Origins of the specificity of inhibitor P218 toward wild-type and mutant PfDHFR: a molecular dynamics analysis

Sheenu Abbat\textsuperscript{a}, Vaibhav Jain\textsuperscript{a} and Prasad V. Bharatam\textsuperscript{b*}

\textsuperscript{a}Department of Pharmacoinformatics, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar, Punjab 160 062, India; \textsuperscript{b}Department of Medicinal Chemistry, National Institute of Pharmaceutical Education and Research, Sector 67, S.A.S. Nagar, Punjab 160 062, India

Communicated by Ramaswamy H. Sarma

(Received 6 July 2014; accepted 17 October 2014)

Molecular dynamics simulations were performed to evaluate the origin of the antimalarial effect of the lead compound P218. The simulations of the ligand in the cavities of wild-type, mutant \textit{Plasmodium falciparum} Dihydrofolate Reductase (PfDHFR) and the human DHFR revealed the differences in the atomic-level interactions and also provided explanation for the specificity of this ligand toward PfDHFR. The binding free energy estimation using Molecular Mechanics Poisson-Boltzmann Surface Area method revealed that P218 has higher binding affinity (\textasciitilde 30 to \textasciitilde 35 kcal/mol) toward PfDHFR (both in wild-type and mutant forms) than human DHFR (\textasciitilde 22 kcal/mol), corroborating the experimental observations. Intermolecular hydrogen bonding analysis of the trajectories showed that P218 formed two stable hydrogen bonds with human DHFR (Ile7 and Glu30), wild-type and double-mutant PfDHFR's (Asp54 and Arg122), while it formed three stable hydrogen bonds with quadruple-mutant PfDHFR (Asp54, Arg59, and Arg122). Additionally, P218 binding in PfDHFR is stabilized by hydrogen bonds with residues Ile14 and Ile164. It was found that mutant residues do not reduce the binding affinity of P218 to PfDHFR, in contrast, Cys59Arg mutation strongly favors inhibitor binding to quadruple-mutant PfDHFR. The atomistic-level details explored in this work will be highly useful for the design of non-resistant novel PfDHFR inhibitors as antimalarial agents.

Keywords: \textit{Plasmodium falciparum} DHFR; human DHFR; molecular docking; molecular dynamics; molecular recognition interactions; MM-PBSA; normal mode analysis

Introduction

Malaria is an important cause of death and illness in children and adults in the tropical and sub-tropical countries, causing 300–500 million clinical cases and 1.2 million deaths annually (Murray et al., 2012). Many malaria-control strategies exist, but none are appropriate and affordable in all contexts. Resistance to antimalarials has been observed for all currently used antimalarials (amodiaquine, chloroquine, mefloquine, quinine, and sulfadoxine-pyrimethamine, cycloguanil) and more recently, in artemisinin derivatives (Dondorp et al., 2009; Gregson & Plowe, 2005). The development and spread of drug-resistant strains of malaria parasites have been identified as a key factor in this resurgence and is one of the greatest challenges for the design of an effective antimalarial compound.

\textit{Plasmodium falciparum} Dihydrofolate Reductase (PfDHFR) is a well-known target for combating malaria. Its function is to catalyze the biochemical reaction which involves the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF), using the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Hence, it plays a key role in the folate biosynthesis pathway and is responsible for the generation of the DNA base, deoxythymidine monophosphate. A difference between host (human) and parasite (\textit{P. falciparum}) DHFR active sites was reported and this difference offers opportunities for the design of selectively binding drugs (Nirmalan, Sims, & Hyde, 2004; Zhang & Rathod, 2002). Two enzymes of the parasite (DHFR and TS (Thymidylate Synthase)) reside on the same polypeptide chain as a DHFR-TS bifunctional protein (\textit{P. falciparum} DHFR-TS). As demonstrated by X-ray crystallographic structure of \textit{P. falciparum} DHFR-TS, it contains 608 amino acids, the first 231 comprising the DHFR domain, the next 89 residues forming the junction region, which joins the remaining 288 residues of the TS domain (Ivanetich & Santi, 1990).

Antifolate drugs known to inhibit the normal function of PfDHFR are Pyrimethamine (Pyr) and Cycloguanil (Cyc) (Figure 1). The triazine proguanil was discovered in the mid 1940s as an effective antimalarial compound (Curd, Davey, & Rose, 1945). Proguanil is converted \textit{in vivo} to its active metabolite, cycloguanil, which

*Corresponding author. Email: pvbharatam@niper.ac.in

© 2014 Taylor & Francis
selectively binds to and inhibits PfDHFR (Carrington, Crowther, Davey, Levi, & Rose, 1951). Recently, quantum chemical method has been employed to study the possible mechanisms for cytochrome-mediated Proguanil to cycloguanil conversion (Arfeen, Patel, Abbat, Taxak, & Bharatam, 2014). Pyrimethamine is also a potent and selective inhibitor of PfDHFR, and has been used as an antimalarial agent since 1952. Genetic mutations in the active site of PfDHFR at positions, Ala16Val, Ser108-Asn, Cys59Arg, Asn51Ile, and Ile164Leu have considerably decreased the therapeutic activities of these antifolates (Bras & Durand, 2003; Gregson & Plowe, 2005; Peterson, Milhous, & Wellems, 1990; Peterson, Walliker, & Wellems, 1988; Rastelli et al., 2000; Vanichtanankul et al., 2012; Yuvaniyama et al., 2003). Later, a dihydrotriazine derivative WR99210 (Figure 1) was developed which showed activity in wild as well as double and quadruple-mutant PfDHFR. The flexible side chain of WR99210 enables it to adopt a conformation that can fit into the active site modified by the Ser108Asn mutation (Nzila, 2006; Rastelli et al., 2000). Unfortunately, low bioavailability and inadequate pharmacokinetic profile of WR99210, limits its use in malaria chemotherapy (Rieckmann, Yeo, & Edstein, 1951). All these known antifolates have common structural features which makes essential molecular recognition interactions with the PfDHFR active site residues, to inhibit its activity. The important molecular recognition centers of PfDHFR for these known antifolates include residues Ile14, Asp54, and Ile/Leu164. The carboxylate oxygen atoms of Asp54 form two hydrogen bonds with WR99210 (Figure S1, Supplementary material), Cys, and Pyr. The backbone oxygen atoms of Ile14 and Ile164 also form H-bond with the amino group of antifolates in the active site of PfDHFR.

