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Design and synthesis of guanylthiourea derivatives as potential inhibitors of *Plasmodium falciparum* dihydrofolate reductase enzyme

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ABSTRACT

A new class of compounds based on S-benzylated guanylthiourea has been designed as potential *Pf*DHFR inhibitors using computer aided methods (molecular electrostatic potential, molecular docking). Several compounds in this class have been synthesized starting from guanylthiourea and alkyl bromides. In vitro studies showed that two compounds from this class are active with the IC_{50} value of 100 μM and 400 nM.

Plasmodium falciparum dihydrofolate reductase (*Pf*DHFR) is one of the validated targets to develop potential therapeutic agents for the treatment of malaria. Inhibition of *Pf*DHFR by typical antifolates such as trimethoprim, cycloguanil and pyrimethamine prevents biosynthesis of thymidine, and consequently interrupts DNA biosynthesis.[1] However, point mutations at amino acid residues such as Ala16, Ile51, Cys59, Ser108 and Ile164 in the active site of wild-type *Pf*DHFR enzyme has resulted in widespread resistance of the parasite to these drugs.[2] Thus, discovery of new potential *Pf*DHFR inhibitors to overcome drug-resistant parasites, is an urgent need.

The known antifolate based *Pf*DHFR inhibitors have 2,4-diaminopyrimidine or 1,3,5-triazine moiety that interact with amino acids in the active site via hydrogen bond and hydrophobic interactions. Molecular modeling studies have shown that potential *Pf*DHFR enzyme inhibitors must fulfill at least three criteria required for chemical and geometrical complementarity of ligands with the active site of the enzyme.[3] These are (i) H-bond donor head group that can form H-bond interaction with Asp54, Ile14 and Leu164, (ii) hydrophobic aromatic tail which occupies the hydrophobic pocket of the active site (Phe58, Met55, Phe116, Pro113, Ile112 and Ser111) to enhance inhibitory activity, and (iii) linker unit between the H-bond donor head groups and hydrophobic aromatic tail to provide flexibility, in order to avoid unfavorable steric clashes with Asn108 in the active site of the mutant *Pf*DHFR enzyme.[3,4]

Dasgupta et al.[5] reported the X-ray crystallographic structures of the wild-type (PDB code: 3DGA) and quadruple mutant (PDB code: 3DG8) *Pf*DHFRs with biguanide based bound ligands (RJF01302 and RJF670). Summerfield et al.[6] suggested, based on the results of crystal structure analysis of *Escherichia coli* DHFR enzyme complexed with amidinoisothiouronium salts (PDB code: 2ANO, 2ANQ) that guanylthiourea (GTU) derivatives can mimic

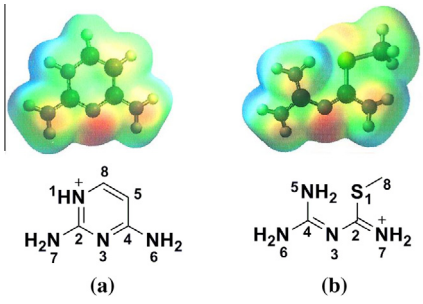

Figure 1. The MESP surfaces of (a) protonated pyrimethamine core and (b) protonated GTU derivative. Red color refers negative potential whereas blue color refers positive potential. All the structures were optimized using B3LYP method and the MESP analysis was generated using SPARTAN software.[10]

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Table 1
The synthesized S-alkylated GTU derivatives (Fig. 3, structure III)

