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Characterization of Potential Drug Targets Farnesyl Diphosphate Synthase and Geranylgeranyl Diphosphate Synthase in *Schistosoma mansoni*

Peter D. Ziniel,[a,b] Janish Desai,[c] Cynthia L. Cass,[a] Craig Gatto,[a] Eric Oldfield,[c,d] David L. Williams[a,b]

School of Biological Sciences, Illinois State University, Normal, Illinois, USA[a]; Department of Immunology/Microbiology, Rush University Medical Center, Chicago, Illinois, USA[b]; Center for Biophysics and Computational Biology, University of Illinois, Urbana, Illinois, USA[c]; Department of Chemistry, University of Illinois, Urbana, Illinois, USA[d]

Schistosomiasis affects over 200 million people worldwide, with over 200,000 deaths annually. Currently, praziquantel is the only drug available against schistosomiasis. We report here that *Schistosoma mansoni* farnesyl diphosphate synthase (*Sm*FPPS) and geranylgeranyl diphosphate synthase (*Sm*GGPPS) are potential drug targets for the treatment of schistosomiasis. We expressed active, recombinant *Sm*FPPS and *Sm*GGPPS for subsequent kinetic characterization and testing against a variety of bisphosphonate inhibitors. Recombinant *Sm*FPPS was found to be a soluble 44.2-kDa protein, while *Sm*GGPPS was a soluble 38.3-kDa protein. Characterization of the substrate utilization of the two enzymes indicates that they have overlapping substrate specificities. Against *Sm*FPPS, several bisphosphonates had 50% inhibitory concentrations (IC_{50}s) in the low micromolar to nanomolar range; these inhibitors had significantly less activity against *Sm*GGPPS. Several lipophilic bisphosphonates were active against *ex vivo* adult worms, with worm death occurring over 4 to 6 days. These results indicate that FPPS and GGPPS could be of interest in the context of the emerging resistance to praziquantel in schistosomiasis therapy.

Human schistosomiasis, also known as bilharzia, is a disease caused by trematodes of three main species: *Schistosoma mansoni*, *S. haematobium*, and *S. japonicum*. Some 230 million individuals are infected, with 800 million at risk, and there are more than 200,000 deaths annually (1, 2). The only current treatment for schistosomiasis is praziquantel. Laboratory strains and clinical isolates of the parasite with resistance to praziquantel have been identified (3). No other drugs are currently available for the treatment of the disease, and no new drug has entered clinical trials for schistosomiasis treatment in more than 30 years (4). Moreover, no vaccines have been developed, and progress is very slow (5). There is therefore a need to develop alternative drugs for schistosomiasis treatment.

One potential target for schistosomiasis drug development is isoprenoid biosynthesis, which is involved in the synthesis of dolichols, quinones, and the isoprenoid diphosphates used in protein prenylation (6, 7). Starting with acetyl-coenzyme A (acetyl-CoA) and acetoacetyl-CoA, 3-hydroxy-3-methylglutaryl-CoA is synthesized by 3-hydroxy-3-methylglutaryl-CoA synthase. 3-Hydroxy-3-methylglutaryl-CoA is then reduced to mevalonate by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) (Fig. 1). A series of phosphorylation and decarboxylation reactions convert mevalonate to the 5-carbon intermediate, isopentenyl diphosphate (IPP) (Fig. 2A). IPP is then isomerized to dimethylallyl diphosphate (DMAPP). The two molecules go through a condensation reaction catalyzed by farnesyl diphosphate synthase (FPPS) to form the 10-carbon geranyl diphosphate (GPP) (Fig. 2A). A second condensation reaction of GPP and IPP catalyzed by FPPS results in the formation of the 15-carbon farnesyl diphosphate (FPP), which then undergoes either a reductive condensation reaction to produce squalene, the first committed intermediate in cholesterol biosynthesis, or a further condensation with IPP to produce the 20-carbon geranylgeranyl diphosphate (GGPP), catalyzed by geranylgeranyl diphosphate synthase (GGPPS). FPP thus serves as the main precursor for synthesis of sterols as well as nonsterol isoprenoids (7–11), including quinones and dolichols.

It has been determined that *S. mansoni* does not make cholesterol (12): the gene for squalene synthase, the enzyme that catalyzes the synthesis of squalene from FPP, is not present in the *S. mansoni* genome (13). Consequently, it has been proposed that the parasite obtains its cholesterol from its human host (14). However, the mevalonate pathway that produces IPP and DMAPP is present in schistosomes (15), and isoprenoids derived from mevalonate have been identified in *S. mansoni* and are thought to be involved in posttranslational prenylation of proteins (with FPP and GGPP), as well as in nonsterol isoprenoid synthesis (16, 17). For *S. mansoni*, work from different groups has shown that schistosome Ras is farnesylated (18), and Rab and Rho orthologs are geranylgeranylated (19, 20).

The mevalonate pathway thus serves as a potential target for schistosomiasis treatment. Statins, which are widely used in reducing hypercholesterolemia in humans via inhibition of HMGR (21–23), have been used in experimental *S. mansoni* infections, resulting in significant worm death (6). Statins such as mevinolin are also reported to inhibit egg production in *S. mansoni*, possibly due to the lack of nonsterol lipids involved in egg production (14, 24). In the absence of mevinolin, parasites produced five to six times more eggs than worms cultured in mevinolin-containing

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Address correspondence to David L. Williams, david_l_williams@rush.edu.
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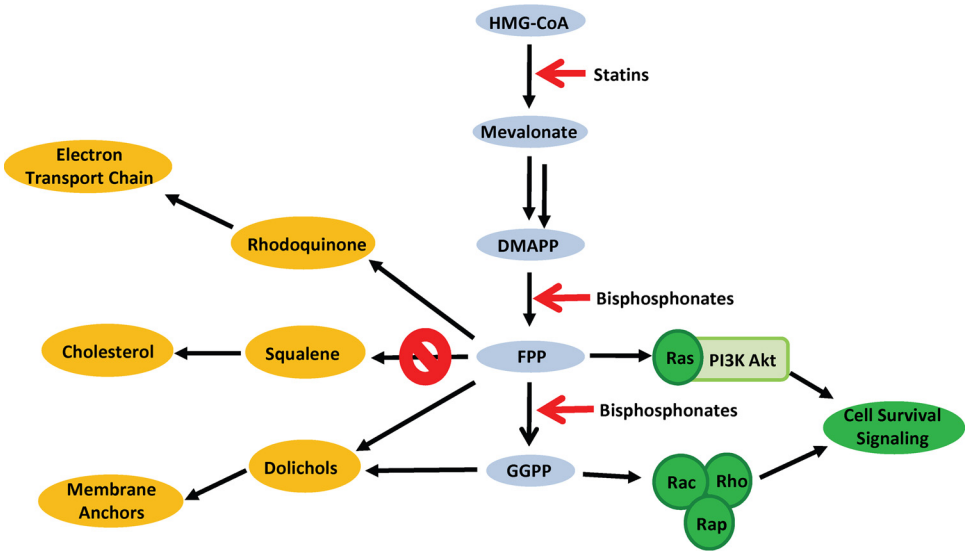

