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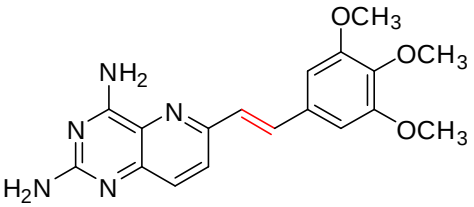
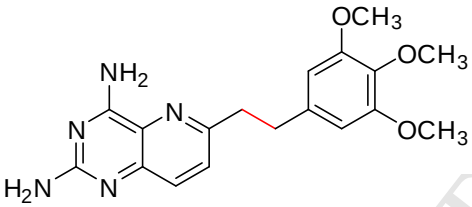
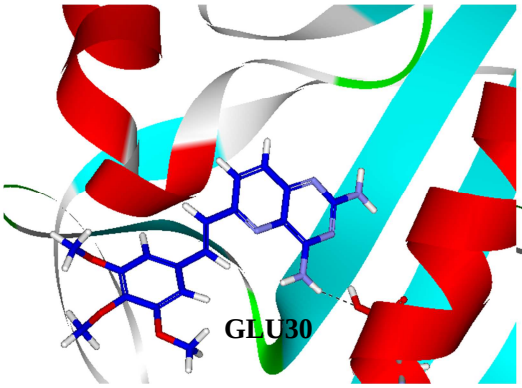
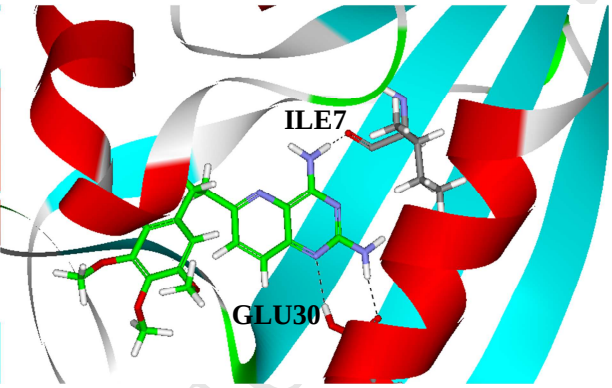


IC_{50}=4.00±0.24 μM



IC_{50}=0.29±0.02 μM





Synthesis and antiproliferative activity of a series of novel 6-substituted pyrido[3,2-*d*]pyrimidines as potential nonclassical lipophilic antifolates targeting dihydrofolate reductase

Meng Wang[a], Jiajia Yang[a], Mengmeng Yuan[a], Liangmin Xue[a], Hao Li[a], Chao Tian[a], Xiaowei Wang[a], Junyi Liu [a,b,**], Zhili Zhang [a*]

[a]*Department of Chemical Biology, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China*
[b]*State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China*

Abstract

Dihydrofolate reductase (DHFR) has been a well-recognized target for the treatment of many diseases. Based on 8,10-dideazaminopterins, which are classical antifolates that potently inhibit DHFR, we have designed a series of novel 2,4-diamino-6-substituted pyrido[3,2-*d*]pyrimidines. By removing the glutamate moiety and introducing lipophilic groups, we hoped to improve passive diffuse through the cell membranes. The target compounds were efficiently synthesized using one-pot procedure and evaluated in vitro for DHFR inhibition and antitumor activity. Compounds **5e**, **5h**, **5i** and **5k** were the most potent inhibitors of recombinant human DHFR (rhDHFR) with IC_{50} values in the range 0.2–1.0 μM. Analysis using flow cytometric indicated that the effect of compound **5k** on cell cycle progression was linked to induction of S phase arrest. Compounds **5g**, **5h**, **5i** and **5k** showed broad spectrum antitumor activity against four different tumor cell lines, with IC_{50} values in the range 0.07–23 μM. Molecular docking investigations showed that the trimethoyphenyl ring of compound **5k** occupied a position near the cofactor-binding site in the rhDHFR-inhibitor complex, with close intermolecular contacts with Asp21, Phe31, Ser59, Ile60 and Pro61.

Keywords: pyrido[3,2-*d*]pyrimidines, antifolate, dihydrofolate reductase inhibitor, anticancer

1. Introduction

Dihydrofolate reductase (DHFR) catalyzes the reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate, using reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. DHFR plays a major role in the biosynthesis of nucleic acids and has become an important target for the treatment of malignant, microbial, parasitic and chronic inflammatory diseases [1–8].

Classical antifolates, such as methotrexate (MTX) [9], raltitrexed (RTX) [10], pemetrexed (PMX) [11] and pralatrexate [12] (**Fig.1**), are analogs of folate containing a pterin ring, an aromatic ring and a glutamate tail. Because of the charged glutamate tail, these inhibitors do not passively diffuse across cell membranes and must be actively transported using the reduced folate carrier system, which entails folate receptors (FR) [13], the reduced folate carrier [14], and/or the proton-coupled folate transporters [15]. Impaired active transport into cells and polyglutamylation of classic antifolate lead to drug resistance [16–18]. To overcome this, nonclassical antifolates, which are liphphilic molecules that passively diffuse into cells and without the need for folate transport systems, have been developed. Lipophilic nonclassical antifolates, such as piritrexim [19], trimetrexate [20] and nolatrexed [21] (**Fig. 1**), have demonstrated significant growth inhibition of tumor cell.

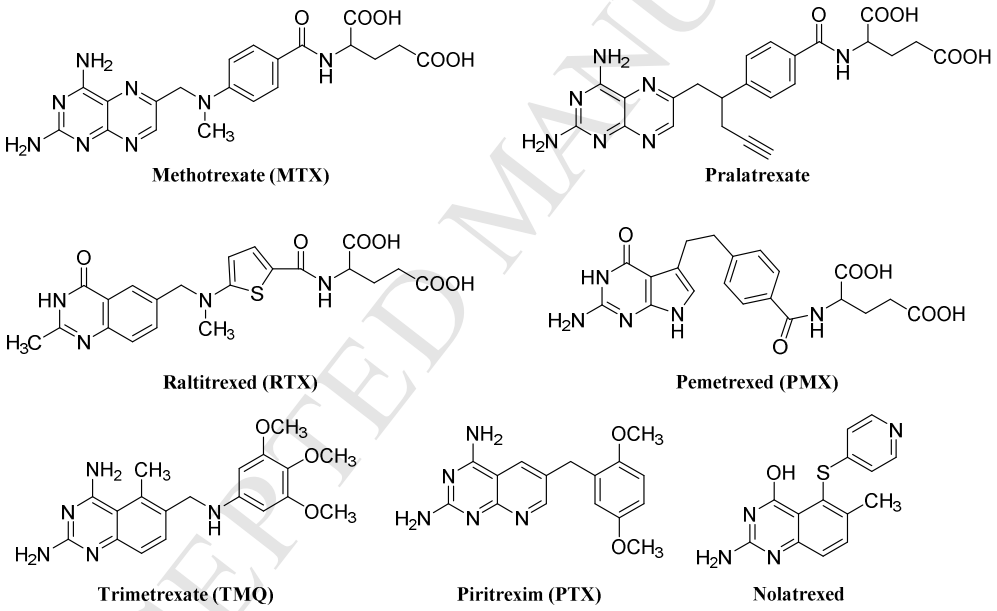


Fig. 1 Representative examples of classical and nonclassical antifolates.

Derivatives of 8,10-dideazaminopterin (**Fig. 2**) are very potent inhibitors of DHFR, but these compounds share the shortcomings of classical antifolates because of their folate-like structures [22]. There has been a continuing effort in our laboratory to develop novel nonclassical antifolates based on 8,10-dideazaminopterins. The aim of the present study was to design and synthesize more lipophilic derivatives that lack a glutamate tail and to explore structure-activity relationships within a series of 6-substituted pyrido[3,2-*d*]pyrimidines. Compounds that inhibit recombinant human DHFR (rhDHFR) may be suitable for treating cancer.

We have now developed an efficient synthesis of 6-phenethyl and 6-phenylethenylpyrido[3,2-*d*]pyrimidines. In addition to removal of the glutamate moiety, different

lipophilic substituents were introduced into the benzene ring to improve membrane permeability. To identify more effective anticancer agents, and to investigate the structure activity relationships of 6-phenylethenyl and phenethyl derivatives of 2,4-diaminopyrido[3,2-*d*]pyrimidine, both electron-donating substituents (methyl, ethyl, isopropyl, tert-butyl, methoxy) and electron-withdrawing (halogen) groups were chosen as the substituent **R**. Different substituents were attached at the same position on the benzene ring and same substituent was attached at different positions on the benzene ring to examine both electronic and spatial effects of substitution on antiproliferative activity. The activities of compounds containing carbon-carbon double bonds and single bonds were compared to explore the effect of conformational flexibility on activity against rhDHFR. The antiproliferative activity of the compounds was evaluated in four different cancer cell lines. Molecular docking studies were conducted to elucidate the structure-activity relationships.

