Trypanosoma brucei prenylated-protein carboxyl methyltransferase prefers farnesylated substrates

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Carboxyl methylation of the C-terminal prenylated cysteine, which occurs in most farnesylated and geranylgeranylated proteins, is a reversible step and is implicated in the regulation of membrane binding and cellular functions of prenylated proteins such as GTPases. The gene coding for prenylated-protein carboxyl methyltransferase (PPMT) of the protozoan parasite *Trypanosoma brucei* has been cloned and expressed in the baculovirus/Sf9 cell system. The protein of 245 amino acids has 24–28 % sequence identity to the orthologues from other species including human and *Saccharomyces cerevisiae*. Methyltransferase activity was detected in the membrane fraction from Sf9 cells infected with the recombinant baculovirus using *N*-acetyl-*S*-farnesylcysteine (AFC) and *S*-adenosyl[*methyl*-^{3}H]methionine ([^{3}H]AdoMet) as substrates. Recombinant *T. brucei* PPMT prefers AFC to *N*-acetyl-*S*-geranylgeranylcysteine (AGGC) by 10–50-fold based on the V_{max}/K_m values. Native PPMT activity detected in the membrane fraction from *T. brucei* procyclics displays similar substrate specificity ($\approx$ 40-fold preference for AFC over AGGC). In contrast, mouse liver PPMT utilizes both AFC and AGGC as substrates with similar catalytic efficiencies. Several cellular proteins of the *T. brucei* bloodstream form were shown to be carboxyl methylated in a cell-free system. Incorporation of [^{3}H]methyl group from [^{3}H]AdoMet into most of the proteins was significantly inhibited by AFC but not AGGC at 20 μM, suggesting that *T. brucei* PPMT acts on farnesylated proteins in the cell. Cells of the *T. brucei* bloodstream form show higher sensitivity to AFC and AGGC (EC_{50} = 70–80 μM) compared with mouse 3T3 cells ($EC_{50} > 150$ μM).

Key words: anti-parasitic agent, baculovirus expression system, farnesylcysteine, post-translational modification.

INTRODUCTION

The protozoan parasite *Trypanosoma brucei* is the causative agent of African sleeping sickness and the cattle disease nagana. Prenyl modification of proteins by C_{15} farnesyl and C_{20} geranylgeranyl groups occurs in most eukaryotes, including protozoan parasites such as trypanosomatids [1–3]. We have previously shown that protein farnesyltransferase inhibitors are generally much more toxic to protozoan parasites than to mammalian cells [4,5]. This strongly suggests that blocking functional maturation of farnesylated proteins is an excellent strategy for the development of anti-parasite therapeutics.

Membrane targeting and cellular functioning of most prenylated proteins such as Ras superfamily GTPases require further processing steps following prenyl modification [6–9]. After modification with a farnesyl or a geranylgeranyl group, proteins that contain the C-terminal CaaX motif (C is cysteine, a is usually but not always an aliphatic amino acid, X is a variety of 20 different amino acids) undergo removal of the last three amino acids (aaX) by specific endoprotease(s), Ras-converting enzyme 1 and/or a-factor-converting enzyme 1, present in the endoplasmic reticulum [9,10]. Subsequently, the α carboxyl group of the newly exposed prenylcysteine is methylated by prenylated-protein carboxyl methyltransferase (PPMT) that is also associated with the endoplasmic reticulum [11,12]. MS analysis and metabolic labelling studies with radioactive methionine have shown that most of the prenylated proteins analysed, except for Rab proteins with the C-terminal doubly geranylgeranylated CC motif, are carboxyl methylated [13]. For some proteins, the additional modification with a palmitoyl ester at their C-termini is required for membrane translocation [14]. C-terminal carboxyl methylation and palmitoylation are thought to be reversible steps and therefore have been implicated in the regulation of membrane binding and cellular functions of prenylated proteins. Studies with farnesylated and geranylgeranylated short peptides have shown that tight membrane binding of farnesylated peptides requires carboxyl methylation [15,16]. Carboxyl methylation of farnesylated transducin γ subunit is important for membrane binding of the $\beta\gamma$ dimer and for its association with the α subunit and effector proteins [17]. However, no significant effect of carboxyl methylation was observed for doubly geranylgeranylated Rab 3A protein on its membrane binding and on its interaction with regulatory proteins [18].

Human and *Saccharomyces cerevisiae* PPMT genes have been cloned [11,19]. These enzymes are predicted to contain multiple transmembrane regions. Homozygous gene knockout of PPMT interferes with localization of K-Ras to the plasma membrane in mammalian cells [20] and leads to the death of mouse embryos during gestation [21]. The gene knockout abolishes the enzyme activity in the mouse embryo to methylate both farnesylated and geranylgeranylated protein substrates including doubly geranylgeranylated Rab proteins. This suggests that only one

Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; AFC, *N*-acetyl-*S*-farnesyl-L-cysteine; AGC, *N*-acetyl-*S*-geranyl-L-cysteine; AGGC, *N*-acetyl-*S*-geranylgeranyl-L-cysteine; DTT, dithiothreitol; [^{3}H]AdoMet, *S*-adenosyl[*methyl*-^{3}H]methionine; PPMT, prenylated-protein carboxyl methyltransferase.

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The nucleotide sequence data reported here will appear in the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under accession number AF480450.

enzyme is responsible for the carboxyl methylation of all types of prenylated protein.

In the present study, we have cloned PPMT from *T. brucei*. We also report the expression of the enzyme in the baculovirus/Sf9 cell system and the distinct substrate specificity of *T. brucei* PPMT compared with that of the mammalian enzyme.

