylene group replaces the oxygen atom bridge between the two phosphorus atoms of the pyrophosphate (Fig. 1). Substitution at the carbon atom 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 and prevention of osteoporosis, Paget's disease, hypercalcemia, tumor bone metastases, and other bone diseases (15, 16). Selective action on bone is based on the binding of the bisphosphonate to the bone mineral (17). Apart from their ability as inhibitors of bone resorption, bisphosphonates have also antibacterial (17), and anticancer activity (18), and stimulate γδ T cells (19). Interestingly, these compounds have also antiparasitic action (9, 20). Aminobisphosphonates such as pamidronate (**1**), alendronate (**2**), and risedronate (**3**) were first found to be effective in the inhibition of *Trypanosoma cruzi in vitro* and *in vivo* without toxicity to the host cells (Fig. 1) (21). The usefulness of these compounds was broadened by the finding that some bisphosphonates were also growth inhibitors of *T. gondii*, *T. brucei*, *Leishmania donovani* and *Plasmodium falciparum* (22). The primary molecular target of bisphosphonates is the farnesyl diphosphate synthase (FPPS) (23).

The *T. gondii* FPPS (TgFPPS) is potently inhibited by bisphosphonates (12). Our laboratory has found that linear bisphosphonates are more efficient antiparasitic agents than aminobisphosphonates (24-27). In some cases, the hydroxyl group at the C-1 position found in aminobisphophonates currently employed for the treatment of bone disorders and essential for bone binding (28), is absent. Compounds **4**–**7** were the first examples of linear 1-hydroxy-, 1-alkyl-, and 1-amino-1,1-bisphosphonates (Fig. 2), which were effective agents against trypanosomatids and Apicomplexan parasites targeting parasite FPPSs (24-27).

In previous work, we reported that linear sulfur-containing bisphosphonates have selective anti-*Toxoplasma* action (29). In this work, we report the synthesis of novel sulfone linear bisphosphonates and their *in vitro* and *in vivo* activity against *T. gondii*, *Plasmodium falciparum* and *Cryptosporidium parvum*. In addition, the compounds were tested against the isoprenoid enzymes, TgFPPS and HsFPPS.

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93 **MATERIALS AND METHODS**

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95 **Ethics Statement.** All animal care and therapy studies were carried out in strict accordance with
96 the NIH guidelines. The animal use protocol was reviewed and approved by the Institutional
97 Animal Care and Use Committee (IACUC) of the University of Georgia.

98 **Inhibitors.** The methyl(alkyl)sulfonium derivatives **13** and **14** were obtained starting from the
99 already described free bisphosphonic acids **11** and **12** (29) by treatment with methyl iodide and
100 silver tetrafluoroborate in acetronitrile to give the title compounds in good yields (30). Similarly,
101 sulfones **18**–**22** were prepared from the respective free acids **8**, **12, 15**–**17**[40] employing hydrogen
102 peroxide as the oxidizing agent as illustrated in Figure 2C. The glassware used in air- and/or
103 moisture-sensitive reactions was flame-dried and reactions were carried out under dry argon.
104 Unless otherwise noted, chemicals were commercially available and used without further
105 purification. Solvents were distilled before use. Dichloromethane was distilled from phosphorus
106 pentoxide. Nuclear magnetic resonance spectra were recorded with a Bruker AM-500 MHz
107 spectrometer. The ^{1}H NMR spectra are referenced with respect to the residual $CHCl_3$ proton of
108 the solvent $CDCl_3$ at δ = 7.26 ppm. Coupling constants are reported in Hz. ^{13}C NMR spectra
109 were fully decoupled and are referenced to the middle peak of the solvent $CDCl_3$ at δ = 77.0
110 ppm. ^{31}P NMR spectra are referenced with respect to the peak of 85% H_3PO_4 as external
111 reference. Splitting patterns are designated as s, singlet; d, doublet; t, triplet; q, quadruplet; dd,
112 double doublet, etc. Melting points were determined with a Fisher–Johns apparatus and are
113 uncorrected. IR spectra were recorded with a Nicolet Magna 550 spectrometer. Elemental
114 analyses were performed with an Exeter CE-440 Elemental Analyzer. Analytical TLC was

performed on commercial 0.2 mm aluminum-coated silica gel plates (F254) and visualized by 254 nm UV or immersion in an aqueous solution of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (0.04 M), $Ce(SO_4)_2$ (0.003 M) in concentrated H_2SO_4 (10%).

As judged from the homogeneity of the ^{1}H, ^{13}C, ^{31}P NMR spectra and HPLC analyses of the title compounds employing a Beckmann Ultrasphere ODS-2 column 5 µM, 250 × 10 mm eluting with water–acetonitrile (9:1) at 3.00 mL/min with a refractive index detector indicated a purity >97%.