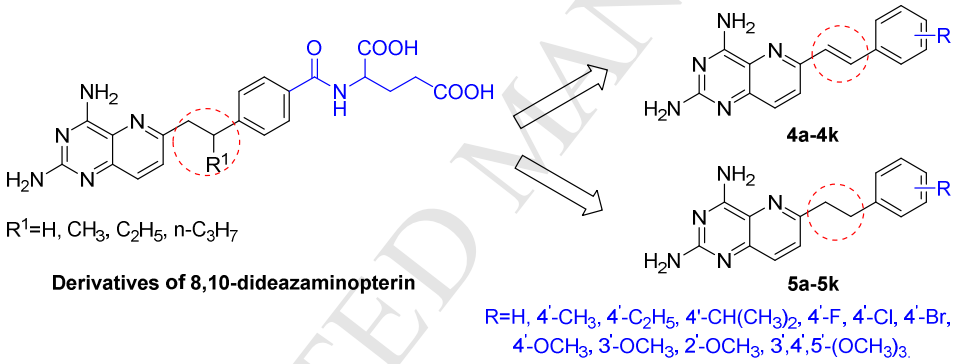
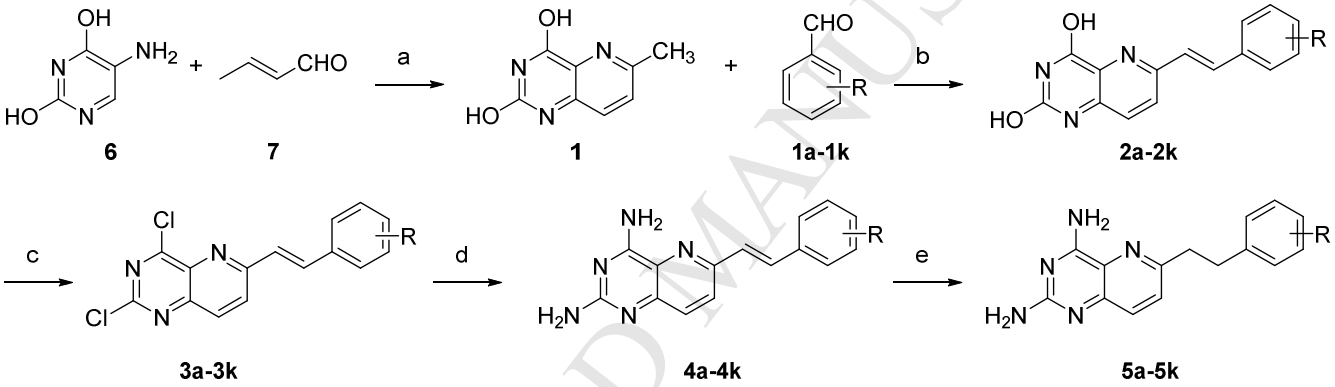


Fig. 2 Design of 6-substituted pyrido[3,2-*d*]pyrimidines **4a-4k** and **5a-5k**.

2. Chemistry

The key to the synthesis of the target compounds is carbon-carbon bond formation, an important and challenging procedure in organic synthesis. Previously reported syntheses of the carbon-carbon bond of 8,10-dideazaaminopterins involve reaction of 2,4-diamino-6-bromomethylpyrido[3,2-*d*]pyrimidine with methyl phenylacetate or benzaldehyde derivatives [22-24], but activation of 6-methyl group of pteridine to 6-bromomethyl is a multi-step procedure [24,25]. We have previously reported we an efficient one-pot reaction for construction of carbon-carbon bonds through C(sp^3)-H bond functionalization of 2,4-dihydroxy-6-methylpyrido[3,2-*d*]pyrimidine [26]. The synthetic strategy to obtain targets of general formulae **4** and **5** (**Fig. 2)** is depicted in **Scheme 1**. Commercially available 5-aminouracil (**6**) and crotonaldehyde were cyclized in 20% HCl using the Skraup reaction to give

2,4-dihydroxy-6-methylpyrido[3,2-*d*]pyrimidine (**1**) as previously described [27]. Compound **1** was then reacted with substituted benzaldehydes in the presence of *p*-toluenesulfonamide using *N*,*N*-dimethylacetamide as solvent to form (*E)*-2,4-dihydroxy-6-phenylethylenylpyrido[3,2-*d*]pyrimidines (**2a-2k**). Intermediates (**2a-2k**) were treated with an excess of $POCl_3$ in the presence of catalytic $(C_2H_5)_3N$ to provide the 2,4-dichloro derivatives (**3a-3k)**. Conversion of **3a-3k** to the corresponding 2,4-diamino derivatives (**4a-4k**) was achieved using a saturated solution of ammonia in dry methanol in a sealed vessel at 150 °C for 8 h. The products were isolated as light yellow solids. Hydrogenation of the carbon-carbon double bonds in compounds **4a-4k** was carried in ethanol using 10% Pd/C as the catalyst.



1a-5a: R=H, **1b-5b:** R=4'-CH_3, **1c-5c:** R=4'-C_2H_5, **1d-5d:** R=4'-$CH(CH_3)_2$, **1e-5e:** R=4'-F, **1f-5f:** R=4'-Cl, **1g-5g:** R=4'-Br, **1h-5h:** R= 4'-OCH_3, **1i-5i:** R=3'-OCH_3, **1j-5j:** R= 2'-OCH_3, **1k-5k: R=** 3',4',5'-$(OCH_3)_3$

Scheme 1. Reagents and conditions: (a) 20% HCl, reflux, 4 h (b) *p*-toluenesulfonamide, *N*,*N*-dimethylacetamide, 160 ^oC, 36 h; (c) $POCl_3$, Et_3N, reflux, 8 h; (d) saturated NH_3 in dry methanol, 150 °C, 8 h; (e) Pd/C, ethanol, rt, 12 h.

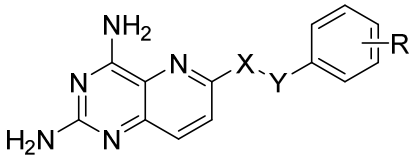
3. Results and discussion

3.1. In vitro antitumor screening

The antiproliferative activities of the compounds were evaluated using four human tumor cell lines: HL-60, HeLa, A549 and H1299.

Table 1

IC_{50} values (μM)[a] of 6-substituted pyrido[3,2-*d*]pyrimidines **4a-4k** and **5a-5k** against HL-60, HeLa, A549 and H1299 cell lines.



Compounds	X-Y	R	HL-60	HeLa	A549	H1299

4a	CH=CH	H	3.85±0.05	13.4±0.9	13.9±0.7	15.2±0.4
4b	CH=CH	4'-CH_3	4.54±0.50	14.5±3.1	18.9±1.5	ND
4c	CH=CH	4'-C_2H_5	1.19±0.04	9.86±0.48	9.13±0.24	4.86±0.11
4d	CH=CH	4'-$CH(CH_3)_2$	1.04±0.23	10.5±0.1	10.7±0.9	11.2±0.3
4e	CH=CH	4'-F	2.61±0.04	7.98±0.17	6.78±0.06	5.99±0.06
4f	CH=CH	4'-Cl	3.80±0.40	7.60±0.85	8.17±0.96	9.39±0.94
4g	CH=CH	4'-Br	2.51±0.84	9.20±0.47	11.9±0.9	6.19±0.02
4h	CH=CH	4'-OCH_3	1.63±0.13	6.50±0.34	5.68±0.21	3.73±0.27
4i	CH=CH	3'-OCH_3	2.03±0.04	18.8±0.9	9.10±0.72	12.2±0.5
4j	CH=CH	2'-OCH_3	3.60±0.28	13.8±0.6	7.56±0.24	8.63±0.13
4k	CH=CH	3',4',5'-$(OCH_3)_3$	2.33±0.59	2.10±0.01	5.34±0.10	3.99±0.23
5a	CH_2CH_2	H	0.36±0.01	8.61±0.15	9.78±0.45	9.16±0.36
5b	CH_2CH_2	4'-CH_3	0.31±0.03	52.4±1.5	34.1±0.9	1.61±0.09
5c	CH_2CH_2	4'-C_2H_5	0.21±0.02	49.4±1.8	15.4±0.7	2.76±0.01
5d	CH_2CH_2	4'-$CH(CH_3)_2$	0.49±0.07	14.0±2.4	15.9±1.3	ND
5e	CH_2CH_2	4'-F	0.58±0.03	54.0±2.1	4.98±0.46	14.8±2.0
5f	CH_2CH_2	4'-Cl	0.27±0.09	5.30±0.07	16.3±1.5	1.06±0.04
5g	CH_2CH_2	4'-Br	0.12±0.01	4.30±0.81	22.6±2.1	1.49±0.03
5h	CH_2CH_2	4'-OCH_3	0.0720±0.009	10.5±0.2	18.0±0.3	2.15±0.13
5i	CH_2CH_2	3'-OCH_3	0.42±0.01	14.3±0.2	14.4±0.4	7.03±0.05
5j	CH_2CH_2	2'-OCH_3	0.80±0.01	10.4±0.1	29.9±0.8	4.73±0.21
5k	CH_2CH_2	3',4',5'-$(OCH_3)_3$	0.42±0.01	2.90±0.20	11.6±0.5	2.17±0.04
MTX			0.0227±0.0004	>100	0.0135±0.0020	0.0720±0.0023
5-FU			1.62±0.03	62.2±1.2	ND	8.91±0.11

[a]Calculated from three replicates

ND (not detected)

Both the position and nature of the substituent influenced the antiproliferative activity (**Table 1)**. 2,4-Diamino-6-phenethylpyrido[3,2-*d*]pyrimidine **5a** had greater antitumor activity than (*E*)-2,4-diamino-6-styrylpyrido[3,2-*d*]pyrimidine **4a** against all four cancer cell lines. With the exception of compound **4b**, alkyl groups (-CH_3, -C_2H_5 and -$CH(CH_3)_2$) at the 4'-position of the benzene

ring in the 6-styryl derivatives were found to enhanced antitumor activity. Introduction of electron-withdrawing substituents at the 4'-position of the benzene ring (F, Cl, Br, **4e-4g**) or electron-donating substituents (4'-OCH_3, 3'-OCH_3, 2'-OCH_3, **4h-4j**) also increased antiproliferative activity in all cell lines. Compound **4k** with three methoxy groups on the benzene ring had the best overall activity with IC_{50} values of 2.33, 2.10, 5.34 and 3.99 μM against HL-60, HeLa, A549 and H1299 cells, respectively.

