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SOME PHOSPHONIC ACID ANALOGS AS INHIBITORS OF PYROPHOSPHATE-DEPENDENT PHOSPHOFRUCTOKINASE, A NOVEL TARGET IN *TOXOPLASMA GONDII*

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Abstract—Pyrophosphate-dependent phosphofructokinase (PP_i-PFK) was identified previously in *Toxoplasma gondii* as the only kinase that phosphorylates fructose-6-P to fructose-1,6-bisP. Since such an enzyme is not present in mammals, it was considered to be a good target for prospective selective inhibitors of the parasite. We have examined the effects of several phosphonic acid derivatives, analogs of pyrophosphate, on PP_i-PFK activity, as well as on the replication of *T. gondii* in human foreskin fibroblast (HFF) cells. The most active compound in inhibiting PP_i-PFK was tetrasodium carbonyldiphosphonate. Several bisphosphonates and related arylhydrazones showed inhibition of the enzyme, but with higher IC_{50} values. Although several phosphonoacetic acid derivatives also inhibited PP_i-PFK, as a group they were less potent than the bisphosphonate derivatives. Comparison among the structures of various inhibitors and their effects against PP_i-PFK indicates that a carbonyl (C=O) or imino (C=N) group between two phosphoryl moieties is associated with more potent enzyme inhibition. Tetrasodium carbonyldiphosphonate did not show a significant effect against replication of *T. gondii* cells, probably because, as a charged molecule, it could not cross the cell membrane to reach the intracellular parasite. Tetraisopropyl carbonyldiphosphonate 2,4-dinitrophenylhydrazone showed some selective inhibitory effect against replication of the parasite in the HFF cells and protected the mammalian cells from damage by *T. gondii*. The results indicate that carbonyldiphosphonic acid is a good prototype compound that is amenable to chemical manipulation, which, in turn, may optimize selective inhibition of *T. gondii* PP_i-PFK and increase accessibility to the intracellular parasite.

Key words: Toxoplasma gondii; pyrophosphate-dependent phosphofructokinase; phosphonic acid derivatives

Toxoplasma gondii is a protozoan parasite that infects humans and domestic animals. The parasite is particularly infectious to AIDS patients or others whose immunity is severely compromised. If not treated in immunosuppressed patients, it is fatal. Current chemotherapy in immunodepressed individuals is limited by the development of severe adverse effects, which may prevent conclusion of the therapy [1]. A rational approach to the problem of chemotherapy is to identify biochemical processes that are unique to the parasite. Chemical agents that influence these sites may become new chemotherapeutic agents that are relatively more toxic to the parasite than to the host. Glycolytic enzymes in parasitic protozoa and helminths play an important role in the survival of these organisms [2, 3]. Studies on these enzymes have revealed kinetic differences between homologous enzymes in the parasite and the host. Enzymes with such differences have been found to be targets for chemotherapeutic agents. One enzyme that is of particular interest to chemotherapists is PFK‖. The enzyme in the host catalyzes the physiologically irreversible phosphorylation of fructose-6-P to fructose-1,6-bisP with ATP as the phosphoryl donor. Because of its critical role in the regulation of glycolysis, PFK has been implicated as the site of action of some antiparasitic agents [3]. We have discovered that this enzyme in *T. gondii* requires PP_i as a phosphoryl donor instead of ATP.

$$\text{Fructose-6-P} + PP_i \longleftrightarrow \text{Fructose-1,6-bisP} + P_i$$

The enzyme, therefore, is designated PP_i-PFK instead of ATP-PFK, the host enzyme. Several other protists also have the pyrophosphate PFK rather than the ATP-PFK [4]. We have purified the enzyme from *T. gondii* almost to homogeneity [5]. The *T. gondii* enzyme is completely different from the host enzyme. In addition to using PP_i instead of ATP as a phosphoryl donor, the *T. gondii* enzyme has different kinetics and different subunit structure.

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‖ Abbreviations: PFK, phosphofructokinase; PP_i, pyrophosphate; PP_i-PFK, pyrophosphate-dependent phosphofructokinase; ATP-PFK, ATP-dependent phosphofructokinase; HFF, human foreskin fibroblast; DMEM, Dulbecco's Minimum Essential Medium; and FBS, fetal bovine serum.

In designing selective inhibitors against the parasite PFK, we have examined several analogs of pyrophosphate. Pyrophosphate analogs, such as diphosphonic acid, phosphonoacetic acid or phosphonoformic acid, have been found previously to have anti-viral activity by inhibiting viral and other DNA polymerases [6, 7]. Hydrazones of varying structures have been studied recently for useful pharmacological properties. Pyridoxal isonicotinoyl hydrazone has been reported to be an orally effective iron chelation therapy [8]. Other hydrazones have been investigated as potential antidiabetic [9] or anti-tumor [10] agents. The present investigation examines several derivatives of methanediphosphonic acid and phosphonoacetic acid, among them several hydrazones of the corresponding α-keto derivatives, to determine whether they inhibit the PP_i-PFK of *T. gondii* and whether they have an effect on intact parasites in cell culture.

