We report the cloning and sequencing of a gene encoding the farnesyl pyrophosphate synthase (FPPS) of Trypanosoma brucei. The protein (TbFPPS) is an attractive target for drug development because the growth of T. brucei has been shown to be inhibited by analogs of its putative target for drug development because the growth of TbFPPS, including an 11-mer peptide insertion present in the Trypanosoma cruzi FPPS. Heterologous expression of TbFPPS in Escherichia coli produced a functional enzyme that was inhibited by several nitrogen-containing bisphosphonates, such as pamidronate and risedronate. Risedronate was active in vivo against T. brucei infection in mice (giving a 60% survival rate), but pamidronate was not effective. The essential nature of TbFPPS was studied using RNA interference (RNAi) to inhibit the expression of the gene. Expression of TbFPPS double-stranded RNA in procyclic trypomastigotes caused specific degradation of mRNA. After 4 days of RNAi, the parasite growth rate declined and the cells subsequently died. Similar results were obtained with bloodstream form trypomastigotes, except that the RNAi system in this case was leaky and mRNA levels and parasites recovered with time. Molecular modeling and structure-activity investigations of enzyme and in vitro growth inhibition data resulted in similar pharmacophores, further validating TbFPPS as the target for bisphosphonates. These results establish that FPPS is essential for parasite viability and validate this enzyme as a target for drug development.

The Trypanosoma brucei group of parasites causes African trypanosomiasis (sleeping sickness) in humans, and nagana in animals and is responsible for heavy socioeconomic losses in most countries of sub-Saharan Africa (1). Therapy against African sleeping sickness is unsatisfactory because of the toxicity of currently available drugs, together with the development of drug resistance (2).

A number of bisphosphonates have recently been shown to have significant activity against the proliferation of T. brucei and other parasites in vitro (3, 4) and also have curative effects in in vivo models of visceral (5) and cutaneous (6) leishmaniasis. Bisphosphonates are pyrophosphate analogs in which the oxygen bridge between the two phosphorus atoms has been replaced by a carbon substituted with various side chains. Several bisphosphonates are potent inhibitors of bone resorption and are in clinical use for the treatment and prevention of osteoporosis, Paget’s disease, hypercalcemia caused by malignancy, and tumor metastases in bone (7–10). Many bisphosphonates, such as pamidronate, alendronate, and risedronate, are known to inhibit farnesyl pyrophosphate synthase (FPPS)1 (11–16), and in so doing, they inhibit the formation of farnesyl pyrophosphate, a compound used in protein prenylation and in the synthesis of dolichols, ubiquinones, heme a, and steroids. Although a recombinant FPPS from Trypanosoma cruzi has recently been shown to be potently inhibited by bisphosphonates (17), the question as to whether or not this enzyme is essential for trypanosomatid viability has remained unresolved. Here, we report the results of RNA interference (RNAi) studies together with in vitro and in vivo inhibition studies that demonstrate that FPPS is indeed an essential cellular component in T. brucei.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum, Dulbecco’s phosphate-buffered saline, protease inhibitor mixture, geranyl pyrophosphate (GPP), and isopentenyl pyrophosphate (IPP) were purchased from Sigma. Fetal bovine serum (normal and dialyzed) was from Atlanta Biologicals (Norcross, GA) and Serum Plus was from JRH Biosciences (Lenex, KS). Restriction enzymes, T4 DNA ligase, Tsoq polymerase, the Klenow fragment of DNA polymerase, Trizol reagent, and 0.24–9.5-kb RNA ladder were from Invitrogen. pCR2.1-TOPO cloning kit, and Superscript II RT were from Invitrogen. Hybond-N nylon membrane, PD-10 desalting column, and [α-32P]dCTP (3000 Ci/mmol) were obtained from Amer sham Biosciences. [1-14C]IPP (57.5 mCi/mmol) was from PerkinElmer Life Sciences. The pET-28a+ expression system and Benzonase1 were used.

