Effects of Bisphosphonates on the Growth of *Entamoeba histolytica* and *Plasmodium* Species in Vitro and in Vivo

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The effects of a series of 102 bisphosphonates on the inhibition of growth of *Entamoeba histolytica* and *Plasmodium falciparum* in vitro have been determined, and selected compounds were further investigated for their in vivo activity. Forty-seven compounds tested were active (IC_{50} < 200 μM) versus *E. histolytica* growth in vitro. The most active compounds (IC_{50} ∼ 4−9 μM) were nitrogen-containing bisphosphonates with relatively large aromatic side chains. Simple *n*-alkyl-1-hydroxy-1,1-bisphosphonates, known inhibitors of the enzyme farnesylpyrophosphate (FPP) synthase, were also active, with optimal activity being found with C9−C10 side chains. However, numerous other nitrogen-containing bisphosphonates known to be potent FPP synthase inhibitors, such as risedronate or pamidronate, had little or no activity. Several pyridine-derived bisphosphonates were quite active (IC_{50} ∼ 10−20 μM), and this activity was shown to correlate with the basicity of the aromatic group, with activity decreasing with increasing pK_a values. The activities of all compounds were tested versus a human nasopharyngeal carcinoma (KB) cell line to enable an estimate of the therapeutic index (TI). Five bisphosphonates were selected and then screened for their ability to delay the development of amebic liver abscess formation in an *E. histolytica* infected hamster model. Two compounds were found to decrease liver abscess formation at 10 mg/kg ip with little or no effect on normal liver mass. With *P. falciparum*, 35 compounds had IC_{50} values <200 μM in an in vitro assay. The most active compounds were also simple *n*-alkyl-1-hydroxy-1,1-bisphosphonates, having IC_{50} values around 1 μM. Five compounds were again selected for in vivo investigation in a *Plasmodium berghei* ANKA BALB/c mouse suppressive test. The most active compound, a C9 *n*-alkyl side chain containing bisphosphonate, caused an 80% reduction in parasitemia with no overt toxicity. Taken together, these results show that bisphosphonates appear to be useful lead compounds for the development of novel antiamebic and antimalarial drugs.

Introduction

Plasmodium falciparum is the major cause of malaria in humans, with 300−500 million individuals affected annually, causing ∼2−3 million deaths, while *Entamoeba histolytica* is the cause of tens of millions of cases of amebic dysentery and is the third leading cause of morbidity and mortality in humans, due to parasitic protozoa.[1,2] Conventional therapy for *P. falciparum* involves use of a range of compounds including mefloquine, artesunate, chloroquine, and quinine, while treatment for *E. histolytica* involves use of the drugs metronidazole or iodoquinol. However, resistance to many antimalarials is widespread and resistance to metronidazole is known in many pathogenic bacteria and protozoa,[3] so there is interest in the development of new and inexpensive drugs. In early work, Eubank and Reeves[4] found that hydrolytically stable analogues of pyrophosphate, bisphosphonates, had activity against *E. histolytica*, and they proposed that these compounds inhibited the parasite's pyrophosphate-dependent phosphofructokinase (PFK).[4] More recently, other results on *E. histolytica* were reported using several nitrogen-containing bisphosphonates.[5] These compounds are now known to be potent, nanomolar inhibitors of the enzyme

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farnesyl pyrophosphate synthase[6−12] (FPPS) and are used clinically to treat osteoporosis, Paget's disease, and hypercalcemia due to malignancy.[13] In addition, nitrogen-containing bisphosphonates have activity as herbicides,[6] a direct effect on some tumor cells,[14−17] as well as activity against several apicomplexan and trypanosomatid parasites. These include *Trypanosoma cruzi*,[18] *Leishmania donovani*,[19] *Leishmania mexicana*,[20] *Toxoplasma gondii*,[19,21] and *Cryptosporidum parvum*.[22] In some cases, bisphosphonate effects appear to be relatively specific against the protozoan, a phenomenon that is thought to be related to the presence of a pyrophosphate-rich metabolism in these cells and the presence of acidocalcisomes, electron-rich dense granules containing condensed phosphates.[18]

In our laboratories, we recently investigated the effects of 19 bisphosphonates on the intraerythrocytic growth of *P. falciparum*.[21] The results obtained were interesting in that only five of the bisphosphonates tested had measurable activity (IC_{50} < 200 μM), and the most active compounds were not the nitrogen-containing bisphosphonates used clinically for treating bone resorption disorders. Rather, our results showed that the more hydrophobic de-aza analogue of risedronate (2-phenyl-1-hydroxyethane-1,1-bisphosphonic acid), as well as simple alkyl bisphosphonates, were the most active species.[21] This observation is reminiscent of the earlier observation of Eubank and Reeves that 1-hydroxynonane-1,1-bisphosphonic acid is an inhibitor of the growth of *E. histolytica*[4] and the observation that other *n*-alkyl bisphosphonates have activity versus *T. cruzi*,[23] mediated most likely by inhibition of the parasites' farnesylpyrophosphate synthase.[24] Since it is known that small, very hydrophilic bisphosphonates, such as 1-hydroxyethane-1,1-bisphosphonate (etidronate, Didronel) and dichloromethane-1,1-bisphosphonate (clodronate), do not readily move across cell membranes,[25] we reasoned that the poor activity of the nitrogen-containing bisphosphonates in *P. falciparum* might arise, at least in part, due to poor transport properties because of the presence of a charged nitrogen atom in the alkyl/aryl side chain. That is, the more lipophilic bisphosphonates (containing *n*-alkyl side chains) might have improved uptake across the erythrocyte membrane, resulting in improved IC_{50} values, and perhaps, the activity of the alkylbisphosphonates in some systems might be due to enhanced uptake.

In this paper, we report on the activity of a broad variety of nitrogen-containing and non-nitrogen-containing bisphosphonates against both *E. histolytica* and *P. falciparum*. A pattern of activity emerges in which simple *n*-alkyl bisphosphonates and nitrogen-containing bisphosphonates containing large and/or uncharged side chains have in general much greater activity in inhibiting cell growth than do the nitrogen-containing bisphosphonates that are currently used in bone resorption therapy, such as risedronate, alendronate, and pamidronate. The toxicity of the compounds investigated was estimated using a human cell line, and based in part on these results, several bisphosphonates were evaluated for their ability to reduce amebic liver abscess formation in an *E. histolytica* infected hamster model and for their activity in a *Plasmodium berghei* BALB/c mouse suppressive test.

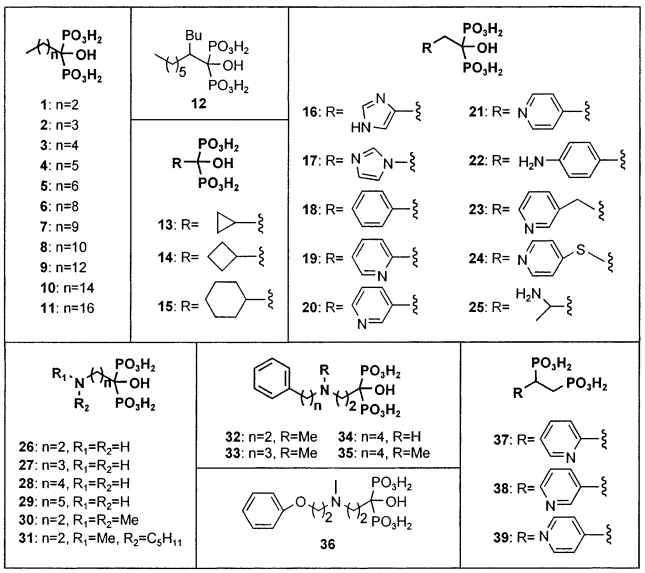


Figure 1. Structures of the 1-hydroxy-1,1-bisphosphonates and 1,2-bisphosphonates investigated.

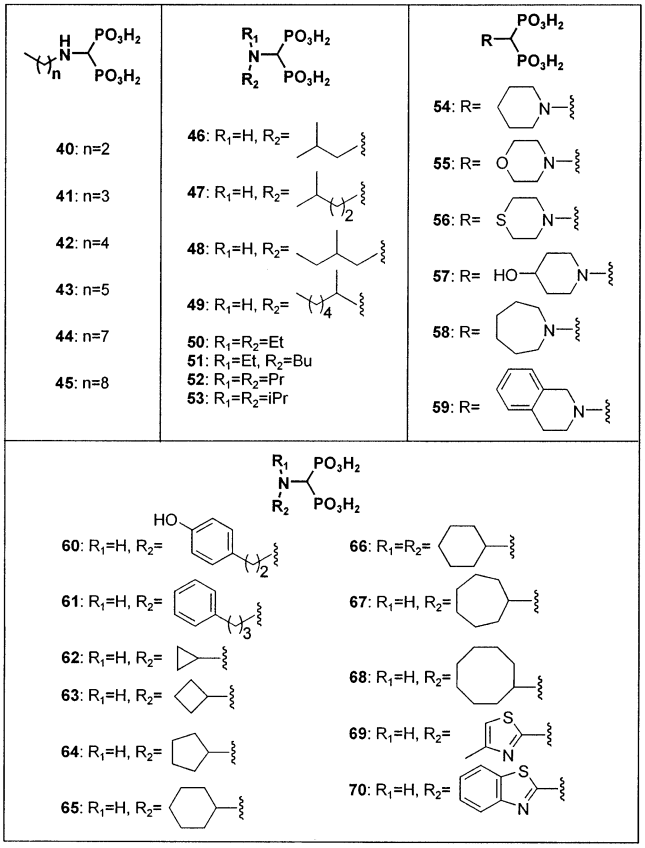


Figure 2. Structures of the aminomethylene bisphosphonates investigated.

