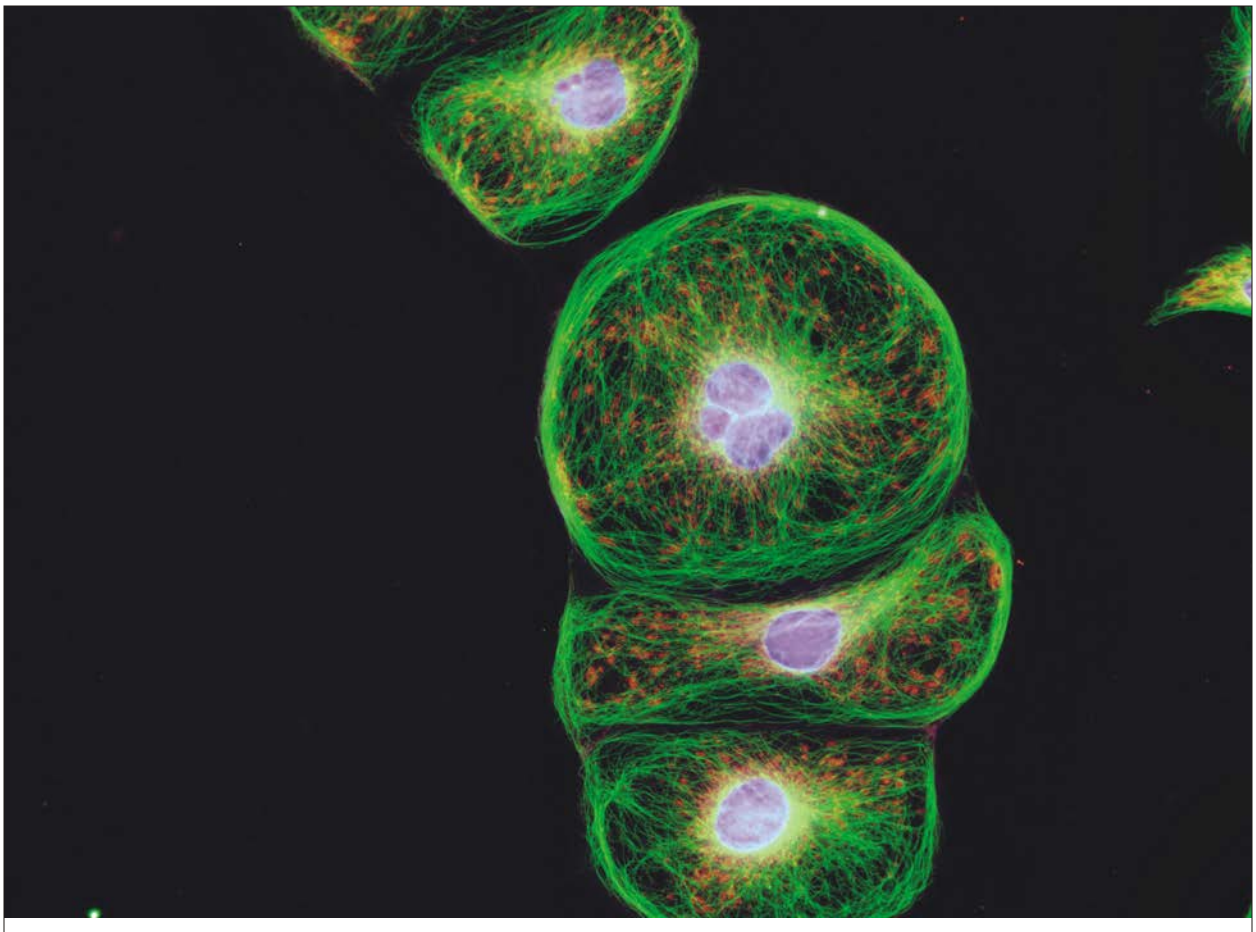

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Dihydrofolate Reductase Enzyme: A Potent Target for Antimalarial Research