Several efforts have been made to discover novel potent PfDHFR inhibitors by chemical modifications of available drugs as well as by designing new antimalarial lead molecules. Modifications in the structure of known antifolates resulted in several compounds with improved activity (McKie et al., 1998; Rastelli et al., 2000; Sardarian et al., 2003). In the past, our research group has developed shape- and chemical feature-based 3D-pharmacophore model based on the bioactive conformation of WR99210 and a common feature-based pharmacophore model from a set of 24 compounds active against quadruple-mutant PfDHFR and carried out virtual screening to identify potential PfDHFR inhibitors (Adane, Bharatam, & Sharma, 2010; Adane, Patel, & Bharatam, 2010). Further, by using molecular docking approach, 2,4-diaminoquinazoline and 2,4-diaminopteridine analogs were reported as potential inhibitors of PfDHFR (Adane & Bharatam, 2010). Multiple computational approaches like ab initio molecular orbital and density functional theory calculations, along with the molecular electrostatic potential analysis, and molecular docking were also utilized by our research group for the design of 1H-imidazole-2,4-diamine derivatives as potential inhibitors of PfDHFR enzyme (Adane & Bharatam, 2011). Many scientific groups have also identified many novel classes of PfDHFR inhibitors such as 2,4-diaminopyrimidines (Falco, Goodwin, Hitchings, Rollo, & Russell, 1951), 2-amino-1,4-dihydro-4,4,7,8-tetramethyl-s-triazino(1,2-a)benzimidazole and Trp-P-2 series (Toyoda et al., 1997), 5-Benzyl-2,4-diamonopyrimidines (Sirichaivat et al., 2004), novel biguanide analogs (RJF001302, RJF00670 and RJF00719) (Dasgupta et al., 2009), 2-methyl-6-ureido-4-quinolinamides (Madapa et al., 2009), 4-anilinoquinoline triazines (Kumar et al., 2011), 1,2,3-Triazole tethered β-lactam and 7-chloroquinoline bifunctional hybrids (Singh, Singh et al., 2012), hybrid phenylthiazolyl-1,3,5-triazine derivatives (Gahtori et al., 2012), 4-aminopyrimidine and 1,3,5-triazine derivatives (Bhat, Ghosh, Prakash, Gogoi, & Singh, 2012; Bhat et al., 2013), and 2-Aminopyrimidine-based 4-aminopyrimidine antiplasmodial agents (Singh, Kaur et al., 2012). More recently, our research group has implemented computer-aided methods for the design of

Figure 1. Structures of cycloguanil, pyrimethamine, WR99210, guanylthiourea, and P218. The atom numbering for P218 atoms is used to define H-bond analysis later in this article.
new class of S-benzylated guanyliothiourea compounds (Figure 1), which were synthesized later and tested for their biological activity. Two compounds from this class of series were found to be active with the EC$_{50}$ values of 100 μM and 400 nM against wild-type PfDHFR (Adane et al., 2014).

Quantitative structure–activity relationship (QSAR) studies have also been extensively carried out on these known antifolates and their derivatives to identify structural features responsible for the differences in anti-plasmodial activities with respect to their electrostatic, steric, and hydrophobic nature (Adane & Bharatam, 2009; Basak & Mills, 2010; Basak, Mills, & Hawkins, 2011; Hecht, Cheung, & Fogel, 2008; Maitarad, Kamchonwongpaisan, et al., 2009; Maitarad, Saparpakorn, et al., 2009; Ojha & Roy, 2011; Santos-Filho & Hopfinger, 2001; Santos-Filho, Mishra, & Hopfinger, 2001; Sivaprakasam, Tosso, & Doerksen, 2009). The studies reported slightly different binding alignment of the ligands to the mutant form of an enzyme as compared to the wild-type form. Asn108 of the mutant enzyme was found to cause steric clash with Pyr and Cyc and is considered as the cause of resistance to these drugs.

Recently, structure-based drug design approach was adopted to discover an antimalarial compound P218, currently in its preclinical trial (Figure 1); to be a selective and efficient inhibitor of both wild-type and quadruplet-mutant form of PfDHFR (Yuthavong et al., 2012). P218 is designed to have basic functional groups required for an inhibitor to bind in PfDHFR cavity as well as it includes pyrimidine side chain flexibility and a carboxylate group which can make hydrogen bonds with conserved Arg122 (similar to natural substrate DHF). Sequence and X-ray crystal structure analysis showed that Met55, Cys/Arg59, and Phe116 in PfDHFR are replaced at structurally equivalent positions in human DHFR by Phe31, Gln35, and Asn64 which causes significant difference in the active sites of human DHFR (hDHFR) and PfDHFR (Yuthavong et al., 2012). These differences in the active sites of PfDHFR and hDHFR lead to the loss of P218 interaction with Arg70 (a conserved residue in hDHFR), as observed from the X-ray crystal structure of hDHFR. The selectivity of P218 for PfDHFR is clear from the significant difference in experimentally determined binding constant values of P218 for PfDHFR (wild-type 51 ± 03 nM and quadruplet-mutant 53 ± 13 nM) and hDHFR (2841 ± 319 nM) (Yuthavong et al., 2012). P218 is reported to have in vitro and in vivo antimalarial activity against wild-type and pyrimethamine-resistant PfDHFR, as well as it is reported to possess suitable pharmacological, metabolic, and safety profiles for its consideration for clinical development (Yuthavong et al., 2012).

Structural details of PfDHFR became available for the past decade and provided clues for the interaction between the catalytic site of an enzyme and also the clues regarding the inhibition as well as the reasons for resistance by the mutated enzyme. As mentioned above, many studies using computer-aided methods have been carried out to understand the enzyme–inhibitor interaction in the past one decade. All these efforts (X-ray, molecular docking, and QSAR) are under static structural conditions. Only two reports which involved the dynamics of enzyme–inhibitor interaction appeared. In 2000, Rastelli et al. built the homology models of wild-type and mutant PfDHFR and carried out molecular docking of Pyr, Cyc, and WR99210 and performed molecular dynamics (MD) to study the enzyme–inhibitor interactions (Rastelli et al., 2000). More recently, MD studies were carried out to evaluate the flexible environment of PfDHFR (Mokmak et al., 2014). The authors observed that the flexibility given to the enzyme during dynamical conditions helped in elucidating the binding patterns of drug (Pyr) and the lead (WR99210) in the active sites of wild-type and quadruple-mutant PfDHFR. Similarly, the dynamical environment of enzyme needs to be taken into account to understand the behavior of various inhibitors and the origin of specificity of the inhibitors and the origin of resistance to these inhibitors. Particularly, the recently developed lead compound P218 and the origin of specificity of this toward wild-type and mutant PfDHFR as against hDHFR is required to be explored. Such study becomes important in the wake of the fact that no new antifolate was introduced in the recent past despite extensive studies. This information gain might help researchers to design potent and specific inhibitors of PfDHFR. Hence, present computational study explores the binding mode and interaction profile of P218 to PfDHFR (wild and mutant) and human DHFR using MD simulations approach, which could be helpful for the design and optimization of novel antimalarials.

