

# A pathway for phosphatidylcholine biosynthesis in *Plasmodium falciparum* involving phosphoethanolamine methylation

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*Plasmodium falciparum* is the causative agent of the most severe form of human malaria. The rapid multiplication of the parasite within human erythrocytes requires an active production of new membranes. Phosphatidylcholine is the most abundant phospholipid in *Plasmodium* membranes, and the pathways leading to its synthesis are attractive targets for chemotherapy. In addition to its synthesis from choline, phosphatidylcholine is synthesized from serine via an unknown pathway. Serine, which is actively transported by *Plasmodium* from human serum and readily available in the parasite, is subsequently converted into phosphoethanolamine. Here, we describe in *P. falciparum* a plant-like S-adenosyl-L-methionine-dependent three-step methylation reaction that converts phosphoethanolamine into phosphocholine, a precursor for the synthesis of phosphatidylcholine. We have identified the gene, *PfPMT*, encoding this activity and shown that its product is an unusual phosphoethanolamine methyltransferase with no human homologs. *P. falciparum* phosphoethanolamine methyltransferase (*PfPmt*) is a monopartite enzyme with a single catalytic domain that is responsible for the three-step methylation reaction. Interestingly, *PfPmt* activity is inhibited by its product phosphocholine and by the phosphocholine analog, miltefosine. We show that miltefosine can also inhibit parasite proliferation within human erythrocytes. The importance of this enzyme in *P. falciparum* membrane biogenesis makes it a potential target for malaria chemotherapy.

Malaria, the world's most important parasitic disease, is caused by intraerythrocytic protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* is responsible for the most severe clinical cases of human malaria and kills >1 million children annually (1). The worldwide emergence of drug-resistant *P. falciparum* strains has made treatment and prophylaxis of malaria increasingly difficult, thus emphasizing the need for new chemotherapeutic strategies to combat this disease. Previous studies in *P. falciparum* have indicated that the enzymes for synthesis of the major phospholipids are critical for the rapid multiplication of the parasite within human erythrocytes and display properties that are different enough from their human counterparts to be considered good targets for chemotherapy (2–5). Accordingly, compounds that interfere with membrane biogenesis inhibit parasite multiplication *in vitro* and clear malaria infection in mice and monkeys (3). In most eukaryotic organisms, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) are the major phospholipids of cellular membranes. Whereas PtdCho and PtdEtn represent 44% and 18% of total phospholipids in yeast, respectively (6), these two phospholipids represent 40–50% and 35–40% of the total phospholipids in *P. falciparum* (5). How the parasite maintains such unusually high levels of PtdEtn and the implications of such a lipid composition on parasite development and survival are not known.

Genetic and biochemical studies in various organisms revealed three major routes for synthesis of PtdCho and PtdEtn: *de novo* CDP-choline and CDP-ethanolamine (Kennedy) pathways and

the CDP-diacylglycerol pathway (7–9). The Kennedy pathways synthesize PtdCho and PtdEtn from choline and ethanolamine, respectively. The CDP-diacylglycerol pathway initiates from serine and CDP-diacylglycerol to form phosphatidylserine, which is then converted into PtdEtn via the activity of phosphatidylserine decarboxylase enzymes. PtdEtn is subsequently methylated into PtdCho by PtdEtn methyltransferases (10, 11). Whereas in most mammalian cells the *de novo* CDP-choline pathway is the major route for synthesis of PtdCho (9), in yeast cells and mammalian hepatocytes, the CDP-diacylglycerol pathway is the primary route for synthesis of PtdCho. Plant cells, however, lack phosphatidylserine decarboxylases but instead catalyze the decarboxylation of serine into ethanolamine (12), which is subsequently phosphorylated into phosphoethanolamine (P-Etn). The P-Etn formed is either incorporated into PtdEtn via the CDP-ethanolamine pathway or converted into phosphocholine (P-Cho) by a P-Etn methyltransferase (PEAMT) (13–15). P-Cho then serves as a precursor for the synthesis of PtdCho.

Available data and the finished genome sequence of *P. falciparum* indicated that the parasite possesses various enzymes that are important for synthesis of phospholipids from precursors produced by the parasite metabolic machineries or scavenged from human serum (fatty acids, serine, inositol, choline) (16, 17). The genes involved in the synthesis of phospholipids have only recently started to be elucidated (18–21). The *de novo* CDP-choline pathway has been proposed to be the primary route for synthesis of PtdCho in *Plasmodium* (16, 17); however, *in vitro* growth assays using dialyzed serum indicated that choline was not essential for parasite intraerythrocytic development and survival (22, 23). These results thus suggest that the CDP-choline is not the sole route for synthesis of PtdCho in *P. falciparum* and indicate that alternative pathways for synthesis of this phospholipid from precursors other than choline must exist in this parasite.