EXPERIMENTAL

Materials

S-Adenosyl-L-[*methyl*-^{3}H]methionine ([^{3}H]AdoMet; 78 Ci/mmol) was from American Radiolabelled Chemicals. *N*-Acetyl-*S*-geranyl-L-cysteine (AGC), *N*-acetyl-*S*-farnesyl-L-cysteine (AFC) and *N*-acetyl-*S*-geranylgeranyl-L-cysteine (AGGC) were from BIOMOL.

Cloning of *T. brucei* PPMT

A BLAST search of the GenBank database was carried out with the gene coding for human PPMT (GenBank accession number AF064084). A cDNA fragment that contains high homology to the 3′-end of the human PPMT gene was identified (accession number AQ641910). This fragment includes 689 bp of coding sequence containing a stop codon. The 5′-end of the *T. brucei* PPMT gene was amplified by PCR using cDNA made with Superscript II reverse transcriptase (Gibco BRL) from the bloodstream form of *T. brucei* strain 427. The oligonucleotide primers were made to the *T. brucei* splice leader, 5′-AACG-CTATTATTAGAACAGTTTCT-3′ (sense), and to the 5′ end of the GenBank® sequence, 5′-CACCATAGTGTAGTTGAGA-CG-3′ (antisense). The PCR product ($\approx$ 550 bp) was sequenced and found to contain an initiation codon downstream of the splice leader sequence and a completely overlapping sequence of a 152 bp 3′-end sequence which is identical to the 5′-end sequence of the gene with accession number AQ641910. The full-length DNA of *T. brucei* PPMT was amplified by PCR based on these sequences using *T. brucei* genomic DNA as template and the high-fidelity DNA polymerase *Pwo* (Roche Molecular Biochemicals).

Expression of *T. brucei* PPMT in the Sf9/baculovirus system

The plasmid containing full-length *T. brucei* PPMT DNA was shuttled into pFastBAC (Invitrogen) using *Bam*HI and *Xba*I. The pFastBAC was subjected to transposition into the bacmid in DH10Bac competent cells (Gibco BRL), and PPMT insertion in the bacmid was confirmed by PCR, according to the manufacturer's instructions. After amplification, the baculovirus was subjected to plaque purification by the procedure described in [22]. The purified baculovirus was amplified three times to a final titre of 3×10^8 plaque-forming units/ml.

For large-scale expression of recombinant *T. brucei* PPMT, Sf9 insect cells were cultured at 27 °C with stirring at 120 rev./min in 200 ml of Grace's medium supplemented with 10% fetal bovine serum and 10 ml/l chemically defined lipid concentrate (Gibco BRL) in a 500 ml spinner flask. The baculovirus carrying the *T. brucei* PPMT gene was infected into Sf9 cells at a multiplicity of infection of 3. After culturing for 60 h, the cells were collected by centrifugation and frozen at −80 °C. The frozen cells were thawed on ice in 20 ml of lysis buffer (50 mM Tris/HCl/1 mM EDTA, pH 7.5) containing freshly added 1 mM dithiothreitol (DTT) and protease inhibitors (1 mM PMSF and 10 μg/ml each of aprotinin, leupeptin and pepstatin A). The cells were disrupted on ice with a Dounce homogenizer (10 strokes). The homogenate was centrifuged at 1000 ***g*** for 10 min followed by 10000 ***g*** for 10 min at 4 °C. The supernatant was centrifuged at 120000 ***g*** for 80 min at 4 °C, and the resulting pellet (membrane fraction) was suspended in 1.5 ml of buffer (50 mM Tris/HCl, 1 mM EDTA and 1 mM DTT, pH 7.5) using a Dounce homogenizer. The membrane suspension contained 10.7 mg of protein/ml, and aliquots of the suspension were frozen in liquid nitrogen and stored at −80 °C. The membrane fraction of uninfected Sf9 insect cells was prepared in the same way.

Preparation of *T. brucei* membranes

The *T. brucei brucei* EATRO 140 procyclic form was cultured as described in [4]. The cells from 500 ml of culture (mid-exponential phase) were harvested by centrifugation, washed once with PBS and frozen at −80 °C. The frozen cells were thawed on ice in 10 ml of lysis buffer as described above. The cells were disrupted at 0 °C in a Branson sonicator using a microtip (10×6 s pulses). The lysate was centrifuged at 10000 ***g*** for 10 min, and the supernatant was centrifuged at 120000 ***g*** for 80 min at 4 °C. The resulting pellet (membrane fraction) was suspended in 1 ml of buffer (50 mM Tris/HCl, 1 mM DTT and 1 mM EDTA, pH 7.5) using a Dounce homogenizer at 0 °C. The suspended *T. brucei* membranes contained 13 mg of protein/ml, and aliquots of the suspension were frozen in liquid nitrogen and stored at −80 °C.

Preparation of mouse liver membranes

Liver (6 g) from four freshly killed C57BL6 mice was minced and homogenized at 0 °C in 18 ml of a lysis buffer (10 mM Tris/HCl/1 mM EDTA/1 mM DTT/0.25 M sucrose, pH 7.4, containing protease inhibitors as above) using a Potter–Elvehjem homogenizer with a motorized Teflon pestle (three strokes) as described in [23]. The homogenate was centrifuged at 600 ***g*** for 10 min, and the supernatant was centrifuged at 12000 ***g*** for 10 min at 4 °C. The resulting supernatant was centrifuged at 120000 ***g*** for 80 min at 4 °C. The pellet was suspended in 1 ml of buffer (50 mM Tris/HCl, 1 mM EDTA and 1 mM DTT, pH 7.5). The suspension of mouse liver membrane contained 27.2 mg of protein/ml, and aliquots of the suspension were frozen in liquid nitrogen and stored at −80 °C.