Results and Discussion

***E. histolytica* in Vitro Testing.** We show in Figures 1−3 the structures of the compounds tested. A wide range of structural motifs were investigated, and the basic structures are shown in bold in the figures. Figure 1 is composed primarily of 1-hydroxy-1,1-bisphosphonates, where the side chain extends from a backbone carbon that is also bonded to two phosphonate groups and one hydroxyl group. We also made three novel 1,2-bisphosphonates, in which the phosphonate groups are

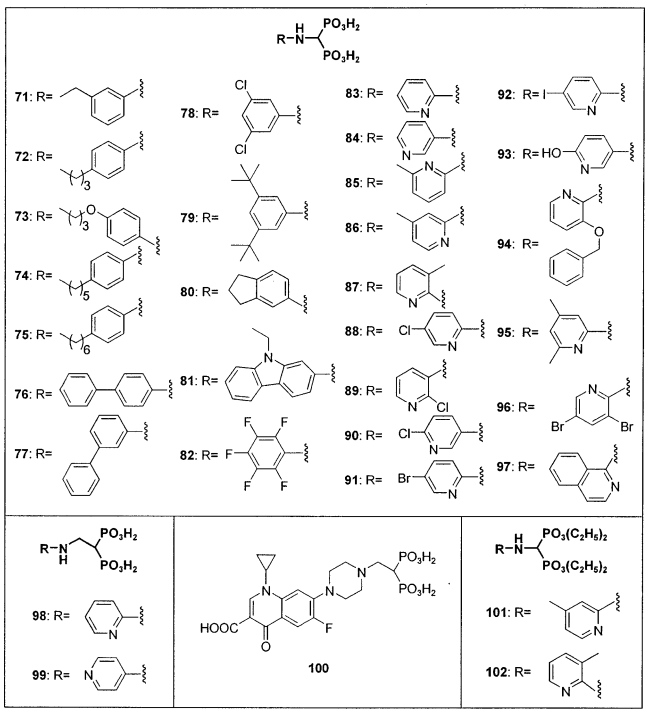


Figure 3. Structures of the arylaminomethylene bisphosphonates, aminoethylene bisphosphonates, and bisphosphonate tetraethyl-esters investigated.

attached to adjacent carbons. Figure 2 consists of aminomethylene 1,1-bisphosphonates, in which the backbone carbon is bonded to a nitrogen in the side chain and two phosphonate groups. Figure 3 shows additional aminomethylene 1,1-bisphosphonates tested, derived from anilines and aminopyridines, as well as three amino*ethylene* 1,1-bisphosphonates and two tetraethyl bisphosphonate esters, which were investigated to see if they had enhanced activity due to enhanced lipophilicity.

Table 1 shows the IC_{50} values for all compounds (**1**−**102**) tested. The experimental concentrations required to reduce parasite proliferation by 50%, the IC_{50} values, were extracted from experimental growth-versus-inhibitor concentration assays by fitting the experimental data to the rectangular hyperbolic function

$$I = \frac{I_{max} C}{IC_{50} + C} \quad (1)$$

where I is the percent inhibition, $I_{max} = 100\%$, and C is the concentration of the inhibitor (μM). Representative graphical results (for compounds **5**, **8**, **31**, and **44**) are shown in Figure S1 of the Supporting Information.

The 47 bisphosphonates that showed activity (IC_{50} < 200 μM) against *E. histolytica* arise from several different classes of compound. Among the *n*-alkyl side chain containing bisphosphonates, eight of the 11 compounds tested showed activity (Table 1 and Figure 4). The C5 compound (**3**) was the least active species ($IC_{50} = 157$ μM). Activity increased as the chain length increased, to a maximum activity at C10 (**7**, 11.0 μM), before dropping off once again for the longer chain compounds (Figure 4). The branched alkane side chain, **12**, also showed good activity, at 12.4 μM. The three alicyclic compounds (**13**−**15**, Table 1) were inactive. The

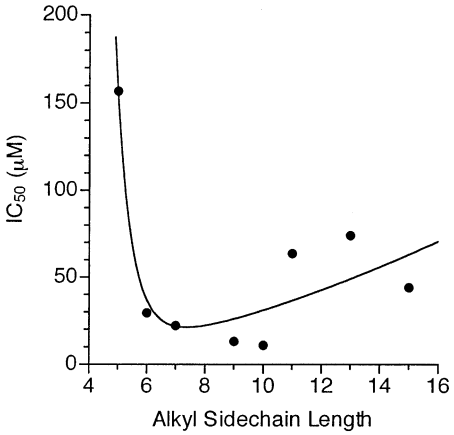
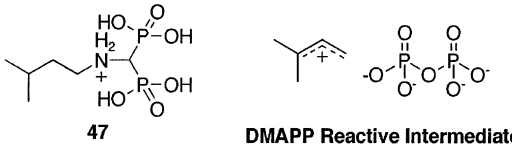


Figure 4. Graph showing the effect of *n*-alkylbisphosphonate side chain length on IC_{50} values against *E. histolytica*. The line is to guide the eye.

commercially available bisphosphonates zoledronate (**17**) and risedronate (**20**) were active, with IC_{50}s of 12.0 and 73.5 μM, respectively, but the chemically similar bisphosphonates **16**, **19**, and **21,** were all inactive. Of the alkyl, nitrogen-containing 1-hydroxy bisphosphonates, only the long tertiary amine compound **31** (ibandronate) had measurable activity, at 53.6 μM. All of the aminopropylidene bisphosphonates containing a phenyl ring, **32**−**36**, were active, with IC_{50}s of 143, 45.1, 20.5, 23.4, and 6.49 μM, respectively. None of the 1,2-bisphosphonates (all with pyridyl side chains), **37**−**39**, had any measurable activity.

Among the *n*-alkyl secondary amine side chain bisphosphonates, only the two longest compounds, **44** (31.1 μM) and **45** (136 μM), had activity against *E. histolytica*. The branched alkane secondary amines, **47**−**49**, were active compounds with respective IC_{50}s of 36.9, 135, and 65.3 μM, yet the structurally similar compound **46** was not. Why is **46** inactive? Clearly, **46** has the smallest alkyl side chain in the monoalkyl species **46**−**49**, so this could be a factor, and indeed, as shown in Figure 4 (in another homologous series), large effects can be seen on increasing chain size. Interestingly, we found compound **47** to be the most active of compounds **46**−**49**, and it certainly seems possible that these compounds might be FPP synthase inhibitors in *E. histolytica* since, as shown in Table 2, on average their IC_{50} values versus *Leishmania major* FPPS are only 780 nM. Plus, the most active species found here would appear to most closely resemble the dimethylallyl pyrophosphate (DMAPP) transition state/reactive intermediate, since it has a branched C5-side chain:



47 **DMAPP Reactive Intermediate**

The tertiary amine side chain compounds, **50**, **51**, and **53**, all had measurable activity (62.6, 47.3, and 194 μM, respectively), but the closely related compound **52** was inactive (IC_{50} > 200 μM). For these dialkylated compounds, the presence of two C3 side chains (**52**, **53**) results in low activity, presumably due to a steric effect, but species containing at least one smaller ethyl group

Table 1. Growth Inhibition (IC_{50}) Data for *P. falciparum* and *E. histolytica*, Toxicity Data (LD_{50}) for a KB Cell Line, and Computed Therapeutic Index (TI) Results for Bisphosphonates