Methods

Protein structure preparation

The X-ray crystallographic structures of wild-type Pf/DHFR (wtP/DHFR, protein data bank (PDB) ID: 4DPD, co-crystallized ligand: DHF), quadruple-mutant Pf/DHFR (qmP/DHFR, PDB ID: 4DP3, co-crystallized ligand: P218), double-mutant Pf/DHFR (dm1P/DHFR, PDB ID: 1J3J, co-crystallized ligand: Pyr), and double-mutant Pf/DHFR (dm2P/DHFR, PDB ID: 3UM5, co-crystallized ligand: Pyr) enzymes for this molecular modeling exercise were obtained from RCSB protein databank (www.rcsb.org). Only DHFR domain (1–231 amino acids) of chain-A (having bound ligand in its active site) was considered, for both wild and mutant enzymes and water coordinates were removed. The missing residues in the chain-A for wtP/DHFR (residue numbers 22–29 and 84–96); qmP/DHFR (residue
numbers 86–95; dm1P/DHFR (residue numbers 86–95); and dm2P/DHFR (residue numbers 86–95) were identified. For a successful dynamics run, it is necessary to add these missing residues. Modeller9v7 (Fiser & Šali, 2003; Šali & Blundell, 1993) software was used to add these missing residues. In order to add missing residues, sequence of P/DHFR enzyme in FASTA format was obtained from Universal Protein Resource. The FASTA file was used as input in the alignment script of modeller9v7 and an alignment file was created. Then the script for adding missing residues was run and the 3D model with the added residues was generated from alignment information. The model was then validated by comparing the root mean square deviation (RMSD) of models with the corresponding crystal structures. The all atom RMSD values for wtP/DHFR (.09 Å), qmP/DHFR (.05 Å), dm1P/DHFR (.03 Å), and dm2P/DHFR (.01 Å) as compared to their corresponding crystal structures were found to be in acceptable range which signify that the model did not vary much as compared to crystal structure. Further, protein preparation of the hDHFR (X-ray crystal structure) and of P/DHFR (missing residues added structures) was done using Maestro (Maestro, 2011). Hydrogen atoms were added during protein preparation wizard. Receptor Grid Generation Panel within Glide suite (Glide, 2011) was used to set up receptor grid for the prepared structures. The grid was defined by 10 Å inner-box and 30 Å outer-box on each side of the centroid of bound ligand. The centroid of the grid box was same as the centroid of the bound ligand. The OPLS_2005 force field was employed for the grid generation.

**Molecular docking**

The 3D structures of P218 (bound ligand in X-ray crystal structures of hDHFR and qmP/DHFR), DHF (bound ligand in X-ray crystal structure of wtP/DHFR) and pyrimethamine (bound ligand in X-ray crystal structure of dm1P/DHFR and dm2P/DHFR) were built using sketch module of SYBYL7.1 and subjected to 1000 cycle minimization with standard Tripos force field and .005 kcal/mol energy gradient convergence criterion using Powell’s method (Powell, 1964). The 3D structure of P218 built as above adopts a linear structure whereas its bioactive conformation (PDB ID: 4DP3) adopts a bent shape bringing the C_2H_2 and C_6H_4 groups into close proximity (Figure S2, Supplementary material). Optimization of the bioactive conformation leads to a macrocyclic structure involving two intramolecular hydrogen bonds between carboxylic group and diamino pyrimidine ring, in a zwitterionic state. Further, conformational analysis using molecular mechanics-based systematic search followed by quantum chemical analysis showed that P218 adopts two macrocyclic arrangements: chair and boat. The macrocyclic boat structure is more stable than the macrocyclic chair structure by 3.15 kcal/mol (see Supplementary material Figure S3 for further details). Three-dimensional structures of these compounds were then prepared using LigPrep module of maestro (LigPrep, 2011) implementing OPLS_2005 force field and ionic states (carboxylates) for the ligands at pH values of 7.0 ± 2.0 were generated. Docking was performed using Glide software (Grid-based Ligand Docking with Energetics), (Glide, 2011) with the standard precision (SP) mode to estimate protein–ligand binding affinities and static intermolecular interactions (Friesner et al., 2004, 2006). Initially, to check the reliability of docking protocol, the bound ligands (as given in X-ray crystal structures) were redocked in the active sites of respective enzymes and RMSD values of the docked poses were compared to the X-ray crystallographic conformation of the ligand. After the validation of docking protocol, P218 was docked flexibly into the active site of the wtP/DHFR, dm1P/DHFR, and dm2P/DHFR enzymes. A maximum of 10 docking poses per ligand were generated in each case and analyzed further for the binding mode and intermolecular interactions.

**MD simulations**

MD simulations were performed to get detailed insight into the stability of hDHFR-P218 and P/DHFR-P218 complexes (both wild-type and mutants), intermolecular interactions of ligands in dynamical conditions, and to estimate the binding free energy of P218 in all the complexes. Full atomistic MD simulations were performed at the molecular mechanics level using the Amber 11 program package with the AMBERff99SB force field (Case et al., 2005, 2010). For the purpose of MD simulations, X-ray co-crystal structures of hDHFR (PDB ID: 4DDR) and qmP/DHFR (PDB ID: 4DP3) and the best docking poses of P218 in wtP/DHFR (PDB ID: 4DP3), dm1P/DHFR (PDB ID: 1J3J), and dm2P/DHFR (PDB ID: 3UM5) were selected. Enzyme–ligand complex structures were solvated in a truncated octahedral box of TIP3P water model that extended up to 12 Å in each direction of the solute. To calculate the long-range electrostatic interactions, the cut-off distance was kept at 12 Å and they were treated using the Particle-Mesh-Ewald method. The appropriate ionization states of all the amino acids were taken into consideration during the simulation. Before performing MD simulations, the systems were relaxed by a series of steepest descent (SD) and conjugate gradient (CG) minimizations. The minimized system was then gradually heated from 0 to 300 K for about 50 ps under constant volume–constant temperature (NVT) conditions, while keeping the solute atoms fixed by a weak harmonic restraint of 2 kcal/mol/Å². Then, 200 ps density equilibration with the same
restraints on the solute was carried out. Subsequently, constant pressure equilibrium (NPT) for 2 ns at 300 K was carried out (relaxing the restraints), followed by the production run for 35 ns at 300 K under periodic boundary conditions. Isotropic position scaling was used to maintain the pressure (NPT = 1) and a constant pressure periodic boundary with an average pressure of 1 atm (PRES0) and a relaxation time of 2 ps were used (TAUp = 2.0). The five complexes in the study were large, especially when the water molecules were added to solvate the complex. For reasons of computational time and resource use, the simulation for each of the complexes was limited to 35 ns to explore the binding difference of P218 in the studied enzyme systems. Verlet algorithm (Verlet, 1967) was used to solve the equations of motion, with an integration time step of 2 fs and the trajectory was recorded at 2 ps interval. SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1977) was used to constrain all the covalent bonds containing hydrogen atoms. Langevin thermostat (NTT = 3) was used to maintain the temperature of the system at 300 K.

**MM-PBSA calculation and normal mode analysis**

The binding free energy between the enzyme and P218 was estimated using the Molecular Mechanics-Poisson Boltzmann Surface Area (MM-PBSA) approach incorporated in AMBER software. MM-PBSA calculations were performed on the last 5 ns trajectories of the total simulation run (35 ns). By using snapshot frequency of 2, total 1250 representative snapshots were extracted and the binding energies were calculated by running the python script in parallel (eight processors) (Miller et al., 2012). MM-PBSA analysis was performed to evaluate the binding affinity of the ligand toward hDHFR and PfDHFR (wild-type and mutants), which corresponds only to the enthalpy contributions of binding free energy. Hence, the binding energy value obtained from MM-PBSA analysis is not the real binding free energy since entropy contribution (unfavorable) to binding is not estimated. Normal mode analysis was carried out to calculate the binding entropies of the ligands. The average of the results from normal modes for the complex, receptor and ligand, was carried out to obtain an estimate of the binding entropy using SANDER module of AMBER. Since these calculations are computationally expensive, only 100 snapshots from last 5 ns simulation run were selected for the binding entropy (TAS) calculation of the ligands. The binding free energy was estimated by taking into account enthalpy (from MM-PBSA calculations) and entropy (from normal mode analysis) contributions to binding free energy.