MATERIALS AND METHODS

Materials. Fructose-6-P was obtained from Sigma. Aldolase, glycerophosphate dehydrogenase and triose phosphate isomerase were from Boehringer Mannheim. Cell culture medium was purchased from BRL-Gibco and fetal bovine serum from Hyclone. Radioactive isotopes were from Amersham.

Syntheses of bisphosphonic acid and phosphonoacetic acid derivatives. The bisphosphonic acid derivatives are: tetrasodium carbonyldiphosphonate, compound **1** [7]; tetraisopropyl carbonyldiphosphonate, compound **2** [11]; tetrasodium ethenylidenebisphosphonate, compound **3** [12]; tetraethyl and tetraisopropyl carbonyldiphosphonate 2,4-dinitrophenylhydrazones, compounds **4** and **5** (prepared from the corresponding ketones as described previously [11]); monosodium trimethyl carbonyldiphosphonate 2-methoxy-4-nitrophenylhydrazone, compound **7** (prepared by monodemethylation of the corresponding tetramethyl ester, compound **6** [11]); methanediphosphonic acid, compound **8**; and its tetraisopropyl ester, compound **9** (purchased from the Aldrich Chemical Co.); the tris(dicyclohexylammonium) salts of chloromethanediphosphonic acid, compound **10**; dibromomethanediphosphonic acid, compound **11**; chlorofluoromethanediphosphonic acid, compound **12** (prepared as described previously [13]); and pyridinium dichloro[hydroxyphenylphosphinyl]-methylphosphonate, compound **13** (synthesis described previously [14]).

The phosphonoacetic acid derivatives included: triethyl *E*-phosphonoglyoxylate 2-methoxy-4-nitrophenylhydrazone, compound **14**; and 2-nitro-4-methoxyphenylhydrazone, compound **15** (both prepared by a diazonium condensation method [11]); the bis(dicyclohexylammonium) salts of *E*-phosphonoglyoxylate: phenylhydrazone, compound **16**; 4-fluorophenylhydrazone, compound **17**; 2,4-dinitrophenylhydrazone, compound **18**; 4-nitrophenylhydrazone, compound **19**; and 2-methoxy-4-nitrophenylhydrazone, compound **20** (prepared by direct reaction of the parent ketone dicyclohexylammonium salt with the appropriate aryl hydrazine [11]); bis(dicyclohexylammonium) phosphonoglyoxylate, compound **21** (prepared as described previously [15]); the corresponding trisodium salt, compound **22**; and trisodium bromophosphonoacetate, compound **23** (prepared from dicyclohexylammonium salts by ion exchange [15, 16]).

Determination of PP_i-PFK and ATP-PFK activity. PP_i-PFK activity was determined spectrophotometrically at a wavelength of 340 nm at 24° as described previously [5]. The standard reaction mixture contained 50 mM Tris–Cl, pH 7.0, 1 mM PP_i, 1 mM fructose-6-P, 0.1 mg NADH, 1 mM dithiothreitol, 1.5 U aldolase, and 15 U of triose phosphate isomerase with 5.1 U glycerophosphate dehydrogenase in a total volume of 0.5 mL. Control assays, when needed, were run either without PP_i or fructose-6-P. One unit of PP_i-PFK activity was defined as the amount of enzyme that catalyzed the phosphorylation of 1 μmol of fructose-6-P/min.

Mammalian heart ATP-PFK activity was determined either at optimal pH 8.2 [17] or under conditions optimal for allosteric interaction at pH 6.9 according to our previously published procedures [18].

Measurement of inhibition. Inhibition of PP_i-PFK or ATP-PFK by phosphonate compounds was measured by adding various concentrations of the drug dissolved in either water or DMSO to the reaction mixture before starting the assay. Controls were run with the corresponding solvent.

Purification of PP_i-PFK. PP_i-PFK was purified as described previously [5] from fresh tachyzoites of *T. gondii* (RH strain) collected from mouse peritoneal fluid obtained from the laboratory of Dr. Jack Remington of the Palo Alto Medical Foundation, Research Institute.

Preparation of tachyzoites. The tachyzoites used to infect the HFF cells were obtained from the same source. The peritoneal fluid containing the tachyzoites was centrifuged at 500 *g* for 10 min, the supernatant was discarded, and the toxoplasma was resuspended in an equal volume of phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4, pH 7.5). The centrifugation was repeated, and the tachyzoites were suspended in DMEM containing 10% heat-inactivated FBS. The tachyzoites were counted, diluted to the correct concentration, and used to infect the HFF cells.

Culture of HFF cells. HFF cells were obtained from the American Type Culture Collection (Rockville, MD). They were grown in DMEM supplemented with 10% heat-inactivated FBS and containing penicillin, streptomycin and fungizone.

