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Identification of Novel Dihydrofolate Reductase Inhibitor as Potential Antimalarial Drug: *In silico* Studies

Zeeshan Iqbal,[1] Mubashir Hassan,[2] Jawaria Munir,[1] Irfana Mariam,[2] Farah Rauf Shakoori,[3] Abdul Rauf Shakoori[4] and Nasir-ud-Din[1,5*]

[1]*Institute of Molecular Sciences & Bioinformatics Lahore, Pakistan*
[2]*Department of Chemistry, GC University, Lahore, Pakistan*
[3]*Department of Zoology, University of the Punjab, Lahore, Pakistan*
[4]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*
[5]*HEJ Research Institute of Chemistry, University of Karachi, Karachi, Pakistan*

Abstract.-Advancement in computational biology leads to improve the efficacy for new compounds to cure the diseases. Malaria is the most virulent diseases and causing millions of deaths annually, especially in developing and under-developed countries. *Plasmodium falciparum* dihydrofolate reductase (*Pf*DHFR) is one of the most important drug target for different antifolates. Pyrimethamine with sulphadoxine complex is the most recommended and efficient antifolate prescribed against *Pf*DHFR. But malarial parasites have developed resistance against this drug due to the point mutations in *Pf*DHFR. This study focus to design a novel antimalarial drug (analog) against mutated *Pf*DHFR by considering the *in silico* approaches. The new antimalarial drugs were designed by the addition/substitution of different functional groups and molecules in parent compound of pyrimethamine. The docking studies of newly designed compound and pyrimethamine with mutated receptor protein of *Pf*DHFR were performed by using different docking servers. Various *in silico* therapeutic calculations for novel antimalarial compound and pyrimethamine were executed using computational approaches. The basic of ligand properties, docking results, energy calculations and drug score favor indicated that the new antimalarial drug compound have potential to show better efficacy than pyrimethamine. This designed analog could be used for preclinical test and have the potential to eradicate *P. falciparum*.

Key words: *In silico* drug designing, malaria, *Pf*DHFR, molecular docking, pyrimethamine

INTRODUCTION

Malaria is a lethal disease causing one million deaths annually in the world (Muerhoff *et al.*, 2010). *Plasmodium falciparum* is the most lethal amongst all *Plasmodium* species. *P. malariae* and *P. vivax* are the most virulent in Pakistan but *P. falciparum* has more lethality in rest of the world (Omonuwa and Omonuva, 2002; Gupta *et al.*, 2009). The life cycle of *Plasmodium* species involve two alternating hosts such as insect vector and vertebrate host (Florens *et al*., 2002; Zakeri *et al.*, 2010). The viability of malarial parasite depends upon folate metabolism. This pathway is important for purine and pyrimidine production in DNA replication (Gregson and Plowe 2005). In folate pathway mechanism deoxythymidine monophosphate (dTMP) (dTMP) is produced from deoxyuridine monophosphate (dUMP) by oxidation of tetrahydrofolate to dihydrofolate catalysed by dihydrofolate reductase (DHFR) (Hyde, 2005; Abali *et al.*, 2008).

*Pf*DHFR-TS is a homodimeric protein comprising DHFR domain, junction region (JR) and TS domain. The JR associates the DHFR and TS domains in most of the parasitic protozoa (Yuvaniyama *et al*., 2003). In *P. falciparum* JR acts as a bridge and assembles DHFR domains by making a strong interaction between these domains. This JR interaction is also significant in the conformation of DHFR domain which helps in the development of novel effective anti-malarial drugs (Chaianantakul *et al.*, 2013). Antifolates prevent malaria by inhibiting the activity of dihydropteroate synthase (DHPS) and DHFR enzymes.