(2,2-Diphosphonoethyl)(methyl)(pentyl)sulfonium Tetrafluoroborate (13). Silver tetrafluoroborate (148 mg, 0.87 mmol), under argon atmosphere, was added to a mixture of compound **11** (255 mg, 0.87 mmol) and iodomethane (0.5 mL) in acetonitrile (20 mL). The reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and the product was purified by column chromatography (silica gel C18-reversed phase) eluting with a mixture of water−methanol (7:1) to produce 219 mg (64% yield) of pure **13** as an amorphous solid: ^{1}H NMR (500.13 MHz, D_2O) δ 0.82 (t, J = 7.3 Hz, 3H, H-8), 1.30 (m, 2H, H-7), 1.38 (m, 2H, H-6), 1.76 (m, 2H, H-5), 2.56 (m, 1H, H-1), 2.86 (s, 3H, S(+)CH_3), 3.21 (ddd, J = 16.1, 9.0, 6.4 Hz, 1H, H-4_a), 3.35 (ddd, J = 12.8, 9.2, 6.8 Hz, 1H, H-4_b), 3.52 (m, 1H, H-2_a), 3.60 (m, 1H, H-2_b); ^{13}C NMR (125.77 MHz, D_2O) δ 12.9 (C-8), 21.3 (C-7), 23.2 (C-5, S(+)CH_3), 29.6 (C-6), 35.6 (t, J = 114.2 Hz, C-1), 40.9 (t, J = 3.4 Hz, C-2), 42.7 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 14.86.

(2,2-Diphosphonoethyl)(methyl)(hexyl)sulfonium Tetrafluoroborate (14). Silver tetrafluoroborate (150 mg, 0.9 mmol) was added to a mixture of **12** (270 mg, 0.9 mmol) and iodomethane (0.6 mL) in acetonitrile (20 mL). The reaction mixture was treated according to the method described for the preparation of **13** to give 272 mg (74% yield) of pure **14** as an

amorphous solid: ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.1 Hz, 3H, H-9), 1.23 (m, 4H, H-7, H-8), 1.40 (p, *J* = 7.3 Hz, 2H, H-6), 1.75 (m, 2H, H-5), 2.55 (m, 1H, H-1), 2.86 (s, 3H, S(+)CH_3), 3.21 (ddd, *J* = 12.8, 9.1, 6.3 Hz, 1H, H-4_a), 3.35 (ddd, *J* = 12.8, 9.1, 6.9 Hz, 1H, H-4_b), 3.51 (m, 1H, H-2_a), 3.58 (m, 1H, H-2_b); ^{13}C NMR (125.77 MHz, D_2O) δ 13.2 (C-9), 21.6 (C-8), 23.1 (C-5), 23.2 (S(+)CH_3), 27.1 (C-7), 29.6 (C-6), 35.4 (t, *J* = 121.2 Hz, C-1), 40.8 (C-2), 42.7 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 14.99. HRMS (ESI) cald for ($C_9H_{23}O_6SP_2^+$) $[M]^+$ 321.0685; found 321.0677.

Synthesis of 1-[(*n*-Alkylsulfonyl)ethyl]-1,1-biphosphonic acid (18−22). *General procedure*. To a solution of the corresponding 2-(alkylthio)ethyl-1,1-biphosphonic acid (1 mmol) in water (5 mL) was added 30% hydrogen peroxide dropwise (2.2 mmol) and the mixture stirred at room temperature. The reaction was monitored by proton NMR until it was complete. The reaction mixture was frozen and lyophilized.

1-[(*n*-Hexylsulfonyl)ethyl]-1,1-biphosphonic acid (18). 83% Yield; amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.77 (t, *J* = 7.2 Hz, 3H, H-9), 1.22 (m, 4H, CH_2), 1.36 (p, *J* = 7.4 Hz, 2H, H-6), 1.73 (p, *J* = 7.7 Hz, 2H, H-5), 2.71 (tt, *J* = 23.3, 5.1 Hz, 2H, H-1), 3.24 (m, 2H, H-4), 3.56 (dt, *J* = 16.0, 5.2 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.2 (C-9), 21.0 (C-8), 21.6 (C-5), 27.2 (C-6), 30.4 (C-7), 32.2 (t, *J* = 126.9 Hz, C-1), 48.8 (t, *J* = 3.3 Hz, C-2), 53.0 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.58. HRMS (ESI) cald for ($C_8H_{20}O_8P_2SNa$) $[M+H]^+$ 361.0246; found 361.0237.

1-[(*n*-Heptylsulfonyl)ethyl]-1,1-biphosphonic acid (19). 80% Yield; amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.78 (t, *J* = 7.0 Hz, 3H, H-10), 1.21 (m, 4H, CH_2), 1.26 (m, 2H, CH_2), 1.37 (p, *J* = 7.4 Hz, 2H, H-6), 1.75 (p, *J* = 7.7 Hz, 2H, H-5), 2.65 (tt, *J* = 23.0, 5.2 Hz, 2H, H-1), 3.26 (m, 2H, H-4), 3.57 (dt, *J* = 15.8, 5.1 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ

13.3 (C-10), 21.0 (C-9), 21.8 (C-5), 27.4 (C-6), 27.7 (C-7), 30.7 (C-8), 32.4 (t, J = 123.4 Hz, C-1), 49.1 (t, J = 3.2 Hz, C-2), 53.0 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.64.