Here, we provide molecular and biochemical evidence for the presence in *P. falciparum* of an alternative pathway for PtdCho biosynthesis. Serine, which is transported from human serum and readily available in the parasite cytoplasm (24), is converted into ethanolamine and then phosphorylated into P-Etn (25). We show that *P. falciparum* catalyzes a PEAMT reaction that converts P-Etn into P-Cho, which is then incorporated into PtdCho. We suggest that this alternative pathway for PtdCho biosynthesis plays a critical role in *P. falciparum* membrane

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Abbreviations: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; *PfPmt*, *P. falciparum* phosphoethanolamine methyltransferase; SAM, S-adenosyl-L-methionine; P-Cho, phosphocholine; P-Etn, phosphoethanolamine; PEAMT, P-Etn methyltransferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY429590).

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biogenesis and could potentially be a good target for design of new antimalarial compounds.

## Materials and Methods

**Strain, Growth Conditions, and Media.** Human blood was obtained from the General Clinical Research Center at the University of Connecticut Health Center. *P. falciparum* (strain 3D7) was grown by using the method developed by Trager and Jensen (26). Albumax I (0.5%; Invitrogen) replaced serum in the culture medium.

**Drug Response Assay.** Hypoxanthine proliferation assay with increasing concentrations of miltefosine (0–180  $\mu$ M) (Cayman) in the *P. falciparum* strain 3D7 grown in RPMI–Albumax medium was performed in triplicate according to the method of Desjardins *et al.* (27).

**Labeling Assays and Pfpmt Activity in *P. falciparum*.** An asynchronous *P. falciparum* 3D7-infected erythrocyte culture (7 ml, 15–16% hematocrit, 18–20% parasitemia with 9–10% trophozoites) was incubated at 37°C in the presence of 5.1  $\mu$ M [<sup>14</sup>C]ethanolamine (55 mCi/mmol; ARC, St. Louis; 1 Ci = 37 GBq) in RPMI–Albumax medium, which contains 20  $\mu$ M choline. After a 3-min incubation, the culture was chased with 28 ml of RPMI medium containing 20 mM ethanolamine (Etn) for 15 min. The *Plasmodium*-infected erythrocytes were then washed twice, and organic and aqueous phases were extracted by using standard procedures (28). The organic and aqueous phases of the Folch extract were evaporated, and the dried materials were dissolved in 200  $\mu$ l of chloroform/methanol (2:1) and 200  $\mu$ l of water/ethanol (1:1), respectively. The organic phase was analyzed by TLC using UV-coated silica gel (Whatman) and chloroform/methanol/acetic acid/0.1 M sodium borate (75:45:12:4.5) as mobile phase. The identity of the radioactive spots was determined by TLC analysis using appropriate standards and by exposure to iodine stain. Aqueous-phase material was separated on cellulose plates (Selecto Scientific, Suwanee, GA) by using a solvent system consisting of 2.7 M ammonium acetate/95% ethanol (3:7, vol/vol) adjusted to pH 10 with 28% ammonia and detected by autoradiography (25). This solvent system allows clear separation of the aqueous compounds Etn ( $R_F$  = 0.82), choline ( $R_F$  = 0.85), P-Etn ( $R_F$  = 0.36), and P-Cho ( $R_F$  = 0.6). The standards Etn, P-Etn, and CDP-Etn were detected by spraying the plates with 0.3% ninhydrin in butanol:acetic acid (100:3) and heating to 110°C for 3 min. In this solvent, P-Etn and CDP-Etn were not separated. To assay native PEAMT activity, *P. falciparum*-infected erythrocytes (15% parasitemia, 12% trophozoites) were grown in RPMI–Albumax medium and then treated with 0.05% saponin in PBS for 15 min on ice. The released parasites were washed twice in PBS, and the pellet was resuspended in a 500- $\mu$ l reaction buffer (100 mM Hepes-KOH, pH 7.8/5 mM DTT/2 mM EDTA/10% glycerol, protease inhibitor mix). *P. falciparum* protein extracts (600  $\mu$ g) were prepared by sonication. After centrifugation, the supernatant was incubated for 30 min at 37°C with 100 nCi of [<sup>14</sup>C]adenosyl-L-methionine (SAM) (52 mCi/mmol, NEN) in the presence of 100  $\mu$ M P-Etn (substrate) and 100  $\mu$ M SAM (methyl donor). The reaction products were purified by ion-exchange chromatography as described by Nuccio *et al.* (13).

**Expression and Cloning of PfpMT.** cDNA was obtained from RNA isolated from the different blood stages (rings, trophozoites, and schizonts) of *P. falciparum*. RNA (2  $\mu$ g) was reverse-transcribed by using SuperScript II and random primers (Life Technologies, Grand Island, NY) as described (29). Quantitative RT-PCR was performed by using primers ocho31 5'-GTGGTTAAAC-CAACAGGTACC-3' and Pfpmt-B 5'-CCGCGGATCCAA-TTTTGGTGGCCTTAAATAAC-3' to amplify a 300-bp

