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## Two New Plant-Like Pathways Link Hemoglobin Degradation to Lipid Biogenesis in *Falciparum* Malaria: Novel Targets for Anti-Malarial Chemotherapy

### **INTRODUCTION:**

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 more than one 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 (5). 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* (7). 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-DAG pathway (8,9). The Kennedy pathways synthesize PtdCho and PtdEtn from choline and ethanolamine, respectively. The CDP-DAG pathway initiates from serine and CDP-DAG to form phosphatidylserine (PtdSer), which is then converted into PtdEtn via the activity of PtdSer decarboxylase enzymes. PtdEtn is subsequently methylated into PtdCho by PtdEtn methyltransferases (10). Whereas in most mammalian cells, the *de novo* CDP-choline pathway is the major route for synthesis of PtdCho in yeast cells and mammalian hepatocytes, the CDP-DAG pathway is the primary route for synthesis of PtdCho. Plant cells, however, lack PtdSer decarboxylases but instead catalyze the decarboxylation of serine into ethanolamine (11), which is subsequently phosphorylated into phosphoethanolamine. The phosphoethanolamine formed is either incorporated into PtdEtn via the CDP-ethanolamine pathway or converted into phosphocholine by a phosphoethanolamine methyltransferase (PEAMT) (12-14). Phosphocholine 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) (7). The genes involved in the synthesis of phospholipids have only recently started to be elucidated. The *de novo* CDP-choline pathway has been proposed to be the primary route for synthesis of PtdCho in *Plasmodium* (7); however *in vitro* growth assays using dialyzed serum indicated that choline was not essential for parasite intraerythrocytic development and survival (15,16). 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.

In this first progress report, we describe our molecular and biochemical studies that provide evidence for the presence in *P. falciparum* of an alternative pathway for PtdCho biosynthesis (**Appendix I**). Serine, which is transported from human serum, and readily available in the parasite cytoplasm, is converted into ethanolamine and then phosphorylated into phosphoethanolamine. We show that *P. falciparum* catalyzes a phosphoethanolamine methyltransferase reaction that converts phosphoethanolamine into phosphocholine, which is then incorporated into PtdCho (**Reprint I**). We will also describe genetic studies to disrupt the contiguity of the *PfPMT* genomic locus and studies to solve the structure of the Pfpmt enzyme.

## **BODY**

Several lines of evidence suggest that the pathways for membrane biogenesis are unique in the human malaria parasite *P. falciparum* and are excellent targets for antimalarial chemotherapy. First, invasion of human red blood cells by *P. falciparum* results in over 600% increase in the lipid content of infected erythrocytes and major uptake of phospholipid precursors from the host serum. Secondly, the pool of phosphatidylethanolamine (PE) is unusually high (~ 40% of total phospholipids) in this parasite. Thirdly, the parasite expresses plant-like type II fatty acid synthetic (FAS) enzymes in the apicoplast. Accordingly, choline analogs and inhibitors of type II FAS enzymes have been shown to inhibit the growth of *P. falciparum in vitro* and *in vivo* and are already considered for future use as anti-malarial drugs. Little is known about the biosynthetic pathways of the glycerophospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in *P. falciparum*. In mammals and lower eukaryotes two PE-methyltransferase (PEM) enzymes catalyze the three steps AdoMet-dependent methylation of PE into PC. Research in our laboratory indicated that *P. falciparum* lacks PEM enzymes, but instead the parasite possesses a plant-like phosphoethanolamine-methyltransferase that we named Pfpmt. Furthermore, *P. falciparum* has evolved a novel way to synthesize

ethanolamine from serine, which is readily available in the parasite cytoplasm as a result of active hemoglobin degradation and transport. This reaction is catalyzed by a serine decarboxylase encoded by a putative *PfSDC1* gene.

#### The Specific Aims of our research are:

1. Characterize *PfPMT* and *PfSDC1* genes.
2. Validate *PfPMT* and *PfSDC1* as malarial drug targets.
3. Determine the structure of recombinant proteins with an eye toward drug development.
4. Analyze the effect of imipramine and phosphocholine analogs against *PfPMT* and *P. falciparum* and Develop high-throughput inhibitory assays to screen chemical databases.

During the first year of funding we have focused our studies on the molecular, genetic and biochemical characterization of *PfPMT*. In this first progress report we will provide a detailed description of four important studies:

- (i) Metabolic studies in *P. falciparum* indicating that the parasite uses ethanolamine and serine as precursors for the synthesis of the major phospholipid, phosphatidylcholine.
- (ii) Biochemical evidence indicating that *PfPMT* encodes a methyltransferase with strong specificity for phosphoethanolamine and no preference for ethanolamine or phosphatidylethanolamine.
- (iii) Inhibition studies indicating that phosphocholine analogs inhibit *PfPMT* activity and have antimalarial activity.
- (iv) Genetic studies to knockout *PfPMT* gene.