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Abstract: Dihydrofolate reductase (DHFR) is an enzyme catalyses NADPH dependent reduction of the 5,6-double bond of dihydrofolate to tetrahydrofolate in malaria parasite. The specificity, i.e., the synthesis of pyrimidine in plasmodium by *de novo* pathway, which does not occur in human, makes the enzyme a valuable target for the therapeutic agents. Antifolate antimalarial drugs interfere with folate metabolism, a pathway essential to malaria parasite survival and can develop resistance to many class of drugs, which is associated with mutations in the active site. Resistance to DHFR inhibitory antimalarial drugs (pyrimethamine and cycloguanil) is found to be associated with point mutations in the parasite DHFR gene sequence. A mutation of 108th residue leads to pyrimethamine resistance whereas resistance to cycloguanil occurs due to mutation at 16th residue. The resistance of the parasite against the single as well as the combination therapy has led to search new effective inhibitors for combating the resistant parasites. Insights from the knowledge of active site of DHFR structure suggest certain requirements for the design of new inhibitors. The structure of the active site of enzyme DHFR reveals that binding the inhibitors to the enzyme, the molecule must contain a heterocyclic like nucleus with groups capable of hydrogen bonding and non-polar group to fit into the hydrophobic core of the active site.

Key words: Dihydrofolate reductase, *Plasmodium falciparum,* homology modeling, antifolate

Introduction

Malaria, the protozoal infection, has become a potential health hazard especially for the third world countries of Africa, Latin America and Asia (Wirth, 1998) and is also one of the most widespread diseases in the world. Each year, more than 100 million people are infected and close to one million die because they don't receive adequate treatment (WHO, 1993). The problems of controlling malaria in these countries are aggravated by inadequate health structures and poor socioeconomic conditions. Malaria is caused by different parasites of the genus plasmodium, of which *Plasmodium falciparum* is the most ferocious one. It causes malaria tropica, which, if not treated, are very frequently leads to lethal to the infected patient. The hope of global eradication of malaria was finally abandoned in 1969 when it was recognized that this was unlikely ever to be achieved. Ongoing control programs remain essential in endemic areas. Malaria is currently endemic in 91 countries with small pockets of transmission occurring further eight countries.

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The parasitic disease involves the infection of the RBC's (Marrie *et al.*, 2000). Life cycle of the malaria parasite (http://www/micro.msb.le.ac.uk/224/malaria/html), i.e., protozoan of the genus *plasmodium*, has two distinct phases, viz. asexual in host and sexual in the vector (female anopheles mosquito). The unique life cycle and resulting microenvironments of the parasite has led to the evolution of metabolic pathways, which differ, from the human host.

The infection begins with ingestion of the parasite in the host cytoplasm followed by the formation of food vacuole and degradation of host hemoglobin ultimately leading to the infection. The three stages of the *plasmodium* life cycle are invasive for cells: ookinetes, merozoites and sporozoites (Sidjanski *et al.*, 1997).

From the biochemical and molecular biological knowledge of plasmodium, many biochemical targets for the successful development of therapeutic agents have been identified (White *et al*., 1986; Blackley *et al*., 1984). Among that some important target are; energy metabolism, protein metabolism, lipid biosynthesis, nucleic acid synthesis and folate metabolism.

Energy Metabolism

The active site of the glucose metabolizing enzyme lactate dehydrogenase was found to be distinct from that of the host enzyme. This can therefore be a good target for parasite growth inhibition.

Protein Synthesis

Malaria parasites obtain majority of their aminoacids by proteolysis of hemoglobin. Therefore, parasite specific cathepsin D, aminopeptidases and parasite-specific cysteine proteases, which have been identified in various malaria parasites, could be specific targets for chemotherapy.

Lipid Biosynthesis

Malaria parasites obtain phospholipids, lysophosphoilipids, cholesterol, fatty acids and phospholipid precursors mainly by participating in a dynamic exchange with components of plasma. Membrane bound parasite specific enzymes such as phophatidylserine decarboxylase and phosphatidyl ethanolamine methyl transferase has been identified as a target for antimalarial therapy.

