Synthesis, Dihydrofolate Reductase Inhibition, Anti-proliferative Testing, and Saturation Transfer Difference ^{1}H-NMR Study of Some New 2-Substituted-4,6-diaminopyrimidine Derivatives

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A series of 2-substituted-4,6-diaminipyrimidine derivatives were synthesized and evaluated for their dihydrofolate reductase (DHFR) inhibitory activity. Saturation transfer difference (STD) ^{1}H-NMR experiments were used to probe the binding characteristics of the compounds with human DHFR enzyme. The most potent molecules, 12 and 15, in enzyme assay study showed the best results in STD experiments indicating their intimate interaction with the receptor. The docking studies were followed to explain the structural basis for the observed interaction between the ligands and DHFR. All the compounds were also assayed *in vitro* for their growth inhibitory activity on MCF-7, HepG2, SKHep1, and Hela tumor cell lines. Compounds 16, 17, and 22 demonstrated the most potent *in vitro* anti-proliferative activity among the others.

Key words diaminopyrimidine; dihydrofolate reductase inhibitory activity; synthesis

Interfering with folate metabolism in cancerous cells could provide a useful mean in cancer chemotherapy as a result of the inhibition of the biosynthesis of nucleic acid precursors.[1,2)]

The cofactor tetrahydrofolate (THF) is formed by the reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of 7,8-dihydrofolate (7,8-DHF) by the enzyme dihydrofolate reductase (DHFR) and serves as the principle component in folate metabolism as a carrier of one-carbon units in its various cofactor forms.[3)] Tetrahydrofolate (THF) picks up one carbon unit from L-serine and is converted to coenzyme N^5,N^{10}-methylenetetrahydrofolic acid where CH_2 group bridges N^5 and N^{10} tetrahydrofolate.[4)] This coenzyme is utilized by thymidylate synthase (TS) for the synthesis of deoxythymidylate monophosphate (dTMP) from deoxyuridylate monophosphate (dUMP).

Therefore thymidylate synthase (TS) coupled with dihydrofolate reductase (DHFR) forms a crucial link responsible for the synthesis of dTMP and hence DNA.

Inhibitors of TS derived from substrate analogs such as 5-fluorouracil[5)] and from folate analogs such as raltitrexed[6)] (*N*-[(5-{methyl[(2-methyl-4-oxo-1,4-dihydroquinazolin-6-yl) methyl]amino}-2-thienyl)carbonyl]-L-glutamic acid have found utility as clinically important antitumor agents. (Fig. 1).

Similarly the DHFR inhibitor methotrexate (MTX) is a mainstay in single and combination cancer chemotherapy. Several TS and DHFR inhibitors which are analogs of folate have also shown antitumor activities *in vitro* and *in vivo* with some currently in clinical trials.[7,8)]

While the presence of 2 amino groups on positions 2 and 4 of pteridine seems to be essential for DHFR inhibition, but there are several examples indicating the insignificant nature of pteridine core. On the other hand, Yamini and Vijjulatha[9)] showed that the replacement of pteridyl group by naphthalene ring in methotrexate could still give molecules of great interest, indicating the not so significant role of nitrogen heteroatoms of pteridine core in methotrexate structure.

Replacement of pyrazine ring with benzene ring in the structure of trimetrexate is another evidence for the insignificant role of pyrazine ring in classical DHFR inhibitors.

It is the aim of this study to make a group of 4,6-diaminopyrimidine derivatives with alkyl/arylalkylthio substituents on position 2 (Fig. 2).

The alkyl/arylalkyl group (R) attached to sulfur atom imparts various degrees of lipophilicity to the compounds which could act in favor of increasing activity against DHFR enzyme of some cells such as *Mycobacterium tuberculosis*

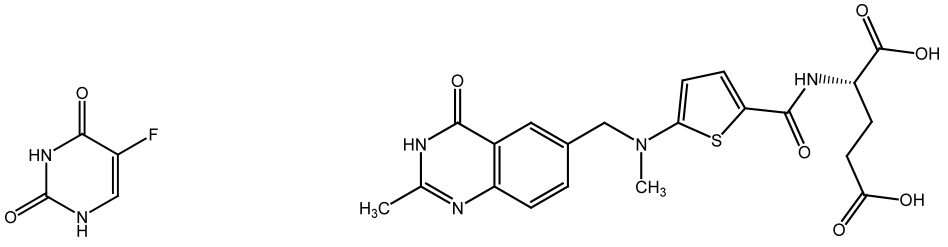

Fig. 1. Chemical Structures of 5FU and Raltitrexed

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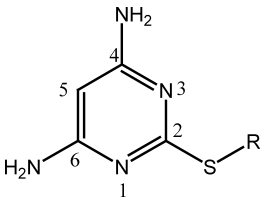


Fig. 2. General Structures of 2-Alkylthio-4,6-diaminopyrimidines

which have more lipophilic characteristics in compared to the same enzymes in human cells.

In order to investigate the DHFR inhibitory activity of the synthesized compounds, enzyme inhibition assay was used according to the protocol described in the manual of DHFR assay kit from Sigma-Aldrich company.

The dynamic interactions of DHFR as the target protein with the synthesized compounds as potential ligands were investigated using saturation transfer difference (STD) NMR technique. The basis of the STD technique relies on the transferred nuclear Overhauser effect (tr-NOE) phenomenon.

To further investigate the structural basis for the ligand-receptor binding, computer-assisted (*in silico*) experiments using the X-ray crystal structure of human DHFR were conducted for docking of the synthesized compounds into their presumed receptor *i.e.* human DHFR.

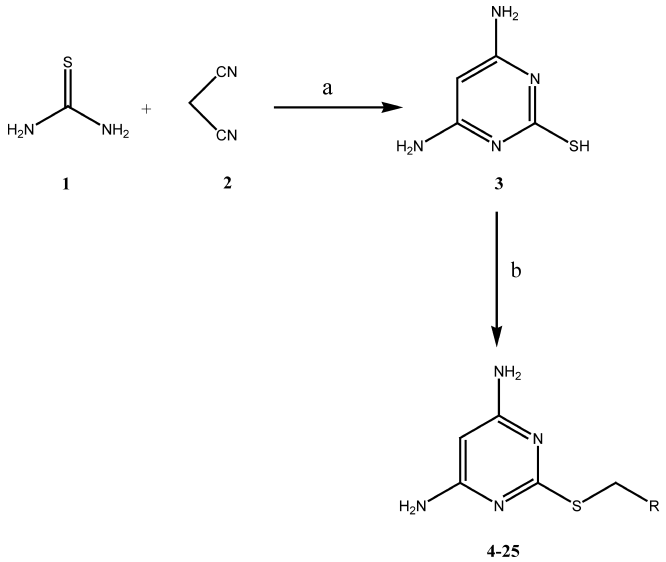
Activities of the compounds were also evaluated using clonogenic test against a selected group of tumor cell lines.

Chemistry The synthetic strategy to synthesize the intermediate **3** and the final compounds **4**—**25** is depicted in Chart 1. 4,6-Diaminopyrimidine-2-thiol (**3**) was obtained as the key intermediate by the reaction of thiourea and malononitrile in absolute ethanol as solvent. Subsequent reaction of compound (**3**) with various alkyl halides at room temperature afforded compounds **4**—**25** in good yields.[10] Structure confirmation of the synthesized intermediate and the final products was performed using IR, NMR, mass spectrometry, and carbon, hydrogen, nitrogen, and sulfur (CHNS) elemental analysis.