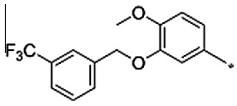
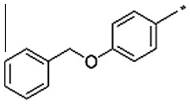
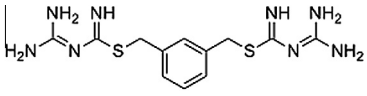
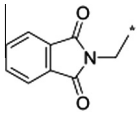
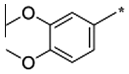
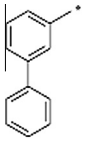
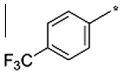
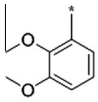
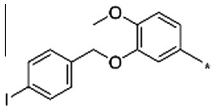
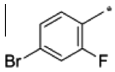
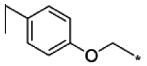
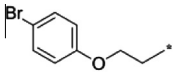
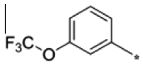
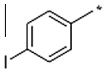
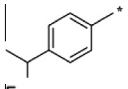
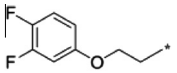
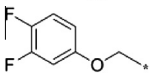
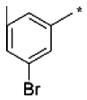
Compounds	R	G_{score}				Yield (%)
		1J3 K	1J3I	3DG8	3DGA	
WR99210	—	−8.708	−8.707	−6.665	−7.837	—
1		−9.075	−8.851	−8.498	−7.327	12.0
2		−8.832	−8.167	−7.514	−6.489	12.0
3		−8.754	−8.430	−8.099	−7.876	90.0
4		−8.620	−8.766	−8.028	−7.031	11.0
5		−8.347	−8.255	−7.102	−6.087	15.0
6		−8.192	−8.041	−7.745	−7.731	54.0
7		−8.160	−7.728	−7.288	−6.319	50.0
8		−8.016	−7.843	−6.673	−6.444	11.5
9		−7.964	−8.271	−6.841	−5.743	57.0
10		−7.878	−7.673	−7.281	−7.026	65.8
11		−7.790	−7.615	−6.750	−6.678	90.0
12		−7.694	−7.932	−6.911	−6.236	40.0
13		−7.651	−7.757	−7.186	−6.975	86.0
14		−7.620	−8.081	−7.540	−7.057	42.0
15		−7.577	−7.467	−6.318	−6.162	72.1
16		−7.526	−7.686	−7.074	−6.344	35.0
17		−7.076	−6.894	−6.414	−6.306	51.0
18		−7.058	−7.528	−6.428	−6.949	48.0

Table 1 (*continued*)

Compounds	R	G_{score}				Yield (%)
		1J3 K	1J3I	3DG8	3DGA	
19		−7.033	−7.883	−6.733	−6.108	90.0
20		−6.977	−6.797	−6.583	−6.512	42.0

* Position of R group.

the binding interactions of 2,4-diaminopyrimidine moieties of known DHFR inhibitors such as methotrexate.

In order to investigate the similarities of GTU moiety with the 2,4-diaminopyrimidine moiety of the known antifolate drugs such as pyrimethamine (Pyr), molecular electrostatic potential (MESP) analysis was carried. Rastelli et al. reported that the antifolate drugs get protonated at physiological pH.[3c] Our results indicated that S-alkylated GTU derivatives are basic in nature and get protonated.[7,8] Also, conformational analysis indicated that the most preferred conformer of the GTU prefers to exist in a conformation comparable to that of pyrimethamine.[7] Therefore, the MESP analysis was carried out on the protonated, most stable conformer of GTU and protonated pyrimidine moiety (representative unit of pyrimethamine) to compare the electrostatic potentials (Fig. 1). The most significant feature of these two MESP surfaces is defined by the $-(H_2N)_2$-C-N-C-N(H_2)– unit of these molecules. The MESP analysis of two protonated species showed that blue color (hydrogen bond donating property) extends over the nitrogen and carbon atom (1st and 7th) in Pyr (Fig. 1a). The similar nature of surface is observed over the nitrogen atoms (5th and 6th) in case of protonated GTU (Fig. 1b). The negative surface (red color, hydrogen bond acceptor) is observed over N(3) in both the cases, while there is partial blue color over N(7) in case of GTU. The alternate electron deficient - electron rich - electron deficient potential surface of $-(H_2N)_2$-C-N-C-N(H_2)– region in these two species is required for molecular recognition interaction with the target macromolecule (*Pf*DHFR). The above analysis showed that under protonated condition the surface of Pyr and GTU are similar in nature. These results are in accordance with our research work on biguanides and other related molecules.[3,9]

Sirichaiwat et al.[11] reported the design and synthesis of trimethoprim derivatives. Derivatives with benzyloxy substituents at the 3 and 4 positions of the benzyl ring of the trimethoprim showed better hydrophobic interactions with amino acids Phe58, Phe116 and Pro113. They showed that antimalarial activity in both the wild-type and mutant varieties of *Pf*DHFR enzymes is improved in case of benzyloxy derivatives as compared to other derivatives with no aromatic substituents. It was suggested that such substituents would increase the binding affinity via hydrophobic interactions with the amino acid residues near the opening of the active site of the enzyme. Based on the above results obtained and suggestions made by Sirichaiwat et al. and Summerfield et al. several GTU derivatives (mono- and bi-subtituted) were designed and their binding potentials were examined using molecular docking methodology.