1-[(*n*-Octylsulfonyl)ethyl]-1,1-biphosphonic acid (20). 79% Yield; amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.76 (t, J = 6.5 Hz, 3H, H-11), 1.21 (m, 8H, CH_2), 1.36 (p, J = 7.1 Hz, 2H, H-6), 1.73 (p, J = 7.6 Hz, 2H, H-5), 2.70 (tt, J = 23.2, 5.0 Hz, 2H, H-1), 3.23 (m, 2H, H-4), 3.56 (dt, J = 15.8, 5.3 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.4 (C-11), 21.1 (C-10), 22.0 (C-5), 27.5 (C-6), 28.07 (C-7), 28.09 (C-8), 31.0 (C-9), 32.5 (t, J = 123.4 Hz, C-1), 49.2 (t, J = 3.4 Hz, C-2), 53.1 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.54. HRMS (ESI) cald for ($C_{10}H_{24}O_8P_2SNa$) $[M+H]^+$ 367.0740; found 367.0745.

1-[(*n*-Nonylsulfonyl)ethyl]-1,1-biphosphonic acid (21). 70% Yield; amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.77 (t, J = 7.0 Hz, 3H, H-12), 1.20 (m, 10H, CH_2), 1.37 (p, J = 7.4 Hz, 2H, H-6), 1.74 (p, J = 7.7 Hz, 2H, H-5), 2.65 (tt, J = 22.8, 5.1 Hz, 2H, H-1), 3.24 (m, 2H, H-4), 3.56 (dt, J = 15.8, 5.3 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.4 (C-12), 21.0 (C-11), 22.0 (C-5), 27.4 (C-6), 28.1 (C-7), 28.3 (C-9), 29.8 (C-5), 31.08 (C-8), 31.10 (C-10), 32.7 (t, J = 121.3 Hz, C-1), 49.5 (t, J = 3.4 Hz, C-2), 53.0 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.23. HRMS (ESI) cald for ($C_{11}H_{26}O_8P_2SNa$) $[M+Na]^+$ 403.0716; found 403.0715.

1-[(*n*-Decylsulfonyl)ethyl]-1,1-biphosphonic acid (22). 64% Yield; amorphous solid; ^{1}H NMR (500.13 MHz, D_2O) δ 0.79 (m, 3H, H-13) 1.22 (m, 12H, CH_2), 1.38 (m, 2H, H-6), 1.74 (m, 2H, H-5), 2.82 (tt, J = 21.1, 6.9 Hz, 2H, H-1), 3.20 (m, 2H, H-4), 3.59 (t, J =14.6 Hz, 2H, H-2); ^{13}C NMR (125.77 MHz, D_2O) δ 13.7 (C-13), 21.2 (C-12), 22.5 (C-5), 28.1 (C-6), 29.0 (C-6), 29.2 (C-7), 29.3 (C-10), 29.4 (C-8), 31.4 (C-9), 31.7 (C-11), 32.4 (t, J = 125.1 Hz, C-1), 49.2 (C-2), 53.1 (C-4); ^{31}P NMR (202.46 MHz, D_2O) δ 17.83. HRMS (ESI) cald for ($C_{12}H_{28}O_8P_2SNa$) $[M+Na]^+$ 417.0878; found 417.0863.

184 **Enzymatic determinations.** Recombinant *Tg*FPPS (12) and *Hs*FPPS (31) were obtained and
185 their activities determined exactly as described in the references. Briefly, DMAPP and ^{14}C IPP
186 were mixed with inhibitors at different concentrations and reactions initiated by the addition of
187 *Tg*FPPS. The reaction was allowed to proceed at 37^{o}C for 30 minutes and followed by extraction
188 of the prenylated products with hexane. ^{14}C labeled products were measured in a scintillation
189 counter and IC_{50} determined.
190
191 **In vitro drug screening**
192 Experiments on *T. gondii* tachyzoites were carried out as described previously using *T. gondii*
193 tachyzoites expressing red fluorescent protein with the modifications described by Recher et al.,
194 2013 (29). Tachyzoites expressing red fluorescent protein (RFP) (32) were maintained in human
195 fibroblasts (hTert cells). For drug testing, parasites were purified by passing through a 27 G
196 needle followed by filtration through a 3 µm filter. Human fibroblasts cells were cultured in 96
197 well plates 24 h before infection. Each well was seeded with 10^4 tachyzoites and fluorescence
198 values followed for 3-4 days. We also measured growth inhibition of *T. gondii* tachyzoites of the
199 Prugniard strain expressing RFP. In this case 10^4 parasites per well were used and the EC_{50} was
200 calculated at day 5. Plates were read with covered lids, and both excitation (544 nm) and
201 emission (590 nm) were read from the bottom. For studies of synergism in vitro, checkerboard
202 studies were done exactly as described before (14). Results were expressed as the sums of the
203 fractional inhibitory concentration [sum FIC = (IC_{50} of drug A in mixture/ IC_{50} of drug A alone)
204 + (IC_{50} of drug B in mixture/ IC_{50} of drug B alone)], as described by Berenbaum (33). Sum FIC
205 values indicate the kinds of interactions as follows: < 0.5, synergy; 1, addition; > 2, antagonism.