Mutations in *Pf*DHFR (3D7) at different residues like Ala16, Asn51/Cys59, Ser108, and Ile164 causes resistance against antifolates (Lynch *et al*., 2008). In *P. falciparum* clone, Ser108Asn is

* Corresponding author: prof_nasir@yahoo.com, professor_nasir@yahoo.com,
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the most effective mutation at DHFR domain (Cowman *et al*., 1988; Sirawaraporn *et al*., 1997; Shallom *et al*., 1999; Basco, 2003; Kamchonwongpaisan *et al*., 2004). These mutations cause resistance to antifolates like pyrimethamine in *P. falciparum*. It has been observed that the increased number of mutations in DHFR protein inhibit the binding of pyrimethamine with DHFR (Sirawaraporn *et al*., 2002).

Several antimalarial drugs have been successfully used against *Pf*DHFR (Plowe *et al*., 1997; Nzila, 2006). The DHFR is an effective target for various antimalarial drugs (Peterson *et al*., 1990; Yuthavong *et al*., 2005), such as proguanil (Färnert *et al*., 2002), cycloguanil (Khan *et al*., 1997) and chloroguanil/chlorproguanil (Fidock and Wellems, 1997; Fidock *et al*., 1998; Maitarad *et al*., 2009). Chloroguanil and cycloguanil both were derived from proguanil. The most important antifolate is pyrimethamine which belongs to the 2, 4-diaminopyrimidine families. The pyrimethamine (5-(4-chlorophenyl)-6-ethyl- 2,4-pyrimidinediamine) is a well reported antifolate drug which has been utilized against malaria to target DHFR protein.

The designing of novel antimalarial drugs against mutated *Pf*DHFR are required to cure the malaria. *In silico* drug designing approaches have played significant role in developing novel compounds against resistant proteins (Sehgal *et al*., 2013). In current study, novel antimalarial compounds were designed against mutated *Pf*DHFR. The pyrimethamine was considered as a parent compound and various modifications by addition/deletion and/or substitution of different functional groups. The designed analogs were characterized, analyzed by computational models and finally docked with receptor protein of *Pf*DHFR. The analog drug scoring values and ligand protein docking results suggested the superiority of newly designed analog over pyrimethamine.

MATERIALS AND METHODS

Receptor protein selection

The crystal structure of the *Pf*DHFR was retrieved from the PDBSum (Laskowski, 2001). Various 3D structures are available against *Pf*DHFR with different PDBIDs. The selected structure for *Pf*DHFR has PDBID IJ3J which is the most recently reported crystal structure and has two mutations at C59R and S108N. It exists in dimeric form with four chains (A, B, C and D). The chains A and B are for the DHFR domain and chains C and D for TS domain. The chains A, C and D were removed from the original pdb file with the help of edit tools of Discovery Studio 3.5 Visualizer for docking analyses. Additionally, co-crystallized ligands were also removed by using Discovery Studio 3.5 Visualizer. The finally prepared coordinate file was used for docking studies.

Designing of novel analog

To design a novel antimalarial drug compound, various drug databases such as Drug Bank (http://www.drugbank.ca/) PubChem (http://pubchem.ncbi.nlm.nih.gov/) Chembank (http://chembank.broad.harvard.edu/welcome.htm) were screened to check the basic properties of available antifolates and its mode of action against malaria. The pyrimidinediamine was considered as the basic skeleton to design new analog by considering different functional groups, benzene rings and long chains. The biochemical activity and molecular properties like molecular weight (MW), hydrogen bond acceptor (HBA), hydrogen bond donor (HBD), pH, drug likeness values, volume, chemical structure and optimal docking area (ODA) of each designed analog were calculated by Molsoft (http://www.molinsoft.com/) and Molinspiration (http://www.molinspiration.com). The finally selected compound was 3-(4-hydroxy-2-{3-[(2,4,6-triaminopyrimidin-5-yl)oxy]propoxy}phenyl)propanoic acid (Fig. 1) and was used for further computational studies.

Molecular docking

Molecular docking of finally selected analog and pyrimethamine with DHFR protein were performed separately by utilizing two different docking programs PatchDock and Molecular Docking Server (Schneidman-Duhovny *et al*., 2005). Several complexes were generated by these docking servers but the highest scoring complexes were selected for further post-docking analyses.