**Results and discussion**

**Multiple sequence alignment**

Multiple sequence alignment of human DHFR (hDHFR), wild-type P/DHFR (wt/P/DHFR) quadruple-mutant P/DHFR (qm/P/DHFR; Asn51Ile, Cys59Arg, Ser108Asn, and Ile164Leu) double-mutant P/DHFR (dm1/P/DHFR; Cys59Arg and Ser108Asn), and double-mutant P/DHFR (dm2/P/DHFR; Ala16Val and Ser108Thr) was carried out using ClustalX (Jeanmougin, Thompson, Gouy, Higgins, & Gibson, 1998; Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997). The sequence alignment was carried out for the DHFR domain (231 residues) of the bifunctional DHFR-TS enzyme. This analysis was carried out to study the degrees of similarity and identity among hDHFR and P/DHFR and also to identify structurally homologous residues in hDHFR corresponding to P/DHFR. The alignment results show that hDHFR and wt/P/DHFR share 30% identity and the regions (highlighted in box) of non-conservative amino acid differences in hDHFR as compared to P/DHFR which are shown in Figure 2. It has also been reported that the active site residues Met55, Cys/Arg59, and Phe116 in P/DHFR are substituted by non-conservative amino acids Phe31, Gln35, and Asn64 in the active site of hDHFR (highlighted with #, Figure 2). These amino acids changeover is reported to lead significant change in the active site among the host and parasite DHFR, such that P218 binds differently to hDHFR and P/DHFR (Yuthavong et al., 2012).

**Molecular docking analysis**

To study the binding mode and interactions of P218 with human and P/DHFR (wild-type and mutants), the protein–ligand complex having P218 bound in their active site was required. X-ray crystal structure for P218-hDHFR and P218-qm/P/DHFR complexes is reported in PDB. Initially, to validate the docking exercise, P218 was redocked in the active site of hDHFR and qm/P/DHFR, to calculate its RMSD with respect to pose observed in X-ray crystallography (Figure S4(A)–(B), Supplementary material). The calculated RMSD for hDHFR (31 Å) and qm/P/DHFR (29 Å) was found to be within acceptable range. P218 binding mode in hDHFR and qm/P/DHFR was further analyzed to identify the key molecular recognition interactions required for P218 to bind in the active site of these enzymes. It was found that diaminopyrimidine ring of P218 interacts with Ile7 and Glu30 in hDHFR (Figure 3(A) and (B)), whereas in qm/P/DHFR, diaminopyrimidine ring forms hydrogen bonds with Ile14, Asp54, and Leu164 (Figure 3(C)). The terminal α-carboxylic group in qm/P/DHFR forms hydrogen bonds with Arg122 and Arg59, similar interaction of α-carboxylic group with hDHFR residue Arg70.
The structural homolog of Arg122 in PfDHFR does not occur in X-ray crystal structure (Figure 3(A)) as well as in the docked pose of P218 in the active site of PfDHFR (Figure 3(B)). P218 docked in the active site of PfDHFR was found to show hydrogen bonding interaction (3.2 Å) between α-carboxylic group and Asn64.

After validation of docking protocol, P218 was docked in the active site of wild-type P7DHFR and double-mutant P7DHFR structures (dm1/P7DHFR and dm2/P7DHFR) using GLIDE, molecular docking software. Docking pose analysis was carried out to examine the binding mode and key protein–ligand interactions.
and the pose with high docking score and which showed key molecular recognition interactions was considered for further studies. The diaminopyrimidine ring of P218 docked in the active site of wtPfDHFR, dm1PfDHFR and dm2PfDHFR showed hydrogen bond interaction with Ile14, Asp54, Ile164, and also α-carboxylic group of P218 formed hydrogen bonds with Arg122 (Figure 3(D)–(F)).

To study the structural features that lead to the selectivity of P218 for PfDHFR and also to study the affect of PfDHFR mutations on P218 binding, all the above-mentioned five complexes were superimposed. From the superposition of X-ray crystal structures of P218 in the active sites of hDHFR and qmPfDHFR and docked poses of P218 in the active-sites of wtPfDHFR, dm1PfDHFR, and dm2PfDHFR (Figure 4), it can be realized that P218 binding mode in hDHFR and PfDHFR is significantly different. The terminal carboxylic group of P218 makes hydrogen bond with Arg122 in the case of PfDHFR but it does not form hydrogen bond with Arg70 (structural homolog of Arg122 in P/DHFR) in hDHFR. The docking results also suggested that P218 binding to various forms of PfDHFR is not affected by existing mutations, as P218 binds in a similar fashion to wild-type and mutant PfDHFR (double and quadruple-mutant PfDHFR). To further analyze whether the interactions observed in all the five complexes were stable over a period of time, MD analysis was carried out.

**MD analysis**

Five different enzyme–ligand structures (i) X-ray crystal structure of human DHFR (PDB ID: 4DDR), (ii) X-ray crystal structure of quadruple-mutant PfDHFR (PDB ID: 4DP3), (iii) docked complex of wild-type PfDHFR, (iv) docked complex of double-mutant PfDHFR (dm1PfDHFR), and (v) docked complex of double-mutant PfDHFR (dm2PfDHFR), all carrying NADPH as the cofactor and P218 as the bound ligand in their respective sites were used for the MD analysis. MD simulations were carried out for 35 ns to ensure that the equilibration state of the complex lasted long enough for further hydrogen bonding and binding energy analysis. The convergence of all the simulations was analyzed in terms of the energy components, B-factor analysis, and RMSD from the initial X-ray crystal structure.

Initially, the stability of the complexes was evaluated by analyzing the kinetic energy, potential energy, and total energy for all the five complexes during the entire simulation run of 35 ns (Figure S5, Supplementary material). The energy plots reveal that the changes in total energy, potential energy, and kinetic energy were smooth. The stability of all the five complexes was also analyzed by studying the complex RMSD values of the backbone atoms (Figure 5(A)) relative to those in the starting structure. The trajectory analysis in Figure 5(A) shows that all the complexes attain equilibrium after 10 ns with acceptable RMSD fluctuations (<2 Å). From the RMSD plot of the ligand P218 (Figure 5(B)), it can be inferred that P218 binds stably (has very low flexibility) in all the complexes.

**P218 binding energies**

MM-PBSA and normal mode analysis was performed to evaluate the binding free energies (ΔGbind) of P218 in all the above-mentioned complexes (Table 1). The experimental binding constants K_i for P218 in hDHFR,
be more than estimated total binding free energy calculated from above terms. Overall, from the Δ of P218 for all the dm1/Pf DHFR, whereas a decrease in binding affinity of P218 can bind selectively and efficiently to P218 binding to human DHFR and envisaged that there is a significant difference between P218 binding to human DHFR and P218 with wt/Pf DHFR, qm/Pf DHFR, and dm2/Pf DHFR are almost comparable (−34.90, −34.15 and −34.93 kcal/mol, respectively), while increase in binding energy of P218 with dm1/Pf DHFR (−29.53 kcal/mol) was noticed. In contrary, ΔGbind value of P218 in hDHFR was −22.14 kcal/mol. The difference between the overall binding energy for hDHFR as compared to wt/Pf DHFR (12.76 kcal/mol), qm/Pf DHFR (12.01 kcal/mol), dm1/Pf DHFR (7.39 kcal/mol), and dm2/Pf DHFR (12.79 kcal/mol) is significantly high. As a result of this binding energy difference between hDHFR and P218, P218 can bind selectively and efficiently to P218 and prevent it from executing its function of DNA synthesis in wild-type P218 and mutant P218 DHFR and subsequently kill the parasite, but does not affect the same process in human DHFR.