The 3D7 strain of *Plasmodium falciparum* was grown in 2% human red blood cells and RPMI 1640 supplemented with 25 mM HEPES, 5 mg/l thiamine, 30mg/l hypoxanthine, 0.225% $NaHCO_3$, and 0.25% Albumax I (Gibco). The parasites were kept under 5% CO_2, 5% O_2 and 90% N_2. The growth of *P. falciparum* was measured using flow cytometry (34). IC_{50}s were determined using asynchronous parasite cultures grown for 72 hours at different drug concentrations. These experiments were performed twice (biological replicates) with technical triplicates in each experiment. Aliquots of parasites (5 µl) grown at different drug concentrations were incubated in acridine orange (1.5 µg/ml) for 20 minutes before being counted on a Beckman Coulter HyperCyAn flow cytometer. At each drug concentration, the parasitemia (or the percentage of infected red blood cells in the culture) was determined. The growth of the parasites was measured every day for three days. The growth of the parasites was normalized to growth in the absence of **22**. Data was analyzed with the software package Prism. The normalized growth at day 3 was fit to a standard dose response equation.

Growth of *Cryptosporidium parvum* was followed using the luciferase assay as described (35). Oocysts were obtained from Bunch Grass Farm and allowed to excyst after washing to remove the HCl used for storage. Oocysts were incubated at 37°C for 1-1:30 hours and excysted parasites observed by microscopy (36). Purified sporozoites (1 x 10^7) were transfected with 10 µg of luciferase plasmid DNA using a 4D-Nucleofector™ System from Lonza. Transfected parasites were transferred to plates containing 70% confluent human ileocecal adenocarcinoma (HCT-8) cells and inhibitors added at different concentrations. Plates were incubated for 48 hours at 37°C. Growth was stopped by aspirating the media and addition of the Nano-Glo Luciferase Assay Reagent from Promega. Luminescence was measured with a plate reader from BioTek. Growth experiments were repeated three times with three technical replicates for each

one. The results shown in Figure 4 are from one representative experiment as the luminiscence varied with different batches of oocytes.

Cytotoxicity to hTert cells. The cytotoxicity was tested using the Alamar Blue™ assay as described by Recher et al., (29). We did not observe cytotoxicity of compound 22 at concentrations up to 200 µM. We observed some swelling of the cells at 500 µM but toxicity was not high enough to be able to detect it with Alamar Blue.

In vivo drug screening

Experiments were carried out as described previously (14) using 20 or 100 *T. gondii* tachyzoites (indicated in the figure legends) of the RH strain to infect Webster mice. Drugs were dissolved in 10% Kolliphor® HS 15 and were inoculated i.p. Treatment was initiated 6 hours after infection and administered daily (or every 12 h) for 10 days. Surviving mice were challenged with 5,000 RH tachyzoites 30 days after infection.

Statistics

All statistical analysis was done using the Student's t-test. P value < 0.05 was considered statistically significant

RESULTS

Activity of sulfur-containing bisphosphonates against parasite growth and enzyme activity

Based on preliminary data showing that bisphosphonates with a methyl sulfonium group at C-3, such as **10** (Fig. 2A), have strong inhibitory action against the target enzyme TgFPPS (29), we designed structural variants of this compound, such as compounds **13** and **14** (Fig. 2B). We previously found that a sulfone-containing bisphosphonate derivative of short chain length, 1-[(*n*-pentylsulfonyl)ethyl]-1,1-biphosphonate, was practically devoid of antiparasitic activity (29). Taking into account this information we prepared bisphosphonates possessing long linear aliphatic chains considering that the favorable entropy that results from burying the hydrophobic alkyl chain is the main binding driving force for inhibition of enzyme activity by closely related bisphosphonates (37). Therefore, sulfone derivatives **18**–**22** were produced (Fig. 2C). We investigated the activity of the compounds whose structures are shown in Fig. 2 against intracellular *T. gondii* tachyzoites as well as against hTert cells, as a counter screen for toxicity (Table 1). The methyl(alkyl)sulfonium bisphosphonates **13** and **14** had potent activity against *T. gondii* having EC_{50} values of 2.18 ± 0.5 µM and 2.8 ± 1.1 µM, respectively. They also showed a moderate action against the target enzyme TgFPPS (Table 1). Remarkably, sulfones **20**–**22** exhibited excellent antiparasitic activity. Compound **22** exhibited an EC_{50} value of 0.11 ± 0.02 µM when tested against the type I strain RH and an EC_{50} value of 0.24 ± 0.08 µM against the type II strain Prugniard (Table 1 and Fig. 3A-D). This compound had little toxicity against hTert cells with an EC_{50} > 200 µM, corresponding to a selectivity index of >2,000. The compound was also active against the recombinant target enzyme, TgFPPS (IC_{50} = 0.30 ± 0.03 µM) (Fig. 3E) at a concentration inhibiting 50% of the activity higher than the concentration needed to inhibit 50% growth. Interestingly, two related compounds (**20** and **21**), which differ in the aliphatic chain length, had good activity against intracellular tachyzoites (EC_{50} of 0.39 ± 0.04, and 0.16 ± 0.03 µM, respectively), and also showed activity against TgFPPS (IC_{50} of 0.27 ±