Received for publication, October 11, 2002, and in revised form, February 19, 2003 Published, JBC Papers in Press, March 4, 2003, DOI 10.1074/jbc.M210467200
Cloning of the *T. brucei* FPPS Gene and DNA Sequencing—A fragment of 428 bp of the *TbFPPS* gene was amplified using the oligonucleotide primers *TbFPPS*1 (5'-GCTATTCTGGATGGAGGA-3') and *TbFPPS*2 (5'-CTTTGGCGGCTAGTAACTC-3') derived from a *T. brucei* genomic survey sequence (GenBank™ accession number A651702) that showed pronounced similarity to FPPSs of other organisms. Based on this sequence, we designed specific primers to obtain the entire genomic survey sequence (GenBank™ accession number AQ651702) of other FPPSs by using the Biology Workbench 3.2 utility. 2 The selected amino acid sequence of *TbFPPS* was aligned with the sequences of other FPPSs by using the ClustalW 2.0 method of Saitou and Nei (25).

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Expression and Purification of *TbFPPS* from *E. coli*—For expression in *E. coli*, the entire coding sequence of the *TbFPPS* gene was amplified by PCR. Oligonucleotide primers for amplification of the FPPS coding region, *ATG*-*TbFPPS*-1 (5'-CTAGCTAGCATGCCAATGCAAATG-3') and *TGA*-*TbFPPS*-2 (5'-CCCAAGCTTCACTTCTGGCGCTTGTA-3'), were designed so that *NheI* and HindIII restriction sites were introduced at the 5' and 3' ends for convenient cloning in the expression vector pET-28a. Double-stranded DNA sequencing was performed to confirm that the correct reading frame was used, with the polyhistidine tag placed in the N-terminal position. Subsequently, pET*-TbFPPS* was used to transform *E. coli* BL21(DE3). Bacterial clones were grown in LB medium containing 50 μg/ml kanamycin. When induction was performed, bacterial cells transformed with pET*-TbFPPS* were first grown to an *A* of 0.6 at 37 °C and then 1 mM isopropyl- β-D-thiogalactoside was added. After 5 h of growth at 37 °C, cells were pelleted by centrifugation and resuspended in lysis buffer (5 mM imidazole, 300 mM NaCl, 250 mM sucrose, 50 mM Tris-HCl, pH 7.2), incubated with 10 mg/ml lysozyme for 15 min on ice, then sonicated. The lysate was incubated with Benzonase® nuclelease for 15 min on ice, then centrifuged at 16,000 × g.
for 15 min. The supernatant was mixed with nickel-nitrilotriacetic acid-agarose for 1 h at 4°C, then loaded onto a column and washed with lysis buffer in which the concentration of imidazole increased from 15 and 25 mM. Protein was eluted from the column with the same buffer but containing 500 mM imidazole. The eluted fraction was desalted with a PD-10 desalting column. Proteins were determined by the method of Bradford (28) with bovine serum albumin as a standard and analyzed for purity by SDS-polyacrylamide gel electrophoresis.

Western Blotting and Generation of Antibodies against T. cruzi FPPS—Total trypanosome proteins (30 μg of protein/lane) were subjected to SDS-polyacrylamide gel electrophoresis (10%). Electrophoresed proteins were transferred to nitrocellulose using a Bio-Rad Transblot apparatus. Membranes were probed with a 1:1,000 dilution of a rabbit anti-FPPS polyclonal antiserum prepared against recombinant T. cruzi FPPS (17). Bound antibodies were revealed by using goat anti-rabbit IgG (1:20,000) and the ECL TM chemiluminescent detection kit (Amersham Biosciences). To purify the T. cruzi FPPS antibodies, an affinity matrix was prepared by coupling 5 mg of T. cruzi FPPS to 1 ml of cyanogen bromide-activated Sepharose (Sigma). Immune serum (3 ml) was diluted 2-fold with phosphate-buffered saline and incubated with the affinity matrix overnight at 4°C. Antibodies were eluted with 100 mM glycine, pH 2.5, and the eluate was neutralized with 0.1 volume of 2 M Tris-HCl, pH 8.0.

FPPS Assay and Product Analysis—The activity of the enzyme was determined by using the radiometric assay described previously (17). One unit of enzyme activity is defined as the activity required to incorporate 1 nmol of 4-[14C]IPP into 4-[14C]FPP in 1 min.