FIG 1 Schematic representation of the mevalonate pathway in *Schistosoma mansoni*. 3-Hydroxy-3-methylglutaryl-coenzymeA (HMG-CoA), the product of HMG-CoA synthase, is converted to mevalonate by HMG-CoA reductase, the target of the cholesterol-reducing statins. A series of reactions converts mevalonate to dimethylallyl diphosphate (DMAPP), which is converted to its isomer, isopentenyl diphosphate (IPP). Condensations of one DMAPP and one IPP to produce geranyl diphosphate (GPP) and of IPP with GPP to form farnesyl diphosphate (FPP) are carried out by FPP synthase (FPPS) and are the targets of bisphosphonate therapies. Condensation of FPP and IPP leads to the production of geranylgeranyl diphosphate (GGPP) by GGPP synthase (GGPPS). FPP is used to synthesize various quinones, GGPP is used to synthesize dolichols, and both are essential in the posttranslational prenylation of a variety of proteins (e.g., the small GTPases Ras, Rab, and Rho) that are important for cell signaling and survival. Rhodoquinone plays a crucial role in the electron transport chain during cellular respiration in *S. mansoni* mitochondria. Conversion of FPP to squalene does not occur in *S. mansoni*, and therefore, there is no cholesterol synthesis.

media. Mevinolin also caused worm death in infected mice, but it required administration for 14 days to achieve high efficacy (6), so HMGR may not be a good target for schistosomiasis therapy.

Although the importance of the mevalonate pathway for schistosomes and its potential as a drug target have been demonstrated, there is no information available on the other enzymes in this pathway. We thus undertook a study to characterize FPPS and GGPPS in *S. mansoni* to determine if they might be drug targets. Bisphosphonates (Fig. 2B), which have been used to treat osteoporosis and similar diseases in millions of people (25), have been shown to be efficient FPPS (26) and, in some cases, GGPPS (27) inhibitors, and one approach for the development of drugs for neglected diseases is the repositioning of drugs currently in use (28). Bisphosphonates have also been found to be active against a variety of protozoan parasites, for example, *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani* (26, 29–31), *Cryptosporidium parvum* (32), *Entamoeba histolytica* (33), *Toxoplasma gondii* (34), and *Plasmodium* species (26, 33, 35–37). However, no previous investigations on FPPS or GGPPS from a parasitic helminth have been reported. Here we show that FPPS and GGPPS from *S. mansoni* share similarities with orthologs from other species: both schistosome enzymes are inhibited by bisphosphonates, although to differing degrees. Investigations on the substrate specificities of FPPS and GGPPS indicate that their activities are redundant, indicating that inhibition of both enzymes will be necessary for effective worm killing. Pyridinium group-containing lipophilic bisphosphonates tested against cultured *ex vivo* adult worms were able to kill worms, providing evidence that *Sm*FPPS and *Sm*GGPPS are essential for worm survival and that they are druggable targets.

MATERIALS AND METHODS

Materials. Radioactive ^{3}H-IPP was obtained from American Radiolabeled Chemicals (St. Louis, MO). *Escherichia coli* BL21 Star(DE3) cells were obtained from Invitrogen (Carlsbad, CA). Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was obtained from Gold Biotechnology (St. Louis, MO). RPMI 1640 medium was obtained from Sigma-Aldrich (St. Louis, MO).

Clones and plasmids. Total RNA was extracted from *S. mansoni* eggs isolated from mouse livers (38) by using TriReagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's instructions. First-strand cDNA synthesis was performed on 1 μg total RNA, using SuperScript II (Stratagene, La Jolla, CA). PCR amplification of FPPS and GGPPS was accomplished using *Pfu* or Vent DNA polymerase and the following gene-specific primers (IDT, Coralville, IA) designed using sequences identified in the *S. mansoni* genome and expressed sequence tag (EST) databases after queries with known FPPS and GGPPS sequences: *Sm*FPPS forward, 5′-caccATGCCAACCTCTGATATACATA; *Sm*FPPS reverse, 5′-TTAAC AAGCACGTTGAAAAAGTAATTC; *Sm*GGPPS forward, 5′-caccATGGA TGCAGAAAGAAGTGG; and *Sm*GGPPS reverse, 5′-TTATCTCTCATTT AGAAACAGG. The PCR products were cloned into pET100 (Invitrogen, Carlsbad, CA), transformed into *E. coli* BL21 Star(DE3) cells, plated on LB containing 50 μg/ml ampicillin, and cultured overnight at 37°C to select for ampicillin-resistant clones. The clones were confirmed by DNA sequencing.

Recombinant protein expression and purification. A single colony was used to inoculate 1 ml liquid broth containing 50 μg/ml ampicillin at 37°C overnight. The overnight culture was transferred to a 500-ml culture of LB containing 50 μg/ml ampicillin at 37°C and shaken at 200 rpm to an optical density at 600 nm (OD_{600}) of 0.5. Recombinant protein expression was induced in the culture with 1 mM IPTG, and the culture was incubated for a further 3 h. The cells were harvested by centrifugation (Sorvall Evolution RC) at 9,000 rpm for 15 min. Protein was obtained by lysing cells by freeze-thaw cycles followed by sonication (Branson digital sonifier) for 6 min. Sonicated cells were centri-