Table 1. Binding free energies along with the different energy components for P218 complexed with the five enzyme structures of hDHFR, wt/Pf DHFR, qm/Pf DHFR, dm1/Pf DHFR, and dm2/Pf DHFR. All values are given in kcal/mol.

| Method | Contribution | hDHFR | wt/Pf DHFR | qm/Pf DHFR | dm1/Pf DHFR | dm2/Pf DHFR |
|--------|--------------|-------|------------|------------|-------------|-------------|
| K_i (nM) | 2841 ± 319 | 51 ± 0.3 | 53 ± 0.13 | – | – | – |
| MM VDW | −45.36 | −49.06 | −40.29 | −47.79 | −46.78 | – |
| ELE | −131.46 | −185.44 | −242.81 | −208.07 | −178.86 | – |
| GAS | −176.82 | −234.50 | −283.10 | −255.86 | −225.65 | – |
| PBSUR | −3.59 | −3.75 | −3.59 | −3.68 | −3.76 | – |
| EPB | 132.68 | 178.08 | 226.96 | 204.38 | 169.17 | – |
| PBELE | 1.22 | −7.36 | −15.85 | −3.69 | −9.69 | – |
| PBSOL | 129.07 | 174.33 | 223.37 | 200.70 | 165.41 | – |
| PBTOT | −47.75 ± 4.14 | −60.17 ± 4.69 | −59.73 ± 5.49 | −55.16 ± 5.94 | −60.24 ± 4.19 | – |
| −ΔTΔS | 25.61 ± 3.35 | 25.27 ± 2.37 | 25.58 ± 2.79 | 25.63 ± 2.44 | 25.31 ± 2.59 | – |
| ΔGbind | −22.14 ± 7.49 | −34.90 ± 7.06 | −34.15 ± 8.28 | −29.53 ± 8.38 | −34.93 ± 6.78 | – |

Abbreviations: K_i experimental binding constant; VDW, VDW contribution from MM force field; ELE, electrostatic energy as calculated by the MM force field; EPB, electrostatic contribution to the solvation free energy calculated by PB; GAS, sum of VDW and ELE; PBSUR, non-polar contribution to the solvation free energy calculated by the empirical models; PBELE, sum of ELE and electrostatic contribution to solvation free energy; PBSOL, sum of PBSUR, and EPB; PBTOT, total binding energy calculated by MM-PBSA; ΔTΔS, binding entropy calculated by normal mode analysis; ΔGbind, estimated total binding free energy calculated from above terms.

Figure 5. RMSD values for human DHFR (hDHFR), wild-type P/Pf DHFR, (wt/Pf DHFR), and mutant P/Pf DHFR’s (qm/Pf DHFR, dm1/Pf DHFR, and dm2/Pf DHFR), relative to the starting structure during MD simulation. (A) The whole backbone atoms of enzyme structures (B) P218 molecule in various complexes.
In the P218-PfDHFR complexes (wild-type and mutant), the contributions favoring P218 binding are VDW (van der Waals [VDW] contribution), ranging from $-40$ to $-49$ kcal/mol, and ELE (electrostatic energy), ranging from $-179$ to $-243$ kcal/mol. The non-polar interaction with the solvent (PBSUR) yielded contributions of $\sim -4$ kcal/mol and the polar interaction with the solvent (EPB) yielded contributions in the range of $169$–$207$ kcal/mol. Overall electrostatic contribution favors P218 binding in P/DHFR, with PBELE (sum of electrostatic solvation free energy and MM electrostatic energy) values ranging from $-4$ to $-16$ kcal/mol, dm1/P/DHFR having the least value of $\sim -4$ kcal/mol. This reduction in PBELE value in the case of dm1/P/DHFR may be the possible reason for the observed lower P218 binding affinity for dm1/P/DHFR as compared to other P/DHFR structures. However, the binding free energy of P218 to hDHFR is comparatively less favorable ($-22.14$ kcal/mol). The main contribution leading to lower binding affinity in hDHFR originates from the unfavorable energy of desolvation of polar groups having PBELE value of $1.22$ kcal/mol (as compared to $-4$ to $-16$ kcal/mol in P/DHFR). Additionally, the binding of P218 to hDHFR had the least favorable electrostatic interactions, as compared to P218-P/DHFR complexes; its ELE is $-131.46$ kcal/mol, whereas its non-polar contribution to solvation free energy (PBSUR) was found to be comparable to the P/DHFR complexes.

The VDW contribution is lower in qm/P/DHFR than the other complexes but its electrostatic interaction (ELE) and PBELE contribute significantly more as compared to all the other four complexes, with least contribution in hDHFR (Table 1). This difference in electrostatic and VDW terms leads to difference in binding affinities among the studied complexes (Table 1).