Measurement of replication of T. gondii. Replication of *T. gondii* was measured by incorporation of [^{3}H]uracil while the tachyzoites were growing in confluent HFF cells. It has been shown by Pfefferkorn and Pfefferkorn [19] that the parasites incorporate uracil from the culture medium into their DNA, whereas the mammalian cells showed no significant incorporation. Drugs were dissolved in either water or DMSO and sterilized by filtration. The absorbance spectra of the filter-sterilized compounds were checked to make sure that the compounds had not been adsorbed to the filter. The drugs that were dissolved in DMSO were diluted subsequently with

DMEM + FBS so that the concentration of DMSO in the culture was 0.1%. Separate controls were run for drugs dissolved in DMSO or in water. For toxoplasma proliferation assays, 4×10^4 HFF cells were plated into 96-well plates and allowed to grow to confluence (~40 hr). The medium was removed, and 10^5 freshly harvested and washed tachyzoites suspended in medium were added to each well. After 4 hr the medium (containing any tachyzoites that had not penetrated the cells) was removed, and the drug solutions in medium were added. After 24 hr (for day 2) or 48 hr (for day 3) 1 mCi [^{3}H]uracil was added. Twenty hours after addition of the radiolabeled uracil, the medium was removed from the cells, and 0.1% SDS containing 1 mg uracil was added to the plates to dislodge the cells. Trichloroacetic acid (10%) was added to precipitate the acid-insoluble portions of the cells that were collected on glass fiber filters with a Cambridge cell harvester. The filters were placed in vials with Optifluor (Packard Instruments), and the radioactivity was measured in a scintillation spectrometer. The procedure was adapted from Mack and McLeod [20].

Measurement of replication of HFF cells. Replication of the HFF cells without parasites was measured by incorporation of [^{3}H]thymidine over 20-hr periods when the cells were in the log phase of growth. HFF cells in DMEM + 10% FBS were plated into 96-well plates at a concentration of 1.5×10^4 cells per well and allowed to adhere for 24 hr. Medium was removed, and test compounds were added as above. In the uninfected mammalian cells, [^{3}H]thymidine was substituted for uracil, and cells were harvested as described above.

RESULTS

Effect of bisphosphonates on PP_i-PFK. Methanediphosphonic acid derivatives (bisphosphonates) were chosen because of their close similarity to the enzyme substrate pyrophosphate. In these analogs, the hydrolytically labile P—O—P bonds of pyrophosphate are replaced by the more stable P—C—P bonds [6]. Thirteen compounds were tested on PP_i-PFK activity using the spectrophotometric assay described under Materials and Methods. Their complete chemical names are listed in Materials and Methods. Eight compounds (**1**, **3**, **7**, **8**, **10–13**) resemble pyrophosphate anion in being negatively charged at the assay pH, whereas the other five (**2**, **4–6**, **9**) are neutral esters.

Figure 1 shows the inhibition curves of *T. gondii* PP_i-PFK by bisphosphonates. Only those compounds that inhibited the enzyme at a concentration lower than 200 μM are shown. Table 1 lists the structures of all compounds tested and the concentrations that caused a 50% inhibition of enzyme activity (IC_{50} values). The most inhibitory of these compounds was shown to be tetrasodium carbonyldiphosphonate, compound **1**. The curve showing inhibition of enzyme activity was very steep and gave an IC_{50} of 13 μM. The un-ionized tetraisopropyl ester, **2**, had little ability to inhibit PP_i-PFK (IC_{50} = 170 μM). Substitution of a vinylidene group for the oxygen on compound **1** (compound **3**) caused a reduction in

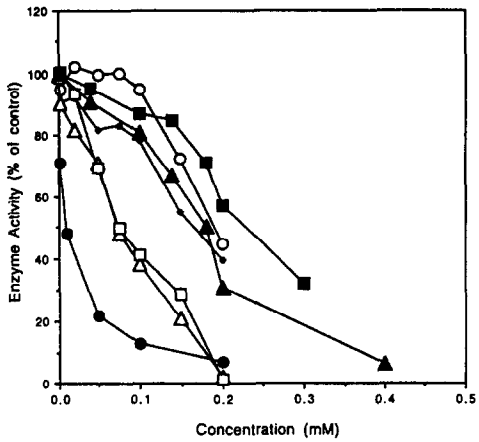
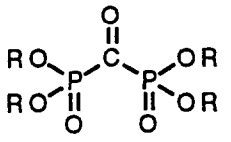


Fig. 1. Inhibition of PP_i-PFK activity from *T. gondii* by methanediphosphonic acid derivatives. Control (no additions) values varied from 0.01 to 0.02 enzyme units per 0.5 mL of reaction mixture. The structures of compounds **1–7** are given in Table 1. Key: (●) **1**, (•) **2**, (○) **3**, (△) **4**, (□) **5**, (▲) **6**, and (■) **7**.

inhibition of PP_i-PFK similar to that of compound **2**. The effects of several arylhydrazone esters of compound **1** with various substitutions on the aryl ring were tested on PP_i-PFK activity. Two of these, compounds **4** and **5** (both IC_{50} values = 75 μM), were less potent inhibitors of PP_i-PFK than **1**. Two other nitrophenylhydrazone derivatives (compounds **6** and **7**) had much higher IC_{50} values when compared with **1** (13- and 17-fold more). Several other bisphosphonates with different α-substitution had little or no inhibitory effect (compounds **8–13**). It appears, therefore, that the carbonyl group between the two phosphoryl moieties significantly enhances inhibitory activity against PP_i-PFK. If the carbonyl group (C=O) was replaced with a hydrazone imino group (C=NNHAr) or a vinylidene group ($C=CH_2$), there was a significant reduction in the inhibitory effect. Although it was expected that acid salts rather than esters would be the most appropriate analogs of pyrophosphate for inhibition of PP_i-PFK, several esters and related arylhydrazones (compounds **4–7**) produced inhibition of enzyme activity with IC_{50} values of 75–220 μM.