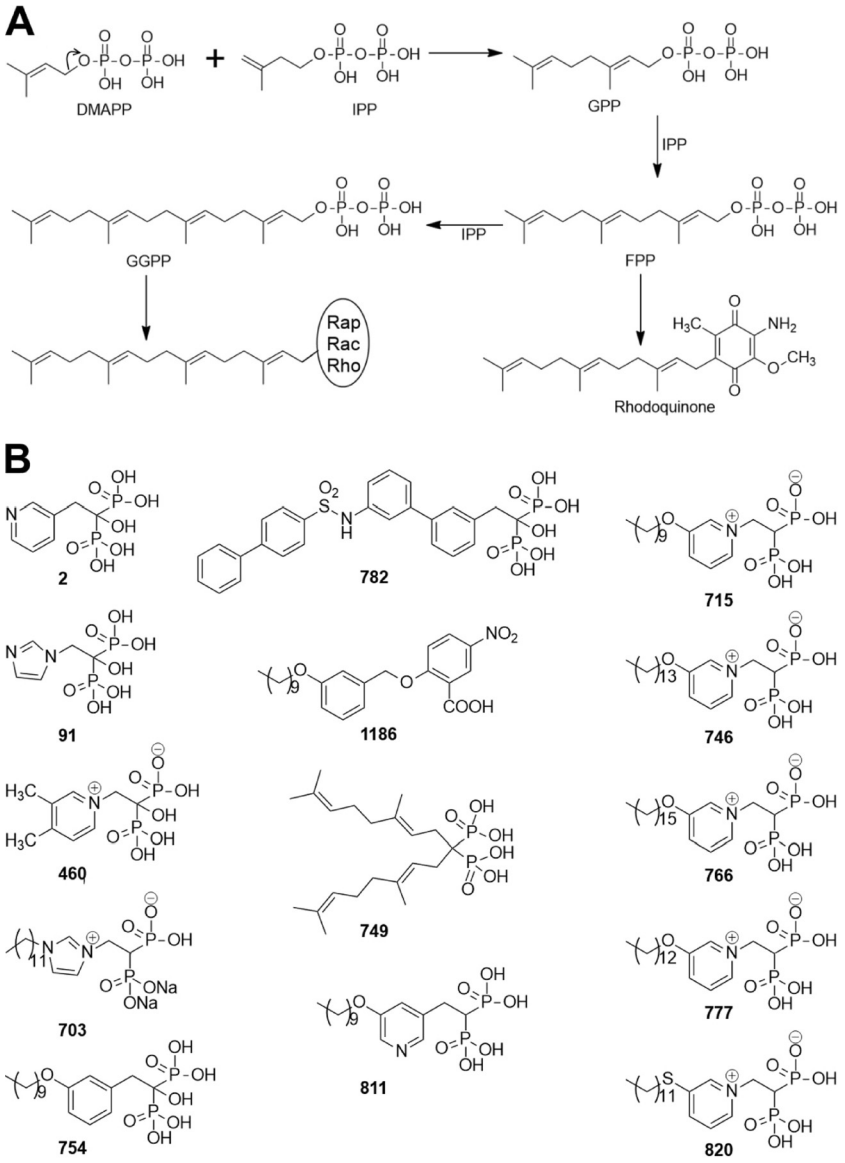

FIG 2 (A) Structures of substrates and products of *Sm*FPPS and *Sm*GGPPS catalysis. DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate; GPP, geranyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate. (B) Structures of bisphosphonate compounds used in this study.

fuged again at 23,000 rpm for 30 min. The recombinant proteins were purified from the supernatant by nickel-affinity chromatography (GE Healthcare) using 10 mM phosphate buffer, pH 7.4, containing 10 mM imidazole, and were eluted with increasing concentrations of imidazole (100 mM, 300 mM, and 500 mM) in 10 mM phosphate buffer, pH 7.4. The purified protein was run in a 16% SDS-polyacrylamide gel to ascertain its size and purity, after which glycerol stocks of BL21 Star(DE3) containing the correct gene insert of each enzyme were made and stored at −80°C for future use.

Radiometric assays for *Sm*FPPS and *Sm*GGPPS. The FPPS enzyme assays were performed essentially as described previously (31), using ^{14}C-labeled IPP with DMAPP, GPP, and FPP. One hundred microliters of assay buffer containing 10 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 2 mM dithiothreitol, 47 μM [4-^{14}C]IPP (55 mCi/mmol), and 55 μM allylic diphosphate was prewarmed to 37°C. The assay was initiated by addition of 20 ng of recombinant *Sm*FPPS and allowed to proceed for 30 min. The reaction was terminated by addition of 10 μl of 6 M HCl, and the mixture was made alkaline by addition of 15 μl of 6 M NaOH diluted with 0.7 ml of water and vortexed with 1 ml of hexane to separate the labeled alcohol products. The hexane solution was washed with water and transferred to a scintillation vial for counting. One unit of enzyme activity is defined as the activity required to incorporate 1 nmol of [4-^{14}C]IPP into the ^{14}C-labeled product in 1 min.

The GGPPS assays were performed as described previously (39, 40), using ^{14}C-labeled IPP with DMAPP, GPP, and FPP. The 20-μl assay setup consisted of 25 mM HEPES, 2 mM $MgCl_2$, 0.5% *n*-octyl-β-glycopyranoside, 10 μM [4-^{14}C]IPP (55 mCi/mmol), and 10 μM allylic diphosphate. The reactions were initiated by addition of 200 ng of recombinant *Sm*GGPPS and were allowed to proceed for 15 min at 37°C. The reactions were stopped by the addition of 2 ml of *n*-butanol saturated with water, followed by 1 ml of 6 M KCl, and mixtures were then vortexed for 1 min to separate the less polar products (FPP and GGPP) from the more polar, ^{14}C-labeled substrate. The butanol phase was transferred to a scintillation vial for counting. One unit of enzyme activity was defined as the amount of protein needed to incorporate 1 nmol of [4-^{14}C]IPP into the ^{14}C-labeled product in 1 min.

TABLE 1 Comparison of *Sm*FPPS and *Sm*GGPPS with orthologs from other species[a]

Organism	Enzyme	% Identity	% Similarity	Accession no.
Xenopus laevis	FPPS	36	53	NP_001084626
Danio rerio	FPPS	37	55	AAH83515
Homo sapiens	FPPS	35	54	NP_001995
Drosophila melanogaster	FPPS	34	54	NP_477380
Trypanosoma brucei gambiense	FPPS	36	54	CBH12431
Plasmodium vivax	FPPS	37	60	XP_001615401
Xenopus laevis	GGPPS	53	71	NP_001091413
Danio rerio	GGPPS	56	72	XP_002664282
Homo sapiens	GGPPS	53	71	NP_004828
Drosophila melanogaster	GGPPS	46	66	NP_523958
Mus musculus	GGPPS	55	72	NP_034412

[a] *Schistosoma mansoni* FPPS, accession no. CCD78373; and *S. mansoni* GGPPS, accession no. XP_002573680.

Kinetic analysis of K_m and V_{max}. The Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) of *Sm*FPPS were determined at an IPP concentration of 47 μM, with concentrations of DMAPP, GPP, and FPP varying from 0 μM to 100 μM, or with IPP from 0 μM to 100 μM while keeping the concentrations of DMAPP, GPP, and FPP constant at 55 μM. For *Sm*GGPPS, IPP or DMAPP/GPP/FPP was maintained at 10 μM, while the second substrate was varied from 0 μM to 50 μM. The concentration ranges used to determine kinetic constants were determined in three independent preliminary experiments using a wide range of concentrations of IPP and the corresponding allylic pyrophosphate (1 μM to 100 μM). K_m and V_{max} for all substrate and enzyme combinations were determined by plotting velocity versus substrate concentration, using SigmaPlot (Systat Software Inc., Chicago, IL).

Inhibition of *Sm*FPPS and *Sm*GGPPS by bisphosphonates. Depending on solubility, bisphosphonates were typically prepared as 500 μM stock solutions in dimethyl sulfoxide (DMSO) or an aqueous solution of sodium bicarbonate (pH of ~8 to 10). Gradients of bisphosphonate concentrations (100 μM to 5 nM) were prepared by serial dilution. Inhibitors and 200 ng of enzyme were incubated at room temperature for 10 min in the assay buffer (10 mM HEPES, 150 mM NaCl, 1 mM $MgCl_2$, pH 7.5) before adding GPP and ^{3}H-IPP to a 50 μM final concentration. The reactions were allowed to proceed for 20 min in a water bath at 37°C and then terminated by addition of 3 M HCl in methanol. The reaction mixtures were neutralized by NaOH-hexane, and the organic phases were collected for radioactive counting. If significant inhibition (>80%) was seen with 50 μM inhibitor, the inhibitor concentration was titrated to determine the 50% inhibitory concentration (IC_{50}), using Prism 5 (GraphPad Software, Inc., La Jolla, CA).