Result and Discussion

A group of 4,6-diaminopyrimidine bearing a thioether side chain on the position 2 of pyrimidine ring were synthesized. The compounds were tested for their ability to inhibit human DHFR and their potencies (IC_{50} values) were measured *in vitro*. The results are presented in Table 1.

The most potent compounds were **15** (9 μM), **12** (11 μM), **23** (12 μM), **25** (12 μM), **20** (13 μM), and **22** (14 μM). The 2 most potent compounds **15** and **12** both have *o*-substituted benzyl thioether on their position 2. Replacement of the substituent on the benzyl group by an *ortho*-nitro group has been detrimental to the DHFR inhibitory activity. Changing the benzyl side chain with aliphatic or alicyclic groups both resulted in decreased activity. However phenethyl and phenpropyl side



1 **2** **3** **4-25**

4: R=H
5: R= methyl
6: R= ethyl
7: R= propyl
8: R= butyl
9: R= pentyl
10:R= cyclobutyl
11:R= morpholinomethyl
12:R= 2-methylphenyl
13:R= 3-methylphenyl
14:R=2-nitrophenyl
15:R=2-chlorophenyl
16:R=2-fluorophenyl
17:R=3-fluorophenyl
18:R=4-fluorophenyl
19:R=3-methoxyphenyl
20:R=4-cyanophenyl
21:R=3-(trifluoromethyl)phenyl
22:R=benzyl
23:R=2-phenethyl
24:R=1-naphthalenyl
25:R=1-(1-methylpiperidin-2-yl)methyl

Reagents and conditions: (a) EtONa, reflux, 3 h; (b) NaOH 0.1 M, CH_3OH, R-CH_2-X (X=Cl, Br), rt, 18 h[10]

Chart 1

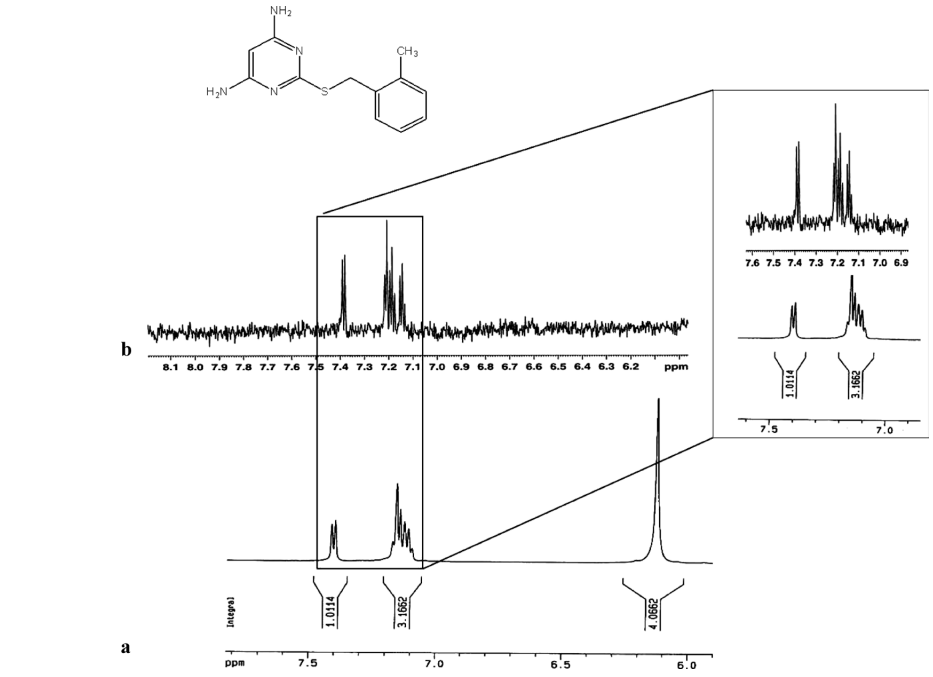


Fig. 3. (a) 500 MHz ^{1}H-NMR Spectrum of Compound **12** in Absence of Human DHFR; (b) 800 MHz STD ^{1}H-NMR Spectrum of Compound **12** in Complex with Human DHFR Protein after Saturation of DHFR

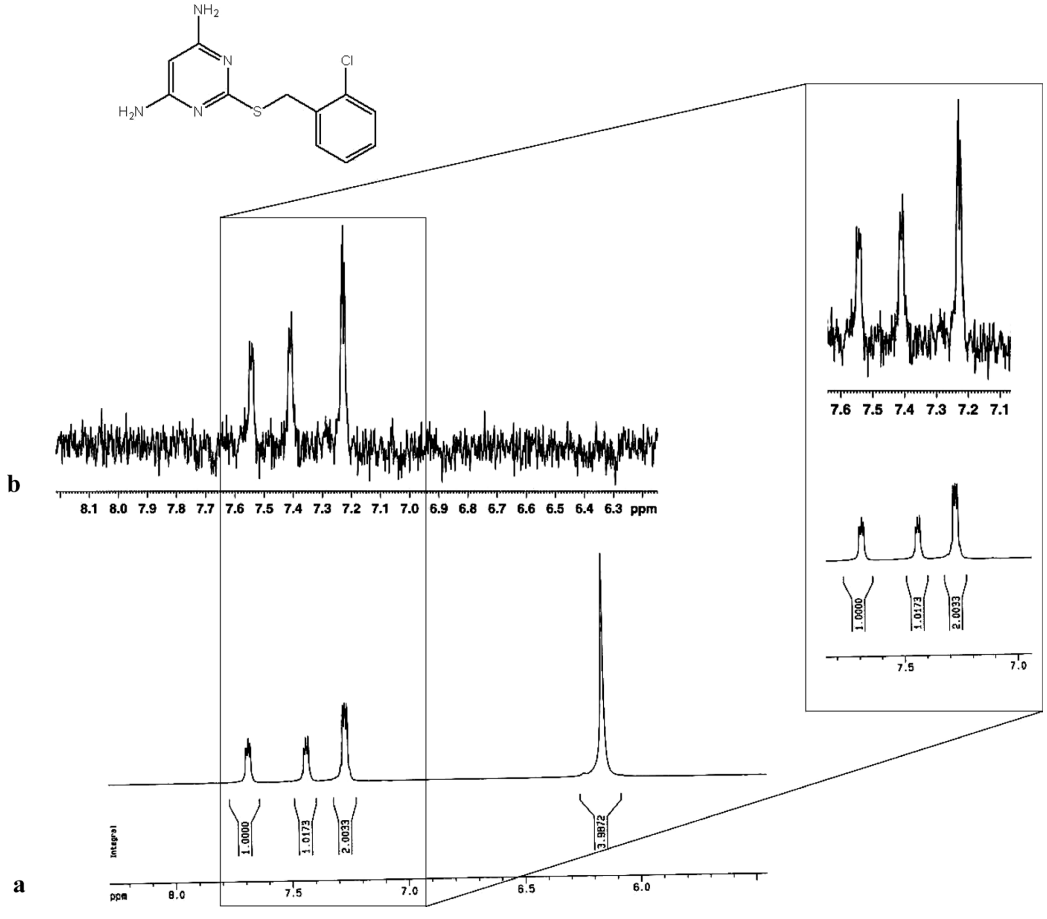


Fig. 4. (a) 500 MHz ^{1}H-NMR Spectrum of Compound **15** in Absence of Human DHFR; (b) 800 MHz STD ^{1}H-NMR Spectrum of Compound **15** in Complex with Human DHFR Protein after Saturation of DHFR