Effects of phosphonoacetic acid derivatives on PP_i-PFK. We have tested several derivatives of phosphonoacetate (Table 2). Only those compounds with a nitrophenylhydrazone moiety had a significant inhibitory effect against PP_i-PFK. Inhibition curves for those compounds (**14–18**), which had effects at a concentration of less than 500 μM, are shown in Fig. 2. Triethyl phosphonoacetate phenylhydrazones (compounds **14** and **15**) had the highest inhibitory potencies among these derivatives (IC_{50} values = 60 and 100 μM). These two compounds were not soluble in the reaction mixture above 250 μM, which limited our ability to test them further. Dealkylation of the phosphonoacetate arylhydrazone esters (compounds **16–18**) markedly increased their IC_{50} values. The

Table 1. Inhibition of PP_i-PFK from *T. gondii* by diphosphonic acid derivatives



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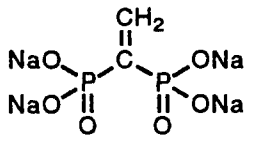

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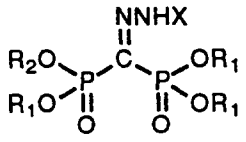
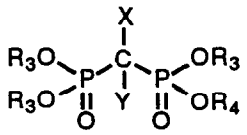
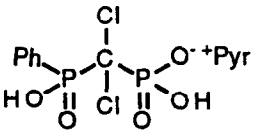


4-7



8-12

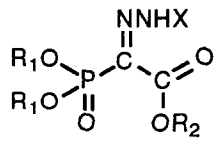


13

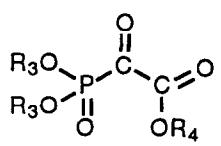
Compound number	R	X	Y	IC_{50} (μM)
1	Na			13
2	iPr			170
3				195
4	Et	2,4-$(NO_2)_2$Ph		75
5	iPr	2,4-$(NO_2)_2$Ph		75
6	Me	2-MeO-4-NO_2Ph		180
7	R_1 = Me; R_2 = Na	2-MeO-4-NO_2Ph		220
8	R_3 = R_4 = H	H	H	>2000
9	R_3 = R_4 = iPr	H	H	>2000
10	R_3 = DCHA*; R_4 = H	Cl	H	>2000
11	R_3 = DCHA; R_4 = H	Br	Br	>2000
12	R_3 = DCHA; R_4 = H	Cl	F	>2000
13				>2000

* DCHA = dicyclohexylammonium.

Table 2. Inhibition of PP_i-PFK from *T. gondii* by phosphonoacetic acid derivatives



14-20



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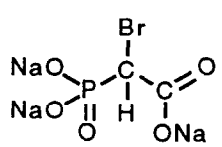

23

Compound number	R	X	IC_{50} (μM)
14	R_1 = R_2 = Et	2-MeO-4-NO_2Ph	60
15	R_1 = R_2 = Et	2-NO_2-4-MeOPh	100
16	R_1 = DCHA*; R_2 = H	Ph	440
17	R_1 = DCHA; R_2 = H	4-FPh	320
18	R_1 = DCHA; R_2 = H	2,4-$(NO_2)_2$Ph	350
19	R_1 = DCHA; R_2 = H	4-NO_2Ph	>2000
20	R_1 = DCHA; R_2 = H	2-MeO-4-NO_2Ph	>2000
21	R_3 = DCHA; R_4 = H		>2000
22	R_3 = R_4 = Na		>2000
23			>2000

* DCHA = dicyclohexylammonium.

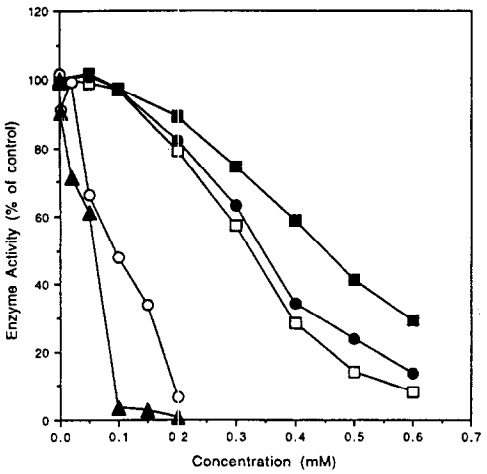

Fig. 2. Inhibition of PP_i-PFK activity from *T. gondii* by phosphonoacetic acid derivatives. Control (no additions) values varied from 0.01 to 0.02 enzyme units per 0.5 mL of reaction mixture. Structures of compounds **14–18** are given in Table 2. Key: (▲) **14**, (○) **15**, (■) **16**, (□) **17**, and (●) **18**.