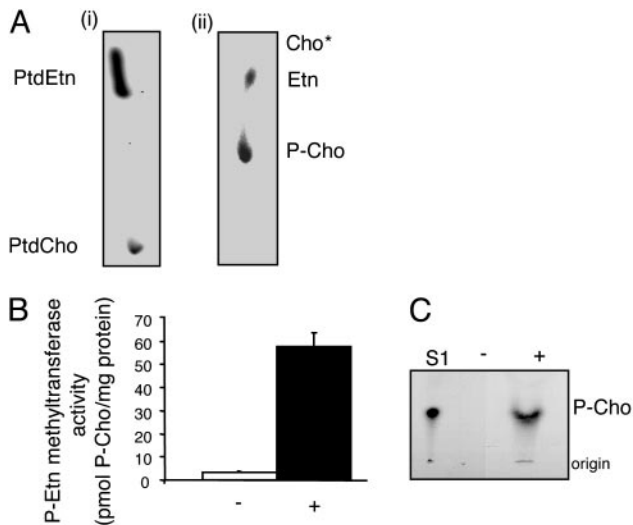
*PfpMT* fragment, and primers 5'C2143 5'-GAGGAATTTTACGTGTTTCATCAA-3' and 3'C2143 5'-GATTACTTGTAG-GAAAGAATCCTTC-3' were used to amplify a 300-bp fragment corresponding to the seryl tRNA synthetase-encoding gene (29). The real-time PCR reaction was performed in duplicate following the manufacturer's instructions (LightCycler-FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals). Data were normalized to expression levels of the seryl tRNA synthetase-encoding gene, which is constitutively expressed during the intraerythrocytic life cycle (29). Using the primers Pfpmt-B and Pfpmt-X 5'-CCGCGCTCGAGATGAC-TTTGATTGAAAACCTTA-3', an 880-bp fragment corresponding to *PfpMT* cDNA was PCR-amplified and cloned in the expression vector pET-15b (Novagen).

**Isolation and Purification of Recombinant Pfpmt.** Pfpmt was expressed in the *Escherichia coli* BL21-CodonPlus strain (Stratagene). Expression clones were grown at 37°C in Luria broth medium containing 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol. A 1-liter culture of *E. coli* was grown to an  $A_{600}$  of 0.6, and Pfpmt expression was induced by addition of 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Recombinant protein was extracted under native conditions by using the BugBuster protein extraction reagent (Novagen, 6 ml/g of cell pellet) containing a protease inhibitor mix (Roche Diagnostics) and 10  $\mu$ g/ml lysozyme. The His-tagged Pfpmt was purified by using nickel-affinity chromatography according to the manufacturer's instructions (Qiagen, Valencia, CA). The eluate was dialyzed by using a buffer containing 5 mM Hepes-KOH (pH 7.8) and 0.5 mM DTT and then kept at 4°C. The purity of the recombinant enzyme was determined by SDS/PAGE, and the protein concentration was measured by the method of Bradford using BSA as a standard.

**Enzyme Assays.** Recombinant Pfpmt activity was determined by measuring the incorporation of a radioactive labeled methyl donor [<sup>14</sup>C]SAM into P-Etn. The incubation mixture contained 100 mM Hepes-KOH buffer, pH 8.6, 2 mM EDTA, 10% glycerol, 100  $\mu$ M P-Etn, 100  $\mu$ M SAM (100 nCi [<sup>14</sup>C]SAM), and 10  $\mu$ g of recombinant enzyme in a final volume of 100  $\mu$ l. The reaction was incubated for 30 min at 37°C and terminated by the addition of 1 ml of ice-cold H<sub>2</sub>O. The product was purified through a AG (H+) resin as described by Nuccio *et al.* (13). The identity of the reaction product was confirmed by TLC using [<sup>14</sup>C]P-Cho (55 mCi/mmol, ARC) as a standard. Control reaction mixtures lacking the enzyme were always included. Pfpmt activity assay using the alternative substrates ethanolamine and PtdEtn were performed as described (13, 30, 31), using 100  $\mu$ M of each substrate. The kinetic properties of Pfpmt for the P-Etn substrate were determined under a saturating concentration of the cosubstrate SAM (3 mM) and with increasing concentrations of P-Etn (20  $\mu$ M to 1 mM). Similarly, the affinity of Pfpmt for its cosubstrate SAM was determined by using different concentrations of SAM (20  $\mu$ M to 1 mM) and a saturating concentration of P-Etn (3 mM). Enzyme inhibition assays were performed by adding various concentrations of P-Cho (0, 10, 25, 50, 75, 100, 200, and 500  $\mu$ M) or miltefosine (0, 1, 10, 50, and 100  $\mu$ M) or 50  $\mu$ M S-adenosyl-L-homocysteine to the reaction mixture containing 100  $\mu$ M P-Etn, 100  $\mu$ M SAM (100 nCi of [<sup>14</sup>C]SAM), and 10  $\mu$ g of recombinant Pfpmt enzyme. The reaction was incubated for 30 min at 37°C and terminated by the addition of 1 ml of ice-cold H<sub>2</sub>O. The product was analyzed and counted as described above.