Activity of bisphosphonates against adult *S. mansoni* worms. Experiments were performed as described previously (41). Infection of mice (NIH Swiss; National Cancer Institute, Rockville, MD) with *S. mansoni* cercariae (NMRI strain) obtained from infected *Biomphalaria glabrata* snails and perfusion of adult worms (6 to 7 weeks) from mice were carried out as described previously (38). Adult worms were obtained by perfusing the portal veins of mice at 45 to 50 days postinfection, using Dulbecco's modified Eagle's medium (DMEM). Live worms were washed thoroughly with DMEM and incubated in 5 ml RPMI medium (Sigma-Aldrich) supplemented with 100 units/ml penicillin, 50 μg/ml streptomycin sulfate, 2 mM L-glutamine, and 10% fetal calf serum (Invitrogen). Bisphosphonates (50 μM) were added to each well, and the cultures were replenished every day with fresh medium and inhibitors. Worms were checked and counted every day for worm pairing, motility, and the presence of live and dead worms. Dead worms were identified as those worms that showed zero motility for several minutes. This study was approved by the Institutional Animal Care and Use Committee at Rush University Medical Center (IACUC number 08-058; DHHS animal welfare assurance number A3120-01).

RESULTS

Cloning and analysis of *S. mansoni* FPPS and GGPPS. Nucleotide sequences with high similarities to FPPS and GGPPS genes from other organisms were amplified from adult worm cDNA, cloned, and sequenced. The sequences obtained were identical to accessions currently in GenBank: accession number CCD78373 for FPPS and accession number XP_002573680 for GGPPS. Multiple-sequence alignments of FPPS and GGPPS were generated using CLUSTAL W 2.0.12 in order to compare the schistosome proteins to orthologs present in other species. The results indicate that the schistosome proteins share the active site motifs found in orthologous proteins (Table 1; see Fig. S1 in the supplemental material). The first aspartate-rich motif (FARM) and second aspartate-rich motif (SARM), which have been demonstrated to be the active sites in avian FPPS (42), are present in *Sm*FPPS and *Sm*GGPPS. In addition to these conserved motifs, *Sm*FPPS and *Sm*GGPPS share significant overall similarities and identities with other FPPS and GGPPS proteins (Fig. 3). For example, *Sm*FPPS has 35% identity to human FPPS and 34% identity to *Drosophila melanogaster* FPPS, while *Sm*GGPPS has 53% identity to human GGPPS and 46% identity to *D. melanogaster* GGPPS. Note that the amino acids proximal to the FARM are involved in determining and limiting the length of the allylic diphosphates able to bind in the active site (Fig. 3). In type I and type III FPPS, there are two highly conserved DDXXD (FARM and SARM) repeats, whereas in type II FPPS, the FARM is DDXXXXD (Fig. 3) (43). Type I and type II FPPS primarily produce FPP, but the type III FPPS, having an aromatic amino acid (Y or F) at the fourth position and a small residue (G, A, or C) at the fifth position upstream from the FARM, is bifunctional, producing both FPP and GGPP (44). This motif in *Sm*FPPS is similar to that seen with the type III FPPS proteins, with Gly-Phe present in the chain-length-determining region. Like other GGPPS enzymes, *Sm*GGPPS has neither the aromatic amino acids at the fourth and/or fifth position before the FARM found in FPPS enzymes nor the two amino acid insertions that occur in FPPS and GGPPS from plants and bacteria. Instead, product chain length is determined by a bulky residue two amino

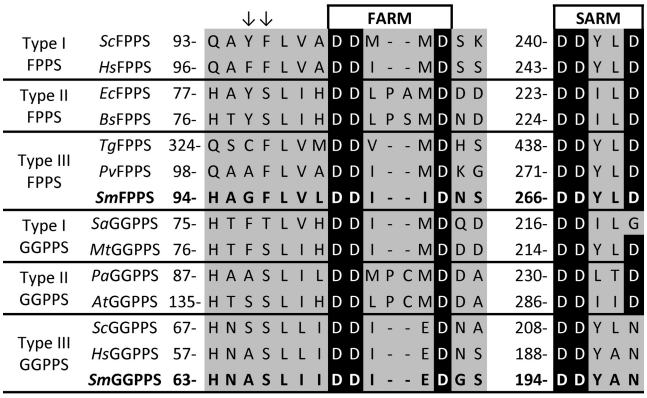

FIG 3 The most conserved motifs, i.e., the first aspartate-rich motif (FARM) and the second aspartate-rich motif (SARM), in three different types of FPPS and GGPPS enzymes from various species. Residues that form a "lid," effectively limiting binding to shorter allylic diphosphates, are shown with arrows. *Sc*, *Saccharomyces cerevisiae*; *Hs*, human; *Ec*, *Escherichia coli*; *Bs*, *Bacillus stearothermophilus*; *Tg*, *Toxoplasma gondii*; *Pv*, *Plasmodium vivax*; *Sm*, *Schistosoma mansoni*; *Sa*, *Sulfolobus acidocaldarius*; *Mt*, *Methanobacterium thermoautotrophicum*; *Pa*, *Pantoea ananatis*; *At*, *Arabidopsis thaliana*.

TABLE 2 Catalytic activities of *Schistosoma mansoni* FPPS and GGPPS[a]

Enzyme	Reaction	V_{max} (units/mg)	K_m (μM)
*Sm*FPPS	IPP + DMAPP	1,100 ± 90	18 ± 5
	DMAPP + IPP	1,100 ± 50	17 ± 2
	GPP + IPP	400 ± 20	19 ± 3
	IPP + GPP	750 ± 40	17 ± 2
	FPP + IPP	15 ± 1	12 ± 2
	IPP + FPP	22 ± 1	19 ± 1
*Sm*GGPPS	IPP + DMAPP	70 ± 4	2.5 ± 1
	DMAPP + IPP	67 ± 5	3.0 ± 1
	GPP + IPP	370 ± 30	7.0 ± 1
	IPP + GPP	180 ± 10	6.0 ± 2
	FPP + IPP	120 ± 9	3.6 ± 1
	IPP + FPP	180 ± 1	7.0 ± 2

[a] In each reaction, the substrate being varied appears first. All V_{max} and K_m values were calculated from the means for three independent reactions ± standard errors. One active unit is defined as one nmol of product formed per minute.

TABLE 3 Activities of bisphosphonates against *Schistosoma mansoni* FPPS and GGPPS

Compound	IC_{50} (μM)[a]			
	*Sm*FPPS	*Sm*GGPPS	*Hs*FPPS	*Hs*GGPPS
2 (risedronate)	0.19	6.4	0.24	350
91 (zoledronate)	0.14	5.0	0.11	100
460	0.53	32	1.72	ND
703	0.038	3.4	1.0	2.0
715	0.65	4.4	2.0	0.28
749	9.9	2.1	35	0.98
754	1.9	9.1	550	0.59
782	1.9	4.1	ND	ND
811	0.037	35	ND	4.5
1186	1.9	23	7	ND

[a] Activities of the bisphosphonates against human FPPS and GGPPS are shown for ease of comparison (53). ND, not determined.

acids before a conserved Gly-Gln (GQ) motif (45); this motif is conserved in *Sm*GGPPS (see Fig. S1B).