Treatment of T. brucei-infected Mice—BALBc mice (8 weeks old) were infected intraperitoneally with 10,000 T. brucei bloodstream trypanosome (monomorphic strain 427 from clone MITat 1.4, otherwise known as variant 117). Starting the next day, mice were then treated intraperitoneally with pentamidine or bisphosphonates in 200 μl of

Fig. 2. Sequence analysis of the FPPS from T. brucei. A, comparison of the deduced amino acid sequence of T. brucei with other FPPSs. The deduced amino acid sequence of T. brucei FPPS (GenBank™ accession number AY158342) is compared with the sequences of T. cruzi (AF312890), human (P14324), and S. cerevisiae (J05091) synthases. Similar residues are shaded. The seven conserved regions I to VII are underlined. B, unrooted tree based upon FPPS amino acid sequences. The bar indicates a branch length corresponding to 0.2 substitutions per site. Distances were calculated using the Neighbor Joining (NJ) method of Saitou and Nei (25).
T. brucei Farnesyl Pyrophosphate Synthase

Fig. 3. Southern blot analysis and purification of FPPS from E. coli. A, total genomic DNA was digested with different endonucleases. The DNA fragments were separated in 1% (w/v) agarose, transferred to a nylon membrane, and hybridized with a 32P-labeled probe corresponding to the FPPS coding sequence. B, a SDS-polyacrylamide gel was stained with Coomassie Brilliant Blue. Lane 1, crude extract from pET-28a- transformed cell; lane 2, crude extract of E. coli BL21(DE3)/pETbFPPS; lane 3, soluble fraction from extract of E. coli BL21(DE3)/pETbFPPS; lane 4, nickel column purified fraction.

Table I

<table>
<thead>
<tr>
<th>Concentration</th>
<th>MgCl2</th>
<th>MnCl2</th>
</tr>
</thead>
<tbody>
<tr>
<td>max units/mg</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>660 ± 30</td>
<td>19 ± 2.5</td>
</tr>
<tr>
<td>1</td>
<td>740 ± 2</td>
<td>9.6 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>840 ± 5</td>
<td>4.6 ± 2.2</td>
</tr>
<tr>
<td>10</td>
<td>840 ± 5</td>
<td>14 ± 0.6</td>
</tr>
<tr>
<td>20</td>
<td>670 ± 30</td>
<td>5.1 ± 2.4</td>
</tr>
</tbody>
</table>

Efflux of divalent cations on FPPS from T. brucei

FPPS activity was measured in the presence of different concentrations of MgCl2 or MnCl2 as indicated, in a reaction medium containing 10 mM Hapes (pH 7.4), 2 mM dithiothreitol, 47 μM 4-[14C]IPP (10 μCi/μmol), 200 μM GPP, and 10 μg of recombinant protein (final volume 100 μl). Reactions were incubated for 30 min at 37 °C stopped by the addition of HCl, and made alkaline with NaOH. The radioactive prenyl product was extracted with hexane. No activity was detected in the absence of MgCl2 or MnCl2 and presence of 10 mM EDTA. Values shown are mean ± S.D. of two experiments.

0.9% sodium chloride solution for the times and doses indicated under “Results.” Control mice received only the vehicle.

Molecular Modeling—Using an avian FPPS X-ray structure as a template (Protein Data Bank number 1UBW (29)), a homology model of TbFPPS was built using default settings in the Modeler module of Insight II (30). The majority of models constructed in this way were immediately discounted because of energy considerations and restraint violations, leaving a small group of structures that was further evaluated using the Procheck program (31). In the reported model, the number of residues in the most favored region of the Ramachandran plot and the overall G-factor were significantly better than typical for 2.6-Å resolution structures (91% and 0.0, respectively). This, together with the fact that the root mean square deviation of the homology model from the template for non-loop atoms was only 0.41 Å, lends confidence to the quality of the reported structure.

To model GPP in the TbFPPS active site, we transferred the GPP coordinates from the template model into the TbFPPS structure. This resulted in steric clashes between GPP and Tyr99, because the template for His98 is also a mutant (F112A) and hence also lacks important side chain information. Rotamer searches for His98 and Tyr99 were performed to minimize the GPP-Tyr99 and His98-Tyr99 interactions. Hydrogen atoms were added within Insight II to simulate a pH of 7.0, and GPP was taken to be deprotonated. CHARMM27 potentials and partial charges were applied to all atoms, except for the vinylic hydrogens in GPP, which were set manually. Charges for GPP were obtained using the Gaussian 98 program (32), using the x-ray coordinates of GPP to calculate Merz-Singh-Kollman charges at the HF/6-31+G* level. The coordinates of His98 and Tyr99 were then optimized by using 1000 steps of steepest descents minimization, then further optimized by using the conjugate gradient algorithm to a gradient tolerance of 0.0001 kcal mol−1 Å−1. This structure was then uploaded to the Consurf server3 and Rasmol (33) was used to visualize the three-dimensional locations of highly conserved residues in the TbFPPS model.