	IC_{50} (μM)			TI			IC_{50} (μM)			TI	
compd	*E. his.*[a]	*P. fal*[b]	LD_{50} (μM)[c]	*E. his.*[d]	*P. fal*[e]	compd	*E. his.*[a]	*P. fal*[b]	LD_{50} (μM)[c]	*E. his.*[d]	*P. fal*[e]
1	>200	130	>967		>7.44	**52**	>200	>200	322		
2	>200	56.7	>1041		>18.4	**53**	194	>200	852	4.39	
3	157	5.53	>993	>6.33	>180	**54**	>200	>200	>1090		
4	29.5	4.34	109	3.69	25.1	**55**	>200	18.4	964		52.4
5	22.3	26.7	308	13.8	11.5	**56**	>200	>200	>1119		
6	13.3	0.83	>775	>58.3	>933	**57**	>200	>200	>1009		
7	11.0	5.23	>806	>73.3	>154	**58**	>200	>200	>1098		
8	63.7	1.07	492	7.72	460	**59**	71.0	nd[g]	>922	>13.0	
9	74.0	2.12	36.8	0.50	17.4	**60**	>200	>200	99.8		
10	44.0	28.8	>729	>16.6	>25.3	**61**	15.6	>200	>970	>62.2	
11	>200	>200	<0.65			**62**	113	>200	246	2.18	
12	12.4	>200	228.1	19.1		**63**	>200	>200	260		
13	>200	>200	>1244			**64**	35.9[f]	>200	629	17.5	
14	>200	>200	>1219			**65**	16.0	>200	272	17.0	
15	>200	>200	1086			**66**	>200	>200	>844		
16	>200	>200	41.0			**67**	>200	>200	166		
17	12.0[f]	167	63.7	5.31	0.38	**68**	15.5	>200	354	22.9	
18	>200	7.7	>931		>121	**69**	>200	>200	150		
19	>200	>200	13.9			**70**	nd	>200	<0.93		
20	73.5	123	249	3.46	2.02	**71**	>200	>200	>1016		
21	>200	>200	171			**72**	172[f]	31.3	278	1.62	8.89
22	>200	>200	218			**73**	3.98	>200	210	52.7	
23	>200	>200	>804			**74**	132	46.3	373	2.83	8.06
24	>200	158	>746		>4.72	**75**	36.2	103	211	5.83	2.05
25	>200	>200	>977			**76**	8.76	76.6	495	56.5	6.46
26	>200	>200	156			**77**	6.60	>200	749	113	
27	>200	>200	145			**78**	>200	>200	314		
28	>200	>200	91.0			**79**	19.2	>200	634	33.0	
29	>200	>200	680			**80**	>200	>200	>976		
30	>200	>200	>1052			**81**	>200	104	710		6.83
31	53.6	59.1	10.1	0.19	0.17	**82**	>200	>200	>779		
32	143	33.55	>739	>5.20	>22.0	**83**	>200	>200	465		
33	45.1	22.8	379	8.40	16.6	**84**	60.0	>200	782	13.0	
34	20.5	>200	>727	>35.5		**85**	64.0[f]	>200	18.6	0.29	
35	23.4	2.90	>690	>29.5	>238	**86**	>200	>200	127		
36	6.49	31.4	312	48.1	9.94	**87**	>200	>200	187		
37	>200	>200	<1.1			**88**	87.0	10.6	>991	>11.4	>93.5
38	>200	>200	1021			**89**	>200	>200	>963		
39	>200	>200	>1123			**90**	21.7	>200	>992	>45.7	
40	>200	>200	499			**91**	44.2	>200	86.7	1.96	
41	>200	>200	212			**92**	39.5	>200	466	11.8	
42	>200	4.8	>1148		>240	**93**	96.1	197	257	2.67	1.30
43	>200	56.5	>966		>17.1	**94**	>200	>200	>764		
44	31.1	13.3	66.8	2.15	5.02	**95**	177	>200	633	3.58	
45	136[f]	112	>919	>6.76	>8.18	**96**	28.5[f]	>200	520	18.2	
46	>200	>200	50.5			**97**	>200	50.4	673		13.3
47	36.9	>200	480	13.0		**98**	>200	17.0	144		5.12
48	135	>200	394	2.92		**99**	>200	>200	386		
49	65.3[f]	103	>989	>15.1	>9.64	**100**	>200	>200	>568		
50	62.6[f]	90.1	809	12.9	8.98	**101**	>200	>200	127		
51	47.3	>200	>1055	>22.3		**102**	>200	>200	>790		

[a] IC_{50} (μM) for *E. histolytica* (metronidazole $IC_{50} = 3.2$ μM). [b] IC_{50} (μM) for *P. falciparum* (chloroquine $IC_{50} = 0.012$ μM). [c] LD_{50} (μM) for a KB (human nasopharyngeal carcinoma) cell line. [d] Therapeutic index for *E. histolytica*. [e] Therapeutic index for *P. falciparum*. [f] IC_{50} determined analytically, utilizing eq 1, from the average inhibition of multiple trials (≥3) at 100 μM. [g] nd denotes value not determined.

(**50**, **51**) are more active. The only fused ring tertiary amine active against *E. histolytica* was the tetrahydroisoquinoline derivative **59** (71.0 μM). The arylalkyl side chain of compound **61** confered relatively good activity, at 15.6 μM. Among the cycloalkane-containing aminomethylene bisphosphonates, the propyl (**62**, 113 μM), pentyl (**64**, 35.9 μM), hexyl (**65**, 16.0 μM), and octyl (**68**, 15.5 μM) species were all active, in sharp contrast to the lack of activity of the nitrogen-free alicyclic compounds, **13**−**15**.

Seven of the 12 aniline derivatives were active, three of which had alkyl substitutions on the ring. These compounds, **72** (172 μM), **74** (132 μM), and **75** (36.2 μM), have increasing activity as the substitutent chain length increased, i.e., as the molecules became more hydrophobic. Compound **73**, which contains a butoxy subtituent on the ring, was the most active compound screened, at 3.98 μM. The biphenyl side chain containing bisphosphonates (**76**, **77**) were also very active, with the *para* derivative (**76**, 8.70 μM) being slightly less active than the *meta* species (**77**, 6.60 μM). Compound **79**, the *meta* di-*tert*-butyl aniline, also showed activity, at 19.2 μM.

Of the 14 aminopyridine derivatives, nine had activity (IC_{50} < 200 μM), including the 3-pyridine-derived compound **84** (60.0 μM), but not the 2-pyridine isomer, **83**. Conversely, the two active alkyl-substituted aminopyridines were both 2-pyridine derivatives. Compound **85** (64.0 μM) has a methyl substitution in the 3

Table 2. Activity against *E. histolytica* and *L. major* FPPS

	IC_{50} (μM)			IC_{50} (μM)	
no.	*E. histolytica*	*L. major* FPPS	no.	*E. histolytica*	*L. major* FPPS
73	3.98	>100	**91**	44.2	1.4
36	6.49	0.45	**33**	45.1	0.50
77	6.60	10−100	**51**	47.3	nd
76	8.76	nd[a]	**31**	53.6	0.48
7	11.0	3.42	**84**	60.0	0.16
17	12.0	0.11	**50**	62.6	18.2
12	12.4	6.75	**8**	63.7	7.75
6	13.3	2.37	**85**	64.0	1.29
68	15.5	2.44	**49**	65.3	1.14
61	15.6	10.74	**59**	71.0	nd
65	16.0	0.16	**20**	73.5	0.17
79	19.2	>100	**9**	74.0	10−100
34	20.5	0.25	**88**	87.0	0.24
90	21.7	>100	**93**	96.1	nd
5	22.3	19.94	**62**	113	40.5
35	23.4	0.49	**74**	132	nd
96	28.5	nd	**48**	135	0.36
4	29.5	5.88	**45**	136	0.90
44	31.1	1.06	**32**	143	0.31
64	35.9	0.54	**3**	157	9.6
75	36.2	10−100	**72**	172	10−100
47	36.9	0.83	**95**	177	>100
92	39.5	nd	**53**	194	>100
10	44.0	6.5			

[a] nd denotes not determined.

position on the ring and compound **95** (177 μM) has methyl groups at positions 3 and 5 on the ring, yet compounds **86** (methyl substitution at the 5 position) and **87** (methyl substitution at position 6) had no discernible activity, although all four were derived from 2-pyridine. Compound **93**, the 4-hydroxy-3-pyridine derivative, had an IC_{50} of 96.1 μM. The remaining five active aminopyridines all contained halogen substituents. The activity of compounds **88** (87.0 μM), **91** (44.2 μM), and **92** (39.5 μM), which are all derived from 2-pyridine with a halogen (chlorine, bromine, and iodine, respectively) substitution at the para position, increased with the atomic weight of the halogen. Compound **96** (28.5 μM) is also a 2-pyridine derivative, but it has a dibromo substitution, at positions 4 and 6 on the ring. Of the halogenated 3-pyridine derivatives, **89** (chlorine at position 2) was inactive, while **90** (chlorine at position 4) was active, with an IC_{50} of 21.7 μM.

The aminoethyl bisphosphonates (**98**−**100**) were all inactive, including compound **100**, which is derived from the antibiotic ciprofloxacin. The tetraethyl ester bisphosphonates (**101**, **102**) were also inactive, as were their parent bisphosphonates (**86**, **87** respectively).

The results shown in Table 1 and as outlined above were quite surprising, since in previous work it has been shown that the enzyme farnesyl pyrophosphate synthase (FPPS) is the primary target of bisphosphonates in the (trypanosomatid) parasites *T. cruzi* and *T. brucei*,[24,26] as well as in bone resorption therapy.[12] Our hypothesis was, therefore, that FPPS would be the target for most of the bisphosphonates tested in these systems as well. However, from the results we have obtained here, this hypothesis may be incorrect, especially for the most active compounds.