PPMT assay

Standard reaction mixtures contained 10 μM AFC (1 μl of DMSO solution), 0.625 μM (0.5 μCi) [^{3}H]AdoMet and membrane fraction in 20 μl of buffer (50 mM Tris/HCl, 1 mM EDTA and 5 mM DTT, pH 7.5). Immediately after incubation at 30 °C for 20 min, the reaction mixture was quickly mixed with 200 μl of ice-cold n-heptane by vortexing. After centrifugation at 10000 ***g*** for 1 min, a 150 μl portion of the upper layer was transferred to a new tube. The solvent was removed *in vacuo*, and the dried sample was dissolved in a 30 μl solution of 1 M NaOH/2% Triton X-100 by brief sonication in a bath sonicator and vortexing. The solution was spotted on a piece of Whatman 3MM filter paper and subjected to [^{3}H]methanol vapour diffusion into 6 ml of scintillation fluid in a glass vial sealed with Parafilm as described in [24]. After incubation for 2–3 h at room temperature, the filter paper was removed, and the vial was subjected to scintillation counting for radioactivity.

Studies on carboxyl methylation of *T. brucei* cellular proteins in a cell-free system

Cells of the *T. brucei* bloodstream form were cultured as described in [5]. The cells (2×10^8) were suspended in 200 μl of buffer (50 mM Tris/HCl, 1 mM EDTA and 5 mM DTT, pH 7.5) containing protease inhibitors (0.1 mM PMSF and 1 μg/ml each

of aprotinin, leupeptin and pepstatin A) and lysed by brief sonication on ice in a Branson sonicator using a microtip. Immediately after sonication, the lysate (10 μl, 8 μg of protein) was incubated at 30 °C for 1 h with 2.5 μM (2 μCi) [^{3}H]AdoMet with vehicle (1 μl of DMSO) or AFC, AGGC or AGC (added as 1 μl of stock solutions in DMSO) in 20 μl of buffer as above. The reaction was terminated by adding 500 μl of ice-cold acetone. The mixture was vortexed and centrifuged at 14000 ***g*** for 10 min at 4 °C. The resulting pellet was air-dried and dissolved in 10 μl of 1 % SDS. After treatment at 100 °C for 3 min with Laemmli sample buffer containing 2 % β-mercaptoethanol, the sample was subjected to SDS/PAGE analysis using a 12.5 % gel. The radioactive protein bands were visualized by fluorography as described in [2].

Cell growth inhibition assays with *T. brucei* bloodstream form and mouse 3T3 fibroblasts

AFC, AGGC and AGC were tested for their ability to inhibit the growth of the *T. brucei* bloodstream form as described in [5]. The effect of the compounds on the growth of 3T3 fibroblasts was quantified with Alamar Blue as described [25]. Stock solutions of the compounds were prepared in DMSO. The final concentration of DMSO in the culture media was 0.25 %, which did not significantly affect growth of *T. brucei* or fibroblast cells.

RESULTS AND DISCUSSION

Cloning of *T. brucei* PPMT

By BLAST search of the GenBank database, we identified a 689 bp *T. brucei* DNA sequence (GenBank accession number AQ641910) whose translated amino acid sequence showed high homology (36 % identity and 57 % homology) to human PPMT (accession number AF064084). This sequence contains a 3′-end fragment of the putative *T. brucei* PPMT gene. The 5′-end of the gene was amplified from *T. brucei* cDNA by splice leader RACE (rapid amplification of cDNA ends) [26], which yielded a product of $\approx$ 550 bp. This fragment contains an ATG at 143 bp downstream from the splice leader, which was assumed to be the initiation codon of the gene. Compilation of these overlapping sequences revealed an open reading frame containing 735 bp of the *T. brucei* PPMT gene. This gene encodes a protein of 245 amino acids with a calculated molecular mass of 28301 Da (Figure 1). Two differences of nucleotide sequence occur in another clone that result in amino acid changes at His-2 to Arg and at Ile-30 to Val. Multiple amino acid sequence alignment of *T. brucei* PPMT and the orthologues from other species shows extensive homology along most of the length of PPMT (Figure 1). Amino acid sequence identities between *T. brucei* PPMT and the enzymes from other species were 24–28 %: *S. cerevisiae* (known as Ste14p; accession number AAA16840), human (known as pcCMT; AF064084), rat (AAD42926), *Xenopus laevis* (BAA19000), *Drosophila melanogaster* (AE003539), *Caenorhabditis elegans* (AAB42280) and *Dictyostelium discoideum* (AAL99548). After completion of this work, the full-length sequence of *Leishmania major* PPMT appeared in GenBank under accession number AC098797. The *Leishmania* sequence is 41 % identical to the sequence of *T. brucei* PPMT.