Recombinant protein expression and purification. *Sm*FPPS and *Sm*GGPPS were expressed in *E. coli* BL21 Star(DE3) cells, generating 6-His-tagged fusion proteins. The supernatants obtained from cell lysates were purified through a nickel column, and elution fractions were analyzed in a 16% SDS gel. *Sm*FPPS eluted in the 300 mM imidazole fractions and had a molecular mass of ~44.2 kDa (see Fig. S2 in the supplemental material), while recombinant *Sm*GGPPS eluted in the 300 mM and 500 mM imidazole fractions and had a molecular mass of ~38.3 kDa (see Fig. S2).

Kinetic analysis of recombinant *S. mansoni* FPPS and GGPPS. Recombinant *Sm*FPPS and *Sm*GGPPS proteins were analyzed for activity without removal of the 6-His tag. Both proteins were tested in reactions with ^{3}H-IPP and allylic diphosphates (DMAPP, GPP, or FPP). Both proteins were active with all three allylic diphosphates. The V_{max} values for *Sm*FPPS ranged from 1,077 units/mg protein with DMAPP plus IPP to 22 units/mg protein with FPP plus IPP (Table 2). The K_m for all substrates was ~17 μM (Table 2). The V_{max} values for *Sm*GGPPS ranged from 67 to 371 units/mg for the different substrates, while the K_m values ranged from 2.5 to 7 μM (Table 2).

Inhibition of *S. mansoni* FPPS and GGPPS by bisphosphonates. Ten bisphosphonates (Fig. 2B) were tested against recombinant *Sm*FPPS and *Sm*GGPPS. The IC_{50}s of each inhibitor were determined by plotting curves of inhibitor concentration versus percent enzyme inhibition. IC_{50}s with *Sm*FPPS ranged from 0.04 μM (compounds 703 and 811) to 10 μM (compound 749), while those for *Sm*GGPPS ranged from 2.1 μM (compound 749) to 35.5 μM (compound 811) (Table 3; Fig. 4A and B).

Effects of inhibitors on *S. mansoni* worms cultured *in vitro*. To determine the effects of bisphosphonates on *S. mansoni* worms, *ex vivo* adult worms, in 10 pairs per treatment, were cultured in the presence of a bisphosphonate at 50 μM and monitored visually for 7 days. The worm culture was replenished daily with fresh medium and 50 μM inhibitor. Of the 10 bisphosphonates tested (Table 3), only compound 715 was active, with worm death beginning after 4 days of exposure and with all worms dead on day 7 (Fig. 4C). Ten other lipophilic bisphosphonates with pyridinium ring structures, which were analogs of compound 715, were then tested on adult worms, with compounds 746, 766, 777, and 820 (Fig. 4D) resulting in worm death beginning after 4 days. Compounds affected male and female worms equally.

DISCUSSION

Schistosomiasis is a disease that affects approximately 200 million people worldwide, with more than 200,000 deaths annually (1). There is only one drug, praziquantel, available for treatment. In order to identify new schistosome targets for drug development, we characterized two enzymes of the mevalonate pathway. These studies were justified by previous studies with statins, inhibitors of HMGR, which result in both reductions in parasite egg production and increased worm death (6). FPPS and GGPPS are downstream of HMGR in the isoprenoid biosynthesis pathways. Their activities yield important molecules, such as dolichols, quinones, and long-chain isoprenoids involved in protein prenylation (23). Inhibition of FPPS/GGPPS enzymes is expected to reduce the amounts of these essential isoprenoid molecules and to have deleterious effects on worm metabolism. Bisphosphonates, including risedronate and zoledronate, are widely used to prevent and treat osteoporosis and have been shown to inhibit FPPS in many organisms, including humans (25) and some parasites (26). Digeranyl bisphosphonates are known to inhibit human GGPPS (46). No studies have been done on schistosome FPPS or GGPPS or on the activity of bisphosphonates against schistosomes. In this study, we expressed and characterized recombinant *Sm*FPPS and *Sm*GGPPS to determine if it might be possible to use "new-generation" lipophilic bisphosphonates for schistosomiasis treatment. We found that recombinant *Sm*FPPS and *Sm*GGPPS are inhibited by several bisphosphonates and that some bisphosphonates have activity against cultured, *ex vivo* adult worms, suggesting that schistosome FPPS and GGPPS may be appropriate targets for drug development for schistosomiasis.

Comparisons of *Sm*FPPS and *Sm*GGPPS to orthologous FPPS and GGPPS proteins indicate that they have similar conserved active site motifs. For instance, the active sites of both proteins typically contain two "aspartate-rich" motifs (40, 46). These are both present in the amino acid sequences of *Sm*FPPS and *Sm*GGPPS. Interestingly, from a three-dimensional structural perspective, *Sm*FPPS is predicted to have the closest structural homology to human FPPS (based on Phyre2 [47] structure prediction), with which it has the highest (38%) sequence identity