**Energy decomposition of each amino acid residue**

To find the key residues that play important role in P218 binding in the active site of hDHFR and P/DHFR, and also to find out the residues responsible for decreased binding affinity of dm1/P/DHFR as compared to other P/DHFR complexes, the interactions were quantified in terms of the pair interaction decomposition of free
energy. The ‘per-residue’ binding energy of important amino acids that interact with P218, in all the five complexes is shown in Figure 6. From the figure, it can be seen that the patterns of the energy decomposition in \( \text{wtPfDHFR} \) and \( \text{dm2PfDHFR} \) are similar and their total binding free energies were also found to be almost equal (Table 1). The patterns of the energy decomposition in \( \text{wtPfDHFR} \) and \( \text{dm1PfDHFR} \) differ by strong interactions of P218 with Asp54 and Cys15 in \( \text{wtPfDHFR} \) as compared to \( \text{dm1PfDHFR} \). Residues Ile14, Arg122, and Ile164 slightly favor P218 binding to \( \text{dm1PfDHFR} \) and the mutated residue Arg59 significantly favors P218 binding to \( \text{dm1PfDHFR} \) as compared to \( \text{wtPfDHFR} \), whereas, in all other cases, the ‘per-residue’ contribution is decreased for \( \text{dm1PfDHFR} \). It can be noticed from the figure that in comparison to \( \text{qmPfDHFR} \), the mutation at position 59 from Cys to Arg, does not strongly favor P218 binding in double-mutant \( \text{PfDHFR} \) (\( \text{dm1PfDHFR} \)). Visualization of binding mode of P218 in the active site of \( \text{dm1PfDHFR} \) revealed that Arg59 in \( \text{dm1PfDHFR} \) was oriented in such a way that it does not interact stably with P218 (Figure S6, Supplementary material). This finding can also be related to comparatively low binding affinity of P218 in \( \text{dm1PfDHFR} \) and low electrostatic contribution to overall binding free energy (Table 1) as compared to other mutant and wild \( \text{PfDHFR} \) forms. Although the binding affinity of P218 in \( \text{qmPfDHFR} \) is similar to \( \text{wtPfDHFR} \), differences are clear in the ‘per-residue’ contribution to total binding energy. The residue wise major difference in binding of P218 to \( \text{qmPfDHFR} \) and \( \text{wtPfDHFR} \) can be attributed to residues Ile14, Cys15, Arg59, Arg122, and Ile/Leu164, where on a comparative scale Ile14, Cys15, and Ile164 favor P218 binding in \( \text{wtPfDHFR} \) and residues Arg59 and Arg122 strongly favor P218 binding in \( \text{qmPfDHFR} \). Unlike in \( \text{dm1PfDHFR} \), the mutation at position 59 from Cys to Arg, strongly stabilize P218 binding to \( \text{qmPfDHFR} \), whereas, mutation at position 164 from Ile-Leu in \( \text{qmPfDHFR} \) is not favorable and slightly destabilizes P218 binding to the enzyme. On the other hand, the patterns of the energy decomposition of P218-\( \text{hDHFR} \) are quite different from that of P218-\( \text{PfDHFR} \) complexes. Figure 6 shows the ‘per-residue’ decomposition energy of residues in \( \text{hDHFR} \) that are structurally equivalent homologs corresponding to \( \text{PfDHFR} \), as studied from sequence alignment exercise (Figure 3). The residues Gln35, Pro60, and Leu67 in \( \text{hDHFR} \) corresponding to residues Cys59/Arg59, Pro113, and Leu119 of \( \text{PfDHFR} \) have significantly low ‘per-residue’ decomposition energy as compared to \( \text{PfDHFR} \). The residues Phe31 and
Arg70 in hDHFR corresponding to residues Met55 and Arg122 in PfDHFR, disfavors and might destabilize P218 binding in hDHFR. This difference in ‘per-residue’ decomposition energy in hDHFR and PfDHFR (wild-type and mutant) can be correlated with poor overall binding affinity of P218 to hDHFR as compared to PfDHFR. These findings support the experimental results (Yuthavong et al., 2012) and also provide additional insights into the unfavorable binding of P218 to hDHFR.

Hydrogen bond analysis

Binding affinity of a ligand in the active site of an enzyme can be correlated to hydrogen bonding interactions. Hydrogen bond (H-bond) occupancy between P218 and residues Ile14, Asp54, Cys/Arg59, Arg122, and Ile164 of PfDHFR and residues Ile7 and Glu30 of hDHFR, during the last 5 ns simulation run are shown in Figure 7. Hydrogen bond occupancies of >75%, 50–75% and <50% were used to define strong, medium, and weak H-bond interactions, respectively (Khuntawee, Rungrothmongkol, & Hannongbua, 2011; Mokmak et al., 2014). Any residue having hydrogen bond occupancy of >100% means that it can form more than one hydrogen bond at a time. In the case of PfDHFR (wild-type and mutant), P218 forms two strong hydrogen bonds with Asp54 (between the N7 amino group of P218 and O1 of Asp54 and between N1 of P218 and O2 of Asp54) having occupancy >150% and two hydrogen bonds with Arg122 (between the phenolic carboxylic O1 of P218 and N1H of Arg122 and between phenolic carboxylic O2 of P218 and N2H of Arg122) with occupancy >100% (Figure 7). The H-bond distances of the above-mentioned hydrogen bonds during the last 5 ns simulation run (Figure S7(A)–(D), Supplementary material) also depict that these bonds were stable during the simulation and thus strongly stabilize P218 binding in the active site of PfDHFR (wild-type and mutant). In case of wtPfDHFR, dm1PfDHFR and dm2PfDHFR, N8 amino group of ligand and backbone carboxylic group of Ile14 formed medium H-bond while it formed weak H-bond with the backbone carboxylic group of Ile164 (Figure 7) as also shown by hydrogen bond distances over last 5 ns simulation run (Figure S7(E)–(F), Supplementary material). Quadruple-mutant PfDHFR formed weak hydrogen bond between N8 amino group and backbone carboxylic group of Ile14, while Leu164 does not form H-bond interaction with P218 (Figure S7(E)–(F), Supplementary material).
Double-mutant dm1/PfDHFR and qm/PfDHFR have Cys59Arg mutation. Unlike dm1/PfDHFR, qm/PfDHFR forms additional strong and stable hydrogen bonds between phenolic carboxyl group and Arg59 (Figure S7(G), Supplementary material). Cys15 is the other amino acid involved in weak hydrogen bond in case of PfDHFR. The pattern of strong H-bonds in all the P218-PfDHFR complexes is similar except for extra strong hydrogen bonds involving Arg59 in qm/PfDHFR. For the P218-hDHFR complex, strong H-bonds are formed between the N7 amino group and Glu30 (structural homolog of Asp54 in P/PfDHFR), similar to the observation in P/PfDHFR (Figure 7 and S7(A)–(B), Supplementary material), whereas it forms medium hydrogen bond between N8 amino group and Ile7 (Figures 7 and S7(E), Supplementary material). Additionally, Asn64 (structural homolog of Cys59 in P/PfDHFR) was found to form weak hydrogen bond interaction with phenolic carboxyl group of P218 during the simulation (Figure S7(H), Supplementary material). These results showed that the wild-type P/PfDHFR and dm2/PfDHFR have similar H-bond profile which involves strong hydrogen bond with Asp54 and Arg122; medium hydrogen bond with Ile14 and weak hydrogen bond with Ile164, while dm1/PfDHFR differs only in terms of extra weak hydrogen bond with Arg59. H-bonding profile of qm/PfDHFR involves strong H-bond with Asp54, Arg59, and Arg122, weak hydrogen bond with Ile14, and no hydrogen bond with Leu164. Human DHFR forms strong hydrogen bond only with Glu30, medium H-bond with Ile7, and weak H-bond with Asn64 (this interaction was also demonstrated in the docked pose of P218 in the active site of hDHFR, Figure 3) during the simulation run; hence, the binding affinity of P218 in hDHFR is reduced as compared to P/PfDHFR. This result is in agreement with the contribution of individual energy components to binding energy (Table 1), where major difference to P218 binding to hDHFR and P/PfDHFR is due to electrostatic component. On a relative scale, it was noticed that hydrogen bond occupancy in dm1/PfDHFR for residues Ile14, Asp54, Arg122, and Ile164 was lower than wt/PfDHFR. The hydrogen bond occupancy in dm1/PfDHFR for Arg59 was also significantly reduced as compared to qm/PfDHFR. This may also account to decreased electrostatic contribution to binding free energy of P218 for dm1/PfDHFR as compared to other P/PfDHFR complexes. Based on the hydrogen bond occupancies during the last 5 ns simulation, it was noticed that P218
formed three–four and five–six hydrogen bonds with hDHFR and P/DHFR, on an average, respectively (Figure S8, Supplementary material).