0.10, and 0.26 ± 0.05 μM, respectively) (Table 1). The IC_{50}s of compounds **20** and **21** for TgFPPS inhibition are lower than the IC_{50} of **22**. However, **22** is more effective when tested against growth. This could be because the longer aliphatic chain of **22** favors its permeability into the cell.

Based on previous findings from our laboratory on the synergistic effect of combining bisphosphonates with statins, we tested compounds **20, 21** or **22** in combination with atorvastatin (an inhibitor of the host HMG-CoA-reductase) or with WC-9 (an inhibitor of squalene synthase)(38-41) but we found no synergy *in vitro* (Table 2).

In light of its high potency against *Toxoplasma* and complete lack of activity against trypanosomes (not shown) we investigated the effect of compound **22** against other Apicomplexan parasites. We found that compound **22** has excellent activity against asexual stages of *P. falciparum* (EC_{50} = 0.6 ± 0.01 μM) (Table 3 and Fig. 4) and also showed activity against *C. parvum* growth *in vitro* (EC_{50} ~ 65 μM) (Table 3 and Fig. 5). This last value compares favorably with that of paromomycin, which has an EC_{50} of ~1 mM against *C. parvum* under these conditions.

In vivo activity of sulfur-containing bisphosphonates.

We tested the efficacy of **22** against *T. gondii* infection using the hypervirulent strain RH. Fig. 6A shows two experiments using groups of 5 mice treated with different doses of **22**. While 100% of control mice died between 9-12 days post-infection, 80-100% of mice treated with the higher 1, 0.5 or 0.1 mg/kg per day doses survived more than 30 days. A **22** ED_{50} of 0.02 ± 0.004 mg/kg/day was calculated. We also tested lower doses of **22** applied every 12 h and infected mice with 100 RH tachzoites. In this case while 100% of control mice died by day 10, 80% of

297 mice treated with 0.05 mg/kg every 12 h survived more than 30 days (Fig. 6B). The survival rate
298 of the twice a day treatment was higher than with the treatment using a singly daily dose.
299 Surviving mice were challenged at 30 days post-infection with a lethal dose of 5,000 tachyzoites.
300 All previously infected mice survived the challenge demonstrating that they had been initially
301 infected.
302
303 **DISCUSSION**
304 Our results indicate that compound **22** could be a lead for developing new drugs against
305 Apicomplexan parasites. Interestingly, **22** and related compounds **20** and **21** had no activity
306 against other parasites like *T. cruzi* suggesting a specific target in Apicomplexan parasites.
307 Compound **22** had the highest activity against *T. gondii* growth, which could be the result of the
308 longer aliphatic chain favoring permeability or because of its potential targeting of more than one
309 enzyme of the isoprenoid pathway. Compound **22** showed activity against the *Toxoplasma*
310 TgFPPS although the IC_{50} for its inhibition was almost double than the concentration of drug
311 needed to inhibit 50% of parasite growth. In general, bisphosphonates show lower IC_{50}s toward
312 the enzyme target than the EC_{50}s against parasite growth. For example compound **15**, which was
313 previously tested (29), inhibits *T. gondii* growth with an EC_{50} of 1.5 µM, and the TgFPPS with an
314 IC_{50} = 0.031 µM (TgFPPS) (29). It is possible that host cells actively incorporate compound **22**
315 into the intracellular environment where the parasite resides and replicates exposing them to
316 higher concentration of the compounds. Something similar could happen if the compound is
317 actively taken up by the parasites, and it could concentrate creating an environment in which the
318 target enzyme(s) would be exposed to higher concentrations of the drug. The longer alkyl chain of
319 compound **22** would favor its uptake by cells. Another possibility would be that these compounds