## Results

***P. falciparum* Converts P-Etn into P-Cho for PtdCho Synthesis.** To elucidate the mechanism of PtdCho synthesis from phospholipid precursors other than choline, pulse–chase labeling studies with

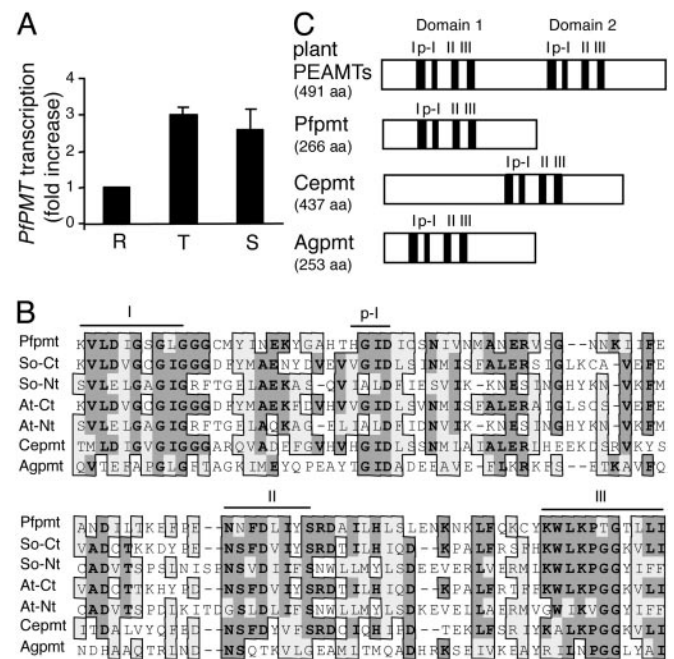


**Fig. 1.** Evidence for a PEAMT (Pfpmt) activity in *P. falciparum*. (A) TLC analysis of the extracted lipid (i) and aqueous (ii) phase after labeling of *P. falciparum* infected erythrocytes with [<sup>14</sup>C]-Etn. The identity of phospholipids (i) and aqueous compounds (ii) was determined by using appropriate standards. \*, the theoretical position of choline. (B) Pfpmt activity in *P. falciparum* extracts using 100 μM P-Etn and 100 μM SAM as substrate and cosubstrate, respectively. The activity was measured at 37°C in the absence (–) or presence (+) of 600 μg of *P. falciparum* protein extract, and the product P-Cho was purified by using a AG-50(H<sup>+</sup>) ion-exchange resin. Each value is the mean ± SD of duplicate experiments. (C) TLC analysis of the reaction product when reaction was performed in the absence (–) or presence (+) of 600 μg of *P. falciparum* protein extract; lane S1, standard [<sup>14</sup>C]-P-Cho. The origin of the migration is indicated.

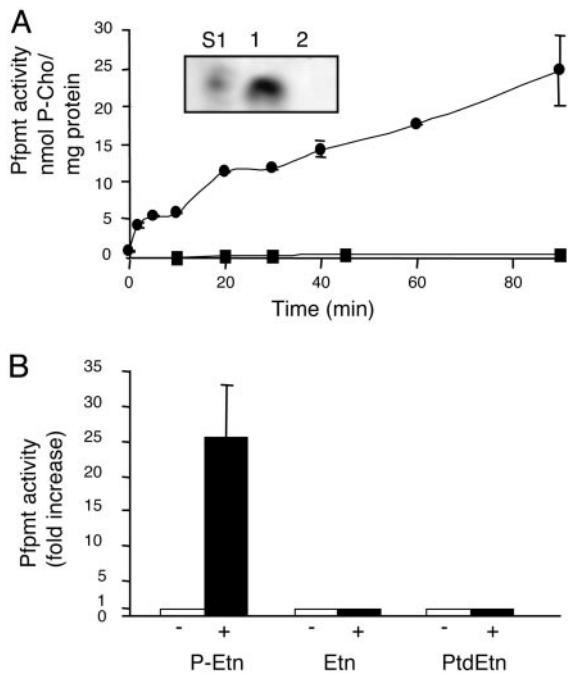
[<sup>14</sup>C]ethanolamine in *P. falciparum*-infected erythrocytes were performed in RPMI medium as described in *Materials and Methods*. After cell lysis, the lipid fraction and the water-soluble metabolites were analyzed by TLC. Analysis of the lipid fraction showed the formation of both PtdEtn and PtdCho (Fig. 1A), indicating the presence of a functional CDP-ethanolamine pathway for PtdEtn synthesis and suggesting the presence of an alternative pathway for PtdCho synthesis from ethanolamine. Interestingly, analysis of the water-soluble fraction showed the formation of P-Cho (Fig. 1A). No choline could be detected in the soluble fraction, indicating that *P. falciparum* does not catalyze the methylation of ethanolamine into choline and rule out the possibility of P-Cho formation from direct phosphorylation of choline. Similar results were obtained when *P. falciparum* parasites were continuously labeled with [<sup>14</sup>C]ethanolamine (data not shown). Together, these results suggest that *P. falciparum* catalyzes the formation of P-Cho from direct methylation of P-Etn, an enzymatic reaction that is similar to that of plant PEAMTs. To analyze this activity in a cell-free system, a *P. falciparum* lysate was prepared and examined for its ability to catalyze the methylation of P-Etn into P-Cho by using SAM as a methyl donor. Our results showed that *P. falciparum* protein extracts catalyze the SAM-dependent methylation of P-Etn into P-Cho (Fig. 1B). The P-Cho product was purified by ion-exchange chromatography and was found to comigrate with a P-Cho standard by TLC analysis (Fig. 1C). No P-Cho could be purified or detected by TLC when parasite extract was omitted from the PEAMT reaction (Fig. 1C).