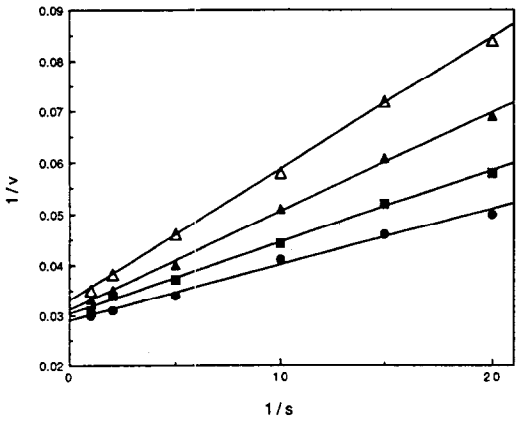

Fig. 3. Lineweaver–Burk plot of the inhibition of PP_i-PFK from *T. gondii* by various concentrations of compound **1**. Key: (●) no inhibitor, (■) 15 μM, (▲) 30 μM, and (△) 60 μM. s = $[PP_i]$ in mM. v = μmol fructose-1,6-bisP formed/min.

two fully dealkylated phenylhydrazone derivatives (compounds **19** and **20**) did not inhibit PP_i-PFK. Several other phosphonoacetic acid derivatives that showed no significant inhibition up to a concentration of 2 mM (compounds **21–23**) were also examined. It appears in comparing **14** with **20** that the triethyl group is important for inhibiting the enzyme.

Nature of inhibition by carbonyldiphosphonate, compound **1**. The most inhibitory compound, carbonyldiphosphonate, was tested against PP_i-PFK activity at various concentrations of PP_i to determine the nature of the inhibition. Lineweaver–Burk plots of enzyme activity in the presence of increasing concentrations of PP_i and at fixed concentrations of inhibitor are shown in Fig. 3. The kinetic data show that the regression lines did not intersect on the y-axis, indicating mixed-type inhibition.

Effect of compound **1** *on mammalian ATP-PFK.* When compound **1** was tested against sheep heart ATP-PFK, it did not inhibit this enzyme at concentrations up to 500 μM when the assays were done under optimal conditions (pH 8.2) [17]. Under conditions optimal for allosteric kinetics (pH 6.9) [18], compound **1** inhibited only at concentrations higher than 400 μM. These results (data not shown) when compared with its high inhibitory potency against PP_i-PFK (IC_{50} = 13 μM) showed compound **1** to be highly selective against the parasite enzyme.

Effects of compound **1** *and its derivatives on replication of* T. gondii *and human fibroblasts.* Tetrasodium carbonyldiphosphonate (compound **1**) and several of its derivatives that had low IC_{50} values were tested against replication of *T. gondii* in confluent HFF cells by measuring incorporation of [^{3}H]uracil [19, 20]. Experiments were done with the same compounds to determine proliferation of the HFF cells by measuring the incorporation of [^{3}H]-thymidine (see Materials and Methods). Representative results of a 2-day exposure to some of these compounds are shown in Fig. 4. Compound **1** appeared to have no significant inhibitory effect on the replication of *T. gondii* and only a minor inhibition of human fibroblast cells at 500 μM (Fig. 4A). In Fig. 4B, it can be seen that compound **4** inhibited HFF cells almost as much as it inhibited *T. gondii*. There was no selective inhibition shown. Inhibition of the replication of toxoplasma by compound **5** was greater than its inhibition of the HFF cells (Fig. 4C). This compound showed a more significant difference between toxoplasma inhibition and HFF cell inhibition than did compound **4**. Results for compound **6** were similar to those for **4**, and results for compound **7** were similar to those for compound **1** (data not shown). Thus, the two water-soluble compounds (**1** and **7**) did not appear to inhibit replication of the HFF cells or the toxoplasma, whereas the DMSO-soluble analogs (compounds **4–6**) inhibited both to varying degrees. The presence of negative charges in these water-soluble compounds may have impeded their entry into the cells.

Compounds **2** and **3**, which had higher IC_{50} values than compound **1**, were tested once on the proliferation of toxoplasma. They did not appear to have a significant inhibitory effect and were not tested further. The two oxophosphonoacetate arylhydrazones (compounds **14** and **15**), which had low IC_{50} values, were also tested once on the proliferation of toxoplasma (data not shown). At a concentration of 250 μM they inhibited much less than the arylhydrazone derivatives **4–6**. Compounds **14** and **15** were not soluble enough in the cell culture medium to warrant further experiments.

Microscopic examination of HFF cells treated with bisphosphonates. We examined Giemsa-stained culture plates of control HFF cells and those treated for 3 days with one of the following compounds: **1**, **4**, and **5**. Figure 5 contains pictures of these cells. Control fibroblast cells are shown in Fig. 5A, while