With the exception of compound **5e**, 6-phenethyl derivatives (**5a-5k**) showed better antitumor activity than the corresponding 6-phenylethenyl derivatives (**4a-4k**) against HL-60 and H1299 cells. For example, compounds **5f-5h** inhibited growth of HL-60 with IC_{50} values of 0.27, 0.12 and 0.072 μM, respectively. These 6-phenethyl derivatives are thus approximately 14-, 21- and 23-fold more active than the corresponding 6-phenylethenyl derivatives **4f-4h**. Similarly, compounds **5f** and **5g**, with IC_{50} values against H1299 cells of 1.49 and 2.15 μM respectively are approximately 9 and 4 times more potent than compounds **4f** and **4g** in this cell line. Incorporation of alkyl groups at the 4'-position of the benzene ring in the 6-phenethyl derivatives (**5b-5d**) decreased antitumor activity against HeLa and A549 cells but improved activity against H1299 cells. In comparison with compound **5a**, compounds **5f** (4'-Cl) and **5g** (4'-Br) exhibited higher antiproliferative activity against all except A549 cells with IC_{50} values in the range 0.12-5.30 μM. The presence of a methoxy group at the 4', 3'or 2'-position of the benzene ring in the 6-phenethyl derivatives decreased antiproliferative activity against HeLa and A549 cells, but increased activity against H1299 cells. Compound **5h** inhibited the growth of HL-60 cells with an IC_{50} value of 0.072 μM and was thus approximately 6-fold more potent than compound **5k** (**Fig. 3**).

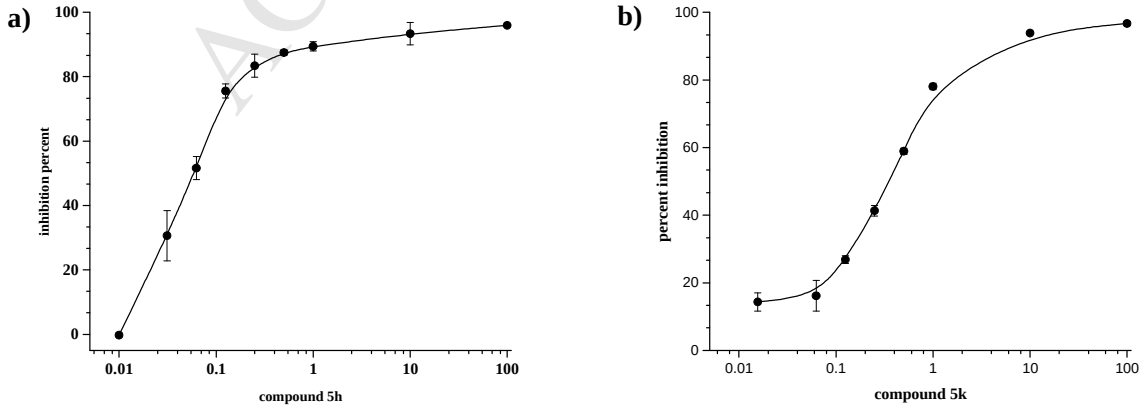


Fig. 3 Inhibition growth of HL-60 cells by compounds **5h** (a) and **5k** (b). Cell proliferation inhibition was measured over a range of

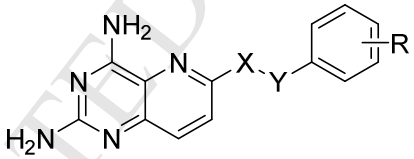
concentrations of compounds **5h** and **5k** in RPMI1640 medium. Cell densities were measured using an MTS assay. Results were normalized to cell density in the absence of drug. Results shown are representative data of experiments performed in triplicate.

3.2. DHFR inhibition

Compounds **4a-4k** and **5a-5k** with structural modifications of the benzene ring were evaluated as inhibitors of rhDHFR as previously described [28-30]. Results are reported as IC_{50} values (**Table 2**). 6-Phenethyl derivatives with a flexible saturated carbon-carbon linker showed better rhDHFR inhibition than 6-phenylethenyl derivatives. For example, compounds **5f**, **5g**, **5h**, **5i** and **5k** showed rhDHFR inhibitory potency with IC_{50} values of 2.84, 1.26, 0.71, 0.45 and 0.29 μM, respectively, and were thus approximately 6-, 12-, 6-, 9- and 14-fold more potent than the corresponding compounds **4f**, **4g**, **4h**, **4i** and **4k** with a less flexible unsaturated linker. The inhibitory activity of other 6-phenethyl derivatives was also improved to varying degrees, relative to the corresponding 6-phenylethenyl derivatives. 6-Phenethyl derivatives with a methoxyl group at the 4'- or 3'-position of the benzene ring (compounds **5h** and **5i**) were more potent inhibitors of rhDHFR than compound **5a**.

Table 2

IC_{50} values (μM)[a] of 6-substituted pyrido[3,2-*d*]pyrimidines **4a-4k** and **5a-5k** against rhDHFR.



Compounds	X-Y	R	DHFR inhibition	Compounds	X-Y	R	DHFR inhibition
4a	CH=CH	H	5.33±0.41	**5a**	CH_2CH_2	H	2.25±0.20
4b	CH=CH	4'-CH_3	3.19±0.76	**5b**	CH_2CH_2	4'-CH_3	2.55±0.45
4c	CH=CH	4'-C_2H_5	5.28±1.12	**5c**	CH_2CH_2	4'-C_2H_5	2.48±0.10
4d	CH=CH	4'-$CH(CH_3)_2$	20.1±1.1	**5d**	CH_2CH_2	4'-$CH(CH_3)_2$	19.0±0.6
4e	CH=CH	4'-F	4.78±0.98	**5e**	CH_2CH_2	4'-F	0.95±0.21
4f	CH=CH	4'-Cl	16.9±3.2	**5f**	CH_2CH_2	4'-Cl	2.84±0.52
4g	CH=CH	4'-Br	14.8±3.5	**5g**	CH_2CH_2	4'-Br	1.26±0.08

4h	CH=CH	4'-OCH_3	4.34±0.71	**5h**	CH_2CH_2	4'-OCH_3	0.71±0.10
4i	CH=CH	3'-OCH_3	4.13±0.34	**5i**	CH_2CH_2	3'-OCH_3	0.45±0.06
4j	CH=CH	2'-OCH_3	3.61±0.92	**5j**	CH_2CH_2	2'-OCH_3	1.67±0.18
4k	CH=CH	3',4',5'-$(OCH_3)_3$	4.00±0.24	**5k**	CH_2CH_2	3',4',5'-$(OCH_3)_3$	0.29±0.02
MTX			0.0309±0.0010				

[a]Calculated from three replicates

*3.3. Docking studies of compounds **4k** and **5k***

To explore possible structural reasons for the different rhDHFR inhibitory activities of 6-phenylethenylpyrido[3,2-*d*]pyrimidine (**4k**) and 6-phenethylpyrido[3,2-*d*]pyrimidine (**5k**), the two compounds were docked into the binding site of the known structure of rhDHFR. Compounds **4k** and **5k** have the same trimethoxy substitution pattern on the benzene ring and differ only in the nature of the 9-10 carbon-carbon bond (double bond in **4k** and single bond in **5k**). The docked poses of compound **5k** (green) in human DHFR (PDB 4QJC) (**Fig. 4** A) shows the 6-phenethylpyrido[3,2-*d*]pyrimidine scaffold buried deep in the active site and occupying the same location as N^6-methyl-N^6-(3,4,5-trifluorophenyl)-2,4,6-triamine pyrido[2,3-*d*]pyrimidine [31], (Supplementary material, Fig. 1). This orientation of the scaffold permits the N1 nitrogen and 2-NH_2 moiety to form hydrogen bonds to the backbone Glu30. The 4-NH_2 group also forms a hydrogen bond with the side chain of Ile7. The trimethoxyphenyl ring of compound **5k** occupies a position near the cofactor-binding site, with close intermolecular contacts with Asp21, Phe31, Ser59, Ile60 and Pro61 **(**Supplementary material, Fig. 2). The docked pose of compound **4k (Fig. 4** B) shows that the 6-phenylethenylpyrido[3,2-*d*]pyrimidine group is less well accommodated in the active site, compared with compound **5k** which contains a flexible carbon-carbon linker. This could partially explain the greater potency of compound **5k** against DHFR compared with compound **4k**.