**Characterization of the *P. falciparum* PEAMT, Pfpmt.** By searching for proteins with sequence homology to plant PEAMTs and containing a SAM binding domain, we identified a homolog, Pfpmt, in the *P. falciparum* genome database (32, 33) and cloned its cDNA. Quantitative RT-PCR analysis using RNA purified from

the three intraerythrocytic developmental stages (rings, trophozoites, and schizonts) of *P. falciparum* showed that Pfpmt was expressed throughout the intraerythrocytic life cycle of the parasite (Fig. 2A). Transition from the ring stage to the trophozoite stage, during which an active synthesis of new membranes takes place, resulted in a 3-fold increase in Pfpmt transcription. This expression remained constant during the later stages of the parasite life cycle. The ORF of Pfpmt is interrupted by three introns and encodes a polypeptide of 266 amino acid residues with a predicted molecular mass of 31 kDa and a theoretical pI of 5.43. Pfpmt shares high sequence identity with plant PEAMTs (24–27% with the N-terminal domain and 48–49% with the C-terminal domain), and two putative proteins from *Caenorhabditis elegans* (41%) and *Anopheles gambiae* (20%) (Fig. 2B). Pfpmt protein does not show any recognizable transmembrane domains or specific organellar targeting signals. Importantly, Pfpmt does not share homology with PtdEtn methyltransferases from lower and higher eukaryotes, and no other homologs of this protein could be found in human or other mammalian databases. Whereas plant PEAMTs are bipartite enzymes of 57 kDa with two SAM-dependent catalytic domains, each containing four consensus motifs (I, p-I, II, and III) important for catalysis, the malarial Pfpmt is only half the size of plant PEAMTs and possesses a single catalytic domain (Fig. 2C). The N-terminal domain of the *Spinacia oleracea* PEAMT is responsible for the addition of the first methylation step, whereas the C-terminal domain catalyzes the two following methylation reactions. The



**Fig. 2.** Expression and sequence analysis of Pfpmt. (A) Quantification of Pfpmt expression by real-time PCR analysis in ring (R), trophozoite (T), and schizont (S) stages. The indicated fold change in Pfpmt transcription was calculated relative to the ring stage expression. Data were normalized to expressed level of the seryl-tRNA synthetase encoding gene. The standard deviation of two independent experiments is given. (B) Sequence alignment of the polypeptide sequence encompassing the PEAMT catalytic motifs (I, p-I, II, and III) from *P. falciparum* (Pfpmt; AN AY429590) and *S. oleracea* (So-Nt and So-Ct, AN AF237633), *Arabidopsis thaliana* (At-Nt and At-Ct, AN AAG41121) PEAMT, *C. elegans* (Cepmt; AN AAB04824), and *A. gambiae* (Agpmt). Sequence identity is indicated in dark gray, and similarity is indicated in light gray. (C) Schematic representation of the bipartite structure of plant PEAMTs and monopartite structures of Pfpmt, Cepmt, and Agpmt. The four motifs (I, p-I, II, and III) of each PEAMT catalytic domain(s) are indicated as black boxes. The size of the protein in amino acid (aa) is indicated.



**Fig. 3.** Recombinant Pfpmt specifically catalyzes the methylation of P-Etn into P-Cho. (A) Time course of Pfpmt SAM-dependent methylation of 100  $\mu$ M P-Etn at 37°C (●) and 0°C (■) using 10  $\mu$ g of recombinant Pfpmt. The reaction was performed as described in *Materials and Methods*. (Inset) A TLC analysis of the product of the Pfpmt reaction after a 30-min incubation at 37°C (lane 1) and 0°C (lane 2); lane S1, standard [ $^3$ H]P-Cho. (B) Substrate specificity of Pfpmt in the presence (+) or absence (–) of 100  $\mu$ M P-Etn, Etn, and PtdEtn. Each datum represents an average of a duplicate  $\pm$  SD.

monopartite structure of Pfpmt suggests that this unusual enzyme could solely be responsible for the three-step methylation of P-Etn into P-Cho. To investigate this hypothesis, recombinant Pfpmt protein was expressed in *E. coli*, purified by affinity chromatography, and assayed for PEAMT activity *in vitro*, by using P-Etn and SAM as substrate and cosubstrate, respectively. The product of the reaction was purified by ion chromatography and its identity confirmed by TLC (Fig. 3A Inset). Our results showed that the purified enzyme catalyzes the conversion of P-Etn into P-Cho using SAM as a methyl donor (Fig. 3A Inset, lane 1). This activity was linear with time at 37°C for at least 90 min and could not be detected at 0°C (Fig. 3A). To determine the substrate specificity of Pfpmt, ethanolamine and PtdEtn were used in the methylation reactions. None of these molecules was found to be a substrate of Pfpmt, thus suggesting that P-Etn is the primary methyl acceptor of this enzyme (Fig. 3B). The apparent affinity values of Pfpmt for its substrate P-Etn and for its cosubstrate SAM were determined under saturating concentrations of the cosubstrate and increasing concentration of the substrate and vice versa (Fig. 4). The Lineweaver–Burk representation of the saturation curves obtained from both assays produced  $K_m$  values of  $\approx$ 79  $\mu$ M and 153  $\mu$ M for P-Etn and SAM, respectively, and a  $V_{max}$  of 1.2 nmol $\cdot$ mg $^{-1}\cdot$ min $^{-1}$  for both substrates.