To further understand the activities of the bisphosphonates investigated (Fig. 1), we applied pharmacophore modeling techniques to the

T. brucei Farnesyl Pyrophosphate Synthase

**Table II. The effects of bisphosphonates on FPPS activity**

The activity of the T. brucei enzyme was assayed in the presence of bisphosphonates in mixtures containing 10 mM Hepes (pH 7.4), 5 mM MgCl₂, 2 mM dithiothreitol, 47 μM 4-¹⁴C]IPP (10 μCi/μmol), 54 μM GPP, and 10 ng of protein in a final volume of 100 μL. Reactions were incubated for 30 min at 37°C and the prenyl product was extracted and measured by liquid scintillation counting.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kᵢ* (μM)</th>
<th>IC₅₀* (μM)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.007 ± 0.0006</td>
<td>0.046 ± 0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.010 ± 0.002</td>
<td>0.054 ± 0.003</td>
</tr>
<tr>
<td>3 (Bisedronate)</td>
<td>0.010 ± 0.002</td>
<td>0.042 ± 0.002</td>
</tr>
<tr>
<td>4 (Incadronate)</td>
<td>0.011 ± 0.002</td>
<td>0.035 ± 0.009</td>
</tr>
<tr>
<td>5</td>
<td>0.058 ± 0.022</td>
<td>0.15 ± 0.047</td>
</tr>
<tr>
<td>6 (Pamidronate)</td>
<td>0.40 ± 0.12</td>
<td>1.2 ± 0.24</td>
</tr>
<tr>
<td>7</td>
<td>0.57 ± 0.07</td>
<td>3.5 ± 0.41</td>
</tr>
<tr>
<td>8</td>
<td>1.3 ± 0.16</td>
<td>4.9 ± 0.06</td>
</tr>
<tr>
<td>9</td>
<td>8.5 ± 4.3</td>
<td>28 ± 10.4</td>
</tr>
<tr>
<td>10</td>
<td>105</td>
<td></td>
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*Values are the mean ± S.D. of three independent experiments in duplicate.

**RESULTS**

Cloning of the TbFPPS Gene—To screen for genes encoding FPPS in T. brucei, the amino acid sequence of T. cruzi FPPS (AF312690) was used to search the T. brucei data base of The Institute for Genome Research (TIGR) using tblASTn. This search yielded six T. brucei genomic survey sequence clones. The predicted amino acid sequence encoded by clone 6A5 (accession number AY965702) contained the C terminus of this protein. The fragment of the T. brucei FPPS gene was amplified by PCR using oligonucleotide primers complementary to the genomic survey sequence with genomic T. brucei DNA acting as a template. The product of the amplification (428 bp) was ligated into vector pCR2.1TOPO for sequence analysis. This sequence enabled appropriate gene-specific primers to be designed for the generation of 5’- and 3’-end DNA fragments using the 5’-rapid amplification of cDNA ends method (35) and reconstruction of a full-length cDNA. The nucleotide sequence of 2070 bp revealed an open reading frame of 1101 bp, which encodes a 367-amino acid protein with a predicted molecular mass of 42 kDa. Two oligonucleotide primers based in the 5’- and 3’-untranslated regions of this gene were then used to amplify the open reading frame from genomic T. brucei DNA, to obtain a genomic clone. The open reading frame was identical with the full-length cDNA. A BLAST search of the protein data base showed that the amino acid sequence from T. brucei has 32–68% identity and 50–81% similarity with other representative FPPSs. The amino acid sequence of the T. brucei enzyme was aligned with the sequences of T. cruzi, human, and Saccharomyces cerevisiae FPPSs as shown in Fig. 2A. All residues involved in catalysis or binding (regions I–VII) identified in other FPPSs (36) are present in the T. brucei enzyme.

A CLUSTALW alignment of FPPS peptide sequences from avian, some mammalian, fungal, plant, and bacterial FPPSs was then used as the basis for the generation of a phylogenetic tree (Fig. 2B). The results suggested that T. brucei and T. cruzi enzymes shared a most recent common ancestor. Plasmodium falciparum and fungal and plant FPPSs were the closest outgroups to the trypanosomatid sequences.