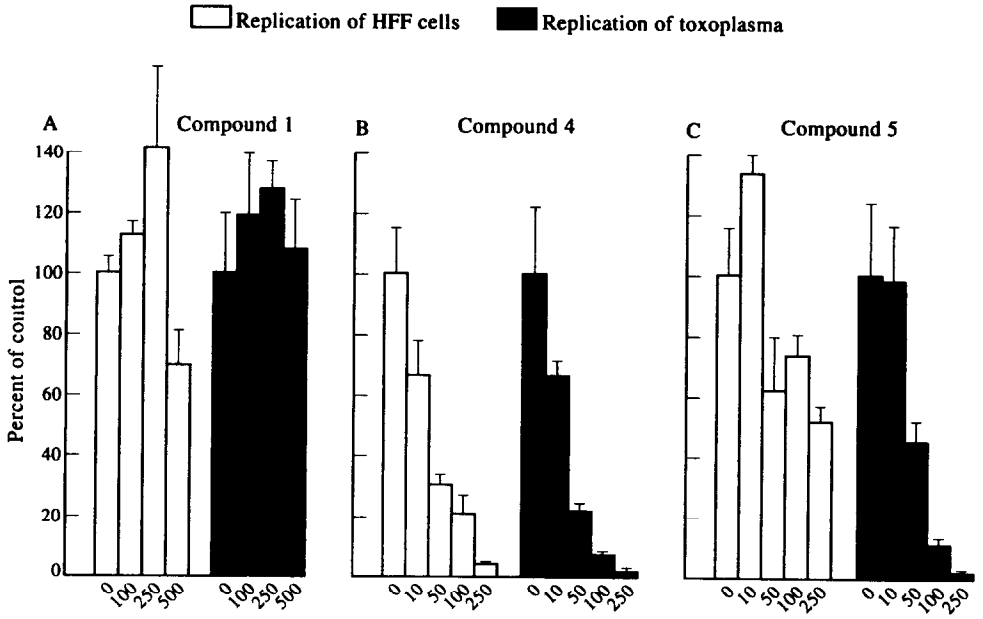

Fig. 4. Effects of bisphosphonates on the replication of human foreskin fibroblast cells measured by incorporation of [^{3}H]thymidine and on the replication of *T. gondii* tachyzoites growing in the human cells measured by the incorporation of [^{3}H]uracil (see Materials and Methods). Replication is shown as a percent of control (100%) at different drug concentrations of compounds **1** (A), **4** (B), and **5** (C). Values are means ± SD of 3–5 determinations. The control value for replication of HFF cells was 8120 cpm. The control value for replication of toxoplasma was 7630 cpm. Values on the x-axis are concentrations of compounds (μM). Structures of these compounds are given in Table 1.

those infected with *T. gondii* are shown in Fig. 5E. Almost complete destruction of the mammalian cells was the result of *T. gondii* infection (Fig. 5E). The effects of compound **1** are shown in uninfected (Fig. 5B) and *T. gondii*-infected cells (Fig. 5F). The infected cells (Fig. 5F) exhibited slightly less cell destruction than untreated cells (Fig. 5E). It appears that compound **1** offered only minor protection to the mammalian cells from toxoplasma infection. This is due to the charged nature of the compound. As would be expected, the charges hinder its transport through mammalian and parasite membranes. Cells treated with compound **7** looked similar to cells treated with compound **1** (results not shown). It was observed that *T. gondii*-infected cells that were treated with either compound **4** or **5** (Fig. 5, G and H) were relatively free of *T. gondii* foci. These infected HFF cells appeared to be somewhat more clumped when compared with control cells that had not been infected (Fig. 5A). However, when the infected cells treated with compound **4** or **5** were compared with the untreated cells (Fig. 5E), it could be seen that these compounds offered almost complete protection from the cell damage seen in the untreated cells. Compound **6** was almost as protective as compound **5** (results not shown). Compounds **4–6**, which are non-charged esters of compound **1**, would be expected to penetrate cell membranes more easily. When the uninfected and untreated HFF cells in (A) were compared with the HFF cells treated with compounds **1** (B), **4** (C), or **5** (D), it can be seen that there were no apparent morphological changes in the HFF cells. These microscopic observations indicate that although compounds **4** and **5** inhibited replication of the HFF cells (see Fig. 4), they did not appear to cause observable morphological changes to the HFF cells and protected the cells from destruction by *T. gondii*.

Reversibility of the inhibition of replication by compound **5** *of the HFF cells or the toxoplasma.* To determine if the inhibitory effects of compound **5** on replication in fibroblasts and *T. gondii* cells were permanent or reversible, we cultured HFF cells and toxoplasma-infected cells with or without drug. After a 2-day exposure to drug, we removed all medium from the cells. In half the cells, the fresh medium contained no drug and in the other half the medium contained the same concentration of compound **5** that had been present during the first 2 days. The results of a representative experiment are shown in Fig. 6. Comparing continuous drug treatment of the HFF cells (left panel, light grey and dark grey) with continuous treatment of the toxoplasma (right panel, black and diagonal stripes), it can be seen that the inhibition of replication in the HFF cells was less than in the toxoplasma. However, when drug was replaced by medium with no drug on day 2, the inhibition of replication on days 3 and 4 was reversed dramatically in the HFF cells that had been treated (light grey) with a 100 μM concentration of compound **5** and partially reversed even from 250 μM treatment. In contrast, the toxoplasma were not able to recover