Nucleic Acid Synthesis

Purines and pyrimidines are necessary for the formation of nucleic acid. In plasmodium, the synthesis of purines and pyrimidines occur by salvage pathway and *de novo* pathway respectively, which are not present in mammals. The synthesis of pyrimidines is catayzled by the bifunctional enzyme dihydrofolate reductase-thymidylate synthase specific to the parasite.

Folate Metabolism

The enzyme dihydropteroate synthetase (DHPS) is responsible for the conversion of para amino benzoic acid (PABA) to dihydrofolate (FH_2). Inhibition of DHPS could stop parasite growth. This specificity, i.e., the synthesis of pyrimidine in plasmodium by *de novo* pathway, which does not occur in human, makes the enzyme a valuable target for the therapeutic agents. The drugs targeting the enzyme DHFR bind selectively to it in different species (Burchall and Hitchings, 1965; Ferone *et al*., 1969).

In dihydrofolate synthesis, PABA is linked with the pteridine to form dihydropteroate (DHP) by the enzyme dihydropteroate synthetase (DHPS). The conjugation of DHP with glutamate forms dihydrofolate (FH_2). In the biosynthesis of pyrimidines (Casteel Dee, 1995), an important enzyme,

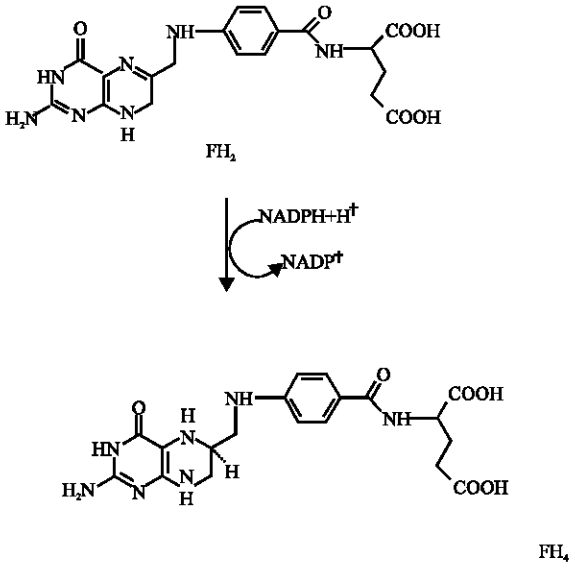


Fig. 1: Conversion of FH_2 in to FH_4

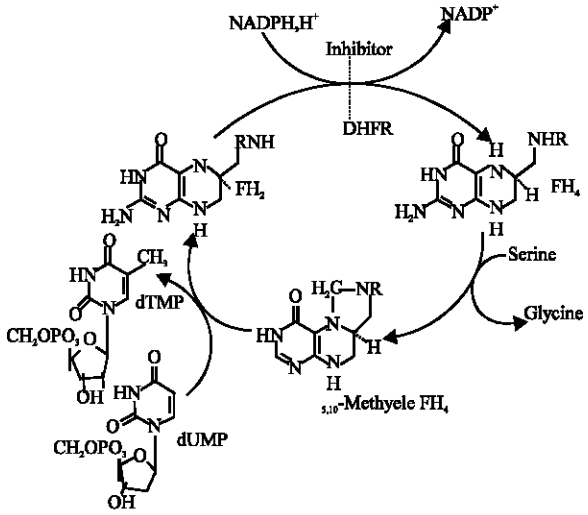


Fig. 2: Role of DHFR in cellular metabolism

dihydro orotate dehydrogenase (DHOD) catalyses the conversion of dihydro orotate to orotate, an intermediate in pyrimidine biosynthetic pathway. In a step further, tetrahydrofolate (FH_4) is required as a cofactor. The bifunctional enzyme DHFR-TS catalyses the conversion of FH_2 to FH_4. Thus by inhibiting this bifunctional enzyme, the nucleic acid synthesis within the plasmodium can be inhibited, ultimately leading to parasite death.