Hydropathy analysis of the full set of PPMTs shown in Figure 1 suggests that all enzymes contain 5–7 predicted transmembrane helices and that two of the N-terminal transmembrane helices are present only in the PPMTs from human, *X. laevis*, *D. melanogaster* and *C. elegans*. Studies by immunofluorescence microscopy and subcellular fractionation have shown that mammalian and yeast PPMTs are localized to the endoplasmic reticulum [11,12,27]. Little is known about the active site of this enzyme except for previous studies showing that specific modification of cysteine and possibly lysine and histidine residues by *N*-ethylmaleimide and modification of arginine by phenylglyoxal inactivate the mammalian enzyme [27,28]. The amino acid sequences of the C-terminal regions are highly homologous among the enzymes from different species (Figure 1), suggesting that the active site of the enzyme resides in the C-terminal region(s). Several C-terminal residues, namely Arg-139, His-162, Arg-174, His-175 and Arg-212 of *T. brucei* PPMT, are conserved among the enzymes from all species, and these are found in segments outside of the predicted transmembrane regions, as would be expected for amino acids involved in the active site.

Expression of *T. brucei* PPMT in the Sf9/baculovirus system

To examine whether the cloned DNA encodes *T. brucei* PPMT, a recombinant baculovirus was constructed with the putative *T. brucei* PPMT open reading frame. SDS/PAGE analysis of membranes prepared from the recombinant PPMT baculovirus-infected cells showed that a protein with an apparent molecular mass of $\approx$ 27 kDa was expressed in the infected cells (results not shown). The observed molecular mass of the expressed protein is similar to the calculated molecular mass (28.3 kDa). The sequence contains a predicted signal peptide with a cleavable site at Leu-24.

When membranes from infected cells were assayed for methyltransferase activity using AFC and [^{3}H]AdoMet as substrates, an over-expressed level of enzyme activity was detected compared with the endogenous PPMT activity present in the uninfected Sf9 cell membranes (Figure 2). The initial velocity measured with membranes from infected cells was $\approx$ 40-fold higher than that detected in the membrane from uninfected cells using the same amount of membrane protein (1 μg). When several baculovirus clones obtained by plaque purification were screened for PPMT expression, levels of the 27 kDa protein expression and the enzyme activity were well correlated (results not shown). The majority of PPMT activity (98.9 % of total activity) was detected in the membrane fraction, and only 1.1 % of the total activity was in the cytosol fraction. These results indicate that the cloned gene encodes a functionally active form of *T. brucei* PPMT and that the recombinant enzyme associates with membranes.

Substrate specificity of *T. brucei* PPMT

In mammals, most of the farnesylated and geranylgeranylated proteins are carboxyl methylated at the C-terminal prenyl cysteine [13]. Substrate specificities of recombinant and native *T. brucei* PPMT with respect to the different prenyl groups (C_{10} geranyl, C_{15} farnesyl and C_{20} geranylgeranyl) were studied using AGC, AFC and AGGC, respectively, as substrates. As shown in Figure 3(A), when Sf9 cell membranes (10 μg of protein) containing recombinant *T. brucei* PPMT were used for the enzyme assays, AFC was found to be a better substrate than AGGC, while AGC was not significantly methylated at concentrations up to 20 μM. The apparent K_m values for AFC and AGGC were determined to be 0.17 and 2.3 μM, respectively, and the V_{max} values with AFC and AGGC were 2.7 and 0.7 pmol/min per mg of protein, respectively (Table 1). Thus recombinant *T. brucei* PPMT prefers AFC to AGGC by $\approx$ 50-fold based on the V_{max}/K_m values. Interestingly, the relative catalytic efficiencies (V_{max}/K_m) for AFC and AGGC varied when different amounts of membranes were used. With 1 μg of the Sf9 cell membrane protein, the V_{max}/K_m for AFC was measured to be $\approx$ 10-fold higher than the value for AGGC (Table 1). It is known that