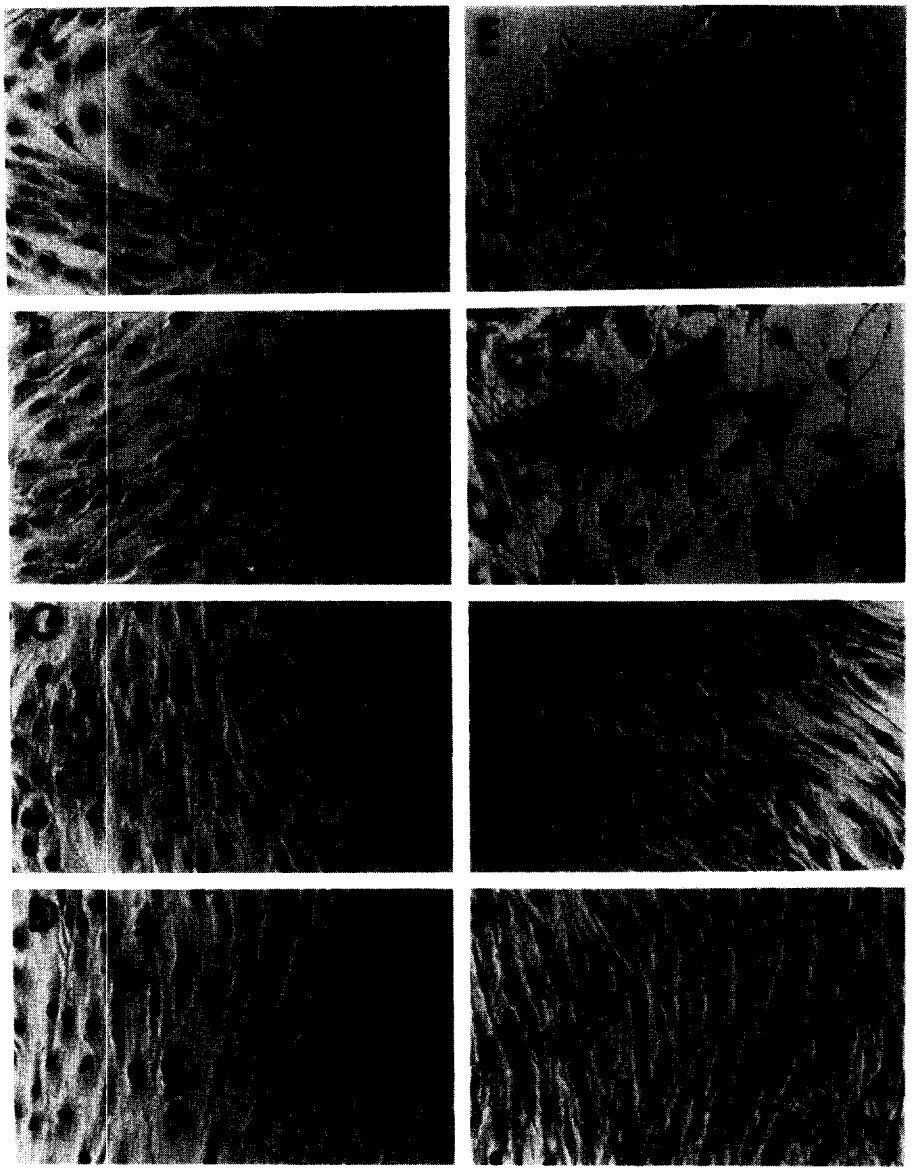

Fig. 5. Microscopic examination of HFF cells (A–D) and toxoplasma-infected HFF cells (E–H) treated with bisphosphonic acid derivatives. The cells were treated with the compounds for 3 days and stained with Giemsa. Key: A, E—no treatment; B, F—500 μM compound **1**; C, G—250 μM compound **4**; D, H—250 μM compound **5**. Structures of these compounds are shown in Table 1.

from 2-day treatment (black) with a 250 μM concentration of compound **5**. On day 4, the toxoplasma showed only a 23% recovery from treatment with 100 μM **5**. On days 3 and 4, some of the toxoplasma treated with 100 μM compound **5** were left alive so that replication could resume. At 250 μM, the toxoplasma did not seem to recover and resume replication.

DISCUSSION

In this paper, we report on the inhibitory effects of phosphonic acid derivatives on PP_i-PFK from *T. gondii*. We have chosen five of these inhibitors and examined their effects on mammalian and parasite cell replication. To assess the selectivity of these inhibitors and their accessibility to the intracellular parasite, we also tested the effects of these inhibitors on the replication of the host mammalian cells. The results show that compound **1** is the most potent inhibitor of PP_i-PFK. Exchanging the carbonyl group with another chemical moiety such as a hydrazone imino group (C=NNHAr) or replacing one phosphonate group by a carboxylic group reduced the inhibition of the enzyme in the series of compounds tested. A series of bisphosphonates in which the α-carbon is tetragonal (**8–13**), rather than