#### Part One: Metabolic studies in *P. falciparum*

##### ***P. falciparum* converts phosphoethanolamine into phosphocholine for PtdCho synthesis.**

To elucidate the mechanism of phosphatidylcholine (PtdCho) synthesis from phospholipid precursors other than choline, pulse-chase labeling studies with [<sup>14</sup>C]-ethanolamine in *P. falciparum*-infected erythrocytes were performed in RPMI medium as described in Material and Methods. After cell lysis, the lipid fraction and the water-soluble metabolites were analyzed by Thin-Layer-Chromatography (TLC). Analysis of the lipid fraction showed the formation of both phosphatidylethanolamine (PtdEtn) and PtdCho (**Appendix II, A**), 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 phosphocholine (**Appendix II, A**). 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 phosphocholine 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 phosphocholine from direct methylation of phosphoethanolamine, an enzymatic reaction that is similar to that of plant phosphoethanolamine methyltransferases. In order 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 phosphoethanolamine into phosphocholine using S-Adenosyl-L-Methionine (SAM) as a methyl donor. Our results showed that *P. falciparum* protein extracts catalyze the SAM-dependent methylation of phosphoethanolamine into phosphocholine (**Appendix II, B**). The phosphocholine product was purified by ion exchange chromatography and was found to comigrate with a phosphocholine standard by TLC analysis (**Appendix II, C**). No phosphocholine could be purified or detected by TLC when parasite extract was omitted from the phosphoethanolamine methyltransferase reaction (**Appendix II, C**).

## **Part Two: Biochemical characterization of *P. falciparum* phosphoethanolamine methyltransferase**

### **Characterization of the *P. falciparum* phosphoethanolamine methyltransferase, Pfpmt.**

By searching for proteins with sequence homology to plant phosphoethanolamine methyltransferases and containing a SAM binding domain, we identified a homolog, Pfpmt, in the *P. falciparum* genome database (17,18), 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 (**Appendix III, A**). 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 open reading frame 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 phosphoethanolamine methyltransferases (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%) (**Appendix III, B**). 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 (**Appendix III, C**). The N-terminal domain of the *S. oleracea* PEAMT is responsible for the addition of the first methylation step, whereas the C-terminal domain catalyzes the two following methylation reactions. The monopartite structure of Pfpmt suggests that this unusual enzyme could solely be responsible for the three-step methylation of phosphoethanolamine into phosphocholine. To investigate this hypothesis, recombinant Pfpmt protein was expressed in *E. coli*, purified by affinity chromatography and assayed for phosphoethanolamine methyltransferase activity *in vitro*, using phosphoethanolamine and SAM as substrate and cosubstrate, respectively. The product of the reaction was purified by ion chromatography and its identity confirmed by TLC (**Appendix IV, A, inset**). Our results showed that the purified enzyme catalyzes the conversion of phosphoethanolamine into phosphocholine using SAM as a methyl donor (**Appendix IV, A, 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 (**Appendix IV, A**). 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 phosphoethanolamine is the primary methyl acceptor of this enzyme (**Appendix IV, B**). The apparent affinity values of Pfpmt for its substrate phosphoethanolamine and for its cosubstrate SAM were determined under saturating concentrations of the cosubstrate and increasing concentration of the substrate and *vice versa* (**Appendix V, A and B**). The Lineweaver-Burk representation of the saturation curves obtained from both assays produced  $K_m$  values of approximately 79  $\mu\text{M}$  and 153  $\mu\text{M}$  for phosphoethanolamine and SAM, respectively, and a  $V_{\text{max}}$  of 1.2  $\text{nmol mg}^{-1} \text{ min}^{-1}$  for both substrates.

### **Part Three: Inhibition studies**

#### **Inhibition of Pfpmt activity and *P. falciparum* growth by phosphocholine and its analog miltefosine.**

The finding that phosphoethanolamine is a substrate for Pfpmt, and knowing that this precursor is also a substrate for CDP-phosphoethanolamine 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 phosphocholine inhibited Pfpmt activity. This effect was concentration-dependent with 50% decrease in Pfpmt activity when phosphocholine

was added at a concentration of 50  $\mu\text{M}$  (**Appendix VI, A and B**). The finding that Pfpmt activity was inhibited by its product phosphocholine suggested that phosphocholine analogs might also inhibit this enzyme. Accordingly, the phosphocholine analog, hexadecylphosphocholine (Miltefosine) was found to inhibit Pfpmt activity with ~50% of this activity reduced when the compound was added at 50  $\mu\text{M}$ , and ~90% inhibition obtained when the compound was added at 100  $\mu\text{M}$  (**Appendix VI, B**). As expected for SAM-dependent enzymatic reactions, addition of S-adenosyl-L-homocysteine (AdoHcy), a known inhibitor of these reactions, affected Pfpmt activity with ~50% of this activity reduced when the compound was added at 50  $\mu\text{M}$  (**Appendix VI, B**). Furthermore, clofibric acid, an inhibitor of PtdEtn methyltransferases (19), had no effect on Pfpmt activity at concentrations up to 150  $\mu\text{M}$  (data not shown).