The Glide docking program was used to study the binding poses of the compounds.[12] The docking calculations were carried out using X-ray crystal structures of wild type (PDB code: 1J3I and 3DGA) and quadruple mutant type (PDB code: 1J3K and 3DG8) *Pf*DHFR enzymes. The crystal structures are in the dimeric form of the DHFR–TS complex and they are co-crystals with cofactors and ligands. During the protein preparation step, only chain A (DHFR) with cofactor NDP610 was retained; all water molecules and the rest of the chains were removed. A radius of 15 Å was selected for active site cavity during receptor grid generation. The reproducibility of the docking calculation was evaluated by docking the bound ligands into the prepared active sites. Table 1 shows G_{score} of important compounds (which show reasonable docking scores) on both protein crystal structures of quadruple mutant type (1J3K and 3DG8) and wild type (1J3I and 3DGA) *Pf*DHFR enzyme. Glide scores of GTU derivatives showing most stable GTU conformation were taken into consideration.

The results of molecular docking analysis indicated that, similar to pyrimethamine (3QG2),[13] WR99210 (1J3K and 1J3I)[14] and biguanide derivatives (3DG8 and 3DGA),[5] GTU moiety was found to form the expected hydrogen bond interactions with Asp54, Leu164, Ile14 and hydrophobic interactions with Phe58 and Phe116 in the active site of *Pf*DHFR enzymes. Flexibility was maintained by introducing linker unit with 1, 2 or 3 carbon atoms between the hydrophobic aromatic tail and GTU moiety in order to prevent potential steric clashes with the amino acid residue Asn108 in the active site of the mutant *Pf*DHFR enzyme. The designed compounds were cross docked on biguanide based crystal structures (PDB code: 3DG8 and 3DGA). The Glide scores obtained were comparable to that of the co-crystallized ligands RJF670 and RJF01302. In Table 1 (arranged according the descending docking scores in 1J3K), compound **1** showed highest G_{score} which may be attributed to additional hydrophobic interactions with Phe116. Bi-substitued guanylthiourea structure (Table 1, compd **3**) showed G_{score} comparable to that of WR99210 (Fig. 2a and c). Compound **3** also showed additional interactions with Ser111, Lys49 and Trp48 (Fig. 2a). These additional interactions are possible only because of the six rotatable bonds between the two guanylthiourea moieties, facilitating stronger interactions. Compound **11** (Table 1) showed hydrogen bonding interaction with Asp54 and hydrophobic interaction with Phe58 (Fig. 2b). The bulky iodine group at the *meta*-position of the benzyl ring occupies the hydrophobic pocket of the enzyme.

Twenty S-alkylated derivatives (Table 1) were taken up for synthesis as per the Schemes 1 and 2. The compounds were synthesized in pure and good to excellent yields. The observation from this experiment also confirmed that the mechanism of S-alkylation of guanylthiourea which was proposed to be based on molecular modeling study is acceptable (Fig. 3).[20]

Synthesized compounds were tested for their inhibitory activity using wild-type and quadruple mutant *Pf*DHFR enzymes expressed in *E. coli*. The expression constructs[2c] harboring the synthetic *Pf*DHFR genes (wild type and quadruple type) were used as a source of recombinant *Pf*DHFR for inhibition testing of the compounds. Briefly the plasmids were transformed into *E. coli* strain BL21 (DE3)pLysS[F^-, *ompT*, $hsdS_B$,(rB^- ,mB^-), dcm, gal, λ9(DE3), pLysSCmR]. The transformed bacteria were then plated on LB agar plate and then supplemented with 100 μg/ml ampicillin and incubated overnight at 37 °C. When *E. coli* colonies appear, an isolated