Post docking and comparative analyses

Docked complexes of both ligand and

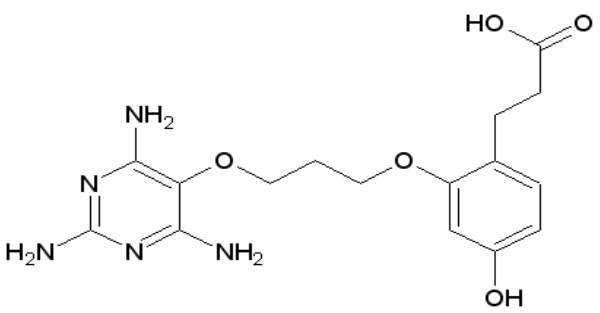

Fig. 1. Modified ligand molecule drawn by ChemSketch considering pyrimethamine as a parent candidate. Different substitutions were employed like a long polar chain with oxygen molecules in between two benzene rings in parent compound. Different functional groups, COOH-, OH- and NH_3- groups are added in basic skeleton of the pyrimethamine that gave better physiochemical result as compared to pyrimethamine.

pyrimethamine with DHFR were analyzed on the basis of energy minimization values. The lowest binding energy values of docked complexes reflect the stability and efficacy of analog molecule. Docked complexes were visualized by Raswin, Discovery Studio 3.5 Visualizer and Pymol softwares. Drug Score[ONLINE] tool was used for further evaluation of docked complexes like drug score (Spyrakis *et al*., 2007) and pseudo binding energy calculations (Gohlke and klebe, 2001).

RESULTS AND DISCUSSION

The *Pf*DHFR is associated with TS domain and acts as a bifunctional enzyme (França e*t al*., 2004). The DHFR plays an important role in folate biosynthetic pathway in protozoans and humans (Gregson, and Plowe, 2005). In *P. falciparum*, the DHFR and TS exist as a single polypeptide with amino and carboxy terminals, respectively (Ivanetich and Santi, 1990). Different protozoans have different lengths of DHFR and TS but in *P. falciparum*, the DHFR domain has 231 amino acids and TS domain has 288 amino acids. The DHFR and TS domain are separated by a JR that consists of 89 amino acids (Chaianantakul *et al*., 2013). The *Pf*DHFR protein used as a receptor molecule has various clinical isolates of wild type and mutants (single, double, triple and quadruple). The selected crystal structure (1J3J) has two mutations (C59R and S108N) and UniProt code of the protein is P13922 (DRTS_PLAFK). The development of the resistance for pyrimethamine against *Pf*DHFR is mainly due to the mutation at 108 position (S108N) (Funanage *et al*., 1984; Das *et al*., 2012). The binding of the pyrimethamine with receptor protein and above described mutations are observed at the B chain of the selected structure. The binding of the pyrimethamine with receptor protein and above described mutations are observed at the B chain of the selected structure. The topology of B chain secondary structure comprises alpha helices (22.6%) and beta sheets (27.6%). Most of mutated residues lie in helices and interaction of our analog with S108N was also confined in helix six (H6) region which may be responsible for minimizing the resistance against antifolates (Fig. 2). These mutated residues induce some conformational changes in its binding pocket that cause hindrance for pyrimethamine binding which favors in the prevalence of malaria (Choowongkomon *et al*., 2010).

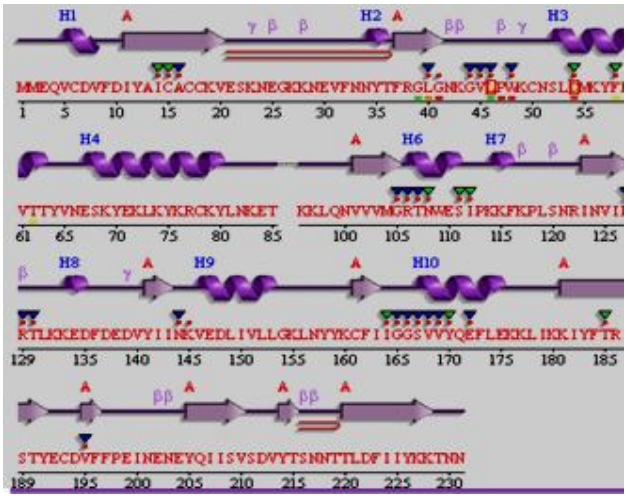

Fig. 2. A secondary structure of B chain of 1J3J is shown with ten helixs in purple color embedded with beta sheets and loops. A long sequence of amino acids is mentioned in red color. Short triangular structures with green and blue color showing the positions of predicted mutated residues.