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

#### **Part Four: Genetic studies to knockout *PfPMT* gene**

**Disruption of *PfPMT* locus by double cross-over.** To determine the importance of *PfPMT* in *P. falciparum* development and survival and validate its function as a target for anti-malarial chemotherapy, we initiated studies to generate knockout lines lacking this gene. The overall strategy, depicted in **Appendix VII**, is a two step process by which the targeted *PfPMT* ORF is first replaced with the *Aspergillus terreus* blasticidin-s-deaminase (*BSD*) gene after a double cross-over event followed by loss of the episome after selection against the *Herpes simplex* thymidine kinase (*TK*) gene. The pRZ/TK/BSD-2 vector constructed in our laboratory was employed in this approach. In this vector, *BSD* gene is under the control of the *PcDT* promoter and *HrpII* terminator, and the *TK* gene is under the control of the *CAM* promoter and the *P. falciparum* *Hsp86* terminator. The pRZ/TK/BSD-2 vector enables selection of blasticidin-resistant parasites in 2.5  $\mu\text{g/ml}$  blasticidin 2 to 3 weeks after transfection. To construct a vector for *PfPMT* gene disruption, a 620 bp fragment of the targeted ORF, corresponding to the N-terminal portion of the enzyme, was amplified by PCR and subcloned by directional cloning into an *HindIII/Bpu1102I* unique site upstream of the *PcDT* promoter. A second round of PCR amplification amplified a 580 bp fragment corresponding to the C-terminal portion of the enzyme, and subcloned by directional cloning into an *EcoRI/NarI* site downstream of the *HrpII* terminator. This targeted gene disruption strategy is expected to result in a large truncation in the middle of the targeted ORF and neither the N-terminal portion nor the C-terminal portion could possibly encode a competent pE-methyltransferase. The resulting targeting construct designated pJJ1234 (**Appendix VII**) was transfected into the blasticidin-sensitive

*P. falciparum* 3D7 clone and blasticidin-resistant transfectants were selected in 2.5  $\mu\text{g/ml}$  blasticidin and screened for integration events by negative selection to generate *pfpmt* $\Delta$  knockout. The growth medium of the parasites was supplemented with 1 mM choline to complement possible choline auxotrophy of *pfpmt* $\Delta$  cells in the events that *PfPMT* is essential and that available amounts of choline in the medium are not sufficient to overcome the low levels of phosphocholine produced from choline for the synthesis of phosphatidylcholine. To promote integration of pJJ1234 targeting vector into the *PfPMT* locus, we first removed blasticidin selection for 2 weeks before applying gancyclovir at 1.5, 3 and 10  $\mu\text{M}$  for 1, 2 or 3 weeks. After each week, blasticidin was added and parasites were maintained until growth was detected by Giemsa stain. Parasites were harvested at different times during this analysis and prepared for genomic DNA extraction. Genomic DNA was then used in PCR and Southern blot analyses to detect integration events into *PfPMT* locus. The result obtained thus far revealed integration of the targeting cassette into the 5' region *PfPMT* locus by single cross-over *PfPMT*. This integration event does not disrupt the gene. However, no integration into the 3' region of integration by double cross-over could be detected, suggesting that *PfPMT* gene might be essential.

Collectively, our data supported by the USAMRMC provide a much better understanding of membrane biogenesis in *P. falciparum* and provide strong support for lipid-based therapies to fight this disease.

## **KEY RESEARCH ACCOMPLISHMENTS:**

1. We have cloned and sequenced the *PfPMT* gene and cDNA and showed that genomic locus of *PfPMT* is interrupted by a single intron.
2. We have demonstrated through extensive biochemical analysis that *PfPMT* encodes a methyltransferase specific for phosphoethanolamine.
3. We showed that *Pfpmt* is a member of a new family of enzymes present in plants, worms and mosquitoes but has no homologs in human or mammalian databases.
4. We demonstrated that phosphocholine and choline analogs inhibit *Pfpmt* activity and parasite growth in the low micromolar range.

## **REPORTABLE OUTCOMES:**

**I. Our laboratory published the following research paper related to the studies described in this report:**

Gabriella PESSI, Guillermo KOCIUBINSKI and Choukri BEN MAMOUN. A Pathway for Phosphatidylcholine Biosynthesis in *Plasmodium falciparum* Involving Phosphoethanolamine Methylation. *Proc. Natl. Acad. Sci. USA.* (2004) 101, 6206 – 6211.

**II. Our findings have been presented at the following research conferences:**

- Molecular and Biochemical Parasitology Meeting XV (2004)
- Molecular, Microbial and Structural Biology Retreat (2004).
- Gordon Research Conference (2003). Molecular Biology of Lipids
- Molecular and Biochemical Parasitology Meeting XIV (2003)