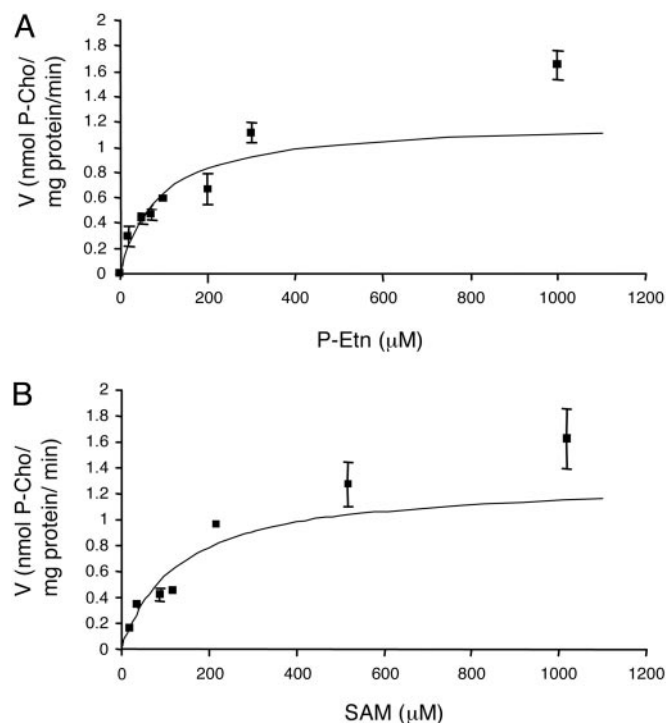
**Inhibition of Pfpmt Activity and *P. falciparum* Growth by P-Cho and Its Analog Miltefosine.** The finding that P-Etn is a substrate for Pfpmt, and knowing that this precursor is also a substrate for CDP–P-Etn transferase, which catalyzes the rate limiting step in the CDP–ethanolamine pathway, suggests that Pfpmt activity might play an important regulatory role in the synthesis of the two major phospholipids PtdEtn and PtdCho. Interestingly, we found that P-Cho inhibited Pfpmt activity. This effect was

concentration dependent, with 50% decrease in Pfpmt activity when P-Cho was added at a concentration of 50  $\mu$ M (Fig. 5A). The finding that Pfpmt activity was inhibited by its product P-Cho suggested that P-Cho analogs might also inhibit this enzyme. Accordingly, the P-Cho analog, hexadecylphosphocholine (miltefosine), was found to inhibit Pfpmt activity with  $\approx$ 50% of this activity reduced when the compound was added at 50  $\mu$ M, and  $\approx$ 90% inhibition obtained when the compound was added at 100  $\mu$ M (Fig. 5B). As expected for SAM-dependent enzymatic reactions, addition of *S*-adenosyl-L-homocysteine, a known inhibitor of these reactions, affected Pfpmt activity with  $\approx$ 50% of this activity reduced when the compound was added at 50  $\mu$ M (Fig. 5B). Furthermore, clofibrate, an inhibitor of PtdEtn methyltransferases (34), had no effect on Pfpmt activity at concentrations up to 150  $\mu$ M (data not shown).

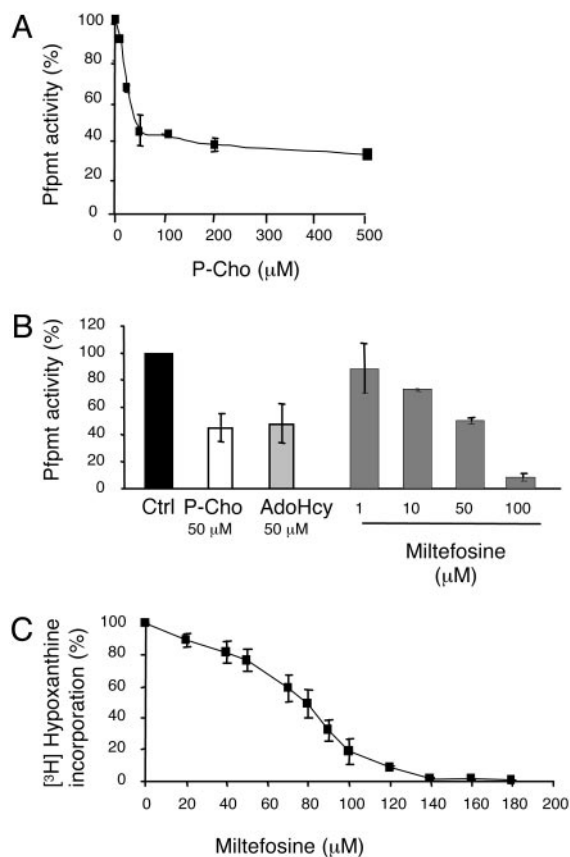
The finding that miltefosine inhibits Pfpmt activity prompted us to examine the antimalarial activity of this compound. Interestingly, when a [ $^3$ H]hypoxanthine incorporation assay was used to measure *P. falciparum* proliferation in human red blood cells, miltefosine was found to possess an antimalarial activity. The IC $_{50}$  value of this compound in the drug-sensitive *P. falciparum* clone 3D7 was  $\approx$ 80  $\mu$ M (Fig. 5C).