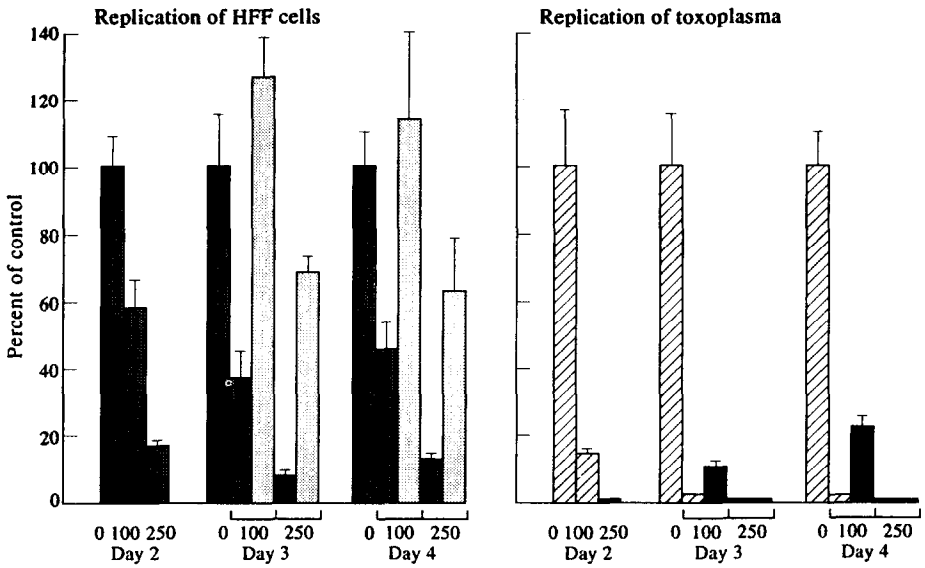

Fig. 6. Reversibility of inhibition of replication of HFF cells and *T. gondii* after a 2-day treatment with compound **5**. In the left panel, day 2 represents replication of HFF cells after 2 days with no drug addition or with a 100 or 250 μM concentration of compound **5** (▩). Day 3 replication is shown after a change in the medium at day 2 with either the same additions as day 2 or with medium containing no drug (▨). Bars shown for day 4 represent replication of HFF cells with no further change in the medium. In the right panel, day 2 represents replication of *T. gondii* either with no drug addition or with compound **5** at concentrations of 100 or 250 μM (▨). Replication in day 3 was measured after changing the medium either with the same drug concentrations as in day 2 or with medium containing no drug (■). Replication in day 4 was measured with no further change in the medium. Replication was determined as described in Materials and Methods. Values are the means ± SD of 3–5 determinations. The control values for replication of HFF cells were (in cpm): day 2, 3460; day 3, 8850; day 4, 10,510. The control values for replication of toxoplasma were (in cpm): day 2, 9570; day 3, 10,230; day 4, 12,570. The structure of compound **5** is given in Table 1.

trigonal as in **1–7**, were inactive. It therefore appears that the chemical structure of compound **1** is a useful prototype for developing more potent analogs as enzyme inhibitors. Compound **1** inhibits PP_i-PFK of *T. gondii* presumably because it is a close analog of the enzyme substrate, pyrophosphate. It probably inhibits the enzyme by competing with the substrate at the active site (Fig. 3).

Our results show that although compound **1** was proven to be the most potent PP_i-PFK inhibitor, it did not show a significant inhibition on proliferation of intracellular *T. gondii* (Fig. 4A), and it therefore did not protect significantly the mammalian cells from destruction by the parasite (Fig. 5). We attribute this to the fact that compound **1** is a charged molecule, and its ability to enter the host and parasite cells is very limited. Methyl, ethyl, or isopropyl esters of compound **1** and their hydrazone derivatives showed reduced inhibition of PP_i-PFK. Since these compounds are more lipophilic, they would be expected to have more accessibility to the parasite growing inside mammalian cells. There is also some selectivity in their ultimate effect on parasite cell proliferation. Furthermore, compounds **4** and **5** showed an impressive protection of mammalian cells from the destruction caused by *T. gondii* infection (Fig. 5). Although the potential of compound **5** as an anti-toxoplasmic agent may be limited by its moderate selectivity against the parasite and possible toxicity, its potency may provide a useful lead for the further development of more selective drugs.

In the present investigation, we have focused on the inhibitory effects of phosphonates on PP_i-PFK activity and the effects of five of these inhibitors on inhibition of *T. gondii* proliferation in mammalian cells. Although we report that several moderately potent phosphonates are able to inhibit both PP_i-PFK and protect mammalian cells from destruction by *T. gondii*, we are not yet in a position to draw a definite linkage between the two events. More studies are needed to determine whether the inhibition of *T. gondii* replication is related to inhibition of PP_i-PFK. Compound **5** may be especially useful in trying to identify the site of action of these derivatives on the parasite cell. Studies testing the effect of this compound on intact *T. gondii* cells may contribute to a better understanding of the cellular function of their PP_i-PFK.

The ultimate goal of this research is to develop a new chemotherapeutic agent against *T. gondii*. The discovery of PP_i-PFK as a target for prospective inhibitors, such as phosphonic acid analogs, is an important step in the rational approach to chemotherapy. Since ATP-PFK is an important rate-limiting enzyme in mammalian cells, we expect that the PP_i-PFK may play an analogous role in the

parasite. Thus, inhibition of PP_i-PFK may deprive toxoplasma of its main source of energy. Since mammals do not have PP_i-PFK, it is an ideal target for chemotherapeutic agents. Future work will emphasize increasing the selectivity of inhibition as well as increasing accessibility to the parasite.

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