Pyrimethamine is a well reported and clinically approved drug compound from Food and Drug Administration (Gutman *et al*., 2012). In this study, pyrimethamine was taken into account as a parent molecule to design new analog. The basic mechanism of pyrimethamine against malaria is to target on folic acid which produces tetrahydrofolates by inhibiting the enzyme dihydrofolate reductase (DHFR) (Yuvaniyama *et al*., 2003). This tetrahydrofolates are the potent machinery for DNA and RNA synthesis in protozoa (Yuthavong, 2002). Pyrimethamine is a most renowned drug that targets B-chain of our selected protein structure. Drug databases such as Drug Bank, PubChem, and ChemBank were used to identify the pyrimethamine characteristics which would help to design the analog molecules.

The best modified analog structure was designed by adding specific functional groups as amino group (NH_2), carboxyl group (COOH), hydroxyl group (OH) and two oxygen molecules embedded in a long polar chain in between two benzene rings (Fig. 1). The NH_2 (basic) and COOH (acidic) group will favor the analog structure because it provides more chances in peptide bond formation by H^+ association and dissociation, respectively. Similarly, OH group also has the affinity in hydrogen bond with different amino acids by hydrolysis or condensation. All these newly introduced functional group properties in analog structure support more chances of good binding against mutated residues as compared to pyrimethamine. The basic molecular and drug likeness properties on the bases of Lipinski rule of five were calculated by utilizing Molsoft and Molinspiration tools. These two web-based software's have the capacity to calculate the molecular formula, molecular weight, number of hydrogen bond acceptor (HBA), number of hydrogen bond donor (HBD), molecular polar surface area (MolPSA), MolLogP, MolLogS and MolVol. The molecular volume and the molecular weight for the novel analog were 322.38 A^3 and 363.15 kD, respectively. Both these values are higher than those of pyrimethamine (Table I). MolPSA justifies the implication that more polar surface area favors and enhances the chances of binding affinity between ligand–protein interactions. The MolPSA values for pyrimethamine and analog were 211.74 $A^3$and 248.08 kD, respectively, which also emphasize the importance of this analog over pyrimethamine. The MolPSA value for the pyrimethamine was 60.88A^2 and for the newly designed analog it was 143.46 A^2. Likewise, the drug likeness score for designed analog was 1.19 whereas for pyrimethamine was 0.98. The calculated values for both molecules showed that this novel compound had better response as drug candidate (Table I).

Table I.- Molecular properties and drug likeness values by Molinspiration, Molsoft and PEARLS.

	Pyrimethamine	Analog
Molecular formula	$C_{12}H_{13}ClN_4$	$C_{16}H_{21}N_5O_5$
Molecular weight	248.08kD	363.15kD
Molecular volume	211.74 A^3	322.38 A^3
Drug-likeness score[1]	0.98	1.19
Binding Energy[2]	-2.24	-2.80
MolPSA	60.88A^2	143.46 A^2

[1]Drug likeness Score The drug score combines drug-likeness, cLogP, logS, molecular weight and toxicity risks in one handy value than may be used to judge the compound's overall potential to qualify for a drug.
[2]Binding Energies calculation depends upon Gibbs free energy. More lower energy more strong interaction between ligand-protein molecules and vice versa.