For example, several of the compounds with the highest activity (<20 μM) against *E. histolytica* (**7**, **12**, **61**, **73**, **77**, **79**) have little activity (>3 μM) against an FPPS enzyme, while others that show potent FPPS activity (such as **23**) are inactive in other systems. This is demonstrated in Table 2, which shows the IC_{50} results for *E. histolytica* in rank order with, for comparison, IC_{50} results for a *Leishmania major* FPPS enzyme.[27] Twenty-five of the 47 compounds active against *E. histolytica* have only moderate to low activity (IC_{50} > 1 μM) against the *L. major* FPPS.[27] Moreover, overall, the activities are uncorrelated ($R^2 = 0.027$, P-value = 0.373, $n = 32$). This could indicate that either the *E. histolytica* FPPS is very different than the *L. major* enzyme or that there is more than one target or, in principle, both could be true.

The isoprene biosynthesis pathway in *E. histolytica* has not been extensively studied. However, it is extremely unusual, since there is no evidence for the presence of either classical mevalonate or nonmevalonate pathway enzymes.[28] There is, however, a sequence that corresponds to an archaeal multifunctional enzyme capable of synthesizing farnesyl pyrophosphate, so FPPS is certainly a possible target. When considering the top three active compounds (**73**, **36**, **77**), **73** and **77** are aniline derivatives that, because of their relatively low pK_a values, are expected to be poor FPPS inhibitors. However, **36** is a known potent FPPS inhibitor[29] and likely to inhibit *E. histolytica* FPPS. We conclude, therefore, that there is more than one target for the bisphosphonates in *E. histolytica*.

The structures of the most active, non-FPPS inhibitors are of some interest. Compounds **73**, **77**, and **79** are all aniline derivatives, containing phenoxylalkyl, biphenyl, and phenyl-di-*tert*-butyl side chains, respectively, but none of them are potent FPPS inhibitors (Table 2). Other actives include **7**, a simple *n*-alkyl; **12**, a branched alkyl; and **61**, a phenylalkyl bisphosphonate. All of these species have in common large, hydrophobic side chains, suggesting that compounds with more lipophilic side chains generally appear to have relatively good activity against *E. histolytica*.

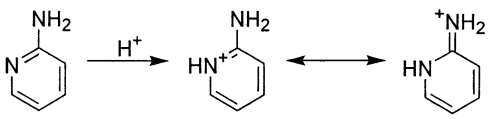
Lipophilicity also clearly plays a significant role in the activity of the *n*-alkylbisphosphonates, compounds that are also known to be moderate inhibitors of FPP synthase. While it might be thought that alkylbisphosphonates would not be FPP synthase inhibitors, since they lack the nitrogen positive charge feature responsible for carbocation transition state/reactive state mimicry, this is an oversimplification, since both electrostatic (positive charge, negative ionizable) as well as hydrophobic (van der Waals or London dispersion forces, i.e., attractive) interactions contribute to the overall potential energy of interaction of these drug molecules with their target. And for FPP synthase, the hydrophobic/steric interactions account for ∼60% of the total interaction energy, while electrostatic interactions (from both the bisphosphonate and positive N charge features) account for only ∼4% of the interaction energy.[21] Thus, removing just one part of the electrostatic potential (due to the positive charge feature) can be expected to have only a relatively small effect, especially if another interaction (the hydrophobic or van der Waals dispersion interaction) increases, as with an increase in chain length.

The activity of the alkylbisphosphonates is, in fact, very strongly dependent on overall side chain length.

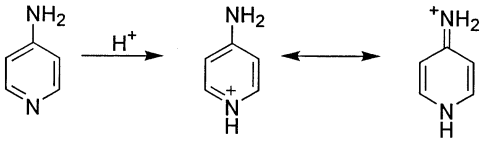
As shown in Table 1 and Figure 4, activity rapidly increases from **1** and **2** (C3, C4: not active) to **3** and **4** (C5, C6: 157 and 29.5 μM, respectively). Optimal activity is seen with the C9 and C10 alkyl side chains (**6**, **7**: 13.3 and 11 μM, respectively), then activity begins to fall off. At C11, C13 and C15, the IC_{50} increases to ~60 μM and for the very long (C17) species (**11**), the IC_{50} is >200 μM. This pattern of activity is not unexpected, however, since the short alkyl species will only have a weak interaction with FPP synthase, and it is well-known that chain elongation of FPP (which has an overall chain length of 12 carbons, excluding the methyl substituents) to GGPP does not occur with FPP synthase, due to the presence of phenylalanine, tyrosine, or histidine groups in the active site, which are thought to block chain elongation.[27] The effect of chain elongation on activity is also seen in enzyme inhibition data for FPP synthase (from *T. brucei*), where the IC_{50} increases from 3.5 μM (at C10) to 4.9 μM (at C11), and from 2.4 μM (C9) to 3.5 μM (C10) to 7.8 μM (C11) for the FPP synthase from *L. major* (see Table 2).[27] These results are all consistent with the early work of Reeves and Eubank, who found that the C8 alkylbisphosphonate was a good inhibitor of *E. histolytica* growth,[4] and based on our chain length dependence results (Figure 4), it appears that this length is close to an optimum one for the *n*-alkyl bisphosphonates. This length is also approximately the overall chain length of the GPP substrate (eight carbons) of FPP synthase. We propose, therefore, that alkylbisphosphonates are good inhibitors of *E. histolytica* growth, due at least in part to the lipophilic nature of their alkyl side chains in enhancing membrane transport, and targeting FPPS.

Another class of bisphosphonates exhibiting interesting growth inhibiton results are the pyridyl aminomethylene bisphosphonates, shown in Figure 3 (compounds **83**−**97**). These bisphosphonates were first developed by Nissan[30] and later by Zeneca[6] as herbicides, and they can be potent FPP synthase inhibitors, having low nanomolar K_i values.[6] However, several of these species (Table 1) appear to be inactive versus *E. histolytica*, while others (such as **85** and **88**), which are good FPP synthase inhibitors,[27] have only low activity. Are there some underlying principles that one might apply to give a general description of the activity of this class of compounds in *E. histolytica* growth inhibition?

Upon inspection, it can be seen that the two most active aminopyridine derivatives, **90** and **96**, are rather unusual (when compared with known FPP synthase inhibitors) in that **90** has a *m*-nitrogen as well as a *p*-chloro substituent, while **96** has a dibromo substitution. These substitutions can be expected to have very major effects on the pK_as of these compounds and, hence, on their protonation state. In the case of *o*-aminopyridines, there is expected to be extensive charge delocalization due to the presence of amidinium-like structures:



A similar resonance stabilization/charge delocalization effect can operate in the case of *p*-aminopyridines as well:



But in the case of the *m*-aminopyridines, there is no such resonance stabilization possible. As a result, the *m*-aminopyridine is the weakest base (computed[31] ortho pK_a = 6.67; meta pK_a = 6.16; para pK_a = 9.25). On halogen substitution, the electron-withdrawing effect of the halogens is pronounced, and results in even lower basicity. For example, in the case of **96**, addition of the two bromine atoms reduces the (computed) pK_a to ~1.90, an extremely weak base, while addition of the single chloro substituent in **90** (which already has a *m*-nitrogen) again results is extremely weak basicity, with a computed pK_a of 2.09. But what does this mean in terms of *E. histolytica* growth inhibition?

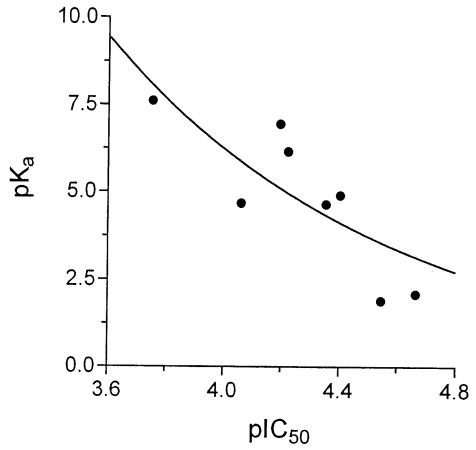


Figure 5. Graph showing correlation between the pIC_{50} [=−log IC_{50} (M)] for *E. histolytica* growth inhibition by bisphosphonates and the computed pK_a value of the parent base. The line is to guide the eye.

As shown in Table 1, **90** and **96** are active, and they will have very low pK_a values. However, five other pyridyl aminomethylene bisphosphonates are less active, and as shown in Figure 5, activity can be correlated with pK_a. In particular, the most active compounds have pK_a values of ~2. and at physiological pH values, their rings will be uncharged, while those of the less active species will be charged (to a greater or lesser extent). Of course, the trend shown in Figure 5 does not consider any steric or electrostatic interactions with the target protein. Rather, it simply indicates that the most active species will have uncharged side chains, just as found with the alkylbisphosphonates (and most likely, the aniline derivatives, **73**, **76** and **77**).