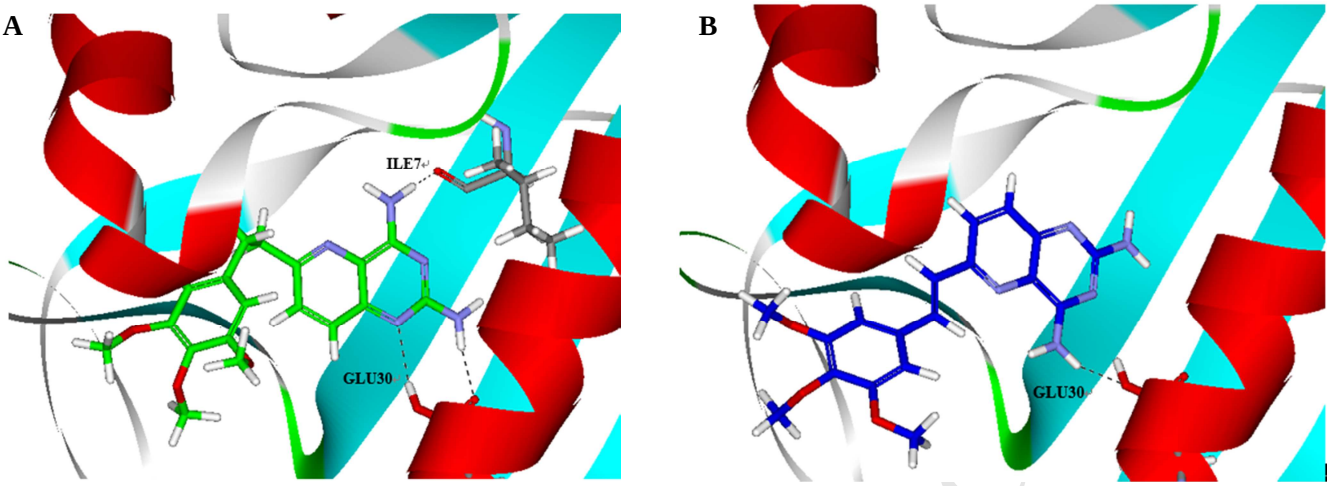
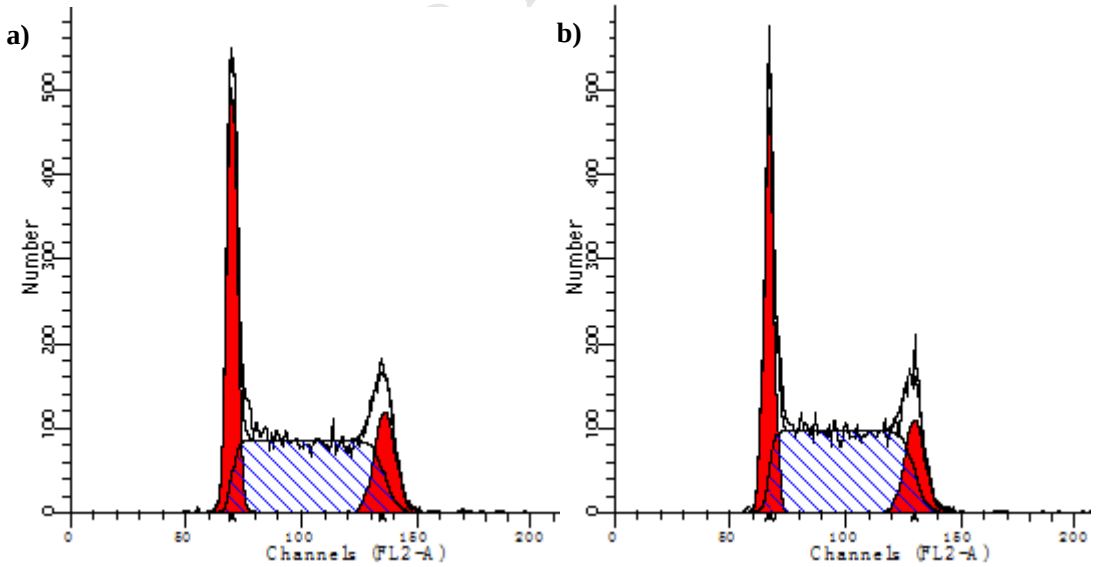
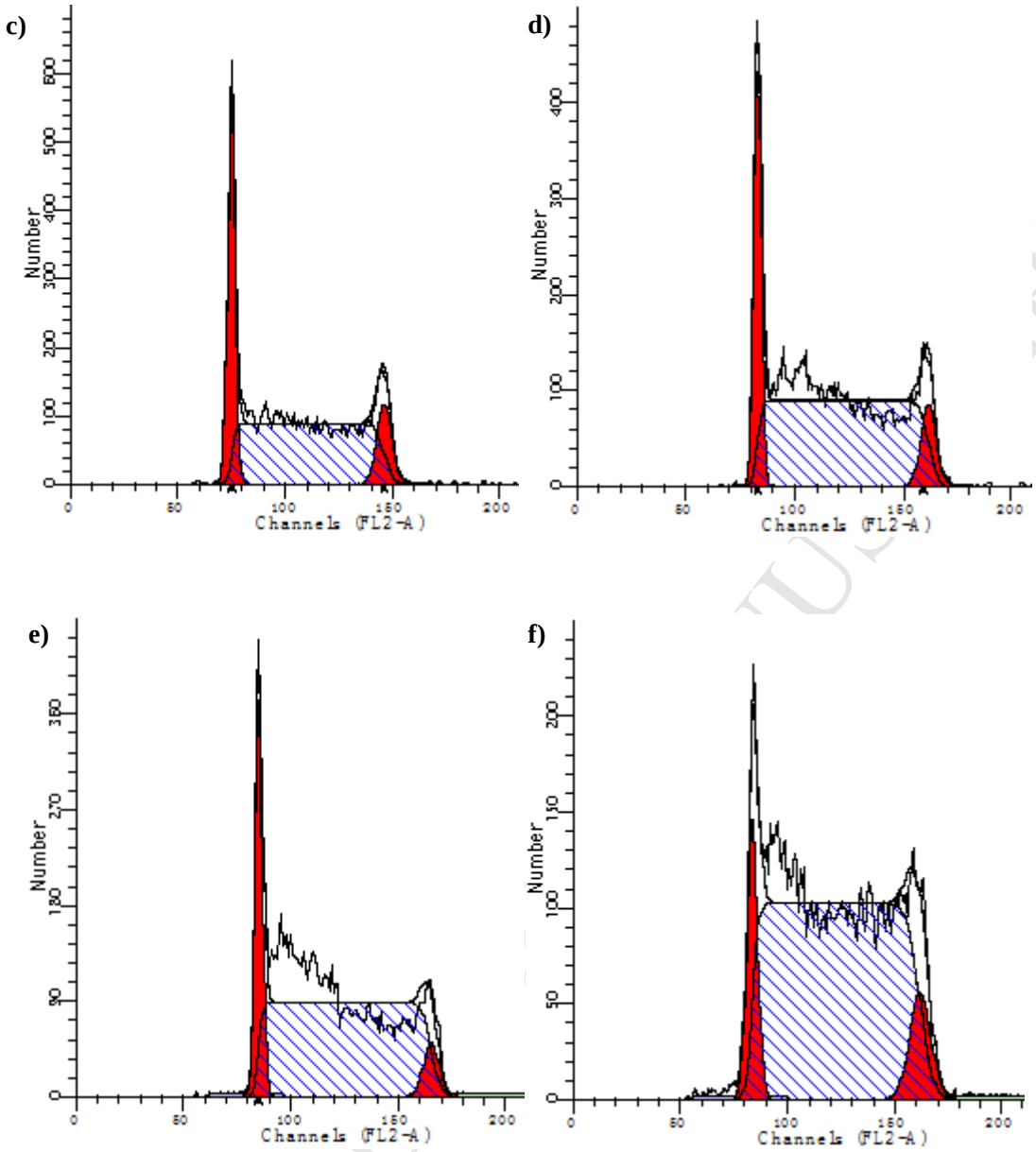


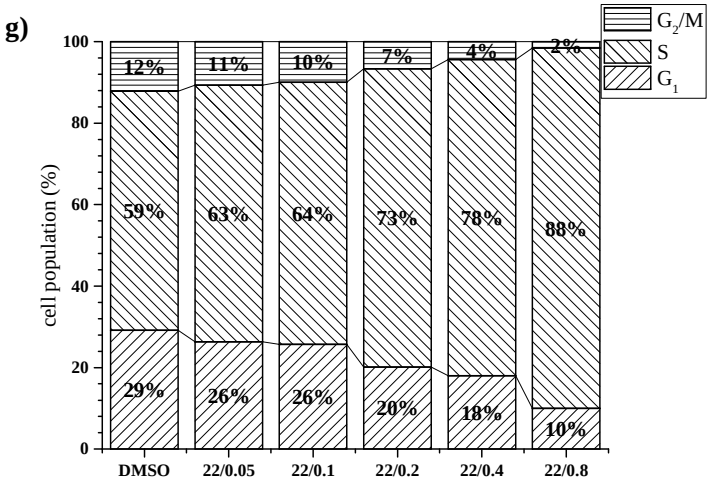
Fig. 4 Docking of compound **5k** (A) and compound **4k** (B) into the active site of rhDHFR (PDB code 4QJC).

*3.4. Effects of compound **5k** on cell cycle progression in HL-60 cells*

To elucidate the mechanism by which compound **5k** inhibits cell proliferation, its effect of on cell cycle progression and apoptosis were evaluated using flow cytometry. Treatment of HL-60 cells with compound **5k** significantly increased the population of cells in S phase (**Fig. 5)**. The proportion of cells in S phase increased by 4.3%, 5.6%, 14.5%, 18.9% and 29.8% with concentrations of 0.05, 0.1, 0.2, 0.4 and 0.8 μM of compound **5k**, respectively. The percentage of cells in G_1/G_2 phase was also markedly reduced suggesting that inhibition of proliferation by compound **5k** may be associated with induction of S phase arrest and induction of apoptosis.