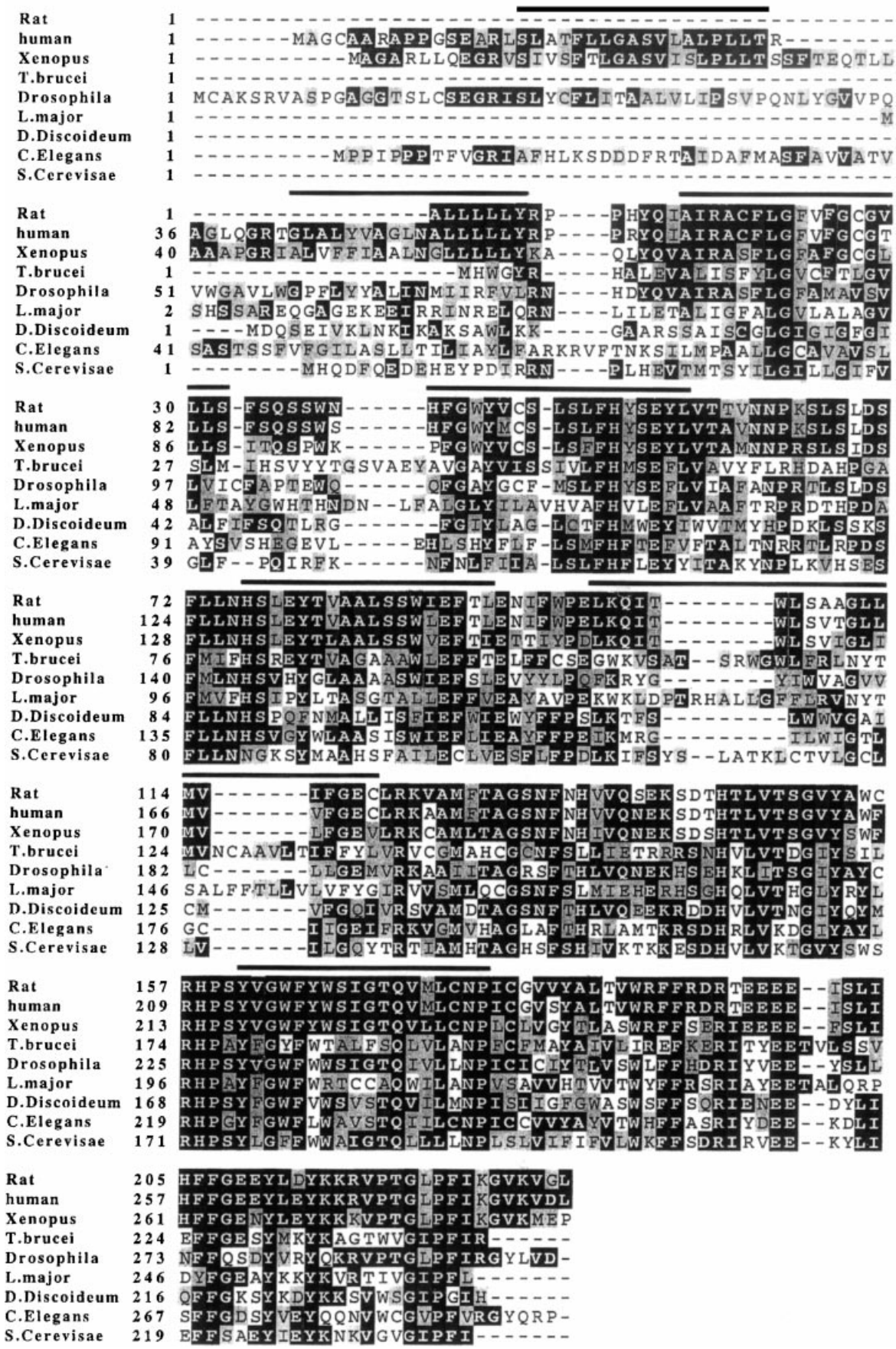

Figure 1 For legend, see facing page.

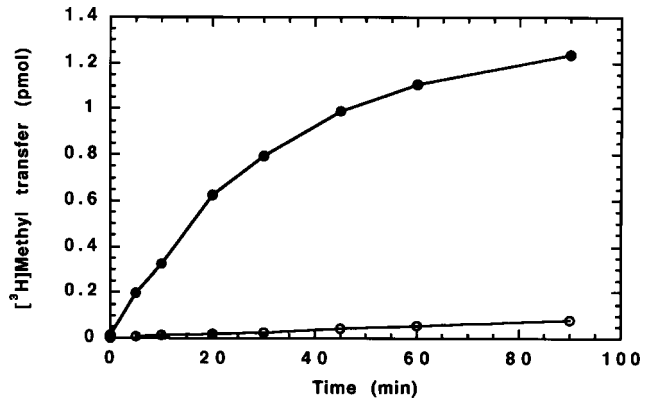

Figure 2 Expression of *T. brucei* PPMT in the baculovirus/Sf9 cell system

Membrane fraction (1 μg of protein/assay) from Sf9 cells expressing recombinant *T. brucei* PPMT (●) or uninfected cells (○) was assayed for PPMT activity with 10 μM AFC and 0.625 μM (0.5 μCi) [^{3}H]AdoMet as substrates under standard conditions. Estimated error was $< 10\%$.

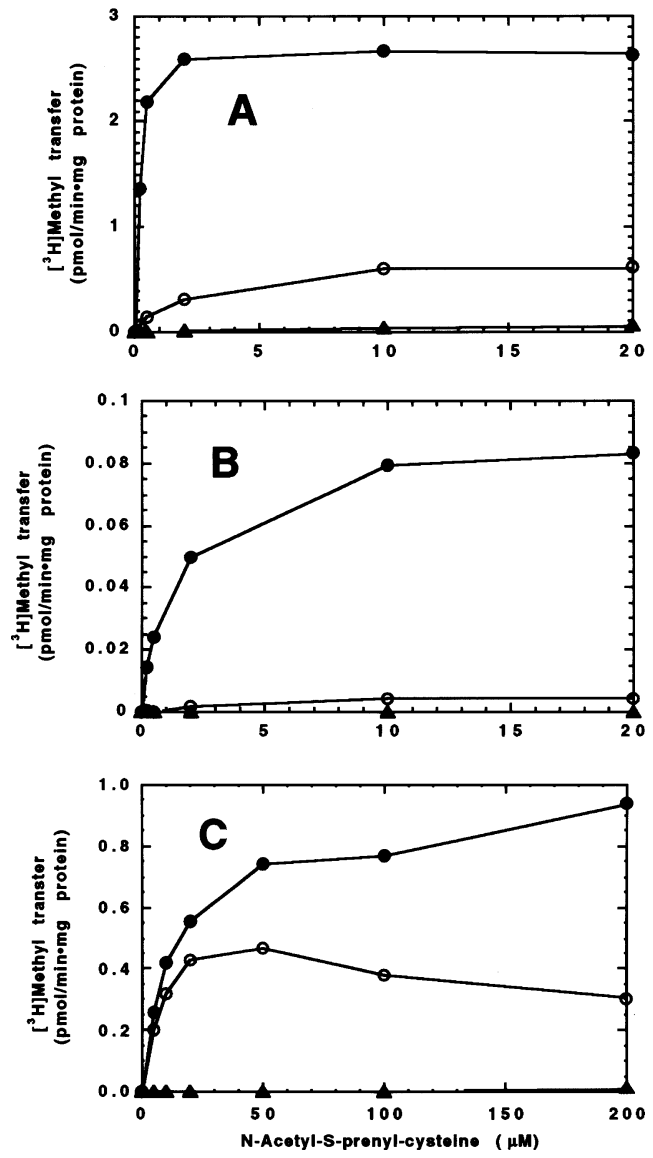

Figure 3 Substrate specificity of recombinant (A) and native (B) *T. brucei* PPMT, and mouse liver PPMT (C)