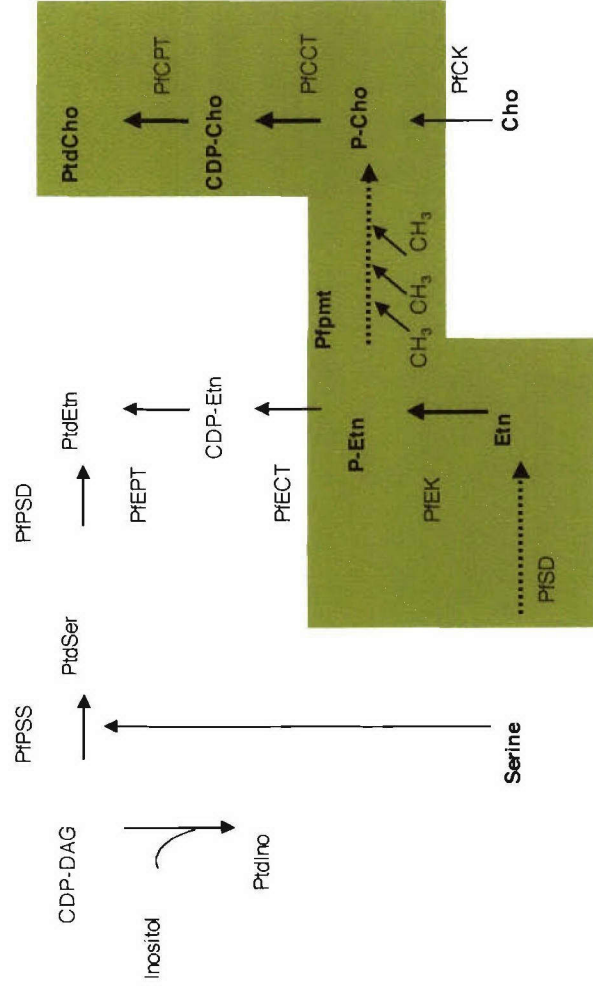
## **CONCLUSIONS:**

During this funding period we have made several key findings that allow us to determine the biological function and examine the importance of *PfPMT* in phospholipid metabolism and parasite physiology. Our future studies will include (1) determining the 3D structure of *PfpmT*, (2) performing structure-function studies to determine the residues that play an important role in its activity, (3) developing a biochemical assay to screen chemical libraries for new classes of compounds that inhibit *PfpmT* activity, (4) disrupting the contiguity of *PfPMT* genomic locus, determining the cellular localization of *PfpmT* in *P. falciparum*, and (5) characterizing the *PfSDC1* gene encoding the malarial serine decarboxylase activity.

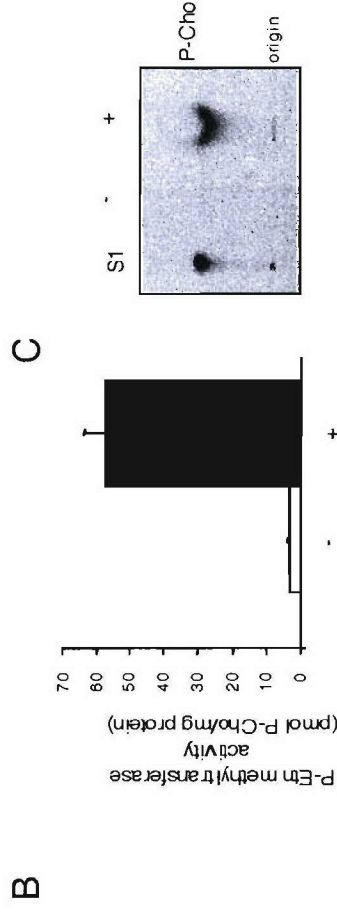
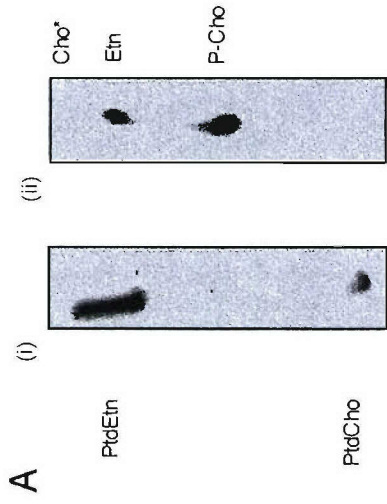
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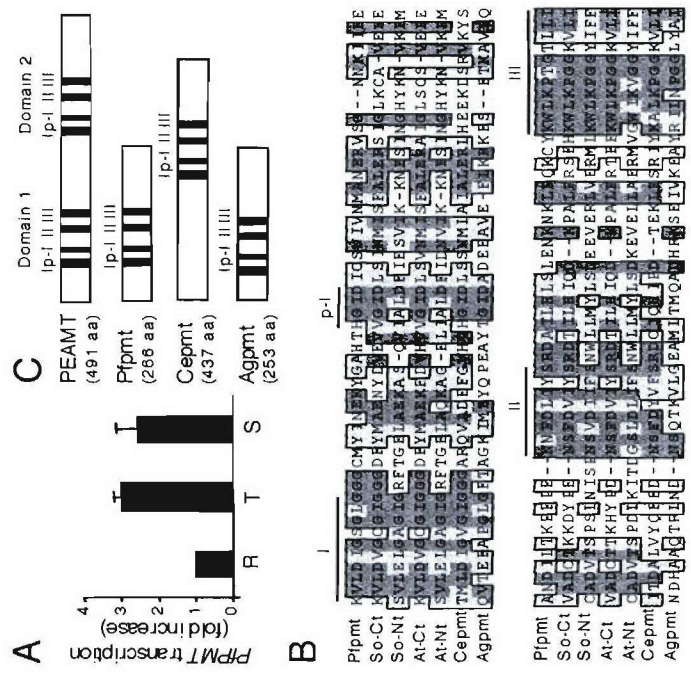
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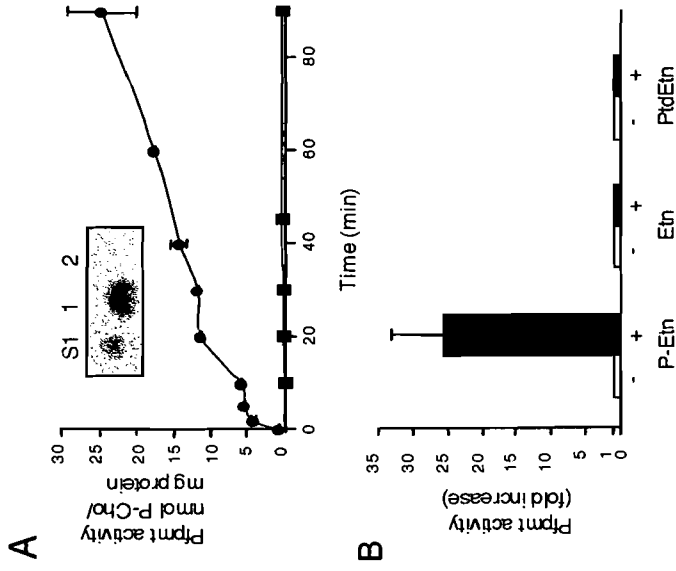
**Appendix I:** Model of the new pathway for PtdCho in *P. falciparum*. Plant-like reactions are indicated as dotted lines and the new identified pathway is shown in gray. CDP-DAG, CDP-Etn, CDP-Cho: cytidine diphospho-diacylglycerol, -ethanolamine, -choline; PtdIno, PtdSer, PtdEtn, PtdCho: phosphatidylinositol, -serine, -ethanolamine, -choline; P-Etn, P-Cho: phosphoethanolamine, -choline; PFPSS: phosphatidylserine synthase, PFEPT and PFCCT: phosphocholine cytidyltransferases; PFCCK: ethanolamine and choline phosphotransferases; PFEK and PFCCT: ethanolamine and choline phosphotransferases; PFS: serine decarboxylase; Pfpmt: phosphoethanolamine methyltransferase.