320 are targeting multiple enzymes within the isoprenoid pathway explaining their high efficacy and
321 their lack of synergy with statins. Their high efficacy would mask the potential synergistic
322 interaction with inhibitors of the host isoprenoid pathway. We tested the activity of these
323 inhibitors against the human FPPS and compound **20-22** all had activity. Interestingly the longer
324 the aliphatic chain the less active they were against the human enzyme, which agrees with the
325 known specificity of the mammalian enzyme for the synthesis of FPP, the 15 C metabolite. The
326 *Toxoplasma* enzyme has the peculiarity of synthesizing both FPP and GGPP (20 C) because of
327 the presence of one small amino acid residue in the active site region of the protein, which
328 controls chain elongation. This peculiar amino acid arrangement is also found in the *Plasmodium*
329 and *Cryptosporidium* enzymes and both enzymes have been found to be bifunctional (42, 43). In
330 summary, even though the inhibition of the parasite synthesis of FPP may not be the only target
331 for this compound, it appears that the Apicomplexan enzymes play an important part in their
332 activity giving these compounds the distinct property of being specific for these intracellular
333 Apicomplexan parasites. Previous work showed that mice infected with *T. gondii* and treated with
334 a dose of 10 mg/kg of an alkyl bisphosphonates (Compound 1 from Ling et al (27)) were
335 protected from death (80%). Compound 2 from the same study, also an alkyl derivative with a
336 shorter chain showed 60% protection against death. These results were the first to show that n-
337 alkyl bisphosphonates could provide protection against death due to *T. gondii* infection.
338 Previously the bisphosphonate risedronate (a Nitrogen derivative) showed only 55% protection
339 from death (44). Compound **22,** from the present study is highly effective (ED_{50} 0.02 mg/kg) *in*
340 *vivo* for the treatment of mice infected with the *Toxoplasma* hypervirulent strain RH and our
341 results with this sulfone bisphosphonate derivative represent a significant improvement.

342

Supporting Information: Copies of the ^{1}H NMR, ^{13}C NMR and ^{31}P NMR spectra of the target molecules and the corresponding intermediates are included as supporting information.

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Table 1. Biological activity of several bisphosphonates against *T. gondii* growth and the enzymatic activities of *Tg*FPPS and *Hs*FPPS.

Compound	*T. gondii* growth EC_{50} (µM)	*Tg*FPPS IC_{50} (µM)	*Hs*FPPS IC_{50} (µM)
13	2.18 ± 0.49	1.06 ± 0.13	1.00 ± 0.60
14	2.82 ± 1.15	0.23 ± 0.01	> 15
15	1.49 ± 0.38	0.03 ± 001	1.31 ± 0.53
18	> 10	NT	NT
19	> 10	NT	NT
20	0.39 ± 0.04	0.27 ± 0.10	1.17 ± 0.27
21	0.16 ± 0.03	0.26 ± 0.06	1.67 ± 0.3
22	0.11 ± 0.03	0.27 ± 0.03	2.73 ± 0.13

Values are means ± SD of three independent experiments (n = 3).

Table 2. Growth Inhibition of Compound 22 against *T. gondii*, *Cryptosporidium* and *Plasmodium falciparum*

T. gondii RH EC_{50} (µM)	*T. gondii* Prugniard EC_{50} (µM)	*P. falciparum* EC_{50} (µM)	*C. parvum* EC_{50} (µM)
0.11 ± 0.03	0.24 ± 0.08	0.6 ± 0.01 µM	65 µM

Values are means ± SD of two (*Plasmodium*) or three (*T. gondii)* independent experiments (n = 3). *C. parvum* shows results of one experiment in triplicate.

Table 3. Combinations of sulfone-containing bisphosphonates and atorvastatin or WC-9 against *T. gondii in vitro*.

Combination	Sum FIC	Synergy
Atorvastatin + **20**	1.38	No
Atorvastatin + **21**	1.60	No
Atorvastatin + **22**	1.64	No
WC-9 + **22**	2.51	No

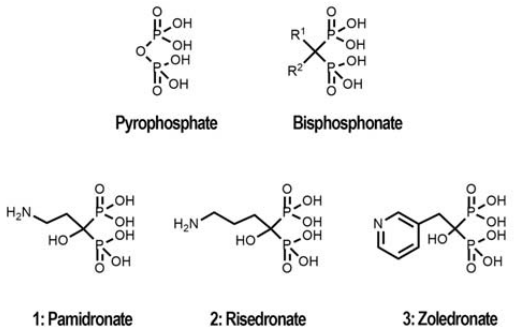

Figure 1. Basic structure of bisphosphonates and aminobisphosphonates clinically used.