Compound	Concentration (μM)	Cell cycle distribution (%)		
		G_1	S	G_2/M
DMSO		29.1±0.5	58.7±0.8	12.2±1.1
5k	0.05	26.3±1.0	63.0±2.3	10.7±1.4
5k	0.1	25.7±0.7	64.3±0.70	10.0±0.3
5k	0.2	20.2±0.5	73.2±0.3	6.6±0.8
5k	0.4	18.0±1.4	77.6±1.5	4.4±0.4
5k	0.8	10.0±0.5	88.5±0.4	1.5±0.3

Fig. 5 Cell cycle assay. HL-60 cells were untreated (DMSO) or treated with compound **5k** (0.05, 0.1, 0.2, 0.4 or 0.8 μM) for 24 h, washed, fixed, and stained with propidium iodide. Cell cycle distributions were analyzed by flow cytometry. Cell cycle distributions: a) DMSO, b) 0.05 μM compound **5k**, c) 0.1 μM compound **5k**, d) 0.2 μM compound **5k**, e) 0.4 μM compound **5k**, f) 0.8 μM compound**5k**, g) Percentage of cells in each phase of the cell cycle (G_1, S, and G_2).

4. Conclusion

A series of novel 2,4-diamino-6-substituted pyrido[3,2-*d*]pyrimidines were designed and synthesized using an efficient one-pot reaction. Compounds **5e**, **5h**, **5i** and **5k** were the most potent rhDHFR inhibitors, with IC_{50} values in the range 0.29–1.0 μM. Compounds **5g-5i** and **5k** showed broad spectrum antitumor activity, with IC_{50} values in the range 0.07-23 μM. Docking studies indicated that a flexible carbon-carbon linker (single bond rather than double bond) was more important for good rhDHFR inhibition than the nature or position of substituents in the benzene ring. Compound **5k** led to G_1/M checkpoint activation and S arrest of HL-60 cells, followed by DNA damage.

5. Experimental section

5.1. Chemistry

All evaporations were carried out in vacuo with a rotary evaporator. Melting points (^{o}C) were determined on SGW® X-4 melting point apparatus and are uncorrected. ^{1}H, ^{13}C NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer; chemical shifts are expressed in δ ppm with reference to TMS; s =singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad singlet. High-resolution electrospray ionization (HR-ESI) mass spectra were recorded on a Waters Xevo G2 Q-TOF mass spectrometer. Thin-layer chromatography (TLC) was carried out on Silica Gel GF254 plates, and the spots were visualized under 254 and 365 nm illumination. Silica gel (200-300 mesh) was employed for routine column chromatography separations.

*5.1.1. General procedure for the synthesis of intermediates **3a-3k***

A mixture of compounds **2a-2k** (1 g), phosphorous oxychloride (20 mL) and triethylamine (1 mL) was heated at reflux for 8 h then evaporated in vacuo. The residue was poured onto ice and extracted with dichloromethane. The organic phase was dried over magnesium sulphate and concentrated in vacuo. Purification by column chromatography (petroleum ether/ethyl acetate, 3/1) afforded the title compound as a yellow solid.

*5.1.1.1 (E)-2,4-Dichloro-6-styrylpyrido[3,2-d]pyrimidine (**3a**)*

Yield 70%, mp: 168-169 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.24 (d, J=8.0 Hz, 1H, 8-CH), 8.08 (d, J=8.0 Hz, 1H, 7-CH), 7.87 (d, J=16.0 Hz, 1H, 10-CH), 7.68 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.48-7.39 (m, 4H, 9-CH, 3',5'-$(CH)_2$ & 4'-CH); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 165.07, 158.95, 154.54, 148.15, 138.39, 136.89, 135.99, 135.52, 129.75, 128.98, 128.66, 127.78, 126.69; LRMS (EI): m/z calcd for $C_{15}H_9Cl_2N_3$ [M^+], 301.0; found, 301.0.

*5.1.1.2 (E)-2,4-Dichloro-6-(4-methylstyryl)pyrido[3,2-d]pyrimidine (**3b**)*

Yield 65%, mp: 215-217 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.22 (d, J=8.0 Hz, 1H, 8-CH), 8.08 (d, J=8.0 Hz, 1H, 7-CH), 7.84 (d, J=16.0 Hz, 1H, 10-CH), 7.58 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.38 (d, J=16.0 Hz, 1H, 9-CH), 7.26 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 2.42 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.92, 159.20, 154.37, 148.14, 140.18, 138.45, 136.89, 135.90, 132.75, 129.73, 128.66, 127.79, 125.73, 21.50; LRMS (EI): m/z calcd for $C_{16}H_{11}Cl_2N_3$ [M^+], 315.0; found, 315.0.

*5.1.1.3 (E)-2,4-Dichloro-6-(4-ethylstyryl)pyrido[3,2-d]pyrimidine (**3c**)*

Yield 70%, mp: 174-175 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.21 (d, J=8.0 Hz, 1H, 8-CH), 8.07 (d,

J=8.0 Hz, 1H, 7-CH), 7.84 (d, *J*=16.0 Hz, 1H, 10-CH), 7.59 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.37 (d, *J*=16.0 Hz, 1H, 9-CH), 7.28 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 2.72 (q, *J*=8.0 Hz, 2H, CH_2), 1.29 (t, *J*=8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 164.92, 159.20, 154.37, 148.13, 146.47, 138.43, 136.92, 135.86, 133.01, 128.60, 128.52, 127.86, 125.75, 28.80, 15.32; LRMS (EI): m/z calcd for $C_{17}H_{13}Cl_2N_3$ [M^+], 329.0; found, 329.0.

*5.1.1.4 (E)-2,4-Dichloro-6-(4-isopropylstyryl)pyrido[3,2-d]pyrimidine (**3d**)*

Yield 72%, mp: 169-170 °C. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 8.22 (d, *J*=8.0 Hz, 1H, 8-CH), 8.08 (d, *J*=8.0 Hz, 1H, 7-CH), 7.84 (d, *J*=16.0 Hz, 1H, 10-CH), 7.61 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.38 (d, *J*=16.0 Hz, 1H, 9-CH), 7.31 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$), 3.01-2.94 (m, 1H, CH), 1.31 (d, *J*=8.0 Hz, 6H, $(CH_3))_2$; ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 164.90, 159.22, 154.36, 151.10, 148.14, 138.43, 136.88, 135.88, 133.13, 128.63, 127.90, 127.12, 125.77, 34.09, 23.82; LRMS (EI): m/z calcd for $C_{18}H_{15}Cl_2N_3$ [M^+], 343.1; found, 343.1.

*5.1.1.5 (E)-2,4-Dichloro-6-(4-fluorostyryl)pyrido[3,2-d]pyrimidine (**3e**)*

Yield 63%, mp: 248-249 °C. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 8.25 (d, *J*=8.0 Hz, 1H, 8-CH), 8.06 (d, *J*=8.0 Hz, 1H, 7-CH), 7.85 (d, *J*=16.0 Hz, 1H, 10-CH), 7.67 (t, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.34 (d, *J*=16.0 Hz, 1H, 9-CH), 7.15 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 165.08, 164.83, 162.33, 158.76, 154.52, 148.07, 137.09, 136.89, 136.01, 131.79, 129.60, 129.51, 128.70, 126.39, 116.24, 116.02; LRMS (EI): m/z calcd for $C_{15}H_8Cl_2FN_3$ [M^+], 319.0; found, 319.0.

*5.1.1.6 (E)-2,4-Dichloro-6-(4-chlorostyryl)pyrido[3,2-d]pyrimidine (**3f**)*

Yield 76%, mp: 260-262 °C. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 8.21 (d, *J*=8.0 Hz, 1H, 8-CH), 8.07 (d, *J*=8.0 Hz, 1H, 7-CH), 7.84 (d, *J*=16.0 Hz, 1H, 10-CH), 7.59 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.37 (d, *J*=16.0 Hz, 1H, 9-CH), 7.31 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 164.15, 161.11, 158.54, 164.75, 148,14, 136.92, 136.13, 135.58, 134.04, 129.24, 128.90, 128.73, 127.13, 111.00; LRMS (EI): m/z calcd for $C_{15}H_8Cl_3N_3$ [M^+], 335.0; found, 335.0.

*5.1.1.7 (E)-2,4-Dichloro-6-(4-bromostyryl)pyrido[3,2-d]pyrimidine (**3g**)*

Yield 75%, mp: 244-246 °C. ^{1}H NMR (400 MHz, $CDCl_3$) *δ*: 8.25 (d, *J*=8.0 Hz, 1H, 8-CH), 8.06 (d, *J*=8.0 Hz, 1H, 7-CH), 7.82 (d, *J*=16.0 Hz, 1H, 10-CH), 7.59 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.54 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.40 (d, *J*=16.0 Hz, 1H, 9-CH); ^{13}C NMR (100 MHz, $CDCl_3$) *δ*: 165.16, 158.52, 154.71, 148.14, 136.98, 136.90, 136.15, 134.47, 132.20, 129.14, 128.74, 127.22, 123.88; LRMS (EI): m/z calcd for $C_{15}H_8BrCl_2N_3$ [M^+], 378.9; found, 378.9.

*5.1.1.8 (E)-2,4-Dichloro-6-(4-methoxylstyryl)pyrido[3,2-d]pyrimidine (**3h**)*

Yield 70%, mp: 225-227 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.19 (d, J=8.0 Hz, 1H, 8-CH), 8.04 (d, J=8.0 Hz, 1H, 7-CH), 7.80 (d, J=16.0 Hz, 1H, 10-CH), 7.61 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.26 (d, J=16.0 Hz, 1H, 9-CH), 6.96 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 3.88 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.75, 161.02, 159.34, 154.17, 148.09, 138.07, 136.90, 135.78, 129.36, 128.61, 128.25, 124.41, 114.43, 55.43; LRMS (EI): m/z calcd for $C_{16}H_{11}Cl_2N_3O$ [M^+], 331.0; found, 331.0.