Southern blot analysis was performed with a PCR fragment that encompasses the entire coding region of TbFPPS (Fig. 3A). Digestions with enzymes that cut at sites not contained within the coding region of the gene gave single bands (EcoRI, HindIII, XhoI), whereas two bands were obtained with enzymes with unique restriction sites in the open reading frame (KpnI, SpaI, Avai, HindII, NsiI), suggesting that the gene was single copy per haploid genome.

**Purification and Reaction Requirements of Recombinant Protein—**TbFPPS was expressed in E. coli BL21(DE3) as a fusion protein with an N-terminal polyhistidine tag. Affinity chromatography on nickel-chelated agarose permitted a simple, one-step purification. Enzyme purity was judged by using SDS-PAGE with Coomassie Blue staining (Fig. 3B). The final protein preparation catalyzed the incorporation of 4-¹⁴C]IPP into a hexane-extractable material when the allylic substrates, dimethylallyl pyrophosphate and GPP, were used (data not shown) (17). 4-¹⁴C]IPP incorporation into the organic solvent extractable material was linear with time for at least 60 min. The radioactive assay was performed in the presence of different concentrations of Mg²⁺ and Mn²⁺, to determine their effect on the T. brucei FPPS. Mg²⁺ and Mn²⁺ were added to the reaction mixture at concentrations of 0.5 and 20 mM. As shown in Table I, optimal levels of activity were obtained by the addition of 5–10 mM Mg²⁺. Enzyme activity was very low when the divalent cation was Mn²⁺. The addition of 10 mM EDTA abolished FPPS activity. Enzyme activity was also assayed between pH 6 and 10.5 using a Tris-HCl (10 mM)/Tris glycine (10 mM) buffer. Optimum activity was observed between pH 7.4 and 8.5 (Fig. 4A).

**Kinetic Analysis—**Standard procedures were used to determine kinetic parameters. Kᵢ and Vₘₐₓ values were obtained by a non-linear regression fit of the data to the Michaelis-Menten equation (SigmaPlot 2000 for Windows). When the rate of FPPS synthesis by the recombinant enzyme (10 ng) was measured in the presence of saturating IPP (47 μM) and varying GPP concentration between 0.15 and 200 μM, a Kᵢ value of 17.2 ± 3.9 μM and a Vₘₐₓ value of 857.0 ± 8.7 units/mg, were calculated (Fig. 4B). When the concentration of GPP was kept at 200 μM and the IPP concentration was varied between 0.5 and 47 μM, the Kᵢ value was 6.2 ± 0.6 μM and the Vₘₐₓ was 1,397.7 ± 1.3 units/mg (Fig. 4C).

**Inhibition by Bisphosphonates—**Nine bisphosphonates (1–9) together with the bromohydrin of isopentenylpyrophosphate (Phosphostim, 10), shown in Fig. 1, were tested for their ability to inhibit the T. brucei enzyme. Kᵢ values were calculated by using the Dixon equation (37) and the IC₅₀ values were obtained as described previously (17) (Table II). BPs are known competitive inhibitors with respect to GPP of FPPSs of different origins (15).