After the selection and preparation of receptor protein and drug molecules PatchDock and Molecular Docking Server were used for the prediction of docking complexes. Both pyrimethamine and newly designed analog docked with mutated DHFR. Several docking complexes were predicted by both docking servers but the selection of the docked complexes were performed on the basis of lower binding energy values and the bindings of both compounds with receptor proteins essentially at mutated residues (Figs. 3, 4). The binding distance between receptor and ligand molecules should be less than the binding distance between receptor and pyrimethamine. The distances between docked molecules and receptor protein were measured by Raswin visualization tool. The binding distance calculated between pyrimethamine and Asn108 was 7.81Å, while it was 3.9Å with the analog (Fig. 5, 6). Novel analog molecule interacts with mutated residue (Asn 108) of *Pf*DHFR which

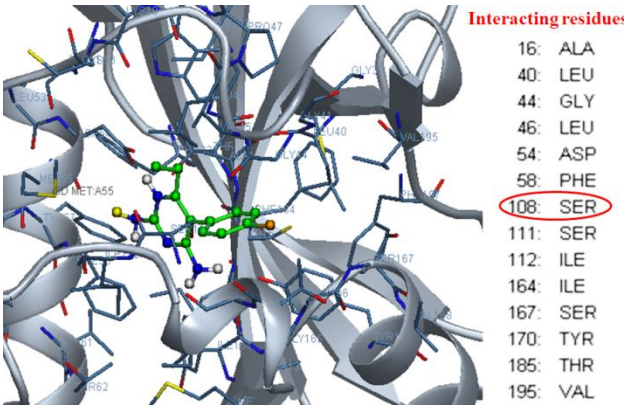

Fig. 3. Pyrimethamine docked complex with DHFR protein, a green color drug (primethamine) molecules shows its interaction with gray color DHFR protein at particuler residues like 16 Ala, 40Leu, 44 Gly, 54 Asp, 108 Ser, 111 Ser, 112 Ile, 164 Ile, 167 Ser, 170 Tyr, 186 Thr and 196 Val. The list of the interacting residues are in right-side of the figure. A red circle shows the interaction of primethamine with Ser108 but unable to bind with the mutated residues at same position with Asn.

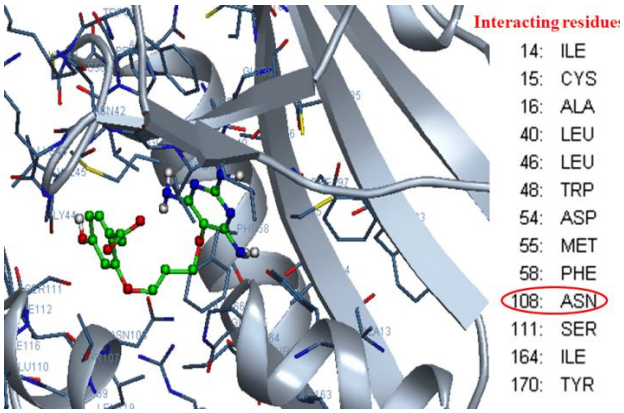

Fig. 4. Novel dock complex with DHFR, a green color analog molecule shows its interaction with gray color DHFR protein at particuler residues like 14 Ile, 15 Cys, 16 Ala, 40 Leu, 46 Leu, 48 Trp, 54 Asp, 55 Met, 58 Phe, 108 Asn, 111 Ser and 164 Ile 170 Tyr. The list of the interacting residues is in right-side of the figure. A red circle shows the interaction of analog with 108 Ans mutated residues at same position where pyrimethamine could not bind.

showed better efficacy compared to pyrimethamine. The bindings of analog with mutated residues of DHFR stretch the advantage towards the novel analog that may give potential results against malaria caused by *P. falciparum.*

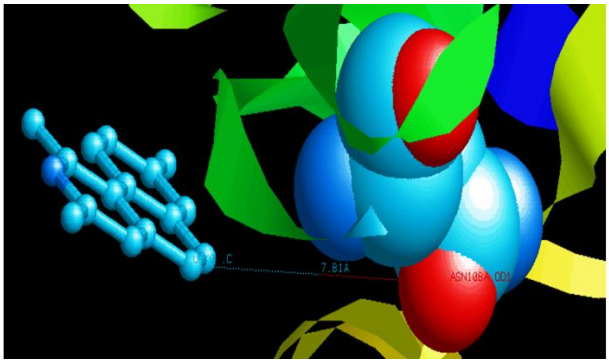

Fig. 5. Docked complex with Asn-108 having distance 7.01Á. The binding distance shows the interacting affinity with a ligand molecule with its target receptor protein. If the distance is more the 5.00Á then interaction will fail.

