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Lipophilic Bisphosphonates Are Potent Inhibitors of
*Plasmodium* Liver-Stage Growth

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Nitrogen-containing bisphosphonates, drugs used to treat bone resorption diseases, also have activity against a broad range of protozoa, including blood-stage *Plasmodium* spp. Here, we show that a new-generation “lipophilic” bisphosphonates designed as anticancer agents that block protein prenylation also have potent activity against *Plasmodium* liver stages, with a high (>100) therapeutic index. Treatment of mice with the bisphosphonate BPH-715 and challenge with *Plasmodium berghei* sporozoites revealed complete protection (no blood-stage parasites after 28 days). There was also activity against blood-stage forms in vitro and a 4-day delay in the prepatent period in vivo. The lipophilic bisphosphonates have activity against a *Plasmodium* geranylgeranyl diphosphate synthase (GGPPS), as well as low nM activity against human farnesyl and geranylgeranyl diphosphate synthases. The most active inhibitor in vitro and in vivo had enzyme inhibitory activity similar to that of the other, less active compounds but was more lipophilic. Lipophilic bisphosphonates are thus promising leads for novel antimalarials that target liver-stage infection.

The malaria parasite *Plasmodium* is transmitted via the bite of an infected female *Anopheles* mosquito that injects sporozoites into the skin. The parasites enter the bloodstream and invade hepatocytes, where the sporozoites transform into liver stages, usually called exoerythrocytic forms (EEFs). These EEFs then undergo multiple rounds of nuclear division and produce thousands of merozoites that enter the blood circulation and infect erythrocytes, leading to malaria symptoms. EEFs are thus targets for prophylactic drugs and vaccines. At present, however, there are a very limited number of drugs that target *Plasmodium* liver stages. Primaquine is one, but its use is limited by its associated toxicity and increased risk of hemolysis when administered to persons with glucose-6-phosphate dehydrogenase (G6PD) deficiency (1). The latter problem also affects two related compounds under development: bulequina and tafenquine (18, 19). Although there has been considerable progress in the treatment of bloodstream infections using artemisinin-based combination therapies, recent reports have indicated the possible development of resistance to artemisinin among Asian isolates (5). This situation is potentially serious considering how widespread the resistance is to the other antimalarials, chloroquine or the pyrimethamine-sulfadoxine combination (13). Under these circumstances, there is therefore a need to identify new lead compounds active against malaria. In particular, there is currently great interest (9) in developing drugs that inhibit the liver-stage forms that precede blood infections, thereby preventing symptomatology and blocking transmission (20).

During the early stages of EEF development, the *Plasmodium* plasma membrane is covered with the circumsporozoite protein (CSP). In infected hepatocytes, parasites are contained inside a parasitophorous membrane, but the CSP is still exported into the hepatocyte cytoplasm and nucleus (16), generating profound changes in the transcriptional program of the host cell. In previous work we noticed that the gene that was most highly upregulated upon CSP expression in infected HeLa cells (16) or HepG2 cells (M. Zhang and V. Nussenzweig, unpublished data) was the Rap1A gene. Since Rap proteins are geranylgeranylated at their C termini and since there are such large changes in Rap1A gene expression upon CSP expression in HeLa cells, it seemed possible that blocking protein geranylgeranylation would have a significant effect on liver-stage parasite proliferation.