**RNA Interference—**The essentiality of TbFPPS was investi-
gated by using RNAi. The inactivating fragment for RNAi was inserted between the two opposing T7 promoters of the pZJM vector that are both regulated by tetracycline repressors (18). Transfection of the procyclic and bloodstream forms of T. brucei that express the tetracycline repressor and the bacterial T7 RNA polymerase with RNAi constructs directed against TbFPPS resulted in cells that survived phleomycin selection. When production of double-stranded RNA was induced by tetracycline in procyclic forms grown in medium supplemented with 15% FBS, cell growth decreased as compared with non-induced parasites after day two (Fig. 5A). When the induction of RNAi was performed in a medium supplemented with 15% dialyzed FBS, instead of normal FBS, the parasites’ growth rate declined after 2 days of tetracycline induction, and the cells subsequently died (Fig. 5B). Northern blot analysis of TbFPPS RNA from cells grown with 15% dialyzed FBS in the absence or presence of tetracycline (1 μg/ml) showed reduction of intact TbFPPS RNA and a broad smear that probably represents degraded RNA (Fig. 5D). No mRNA could be found in these parasites as analyzed by RT-PCR (Fig. 5E). Immunoblot analysis using an affinity-purified antiserum raised against T. cruzi FPPS (17) detected a band of 43.9 kDa in procyclic form lysates, which is very close to the size predicted by its amino acid sequence (42 kDa). This band was not detected in the RNAi-induced cells by day 4 (Fig. 5F, lane 3). Induction of TbFPPS double stranded RNA in bloodstream forms caused an inhibition of cell proliferation, but they still exhibited exponential growth even when cultured in the presence of dialyzed serum.
...bic cleft or active site region, which for the most part is highly conserved across species (mauve color). There are, however, some interesting differences between the trypanosomatid and other FPPS sequences investigated, in particular, the FF or YF residues located toward the distal end of the hydrophobic pocket of most FPPSs (at positions 98 and 99) are changed to HY in both the T. cruzi and T. brucei proteins (and to SF in P. falciparum), offering some potential for the development of novel inhibitors that might hydrogen bond to these residues.

**Structure-Activity Investigation**—To begin to carry out a three-dimensional structure-activity relationship investigation of TbFPPS inhibition by the compounds investigated, we utilized the Catalyst™ technique (34). The Catalyst™ program basically explores a wide range of conformations (up to 255) for a given molecule and correlates particular spatial features with observed experimental activity. In this way, a pharmacophore can be generated that contains specific chemical features (such as aromatic groups, positive charges, hydrogen bond donors/acceptors, and negative ionizable groups) in specific locations, which correlate with activity. When large amounts of Ki information are available, a quantitative structure-activity relationship model can be developed, whereas for a smaller data set, such as that we have obtained, the pharmacophore contains the major features of importance in enzyme inhibition, but is less suitable for quantitative predictions. Using this approach (the HipHop feature of Catalyst™) we obtained the pharmacophore shown in Fig. 8, A–C. This pharmacophore contains four features of key importance in drug activity: two negative ionizable groups (the bisphosphonates, shown in blue); an endocyclic carbon (green) and a positive charge (red). These features are shown superimposed on the stick diagram structures of two of the more active species: 3-picolylaminomethylene bisphosphonate I (Fig. 8A) and risedronate (3, Fig. 8B). The pharmacophore is also shown docked into the active site of TbFPPS (+GPP) in Fig. 8C.

Next, we used the Catalyst™ approach to develop a pharmacophore for *in vitro* T. brucei growth inhibition, using data obtained previously (4), to compare with the enzyme inhibition results. In this case, test results for some 26 compounds are available, which enables use of the more quantitative Hypogen feature of Catalyst™. Using Hypogen, we obtained a pharmacophore for *T. brucei* growth inhibition that is quite similar to that obtained for TbFPPS enzyme inhibition (obtained using HipHop). This similarity can clearly be seen in Fig. 8D in which we show, superimposed, the pharmacophores for enzyme inhibition and for *T. brucei* cell growth inhibition. The *T. brucei* growth inhibition model contains an additional custom hydrophobic feature to account for, in a quantitative manner, the activity of the long chain n-alkyl bisphosphonates (4).

**DISCUSSION**

The sensitivity of trypanosomatid protozoa to isoprenoid biosynthesis inhibitors (39) offers a unique opportunity for drug target identification and the subsequent development of new anti-trypanosomatid agents. FPPS plays a central role in metabolism through the enzymatic generation of FPP, which is used for protein prenylation, and for the synthesis of sterols, dolichols, heme *a*, and ubiquinone, and is potently inhibited by bisphosphonates (11–16). Stringent genetic validation of putative drug targets is desirable before the rational design of inhibitory compounds intended for chemotherapeutic use is undertaken (40). This study validates FPPS as a drug target through the use of RNAi studies. It provides genetic evidence that FPPS plays an essential cellular role in *T. brucei* and demonstrates that the enzyme is vital for parasite survival *in vitro* and *in vivo*. The finding that a similar pharmacophore can be obtained by structure-activity investigations of *in vitro*
growth and enzyme inhibition data further validates TbFPSS as the target of bisphosphonates.