Role of DHFR in Cellular Metabolism (Kuyper, 1989)

The role of DHFR in cellular metabolism and the biochemical basis for its position as a drug target are reasonably well understood. DHFR in protozoa is a part of bifunctional homodimeric protein, where each monomer comprises of a DHFR domain, a junctional peptide and a Thymidylate Synthase (TS) domain (Lemcke *et al*., 1999).

DHFR catalyses NADPH dependent reduction of the 5,6-double bond of FH_2, which produces FH_4 (Fig. 1). Derivatives of FH_4 in which one carbon function have been enzymatically added to the 5 or 10th position or in both serve as one carbon donors in the biosynthesis of purines, pyrimidines and several amino acids (Fig. 2).

The most important transformation involving FH_4, related to DHFR related chemotherapy is the biosynthesis of thymidylate (dTMP). FH_4 is first converted into active factor, 5, 10-methylene tetrahydrofolate by serine hydroxy methyl transferase. The enzyme thymidylate synthase uses this cofactor for conversion of deoxyuridylate (dUMP) to deoxythymidylate (dTMP).

For balanced cell growth, continuous supply of FH_4 is needed, which is possible only by replenishing DHFR. Inhibition of DHFR leads to a cellular deficiency of FH_4 cofactors and hence disrupts the synthesis of purines, pyrimidines and several amino acids. This eventually causes cell death (plasmodial).

Structure of Plasmodium falciparum DHFR (pfDHFR)

The 3-D structure of the plasmodial DHFR is not yet known. In order to design new and more effective antimalarial drugs, the understanding of selectivity and resistance at the molecular level is of utmost importance. Detailed X-ray crystal structures of DHFR in several species and in complex with various ligands have been reported (Warhurst, 1998). Models of *pfDHFR* have also been reported based on the homology of the DHFR sequences (Lemcke *et al*., 1999, Rastelli *et al*., 2000 and Delfino *et al*., 2002). Yuthavong *et al* obtained the 3-D structure of *pf*DHFR in complex with certain drugs to study the antifolate resistance in *Plasmodium falciparum*. The structure is classified by Richardson as a doubly wound mixed β-sheet, is dominated by a central eight-stranded β-sheet flanked on either side by two α-helices (Yuvaniyama *et al*., 2003).

The individual strands of the β-sheet are labeled alphabetically in the order of occurrence in the linear protein sequence. Each helix is assigned the letter of the β-strand that it precedes in the sequence. In the enzyme structure, a deep cleft exists between the B and C helices (Kuyper, 1989). This large cavity serves as the binding site for diaminopyrimidine type inhibitors (pyrimethamine). The nicotinamide portion of NADPH occupies the lower half of the cleft. The adenosine moiety of the cofactor binds in a shallow niche on the opposite side of the β-sheet, with the diphosphate bridge arranged over the edge of the sheet in a position that favours stabilization of the dipoles of helices C and F.

The core of the inhibitor-binding site is hydrophobic. This hydrophobic active site pocket accommodates the heterocyclic ring system of the substrate or the drug. Either end of this hydrophobic pocket is a polar region having hydrogen-bonding sites (Warhurst, 1998). It has methyl group of Thr in one end of the α-helix C (Thr/Ser/Asn 108). The opposite end lying on the α-helix, B contains the carboxylic oxygen of Asp (Asp 54).

The alignment of active site related portion of *Plasmodium falciparum* dihydrofolate reductase sequence is shown (Fig. 3).