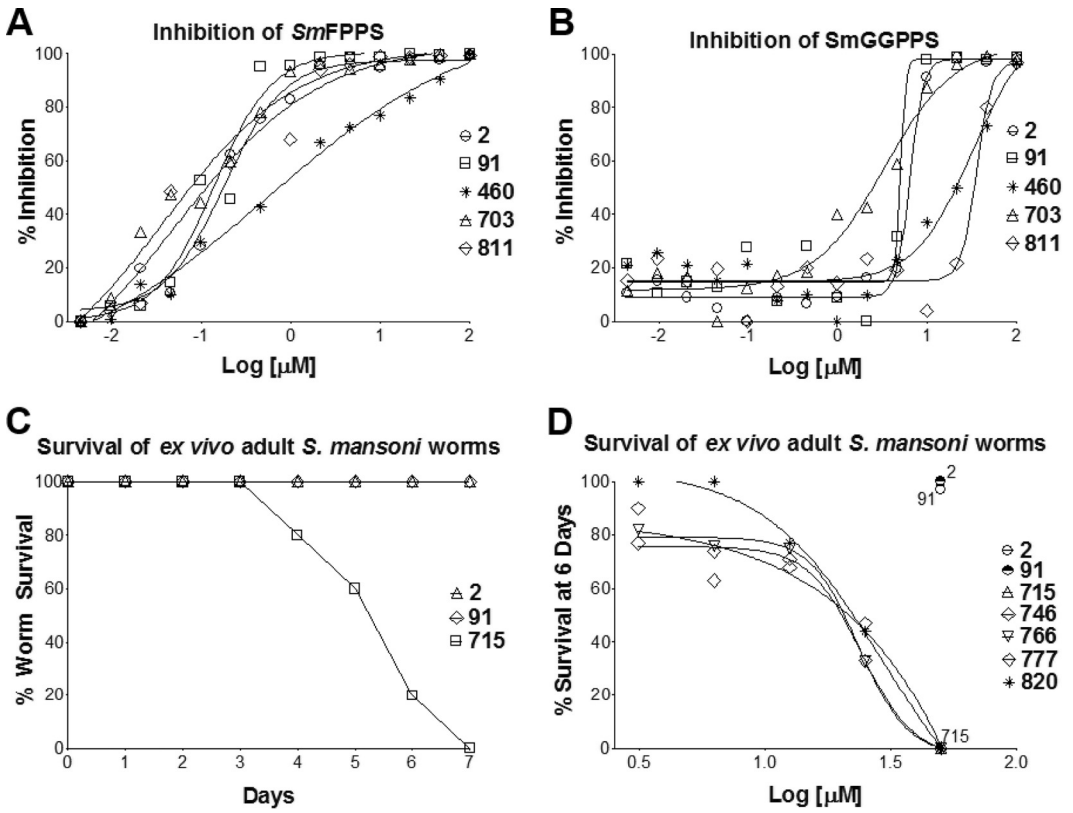

FIG 4 (A) IC_{50} curves for inhibitor concentration versus percent *Schistosoma mansoni* FPPS inhibition. (B) IC_{50} curves for inhibitor concentration versus percent *S. mansoni* GGPPS inhibition. (C) Survival of *ex vivo* adult *S. mansoni* worms cultured in the presence of 50 μM bisphosphonates 2, 91, and 715 as a function of time. (D) Survival of *ex vivo* adult *S. mansoni* worms cultured with 50 μM 2, 91, and 715 and with various concentrations of compounds 746, 766, 777, and 820. Worms were cultured in RPMI medium with inhibitors, with the medium and inhibitors replaced daily. Worms were observed for survival (as assessed by motility) over 6 days. The LD_{50}s of the compounds for adult worms are ~25 μM.

among the prenyltransferases whose structures have been reported. Likewise, *Sm*GGPPS is also predicted to have the closest structural homology to human GGPPS (54% sequence identity). The predicted structural models for *Sm*FPPS (based on *Homo sapiens* FPPS [*Hs*FPPS]; Protein Data Bank [PDB] ID code 2F94) and *Sm*GGPPS (based on *Hs*GGPPS; PDB ID code 2Q80) are shown in Fig. S3 in the supplemental material. However, *Sm*FPPS and *Sm*GGPPS do have significant sequence differences from the human orthologs, which could be exploited for selective enzyme inhibition and drug development.

We went on to investigate if the schistosome proteins have similarity in function by investigating the substrate specificities of the enzymes. We found that *Sm*FPPS was active with DMAPP, GPP, and FPP as substrates, but with substrate preferences of DMAPP > GPP > FPP under similar reaction conditions. In previous studies involving FPPS, the allylic substrates studied were DMAPP and GPP, with FPPS enzymes showing a preference for DMAPP over GPP. FPPS from *Trypanosoma brucei* (*Tb*FPPS) and *T. cruzi* FPPS (*Tc*FPPS) have specific activities of 840 units/mg (31) and 325 units/mg (30), respectively, with IPP and GPP as substrates, and these values are similar to those we determined for *Sm*FPPS. However, there has not been any reported activity of *Tb*FPPS or *Tc*FPPS with FPP. FPPS from the rubber-producing mushroom *Lactarius chrysorrheus* has been shown to be unable to form products with FPP as the substrate (48), and data from the Comprehensive Enzyme Information System (http://www.brenda-enzymes.org/) indicate that only DMAPP and GPP are substrates for FPPS enzymes from over 20 different species. However, FPPS enzymes from a number of parasitic protozoa, including *Toxoplasma gondii* (44), *Plasmodium vivax* (49), and *Cryptosporidium parvum* (50), were recently shown to be "bifunctional" prenyl synthases that are capable of accepting DMAPP, GPP, and FPP as substrates to produce FPP and GGPP, as well as, in some cases, products with even longer chains. It has been proposed that substitution of a small amino acid residue for a bulky hydrophobic residue in the chain-length-determining region of type III FPPS proteins allows them to accept GPP and longer prenyl compounds as substrates. The chain-length-determining region of *Sm*FPPS is similar to that seen in type III FPPS, and indeed, the substrate specificity of *Sm*FPPS is similar as well. In addition to changes in the chain-length-determining region, several amino acid insertions are found in type III FPPS proteins, which may also enlarge the substrate binding pocket to accommodate larger prenyl precursors. Similar insertions are also found in *Sm*FPPS (see Fig. S1A in the supplemental material), which, again, may be responsible for broadening the substrate range of the protein and could be targeted in the development of *Sm*FPPS-specific inhibitors.

The inhibitory effects of bisphosphonates on recombinant *Sm*FPPS and *Sm*GGPPS proteins were determined for both enzymes, using the commercial drugs risedronate (compound 2) and zoledronate (compound 91), as well as a series of other species (compound 460 and the more lipophilic bisphosphonates 703, 715, 749, 754, 782, 811, and 1186). Compounds 2 and 91 are

known to inhibit FPPS. In studies with trypanosomes, compounds 2 and 91 were found to inhibit *Tc*FPPS (30) and *Tb*FPPS (26), with IC_{50}s of 37 nM and 42 nM, respectively, and human FPPS, with IC_{50}s in the 100 to 240 nM range (51). The IC_{50} of compound 2 against *Sm*FPPS was 0.19 μM, while compound 91 had an IC_{50} of 0.14 μM against *Sm*FPPS, which is in the same range as that against human FPPS. The lipophilic bisphosphonates were also efficient *Sm*FPPS inhibitors, with IC_{50}s ranging from 0.04 μM (compound 703) to 10 μM (compound 749). In general, most compounds were less active against *Sm*GGPPS than against *Sm*FPPS (Fig. 4A and B). An exception was compound 749, which contains two geranyl moieties. Compounds 715, 754, and 782, which are known inhibitors of GGPPS (52), had IC_{50}s in the micromolar range (4.1 to 9.1 μM) against *Sm*GGPPS. Compounds 460, 811, and 1186 were the least inhibitory against *Sm*GGPPS (23 to 35 μM).

Since these compounds had some inhibitory activity against the *Sm*FPPS and *Sm*GGPPS proteins, we next investigated if they had activity against cultured *ex vivo* adult *S. mansoni* worms. We initially tested all compounds listed in Table 3 for activity against adult worms. However, only compound 715, a lipophilic pyridinium bisphosphonate, had any activity (100% death at 50 μM) (Fig. 4C). The bone resorption bisphosphonate drugs risedronate (compound 2) and zoledronate (compound 91) had no activity at 50 μM (Fig. 4C). We then tested a series of compound 715 analogs (compounds 746, 766, 777, and 820) for worm-killing activity. The results are shown in Fig. 4D. Clearly, the long alkyl chain bisphosphonates have good activity, with IC_{50}s for worm killing of ~20 μM, while risedronate (compound 2) and zoledronate (compound 91) have no effect (Fig. 4C and D), even though they are good *Sm*FPPS and quite good *Sm*GGPPS inhibitors—pointing again to the need for lipophilicity in order to have worm-killing activity. It is also clear from the results described above that targeting GGPPS alone is unlikely to be a good therapeutic strategy, since FPPS can make GGPP.

Also note that compound 715 was previously reported to have activity in a mouse xenograft system, without apparent toxicity to the host (52). What is not clear at present is why some of the lipophilic bisphosphonates were inactive against worms. They have both FPPS and GGPPS activities, although given the difficulty of correlating enzyme and cell activity (36), extrapolations to animals are expected to be even more challenging. Overall, then, the results provide proof of principle that *Sm*FPPS and *Sm*GGPPS are druggable parasite enzymes and that lipophilic bisphosphonates may serve as starting points for the development of new drugs for the treatment of schistosomiasis.