In conclusion, of the four most active compounds against *E. histolytica*, only one is active against farnesyl pyrophosphate synthase, and overall there is no correlation between *E. histolytica* growth and FPPS enzyme inhibition activity. If FPPS is not the universal target, then what are other likely targets? There are, of course, numerous intermediates in the isoprene and mevalonate pathways that are phosphorylated, where bisphosphonates could act as substrate mimics, and

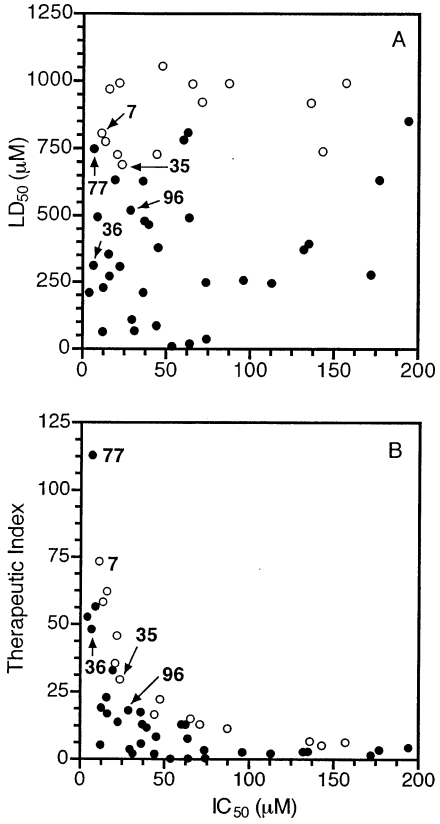


Figure 6. Scatter plots of (A) IC_{50} versus LD_{50} and (B) IC_{50} versus TI for *E. histolytica* growth inhibition by bisphosphonates. The open circles represent data points where only a lower limit to the LD_{50} or TI was determined. Compounds **7**, **35**, **36**, **77**, and **96** were investigated in vivo for their ability to delay the development of liver abscesses.

many more outside this pathway. One system that has been proposed previously is the *E. histolytica* phosphofructokinase, and we describe below additional experiments to test the hypothesis that this enzyme is the target for some of the more active inhibitors. Fortunately, however, even in the absence of a confirmed target for many of the most active species, the observation of quite potent inhibitory activity is, in and of itself, of considerable interest and encouraged us to test a selection of these compounds in vivo.

***E. histolytica* in Vivo Testing.** The patterns of *E. histolytica* growth inhibition by bisphosphonates are of interest since they are different than those seen with mammalian cells and this might, therefore, lead to the development of compounds that have selective activity against *E. histolytica*. For example, while the simple alkylbisphosphonates are active against *E. histolytica* at low micromolar concentrations, their effects on, for example, rat calvaria cells have been reported to be negligible.[30] Likewise, the lack of FPPS inhibition activity of two of the top three growth inhibitors could lead to antiamebic drugs with low toxicity. To further explore this topic in detail, we investigated if there was any correlation between the IC_{50} for parasite growth inhibition (the IC_{50}s) and the LD_{50} for growth of mammalian cells, using a human nasopharyngeal carcinoma (KB) cell line.[21] If the IC_{50} results are correlated, there is unlikely to be good parasite specificity. There is no correlation, however, as may be seen in Table 1 and Figure 6A, although we hasten to add that some data points (open circles) represent only lower limits for the LD_{50}. Nevertheless, there is no correlation either in cases where the LD_{50} values (closed circles) are known. Next we estimated a therapeutic index ratio (TI) for each of the bisphosphonates investigated, using the following definition

$$\mathrm{TI} = \frac{\mathrm{LD}_{50}}{\mathrm{IC}_{50}} \qquad (2)$$

in which LD_{50} is the concentration of drug that killed 50% of a human nasopharyngeal carcinoma (KB) cell line and IC_{50} is that for *E. histolytica* growth inhibition. This approach enables an estimate of which compounds might be efficacious in vivo. The numerical results for each compound are given in Table 1. We then plotted these therapeutic indices versus the IC_{50} values, obtaining the results shown in Figure 6B. Here, the solid circles again indicate data points for which discrete LD_{50} values were obtained, while the open circles indicate only lower limits for the therapeutic index, since no toxicity was observed at the highest bisphosphonate levels tested (300 μg/mL).

We selected a series of five compounds having good IC_{50} and TI values for in vivo testing in a hamster model of *E. histolytica* induced liver abscess formation. We chose the alkyl bisphosphonate **7** (IC_{50} = 11 μM, TI = >73.3), a known[29] potent bone resorption drug (*N*-[methyl(4-phenylbutyl)]-3-aminopropyl-1-hydroxy-1,1-bisphosphonate (**35**, IC_{50} = 23.4 μM; TI = >29.5), the phenoxyethyl analogue of *N*-Me pamidronate (**36**, IC_{50} = 6.49 μM; TI = 48.1), a biphenyl aminomethylene bisphosphonate (**77**, IC_{50} = 6.6 μM; TI = 113), and the dibromopyridyl aminomethylene bisphosphonate (**96**, IC_{50} = 28.5 μM; TI = 18.2). We tested each of these five compounds in pairs of hamsters, together with an infected but otherwise nontreated pair. Drugs were administrered 1 day after infection at 10 mg/kg ip for 5 days. Results for liver abscess weights and the weights of the normal liver fractions (equal to total liver weight minus liver abscess weight, in mg) are given in Table 3. Surprisingly, **36**, the most active compound tested in vitro, was ineffective in vivo. The largest decrease in liver abscess formation was seen with compound **35**, *N*-[methyl(4-phenylbutyl)]-3-aminopropyl-1-hydroxy-1,1-bisphosphonate. However, there was a statistically significant decrease in normal liver weight (26%), together with ascites formation and diarrhea. In contrast, the alkyl bisphosphonate **7** showed a 68% reduction in liver abscess formation but only a 9% reduction in normal liver weight (about the experimental uncertainty), while the biphenyl compound **77** showed a 36% abscess reduction with an insignificant change in normal liver weight. These are very promising initial in vivo results for reduction in liver abscess formation and suggest that **7** and **77** may represent useful new leads for the development of antiamebic drugs. Also of interest is the fact that these two species clearly cluster to the upper left of the TI/IC_{50} plot (Figure 6B), supporting the use of this representation to select the most promising drug leads.

***P. falciparum* Testing in Vitro.** We have also investigated the activity of the compounds shown in Figures 1−3 against *P. falciparum*, in vitro. Table 1

Table 3. Effects of Bisphosphonates on Liver Abscess Formation in *E. histolytica* Infected Hamsters[a]

	liver abscess (mg)				normal liver weight (mg)[b]			
drug	1[c]	2[c]	mean	% decrease	1[c]	2[c]	mean	% decrease
control	816	960	888	0	2552	2178	2365	0
7	516	53	285	68	2564	1761	2163	9
35	10	178	94	89	1667	1845	1756	26
36	1145	970	1058	−19	2314	2275	2295	3
77	561	588	570	36	2320	2597	2459	−4
96	853	680	767	14	2977	2788	2883	−22

[a] Two hamsters were treated at 10 mg/kg of the corresponding drug ip once a day for 5 days. [b] The normal liver weight is the difference between the total liver weight and the liver abscess weight. [c] The numbers refer to the two hamsters used in each experiment.

shows the IC_{50} values for all compounds tested. IC_{50} values were determined as described above using eq 1, and TI values using eq 2. Representative graphical results for compounds **9**, **33**, **42**, and **88** are shown in Figure S2 of the Supporting Information.

The 35 bisphosphonates that had measurable activity (IC_{50} < 200 μM) against *P. falciparum* again consist of a diverse collection of structures. Of the 11 *n*-alkyl side chain compounds, all but the longest (**11**, C17) showed activity. The span of activity for these compounds covers a range from 0.83 μM (**6**, C9) to 130 μM (**1**, C3), with six of the compounds having IC_{50}s below 10 μM. Neither the branched alkyl side chain compound (**12**) nor the cycloalkyl side chain compounds (**13**−**15**) had any discernible activity.

Both of the commercially available bisphosphonates, zoledronate (**17**) and risedronate (**20**), had modest activities (167 and 123 μM, respectively). However, the de-aza analogue of risedronate (**18**, 7.7 μM) was much more active. Isomers of zoledronate (**16**) and risedronate (**19** and **21**) were all inactive. A homologue of risedronate containing an ethyl-3-pyridyl side chain (**23**) was also inactive. Compound **24**, which has a 4-ethylmercaptopyridine side chain, also had measurable activity, with an IC_{50} of 158 μM.

The nitrogen-containing alkyl side chain compounds (**26**−**31**) were all inactive except for **31** (ibandronate), the longest of this set, which had an IC_{50} of 59.1 μM. Of the aminopropylidene bisphosphonates containing a phenyl ring, those that had a methyl group on the nitrogen, **32**, **33**, **35**, **36**, had activities of 33.6, 22.8, 2.90, and 31.4 μM, respectively. Compound **34**, which has a hydrogen instead of a methyl substituent on the nitrogen, was inactive. None of the pyridine-derived 1,2-bisphosphonates (**37**−**39**) showed any measurable activity.