## Discussion

Upon infection of human erythrocytes, the phospholipid content of *P. falciparum* increases by at least 5- to 6-fold (35). PtdCho, which represents 50% of the total phospholipid pool, has been proposed to be synthesized mainly from choline transported from human serum (5, 16). However, *in vitro* growth assays indicated that choline was not essential for parasite development and multiplication (22, 23). Here, our studies provide evidence



**Fig. 4.** Kinetics of Pfpmt reaction using recombinant enzyme. Pfpmt activity was measured in the presence of varying concentrations of P-Etn (A) and SAM (B). In A, SAM was present at a concentration of 2 mM, whereas in B, P-Etn was added at a final concentration of 3 mM. The reaction was carried out as described in *Materials and Methods*. The formation of P-Cho was quantified per mg of protein per minute. The curve for velocity ( $V$ ) versus substrate concentration ( $[S]$ ) was fit to the Michaelis–Menten equation ( $V = V_{max} \times S / [K_m + S]$ ). Each datum point represents an average of a duplicate  $\pm$  SD.

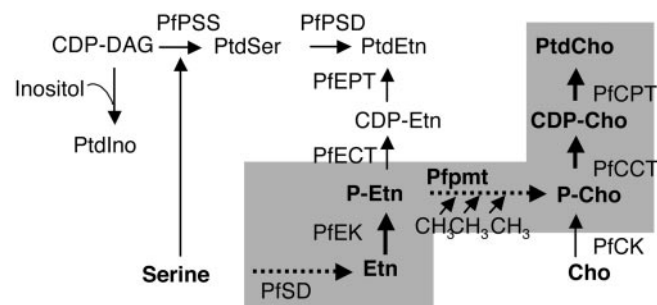


**Fig. 5.** Inhibition of recombinant PfpmT activity and *P. falciparum* growth. (A) Inhibition of PfpmT activity by P-Cho. Inhibition assay was performed by adding various concentrations of P-Cho (0–500  $\mu$ M) as described in *Materials and Methods*. (B) Inhibition of PfpmT activity by 50  $\mu$ M P-Cho, 50  $\mu$ M adenosyl-L-homocysteine (AdoHcy), and 1, 10, 50, and 100  $\mu$ M miltefosine. Control, PfpmT activity without inhibitor. (C) Effect of several concentrations of miltefosine on [ $^3$ H]hypoxanthine incorporation in cultured parasites. Each value is the mean  $\pm$  SD of at least duplicate experiments.

that *P. falciparum* utilizes ethanolamine as an alternative precursor for the synthesis of PtdCho. Ethanolamine, which is absent from plasma (36), is derived from serine by decarboxylation (25). In *Plasmodium*-infected erythrocytes, serine is available via active transport from serum and active degradation of host proteins (37, 38). Previous studies in *P. falciparum* using radiolabeled serine showed that this precursor was readily incorporated into phospholipids (25). In this study, we provide evidence, using pulse–chase (Fig. 1A) and continuous labeling (data not shown) experiments with [ $^{14}$ C]ethanolamine, that PtdCho can be synthesized from ethanolamine in *P. falciparum* infected erythrocytes. Pulse–chase experiments showed that PtdEtn was rapidly formed, and only small amounts of P-Etn could be detected (data not shown), whereas continuous labeling with [ $^{14}$ C]ethanolamine identified large amounts of PtdEtn and P-Etn (data not shown). These data suggest that ethanolamine taken by the parasite is rapidly phosphorylated and incorporated into PtdEtn via the CDP–ethanolamine pathway. Interestingly, both labeling studies revealed the formation from ethanolamine of both P-Cho and PtdCho, but not choline. These results demonstrate that *P. falciparum* does not synthesize choline from ethanolamine and that the P-Cho formed could result from a three-step methylation of P-Etn. Previous reports have suggested that PtdEtn methylation might occur in *P. falciparum* (16). However, the presence of unusually high levels of PtdEtn (35–40%) in *P. falciparum* membranes suggests that, if a PtdEtn