*5.1.1.9 (E)-2,4-Dichloro-6-(3-methoxylstyryl)pyrido[3,2-d]pyrimidine (**3i**)*

Yield 73%, mp: 153-154 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.19 (d, J=8.0 Hz, 1H, 8-CH), 8.05 (d, J=8.0 Hz, 1H, 7-CH), 7.76 (d, J=16.0 Hz, 1H, 10-CH), 7.34 (d, J=16.0 Hz, 1H, 9-CH), 7.30 (t, J=8.0 Hz, 1H, 5'-CH), 7.20 (d, J=8.0 Hz, 1H, 6'-CH), 7.13 (s, 1H, 2'-CH), 3.87 (s, 1H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.97, 159.95, 158.84, 154.48, 148.12, 138.21, 136.81, 136.77, 135.96, 129.92, 128.61, 126.89, 120.53, 115.60, 112.53, 55.37; LRMS (EI): m/z calcd for $C_{16}H_{11}Cl_2N_3O$ [M^+], 331.0; found, 331.0.

*5.1.1.10 (E)-2,4-Dichloro-6-(2-methoxylstyryl)pyrido[3,2-d]pyrimidine (**3j**)*

Yield 67%, mp: 180-181 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.22-8.14 (m, 3H, 8-CH, 10-CH, 6'-CH), 7.72 (d, J=8.0 Hz, 1H, 7-CH), 7.48 (d, J=16.0 Hz, 1H, 9-CH), 7.38 (t, J=8.0 Hz, 1H, 4'-CH), 7.03 (t, J=8.0 Hz, 1H, 5'-CH), 6.97 (d, J=8.0 Hz, 1H, 3'-CH), 3.96 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.88, 159.82, 157.83, 154.30, 148.19, 136.88, 135.70, 133.39, 131.00, 128.44, 127.80, 127.28, 124.45, 120.91, 111.13, 55.60; LRMS (EI): m/z calcd for $C_{16}H_{11}Cl_2N_3O$ [M^+], 331.0; found, 331.0.

*5.1.1.11 (E)-2,4-Dichloro-6-(3,4,5-trimethoxylstyryl)pyrido[3,2-d]pyrimidine (**3k**)*

Yield 58%, mp: 201-203 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.24 (d, J=8.0 Hz, 1H, 8-CH), 8.10 (d, J=8.0 Hz, 1H, 7-CH), 7.78 (d, J=16.0 Hz, 1H, 10-CH), 7.34 (d, J=16.0 Hz, 1H, 9-CH), 6.91 (s, 2H, 2',6'-$(CH)_2$), 3.97 (s, 6H, $(OCH_3)_2$), 3.93 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 164.86, 158.97, 154.49, 153.56, 148.20, 139.78, 138.38, 136.93, 136.72, 136.01, 131.04, 128.48, 126.07, 61.05, 56.27; LRMS (EI): m/z calcd for $C_{18}H_{15}Cl_2N_3O_3$[M^+], 391.0; found, 391.1

*5.1.2. General procedure for the synthesis of intermediates **4a-4k***

A mixture of compounds 3a-3k (0.7 g), absolute methanol saturated by ammonia (15 mL) was heated at 150 ^{o}C for 8 h in sealed vessel. After cooled to room temperature, the light yellow precipitate was filtered and washed with water to give the title compound as a yellow solid.

*5.1.2.1 (E)-2,4-Diamino-6-styrylpyrido[3,2-d]pyrimidine (**4a**)*

Yield 91%, mp: 299-300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.81 (d, J=16.0 Hz, 1H, 10-CH), 7.74 (d, J=8.0 Hz, 1H, 8-CH), 7.65 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.54 (d, J=8.0 Hz, 1H, 7-CH), 7.48 (s, 2H, 4-NH_2), 7.41 (t, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.33 (t, J=8.0 Hz, 1H, 4'-CH), 7.31 (d, J=16.0 Hz, 1H, 9-CH), 6.27 (s, 2H, 2-NH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.53, 161.14, 148.45, 146.85, 137.26, 132.52, 131.85, 129.28, 128.52, 128.25, 128.10, 127.33, 127.26; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{13}N_5$ [$(M+H)^+$], 264.1249; found, 264.1248.

*5.1.2.2 (E)-2,4-Diamino-6-(4-methylstyryl)pyrido[3,2-d]pyrimidine (**4b**)*

Yield 95%, mp: >300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.75 (d, J=20.0 Hz, 1H, 10-CH), 7.73 (d, J=8.0 Hz, 1H, 8-CH), 7.54 (d, J=8.0 Hz, 3H, 2',6'-$(CH)_2$ & 7-CH), 7.42 (s, 2H, 4-NH_2), 7.25 (d, J=16.0 Hz, 1H, 9-CH), 7.23 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.21 (s, 2H, 2-NH_2), 2.33 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.52, 161.08, 148.72, 146.74, 138.04, 134.50, 132.53, 131.87, 129.89, 128.22, 127.23, 127.17, 21.35; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{16}N_5$ [$(M+H)^+$], 278.1406; found, 278.1407.

*5.1.2.3 (E)-2,4-Diamino-6-(4-ethylstyryl)pyrido[3,2-d]pyrimidine (**4c**)*

Yield 95%, mp: 314-316 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.77 (d, J=16.0 Hz, 1H, 10-CH), 7.73 (d, J=8.0 Hz, 1H, 8-CH), 7.56 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.53 (d, J=8.0 Hz, 1H, 7-CH), 7.48 (s, 2H, 4-NH_2), 7.26 (d, J=16.0 Hz, 1H, 9-CH), 7.25 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.28 (s, 2H, 2-NH_2), 2.62 (q, J=8.0 Hz, 2H, CH_2), 1.19 (q, J=8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.51, 161.08, 148.64, 146.75, 144.35, 134.74, 132.50, 131.83, 128.71, 128.20, 127.29, 127.22, 127.16, 28.45, 15.92; HRMS (TOF ES$^+$): m/z calcd for $C_{17}H_{18}N_5$ [$(M+H)^+$], 292.1562; found, 292.1562.

*5.1.2.4 (E)-2,4-Diamino-6-(4-isopropylstyryl)pyrido[3,2-d]pyrimidine (**4d**)*

Yield 94%, mp: 292-294 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.78 (d, J=16.0 Hz, 1H, 10-CH), 7.74 (d, J=8.0 Hz, 1H, 8-CH), 7.57 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.54 (d, J=8.0 Hz, 1H, 7-CH), 7.38 (s, 2H, 4-NH_2), 7.28 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.25 (d, J=16.0 Hz, 1H, 9-CH), 6.30 (s, 2H, 2-NH_2), 2.90 (m, 1H, $CH(CH_3)_2$), 1.21 (d, J=4.0 Hz, 6H, $(CH_3)_2$), ; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.52, 160.89, 148.99, 148.79, 146.38, 134.88, 132.32, 131.89, 128.16, 127.31, 127.25, 127.22, 127.16, 33.71, 24.22; HRMS (TOF ES$^+$): m/z calcd for $C_{18}H_{20}N_5$ [$(M+H)^+$], 306.1719; found, 306.1726.

*5.1.2.5 (E)-2,4-Diamino-6-(4-fluorostyryl)pyrido[3,2-d]pyrimidine (**4e**)*

Yield 92%, mp: 288-290 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.81 (d, J=16.0 Hz, 1H, 10-CH), 7.72 (d, J=8.0 Hz, 1H, 8-CH), 7.70 (dd, J=8.0 Hz, J=4.0 Hz, 2H, 2',6'-$(CH)_2$), 7.55 (d, J=8.0 Hz, 1H, 7-CH),

7.48 (s, 2H, 4-NH_2), 7.26 (d, *J*=16.0 Hz, 1H, 9-CH), 7.25 (t, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.29 (s, 2H, 2-NH_2), ; ^{13}C NMR (100 MHz, DMSO-d_6) δ: 163.59, 162.54, 161.13, 148.38, 146.82, 133.89, 133.86, 132.52, 130.69, 129.21, 129.13, 128.23, 127.97, 127.95, 127.33,116.30, 116.09; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{13}FN_5$ [$(M+H)^+$], 282.1155; found, 282.1156.

*5.1.2.6 (E)-2,4-Diamino-6-(4-chlorostyryl)pyrido[3,2-d]pyrimidine (**4f**)*

Yield 94%, mp: 312-314 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.82 (d, *J*=16.0 Hz, 1H, 10-CH), 7.72 (d, *J*=8.0 Hz, 1H, 8-CH), 7.67 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.54 (d, *J*=8.0 Hz, 1H, 7-CH), 7.48 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$) & 2H, 4-NH_2), 7.34 (d, *J*=16.0 Hz, 1H, 9-CH), 6.27 (s, 2H, 2-NH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.54, 161.19, 148.13, 146.93, 136.26, 132.79, 132.52, 130.48, 129.29, 128.89, 128.30, 127.47; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{13}ClN_5$ [$(M+H)^+$], 298.0895; found, 298.0865.