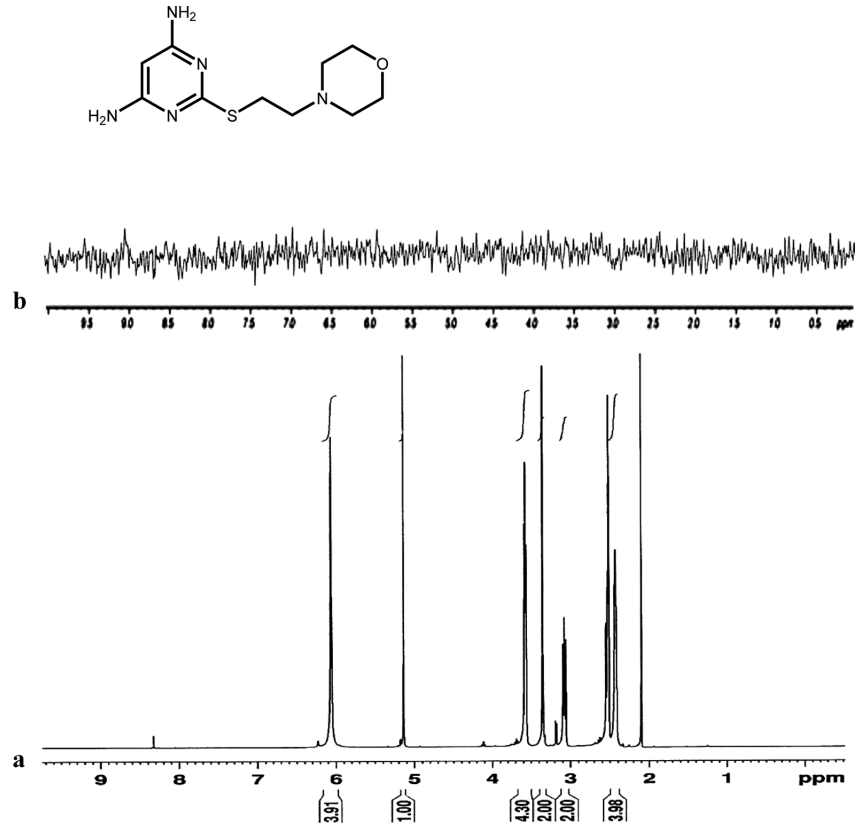


Fig. 5. (a) 500 MHz ^{1}H-NMR Spectrum of Compound **11** in Absence of Human DHFR; (b) 800 MHz STD ^{1}H-NMR Spectrum of Compound **11** in Complex with Human DHFR Protein after Saturation of DHFR

chains have been well tolerated indicating the possible role of phenyl ring in ligand-receptor interaction. None of the compounds with fluorobenzyl side chains showed increased activities against DHFR enzyme. The presence of a basic piperidine ring in the side chain has increased the enzyme inhibition activity.

In order to examine the interaction of these ligands to DHFR, saturation transfer difference (STD) NMR was used. STD is a fast and versatile method for investigating ligand-protein binding.[11] It is based on magnetization transfer by protein signal saturation and its relayed effect to the ligand. Saturation of a single protein resonance can result in a rapid spread of the saturation over the entire protein if spin diffusion within the protein is efficient. During the saturation period, progressive saturation will transfer from the protein (DHFR in this study) to the ligand protons if the ligand binds to the target. If the ligand doesn't bind to the protein, the magnetization transfer will not occur and the difference spectrum thus obtained will not show any signal of the ligand.

Figure 3a shows the ^{1}H-NMR spectrum of compound **12** in absence of DHFR while Figure 3b shows the STD NMR spectrum of compound **12** in complex with human DHFR protein after saturation of DHFR. The appearance of aromatic signals of compound **12** in STD NMR indicates the intimate interaction of this compound with protein DHFR. Compound **15** demonstrated the same pattern in STD study. Figure 4 shows ^{1}H-NMR spectrum of this compound in absence of DHFR (Fig. 4a) and STD NMR spectrum after saturation of the enzyme (Fig. 4b). Compound **11** which showed no inhibitory activity against DHFR was also subjected to STD NMR study. The non-existence of any peak in difference spectrum indicated the lack of formation of ligand–receptor complex for this compound. Figure 5a shows the ^{1}H-NMR spectrum of compound **11** in absence of DHFR and Fig. 5b shows the STD NMR spectrum of compound **11** in complex with human DHFR protein after saturation of DHFR.

To investigate the structural basis for the observed interaction between compound **12** and DHFR, a docking study was conducted using AutoDock 3.05 program. The binding mode of one of the most active compounds, **12**, has been shown in Fig. 6. It was found that the amine groups in positions 4 and 6 of pyrimidine ring were the main keys for hydrogen bond formation with =NH group of Arg 70 residue with distance: 2.27 Å and –C=O group of Asn 64 with distance = 2.36 Å respectively. This confirms the important role of diaminopyrimidine moiety in ligand-receptor interaction. Furthermore, the carbon atoms in positions 5 and 6 of the phenyl ring and the sulfur atom form hydrophobic contacts with the phenyl ring of Phe 31 residue with distances: 3.92, 3.37, and 3.47 Å, respectively. Conducting a docking study for compound **15** shows the same binding characteristics.

Anti-proliferative activity of the compounds were also evaluated using clonogenic method against 4 tumor cell lines MCF-7, HepG2, SK-Hep1, and HeLa (Table 1). Surprisingly the most active DHFR inhibitors (**12**, **15**) are not the most potent compounds against the 4 tumor cell lines. This could be due to the different distribution and accessibility of these compounds to their target inside the cells. On the other hand, there are few compounds (**16**, **17**) with fair anti-proliferative activity against tumor cell lines but low DHFR inhibitory

