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Crystal structure of phosphoethanolamine methyltransferase from *Plasmodium falciparum* in complex with amodiaquine

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Abstract

Phosphoethanolamine *N*-methyltransferase (PMT) is essential for phospholipid biogenesis in the malarial parasite *Plasmodium falciparum*. PfPMT catalyzes the triple methylation of phosphoethanolamine to produce phosphocholine, which is then used for phosphatidylcholine synthesis. Here we describe the 2.0 Å resolution x-ray crystal structure of PfPMT in complex with amodiaquine. To better characterize inhibition of PfPMT by amodiaquine, we determined the IC₅₀ values of a series of aminoquinolines using a direct radiochemical assay. Both structural and functional analyses provide a possible approach for the development of new small molecule inhibitors of PfPMT.

Keywords

Methyltransferase; *Plasmodium falciparum*; malaria; crystallography; aminoquinolines

Malaria is a major disease caused by the protozoan parasites of genus *Plasmodium* with an estimated 3.3 billion people at risk¹. *P. falciparum* causes the most severe disease and is predominant in Africa, where more than 90% of all malarial deaths were reported in 2010¹. Several conventional anti-malarials, such as chloroquine and sulphadoxine-pyrimethamine, have been extensively used because of their availability and low cost²; however, due to the increasing emergence of drug resistance, artemisinin-based combination therapies (ACTs) are recommended as a first-line therapy³. Although the efficacy of ACT remains high, resistance to artemisinin has also been reported⁴. With the threat of artemisinin resistance and rapid spread of resistant parasites, identification of new biochemical targets for therapeutics against *Plasmodium* is required.

Phosphoethanolamine *N*-methyltransferase (PMT) is an emerging biochemical target in *P. falciparum*^{5–6}. *Plasmodium* PMT (PfPMT) is an S-adenosylmethionine (SAM)-dependent methyltransferase that converts phosphoethanolamine (pEA) into phosphocholine (pCho)⁵. In the case of *Plasmodium*, rapid membrane biogenesis and phospholipid biosynthesis are required for growth and replication when it infects human erythrocytes^{7–8}. *In vivo* studies show that disruption of PfPMT causes severe defects in growth, reproduction, and viability

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Supplementary Material Figures S1–S4 showing the view of the crystallographically-related amodiaquine binding site (S1), IC₅₀ data (S2), docking of chloroquine and primaquine in PfPMT (S3), and a structural comparison of PfPMT and HNMT (S4) are included as supplementary material.

in *P. falciparum*, suggesting an important metabolic role for the phosphobase methylation pathway in the biosynthesis of phospholipids⁹⁻¹¹. Recent biochemical analyses of PMT from *Plasmodium* and nematodes provide insight on substrate recognition and the catalytic mechanism of these enzymes¹²⁻¹³. A series of high resolution x-ray crystal structures of PfPMT in complex with substrates, products, and the inhibitor sinefungin reveal the active site architecture of PfPMT¹³. Since PMT homologs are not found in mammals and are present only in *Plasmodium*, plants, nematodes, and some lower vertebrates, it is a potential anti-parasitic target^{5-6,14-16}. Biochemical studies show that sinefungin, a SAM analog, and the pCho analogs hexadecylphosphocholine (miltefosine) and hexadecyltrimethylammonium bromide inhibit PfPMT at micromolar concentrations^{5,12-13}. Recent studies suggest that amodiaquine (4-[(7-chloroquinolin-4-yl)amino]-2-[(diethylamino)methyl]phenol; Fig. 1A) may also be an inhibitor, although other related 4-aminoquinolines (i.e., chloroquine) are weak (i.e., millimolar) inhibitors of PfPMT¹⁷. To better understand amodiaquine inhibition of PfPMT, we determined the x-ray crystal structure of the enzyme in complex with S-adenosylhomocysteine (SAH) and amodiaquine¹⁸.