In the sequence, two loops of 17 and 35 residues are present which are inserted between active site residues. The letter h marks residues interacting by H-bonding and o marks residues showing

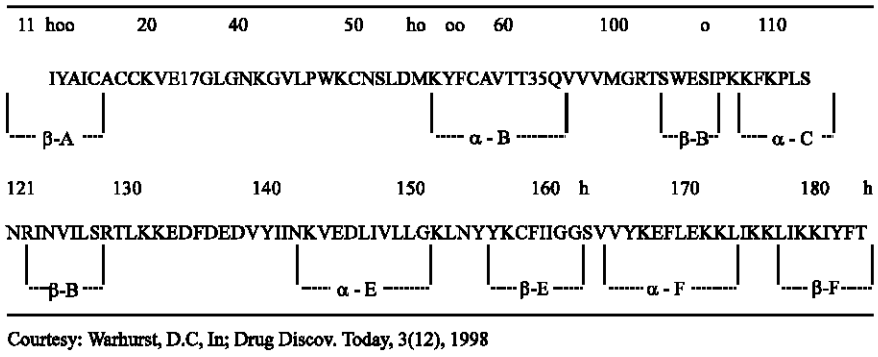


Fig. 3: Alignment of active site-related portion of *plasmodium falciparum*

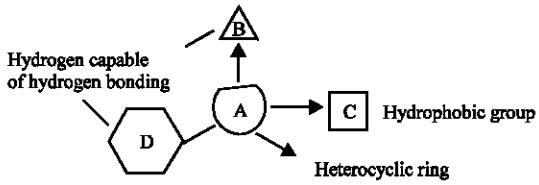


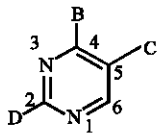
Fig. 4: Requirements for the design of new DHFR inhibitors

Vander Waals interactions. The residues in bold italics indicate the amino acids associated with mutations responsible for resistance.

Structural Requirements for DHFR Inhibitors (Warhurst, 1998)

Insights from the knowledge of active site of DHFR structure suggest certain requirements for the design of new inhibitors (Fig. 4).

Assuming pyrimidine to be the heterocyclic nucleus, the structural requirements would be as under:



At 2-position, a hydrogen donor group donates hydrogen bond to buried water molecules of carboxylic group of Asp 54. At 4-position, a hydrogen acceptor group forms a hydrogen bond to the backbone carbonyl oxygen of aminoacid residues. The protonated pyrimidine binds ionically to carboxyl group of Asp54 through two hydrogen bonds: one from protonated N-1 and the other from the hydrogen donor group at the 2-position. Substitution at 5-position should be non-polar as it is located in the hydrophobic core of the enzyme active site.

Resistance to DHFR Inhibitors

The malaria parasites rapidly develop resistance against different subclasses of the drugs. Hence there is a great urge to develop new and effective drugs (Sirawaraporn *et al*., 1997a). Resistance to

DHFR inhibitory antimalarial drugs (pyrimethamine and proguanil) is found to be associated with point mutations in dihydrofolate reductase and dihydropteroate synthase gene sequence, the two key enzymes in the folate biosynthetic pathway (Tarnchompoo *et al*., 2002). Sometimes the resistance is also due to the steric clash of the substituents at a particular position with the active site of the enzyme.

Serine residue at position 108 is found in the sensitive strains of *pf*DHFR whereas mutation to Asn108 leads to resistance to PYR (Peterson *et al*., 1990; Yuthavon, 2002) and a moderate loss of response to cycloguanil (CYC), the active metabolite of proguanil. A mutation of Thr 108 along with Ala16 to Val 16 mutation provides resistance to CYC. When multiple mutation occur, i.e., S108N+Cys59 to Arg and Asp51 to Ile, it causes higher level of resistance against PYR. Mutations from Ile164 to Leu164 in combination with Asp108 and one or both of the Ile51 or Arg 59 have led to resistance against both PYR and CYC in *Plasmodium falciparum* strains (Foote *et al*., 1990).

Residue 108 occurs in the C-α-helix of the enzyme; residues 51 and 59 align in or near the B-α-helix at the back of the cavity; and residues 16 and 164 are located in the A-β-strand and E-β-strand, respectively. Residues at or near these positions are reported to be involved in the binding of many inhibitors of DHFR (Volz *et al*., 1982; Matthews *et al*., 1978; Buccanari *et al*., 1981; Simonsen *et al*., 1983). The resistance to sulfadoxine has been associated to the mutations in the gene encoding DHPS (Wag *et al*., 1997). All the studies have highlighted that the steric constraint hypothesis as a fundamental basis of antifolate resistance and provided rational approaches for designing inhibitors devoid of substituents at position where steric clash can likely be formed (Yuthavong *et al*., 2000). Figure 5 shows the various point mutations in the active site of DHFR, which are responsible for resistance.