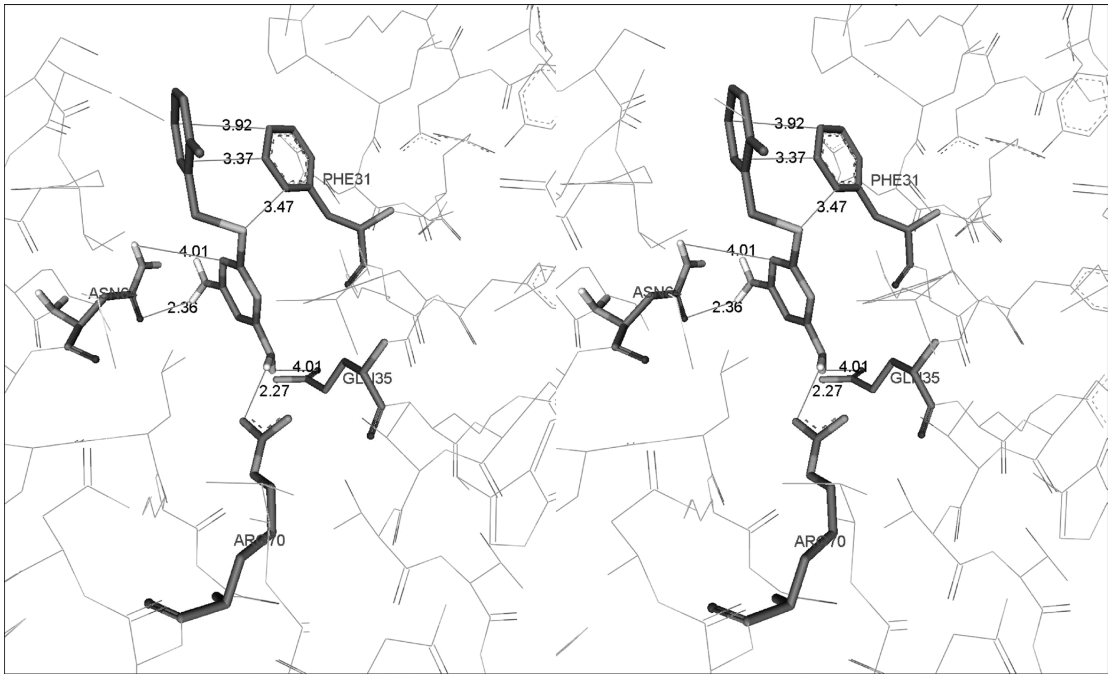


Fig. 6. Stereoview of Compound **12** Docked into the Active Site of Human DHFR (PDB ID Code 1OHJ)[12]

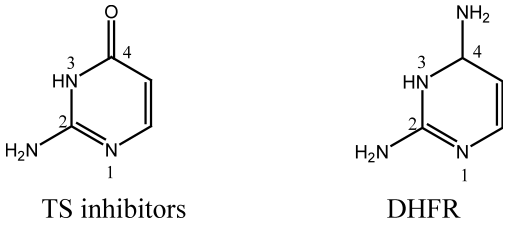


Fig. 7. General Structures of TS and DHFR Inhibitors

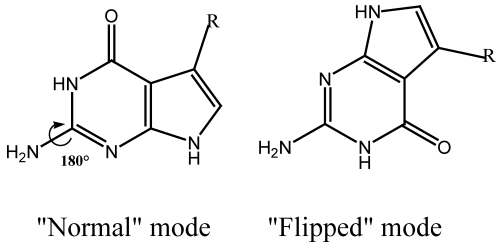


Fig. 8. Pemetrexed in Normal and Flipped Modes

activity. Other anti-proliferative mechanisms of action rather than DHFR inhibition could be responsible for this phenomenon such as thymidylate synthase inhibition.

As shown in Fig. 7, folate analogs that inhibit TS generally contain a 2-amino-4-oxo or 2-methyl-4-oxo substitution in their pyrimidine ring. For example PDDF(CB3717)[13] and raltitrexed.[14]

In contrast, inhibitors of DHFR generally contain a 2,4-diamino substitution in the pyrimidine ring[13,14] as typified by methotrexate (MTX). Gangjee *et al.*[15] have hypothesized that pemetrexed could bind to TS in a "normal" mode (Fig. 8) which is similar to way that classic TS substrates or inhibitors bind, or bind to DHFR in a "flipped" mode which is achieved by rotating the analogs in the normal binding mode by 180° about its NH_2–C_2 bond.

The alkylthio/arylalkylthio derivatives made in the present study could also be speculates to mimic the "Normal" and "Flipped" forms of pemetrexed (Fig. 9). In their normal form, they are similar to the normal form of pemetrexed and thus are expected to be DHFR inhibitors. By 180° rotating about C_6–NH_2 bond, the sulfur atom could mimic the oxo group of TS inhibitors. Therefore it is expected that these compounds have both TS inhibitor as well.

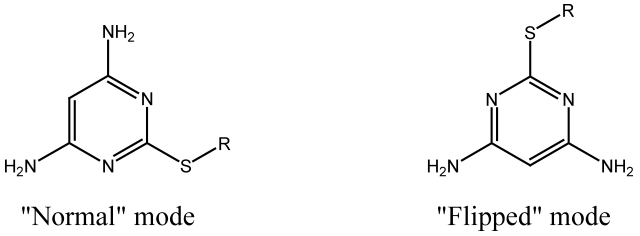


Fig. 9. 4,6-Diaminopyrimidines in Normal and Flipped Modes

Experimental

General All evaporations were carried out *in vacuo* with a rotary evaporator. Melting points (°C) were determined by capillary method on an electrothermal melting point apparatus. Infrared spectra were recorded as thin films on KBr plates with ν_{max} in inverse centimeters. Nuclear magnetic resonance spectra for proton (^{1}H-NMR) were recorded on a Bruker DRX-Avance (500 MHz) spectrometer to confirm the structure of synthesized compounds. Chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane (TMS) as internal standard; s=singlet, d=doublet, dd=double doublet, t=triplet, q=quartet, quin=quintet, sex=sextet, m=multiplet, brs=broad singlet. *J* values are

Table 1. Anti-proliferative Activity of Synthesized Compounds against 4 Human Tumor Cell Lines and Inhibitory Concentrations (IC_{50} in μM) of the Compounds against Human DHFR

Compound	EC_{50}[e] (μM)				Human DHFR inhibition (IC_{50}[f], μM)
	MCF-7[a]	HepG2[b]	SK-Hep1[c]	HeLa[d]	
4	>200	>200	>200	>200	21
5	>200	>200	>200	>200	18
6	>200	>200	>200	160	22
7	90	51	62	110	24
8	150	93	61	>200	>100
9	84	81	65	156	>100
10	>200	135	110	>200	23
11	121	93	85	147	>100
12	95	56	60	138	11
13	90	49	55	151	>100
14	92	65	58	117	19
15	66	41	42	93	9
16	37	19	15	24	22
17	58	25	21	35	18
18	108	61	53	83	19
19	178	121	105	>200	21
20	98	106	134	>200	13
21	63	58	48	93	19
22	36	18	12	45	14
23	65	39	45	93	12
24	80	75	67	115	17
25	60	32	31	28	12
Methotrexate	1.6	25	10	0.9	0.022

a) Human breast adenocarcinoma cell line. *b*) Human hepatocellular carcinoma cell line. *c*) Human liver adenocarcinoma cell line. *d*) Human cervical carcinoma cell line. *e*) EC_{50} value: the concentration, after which 50% of cells retain their clonogenic capacity and form colonies. *f*) IC_{50} value is the concentration of the compound where the activity of the enzyme is reduced by half.

expressed in Hertz. The NMR experiments (STD) were performed on a Bruker Avance III generation (800 MHz) spectrometer. Thin layer chromatography (TLC) was performed on Whatman Sil G/UV_{254} silica gel plates with fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Mass analyses were performed with an Agilent 6400 Series equipped with an electrospray ionization source (capillary voltage at 4000V, nebulizing gas temperature at 300°C, nebulizing gas flow at 12 L/min). All the compounds were analyzed for C, H, N, and S on a Costech model 4010 and agreed with the proposed structures within ±0.4% of the theoretical values.