The T. brucei FPPS gene, TbFPSS, was shown to be present in the T. brucei genome and encodes for a functional enzyme. As shown in the alignment in Fig. 2A, there is considerable sequence similarity between different FPPSs. Heterologous expression of TbFPSS in E. coli resulted in the production of a recombinant enzyme that was generally similar to other FPPSs with respect to its Mg$^{2+}$ requirement, optimum pH, and sensitivity to bisphosphonates. As shown in Table I and Fig. 4A, the protein we have expressed has optimum activity in the presence of 0.5–10 mM MgCl$_2$ and in the pH range 7.4–8.5, basically as found with the human enzyme (41). In addition, our results show that TbFPSS is potently inhibited by a number of bisphosphonates (Table II). The most potent inhibitor found is the aminomethylene bisphosphonate 1, having a $K_i$ of 7 nM, followed closely by its isomer 2 and risedronate (3), both of which have $K_i = 10$ nM. The next most potent inhibitors found were the drugs incadronate (4, $K_i = 11$ nM), the isoxquinoline species (5, $K_i = 58$ nM), and pamidronate (6, $K_i = 400$ nM). The general pattern of reactivity found here is similar to that found for bisphosphate inhibition of a recombinant human FPPS, where the activity profile is again risedronate > incadronate > pamidronate (42). Likewise, the picolyl aminomethylene bisphosphonate 2 has been found to be a potent ($IC_{50} = 25$ nM) inhibitor of a plant FPPS (11) and this species is also a potent inhibitor of bone resorption (43), consistent with activity versus human FPPS. The imidazo[1,2-H]pyridyl species 9 is relatively inactive, consistent with its poor performance as a bone antiresorptive agent (19), presumably because of unfavorable steric interactions in the TbFPSS active site.

In addition to these investigations of aromatic bisphosphonate inhibitors, we also tested whether aliphatic, nitrogen-free bisphosphonates might act as inhibitors of TbFPSS, because these compounds have been found to have activity against the growth of both T. cruzi and Entamoeba histolytica, in vitro (44, 45). The bisphosphonates 7 and 8 contain simple n-alkyl side chains containing 10 and 11 carbon atoms, and were found to have $K_i$ values of 0.57 and 1.3 $\mu$M, respectively. These $K_i$ values are still quite small and approach those found with second generation bone resorption drugs such as pamidronate ($K_i = 0.40$ $\mu$M, Table II). Indeed, in very early work, Shinoda et al. (46) proposed that species such as 7 and 8 might be used in treating bone resorption diseases. Presumably, although these species do not contain the ammonium or pyridinium-like features associated with the most potent inhibitors, they do provide an enhanced hydrophobic interaction (van der Waals dispersion) in the active site of the enzyme, resulting in at least moderate activity. The importance of this effect is also seen on transition from pamidronate to olpadronate to ibandronate (47), where successive N-alkylations and chain elongations result in a potent FPPS inhibitor, having an $IC_{50}$ of 20 nM against a human recombinant FPPS (42). The slightly increased $K_i$ for the longer chain species is not inconsistent with this idea, because it has recently been shown by the Novartis group (48) that increasing the side chain length of N-containing bisphosphonates beyond a certain threshold basically abolishes all activity, most likely because they clash with the aromatic rings located at the distal end of the hydrophobic pocket, thought to be responsible for termination of isoprene chain elongation by FPPS (29). In any case, it is of interest that the simple alkyl species 7 has essentially the same activity as pamidronate.

We also tested the activity of one pyrophosphate species, the bromohydrin of isopentenylpyrophosphate, 10. This compound...
is currently being developed as an anti-cancer drug and is known to stimulate the growth and cytokine expression of γT cells (49), but its precise molecular mechanism of action is unclear. Because FPFPs, in addition to its direct role in FFP synthesis, is also known to be involved in binding to the tyrosine kinase domain of the fibroblast growth factor receptor (50) and affects cell growth, it seemed possible that this alternative role of FPFPs might also occur in γT cells, mediated by pyrophosphate (and indeed, bisphosphonate (51, 52)) binding. We therefore determined the \( K_i \) for the bromobyrnin pyrophosphate. \( \text{We obtained a } K_i = 103 \mu M, \text{which would tend not to support this possibility.} \)

This leads us finally to consider the potential use of these and related compounds in chemotherapy intervention. Here, our initial results indicated that procyclic (insect form) and related compounds in chemotherapeutic intervention.

**REFERENCES**