To inhibit prenylation we investigated the effects of several different bisphosphonates, a class of drugs known to inhibit protein prenylation. Bisphosphonates are potent, low nM inhibitors of the enzyme farnesyl diphosphate synthase (FPPS), which catalyzes the condensation of the isoprenoids dimethyl-allyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), produced in the mevalonate pathway (Fig. 1), to form geranyl diphosphate (GPP). This then condenses with a second
IPP molecule to form farnesyl diphosphate (FPP) and thence geranylgeranyl diphosphate (GGPP) (Fig. 1). As a result of this FPPS inhibition, bisphosphonates block protein prenylation, as well as sterol, ubiquinone, dolichol, and heme biosynthesis (14). Bisphosphonates also stimulate human \( \text{H}2\text{K}3\text{H}2\text{K}4 \) T cells (containing the \( \text{V}2\text{H}2\text{K}2\text{V}2\text{H}2\text{K}4 \) T-cell receptor) (7) and may therefore have antiparasitic activity. Bisphosphonate inhibition of FPPS also results in the accumulation of the substrate IPP, which becomes conjugated to AMP to form a toxic ATP analogue, \( \text{O}^{-}\text{isopentenyl-ATP} \) (ApppI) (Fig. 1), which can inhibit the mitochondrial adenine nucleotide translocase and induce apoptosis (10).

We reasoned that novel, lipophilic bisphosphonates (22) might have enhanced effects on the survival of malaria parasites over the more polar bisphosphonates reported previously (6, 8), and we focus here on a comparison of the activities of three lipophilic bisphosphonates (currently being developed as anticancer drug leads) with the activity of the second- and third-generation bisphosphonates pamidronate and risedro-

FIG. 1. Schematic illustration of isoprenoid biosynthesis pathways (in humans and \textit{Plasmodium} spp.) and sites of action of several inhibitors. Malaria parasites make IPP and DMAPP via the nonmevalonate pathway. Inhibition of isoprenoid biosynthesis affects protein prenylation; IPP buildup activates \( \gamma \delta \) (gd) T cells; and IPP is converted to toxic ATP analogs on FPPS inhibition. ANT, adenine nucleotide translocase; DXP, 1-deoxy-d-xylulose 5-phosphate; MEP, 2-C-methyl-D-erythritol 4-phosphate; HMG, 3-hydroxy-3-methyl-glutaryl-CoA; TNF-\( \alpha \), tumor necrosis factor alpha; IFN-\( \gamma \), gamma interferon.
or, in both liver-stage (EEF) and blood-stage *Plasmodium* infection models.

**MATERIALS AND METHODS**

**Animals.** The methods for mouse use and care were approved by the New York University School of Medicine Institutional Animal Care and Use Committee and are in accordance with the PHS policy on Care and Use of Laboratory Animals under the guidance of the Office of Laboratory Animal Welfare (OLAW) within the NIH.

**Reagents.** The compounds used were pravastatin and pamidronate from Sigma, GTGTI-298 from Calbiochem, risedronate, synthesized as described in reference 5, and BPH-715, BPH-942, and BPH-943, lipophilic bisphosphonates synthesized as described previously (4).

**In vitro growth inhibition assay for liver-stage parasites.** Drug solutions were prepared in 1× sterile phosphate-buffered saline (PBS). The diluted solutions were added to 24-well culture plates containing human HepG2 cells seeded a day prior to the experiment and 0.5 ml complete Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), together with an antibiotic-antimycotic. Infection was initiated by adding 10,000 *Plasmodium berghei* sporozoites (NK65 line). Infected cultures were then allowed to grow at 37°C in a 5% CO2 atmosphere for 48 h. Culture medium was changed 24 h after infection, and fresh compounds were added at the same concentration, to maintain inhibitor pressure throughout the growth period. At the end of the 48-h incubation period, total RNA was extracted using Trizol reagent. Reverse transcription from 4 μg RNA was performed to obtain cDNA. In a real-time PCR mix of 50 μl, a cDNA equivalent of 0.5 μg RNA was used. The real-time PCR mix also contained *P. berghei* 18S rRNA specific primers and SYBR green dye (2). Real-time PCR was performed in a Bio-Rad iCycler, and the copy numbers were calculated, using the known amount of plasmid standard having the amplification target sequence (2). Parasite growth inhibition was calculated by dividing the 18S rRNA copy number of the experimental group by that of the untreated control group. Nonlinear regression analysis was performed using Sigmaplot (version 10) to determine 50% inhibitory concentrations (IC50s).