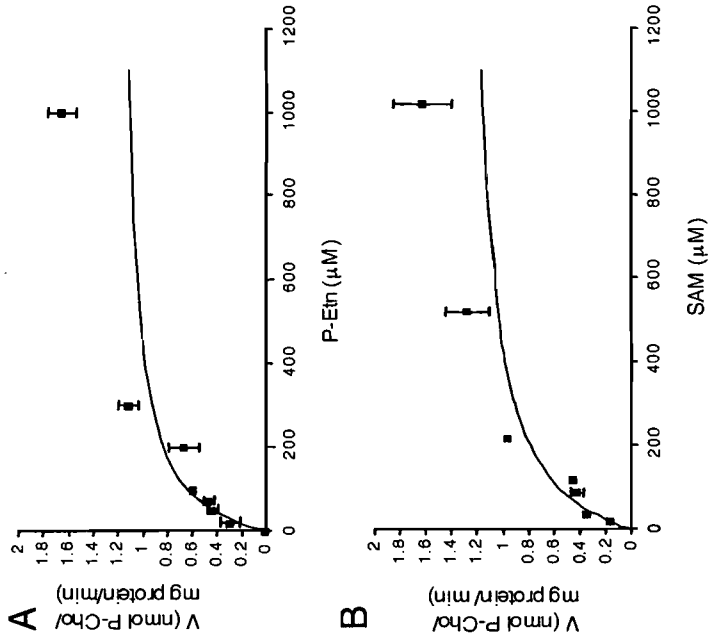
**Appendix II:** Evidence for a phosphoethanolamine methyltransferase (PfpmT) activity in *P. falciparum*. (A) TLC analysis of the extracted lipid (i) and aqueous (ii) phase after labeling of *P. falciparum* infected erythrocytes with [ $^{14}\text{C}$ ]-ethanolamine (Etn). The identity of phospholipids (i) and aqueous compounds (ii) was determined using appropriate standards. \* indicates the theoretical position of choline. (B) PfpmT activity in *P. falciparum* extracts using 100  $\mu\text{M}$  phosphoethanolamine (P-Etn) and 100  $\mu\text{M}$  [*methyl*- $^{14}\text{C}$ ]-S-Adenosyl-L-Methionine (SAM) as substrate and cosubstrate, respectively. The activity was measured at 37°C in the absence (-) or presence (+) of 600  $\mu\text{g}$  *P. falciparum* protein extract and the product phosphocholine (P-Cho) was purified using a AG-50( $\text{H}^+$ ) ion exchange resin. Each value is the mean  $\pm$  standard deviation of duplicate experiments. (C) TLC analysis of the reaction product when reaction was performed in the absence (-) or presence (+) of 600  $\mu\text{g}$  *P. falciparum* protein extract; lane S1, standard [ $^{14}\text{C}$ ]-P-Cho. The origin of the migration is indicated.



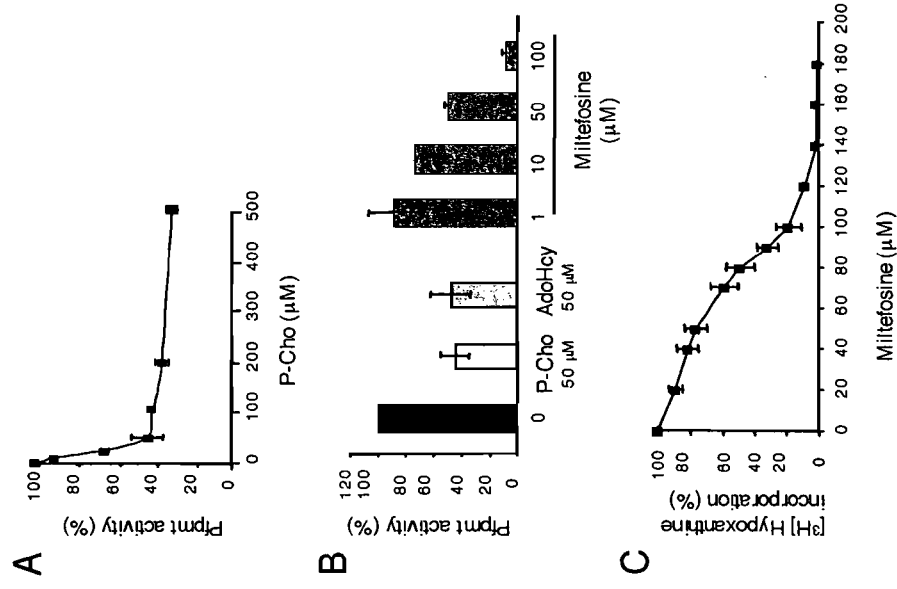
**Appendix III:** Expression and sequence analysis of PfpmT. (A) Quantification of PfpmT expression by real-time PCR analysis in ring (R), trophozoite (T) and schizont (S) stages. The indicated fold change in PfpmT transcription was calculated relative to the ring stage expression. Data were normalized 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 phosphoethanolamine methyltransferase catalytic motifs (I, p-I, II and III) from *P. falciparum* (Pfpmt; AN AY429590) and *Spinacia 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 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 P-Etn methyltransferase catalytic domain(s) are indicated as black boxes. The size of the protein in amino acid (aa) is indicated.