Hydrophobic contact
The VDW term of the MM-PBSA (Table 1) mostly represents the non-polar interaction: the hydrophobic contact between the molecules. This can also be found by the decomposed energy contribution. The non-polar contributions of selected amino acid residues in P/DHFR and their corresponding homologs in hDHFR are given in Figure 8. Residues Met55 and Phe58, in P/DHFR and their corresponding homologous residues Phe31 and Phe34 in hDHFR play an important role in hydrophobic contribution. While the residues in wtP/DHFR, qmP/DHFR, dm1P/DHFR, and dm2P/DHFR generally had comparable values but there exists small differences in the ‘per-residue’ hydrophobic contribution in hDHFR. Almost comparable ‘per-residue’ contribution to hydrophobic interaction justifies the above-mentioned results (Table 1), where hydrophobic contribution to binding energy in all the complexes was found to be similar. Overall, on a comparative scale, the hydrophobic interactions were slightly more prevalent in P/DHFR relative to hDHFR.

B-factor analysis
As indicated by the B-factors calculated over the last 5 ns trajectories (Figure 9), it was noticed that the flexible regions in all the five complexes correspond to loop regions. In the case of wtP/DHFR and qmP/DHFR, the most flexible regions correspond to the loop regions (residues 20–30 and residues 75–100). The flexibility is more pronounced in the case of qmP/DHFR as compared to wtP/DHFR. In the case of double-mutants, the flexibility again corresponds to the loop regions (residues 20–30, 75–100, and 125–140) with more pronounced flexibility in case of dm2P/DHFR.

Conclusions
The rise of resistance in P/DHFR is predominantly attributed to the steric constraints caused by specific mutations, the mutation Ser108Asn, being the prime cause of the loss in binding affinity of rigid antifolates like pyrimethamine, cycloguanil, and chlorcycluguanil. Recently, a new inhibitor P218, of resistant and wild-type P/DHFR strains, which does not inhibit human DHFR, has been reported and is in preclinical trials (Yuthavong et al., 2012). To evaluate the detailed interaction profile of P218 with human DHFR and to find the affect of prevailing mutations in P/DHFR on P218 binding, MD simulations of P218 with human DHFR, wild-type P/DHFR, quadruple-mutant P/DHFR (Asn51Ile, Cys59Arg, Ser108Asn, and Ile164Leu), double-mutant P/DHFR, dm1P/DHFR (Cys59Arg and Ser108Asn), and double-mutant P/DHFR, dm2P/DHFR (Ala16Val and Ser108Thr) were carried out.

The binding mode analysis of P218 in the active sites of P/DHFR and hDHFR, as carried out by molecular docking studies revealed that P218 binds in a similar fashion to wild-type and mutant P/DHFR (quadruple-mutant and double-mutants) but differently to hDHFR. It was noticed that diaminopyrimidine ring of P218 in the active site of P/DHFR forms hydrogen bonds with Ile14, Asp54, and Ile/Leu164, whereas it forms hydrogen bonds with Ile7 and Glu30 in the active site of hDHFR. In addition, the terminal carboxyl group of P218 showed hydrogen bond interactions with Arg122 in case of P/DHFR but no such hydrogen bond was observed between Arg70 of hDHFR (structural homolog of Arg122 in P/DHFR). This may lead to the lower binding affinity of P218 to hDHFR as compared to the affinity in P/DHFR. Quadruple-mutant P/DHFR and dm1P/DHFR have Cys59Arg mutation. In the case of qmP/DHFR, Arg59 showed strong H-bond interaction with terminal carboxyl group of P218 but such interaction was diminished in the case of dm1P/DHFR. As all these interactions were studied in static structures further to check whether these interactions were stable over a period of time, MD simulations for 35 ns were carried out.

The MD simulation studies confirmed the selectivity and efficiency of P218 against wild-type and quadruple-mutant P/DHFR, in agreement with experimental data and also it was found that P218 binding affinity to the double-mutants is not much affected by the mutations. Calculated binding free energy of P218 with hDHFR (−22.14 kcal/mol) is significantly lower than wtP/DHFR (−34.90 kcal/mol); qmP/DHFR (−34.15 kcal/mol); dm1P/DHFR (−29.53 kcal/mol); and dm2P/DHFR (−34.93 kcal/mol). It was also noticed that majorly electrostatic contribution can be attributed to the differences in binding free energies among these complexes.

Detailed hydrogen bonding analysis during the last 5 ns simulation run revealed that P218 in the active site of P/DHFR binds stably, and in the entire simulation run, it was anchored at its both ends i.e. its diaminopyrimidine ring interacts with Ile14, Asp54, and Ile164 and its terminal carboxylic group forms strong hydrogen bonds with Arg122 (and Arg59 in case of qmP/DHFR). Whereas, in human DHFR, P218 binding is stabilized only by the interaction of its diaminopyrimidine ring Ile7, Glu30 at its one end, terminal carboxylic group at opposite end does not interact with Arg70 (structural homolog of Arg122 in P/DHFR). The mutations in P/DHFR cause no significant loss in interaction energy with P218 inhibitor, rather mutated residues additionally forms stable electrostatic and VDW interactions with P218. The atomic-level
details studied in the present work, on binding pattern of P218 can be utilized to design novel and potent inhibitors of PfDHFR which binds specifically to PfDHFR and are not affected by mutations.

Supplementary material
The information on binding mode of WR99210 in wild-type and quadruple-mutant PfDHFR; difference between energy minimized and bioactive conformation of P218 and conformational analysis of P218; RMSD of the re-docked ligands; kinetic energy, potential energy, and total energy graphs of all the five complexes during the simulation run; different orientation of Arg59 in qmPfDHFR and dm1pf/PfDHFR; and detailed H-bond analysis in all the five complexes are given in supplementary material. The supplementary material for this paper is available online at http://dx.doi.1080/07391102.2014.979231.

Acknowledgments
SA acknowledges University Grants Commission (UGC), New Delhi, for providing Senior Research Fellowship [Grant file No. F. 2-101/1998(SA-I)].

References
Adane, L., Bhagat, S., Arfeen, M., Bhatia, S., Sirawaraporn, R., Sirawaraporn, W., … Bharatam, P. V. (2014). Design and synthesis of guanlythiourea derivatives as potential inhibitors of Plasmodium falciparum dihydrofolate reductase enzyme. Bioorganic & Medicinal Chemistry Letters, 24, 613–617.

Adane, L., & Bharatam, P. V. (2009). 3D-QSAR analysis of cycloguanil derivatives as inhibitors of A16V + S108T mutant Plasmodium falciparum dihydrofolate reductase enzyme. Journal of Molecular Graphics and Modelling, 28, 357–367.

Adane, L., & Bharatam, P. V. (2010). Binding modes of 2, 4-diaminoquinoxaline and 2, 4-diaminopteridine analogs to P. falciparum dihydrofolate reductase enzyme: Molecular docking studies. Indian Journal of Pharmaceutical Sciences, 72, 324–333.

Adane, L., & Bharatam, P. V. (2011). Computer-aided molecular design of 1H-imidazole-2, 4-diamine derivatives as potential inhibitors of Plasmodium falciparum DHFR enzyme. Journal of Molecular Modeling, 17, 657–667.