**In vitro growth inhibition assay for liver-stage parasites (EEFs).** Compounds were injected into mice intraperitoneally (i.p.) for a variable number of days, depending on the experiment. Treatments started on day −2, −1, or 0, where day 0 corresponds to sporozoite infection by the intravenous route. Treatments were continued on days +1 and +2 postinfection. EEF development was evaluated by the success of the ensuing blood-stage infection. Blood smears were prepared on day 3 postinfection. Percent parasitemia was monitored until it reached 3 to 5%, and the animals were then sacrificed. Complete protection is defined as the absence of parasites in blood smears up to 2 weeks postchallenge with sporozoites.

**Blood-stage parasite growth inhibition assay with infected mice.** Mice (five per group) were injected i.p. with 5 × 106 blood-stage *P. berghei* parasites/mouse, to initiate infection. Treatments started on day 0 and were continued on days +1 and +2 postinfection. Blood smears were prepared on days 2 and onwards, post-parasite challenge. Parasitemia was determined microscopically until it reached 3 to 5%, and the animals were sacrificed.

**In vitro growth inhibition assay for *P. falciparum* blood-stage parasites.** Bisphosphonates were evaluated for their inhibitory activity against blood stages of *P. falciparum* strains 3D7 (chloroquine sensitive) and W2mcl (chloroquine resistant). Each compound was tested in triplicate in 96-well culture plates in a volume of 100 μl. Drug concentrations of 1, 10, 100, 1,000, and 10,000 nM were tested. Chloroquine at concentrations of 10 and 100 nM was used as a positive control. *P. falciparum* parasites were cultured in O-positive washed erythrocytes in RPMI 1640 medium using the candle jar technique (17). Parasites were added to reach an appropriate parasitemia (0.2 to 0.3%). Flasks were kept in a desiccator, and a low-oxygen environment was produced by flushing with mixed gas (90% N2, 5% CO2, and 5% O2). Cells were incubated at 37°C, and percent parasitemia was determined microscopically.

**Toxicity assays in hepatocytes.** HepG2 (human liver hepatoma) cells were seeded at 70% confluence (with 0.5-mL medium) in 24-well tissue culture plates a day prior to treatment with inhibitors. Various concentrations of inhibitors were added to the HepG2 cells, which were then incubated at 37°C under 5% CO2 for 22 h. After incubation, CellTiter One reagent (Promega) was added to the wells, and two additional hours of incubation followed (11). The plates were briefly spun, and the clear media transferred to 96-well plates. The optical density at 495 nm (OD495) was then determined by using a microplate reader (Molecular Devices), and the IC50 value determined by nonlinear regression (percent viable cells at various concentrations of the individual bisphosphonates).

**RESULTS**

**Growth inhibition of *P. berghei* EEFs in vitro.** We first tested the effects of two bisphosphonate drugs, pamidronate (Aredonel) and risedronate (Actonel) (Fig. 2), together with the effects of pravastatin, an isoprenoid biosynthesis inhibitor that targets 3-hydroxymethyl-glutaryl–coenzyme A (HMG-CoA) reductase (Fig. 1 and 2), on *P. berghei* EEF growth in HepG2 cells. Pamidronate and pravastatin were inactive at the concentrations tested (IC50 of >200 μM) (Table 1). The risedronate IC50 was ~16 μM (Fig. 3B and Table 1), while the lipophilic bisphosphonates (BPH-715, BPH-942, and BPH-943; Table 1 and Fig. 3A) had IC50s in the range of ~8 to 10 μM. We also tested the toxicity of the active bisphosphonates on HepG2 cells, and their 50% toxic concentrations (TC50s) were in the range of 1 to 13 mM (Table 1). All of the active bisphosphonates had therapeutic index values of >100, comparable to that of primaquine (3). In addition, we tested the geranylgeranyl

![Fig. 2. Structures of the inhibitors tested.](image-url)
transferase I inhibitor GGTI-298 (Fig. 2), finding an IC\textsubscript{50} of \( \approx 30 \) \( \mu \)M (Table 1), but this compound was later found to have no activity \textit{in vivo} (Table 2).