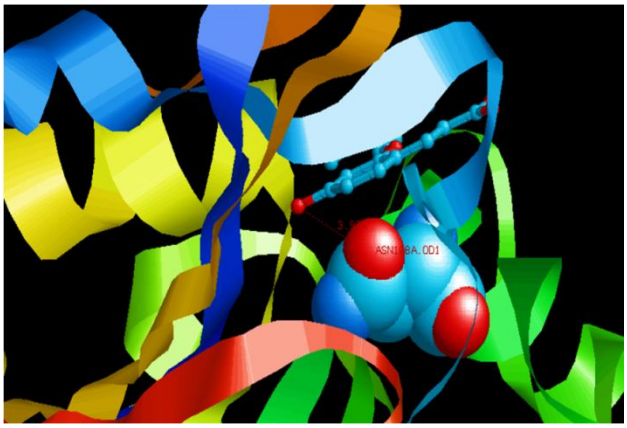

Fig. 6. Analog and Asn-108 complex having distance 3.93 Á. The binding distance shows the interacting affinity with a ligand molecule with its target receptor protein. If the distance is less then 5.00Á so interaction will consider favorable.

Docking analyses depend on binding energy values between the ligand and receptor molecule. Gibbs free energy method was used to calculate energy and to predict the binding efficacy between ligand and receptor molecules. The lowest energy values imply better results and help in evaluating docking complexes. Bioinformatics & Drug Design (BIDD) (Chen, 2006) and Program of energetic analysis of receptor ligand system (PEARL) (Han *et al.*, 2006) softwares were utilized to evaluate the

binding energies of selected docking complexes. The binding energies calculated by PEARL for novel analog (-2.80 kcal/mol) were less compared to that of pyrimethamine (-2.24 kcal/mol). Furthermore, Drug Score[ONLINE] and drug score[CDS] (Gohlke *et al*., 2000) tools were used for estimation of drug score values. The calculated values of drug score for pyrimethamine (0) was higher than novel analog (-7). The drug-likeness score 1.19 of designed ligand and for pyrimethamine (0.98) was analyzed by Molsoft tool. These values indicated that the novel analog has high potential therapeutic values to target the *Pf*DHFR.

Choowongkomon *et al*. (2010) have shown that Ala16, Leu40, Gly44, Leu46, Asp54, Phe58, Ser108, Ser111, Ile112, Ilu164, Ser167, Tyr 170, Thr 185 and Val 195 are the interacting residues of *Pf*DHFR with pyrimethamine. The novel designed analog shown interaction with different residues such as Ile14, Cys15, Ala16, Leu40, Leu46, Trp48, Asp54, Meth55, Phe58, Asn108, Ser111, Ilu164 and Tyr170. These interacting residues indicate the binding pocket of DHFR protein and its vicinity for the analog molecule. The analog molecule that interacts with these residues may have the potential to minimize the resistance against malaria. The mutation of Ser108Asn in DHFR eliminates the resistance of pyrimethamine with DHFR (Sirawaraporn *et al*., 2002). This mutation inhibits the binding of pyrimethamine with receptor molecules whereas designed analog structure binds with Asn108, which might enhance the efficacy of drug binding with target site.

CONCLUSIONS

In this study, a novel antimalarial drug has been proposed against the mutant *Pf*DHFR. In this new drug compound, different functional groups and molecules were attached in the parent compound (pyrimethamine). The assessment of this novel antimalarial drug compound was performed by utilizing different computational tools. The results generated by utilizing computational tools suggested that the novel drug compound have potential to show better efficacy than the previously reported drugs. This *in silico* study will be useful for designing an efficient antimalarial drug compound. Furthermore, the current study will also lead to design an experimental protocol for new drug molecules.

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