*5.1.2.7 (E)-2,4-Diamino-6-(4-bromostyryl)pyrido[3,2-d]pyrimidine (**4g**)*

Yield 88%, mp: 331-333 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.80 (d, *J*=16.0 Hz, 1H, 10-CH), 7.72 (d, *J*=8.0 Hz, 1H, 8-CH), 7.61 (s, 2H, 2',6'-$(CH)_2$ & 2H, 4-NH_2), 7.54 (d, *J*=8.0 Hz, 1H, 7-CH), 7.48 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$)), 7.35 (d, *J*=16.0 Hz, 1H, 9-CH), 6.28 (s, 2H, 2-NH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.53, 161.20, 148.11, 146.94, 136.61, 132.52, 132.20, 130.54, 129.20, 128.95, 128.32, 127.48, 121.40; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{13}BrN_5$ [$(M+H)^+$], 342.0354; found, 344.0342.

*5.1.2.8 (E)-2,4-Diamino-6-(4-methoxylstyryl)pyrido[3,2-d]pyrimidine (**4h**)*

Yield 95%, mp: 297-298 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.74 (d, *J*=16.0 Hz, 1H, 10-CH), 7.70 (d, *J*=8.0 Hz, 1H, 8-CH), 7.59 (d, *J*=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.52 (d, *J*=8.0 Hz, 1H, 7-CH), 7.43 (s, 2H, 4-NH_2), 7.16 (d, *J*=16.0 Hz, 1H, 9-CH), 6.98 (d, *J*=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.22 (s, 2H, 2-NH_2), 3.79 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.48, 161.00, 159.82, 148.93, 146.60, 132.51, 131.64, 129.88, 128.66, 128.15, 127.04, 125.84, 114.78, 55.66; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{16}N_5O$ [$(M+H)^+$], 294.1355; found, 294.1360.

*5.1.2.9 (E)-2,4-Diamino-6-(3-methoxylstyryl)pyrido[3,2-d]pyrimidine (**4i**)*

Yield 91%, mp: 275-277 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.78 (d, *J*=16.0 Hz, 1H, 10-CH), 7.73 (d, *J*=8.0 Hz, 1H, 8-CH), 7.55 (d, *J*=8.0 Hz, 1H, 7-CH), 7.48 (s, 2H, 4-NH_2), 7.33 (d, *J*=16.0 Hz, 1H, 9-CH), 7.32 (d, *J*=8.0 Hz, 1H, 5'-CH), 7.22 (d, *J*=8.0 Hz, 1H, 6'-CH), 7.21 (s, 1H, 2'-CH), 6.88 (dd, *J*=8.0 Hz, *J*=4.0 Hz, 1H, 4'-CH), 6.28 (s, 2H, 2-NH_2), 3.81 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.54, 161.15, 160.11, 148.40, 146.86, 138.72, 132.52, 131.83, 130.26, 128.38, 128.26,

127.36, 119.86, 114.47, 112.21, 55.56; HRMS (TOF ES^+): m/z calcd for $C_{16}H_{16}N_5O$ $[(M+H)^+]$, 294.1355; found, 294.1357.

*5.1.2.10 (E)-2,4-Diamino-6-(2-methoxylstyryl)pyrido[3,2-d]pyrimidine (**4j**)*

Yield 90%, mp: 264-266 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.84 (d, J=16.0 Hz, 1H, 10-CH), 7.72 (d, J=8.0 Hz, 1H, 8-CH), 7.65 (d, J=8.0 Hz, 1H, 6'-CH), 7.54 (d, J=8.0 Hz, 1H, 7-CH), 7.42 (s, 2H, 4-NH_2), 7.33 (d, J=16.0 Hz, 1H, 9-CH), 7.31 (t, J=8.0 Hz, 1H, 4'-CH), 7.07 (d, J=8.0 Hz, 1H, 3'-CH), 7.00 (t, J=8.0 Hz, 1H, 5'-CH), 6.29 (s, 2H, 2-NH_2), 3.89 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.48, 161.00, 157.52, 149.10, 146.69, 132.52, 129.85, 129.19, 128.13, 127.91, 127.04, 126.98, 125.66, 121.14, 111.96, 55.96; HRMS (TOF ES^+): m/z calcd for $C_{16}H_{16}N_5O$ $[(M+H)^+]$, 294.1355; found, 294.1351.

*5.1.2.11 (E)-2,4-Diamino-6-(3,4,5-trimethoxylstyryl)pyrido[3,2-d]pyrimidine (**4k**)*

Yield 86%, mp: 248-250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.76 (d, J=16.0 Hz, 1H, 10-CH), 7.72 (d, J=8.0 Hz, 1H, 8-CH), 7.56 (d, J=8.0 Hz, 1H, 7-CH), 7.54 (s, 2H, 4-NH_2), 7.29 (d, J=16.0 Hz, 1H, 9-CH), 6.97 (s, 2H, 2',6'-$(CH)_2$), 6.37 (s, 2H, 2-NH_2), 3.85 (s, 6H, $(OCH_3)_2$, 3.69 (s, 3H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.53, 160.78, 153.57, 148.74, 146.17, 138.19, 132.91, 132.26, 132.22, 128.16, 127.37, 127.30, 104.68, 60.57, 56.35; HRMS (TOF ES^+): m/z calcd for $C_{18}H_{20}N_5O_3$ $[(M+H)^+]$, 354.1566; found, 354.1567.

*5.1.3. General procedure for the synthesis of intermediates **5a-5k***

A solution of intermediates **4a**-**4k** (100 mg) and Pd/C (10 %) in ethanol (40 mL) in an autoclave. The autoclave was evacuated and backfilled with H_2 three times, pressurized to 0.4 Mpa, and stirred at room temperature for 12 h. After the pressure was released, the reaction mixture was filtered through filter paper and the filtrate was concentrated in vacuo. The resulting residue was purified by using a silica gel column (eluent: dichloromethane/methanol, 9/1) to obtain the title compound as a white solid.

*5.1.3.1 2,4-Diamino-6-phenethylpyrido[3,2-d]pyrimidine (**5a**)*

Yield 92%, mp: 206-207 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.49 (d, J=8.0 Hz, 1H, 8-CH), 7.41 (d, J=8.0 Hz, 1H, 7-CH), 7.35-7.20 (m, 6H, 4-NH_2, 3',5'-$(CH_2)_2$, 2',6'-$(CH_2)_2$), 7.19-7.13 (m, 1H, 4'-CH), 6.15 (s, 2H, 2-NH_2), 3.09 (s, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.39, 160.71, 154.24, 145.76, 142.13, 132.39, 128.85, 128.68, 128.32, 127.58, 126.23, 39.15, 35.04; HRMS (TOF ES^+): m/z calcd for $C_{15}H_{16}N_5$ $[(M+H)^+]$, 266.1406; found, 266.1402.

*5.1.3.2 2,4-Diamino-6-(4-methylstyryl)pyrido[3,2-d]pyrimidine (**5b**)*

Yield 98%, mp: 201-202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.17-8.04 (d, 2H, 4-NH_2), 7.65 (d, J=8.0 Hz, 1H, 8-CH), 7.55 (d, J=8.0 Hz, 1H, 7-CH), 7.13 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.07 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$ & s, 2H, 2-NH_2), 3.10-3.05 (m, 4H, CH_2CH_2), 2.25 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.71, 157.70, 156.47, 138.76, 135.15, 129.29, 129.00, 128.71, 126.82, 39.14, 34.44, 21.07; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{18}N_5$ [(M+H)$^+$],280.1562; found, 280.1563.

*5.1.3.3 2,4-Diamino-6-(4-ethylstyryl)pyrido[3,2-d]pyrimidine (**5c**)*

Yield 95%, mp: 223-224 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.49 (d, J=8.0 Hz, 1H, 8-CH), 7.46 (s, 1H, 4-NH), 7.41 (d, J=8.0 Hz, 1H, 7-CH), 7.29 (s, 1H, 4-NH), 7.15 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.09 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.23 (s, 2H, 2-NH_2), 3.09-3.00 (m, 4H, CH_2CH_2), 2.54 (q, J=8.0 Hz, 2H, CH_2), 1.14 (t, J=8.0 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.41, 160.81, 154.25, 145.98, 141.51, 139.27, 132.51, 128.76, 128.31, 128.06, 127.60, 39.31, 34.70, 28.23, 16.13; HRMS (TOF ES$^+$): m/z calcd for $C_{17}H_{20}N_5$ [(M+H)$^+$], 294.1719; found, 294.1725.

*5.1.3.4 2,4-Diamino-6-(4-isopropylstyryl)pyrido[3,2-d]pyrimidine (**5d**)*

Yield 91%, mp: 223-235 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.51 (d, J=8.0 Hz, 1H, 8-CH), 7.45 (d, J=8.0 Hz, 1H, 7-CH), 7.46-7.36 (2H, 4-NH_2), 7.17 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.13 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 6.29 (s, 2H, 2-NH_2), 3.08-3.01 (m, 4H, CH_2CH_2), 2.86-2.80 (m, 1H, CH), 1.17 (d, 6H, $(CH_3)_2$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.42, 160.34, 154.58, 146.21, 145.07, 139.42, 132.02, 128.74, 128.42, 127.48, 126.58, 39.26, 34.63, 33.48, 24.43; HRMS (TOF ES$^+$): m/z calcd for $C_{18}H_{22}N_5$ [(M+H)$^+$], 308.1875; found, 308.1875.