Synthesis of 4,6-Diamino-pyrimidine-2-thiol (3)[10] To a freshly prepared solution of sodium ethoxide (884 mg, 13 mmol) in absolute ethanol, were added thiourea (**1**) (13 mmol) and malononitrile (**2**) (13 mmol). The mixture was refluxed for 3 h, and the precipitate was then filtered off. The solid was dissolved in water (10 mL), followed by adjusting pH around 7—38, and the resulted precipitate was filtered to give the pure product (**3**) as a white solid (75%).

Representative Procedure for the Preparation of Compounds (4—25)[10] To a solution of 4,6-diaminopyrimidine-2-thiol (**3**) (1.4 mmol) in methanol, was added alkyl halide (3.5 mmol) under basic conditions (NaOH 0.1 M, 14 mL). The mixture was then stirred for 18 h at room temperature. After removing the solvent under reduced pressure, the residue was washed by water and the precipitate was collected as a solid. All compounds were obtained in acceptable purity and no further purification was needed.

4,6-Diamino-pyrimidine-2-thiol (3) Yield 75%, mp 310°C (dec.); IR (KBr) ν_{max} 3430, 3345, 1688, 1315 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 5.05 (s, 1H, ArH), 6.48 (brs, 4H, NH_2), 10.85 (brs, 1H); electrospray ionization (ESI)-MS: Observed ($M+H^+$)=143. Calcd for $C_4H_6N_4S$=142.18. *Anal.* Found: C, 33.80; H, 4.25; N, 39.36; S, 22.59. Calcd for $C_4H_6N_4S$: C, 33.79; H, 4.25; N, 39.40; S, 22.55%.

2-(Methylthio)pyrimidine-4,6-diamine (4) Yield 85%, mp 170—172°C; IR (KBr) ν_{max} 3455, 3312, 3189, 1649, 1552, 1477, 1295, 935, 817 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 2.51(s, 3H, CH_3), 4.57 (brs, 4H, NH_2), 5.30 (s, 1H, ArH); ESI-MS: Observed ($M+H^+$)=157. Calcd for $C_5H_8N_4S$=156.21. Anal. Found: C, 38.36; H, 5.15; N, 35.90; S, 20.59. Calcd for $C_4H_6N_4S$: C, 38.44; H, 5.16; N, 35.87; S, 20.53%.

2-(Ethylthio)pyrimidine-4,6-diamine (5) Yield 83%, mp 142—144°C; IR (KBr) ν_{max} 3522, 3414, 3229, 1658, 1638, 1589, 1481, 1323, 1280, 991, 831, 620 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 1.90 (t, 3H, J=7.06 Hz, CH_3), 2.89 (q, 2H, J=7.30 Hz, CH_2), 4.86 (brs, 4H, NH_2), 5.16 (s, 1H, ArH); ESI-MS: Observed ($M+H^+$)=171. Calcd for $C_6H_{10}N_4S$=170.24. *Anal.* Found: C, 42.28; H, 5.93; N, 32.93; S, 18.86. Calcd for $C_4H_6N_4S$: C, 42.33; H, 5.92; N, 32.91; S, 18.84%.

2-(Propyllthio)pyrimidine-4,6-diamine (6) Yield 81%, mp 140—142°C; IR (KBr) ν_{max} 3458, 3330, 3163, 1651, 1477, 1312, 1235, 991, 819, 652 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.00 (t, 3H, J=7.34 Hz, CH_3), 1.70 (sex, 2H, J=7.32 Hz, S-$CH_2\underline{CH_2}$), 3.02 (t, 2H, J=7.20 Hz, S-$\underline{CH_2}CH_2$), 4.70 (brs, 4H, NH_2), 5.26 (s, 1H, ArH); ESI-MS: Observed ($M+H^+$)=185. Calcd for $C_7H_{12}N_4S$=184.26. *Anal.* Found: C, 45.60; H, 6.55; N, 30.43; S, 17.42. Calcd for $C_4H_6N_4S$: C, 45.63; H, 6.56; N, 30.41; S, 17.40%.

2-(Butylthio)pyrimidine-4,6-diamine (7) Yield 76%, mp 91—93°C; IR (KBr) ν_{max} 3442, 3319, 3163, 2940, 1645, 1578, 1471, 1315, 984, 932, 818, 647 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.97 (t, 3H, J=7.35 Hz, CH_3), 1.49 (sex, 2H, J=7.47 Hz, S-$CH_2CH_2\underline{CH_2}$), 1.71 (quin, 2H, J=7.23 Hz, S-$CH_2\underline{CH_2}$), 3.10 (t, 2H, J=7.33 Hz, S-$\underline{CH_2}$), 4.59 (brs, 4H, NH_2), 5.29 (s, 1H, ArH); ESI-MS: Observed (M+H$^+$)=199. Calcd for $C_8H_{14}N_4S$=198.29. *Anal.* Found: C, 48.40; H, 7.11; N, 28.28; S, 16.21. Calcd for $C_4H_6N_4S$: C, 48.46; H, 7.12; N, 28.26; S, 16.17%.

2-(Pentylthio)pyrimidine-4,6-diamine (8) Yield 74%, mp 92—94°C; IR (KBr) ν_{max} 3500, 3372, 3188, 2947, 1614, 1585, 1468, 1316, 1249, 985, 823 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.93 (t, 3H, J=7.21 Hz, CH_3), 1.36—1.47 (m, 4H, S-$CH_2CH_2CH_2\underline{CH_2}$), 1.72 (quin, 2H, J=7.47 Hz, S-$CH_2\underline{CH_2}$), 3.09 (t, 2H, J=7.34 Hz, S-$\underline{CH_2}$), 4.64 (brs, 4H, NH_2), 5.28 (s, 1H, ArH); ESI-MS: Observed (M+H$^+$)=213. Calcd for $C_9H_{16}N_4S$=212.32. *Anal.* Found: C, 50.94; H, 7.60; N, 26.37; S, 15.09. Calcd for $C_4H_6N_4S$: C, 50.91; H, 7.60; N, 26.39; S, 15.10%.

2-(Hexylthio)pyrimidine-4,6-diamine (9) Yield 68%, mp 80—82°C; IR (KBr) ν_{max} 3487, 3191, 2938, 2857, 1644, 1566, 1464, 1242, 936, 805, 606 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 0.93 (t, 3H, J=6.74 Hz, CH_3), 1.33—1.35 (m, 4H, S-$CH_2CH_2CH_2\underline{CH_2CH_2}$), 1.46 (quin, 2H, J=7.46 Hz, S-CH2$CH_2\underline{CH_2}$), 1.72 (quin, 2H, J=7.45 Hz, S-$CH_2\underline{CH_2}$), 3.08 (t, 2H, J=7.35 Hz,S-$\underline{CH_2}$), 4.61 (brs, 4H, NH_2), 5.28 (s, 1H, ArH); ESI-MS: Observed (M+H$^+$)=227. Calcd for $C_{10}H_{18}N_4S$=226.34. *Anal.* Found: C, 52.98; H, 8.03; N, 24.77; S, 14.22. Calcd for $C_4H_6N_4S$: C, 53.06; H, 8.02; N, 24.75; S, 14.17%.