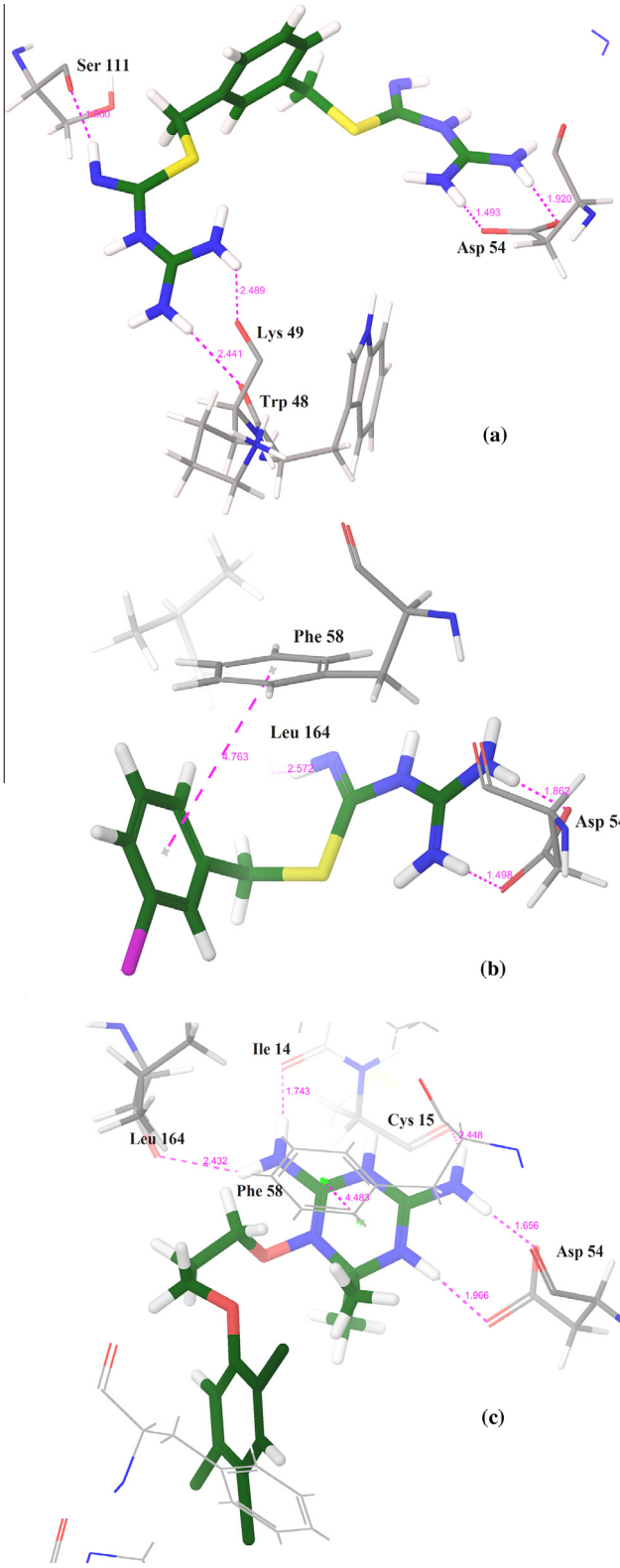
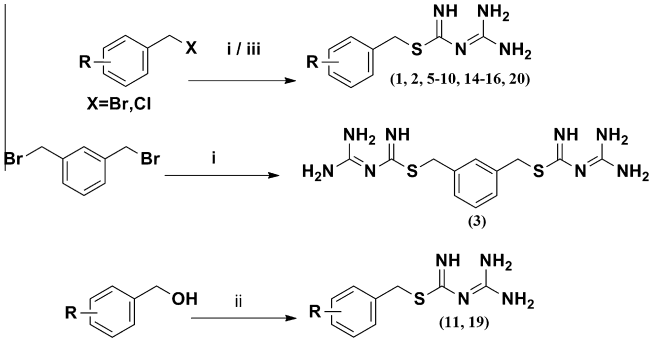
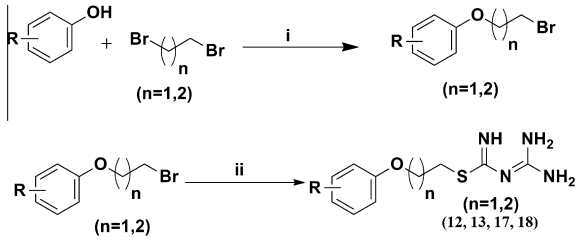