The crystal structure of PfPMT complexed with amodiaquine was determined by molecular replacement using the PfPMT•SAM•PO₄²⁻ structure¹³ with ligands removed as a search model (Table 1). Two PfPMT molecules are in an asymmetric unit and each monomer consists of 258 amino acid residues, SAH, PO₄²⁻, and two amodiaquine molecules with 40.5% solvent content. All the residues and ligands were clearly defined (Fig. 1B). PfPMT consists of a classic SAM-binding domain, which contains a central β -sheet (β 1- β 7) flanked by two α -helical regions (α 3/ α 4 and α 5/ α 7), and a characteristic α -helical “lid” domain (α 1, α 2, α 6, α 8, α 9, and α 10) critical for substrate binding in the active site (Fig. 1C). The solvent inaccessible active site suggests that the lid domain likely changes conformation to allow for substrate binding¹³. As previously shown for PfPMT, both SAH and PO₄²⁻ are bound in the active site. Interestingly, two amodiaquine molecules are found in the PfPMT monomer at two different binding sites on the protein surface (Fig. 1C).

The first amodiaquine molecule is bound to the outer surface of PfPMT at the interface of two crystallographically related monomers (Figs. 1C & 2A-B). The diethylamino group is oriented into the protein with the quinoline ring positioned toward solvent. The exposed quinoline group stacks with a crystallographically-related amodiaquine, which provides an additional interaction likely present only in the crystal because PfPMT is monomeric¹³ (Fig. S1). Within this binding pocket, amodiaquine makes extensive van der Waals contacts with multiple aromatic and hydrophobic amino acid residues (Phe29, Ile30, Phe31, Leu213, Try209, Val216, and Tyr220) and two neutral residues (Gly32 and Gly39) (Fig. 2A-B). In addition, Glu42 and Glu217 form part of the interaction site near the phenol ring of amodiaquine. The N1 nitrogen of the quinoline ring forms a water-mediated hydrogen bond with the hydroxyl group of Tyr209, and the oxygen atom of amodiaquine hydrogen bonds with the side-chain oxygen of Glu217 and backbone oxygen of Ile30. Overall, the predominant hydrophobic site combined with acidic region and stacking interactions with the crystallographic symmetry mate allow for binding of amodiaquine at this site in the crystal lattice (Fig. S1).

The second amodiaquine binding site is adjacent to the active site in a cleft that fits the quinoline ring and makes contacts across the ligand interaction surface (Figs. 1C & 2C-D). In this site, amodiaquine is positioned between residues in α 8 and α 10 and blocks an entrance to the phosphobase binding site. Glu171, Glu174, and Asp250 interact with the diethylamino and phenol portions of amodiaquine. The quinoline ring π -stacks with Tyr175. Additional van der Waals interactions occur between amodiaquine and Glu171, Glu174, Gln178, Gly243, and Arg246 (Fig. 2C-D). Moreover, the N1 nitrogen atom of amodiaquine

forms water-mediated hydrogen bonds with Asp250 and Gln256 (Fig. 2D). The network of polar and hydrophobic interactions retain the amodiaquine molecule in the cleft and orient the chlorine atom of amodiaquine toward the phosphobase binding site defined by the position of the phosphate molecule. The location of this amodiaquine binding site in proximity to the active site and the more extensive protein-ligand interactions in this site suggest this is the primary inhibition site.

The structure of PfPMT with amodiaquine bound shows relatively little structural difference (r.m.s. deviation of C α -atoms less than 0.5 Å²) compared to previously determined substrate, product, and inhibitor complexes (Fig. 3)¹⁴. There are slight shifts in the positions of the four α -helices (α 1, α 2, α 8, and α 10) near the phosphobase site and this amodiaquine binding site. The two largest changes in conformation occur in Arg179 and Arg246. Binding of amodiaquine moves the side-chain of Arg179 away from the bound phosphate, which opens access to the substrate site. The side-chain of Arg246 is completely displaced to provide a pocket for amodiaquine binding.