2-(Cyclobutylmethylthio)pyrimidine-4,6-diamine (10) Yield 70%, mp 107—109°C; IR (KBr) ν_{max} 3484, 3358, 3154, 2979, 1633, 1580, 1470, 1316, 1250, 991, 942, 820 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.65—1.69 (m, 2H, CH_2), 1.77—1.83 (m, 2H, CH_2), 1.99—2.05 (m, 2H, CH_2), 2.54 (d, 1H, J=7.50 Hz, CH), 3.06 (d, 2H, J=7.53 Hz, S-$\underline{CH_2}$), 5.17 (s, 1H, ArH), 6.03 (brs, 4H, NH_2); ESI-MS: Observed (M+H$^+$)=211. Calcd for $C_9H_{14}N_4S$=210.3. *Anal.* Found: C, 51.35; H, 6.70; N, 26.67; S, 15.28. Calcd for $C_4H_6N_4S$: C, 51.40; H, 6.71; N, 26.64; S, 15.25%.

2-(2-Morpholinoethylthio)pyrimidine-4,6-diamine (11) Yield 65%, mp 270°C (dec.); IR (KBr) ν_{max} 3400, 3168, 2832, 1654, 1583, 1305, 1121, 987, 874, 820 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.42—2.44 (m, 4H, CH_2), 3.07 (t, 2H, J=7.60 Hz, S-CH_2-$\underline{CH_2}$), 3.33 (s, 2H, S-$\underline{CH_2}$-CH_2), 3.57 (t, 4H, J=4.80 Hz, CH_2), 5.13 (s, 1H, ArH), 6.06 (brs, 4H, NH_2); ESI-MS: Observed (M+H$^+$)=256. Calcd for $C_{10}H_{17}N_5OS$=255.34. *Anal.* Found: C, 47.00; H, 6.69; N, 27.46; S, 12.57. Calcd for $C_4H_6N_4S$: C, 47.04; H, 6.71; N, 27.43; S, 12.56%.

2-(2-Methylbenzylthio)pyrimidine-4,6-diamine (12) Yield 69%, mp 165—167°C ; IR (KBr) ν_{max} 3478, 3354, 3146, 1649, 1547, 1468, 1304, 1245, 981, 932, 815, 782, 688 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 2.33 (s, 3H, CH_3), 4.25 (s, 2H, S-CH_2), 5.16 (s, 1H, pyrimidine-CH), 6.12 (brs, 4H, NH_2), 7.09—7.17 (m, 3H, ArH), 7.40 (d, 1H, J=7.18 Hz, ArH); ESI-MS: Observed (M+H$^+$)=247. Calcd for $C_{12}H_{14}N_4S$=246.33. *Anal.* Found: C, 58.46; H, 5.75; N, 22.75; S, 13.04. Calcd for $C_{12}H_{14}N_4S$: C, 58.51; H, 5.73; N, 22.74; S, 13.02%.

2-(3-Methylbenzylthio)pyrimidine-4,6-diamine (13) Yield 75%, mp 115—117°C ; IR (KBr) ν_{max} 3462, 3367, 3154, 1643, 1548, 1304, 814, 713, 641 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 2.38 (s, 3H, CH_3), 4.21 (s, 2H, S-CH_2), 5.16 (s, 1H, pyrimidine-CH), 6.14 (brs, 4H, NH_2), 7.03 (d, 1H, J=6.8 Hz, ArH), 7.15—7.21 (m, 3H, ArH); ESI-MS: Observed (M+H$^+$)=247. Calcd for $C_{12}H_{14}N_4S$=246.33. *Anal.* Found: C, 58.46; H, 5.75; N, 22.75; S, 13.04. Calcd for $C_{12}H_{14}N_4S$: C, 58.51; H, 5.73; N, 22.74; S, 13.02%.

2-(2-Nitrobenzylthio)pyrimidine-4,6-diamine (14) Yield 73%, mp 164—166°C; IR (KBr) ν_{max} 3498, 3457, 3390, 1600, 1514, 1350, 959, 751, 704 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.52 (s, 2H, S-CH_2), 5.15 (s, 1H, pyrimidine-CH), 6.16 (brs, 4H, NH_2), 7.50 (t, 1H, J=8.2 Hz, ArH), 7.65 (t, 1H, J=7.6 Hz, ArH), 7.89 (dd, 1H, J_1=7.8 Hz, J_2=0.8 Hz, ArH), 7.99 (dd, 1H, J_1=8.2 Hz, J_2=1.2 Hz, ArH); ESI-MS: Observed (M+H$^+$)=278. Calcd for $C_{11}H_{11}N_5O_2S$=277.3. *Anal.* Found: C, 47.68; H, 4.01; N, 25.25; S, 11.54. Calcd for $C_{11}H_{11}N_5O_2S$: C, 47.64; H, 4.00; N, 25.26; S, 11.56%.

2-(2-Chlorobenzylthio)pyrimidine-4,6-diamine (15) Yield 75%, mp 146—148°C; IR (KBr) ν_{max} 3475, 3354, 3147, 1642, 1574, 1456, 1240, 1052, 980, 931, 812, 774, 748, 674 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 4.33 (s, 2H, S-CH_2), 5.16 (s, 1H, pyrimidine-CH), 6.16 (brs, 4H, NH_2), 7.25—7.28 (m, 2H, ArH), 77.43—7.45 (m, 1H, ArH), 7.67—7.70 (m, 1H, ArH); ESI-MS: Observed (M+H$^+$)=267.6. Calcd for $C_{11}H_{11}ClN_4S$=266.75. *Anal.* Found: C, 49.50; H, 4.15; N, 21.06; S, 11.96. Calcd for $C_{11}H_{11}ClN_4S$: C, 49.53; H, 4.16; N, 21.00; S, 12.02%.

2-(2-Fluorobenzylthio)pyrimidine-4,6-diamine (16) Yield 77%, mp 147—149°C; IR (KBr) ν_{max} 3499, 3462, 3388, 3298, 3148, 1647, 1463, 1305, 1227, 978, 812, 760 cm^{-1}; ^{1}H-NMR (500 MHz, $CDCl_3$) δ: 4.41 (s, 2H, S-CH_2), 4.63 (brs, 4H, NH_2), 5.29 (s, 1H, pyrimidine-CH), 7.03—7.11 (m, 2H, ArH), 7.23 (d, 1H, J=6.20 Hz, ArH), 7.55 (t, 1H, J=7.52 Hz, ArH); ESI-MS: Observed (M+H$^+$)=251. Calcd for $C_{11}H_{11}FN_4S$=250.3. *Anal.* Found: C, 52.75; H, 4.41; N, 22.40; S, 12.83. Calcd for $C_{11}H_{11}FN_4S$: C, 52.78; H, 4.43; N, 22.38; S, 12.81%.