Figure 2. Molecular docking in the active site of quadruple mutant *Pf*DHFR enzyme (a) Guanylthiourea derivatives **3** (Table 1) showing H-bond interaction with Trp48, Lys49, Asp54 and Ser111; (b) compound **11** (Table 1) showing H-bond interaction with Asp54, Phe58 and (c) WR99210 showing H-bond interaction with Asp54, Leu164, Ile14 and Cys15.

colony was picked and inoculated in 5 ml LB medium supplemented with 100 μg/ml ampicillin. Fresh overnight culture of



Scheme 1. Reagents and conditions: (i) GTU, EtOH, NaOH, 80 °C, reflux (Table 1, **15** and **20**);[15] (ii) GTU, conc. HBr, 100 °C, reflux (Table 1, **11** and **19**);[16] (iii) GTU, CH_3CN, MW, 100 °C, 15 min (Table 1, **1**, **2**, **5**–**10**, **14**, **16**).[17,18]



Scheme 2. Reagents and conditions: (i) anhyd K_2CO_3, CH_3CN, 80 °C, reflux;[19] (ii) GTU, EtOH, NaOH, 80 °C, reflux (Table 1 (**13**, **17**, $n = 2$; **12**, **18**, $n = 1$)).[15]

E. coli was then used to inoculate 500-ml culture. Unless otherwise indicated, the culture was grown at 37 °C until A_{600} reached ~0.5-0.6, at which time IPTG was added to a final concentration of 1 mM. The culture was then allowed to grow with shaking at 37 °C for additional 10-12 h. The *E. coli* cells expressing recombinant *Pf*DHFR were harvested by centrifugation at 3,000x*g* for 15 min at 4 °C. The resulting cell pellet obtained was resuspended in 20 ml ice-cold buffer (20 mM potassium phosphate buffer, pH 7.0; 1 mM EDTA; 1 mM DTT; 20% glycerol) and the cells were passed through French pressure cell (American Instrument Co., Inc., Silver Spring, MD, USA) at 15000 psi twice to lyse the cells. Clear extract was separated from the particulate debris by centrifugation at 12000 rpm for 30 min at 4 °C.

The protein concentration of the crude extract was determined[21] and the DHFR activity was determined spectrophotometrically by measuring the decrease in absorbance at 340 nm upon NADPH utilization. The assay reaction (1 ml) is composed of 1×DHFR buffer, 100 μM H_2 folate, 100 μM NADPH, 1 mg/ml BSA, and the reaction was initiated by addition of ~0.01 units of enzyme. One unit of enzyme activity is defined as amount of DHFR that produces 1 μmol of product/min at 25 °C. To determine the inhibition of DHFR activity, stock solution of compounds to be tested were first dissolved with dimethyl sulfoxide (DMSO). Two-fold serial dilutions of the compounds were prepared, and 10 μl of each of the diluted compounds was added in the assay reaction to test for the enzyme inhibition. The IC_{50} values were then calculated as the concentrations of the compounds which inhibit 50% of the DHFR activity.

The preliminary enzyme assay tests using crude enzyme indicated that two compounds (Table 1, **3** and **11**) could inhibit the *Pf*DHFR enzyme at μM range with IC_{50} values ~100 μM and 400 nm respectively.

In conclusion, the in vitro activity showed that the compound which is *meta*-substituted with a bulky atom is active. Bi-substituted guanylthiourea compound showed good activity. This may

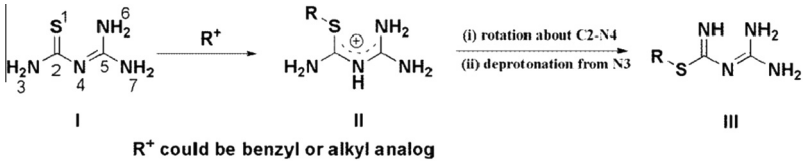


Figure 3. Proposed reaction mechanism for S-alkylation of GTU.[7,20]

be due to increase in hydrogen bond interactions in the active site of *Pf*DHFR enzyme. This additional feature of bi-substituted guanylthiourea derivatives can be further studied to improve the activity of this class of compounds. More work is being carried out in relation to mono-substitued and bi-substituted guanylthiourea derivatives.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.12.009.

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