(**A**) Membrane fraction (10 μg of protein) obtained from Sf9 cells expressing recombinant *T. brucei* PPMT was incubated at 30 °C for 20 min with 0.625 μM (0.5 μCi) [^{3}H]AdoMet and the indicated concentrations of AFC (●), AGGC (○) or AGC (▲) to measure the initial velocity. (**B**) Membrane fraction (10 μg) from *T. brucei*-procyclic-form cells was used for the PPMT assay at 30 °C for 60 min with the indicated concentrations of AFC, AGGC or AGC under the same conditions as for (**A**). (**C**) Mouse liver membrane fraction (10 μg of protein) was incubated at 30 °C for 10 min with 2 μM (3.3 μCi) [^{3}H]AdoMet and the indicated concentrations of AFC, AGGC or AGC. Estimated error was $< 10\%$.

geranylgeranylated peptides bind more tightly to synthetic phospholipid vesicles compared with farnesylated peptides [15,16]. Thus the ratio for the rates of utilization of AFC versus AGGC increases in the presence of a higher amount of membranes, which promotes additional AFC to partition from the aqueous phase into membranes. Therefore, the measured kinetic parameters for the relative catalytic efficiency of AFC versus AGGC are apparent values. Similar substrate specificity was observed with native *T. brucei* PPMT when the amount of membranes (10 μg of protein) obtained from cells of the *T. brucei* procyclic form used for assays was the same as the amount of Sf9 cell membranes containing recombinant enzyme (Figure 3B and Table 1). The native enzyme preferentially utilized AFC over AGGC by $\approx$ 40-fold based on values of V_{max}/K_m (Table 1). No significant activity was detected with AGC at concentrations up to 20 μM using native *T. brucei* PPMT (Figure 3B). These results show that the cloned gene encodes *T. brucei* PPMT. Hasne and Lawrence have detected PPMT activity in membranes from *Leishmania donovani* [29]. As for the *T. brucei* enzyme, *Leishmania* PPMT prefers AFC over AGGC by $\approx$ 7-fold based on values of V_{max}/K_m when 15–20 μg of protein was used for the assay: apparent K_m values for AFC and AGGC are ≈ 60 and ≈ 170 μM, respectively, and the V_{max} values with AFC and AGGC are ≈ 0.23 and ≈ 0.1 pmol/min per mg of protein, respectively.

In contrast to these results with trypanosomatid PPMTs, it has been reported that mammalian PPMT from bovine retinal rod membranes utilizes both AFC and AGGC with similar catalytic efficiencies [30] and that PPMT in human neutrophil membranes prefers AGGC to AFC by $\approx$ 10-fold [31]. We prepared membranes from mouse liver as a source of mammalian PPMT in order to evaluate its substrate specificity using the same assay conditions as used for *T. brucei* PPMT. As shown in Figure 3(C), mouse liver PPMT utilizes AFC slightly better than AGGC. Decreased enzyme activity was observed with high concentrations

Figure 1 Amino acid sequence alignment of PPMTs

Shown are the enzymes from rat, human, *Xenopus laevis*, *T. brucei*, *Drosophila melanogaster*, *Leishmania major*, *Dictyostelium discoideum*, *Caenorhabditis elegans* and *S. cerevisiae*. Letters shaded in black and grey indicate identical and similar amino acids, respectively. Transmembrane helical regions were predicted with the TMHMM transmembrane domain analysis program (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The positions of the predicted transmembrane helical regions for the human enzyme are shown by the bars above the rat sequence. The first two transmembrane helices at the N-terminus are only present in the PPMTs from human, *X. laevis*, *D. melanogaster* and *C. elegans*.

Table 1 Kinetic parameters of recombinant and native *T. brucei* PPMT and mouse liver PPMT

Sf9 cell membranes (10 μg of protein) containing recombinant *T. brucei* PPMT, *T. brucei*-procyclic-form cell membranes (10 μg of protein) and mouse liver membranes (10 μg of protein) were used for PPMT assays to determine the kinetic parameters. Numbers shown in parentheses for recombinant *T. brucei* and native mouse liver PPMTs represent the values obtained using 1 μg of protein of the Sf9 cell membranes and 27 μg of protein of mouse liver membranes, respectively. The K_m and V_{max} values ($\pm$S.E.M.) were determined by fitting the data to the standard Michaelis–Menten equation using the GraphPad Prism program.