\textbf{Bisphosphonates inhibit the growth of} \textit{P. berghei} \textit{EEFs} at an early stage. We next treated sporozoite-infected hepatocytes with one of the lipophilic bisphosphonates (BPH-715), chosen since it has already been tested for safety in mice (22), and examined the cultures 50 h later, using light microscopy (Fig. 4A and B). When BPH-715 was present at 30 \( \mu \)M, there was a 9-fold reduction in the EEF area (Fig. 4C). There was no difference in the total numbers of EEFs in the control and treated groups. BPH-715 thus inhibits EEF growth at an early trophozoite stage.

\textbf{Bisphosphonates also inhibit} \textit{P. berghei} \textit{EEF growth} \textit{in vivo}. To evaluate to what extent the lipophilic bisphosphonates had activity \textit{in vivo}, we injected them i.p. once daily into mice, starting 2 days before and ending 2 days after infection with sporozoites (a total of 5 days). When challenged with 3,000 infective sporozoites, the bisphosphonates showed a wide range of effects, ranging from no activity to complete protection (Table 2; see also Tables S1 and S2 in the supplemental material). The most active compound (BPH-715) protected mice completely at a dose of 1.5 mg/kg body weight, which also represents a very large reduction in parasite burden. Risedronate was less active than was BPH-715, although it did show a 4-day reduction in the prepatent period at 20 mg/kg (Table 2). The lipophilic bisphosphonates were, therefore, far more potent than the commercial bisphosphonate risedronate. We also tested GGTI-298 (Fig. 2), a commercially available inhibitor of geranylgeranyl transferase (GGTI), \textit{in vivo} (up to a dose of 5 mg/kg), but GGTI-298 did not show any activity against the liver-stage parasites \textit{in vivo}, although it did show very modest (\( \approx 30 \) \( \mu \)M) activity \textit{in vitro}. Another GGTI inhibitor (DU40; Fig. 2) also failed to show any \textit{in vivo} activity against the EEFs. So, only the lipophilic bisphosphonates have good \textit{in vivo} activity.

\textbf{Bisphosphonates inhibit} \textit{P. falciparum} \textit{blood-stage parasites} \textit{in vitro}. We next tested risedronate and the three lipophilic bisphosphonates for activity against \textit{P. falciparum} \textit{in vitro}. We found that with the chloroquine-sensitive strain 3D7, the lipophilic bisphosphonates (BPH-715, BPH-942, and BPH-943) had IC\textsubscript{50}s in the submicromolar range (Table 3), while risedronate had an IC\textsubscript{50} of \( \approx 1.2 \mu \)M (Table 3). BPH-942 and BPH-943 showed 3- or 4-fold less activity against the chloroquine-resistant strain (W2mef) than the chloroquine-sensitive strain (3D7), but BPH-715 had good activity against both strains (Table 3).

\begin{table}[h]
\centering
\caption{In vitro activity of compounds against \textit{Plasmodium} EEFs}
\begin{tabular}{|l|l|l|l|}
\hline
Compound & IC\textsubscript{50} for EEFs (\( \mu \)M) & TC\textsubscript{50} for HepG2 cells (mM) & Therapeutic index* \\
\hline
Pamidronate & >200 & ND & NA \\
Risedronate & 16 & 13 & 813 \\
Pravastatin & >200 & ND & NA \\
BPH-715 & 10 & 2 & 200 \\
BPH-942 & 8.8 & 1 & 114 \\
BPH-943 & 8 & 1 & 125 \\
GGTI-298 & \(-30\) & ND & NA \\
\hline
\end{tabular}
\footnote{ND, not determined.}
\footnote{NA, not applicable.}
\end{table}