Adane, L., Bharatam, P. V., & Sharma, V. (2010). A common feature-based 3D-pharmacophore model generation and virtual screening: Identification of potential Pf DHFR inhibitors. Journal of Enzyme Inhibition and Medicinal Chemistry, 25, 635–645.

Adane, L., Patel, D., & Bharatam, P. V. (2010). Shape and chemical feature based 3D pharmacophore model generation and virtual screening: Identification of potential leads for P. falciparum DHFR enzyme inhibition. Chemical Biology & Drug Design, 75, 115–126.

Arfeen, M., Patel, D. S., Abbat, S., Taxak, N., & Bharatam, P. V. (2014). Importance of cytochromes in cyclization reactions: Quantum chemical study on a model reaction of proguanil to cycloguanil. Journal of Computational Chemistry, 35, 2047–2055.

Basak, S. C., & Mills, D. (2010). Quantitative structure-activity relationships for cycloguanil analogs as PfDHFR inhibitors using mathematical molecular descriptors. SAR and QSAR in Environmental Research, 21, 215–229.

Basak, S. C., Mills, D., & Hawkins, D. M. (2011). Characterization of dihydrofolate reductases from multiple strains of Plasmodium falciparum using mathematical descriptors of their inhibitors. Chemistry & Biodiversity, 8, 440–453.

Bhat, H. R., Ghosh, S. K., Prakash, A., Gogoi, K., & Singh, U. P. (2012). In vitro antimalarial activity and molecular docking analysis of 4-aminoquinoline-clubbed 1,3,5-triazine derivatives. Letters in Applied Microbiology, 54, 483–486.

Bhat, H. R., Singh, U. P., Gahtori, P., Ghosh, S. K., Gogoi, K., Prakash, A., & Singh, R. K. (2013). Antimalarial activity and docking studies of novel bi-functional hybrids derived from 4-aminoquinoline and 1, 3, 5-triazine against wild and mutant malaria parasites as pf-DHFR inhibitor. RSC Advances, 3, 2942–2952.

Bras, J. L., & Durand, R. M. (2003). The mechanisms of resistance to antimalarial drugs in Plasmodium falciparum. Fundamental & Clinical Pharmacology, 17, 147–153.

Carrington, H. C., Crowther, A. F., Davey, D. G., Levi, A. A., & Rose, F. L. (1951). A metabolite of ‘Paludrine’ with high antimalarial activity. Nature, 168, 1080.

Case, D. A., Cheatham, T. E., Darden, T., Gohlke, H., Luo, R., Merz, K. M., … Woods, R. J. (2005). The Amber biomolecular simulation programs. Journal of Computational Chemistry, 26, 1668–1688.

Case, D. A., Darden, T. A., Cheatham III, T. E., Sammling, C. L., Wang, J., Duke, R. E., … Roberts, B. P. (2010). AMBER 11. San Francisco: University of California, 142.

Curd, F. H., Davey, D. G., & Rose, F. L. (1945). Studies on synthetic antimalarial drugs; some biguanide derivatives as new types of antimalarial substances with both therapeutic and causal prophylactic activity. Annals of Tropical Medicine and Parasitology, 39, 208–216.

Dasgupta, T., Chitnumsub, P., Kamchonwongpaisan, S., Maneeruttanarungroj, C., Nichols, S. E., Lyons, T. M., … Anderson, K. S. (2009). Exploiting structural analysis, in silico screening, and serendipity to identify novel inhibitors of drug-resistant falciparum malaria. ACS Chemical Biology, 4, 29–40.

Dondorp, A. M., Nosten, F. O., Yi, P., Das, D., Phyo, A. P., Tarning, J., … Lee, S. J. (2009). Artemisinin resistance in Plasmodium falciparum malaria. New England Journal of Medicine, 361, 455–467.

Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., & Russell, P. B. (1951). 2: 4 diamino pyrimidines – A new series of antimalarials. British Journal of Pharmacology and Chemotherapy, 6, 185–200.

Fiser, A., & Šali, A. (2003). Modeller: Generation and refinement of homology-based protein structure models. Methods in Enzymology, 374, 461–491.

Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., … Perry, J. K. (2004). Glide: A new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. Journal of Medicinal Chemistry, 47, 1739–1749.

Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., … Mainz, D. T. (2006). Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. Journal of Medicinal Chemistry, 49, 6177–6196.

Gahtori, P., Ghosh, S. K., Parida, P., Prakash, A., Gogoi, K., Bhat, H. R., & Singh, U. P. (2012). Antimalarial evaluation and docking studies of hybrid phenylthiazolyl-1, 3-triazine derivatives: A novel and potential antifolate lead for PfDHFR-TS inhibition. Experimental Parasitology, 130, 292–299.
derived from β-lactams and 7-chloroquinoline using click chemistry. *Bioorganic & Medicinal Chemistry Letters*, 22, 57–61.

Sirichaiwat, C., Intaraudom, C., Kamchonwongpaisan, S., Vanichtanankul, J., Thebtaranonth, Y., & Yuthavong, Y. (2004). Target guided synthesis of 5-benzyl-2, 4-diaminopyrimidines: their antimalarial activities and binding affinities to wild type and mutant dihydrofolate reductases from *Plasmodium falciparum*. *Journal of Medicinal Chemistry*, 47, 345–354.

Sivaprakasam, P., Tosso, P. N., & Doerksen, R. J. (2009). Structure activity relationship and comparative docking studies for cycloguanil analogs as PfDHFR-TS inhibitors. *Journal of Chemical Information and Modeling*, 49, 1787–1796.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research*, 25, 4876–4882.

Toyoda, T., Brobey, R. K. B., Sano, G.-I., Horii, T., Tomioka, N., & Itai, A. (1997). Lead discovery of inhibitors of the dihydrofolate reductase domain of *Plasmodium falciparum* dihydrofolate reductase-thymidylate synthase. *Biochemical and Biophysical Research Communications*, 235, 515–519.

Vanichtanankul, J., Taweechai, S., Uttamapinant, C., Chitnumsub, P., Vilaivan, T., Yuthavong, Y., & Kamchonwongpaisan, S. (2012). Combined spatial limitation around residues 16 and 108 of *Plasmodium falciparum* dihydrofolate reductase explains resistance to cycloguanil. *Antimicrobial Agents and Chemotherapy*, 56, 3928–3935.

Verlet, L. (1967). Computer “experiments” on classical fluids. I. Thermodynamical properties of Lennard-Jones molecules. *Physical Review*, 159, 98–103.

Yuthavong, Y., Tarnchompoo, B., Vilaivan, T., Chitnumsub, P., Kamchonwongpaisan, S., Charman, S. A., … Bongard, E. (2012). Malarial dihydrofolate reductase as a paradigm for drug development against a resistance-compromised target. *Proceedings of the National Academy of Sciences*, 109, 16823–16828.

Yuvaniyama, J., Chitnumsub, P., Kamchonwongpaisan, S., Vanichtanankul, J., Sirawaraporn, W., Taylor, P., … Yuthavong, Y. (2003). Insights into antifolate resistance from malarial DHFR-TS structures. *Nature Structural & Molecular Biology*, 10, 357–365.

Zhang, K., & Rathod, P. K. (2002). Divergent regulation of dihydrofolate reductase between malaria parasite and human host. *Science*, 296, 545–547.