To characterize inhibition of PfPMT by selected aminoquinolines, we compared the effects of amodiaquine, chloroquine, and primaquine on enzymatic activity using a radiochemical assay that directly monitors product formation¹⁹ (Table 2; Fig. S2). In contrast to a previous report¹⁷, amodiaquine was a weak inhibitor of PfPMT (IC₅₀=1.35 mM). The difference in inhibition effects likely reflect the use of direct assay versus a multicomponent enzyme-coupled system that may be sensitive to amodiaquine. The poor inhibition of PfPMT by amodiaquine was comparable to the ~3 mM IC₅₀ values determined for the two PMT from the nematode parasite *Haemonchus contortus*¹². Chloroquine and primaquine were also millimolar inhibitors of PfPMT with IC₅₀ values that differed by less than 3-fold from that of amodiaquine. Efforts to determine binding affinities of amodiaquine, chloroquine, and primaquine using isothermal titration calorimetry were unsuccessful due to a lack of heat signal, suggesting poor binding affinities consistent with the weak inhibition observed with these compounds. Computational docking of amodiaquine to PfPMT identified the same sites as observed crystallographically (not shown). Similar docking results were obtained using chloroquine and primaquine (Fig. S3), which placed the quinoline moiety of each molecule into the binding cleft between α 8 and α 10. These results are consistent with binding of the aminoquinoline group in the second amodiaquine binding site of PfPMT as the structurally common feature of these molecules. Taken together, the effect on enzymatic activity, lack of binding in ITC experiments, and docking studies suggest that amodiaquine and other commonly used aminoquinolines weakly inhibit PfPMT.

Previous structural modeling and NMR titration studies of PfPMT suggested that Gly32 and Gly68 interacted with amodiaquine¹⁷. In the crystal structure of PfPMT with amodiaquine bound, Gly32 is positioned in the α 2/ α 3 loop near the first amodiaquine binding site and Gly68 is in α 4, which is adjacent to α 2. Based on structural comparison of the PfPMT structures (Fig. 3), we suggest that amodiaquine binding in the second site, which occupies a cleft between α 8 and α 10 near the active site, may affect movement of the lid domain during the reaction sequence and lead to weak inhibition¹³. Alteration of lid domain movement is also consistent with previous NMR titration experiments¹⁷.

Several methyltransferases, such as histamine *N*-methyltransferase (HNMT)²⁰ and catechol-*O*-methyltransferase²¹, are inhibited by aminoquinolines. Comparison of the structures of PfPMT and HNMT in complex with amodiaquine reveals a similar overall fold (r.m.s. deviation of C α -atoms is 3.7 Å²), even though the two proteins share only 13% sequence identity (Fig. S4). In the HNMT•amodiaquine structure, two inhibitor molecules were also bound to the N-terminal domain of the enzyme with one in the active site and the second in a deep pocket on the outer surface (Fig. S4). Both PfPMT and HNMT contain multiple

amodiaquine binding pockets, but the sites are not identical. Moreover, inhibition by amodiaquine differs significantly between PfPMT and HNMT, as this molecule inhibits HNMT with a $K_i = 19 \text{ nM}$ ¹⁹. Although amodiaquine inhibits each structurally-related methyltransferase, the binding locations of amodiaquine in PfPMT (surface site) and HNMT (active site) differ, as does inhibitor effectiveness. Overall, the structural and functional analysis of amodiaquine binding to PfPMT presented here suggests that aminoquinoline compounds are not strong candidates for inhibitor development targeting PfPMT; however, the proximity of the quinoline ring, especially the 7-chloro group, near the phosphobase binding site suggests that development of compounds extending into this site may offer a possible approach for the development of PfPMT inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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18. PfPMT was expressed and purified as previously described¹³. Amodiaquine was obtained from Sigma-Aldrich. PfPMT (7.0 mg mL^{-1}) was pre-incubated with 1.25 mM amodiaquine and 0.5 mM SAH for 2 h before crystallization. Crystals of PfPMT in complex with amodiaquine and SAH were grown by the vapor diffusion method in hanging-drops of a 1:1 mixture of protein and crystallization buffer (30% PEG-8000, 0.1 M sodium cacodylate, pH 6.5, 0.2 M sodium acetate, 5 mM β -mercaptoethanol, and 1.25 mM amodiaquine). Diffraction data were collected at beamline 19-ID of the Argonne National Lab Advanced Photon Source. HKL3000²² was used for indexing, integration, and scaling of diffraction data. The crystal structure of PfPMT•SAH• PO_4^{2-} •amodiaquine was determined by a molecular replacement using PHASER²³ and the native PfPMT•SAM• PO_4^{2-} structure¹³ (PDB: 3UJ7) with ligands removed as the search model. Manual model building and refinement were performed in COOT²⁴ and PHENIX²⁵, respectively. Data collection and refinement statistics are summarized in Table 1. Coordinates and structure factors for the PfPMT•SAH• PO_4^{2-} •amodiaquine complex have been deposited in the Protein Data Bank (4FGZ).
19. Enzyme assays were performed using a direct radiochemical assay for PMT activity¹³⁻¹⁴. Inhibition assays used the same assay under conditions described previously¹² with the determination of IC_{50} values of inhibitors performed at 150 μM SAM and 300 μM pEA. Concentrations of inhibitors were varied from 0-3 mM. Data were plotted as percentage of inhibition versus inhibitor concentration and were fit to $y = 100 / (1 + ([I]/\text{IC}_{50}))$ using Kaleidagraph (Synergy Software), where IC_{50} is the inhibitor concentration at 50% activity.
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Figure 1.