\begin{table}[h]
\centering
\caption{In vivo activity of bisphosphonates against \textit{Plasmodium} EEFs}
\begin{tabular}{|l|l|l|l|}
\hline
Compound & Mouse dose (mg/kg) for 5 days\textsuperscript{c} & Prepatent day\textsuperscript{a} & Delay in prepatent period (days) \\
\hline
Untreated & None & 4 & 0 \\
Risedronate & 20 & 8 & 4 \\
BPH-715 & 1.5 & >28\textsuperscript{b} & >28\textsuperscript{b} \\
BPH-942 & 0.8 & 7 & 3 \\
BPH-943 & 2 & 9 & 5 \\
GGTI-298 & 5 & 4 & 0 \\
\hline
\end{tabular}
\footnote{Day 0 corresponds to day of challenge with 3,000 \textit{P. berghei} sporozoites.}
\footnote{>28, no blood-stage parasites through the 28-day observation period.}
\footnote{The 5 days are days \(-2, -1, 0, +1,\) and \(+2\).}
\end{table}
Bisphosphonates inhibit *P. berghei* blood-stage parasites *in vivo*. Two bisphosphonates (risedronate and the lipophilic bisphosphonate BPH-715) were then tested *in vivo* for activity against a blood-stage parasite challenge. Risedronate was given at 20 mg/kg for 5 days, with the mice being challenged against a blood-stage parasite challenge. Risedronate was active. In vivo, Two bisphosphonates (risedronate and the lipophilic bisphosphonate BPH-715) were then tested *in vivo* for activity against a blood-stage parasite challenge. Risedronate was given at 20 mg/kg for 5 days, with the mice being challenged against a blood-stage parasite challenge. Risedronate was active. However, in the *in vivo* EEF experiments (Table 2), BPH-715 was clearly far more effective than is BPH-943 (at essentially the same dosing: 1.5 mg/kg versus 2.0 mg/kg). A likely explanation for this is that BPH-715 has much better uptake into the liver since it is more lipophilic than is BPH-943. Table 4 shows the SlogP (the logarithm of the oil/water partition coefficient) values computed in MOE (Molecular Operating Environment 2006.08; Chemical Computing Group, Inc., Montreal, Quebec, Canada) for all four bisphosphonates. From these results we see that BPH-715 is clearly more lipophilic than is BPH-943, with an SlogP value of −1.2, compared with −3.4 for BPH-943. The −5.5 SlogP for risedronate represents an extremely hydrophilic species, which together with its lack of activity against HsGGPPS (Table 4) likely explains its lower activity than that of the lipophilic bisphosphonates *in vivo*.

**DISCUSSION**

The results we have described above show that lipophilic bisphosphonates have potent activity against liver-stage *P. berghei* *in vivo*, with the most active compound completely protecting mice against sporozoite challenge (at a dose of 1.5 mg/kg body weight for 5 days) over the 28-day observation period, with no adverse effects. The enhanced activity of this compound over that seen with the other bisphosphonates tested arises, we propose, from enhanced uptake into the liver. The bisphosphonate risedronate has poorer efficacy than does BPH-715 *in vivo* but still provides a modest delay (4 days) in the prepatent period. Interestingly, while BPH-715 is more potent in inhibiting liver stage rather than blood stages *in vivo*, it generates a much smaller effect on blood-stage parasites *in vivo*, even though it is quite potent *in vitro*. One possible explanation is that BPH-715 uptake into the liver may simply be much more effective because of the much higher lipophilicity of this species (an SlogP value 2 to 4 units greater than with any of the other bisphosphonates tested). At present, we cannot quantify the relative importance of host versus pathogen targeting in the liver stages, in which PbGGPPS, HsFPPS, and HsGGPPS could all be inhibited to various degrees. Indeed, in