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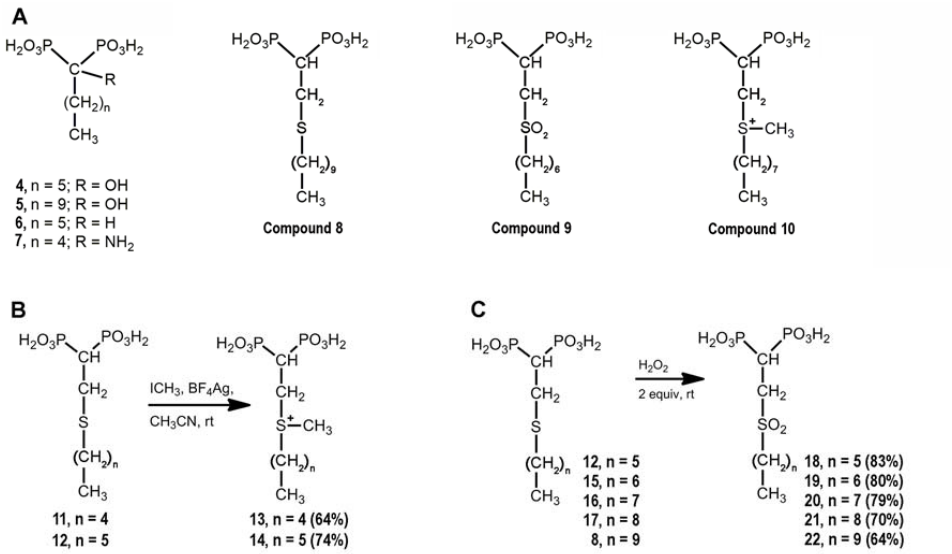

Figure 2. A, chemical structures of representative lineal bisphosphonic acids as putative antiparasitic agents. B, C, synthetic approaches used to produce the designed linear sulfur-containing 1,1-bisphosphonates.

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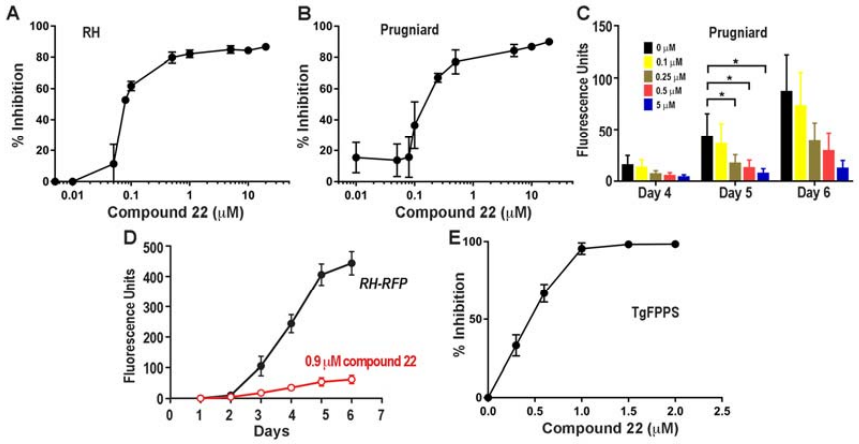

Figure 3: Inhibition curves for compound 22 against growth of *Toxoplasma* RH strain (A), and Prugniard (B and C). C, Fluorescence values at days 3, 4 and 5 of red parasites of the Prugniard strain growing in fibroblasts in 96 well plates. Notice that these cells have a slow growth and they are still replicating at day 5; D, Growth curve of *T. gondii* RH-RFP cells and its inhibition by 22 at 0.9 μM, which is the concentration calculated from A that inhibits 90% of growth; E, FPPS activity with different concentrations of compound 22. The protocol to measure the activity is explained under Materials and Methods. Values in A-D are means ± SD of three independent experiments (n = 3).

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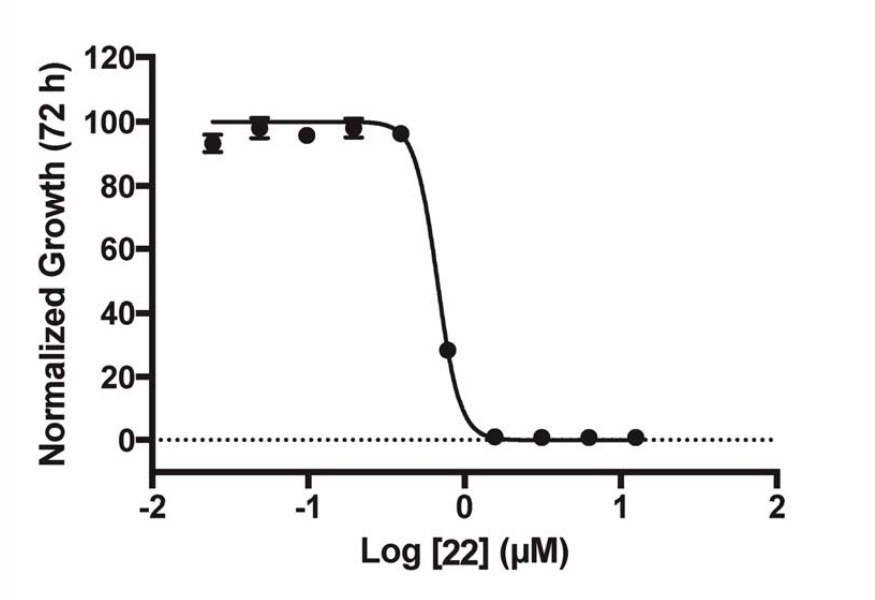

Figure 4. Dose-response showing the inhibition of growth of *Plasmodium falciparum* by compound **22**. R^2 was 0.9935. Details of the experiment are explained under Methods. Values are means ± SD of two independent experiments, each one in triplicate.