methyltransferase activity exists in the parasite, it has little or no contribution to the PtdCho pool. Furthermore, no homologs of higher or lower eukaryotic PtdEtn methyltransferase genes could be identified in the finished genome sequence of *P. falciparum*. Instead, we identified in *P. falciparum* a single gene, *PfPMT*, encoding a protein homologous to plant PEAMTs. We found that *PfPMT* was expressed throughout the intraerythrocytic cycle of the parasite and primarily induced during the mature stages. Furthermore, genome-wide microarray analysis revealed that *PfPMT* is also expressed during the gametocyte and sporozoite stages of the parasite (32, 39). Whereas several *P. falciparum* proteins with plant-like properties have been shown to be targeted to the apicoplast (40), PfpmT lacks signal and transit peptide sequences that could mediate targeting to this organelle. Plant PEAMTs are bipartite enzymes possessing two different SAM-dependent catalytic domains responsible for the three-step methylation reaction. Interestingly, PfpmT is only half the size of plant PEAMTs, with a single catalytic domain sharing homology with both the N- and C-terminal domains of these enzymes. This unique property of PfpmT suggests that the single catalytic domain of PfpmT can either catalyze all of the three methylation reactions to convert P-Etn into P-Cho or that PfpmT dimerizes to compensate for the lack of the second domain. Interestingly, multiangle laser light scattering analysis of PfpmT revealed that this protein was a monomer (unpublished data), and ion exchange and TLC analyses indicated that this monomeric protein was able to perform all three methylation reactions (Fig. 3A). Search of *Plasmodium* database (PlasmoDB) revealed two proteins in *C. elegans* (*Cepmt*) and *A. gambiae* (*Agpmt*) that share homology with PfpmT (32). Like PfpmT, *Agpmt* is half the size of plant PEAMTs and possesses a single putative catalytic domain (Fig. 2B and C). On the other hand, *Cepmt* is of similar size as plant PEAMTs but possesses only one recognizable putative catalytic domain in the C-terminal region of the protein (Fig. 2B and C). It is not yet known whether these proteins are active PEAMTs and what their contribution is to the phospholipid composition of these organisms.

Our labeling studies revealed the incorporation of ethanolamine into PtdEtn and PtdCho, despite the presence of choline in the medium. This suggests that *P. falciparum* utilizes both the CDP–choline pathway and the PfpmT-mediated alternative pathway for PtdCho biosynthesis. Synthesis of PtdCho from alternative pathways has also been shown in *Saccharomyces cerevisiae*. In this organism, although the transmethylation of PtdEtn in the CDP–diacylglycerol pathway is the primary route of synthesis of PtdCho, the CDP–choline pathway, which has long been viewed as an auxiliary or salvage pathway, can also contribute to the



**Fig. 6.** Model of the pathways for PtdCho biosynthesis in *P. falciparum*. Plant-like reactions are indicated as dotted lines, and the new identified pathway is shown in gray. DAG, diacylglycerol; PtdIno, phosphatidylinositol; PfPSS, phosphatidylserine synthase; PfPSD, phosphatidylserine decarboxylase; PfEKT and PfCK, ethanolamine and choline kinases; PfECT and PfCCT, P-Etn and P-Cho cytidyltransferases; PfEPT and PfCPT, ethanolamine and choline phosphotransferases; PfSD, serine decarboxylase.

synthesis of this phospholipid. Choline used by the CDP-choline pathway is either transported from the medium or derived from phospholipase D hydrolysis (41). Unlike yeast cells where phospholipid metabolism is highly regulated by its phospholipid precursors (42, 43), no regulatory mechanisms of the different phospholipid biosynthetic pathways have been described in *P. falciparum*. The finding that Pfpmt activity is inhibited by its product P-Cho suggests that Pfpmt might act as a sensor of phospholipid biosynthesis to determine the metabolic outcome of P-Etn. One possibility is that PtdCho accumulation inhibits Pfpmt activity, thus shifting the equilibrium toward the utilization of P-Etn for the synthesis of PtdEtn. We found that the P-Cho analog miltefosine also inhibited Pfpmt activity. The inhibition of Pfpmt activity by miltefosine was similar to that of *S*-adenosyl-L-homocysteine, a specific inhibitor of SAM-dependent reactions (Fig. 5B). Furthermore, clofibrilic acid, an inhibitor of PtdEtn methyltransferases, had no effect on Pfpmt activity. Together, these data suggest that the inhibition of Pfpmt activity by miltefosine is specific. Interestingly, miltefosine also inhibited *P. falciparum* proliferation with an IC<sub>50</sub> value of ≈80 μM. Miltefosine and its analogs are known to possess antitumor

and antileishmanial activities and are already validated for treatment of cancer and leishmaniasis (44–46). The low solubility of miltefosine and the presence of an active CDP-choline pathway might account for the low antimalarial activity of this compound. Studies to identify new P-Cho analogs with better antimalarial activity are warranted.

In conclusion, we provide evidence for a pathway for PtdCho biosynthesis in *P. falciparum* that includes two plant-like components (Fig. 6). First, *P. falciparum* converts serine into ethanolamine (25); then, the phosphorylated ethanolamine is converted by Pfpmt into P-Cho, which serves as a precursor for the synthesis of PtdCho (Fig. 6).

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