Of the *n*-alkyl aminomethylene bisphosphonates, compounds **42**−**45** were active with IC_{50}s of 4.8, 56.5, 13.3, and 112 μM, respectively, but the only branched alkyl side chain to display activity was compound **49** (103 μM), and of the tertiary amine side chain containing species, the only active compound was the diethylamino species **50**, at 90.1 μM. The morpholino compound (**55**, 18.4 μM) was the only fused-ring tertiary amine to possess activity. None of the alkyl aromatic (**60**, **61**), cycloalkane (**62**−**68**), or thiazole (**69**, **70**) derivatives had any discernible activity.

Five of the 12 aniline derivatives were active, three of which had alkyl substitutions on the ring. Unlike the trend with *E. histolytica*, compounds **72** (31.3 μM), **74** (46.3 μM), and **75** (103 μM) had decreasing activity as the substituted chain length increased, although the effects (in both cases) were small. Of the biphenyl side chain bisphosphonates, the *p*-biphenylamine (**76**, 76.6 μM) was active while the meta isomer (**77**) was not. These activities were also much lower than those found with *E. histolytica*. Compound **81**, the *N*-ethylcarbazolyl derivative, also showed some activity, with an IC_{50} of 104 μM.

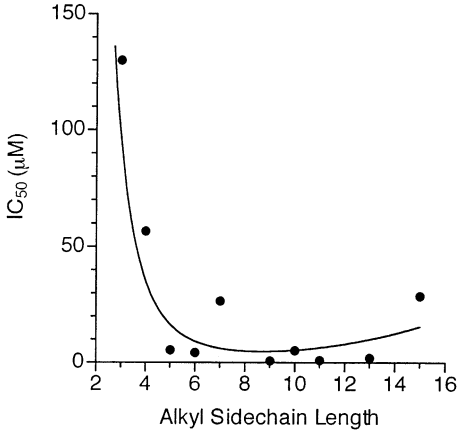


Figure 7. Graph showing the effect of alkyl side chain length of *n*-alkylbisphosphonates on IC_{50} values against *P. falciparum*. The line is to guide the eye.

Only three of the 15 aminopyridine derivatives showed activity. Compound **88** (10.6 μM) has a nitrogen at the 2 position and a chlorine at the 4 position. Compound **93**, the 4-hydroxy-3-pyridine derivative, had an IC_{50} of 197 μM and is essentially inactive (existing primarily as the zwitterionic or pyridone species) The isoquinolyl species, **97**, had an IC_{50} of 50.4 μM. Of the aminoethylene bisphosphonates (**98**−**100**), the 2-pyridine derivative was active (**98**, 17 μM), but the 4-pyidine and the ciprofloxacin derivatives (**99** and **100**, respectively) were not. As with *E. histolytica*, the tetraethyl bisphosphonate esters (**101**, **102**) were inactive, as were their respective parent bisphosphonates (**86**, **87**).

These results, while albeit of some complexity, are nevertheless again of considerable interest, since many of the most active compounds (four of the top six most active) are simple *n*-alkylbisphosphonates (**3**−**6**). For all of the *n*-alkyl compounds (**1**−**11**), we again find that there is a general pattern of increasing activity with increasing chain length for the short chain compounds, as shown in Figure 7, with good activity for the C5−C11 side chains, then generally decreased activity for the very long side chains. Similar results were found for *E. histolytica*, where two (**6**, **7**) of the top eight active compounds were *n*-alkylbisphosphonates, and the same general trend in side chain length vs activity was observed in Figure 4.

There are several similarities between the results obtained with the two organisms. Indeed, for the 35

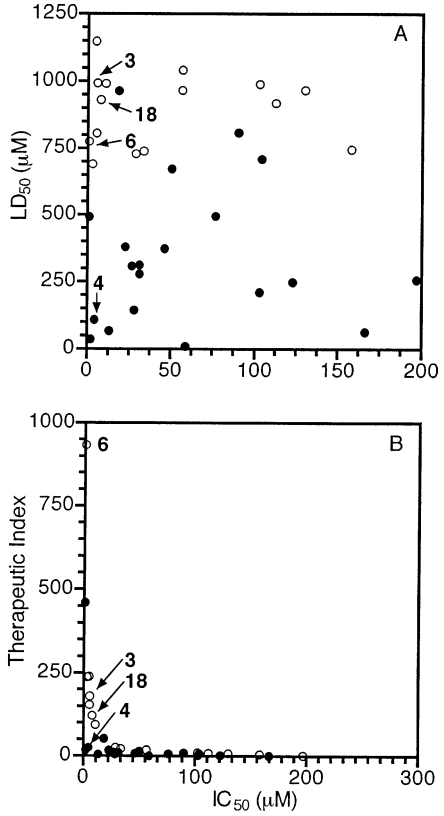


Figure 8. Scatter plots of (A) IC_{50} versus LD_{50} and (B) IC_{50} versus TI, for *P. falciparum*. The open circles represent data points where only a lower limit to the LD_{50} or TI was determined. Compounds **3**, **4**, **6**, and **18** were investigated in the *P. berghei* mouse suppressive test.

compounds active against *P. falciparum*, 25 also have activity versus *E. histolytica*. There are also similarities in the structures of the most active species, with the long, hydrophobic side chain containing species having, in general, higher activity in both organisms. This may suggest that membrane transport plays an important role, so development of additional lipophilic compounds appears to be a worthwhile goal. Indeed, McIntosh and Vaidya have suggested that there might be difficulty with cellular uptake of bisphosphonates in vitro with *Plasmodium* species,[33] citing previous work which concluded that endocytosis (specifically, fluid phase pinocytosis[34]) is the primary cellular uptake method for bisphosphonates. Since erythrocytes are incapable of endocytosis, some bisphosphonates may have difficulty in penetrating the erythrocyte membrane, unlike the situation with the trypanosomatids or macrophages or macrophage-related systems. Nevertheless, the observation that some bisphosphonates have ~1 μM IC_{50} values versus *P. falciparum* in vitro prompted us to investigate the activities of these species in vivo, with promising results.

***P. berghei* Testing in Vivo.** To assess which types of compounds might have potential for further development as antimalarial agents, we derived the therapeutic index values shown in Table 1, and IC_{50} vs LD_{50} and IC_{50} vs TI values are shown graphically in parts A and B of Figure 8, respectively. As may be seen in Figure 8A, there is no correlation between the IC_{50} (*P. falciparum*) and LD_{50} (KB cell line) results. From the therapeutic index/IC_{50} results (Figure 8B), it is clear

Table 4. Activity against *P. falciparum* and *L. major* FPPS

	IC_{50} (μM)			IC_{50} (μM)	
no.	*P. falciparum*	*L. major* FPPS	no.	*P. falciparum*	*L. major* FPPS
6	0.83	2.37	**32**	33.6	0.31
8	1.07	7.75	**74**	46.3	nd[a]
9	2.12	10−100	**97**	50.4	0.43
35	2.90	0.49	**43**	56.5	1.72
4	4.34	5.88	**2**	56.7	>100
42	4.80	0.35	**31**	59.1	0.48
7	5.23	3.42	**76**	76.6	nd
3	5.53	9.80	**50**	90.1	18.2
18	7.70	16.6	**49**	103	1.14
88	10.6	0.24	**75**	103	10−100
44	13.3	1.06	**81**	104	nd
98	17.0	0.17	**45**	112	0.90
55	18.4	>100	**20**	123	0.17
33	22.8	0.50	**1**	130	>100
5	26.7	19.9	**24**	158	>100
10	28.8	6.50	**17**	167	0.11
72	31.3	10−100	**93**	197	nd
36	31.4	0.45			

[a] nd denotes not determined.

Table 5. In Vivo Results for *P. berghei* ANKA Suppressive Test in BALB/c Mice

drug	dose (mg/kg)	schedule (days)	mean reduction in parasitemia (%)
3	25	4	80
4	10	4	48
6	10	2	75
18	25	4	64
26	25	4	5
chloroquine	10	4	100

that compounds **3**, **4**, **6**, and **18** have therapeutic indices >25 and low IC_{50} values (<10 μM). The IC_{50} values for **4** and **6** are particularly low, in the 0.5−5 μM range [**4**, IC_{50} = 500 nM (W2 strain), 500 nM (Ghana strain), 4.34 μM (3D7 strain); **6**, IC_{50} = 1 μM (W2), 2 μM (Ghana) and 830 nM (3D7)].

We selected compounds **3**, **4**, **6**, and **18** for in vivo testing in a *P. berghei* BALB/c mouse suppressive test. In addition, we tested pamidronate (**26**, Aredia), a second-generation bisphosphonate, to see if it had any effects on parasitemia reduction. On day 1, five female BALB/c mice per group were inoculated with 0.2 mL of 1% parasitemia (*P. berghei* ANKA, 1×10^7 infected RBCs). Drug administration commenced 2 h postinfection, ip. On day 5 (day 3 with **6**), tail smears were taken, fixed with methanol, and stained with 10% Giemsa's stain, and parasitemia was determined under oil immersion at 1000× magnification.