Enzyme	Substrate	K_m (μM)	V_{max} (pmol/min per mg)	V_{max}/K_m (pmol/min per mg per μM)	Relative V_{max}/K_m AFC versus AGGC
Recombinant *T. brucei* PPMT (Sf9 cell membranes)	AFC	0.17 $\pm$ 0.03 (0.53 $\pm$ 0.17)	2.7 $\pm$ 0.17 (24.3 $\pm$ 2)	16.2 (46.3)	52 (9.9)
	AGGC	2.3 $\pm$ 0.03 (1.8 $\pm$ 0.13)	0.71 $\pm$ 0.03 (8.2 $\pm$ 0.13)	0.31 (4.7)	1 (1)
Native *T. brucei* PPMT	AFC	1.4 $\pm$ 0.14	0.09 $\pm$ 0.002	0.063	42
	AGGC	4 $\pm$ 1.3	0.006 $\pm$ 0.001	0.0015	1
Native mouse liver PPMT	AFC	11.5 $\pm$ 1.3 (22.2 $\pm$ 3.7)	0.88 $\pm$ 0.05 (0.42 $\pm$ 0.02)	0.076 (0.019)	1.3 (2.3)
	AGGC	11.6 $\pm$ 1.0 (34.4 $\pm$ 6.3)	0.68 $\pm$ 0.03 (0.28 $\pm$ 0.02)	0.059 (0.008)	1 (1)

of AGGC (> 100 μM) and AFC (> 500 μM). These inhibitory effects of high concentrations of AGGC and AFC were also observed with recombinant and native *T. brucei* PPMT (results not shown) and may be due to micelle formation of the compounds or to their effects as detergents. Inactivation of PPMT by detergent solubilization was reported for the enzyme in mammalian membranes [31]. We observed that 0.2% *n*-octyl β-glucoside caused 55–75% inhibition of mouse liver PPMT activity when measured with 50 μM AGGC and AFC. The kinetic parameters for mouse liver PPMT were estimated from initial velocities measured with AGGC concentrations < 50 μM and with AFC concentrations < 200 μM that do not apparently interfere with the enzyme activity. When 10 μg of protein from mouse liver membranes was used for the assay, the apparent K_m values were similar for AFC and AGGC (≈ 12 μM), and the V_{max}/K_m value obtained with AFC was slighly higher (1.3-fold) than the value with AGGC (Table 1). With a larger amount of mouse liver membranes (27 μg of protein), a higher ratio of V_{max}/K_m for AFC versus AGGC utilization (2.3-fold) was observed, as seen with the Sf9 cell membranes containing recombinant *T. brucei* PPMT (Table 1). Thus, it is clear that *T. brucei* PPMT has an intrinsic preference for AFC over AGGC that is distinctly high compared with mammalian PPMT.

It is noteworthy that no significant level of protein geranylgeranyltransferase-I activity was detected in cytosolic fractions from trypanosomatid cells using known substrates of the mammalian enzyme [5,32]. In addition, while several Rho GTPases are geranylgeranylated in mammals [7,8], we have found that a Rho homologue from *Trypanosoma cruzi* is farnesylated *in vivo* [32]. These data led us to speculate that trypanosomatids may lack protein geranylgeranyltransferase-I. This idea is consistent with the above results that the geranylgeranylated substrate is poorly utilized compared with the farnesylated substrate by *T. brucei* PPMT (Figures 3A and 3B, and Table 1). However, several trypanosomatid Rab homologues have been cloned [33,34] that may be doubly geranylgeranylated and carboxyl methylated.

Inhibition of protein carboxyl methylation in the *T. brucei* bloodstream form by AFC in a cell-free system

To detect carboxyl methylation of *T. brucei* cellular proteins, a lysate of the bloodstream-form cells was incubated with [^{3}H]AdoMet, and radiolabelled proteins were analysed by SDS/PAGE/fluorography. As shown in Figure 4 (lane 2), the radioactivity was incorporated from [^{3}H]AdoMet into several cellular proteins with apparent molecular masses of > 113, 58, 52, 45, 40.5, 37.5, 33, 24.5 and 22.5 kDa. Treatment of the protein sample with 0.5 M NaOH resulted in disappearence of most radioactive protein bands except for the band with molecular mass < 21.4 kDa (Figure 4, lane 1), showing that these proteins are modified with [^{3}H]methyl ester. [^{3}H]Methyl incorporation into most of these proteins, except two bands (> 113 and < 21.4 kDa), was significantly inhibited by 20 μM AFC but not AGGC or AGC (Figure 4, lanes 3–9). The results suggest that carboxyl methylation of proteins occurs in *T. brucei*-bloodstream-form cells and that farnesylated proteins are major substrates of *T. brucei* PPMT in the cell. The effective concentration (20 μM) of AFC for inhibition of [^{3}H]methylation of the cellular proteins (Figure 4, lane 5) is significantly higher than the K_m value (0.2–1.4 μM) for AFC of *T. brucei* PPMT (Table 1), indicating that the endogenous proteins in *T. brucei*-bloodstream-form cells are better substrates for the *T. brucei* PPMT than is AFC.

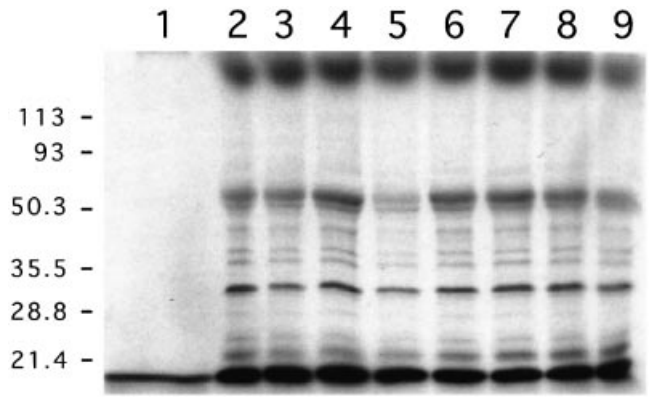

Figure 4 Effects of AFC, AGGC and AGC on carboxyl methylation of *T. brucei*-bloodstream-form cellular proteins in a cell-free system

T. brucei-bloodstream-form cell lysate (8 μg of protein) was incubated at 30 °C for 1 h with 2.5 μM (2 μCi) [^{3}H]AdoMet. Proteins were precipitated with acetone and analysed by SDS/PAGE using a 12.5% gel. Radioactive protein bands were visualized by fluorography. The protein sample in lane 1 was treated at 37 °C for 30 min with 0.5 M NaOH and then neutralized with acetic acid prior to SDS/PAGE analysis. A control with no addition is shown in lane 2. AFC at 1, 5 and 20 μM (lanes 3–5, respectively), AGGC at 1, 5 and 20 μM (lanes 6–8) and AGC at 20 μM (lane 9) were tested for inhibition of the [^{3}H]methyl incorporation into proteins.