**Appendix IV:** Recombinant Pfpmt specifically catalyses the methylation of phosphoethanolamine (P-Etn) into phosphocholine (P-Cho). (A) Time course of Pfpmt SAM-dependent methylation of 100  $\mu$ M P-Etn at 37°C (circles) and 0°C (squares) using 10  $\mu$ g of recombinant Pfpmt. The reaction was performed as described in Material and Methods. Inset shows 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 [ $^{14}$ C]-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  $\pm$  standard deviation.



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



**Appendix VI:** Inhibition of recombinant Pfpmt activity and *P. falciparum* growth. (A) Inhibition of Pfpmt activity by phospholine (P-Cho). Inhibition assay was performed by adding various concentration of P-Cho (0-500 μM) as described in Material and Methods. (B) Inhibition of Pfpmt activity by 50 μM of P-Cho, 50 μM of S-Adenosyl-L-Homocysteine (AdoHcy) and 1, 10, 50, 100 μM miltefosine. (C) Effect of several concentrations of miltefosine on [<sup>3</sup>H]-hypoxanthine incorporation in cultured parasites. Each value is the mean ± standard deviation of at least duplicate experiments.



# 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  $\mu\text{M}$ ) (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  $\mu\text{M}$  [ $^{14}\text{C}$ ]ethanolamine (55 mCi/mmol; ARC, St. Louis; 1 Ci = 37 GBq) in RPMI–Albumax medium, which contains 20  $\mu\text{M}$  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  $\mu\text{l}$  of chloroform/methanol (2:1) and 200  $\mu\text{l}$  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- $\mu\text{l}$  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  $\mu\text{g}$ ) were prepared by sonication. After centrifugation, the supernatant was incubated for 30 min at 37°C with 100 nCi of [ $^{14}\text{C}$ ]adenosyl-L-methionine (SAM) (52 mCi/mmol, NEN) in the presence of 100  $\mu\text{M}$  P-Etn (substrate) and 100  $\mu\text{M}$  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  $\mu\text{g}$ ) 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-TTTTTGGTGGCCTTAAATAAC-3' to amplify a 300-bp

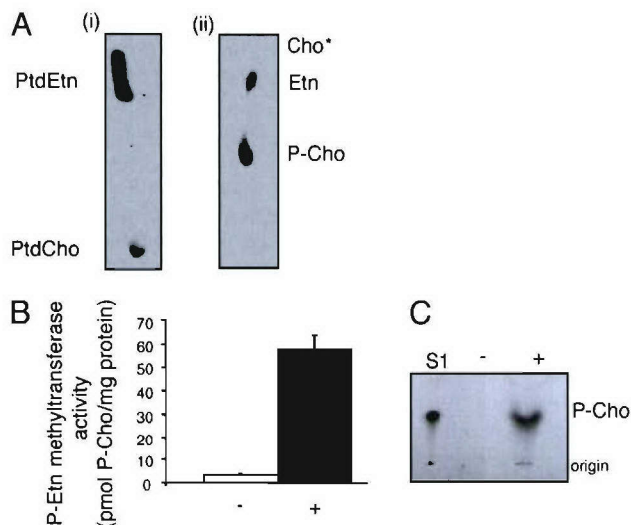
*PfpMT* fragment, and primers 5'C2143 5'-GAGGAATTTTACGTGTTTCATCAA-3' and 3'C2143 5'-GATTACTTGTAG-GAAAGAATCCTTC-3' were used to amplify a 300-bp fragment corresponding to the seryl tRNA synthetase-encoding gene (29). The real-time PCR reaction was performed in duplicate following the manufacturer's instructions (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  $\mu\text{g}/\text{ml}$  ampicillin and 34  $\mu\text{g}/\text{ml}$  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  $\mu\text{g}/\text{ml}$  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 [ $^{14}\text{C}$ ]SAM into P-Etn. The incubation mixture contained 100 mM Hepes-KOH buffer, pH 8.6, 2 mM EDTA, 10% glycerol, 100  $\mu\text{M}$  P-Etn, 100  $\mu\text{M}$  SAM (100 nCi [ $^{14}\text{C}$ ]SAM), and 10  $\mu\text{g}$  of recombinant enzyme in a final volume of 100  $\mu\text{l}$ . The reaction was incubated for 30 min at 37°C and terminated by the addition of 1 ml of ice-cold  $\text{H}_2\text{O}$ . 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 [ $^{14}\text{C}$ ]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  $\mu\text{M}$  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  $\mu\text{M}$  to 1 mM). Similarly, the affinity of Pfpmt for its cosubstrate SAM was determined by using different concentrations of SAM (20  $\mu\text{M}$  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  $\mu\text{M}$ ) or miltefosine (0, 1, 10, 50, and 100  $\mu\text{M}$ ) or 50  $\mu\text{M}$  S-adenosyl-L-homocysteine to the reaction mixture containing 100  $\mu\text{M}$  P-Etn, 100  $\mu\text{M}$  SAM (100 nCi of [ $^{14}\text{C}$ ]SAM), and 10  $\mu\text{g}$  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  $\text{H}_2\text{O}$ . 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