In a first series of experiments, we tested **3**, **18**, and **26** (pamidronate) at 25 mg/kg ip for 4 days. The mean reductions in parasitemia are shown in Table 5, where it can be seen that there is a 64% reduction in parasitemia for the benzyl bisphosphonate **18** and an even larger 80% reduction in parasitemia with **3**, the C5 bisphosphonate. This pattern of activity parallels their in vitro activity, as seen in Table 1. Next, we investigated the more active bisphosphonates **4** and **6**, this time using a reduced dosing scheme (Table 5). With **4**, there was a 48% reduction in parasitemia at 10 mg/kg × 4 ip and with **6** there was a 75% reduction at 10 mg/kg × 2 ip, although toxicity (as evidenced by panting and horripilation) was noted with **6**. Nevertheless, the 64% reduction seen with **18** and the 80% reduction in

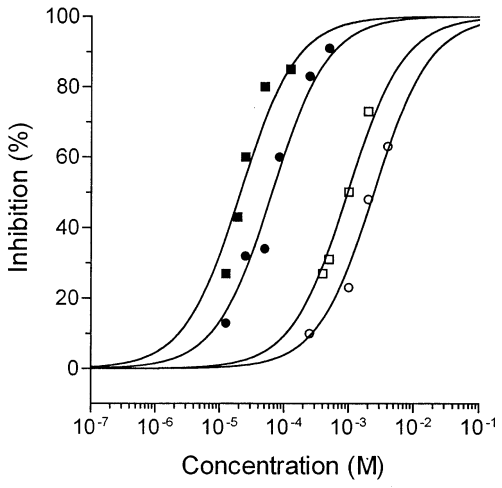


Figure 9. Dose−response curves for selected bisphosphonates against an expressed *E. histolytica* ATP PFK: **8**, ■; **6**, ●; **12**, □; **4**, ○. IC_{50} values deduced from these and similar plots for other compounds are given in Table 4.

Table 6. Effects of Bisphosphonates on the Activity of an Expressed *E. histolytica* Phosphofructokinase

	IC_{50}, μM			IC_{50}, μM	
drug	PFK activity[a]	*E. histolytica*[b] growth	drug	PFK activity[a]	*E. histolytica*[b] growth
7	20	11	**12**	1000	12.4
8	40	63.7	**4**	2500−3000	29.5
6	70	13.3	**44**	>3000	31.1
77	90	6.6	**31**	>3000	53.6
3	600−1200	156.8	**20**	>3000	73.5
18	900−1000	>200			

[a] Assay conditions: 50 M Ktes (pH 7.0), 100 mM KC1, 0.1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 0.5 mM Fru 6-P, 0.1 mM PPi, 30 °C. [b] *E. histolytica* growth inhibition, from Table 1.

parasitemia observed with **3** are, again, very promising initial in vivo results.

Other Possible Bisphosphonate Targets. Finally, we briefly consider the nature of some other possible non-FPP synthase targets for the bisphosphonates. In earlier studies, it was proposed that a pyrophosphate-dependent phosphofructokinase was the bisphosphonate target in *E. histolytica*, with a 40 μM K_i being reported for 1-hydroxynonane-1,1-bisphosphonate.[4] In more recent work, a much more active PFK has been isolated from *E. histolytica*,[35,36] so we investigated the inhibition of this enzyme using 11 different bisphosphonates. Phosphofructokinase is a potentially attractive target for growth inhibition in both *E. histolytica* and *P. falciparum*, since both organisms are reliant on glycolysis for ATP production, and it appears that the recently discovered enzyme is the one that is primarily responsible for PFK activity, at least in *E. histolytica*.[36]

We show in Figure 9 enzyme inhibition as a function of bisphosphonate concentration for a selection of compounds, and in Table 6, we show all the IC_{50} values derived from the experimental data. As may be seen from the results in Table 6, the most active PFK inhibitors are the C9−C11 *n*-alkyl bisphosphonates, which have IC_{50} values in the range 20−70 μM. The two most active species are **7** and **8**, but these IC_{50}s are ≳2 times higher than their IC_{50}s against *E. histolytica*. Likewise, the IC_{50} for *E. histolytica* growth inhibition for e.g. **4** is ∼30 μM, while the IC_{50} for *E. histolytica* PFK inhibition for **4** is ∼3 mM, a factor of 100 times greater. And in another example, the IC_{50} for *E. histolytica* PFK inhibition by risedronate (**20**) is >3 mM, although **20** is a ∼70 μM *E. histolytica* growth inhibitor and a known potent FPP synthase inhibitor (of both human and trypanosomatid enzymes). The IC_{50} values for each of these compounds in inhibiting *E. histolytica* growth (from Table 1) are also shown in Table 6 and are essentially uncorrelated with PFK inhibition (R^2 = 0.207, *P*-value = 0.160, n = 11). Some caution must be used, however, in trying to directly compare IC_{50}s for growth and PFK inhibition, since several compounds tested were found to be competitive inhibitors with respect to pyrophosphate (PPi), so the actual inhibitory potency in vivo will depend on the intracellular concentration of PPi, which is not known. Nevertheless, the observation that the relative potency of the bisphosphonates as PFK inhibitors was uncorrelated with their ability to inhibit growth makes it unlikely that phosphofructokinase is the major bisphosphonate target in *E. histolytica*, although it may be a secondary target for the alkyl bisphosphonates.

E. histolytica also employs a pyrophosphate-dependent pyruvate phosphate dikinase (PPDK) that catalyzes the reversible conversion of pyruvate to phosphoenolpyruvate. Compounds **17**, **36**, **73**, **75**−**77**, **79**, **84**, **93**, and **94** were tested against a recombinant *E. histolytica* PPDK. The lowest resultant IC_{50}s were greater than 60 μM, meaning that this enzyme is unlikely to be the target for the bisphosphosphonates (Ruy Pérez-Montfort, private communication).

Likewise, **4**, **7**, **20**, and **37** were tested for activity against another ATP-producing enzyme, a recombinant *Giardia lamblia* acetyl-CoA synthetase (ADP-forming). This enzyme is known in only two species, *G. lamblia* and *E. histolytica* (NCBI accession numbers AAD44021 and AAF88064, respectively), and there is moderate homology between the two enzymes (38% identity and 58% similarity; BLAST, http://workbench.sdsc.edu). The enzyme converts acetyl-CoA plus ADP to acetate plus ATP. The IC_{50} values were all >1 mM (Lidyá Sanchez, private communication), making this enzyme an unlikely drug target. Thus, while we have not yet fully established the bisphosphonate targets in these organisms, several of the active compounds studied are known to be inhibitors of either bone resorption or human or parasite (trypanosomatid) FPP synthases, while activity against phosphofructokinase, PPDK, and acetyl-CoA synthetase (ADP-forming) are very weak.

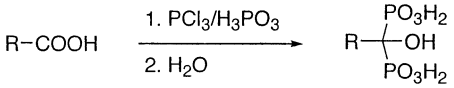
Conclusions

The results we have described above are of interest for several reasons. First, we have investigated the effects of 102 bisphosphonates on the growth of *E. histolytica*. Several of the most promising compounds in in vitro inhibition appear to be those with biphenyl (**76**, **77**), *n*-alkyl (**6**, **7**), arylalkyl (**35**, **36**), and phenoxyalkyl (**73**) side chains. A common characteristic of these compounds is the presence of long side chains (eight or more heavy atoms in a continuous chain). We also found an unusual pattern of reactivity with the bisphosphonates in that many potent nitrogen-containing species (such as pamidronate and risedronate) used as FPPS inhibitors in bone resorption therapy had relatively little