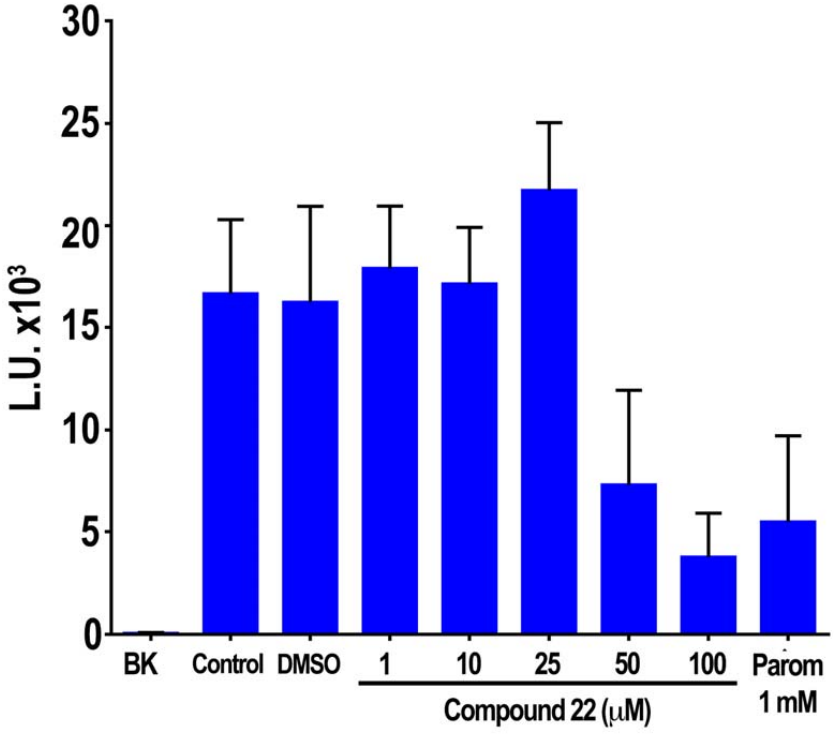

Figure 5. Dose-response curve for **22** against *C. parvum* growth. BK, background counts without parasites; Parom, paromomycin control. The protocol for measuring inhibition of growth and growth of *C. parvum* is explained under Materials and Methods. Results are from one representative experiment out of three.

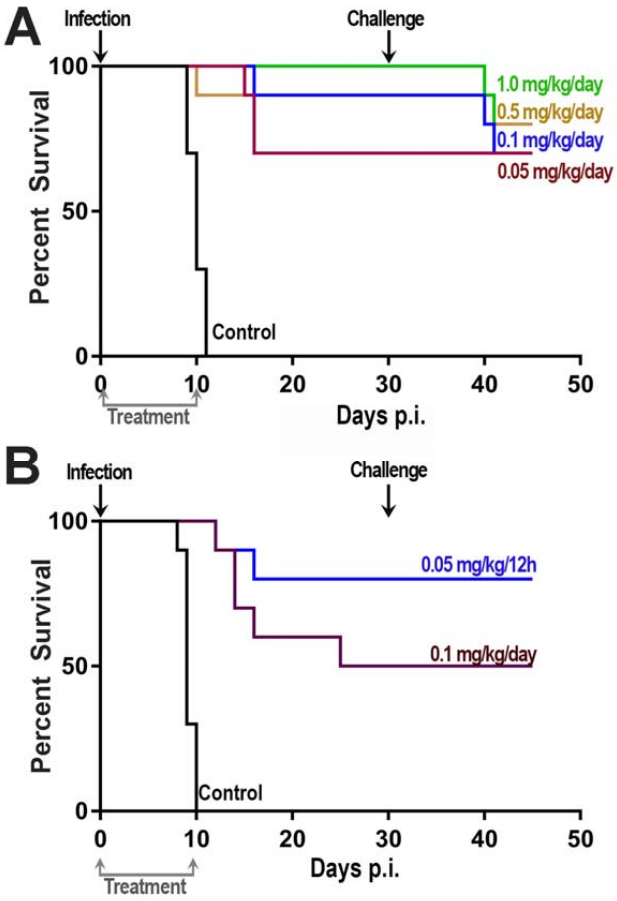

Figure 6. *In vivo* results for the *T. gondii* RH mouse model of infection. A, Mouse survival after treatment with 0.05, 0.1, 0.5, and 1 mg/kg of **22** i.p. for 10 days. Surviving mice were challenged with 5,000 tachyzoites at day 30. B, Mouse survival after treatment with 0.05 mg/kg of **22** i.p. twice a day for 10 days, as compared to treatment with 0.1 mg/kg daily for 10 days. Surviving mice were challenge with 5,000 tachyzoites at day 30. All these experiments were repeated twice with 5 mice for each dose.