2-(3-Fluorobenzylthio)pyrimidine-4,6-diamine (17) Yield 75%, mp 132—134°C; IR (KBr) ν_{max} 3500, 3468, 3372, 3307, 3159, 1648, 1610, 1547, 1463, 1311, 1141, 934, 816, 799 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.25 (s, 2H, S-CH_2), 5.17 (s, 1H, pyrimidine-CH), 6.18 (brs, 4H, NH_2), 7.01—7.06 (m, 1H, ArH), 7.25—7.35 (m, 3H, ArH); ESI-MS: Observed (M+H$^+$)=251. Calcd for $C_{11}H_{11}FN_4S$=250.3. *Anal.* Found: C, 52.75; H, 4.41; N, 22.40; S, 12.83. Calcd for $C_{11}H_{11}FN_4S$: C, 52.78; H, 4.43; N, 22.38; S, 12.81%.

2-(4-Fluorobenzylthio)pyrimidine-4,6-diamine (18) Yield 69%, mp 176—178°C; IR (KBr) ν_{max} 3510, 3476, 3401, 3317, 3172, 1647, 1550, 1470, 1315, 1163, 849, 819 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 4.22 (s, 2H, S-CH_2), 5.15 (s, 1H, pyrimidine-CH), 6.13 (brs, 4H, NH_2), 7.07—7.12 (m, 2H, ArH), 7.44—7.46 (m, 2H, ArH); ESI-MS: Observed (M+H$^+$)=251. Calcd for $C_{11}H_{11}FN_4S$=250.3. *Anal.* Found: C, 52.75; H, 4.41; N, 22.40; S, 12.83. Calcd for $C_{11}H_{11}FN_4S$: C, 52.78; H, 4.43; N, 22.38; S, 12.81%.

2-(3-Methoxybenzylthio)pyrimidine-4,6-diamine (19) Yield 70%, mp 118—120°C; IR (KBr) ν_{max} 3459, 3359, 3155, 1663, 1402, 1149, 1052, 980, 930, 882, 725, 687 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 3.37 (s, 3H, CH_3), 4.22 (s, 2H, S-CH_2), 5.16 (s, 1H, pyrimidine-CH), 6.13 (brs, 4H,

NH_2), 6.78 (dd, 1H, J_1=8.17 Hz, J_2=2.41 Hz, ArH), 6.96 (d, 1H, J=7.56 Hz, ArH), 7.00 (s, 1H, ArH), 7.19 (t, 1H, J=7.76 Hz, ArH); ESI-MS: Observed ($M+H^+$)=263. Calcd for $C_{12}H_{14}N_4OS$=262.33. *Anal.* Found: C, 54.91; H, 5.37; N, 21.38; S, 12.23. Calcd for $C_{12}H_{14}N_4OS$: C, 54.94; H, 5.38; N, 21.36; S, 12.22%.

2-(4-Cyanobenzylthio)pyrimidine-4,6-diamine (20) Yield 65%, mp 200—202°C; IR (KBr) ν_{max} 3484, 3361, 3296, 3136, 2220, 1626, 1565, 1543, 1470, 1240, 976, 878, 644 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.29 (s, 2H, S-CH_2), 5.15 (s, 1H, pyrimidine-CH), 6.18 (br s, 4H, NH_2), 7.63 (d, 2H, J=8.4 Hz, ArH), 7.75 (d, 2H, J=8.4 Hz, ArH); ESI-MS: Observed ($M+H^+$)=258. Calcd for $C_{12}H_{11}N_5S$=257.31. *Anal.* Found: C, 56.04; H, 4.32; N, 27.21; S, 12.43. Calcd for $C_{12}H_{11}N_5S$: C, 56.01; H, 4.31; N, 27.22; S, 12.46%.

2-(3-(Trifluoromethyl)benzylthio)pyrimidine-4,6-diamine (21) Yield 65%, mp 146—148°C; IR (KBr) ν_{max} 3497, 3467, 3395, 3305, 3189, 1648, 1553, 1465, 1336, 1245, 1173, 1121, 916, 813, 705 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.34 (s, 2H, S-CH_2), 5.18 (s, 1H, pyrimidine-CH), 6.19 (br s, 4H, NH_2), 7.50—7.58 (m, 2H, ArH), 7.75—7.79 (m, 2H, ArH); ESI-MS: Observed ($M+H^+$)=301. Calcd for $C_{12}H_{11}F_3N_4S$=300.3. *Anal.* Found: C, 47.96; H, 3.68; N, 18.68; S, 10.69. Calcd for $C_{12}H_{11}F_3N_4S$: C, 47.99; H, 3.69; N, 18.66; S, 10.68%.

2-(Phenylethylthio)pyrimidine-4,6-diamine (22) Yield 71%, mp 154—156°C; IR (KBr) ν_{max} 3503, 3415, 3382, 3323, 3143, 1657, 1629, 1582, 1471, 1316, 1251, 992, 819, 777 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 2.89 (t, 2H, J=8.32 Hz, S-CH_2-CH_2), 3.17 (t, 2H, J=8.35 Hz, S-CH_2-CH_2), 5.16 (s, 1H, pyrimidine-CH), 6.08 (br s, 4H, NH_2), 7.20—7.22 (m, 1H, ArH), 7.29—7.31 (m, 4H, ArH); ESI-MS: Observed ($M+H^+$)=247. Calcd for $C_{12}H_{14}N_4S$=246.33. *Anal.* Found: C, 58.48; H, 5.74; N, 22.76; S, 13.02. Calcd for $C_{12}H_{14}N_4S$: C, 58.51; H, 5.73; N, 22.74; S, 13.02%.

2-(3-Phenylpropylthio)pyrimidine-4,6-diamine (23) Yield 65%, mp 118—120°C; IR (KBr) ν_{max} 3473, 3354, 3140, 1638, 1589, 1466, 1286, 993, 827, 762 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.90 (quin, 2H, J=7.2 Hz, S-CH_2-CH_2-CH_2), 2.69 (t, 2H, J=7.2 Hz, S-CH_2-CH_2-CH_2), 2.96 (t, 2H, J=7.2 Hz, S-CH_2-CH_2-CH_2), 5.14 (s, 1H, pyrimidine-CH), 6.05 (br s, 4H, NH_2), 7.16—7.22 (m, 3H, ArH), 7.27—7.31 (m, 2H, ArH); ESI-MS: Observed ($M+H^+$)=261. Calcd for $C_{13}H_{16}N_4S$=260.36. *Anal.* Found: C, 59.95; H, 6.20; N, 21.53; S, 12.32. Calcd for $C_{13}H_{16}N_4S$: C, 59.97; H, 6.19; N, 21.52; S, 12.32%.

2-((Naphthalen-1-yl)methylthio)pyrimidine-4,6-diamine (24) Yield 55%, mp 190—192°C; IR (KBr) ν_{max} 3520, 3452, 3412, 3327, 3146, 1657, 1619, 1474, 1315, 1297, 800 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 4.78 (s, 2H, S-CH_2), 5.23 (s, 1H, pyrimidine-CH), 6.24 (br s, 4H, NH_2), 7.41—7.53 (m, 3H, ArH), 7.68 (d, 1H, J=6.8 Hz, ArH), 7.83 (d, 1H, J=8.00 Hz, ArH), 7.94 (d, 1H, J=7.8 Hz, ArH), 8.14 (d, 1H, J=8.00 Hz, ArH); ESI-MS: Observed ($M+H^+$)=283. Calcd for $C_{15}H_{14}N_4S$=282.36. *Anal.* Found: C, 63.83; H, 5.01; N, 19.82; S, 11.34. Calcd for $C_{15}H_{14}N_4S$: C, 63.80; H, 5.00; N, 19.84; S, 11.36%.