*5.1.3.5 2,4-Diamino-6-(4-fluorostyryl)pyrido[3,2-d]pyrimidine (**5e**)*

Yield 90%, mp: 229-231 °C ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.76-7.65 (2H, 4-NH_2), 7.56 (d, J=8.0 Hz, 1H, 8-CH), 7.47 (d, J=8.0 Hz, 1H, 7-CH), 7.27 (t, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 7.08 (t, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 6.61 (s, 2H, 4-NH_2), 3.09 (s, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.52, 162.26, 159.86, 159.29, 155.01, 142.93, 138.13, 130.81, 130.63, 130.55, 128.80, 127.22, 115.44, 115.23, 39.10, 33.95; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{15}FN_5$ [(M+H)$^+$], 284.1311; found, 284.1315.

*5.1.3.6 2,4-Diamino-6-(4-chlorostyryl)pyrido[3,2-d]pyrimidine (**5f**)*

Yield 92%, mp: 222-224 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.83-7.70 (2H, 4-NH_2), 7.57 (d, J=8.0 Hz, 1H, 8-CH), 7.47 (d, J=8.0 Hz, 1H, 7-CH), 7.31 (t, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.27 (t, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 6.67 (s, 2H, 4-NH_2), 3.10 (s, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.54, 159.11, 155.00, 141.07, 130.85, 130.81, 130.76, 130.63, 128.86, 128.61, 127.18, 38.76, 34.00; HRMS

(TOF ES$^+$): m/z calcd for $C_{15}H_{15}ClN_5$ [(M+H)$^+$], 300.1016; found, 300.1019.

*5.1.3.7 2,4-Diamino-6-(4-bromostyryl)pyrido[3,2-d]pyrimidine (**5g**)*

Yield 89%, mp: 226-228 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.51 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 7.45-7.41 (m, 4H, 8-CH, 7-CH & 4-NH_2), 7.21 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 6.33 (s, 2H, 2-NH_2), 3.07 (s, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.44, 160.16, 154.25, 144.68, 141.56, 131.80, 131.51, 131.18, 128.52, 127.45, 119.26, 38.71, 34.12; HRMS (TOF ES$^+$): m/z calcd for $C_{15}H_{15}BrN_5$ [(M+H)$^+$], 344.0511; found, 344.0508.

*5.1.3.8 2,4-Diamino-6-(4-methoxylstyryl)pyrido[3,2-d]pyrimidine (**5h**)*

Yield 95%, mp: 218-219 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.48 (d, J=8.0 Hz, 1H, 8-CH), 7.39 (d, J=8.0 Hz, 1H, 7-CH), 7.23 (s, 2H, 4-NH_2), 7.16 (d, J=8.0 Hz, 2H, 2',6'-$(CH)_2$), 6.83 (d, J=8.0 Hz, 2H, 3',5'-$(CH)_2$), 3.71 (s, 3H, OCH_3), 3.03 (m, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.38, 160.83, 157.90, 154.27, 145.99, 133.97, 132.51, 129.76, 128.28, 127.63, 114.14, 55.43, 39.45, 34.20; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{18}N_5O$ [(M+H)$^+$], 296.1511; found, 296.1515.

*5.1.3.9 2,4-Diamino-6-(3-methoxylstyryl)pyrido[3,2-d]pyrimidine (**5i**)*

Yield 89%, mp: 201-202 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.48 (d, J=8.0 Hz, 1H, 8-CH), 7.41 (d, J=8.0 Hz, 1H, 7-CH), 7.30 (s, 2H, 4-NH_2), 7.17 (t, J=8.0 Hz, 1H, 5'-CH), 6.83 (s, 1H, 2'-CH), 6.82 (d, J=8.0 Hz, 1H, 6'-CH), 6.73 (dd, J=8.0 Hz, J=4.0 Hz, 1H, 4'-CH), 6.16 (s, 2H, 2-NH_2), 3.71 (s, 3H, OCH_3), 3.11-3.02 (m, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.38, 160.70, 159.69, 154.20, 145.75, 143.75, 132.37, 129.66, 128.35, 127.56, 121.10, 114.54, 111.73, 55.32, 39.01, 34.99; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{18}N_5O$ [(M+H)$^+$], 296.1511; found, 296.1516.

*5.1.3.10 2,4-Diamino-6-(2-methoxylstyryl)pyrido[3,2-d]pyrimidine (**5j**)*

Yield 88%, mp: 299-300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.49 (d, J=8.0 Hz, 1H, 8-CH), 7.38 (d, J=8.0 Hz, 1H, 6'-CH), 7.25 (s, 2H, 4-NH_2), 7.19-7.15 (m, 2H, 5'-CH & 7-CH), 6.95 (d, J=8.0 Hz, 1H, 3'-CH), 6.83 (t, J=8.0 Hz, 1H, 4'-CH), 6.21 (s, 2H, 2-NH_2), 3.79 (s, 3H, OCH_3), 3.03 (s, 4H, CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.40, 160.59, 157.62, 154.71, 145.55, 132.27, 130.06, 129.83, 128.28, 127.71, 127.49, 120.64, 111.07, 55.73, 37.62, 29.78; HRMS (TOF ES$^+$): m/z calcd for $C_{16}H_{18}N_5O$ [(M+H)$^+$], 296.1511; found, 296.1514.

*5.1.3.11 2,4-Diamino-6-(3,4,5-trimethoxylstyryl)pyrido[3,2-d]pyrimidine (**5k**)*

Yield 93%, mp: 145-146 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.49 (d, J=8.0 Hz, 1H, 8-CH), 7.43 (d, J=8.0 Hz, 1H, 7-CH), 7.28 (s, 2H, 4-NH_2), 6.57 (s, 2H, 2',6'-$(CH)_2$), 6.12 (s, 2H, 2-NH_2), 3.73 (s, 6H,

$(OCH_3)_2$), 3.61 (s, 3H, OCH_3), 3.11-3.07 (m, 2H, CH_2), 3.03-3.00 (m, 2H, CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 162.37, 160.81, 154.20, 153.10, 145.96, 137.85, 136.06, 132.51, 128.34, 127.56, 106.18, 60.41, 56.21, 39.12, 35.31. HRMS (TOF ES$^+$): m/z calcd for $C_{18}H_{22}N_5O_3$ [$(M+H)^+$], 356.1723; found, 356.1722.

5.2. MTS assay

Human cancer cell lines were obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HeLa, HL-60, A549 and H1299 cells were routinely cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum, penicillinestreptomycin solution, at 37 ^{o}C with 5% CO_2. To evaluate antiproliferative properties of the synthesized compounds, the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium, inner salt; (MTS)] assay was used. The cell lines were assessed by trypsinizing each cell line and seeding 1000-3000 cells per well into 96-well plates. Cells were grown for 12 h and then treated with compounds at concentrations ranging from 0.03 μM to 100 μM and incubated for 48 h in 200 μL media. MTS reagent (20 μL) in phosphate buffer solution was added to each well and incubated further for 3 h. A490 nm was measured using a Thermomax Molecular Device plate reader. The experiments were performed in triplicate and repeated at least three times for each compound per cell line. MTX and 5-Fu were used as positive controls.

5.3. Dihydrofolate reductase (DHFR) inhibition assay

The recombinant human (rh) DHFR inhibition assay was performed in a final volume of 100 μL. The assay systerm contained 0.1 mM dihydrofolate, 0.3 mM NADPH, 100 mM KCl and 0.011 unit rh DHFR in phosphate buffer, pH 7.5. After addition of the enzyme, the mixture was incubated at room temperature for 15 min. The absorbance of each well was read in the microplate reader at room temperature at wavelengths of 340 nm.

*5.4. Effects of compound **5k** on cell cycle progression in HL-60 cells*

HL-60 cells at a density of 1.8×10^6 cells per well were cultured in 60 mm petri dish overnight, then they were harvested and washed twice with ice-cold PBS after treating with compound **5k** for 24 h. Next, these cells were fixed in 70% cooled ethanol at 4 ^{o}C until being washing in PBS just before analysis by flow cytometry. 0.5 mL PBS within 10 mg/mL RNase A were add to the fixed cells and incubated at 37 ^{o}C for 30min. After this, 50 mg/mL propidium iodide was added to the cells. The DNA contents were determined using a CellQuest and analyzed by the Modfit software (Becton Dickinson).

5.5. Docking and molecular modeling study

The three-dimensional structures of compounds **5k** and **4k**, which presented best and worst biological profiles, in their neutral forms were constructed using ACCELRYS DISCOVERY STUDIO client 2.5 (DS 2.5) software. Lowest energy conformer of each new analog 'global-minima' was docked into the rhDHFR enzyme-binding domain in tertiary complex with NADPH and N^6-methyl-N^6-(3,4,5-trifluorophenyl)-2,4,6-triamine pyrido[2,3-*d*]pyrimidine [31], code ID 4QJC, was downloaded from the protein data bank at the Research Collaboratory for Structural Bioinformatics (www.rcsb.org) [32]. All of hydrogens were added and enzyme structure was subjected to refinement by DS 2.5 software. All atoms within a 10 Å around the co-crystallized ligand in crystal co-ordinates of DHFR were selected as binding site. For DS 2.5 docking, the default parameters were used if it was not mentioned.

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Appendix A. Supplementary data

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Highlight

- A series of 2,4-diamino-6-substituted pyrido[3,2-*d*]pyrimidines were efficiently synthesized.
- Compounds were evaluated for *in vitro* DHFR inhibition and antitumor activity.
- Compounds **5g**, **5h**, **5i** and **5k** showed antitumor activity with IC_{50} values range of 0.07-23 μM against four cell lines.
- SARs and docking studies were discussed in detail.
- Compound **5k** arrested in S Phase.