β-Sheet A

The backbone oxygen of Ile14 forms hydrogen bond with the 4-amino group of TMP in *pf*DHFR. When we move to two residues further along the β-sheet A, in *pf*DHFR, Ala16 is present which gets mutated causing CYC resistance. The change from Ala16 to Val16 alters the orientation of drug in the active site during its interaction with Asp54 and the residue 108 (Mathews *et al*., 1977).

α-Helix B

In α-helix B, two mutations Asn51 to Ile and Cys59 to Arg occur in *pf*DHFR. Mutation at Asn51 causes alteration of hydrogen bonding with the drug whereas the mutation of Cys59 (Hyde *et al*., 1990) may lead to influence the position of the α-helix.

α-Helix C

Thr/Ser/Asn 108 in α-helix C are located at the opposite end of binding site of Asp54. The methyl carbon of side chain of Thr is an important component of the hydrophobic pocket of the active site. The hydroxyl oxygen of the Thr side chain and amino group of the backbone form hydrogen bond with the adenine nucleotide 5'-phosphate group in the NADPH cofactor (Filman *et al*., 1982). The position and nature of 108 residues can influence the activity of the enzyme and also drug binding (Sirawaraporn *et al*., 1997b).

β-Sheet E

By mutation of Ile164 to Leu, the methyl carbon of the side chain becomes bulky and longer. This change could affect the hydrogen bonding between Asp54 and the drug.

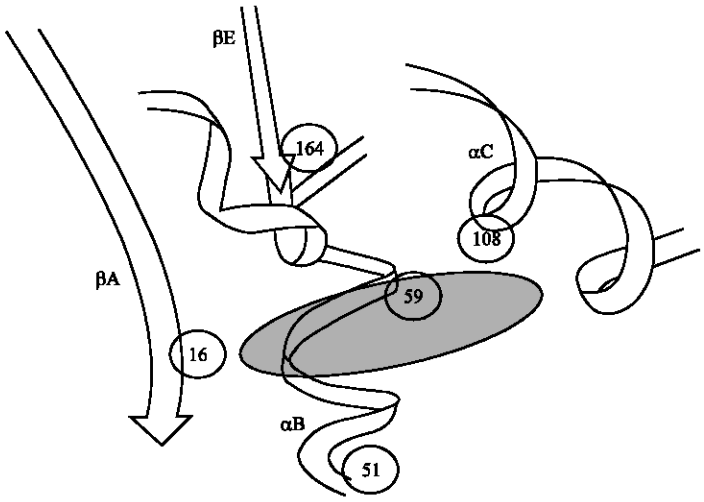


Fig. 5: Point mutations in the pfDHFR active site. The diagram is based on the adaptations from the work of Mathews *et al*. (1977)

β-Sheet F

The Thr 185 in the β-sheet F of *pf*DHFR hydrogen bonds through its side chain with a fixed water molecule which in turn is hydrogen bonded to the 2-amino group of TMP and again with an hydrogen bond helps in the stabilization of the position of Asp54 carboxylic acid group.

Inhibitors of DHFR Used in Antimalarial Therapy

Till date only two of the drugs acting as DHFR inhibitors have been marketed as antimalarial agents for clinical use, viz, pyrimethamine and proguanil (Rosenthal *et al*., 2003). Also combinations of PYR and CYC with sulfonamides (Tracy and Webster, 2001) are available. The sulphadoxine-PYR inhibits the action of DHFR while sulphones and sulphonamide compounds inhibit the action of dihydropteroate synthase. The resistance of the parasite against the single as well as the combination therapy has led to search new effective inhibitors for combating the resistant parasites. The first agent to be tested as antimalarial in this step was TMP (Trimethoprim), an antimetabolite used widely as antibacterial. TMP possessed antimalarial activity at high doses.