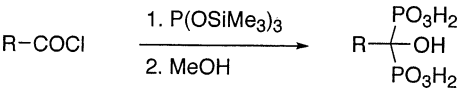
activity against *E. histolytica* proliferation in vitro, while most of the active compounds had low activity versus FPPS. Second, we found that *n*-alkylbisphosphonates have much more pronounced activity, as low as 11 μM in vitro. The results indicate that activity is chain length dependent, with optimal activity being found for a ~C9−C10 chain length. Third, we found a correlation between the activities of a series of pyridyl aminomethylene bisphosphonates and the pK_a values of the arylamine bases. These results suggest that low pK_a/deprotonated side chains are important for activity, consistent with the activity of the alkylbisphosphonates (which likewise do not contain a positive charge feature). Fourth, we investigated if there was any correlation between the activity of a given bisphosphonate in inhibiting the growth of a mammalian cell line and the activity of the given bisphosphonate in inhibiting *E. histolytica* cell growth. No correlation was evident. Fifth, we then used these results to estimate therapeutic indices for each compound. Five compounds found to have relatively low IC_{50} values and relatively high therapeutic indices were tested for their ability to delay the onset of liver abscess formation in a hamster model. At 10 mg/kg per day ip, we obtained a 68% reduction in liver abscess formation for compound **7** with no overt toxicity. For in vivo inhibition, **7** appears to be the best compound we have investigated to date; however, compound **77** also looks promising, since although it resulted in a smaller decrease in abscess formation, there was no effect on the host liver. Sixth, we investigated the activity of all compounds against the growth of *P. falciparum* in vitro. The most active compounds were the *n*-alkylbisphosphonates and we found a broadly similar dependence of activity on chain length to that seen with *E. histolytica* growth inhibition. Seventh, we made therapeutic index/activity plots for *P. falciparum* growth inhibition and used these to choose a small set of compounds for testing in vivo in a *P. berghei* BALB/c mouse suppressive test. The best inhibitor was a simple *n*-alkylbisphosphonate with a C5 side chain (**3**), which gave an 80% reduction in parasitemia in vivo with no overt toxicity. Eighth, we tested the idea that phosphofructokinase might be the principal bisphosphonate drug target in *E. histolytica*. For the eleven compounds studied, the potency of the bisphosphonates as phosphofructokinase inhibitors was uncorrelated with their ability to inhibit growth. The IC_{50} values for phosphofructokinase inhibition were also higher than the IC_{50}s for *E. histolytica* growth inhibition. Similar (negative) results were obtained for PPDK and acetylCoA synthase (ADP-forming). Taken together, these results are in contrast to many previous results obtained on *T. cruzi*, *T. brucei*, *T. gondii*, *L. donovani*, and *L. mexicana*, where nitrogen-containing bisphosphonates, such as pamidronate and risedronate, have been found to have the most potent antiparasitic activity, and suggest the importance of neutral side chains and extended hydrophobic interactions for optimal activity. The observation of considerable in vivo activity with both *E. histolytica* (liver abscess formation) and *P. berghei* (reduction in parasitemia) with some bisphosphonates suggests that these compounds represent potentially interesting leads for the development of antiamebic and antimalarial drugs.

Experimental Section

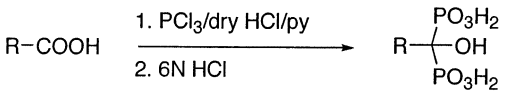
Bisphosphonates. We used the Merck method[37] for production of compounds **1**−**3**, **5**, **7**, **8**, **16**, and **18**−**30**.



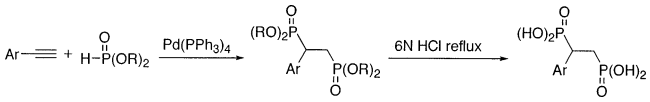
Compounds **4**, **6**, and **9**−**15** were synthesized following the method of Lecouvey et al.:[38]



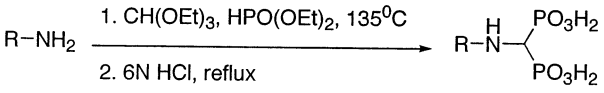
Compounds **17** and **32**−**36** were produced using the method of Mikhalin et al.:[39]



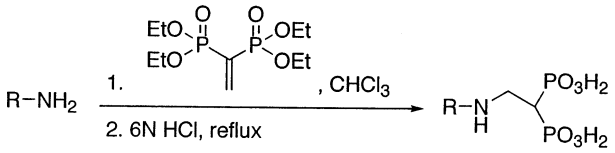
The synthesis of **31** has been described in detail elsewhere.[40] The 1,2-bisphosphonates **37**−**39** were produced using the method described by Allen et al.:[41]



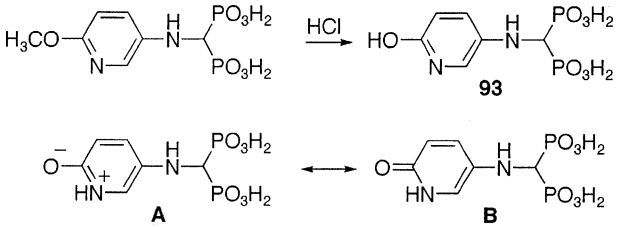
The aminomethylene bisphosphonates **40**−**97, 101**, and **102** were made following the method of Soloducho et al.:[42]



Compounds **98**−**100** were produced via the method of Hutchinson and Thornton:[43]



An exception to the synthesis procedures above is compound **93**, which was orignally intended to be synthesized as the methoxy pyridine derivative. During the hydrolysis step, the methoxy group was removed by HCl, leaving the hydroxy compound **93**, which can also exist as the tautomers **A** and **B**, below:



The purity of compounds **1**−**8**, **16**, **18**−**31**, **42**−**44**, **50**, **65**, **86**−**88**, **91**, and **102** was verified by H/C/N microanalysis and/or ^{1}H, ^{13}C and ^{31}P NMR spectroscopy, as reported previously.[21,24,40] For compounds **9**−**15**, **17**, **32**−**41**, **45**−**49**, **51**−**64**, **66**−**85**, **89**, **90**, and **92**−**101** we used H/C/N microanalysis and ^{1}H and ^{31}P NMR. For convenience, a detailed exemplary

synthesis for each method is described in the Supporting Information.

In Vitro Testing. *E. histolytica.* *E. histolytica* trophozoites of the isolate HM-1:IMSS were cultured axenically in TYI-33 medium.[5] Inocula for experimental tubes and their controls were taken from stocks still in the exponential growth phase. The amoebae were cultured in 96-well plates under anaerobic conditions. Various concentrations of bisphosphonates were added to the growth medium at time $t = 0$ (0, 5, 10, 25, 50, 100, and 150 μM) and the cells incubated for 90 h. At that time, [^{3}H]hypoxanthine (0.1 μCi) was added, and the cells were cultured for a further 6 h. The cells were then lysed by submitting them to two freeze (−70 °C) and thaw (37 °C) cycles and harvested using a Micro Cell Harvester (Skatron Instruments). During harvesting, the ^{3}H-labeled DNA was spotted onto a filter and radioactivity was counted by using a 1205 Betaplate counter (Walac).

P. falciparum. *P. falciparum* (chloroquine-sensitive strain 3D7) was maintained in human A^+ erythrocytes in RPMI1640 medium supplemented with Albumax II at 37 °C in a 5% CO_2-air mixture. *P. falciparum* intraerythrocytic cultures were set up as above, with 1% ring stage parasitemia, 2.5% hematocrit, in triplicate in 100 μL of medium in 96-well, flat-bottomed Microtest III tissue culture plates. Drugs were added in 3-fold dilution series and cultures incubated for a total of 48 h at 37 °C in a 5% CO_2-air mixture. After 24 h, [^{3}H]hypoxanthine (0.2 μCi) was added to each well. At the end of the assay, plates were rapidly freeze−thawed (3×), cells were harvested using a Tomtec Mach III cell harvester onto a 96-well format filtermat, and Meltilex solid scintillant (both Wallac, Finland) was added prior to reading in a Microbeta 1450 scintillation counter (Wallac, Finland) at 1 min per well.

Hamster Experiments. Hamsters were challenged with the direct injection of 5×10^5 *E. histolytica* trophozoites into the left lobe of the liver (day 0). Animals were treated with ip administration of compounds (10 mg/kg) dissolved in 0.1 mL of 100% DMSO once a day for 5 days starting on day 1. Animals were sacrificed on day 6 and amebic liver abscess and normal liver weights determined.

***P. berghei* Experiments.** Female BALB/c mice (18−20 g) infected with *P. berghei* (ANKA strain) were used. Blood was taken from donor mice; the serum was diluted in heat-inactivated fetal calf serum to a parasitemia of 1% (the equivalent of 1×10^7 infected erythrocytes), and 0.2 mL was administered iv to each mouse. Mice were randomly sorted into groups of five and dosing commenced 2 h postinoculation. Compounds were prepared as stock solutions at 10 mg/mL in 10% DMSO/PBS. The control drug, chloroquine, was given ip every day for 4 days. Bisphosphonate drugs were given in a 0.2 mL bolus every day for the number of days indicated and at the concentrations indicated (Table 5).

PKF Expression and Inhibition. Isolation of *E. histolytica* PPi-PFK expressed in *E. coli* bearing a vector consisting of the PPi-PFK gene cloned into the prokaryotic expression plasmid palter-Ex1 (Promega) has been described previously.[44] Homogeneity of the preparation was demonstrated by the presence of a single band upon SDS−PAGE.

Enzyme activity of PPi-PFK was assayed spectrophotometrically at 30 °C in an assay solution that contained 50 mM Tes/KOH (pH 7.0), 2 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 0.5 mM Fru 6-P, 0.1 mM PPi, 0.2 mM NADH, and 2−6 units each of aldolase, triosephosphate isomerase, and glycerol 3-phosphate dehydrogenase. These auxiliary enzymes were dialyzed against 50 mM Tes/KOH (pH 7.0), 1 mM EDTA prior to use. Varying concentrations of potential inhibitors were added to determine IC_{50}s. With several inhibitors, the mode of inhibition was examined by using a fixed concentration of inhibitor with varying concentrations of substrates.

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Supporting Information Available: Typical growth inhibition curves for *E. histolytica* and *P. falciparum* and exemplary syntheses. This material is available free of charge via the Internet at http://pubs.acs.org.

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