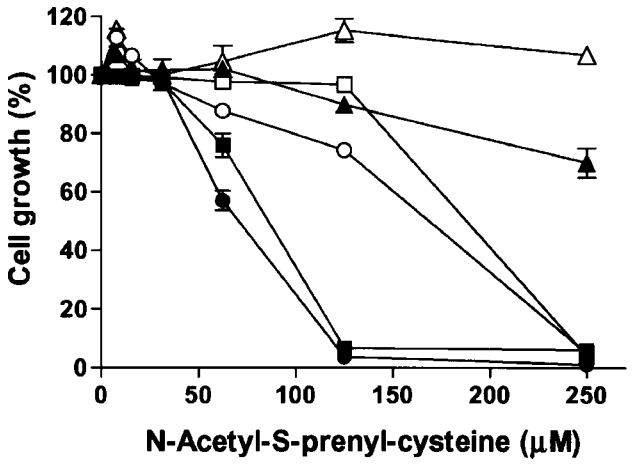

Figure 5 Cell growth inhibition of *T. brucei* bloodstream form and mouse 3T3 fibroblasts by AFC and AGGC

Indicated concentrations of AFC (■, □), AGGC (●, ○) and AGC (▲, △) were tested for cell growth inhibition of *T. brucei* bloodstream form (closed symbols) and of mouse 3T3 fibroblasts (open symbols). Data were collected from three assays. Error bars not displayed are within the symbols.

Effects of AFC and AGGC on cell growth of *T. brucei* bloodstream form

The minimal structural element for mammalian PPMT substrates has been found to be prenylated thiopropionic acid, and the distance between the carboxylic acid and the prenyl group is important [35]. Small compounds, including AFC and AGGC, have been used as competitive substrates for the action of PPMT in cells to investigate the physiological importance of carboxyl methylation of prenylated proteins *in vivo*, although these compounds have also been shown to interfere with other cellular events [36–38]. AFC-selective inhibition of cellular protein carboxyl methylation observed in the cell-free system of the *T. brucei* bloodstream form (Figure 4) is in accordance with the specificity of *T. brucei* PPMT (Figures 3A and 3B, and Table 1). Thus, the distinct substrate specificity and relatively low K_m values for AFC and AGGC of *T. brucei* PPMT (low μM) compared with those for the mammalian enzyme (10–35 μM; Figure 4 and Table 1) suggested the possibility that these compounds could inhibit the methylation of parasite-prenylated proteins at concentrations that do not interfere with the methylation of prenylated proteins in mammalian cells. As shown in Figure 5, AFC and AGGC display > 2-fold higher potencies to block the growth of *T. brucei* bloodstream forms (EC_{50} = 70–80 μM) than of mouse 3T3 fibroblast cells (EC_{50} > 150 μM). AGGC is slightly more toxic than AFC to both the parasite and mammalian cells. One might anticipate that AGGC is more cell permeable than is AFC. At concentrations of 125 μM, AFC and AGGC nearly completely block *T. brucei* cell growth, whereas AFC and AGGC inhibit the mammalian cell growth by only $\approx$ 5 and 25%, respectively (Figure 5). AGC that is virtually inactive as a substrate for both *T. brucei* and mammalian PPMTs (Figure 3), which caused a slight inhibition of *T. brucei* cell growth at concentrations > 125 μM and had no effect on 3T3 cell growth up to 250 μM.

These results suggest that PPMT is a valid anti-parasite drug target. However, it has been shown in mammalian cells that AFC and AGGC can also affect cellular events by mechanisms other than inhibition of PPMT [36–39]. These small prenylated compounds may competitively block the interaction of prenylated proteins with their acceptor proteins and effector proteins, which makes it difficult to distinguish their effects on PPMT inhibition from other effects. For example, Desrosiers and co-workers [39] have shown that AGGC at concentrations below its critical micellar concentration (340 μM) selectively dissociates geranylgeranylated Rho family proteins and Rab1, but not farnesylated Ras, from mammalian cell membranes. Several prenylated cysteine analogues and *S*-adenosyl-L-methionine (AdoMet) analogues have been reported to inhibit PPMT. Among those, the antibiotic sinefungin (an AdoMet analogue) has been shown to be a potent inhibitor of PPMTs from *L. donovani* and human neutrophils with K_i values of 20 and 70 nM, respectively [29,31], although this compound also inhibits other methyltransferases. *L. donovani* is reported to be sensitive to this compound [40]. Together, these results, in addition to previous studies of protein farnesyltransferase inhibitors [4,5], suggest that the growth of protozoan parasite cells is more susceptible than mammalian cell growth to agents that block the cellular functions of prenylated proteins. Future studies will further explore the potential to exploit *T. brucei* PPMT as a drug target alone or in combination with inhibiting other steps in the formation of mature prenylated proteins.

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