2-(2-(1-Methylpiperidin-2-yl)ethylthio)pyrimidine-4,6-diamine (25) Yield 65%, mp 165—167°C; IR (KBr) ν_{max} 3468, 3416, 3302, 3159, 2937, 2788, 1630, 1562, 1460, 1297, 1235, 972, 811 cm^{-1}; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 1.21—1.97 (m, 10H, piperidin-CH_2, S-CH_2-CH_2), 2.16 (s, 3H, CH_3), 2.72—2.75 (m, 1H, piperidin-CH), 2.82—2.88 (m, 1H, S-CH_2), 2.97—3.03 (m, 1H, S-CH_2), 5.14 (s, 1H, ArH), 6.00 (br s, 4H, NH_2); ESI-MS: Observed ($M+H^+$)=268. Calcd for $C_{12}H_{21}N_5S$=267.39. *Anal.* Found: C, 53.88; H, 7.91; N, 26.21; S, 12.00. Calcd for $C_{12}H_{21}N_5S$: C, 53.90; H, 7.92; N, 26.19; S, 11.99%.

Enzyme Inhibition Assay The DHFR enzyme assay is a sensitive and specific method to screen DHFR inhibitors. The assay is based on the ability of dihydrofolate reductase to catalyze the reversible NADPH-dependent reduction of dihydrofolic acid to tertrahydrofolic acid.

$$DHF + NADPH + H^+ \overset{DHFR}{\rightleftharpoons} THF + NADP^+$$

The reduction progress is monitored by the decrease in absorbance at 340 nm that occurs when NADPH is converted to $NADP^+$.

In this study, DHFR enzyme kit from Sigma-Aldrich was used. The spectrophotometer was set at 340 nm, 22°C, and kinetic program (reading every 15 s for 5 min). To a solution of assay buffer (1X) and human DHFR, was added NADPH and mixed well. The test compounds were dissolved in dimethyl sulfoxide (DMSO) and added to the mixture in different concentrations. The final concentration of DMSO in each experiment was 0.4%. The changes in absorbance at 340 nm were followed using the test compounds and methotrexate (as a positive control). The activity under these conditions was linear for 5 min. Results were obtained as % inhibition of enzymatic activity calculated using the following formula:

$$\%\ \text{Inhibition} = \left[1 - \frac{\Delta OD/\min\ (\text{test})}{\Delta OD/\min\ (\text{blank})}\right] \times 100$$

The % inhibition values were plotted *versus* compounds concentrations. The 50% inhibitory concentration (IC_{50}) of each compound was obtained using the GraphPad Prism 3 program.

NMR Experiment (STD) Samples were prepared by dissolving DHFR and a quantity of ligands (**12**, **15**) to give a protein : ligand mole ratio of 1 : 100 in deuterated sodium phosphate buffer, pH 7.3. Prior to use, DHFR was dialyzed extensively and the ligand was dissolved into PBS buffer. All the NMR experiments were conducted on Bruker Avance DRX 800-MHz spectrometer using 5-mm inverse triple resonance (^{15}N/^{13}C/^{1}H) probes equipped with triple axis actively shielded gradient. STD spectra were collected with 16 K data points to cover a sweep width of 10 ppm.

The protein was saturated on its methyl signals around 0.9 ppm at a frequency of 7000 Hz and off-resonance at 20000 Hz with a cascade of 40 selective Gaussian-shaped pulses of 50 ms duration with a 100 μs delay between each pulse, resulting in a total saturation time of 2 s.

STD NMR spectra were recorded at 303 K and 298 K. The watergate pulse train was used to suppress the H_2O signal. The spectra were processed using Bruker xwinnmr. A cosine function prior to zero-filling by a factor of 2 was applied before fourier transform. STD spectrum was obtained by subtraction of saturated spectrum from the reference spectrum and STD intensity of individual signal was measured relative to the corresponding signal intensity in the reference spectrum.

Docking Study The crystal structure of human DHFR

complexed with COP (*N*-(4-carboxy-4-{4-[(2,4-diaminopteridin-6-ylmethyl)-amino]-benzoylamino}-butyl)-phthalamic acid, and NADPH (PDB ID code 1OHJ) was downloaded from protein data bank and the surrounding important residues such as Ile 7, Glu 30, Leu 22, Phe 31, Arg 28, Arg 32, Glu 35, Asn 64, Arg 70, Val 115, Tyr 121, Tyr 136 were recognized and the native ligand was extracted to leave a cavity. Thereafter, the docking simulations were carried out with and without cofactor NADPH and water molecules, to elucidate the role of NADPH and water molecules for the binding of 4,6-diamionpyrimidine derivatives. All compounds were drawn and optimized using Hyperchem 8.0.3 software. The protein and ligands were saved in pdbqs and pdbqt format respectively. Docking was performed as in the AutoDock 3.05 manual and the results were visualized using AutoDock tools. Binding affinities were calculated and the highly ranked compounds were selected and docking was repeated for these selected compounds to confirm their affinity.

The grid maps representing the native ligand in the actual docking target site were calculated. The grid box dimensions were chosen to be sufficiently large to include all the previously mentioned residues.

Antiproliferative Activity (Clonogenic Assay) Four cell lines consisting of HepG2 (human hepatocellular liver carcinoma), MCF-7 (human breast adenocarcinoma), SKHep1 (human liver adenocarcinoma), and Hela (human cervical carcinoma) which had been verified to be negative for mycoplasma contamination were used in this experiment. To apply the method of clonogenic assay,[16] cells were plated in 6-well plates (200 cells/well) for 24 h before treatment with the test compounds to allow attachment of the cells to wells. Seven different concentrations of each compound, methotrexate (as reference), and DMSO 0.5% (applied solvent to dissolve the compound) were then added to the monolayer cells in triplicates. The plates were incubated for 10 d at 37°C in atmosphere of 5% CO_2. The media were removed after 10 d and the formed colonies were stained with a solution of 0.5% crystal violet in ethanol for 10 min and the number of colonies containing more than 50 cells was counted under microscope. The relation between the number of the colonies (as a percentage of the control containing DMSO 0.5%) and the concentrations of each compound was plotted to get survival curve of the cell lines and EC_{50} values were calculated.

Conclusion

A series of 4,6-diaminopyrimidine derivatives bearing various alkyl side chains at position 2 of the ring *via* a sulfur atom were tested for their DHFR inhibitory activity. The compounds with the lowest IC_{50} values demonstrated high affinity to human DHFR enzyme in STD studies. In light of the fact that 4,6-diaminopyrimidine derivatives synthesized in the present study which show acceptable DHFR inhibition and their binding to the enzyme which is confirmed by STD ^{1}H-NMR studies, could be concluded that 4,6-diaminopyrimidine could be considered as new scaffold for design and synthesis of novel DHFR inhibitors. Lipophilic thioether side chains could help to improve the ligand-receptor binding in these group of compounds. Anti-proliferative activity of the compounds were also determined against tumor cell lines. Some of the compounds, despite their high IC_{50} against DHFR enzyme, showed good anti-proliferative activity which could be attributed to the other possible mechanisms of anti-proliferative activity rather than DHFR inhibition.

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