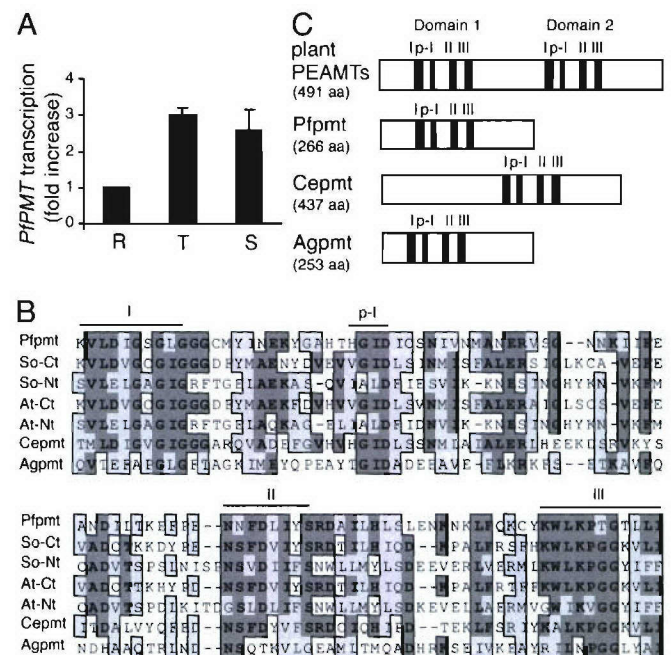


**Fig. 1.** Evidence for a PEAMT (*Pfpmt*) activity in *P. falciparum*. (A) TLC analysis of the extracted lipid (i) and aqueous (ii) phase after labeling of *P. falciparum* infected erythrocytes with [<sup>14</sup>C]-Etn. The identity of phospholipids (i) and aqueous compounds (ii) was determined by 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.

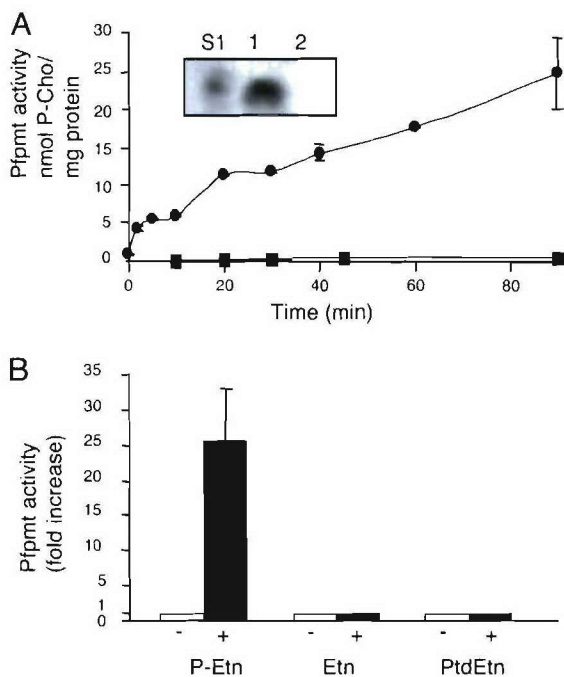
[<sup>14</sup>C]ethanolamine in *P. falciparum*-infected erythrocytes were performed in RPMI medium as described in *Materials and Methods*. After cell lysis, the lipid fraction and the water-soluble metabolites were analyzed by TLC. Analysis of the lipid fraction showed the formation of both PtdEtn 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 [ $^{14}$ C]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  $\text{nmol}\cdot\text{mg}^{-1}\cdot\text{min}^{-1}$  for both substrates.

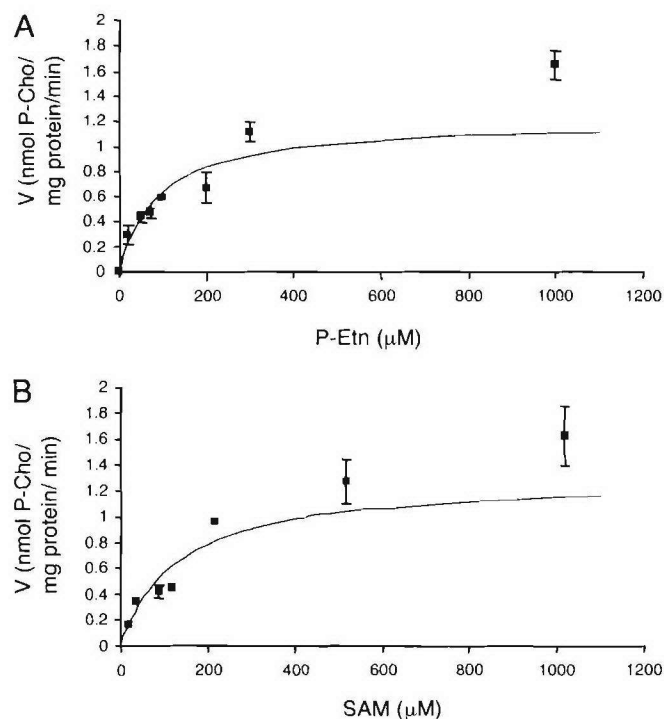
**Inhibition of Pfpmt Activity and *P. falciparum* Growth by P-Cho and Its Analog Miltefosine.** The finding that P-Etn is a substrate for Pfpmt, and knowing that this precursor is also a substrate for CDP-P-Etn transferase, which catalyzes the rate limiting step in the CDP-ethanolamine pathway, suggests 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, clofibric acid, an inhibitor of PtdEtn methyltransferases (34), had no effect on Pfpmt activity at concentrations up to 150  $\mu$ M (data not shown).