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REFERENCES

1. **Hotez PJ, Fenwick A, Savioli L, Molyneux DH.** 2009. Rescuing the bottom billion through control of neglected tropical diseases. Lancet **373:** 1570–1575.
2. **Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J.** 2006. Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. Lancet Infect. Dis. **6:**411–425.
3. **Wang W, Wang L, Liang YS.** 2012. Susceptibility or resistance of praziquantel in human schistosomiasis: a review. Parasitol. Res. **111:**1871–1877.
4. **Dömling A, Khoury K.** 2010. Praziquantel and schistosomiasis. ChemMedChem **5:**1420–1434.
5. **Mutapi F, Billingsley PF, Secor WE.** 2013. Infection and treatment immunizations for successful parasite vaccines. Trends Parasitol. **29:**135–141.
6. **Chen GZ, Foster L, Bennett JL.** 1990. Antischistosomal action of mevinolin: evidence that 3-hydroxy-methylglutaryl-coenzyme A reductase activity in *Schistosoma mansoni* is vital for parasite survival. Naunyn Schmiedebergs Arch. Pharmacol. **342:**477–482.
7. **Edwards PA, Ericsson J.** 1999. Sterols and isoprenoids: signaling molecules derived from the cholesterol biosynthetic pathway. Annu. Rev. Biochem. **68:**157–185.
8. **Clarke S.** 1992. Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu. Rev. Biochem. **61:**355–386.
9. **Oldfield E.** 2010. Targeting isoprenoid biosynthesis for drug discovery: bench to bedside. Acc. Chem. Res. **43:**1216–1226.
10. **Oldfield E, Lin FY.** 2012. Terpene biosynthesis: modularity rules. Angew. Chem. Int. Ed. Engl. **51:**1124–1137.
11. **Shafer WR, Rine J.** 1992. Protein prenylation: genes, enzymes, targets, and functions. Annu. Rev. Genet. **30:**209–237.
12. **Meyer F, Meyer H, Bueding E.** 1970. Lipid metabolism in the parasitic and free-living flatworms, *Schistosoma mansoni* and *Dugesia dorotocephala*. Biochim. Biophys. Acta **210:**257–266.
13. **Berriman M, Haas BJ, LoVerde PT, Wilson RA, Dillon GP, Cerqueira GC, Mashiyama ST, Al-Lazikani B, Andrade LF, Ashton PD, Aslett MA, Bartholomeu DC, Blandin G, Caffrey CR, Coghlan A, Coulson R, Day TA, Delcher A, DeMarco R, Djikeng A, Eyre T, Gamble JA, Ghedin E, Gu Y, Hertz-Fowler C, Hirai H, Hirai Y, Houston R, Ivens A, Johnston DA, Lacerda D, Macedo CD, McVeigh P, Ning Z, Oliveira G, Overington JP, Parkhill J, Pertea M, Pierce RJ, Protasio AV, Quail MA, Rajandream MA, Rogers J, Sajid M, Salzberg SL, Stanke M, Tivey AR, White O, Williams DL, Wortman J, Wu W, Zamanian M, Zerlotini A, Fraser-Liggett CM, Barrell BG, El-Sayed NM.** 2009. The genome of the blood fluke *Schistosoma mansoni*. Nature **460:**352–358.
14. **Vandewaa EA, Mills G, Chen GZ, Foster LA, Bennett JL.** 1989. Physiological role of HMG-CoA reductase in regulating egg production by *Schistosoma mansoni*. Am. J. Physiol. **257:**R618–R625.
15. **Rajkovic A, Simonsen JN, Davis RE, Rottman FM.** 1989. Molecular cloning and sequence analysis of 3-hydroxy-3-methylglutaryl-coenzyme A reductase from the human parasite *Schistosoma mansoni*. Proc. Natl. Acad. Sci. U. S. A. **86:**8217–8221.
16. **Chen GZ, Bennett JL.** 1993. Characterization of mevalonate-labeled lipids isolated from parasite proteins in *Schistosoma mansoni*. Mol. Biochem. Parasitol. **59:**287–292.
17. **Foster JM, Pennock JF, Marshall I, Rees HH.** 1993. Biosynthesis of isoprenoid compounds in *Schistosoma mansoni*. Mol. Biochem. Parasitol. **61:**275–284.
18. **Osman A, Niles EG, LoVerde PT.** 1999. Characterization of the Ras homologue of *Schistosoma mansoni*. Mol. Biochem. Parasitol. **100:**27–41.
19. **Loeffler IK, Bennett JL.** 1996. A rab-related GTP-binding protein in *Schistosoma mansoni*. Mol. Biochem. Parasitol. **77:**31–40.
20. **Vermeire JJ, Osman A, LoVerde PT, Williams DL.** 2003. Characterisation of a Rho homologue of *Schistosoma mansoni*. Int. J. Parasitol. **33:**721–731.
21. **Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hensens O, Hirshfield J, Hoogsteen K, Liesch J, Springer J.** 1980. Mevinolin: a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. U. S. A. **77:**3957–3961.
22. **Brown MS, Faust JR, Goldstein JL.** 1978. Inhibition of 3-hydroxy 3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. J. Biol. Chem. **253:**1121–1128.
23. **Brown MS, Goldstein JL.** 1986. A receptor mediated pathway for cholesterol homeostasis. Science **232:**34–47.
24. **Araújo N, Mattos AC, Sarvel AK, Coelho PM, Katz N.** 2008. Oxamniquine, praziquantel and lovastatin association in the experimental *Schistosomiasis mansoni*. Mem. Inst. Oswaldo Cruz **103:**450–454.