Structure of PfPMT in complex with amodiaquine. **(A)** Chemical structure of amodiaquine with the quinoline group indicated. **(B)** Electron density ($2F_o - F_c$ omit map contoured at 1.0σ) of amodiaquine in binding site 2 of PfPMT. Oxygen, nitrogen, and chloride atoms are shown in red, blue, and green, respectively. **(C)** Ribbon diagram of the PfPMT•SAH•PO₄²⁻•amodiaquine complex with α -helices and β -strands colored gold and blue, respectively. Green stick molecules indicate SAH in the SAM/SAH binding site and PO₄²⁻ in the phosphobase binding site. Pink stick molecules indicate amodiaquine molecule in each binding site.

Figure 2.

View of the amodiaquine binding sites in PfPMT. (A) Surface view of amodiaquine binding site 1. PfPMT is shown as a surface rendering. Surfaces for acidic residues (Glu42 and Glu217) and hydrophobic residues (Phe29, Ile30, Phe31, Tyr209, Leu213, Val216, and Tyr220) are highlighted in red and gold, respectively. (B) Residues in amodiaquine binding site 1. Side-chains for residues in the site are shown as stick drawings. Two water molecules that mediate hydrogen bonds between the ligand and protein are shown as red spheres. (C) Surface view of amodiaquine binding site 2. Surfaces for acidic residues (Glu171 and Glu174) and Tyr175 are highlighted in red and gold, respectively. (D) Residues in amodiaquine binding site 2. Side-chains for residues in the site are shown as stick drawings. The water molecule that mediate hydrogen bonds between the ligand and protein is shown as a red sphere.

Figure 3.
Structure comparison of PfPMT in the presence (white) and absence (gold) of amodiaquine. The region around amodiaquine binding site 2, which is adjacent to the active site is shown.

Table 1

Summary of Crystallographic Data

<u>Crystal</u>	
Space group	P2
Cell dimensions	$a = 66.05 \text{ \AA}$, $b = 60.64 \text{ \AA}$, $c = 68.15 \text{ \AA}$
<u>Data Collection</u>	
Wavelength ($\text{\AA}$)	0.979
Resolution ($\text{\AA}$) (highest shell)	34.1 - 1.99 (2.02 - 1.99)
Reflections (total / unique)	122,156 / 34,139
Completeness (highest shell)	92.4% (65.8%)
$\langle I/\sigma \rangle$ (highest shell)	19.7 (2.0)
R_{sym} (highest shell)	6.7 (35.8%)
<u>Refinement</u>	
$R_{\text{cryst}} / R_{\text{free}}$	18.1% / 22.7%
Protein atoms	4,338
Water molecules	191
Ligand atoms	162
R.m.s.d., bond lengths ($\text{\AA}$)	0.007
R.m.s.d., bond angles ($^{\circ}$)	1.007
Avg. B factor ($\text{\AA}^2$) - protein, water, ligand	52.9, 49.4, 44.5
Stereochemistry: favored, allowed	98.1, 1.9%

Table 2

Analysis of PfPMT Inhibitors

Inhibitor	Chemical Structure	IC ₅₀ values
Amodiaquine		1350 ± 43 μM
Chloroquine		554 ± 35 μM
Primaquine		460 ± 11 μM