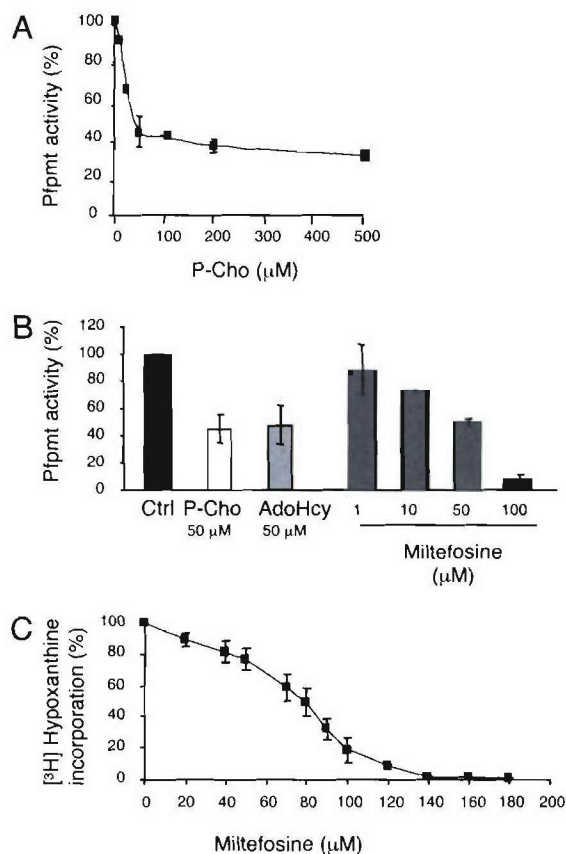
The finding that miltefosine inhibits Pfpmt activity prompted us to examine the antimalarial activity of this compound. Interestingly, when a [ $^3$ H]hypoxanthine incorporation assay was used to measure *P. falciparum* proliferation in human red blood cells, miltefosine was found to possess an antimalarial 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.

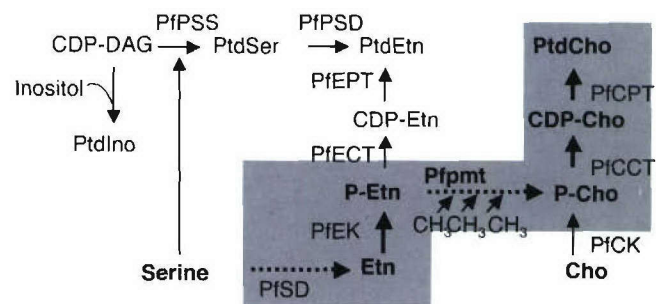


**Fig. 5.** Inhibition of recombinant Pfpmt activity and *P. falciparum* growth. (A) Inhibition of Pfpmt activity by P-Cho. Inhibition assay was performed by adding various concentrations of P-Cho (0–500 μM) as described in *Materials and Methods*. (B) Inhibition of Pfpmt activity by 50 μM P-Cho, 50 μM adenosyl-L-homocysteine (AdoHcy), and 1, 10, 50, and 100 μM miltefosine. Control, Pfpmt activity without inhibitor. (C) Effect of several concentrations of miltefosine on [<sup>3</sup>H]hypoxanthine incorporation in cultured parasites. Each value is the mean ± 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 [<sup>14</sup>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 [<sup>14</sup>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), *Pfpmmt* 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, *Pfpmmt* 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 *Pfpmmt* suggests that the single catalytic domain of *Pfpmmt* can either catalyze all of the three methylation reactions to convert P-Etn into P-Cho or that *Pfpmmt* dimerizes to compensate for the lack of the second domain. Interestingly, multiangle laser light scattering analysis of *Pfpmmt* 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 *Pfpmmt* (32). Like *Pfpmmt*, *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 *Pfpmmt*-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; PfEPT and PfCPT, ethanolamine and choline phosphotransferases; PfECT and PfCCT, P-Etn and P-Cho cytidyltransferases; PfCK and PfCCT, ethanolamine and choline kinases; 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 Pfpm1 activity is inhibited by its product P-Cho suggests that Pfpm1 might act as a sensor of phospholipid biosynthesis to determine the metabolic outcome of P-Etn. One possibility is that PtdCho accumulation inhibits Pfpm1 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 Pfpm1 activity. The inhibition of Pfpm1 activity by miltefosine was similar to that of S-adenosyl-L-homocysteine, a specific inhibitor of SAM-dependent reactions (Fig. 5B). Furthermore, clofibrate, an inhibitor of PtdEtn methyltransferases, had no effect on Pfpm1 activity. Together, these data suggest that the inhibition of Pfpm1 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 Pfpm1 into P-Cho, which serves as a precursor for the synthesis of PtdCho (Fig. 6).

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