25. **Russell RG.** 2006. Bisphosphonates: from bench to bedside. Ann. N. Y. Acad. Sci. **1068:**367–401.
26. **Martin MB, Grimley JS, Lewis JC, Heath HT, 3rd, Bailey BN, Kendrick H, Yardley V, Caldera A, Lira R, Urbina JA, Moreno SN, Docampo R, Croft SL, Oldfield E.** 2001. Bisphosphonates inhibit the growth of *Trypanosoma brucei*, *Trypanosoma cruzi*, *Leishmania donovani*, *Toxoplasma gondii*, and *Plasmodium falciparum*: a potential route to chemotherapy. J. Med. Chem. **44:**909–916.
27. **Goffinet M, Thoulouzan M, Pradines A, Lajoie-Mazenc I, Weinbaum C, Faye JC, Séronie-Vivien S.** 2006. Zoledronic acid treatment impairs protein geranyl-geranylation for biological effects in prostatic cells. BMC Cancer **6:**60. doi:10.1186/1471-2407-6-60.
28. **Ashburn TT, Thor KB.** 2004. Drug repositioning: identifying and developing new uses for existing drugs. Nat. Rev. Drug Discov. **3:**673–683.
29. **Martin MB, Sanders JM, Kendrick H, de Luca-Fradley K, Lewis JC, Grimley JS, Van Brussel EM, Olsen JR, Meints GA, Burzynska A, Kafarski P, Croft SL, Oldfield E.** 2002. Activity of bisphosphonates against *Trypanosoma brucei rhodesiense*. J. Med. Chem. **45:**2904–2914.
30. **Montalvetti A, Bailey BN, Martin MB, Severin GW, Oldfield E, Docampo R.** 2001. Bisphosphonates are potent inhibitors of *Trypanosoma cruzi* farnesyl diphosphate synthase. J. Biol. Chem. **276:**33930–33937.
31. **Montalvetti A, Fernandez A, Sanders JM, Ghosh S, Van Brussel E, Oldfield E, Docampo R.** 2003. Farnesyl diphosphate synthase is an essential enzyme in *Trypanosoma brucei*. *In vitro* RNA interference and *in vivo* inhibition studies. J. Biol. Chem. **278:**17075–17083.
32. **Moreno B, Bailey BN, Luo S, Martin MB, Kuhlenschmidt M, Moreno SN, Docampo R, Oldfield E.** 2001. $^{(31)}$P NMR of apicomplexans and the effects of risedronate on *Cryptosporidium parvum* growth. Biochem. Biophys. Res. Commun. **284:**632–637.
33. **Ghosh S, Chan JM, Lea CR, Meints GA, Lewis JC, Tovian ZS, Flessner RM, Loftus TC, Bruchhaus I, Kendrick H, Croft SL, Kemp RG, Kobayashi S, Nozaki T, Oldfield E.** 2004. Effects of bisphosphonates on the growth of *Entamoeba histolytica* and *Plasmodium* species *in vitro* and *in vivo*. J. Med. Chem. **47:**175–187.
34. **Moreno SN, Li ZH.** 2008. Anti-infectives targeting the isoprenoid pathway of *Toxoplasma gondii*. Expert Opin. Ther. Targets **12:**253–263.
35. **Jordão FM, Saito AY, Miguel DC, de Jesus Peres V, Kimura EA, Katzin AM.** 2011. *In vitro* and *in vivo* antiplasmodial activity of risedronate and its interference with protein prenylation in *Plasmodium falciparum*. Antimicrob. Agents Chemother. **55:**2026–2031.
36. **Mukkamala D, No JH, Cass LM, Chang TK, Oldfield E.** 2008. Bisphosphonate inhibition of a *Plasmodium* farnesyl diphosphate synthase and a general method for predicting cell-based activity from enzyme data. J. Med. Chem. **51:**7827–7833.
37. **Singh AP, Zhang Y, No JH, Docampo R, Nussenzweig V, Oldfield E.** 2010. Lipophilic bisphosphonates are potent inhibitors of *Plasmodium* liver-stage growth. Antimicrob. Agents Chemother. **54:**2987–2993.
38. **Lewis F.** 2001. Schistosomiasis. Curr. Protoc. Immunol. Chapter 19:Unit 19.1. doi:10.1002/0471142735.im1901s28.
39. **Sagami H, Morita Y, Ogura K.** 1994. Purification and properties of geranylgeranyl-diphosphate synthase from bovine brain. J. Biol. Chem. **269:**20561–20566.
40. **Ericsson J, Greene JM, Carter KC, Shell BK, Duan DR, Florence C, Edwards PA.** 1998. Human geranylgeranyl diphosphate synthase: isolation of the cDNA, chromosomal mapping and tissue expression. J. Lipid Res. **39:**1731–1739.
41. **Kuntz AN, Davioud-Charvet E, Sayed AA, Califf LL, Dessolin J, Arnér ES, Williams DL.** 2007. Thioredoxin glutathione reductase from *Schistosoma mansoni*: an essential parasite enzyme and a key drug target. PLoS Med. **4:**e206. doi:10.1371/journal.pmed.0040206.
42. **Tarshis LC, Yan M, Poulter CD, Sacchettini JC.** 1994. Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-A resolution. Biochemistry **33:**10871–10877.
43. **Kawasaki T, Hamano Y, Kuzuyama T, Itoh N, Seto H, Dairi T.** 2003. Interconversion of the product specificity of type I eubacterial farnesyl diphosphate synthase and geranylgeranyl diphosphate synthase through one amino acid substitution. J. Biochem. **133:**83–91.
44. **Li ZH, Cintron R, Koon NA, Moreno SN.** 2012. The N-terminus and the chain-length determination domain play a role in the length of the isoprenoid product of the bifunctional *Toxoplasma gondii* farnesyl diphosphate synthase. Biochemistry **51:**7533–7540.
45. **Hemmi H, Noike M, Nakayama T, Nishino T.** 2003. An alternative mechanism of product chain-length determination in type III geranylgeranyl diphosphate synthase. Eur. J. Biochem. **270:**2186–2194.
46. **Wiemer AJ, Tong H, Swanson KM, Hohl RJ.** 2007. Digeranyl bisphosphonate inhibits geranylgeranyl pyrophosphate synthase. Biochem. Biophys. Res. Commun. **353:**921–925.
47. **Kelley LA, Sternberg MJE.** 2009. Protein structure prediction on the web: a case study using the Phyre server. Nat. Protoc. **4:**363–371.
48. **Mekkriengkrai D, Sando T, Hirooka K, Sakdapipanich J, Tanaka Y, Fukusaki E, Kobayashi A.** 2004. Cloning and characterization of farnesyl diphosphate synthase from the rubber-producing mushroom *Lactarius chrysorrheus*. Biosci. Biotechnol. Biochem. **68:**2360–2368.
49. **Artz JD, Wernimont AK, Dunford JE, Schapira M, Dong A, Zhao Y, Lew J, Russell RG, Ebetino FH, Oppermann U, Hui R.** 2011. Molecular characterization of a novel geranylgeranyl diphosphate synthase from *Plasmodium* parasites. J. Biol. Chem. **286:**3315–3322.
50. **Artz JD, Dunford JE, Arrowood MJ, Dong A, Chruszcz M, Kavanagh KL, Minor W, Russell RG, Ebetino FH, Oppermann U, Hui R.** 2008. Targeting a uniquely nonspecific prenyl synthase with bisphosphonates to combat cryptosporidiosis. Chem. Biol. **15:**1296–1306.
51. **Glickman JF, Schmid A.** 2007. Farnesyl diphosphate synthase: real-time kinetics and inhibition by nitrogen-containing bisphosphonates in a scintillation assay. Assay Drug Dev. Technol. **5:**205–214.
52. **Zhang Y, Cao R, Yin F, Hudock MP, Guo RT, Krysiak K, Mukherjee S, Gao YG, Robinson H, Song Y, No JH, Bergan K, Leon A, Cass L, Goddard A, Chang TK, Lin FY, Van Beek E, Papapoulos S, Wang AH, Kubo T, Ochi M, Mukkamala D, Oldfield E.** 2009. Lipophilic bisphosphonates as dual farnesyl/geranylgeranyl diphosphate synthase inhibitors: an X-ray and NMR investigation. J. Am. Chem. Soc. **131:**5153–5162.
53. **Zhang Y, Cao R, Yin F, Lin FY, Wang H, Krysiak K, No JH, Mukkamala D, Houlihan K, Li J, Morita CT, Oldfield E.** 2010. Lipophilic pyridinium bisphosphonates: potent γδ T cell stimulators. Angew. Chem. Int. Ed. Engl. **49:**1136–1138.