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**TABLE 3.** IC₅₀ for the selected bisphosphonates against blood-stage *Plasmodium falciparum* chloroquine-sensitive and -resistant strains *in vitro*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC₅₀ (nM) (avg ± SD)</th>
<th>3D?</th>
<th>W2mef</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risedronate</td>
<td>1,200 ± 90</td>
<td>1,400 ± 100</td>
<td></td>
</tr>
<tr>
<td>BPH-715</td>
<td>670 ± 40</td>
<td>590 ± 15</td>
<td></td>
</tr>
<tr>
<td>BPH-942</td>
<td>650 ± 90</td>
<td>2,000 ± 75</td>
<td></td>
</tr>
<tr>
<td>BPH-943</td>
<td>450 ± 110</td>
<td>2,200 ± 150</td>
<td></td>
</tr>
</tbody>
</table>
recent work we reported that, in 10 simpler systems, the correlation between enzyme inhibition \(-\log_{10} IC_{50}\) (pIC\textsubscript{50}) and cell activity pIC\textsubscript{50} values for a broad range of assays was only an \(R^2\) value of 0.30 (12). This pointed to a key role of transport or cell permeability, and when this was taken into account, the correlation improved to an \(R^2\) value of 0.70 (12). However, applying the mathematical model used in that approach to liver-stage \textit{Plasmodium} spp. \textit{(in vitro or in vivo)} is a challenge since there are, in principle, at least 3 targets (PbGGPPS, HsFPPS, and HsGGPPS) and at least 3 “permeability” barriers (the host cell, the \textit{Plasmodium} plasma membrane, and the parasitophorous vacuole membrane). In blood-stage \textit{Plasmodium}, this may be more tractable since the HsFPPS/HsGGPPS are not targets, but quite large data sets (>20 compounds) are needed for this sort of analysis (12). In addition, we cannot rule out other possible targets, such as octaprenyl diphosphate synthase, and indeed, in other work (22) we have shown that bisphosphonates can inhibit multiple prenyl synthase targets—something that may contribute to their potent activity.

In any case, the results we have obtained here are clearly of considerable interest since we find complete protection against \textit{P. berghei} liver-stage infection in mice using the lipophilic bisphosphonate BPH-715. Interestingly, this compound has also been found to be the most potent inhibitor of tumor cell growth, both \textit{in vitro} and \textit{in vivo} (22), due, we proposed, to its high cell permeability and its lipophilic nature (compared with other bisphosphonates). This suggests the possibility that BPH-715 does in fact block protein geranylgeranylation, and notably, with three human cell lines, we found that cell growth inhibition could be “rescued” by addition of geranylgeraniol—although, of course, this rescue effect would not have probative value in \textit{Plasmodium} blood-stage experiments since the effects on both PbGGPPS and HsGGPPS inhibition would be “rescued” (by providing the exogenous isoprenoid source).

In the blood-stage \textit{P. falciparum} experiments, the effects of BPH-715 and the more hydrophilic bisphosphonate risedronate are quite similar to each other, with the IC\textsubscript{50} ratio (risedronate/BPH-715) being only about a factor of 2 to 3. Since there is no isoprenoid biosynthesis in the red blood cell, HsFPPS/HsGGPPS inhibition is not involved, so solely the \textit{Plasmodium} GGPPS is being targeted. Notably, this factor of \(\approx 2.5\) is the same as that seen with the \textit{Plasmodium} EEFs (Table 1), for whose inhibition risedronate has an IC\textsubscript{50} of 16 \(\mu\text{M}\), while it has an IC\textsubscript{50} of 10 \(\mu\text{M}\) for BPH-715 inhibition (Table 1), suggesting the possibility that the \textit{Plasmodium} GGPPS is a primary target in both blood- and liver-stage forms \textit{in vitro}. The improved activity of the more lipophilic bisphosphonates is in agreement with results we reported previously (on much less active compounds): that the most lipophilic bisphosphonates have the best activity against \textit{Plasmodium} blood-stage parasites (6).

In summary then, lipophilic bisphosphonates are potential leads against the liver stages of malaria parasites, including the hypnozoites of \textit{P. vivax}, and could have a role in malaria chemoprophylaxis.

### ACKNOWLEDGMENTS

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### REFERENCES