By utilizing the newer techniques of drug development, many new molecules have been designed as antimalarials (WR 238605). The most widely used techniques for discovery of enzyme inhibitors with desired and specific chemical properties are molecular docking and de novo design (Kuntz, 1992; Balbes *et al*., 1994; Kuntz *et al*., 1995). The one in clinical trials is WR99210.

Homology modeling has helped to study the interaction of the existing effective molecules with the active site of DHFR. Docking studies and database screening (Rastelli *et al*., 2003) have revealed many new non-conventional classes of *pf*DHFR inhibitors. This new classes include hydrazine, hydrazides, ureas, (thio)semicarbazides/carbazones, biguanides, 2yl-amino-heterocycles, alkyldiamines, N-amino alkyl sulfonamides, polyols, dithiocarbamic acids, tert-amino derivatives, amino pyrimidines, amino triazines, purines, thiazoles, oxazoles, N-hydroxy amidines, alkyl amino ammonium salts, etc.

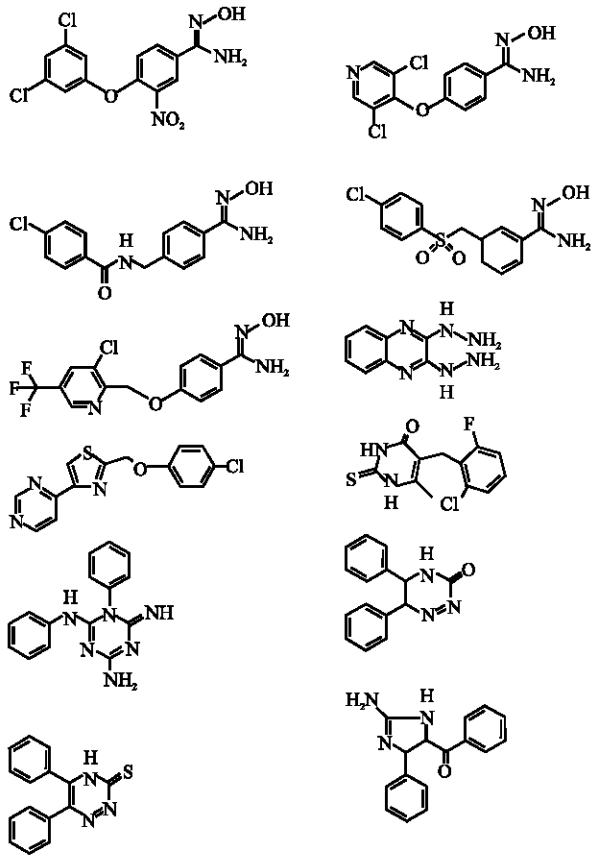


Fig. 6: Top scored antifolate compounds by docking studies

Some compounds giving good score as antimalarials on docking studies from the above classes have also been designed suggesting them to be novel derivatives as potential leads of antimalarial therapy. These top scoring compounds are given in Fig. 6.

Conclusions

The DHFR domain of plasmodium falciparum bifunctional enzyme dihydrofolate reductase-thymidylate synthetase is a validated target of antimalarial antifolates. The structure of the active site of this enzyme obtained by homology modeling studies reveals that for binding to the enzyme DHFR, the molecule must contain a heterocyclic like nucleus with groups capable of hydrogen bonding by accepting/donating hydrogen binds and non-polar group to fit into the hydrophobic core of the active site.

Differential resistance to the antifolates (PYR and CYC) is associated with the mutations in the active site. A mutation of Thr 108 to Asn108 causes PYR resistance whereas Ala16 to Val 16 renders CYC resistance. A cross resistance of CYC and PYR is due to multiple mutations of Thr108 to Asn108 and Cys59 top Arg59, Asp51 to Ile51 and ILE164to Leu164. The molecular modeling studies

have led to development of new classes of *pf*DHFR inhibitors that may be helpful against the resistant forms of the parasites.

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