Accepted Manuscript

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PII: S0223-5234(17)30524-X

DOI: 10.1016/j.ejmech.2017.07.002

Reference: EJMECH 9565

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 5 April 2017

Revised Date: 30 June 2017

Accepted Date: 2 July 2017

Please cite this article as: C. Tian, M. Wang, Z. Han, F. Fang, Z. Zhang, X. Wang, J. Liu, Design, synthesis and biological evaluation of novel 6-substituted pyrrolo [3,2-*d*] pyrimidine analogues as antifolate antitumor agents, *European Journal of Medicinal Chemistry* (2017), doi: 10.1016/j.ejmech.2017.07.002.

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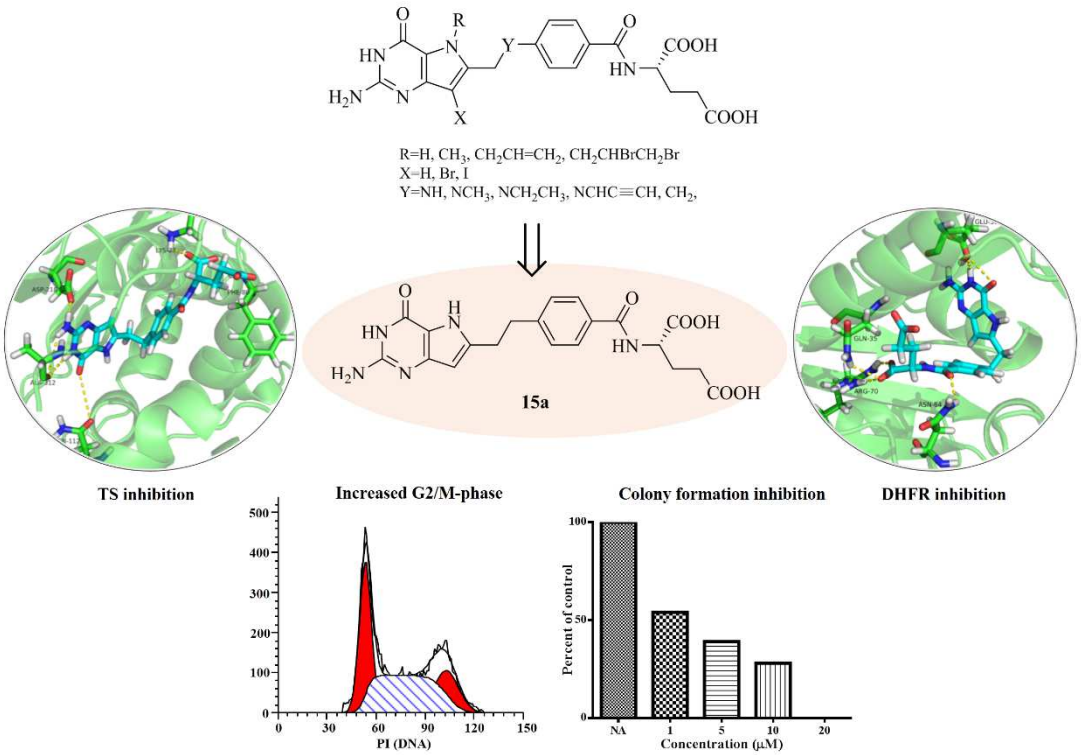

Design, Synthesis and Biological Evaluation of Novel 6-Substituted Pyrrolo [3,2-*d*] Pyrimidine Analogues as Antifolate Antitumor Agents

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Abstract

A series of novel 6-substituted pyrrolo[3,2-*d*]pyrimidine analogues (**10a**, **11a**-**13a**, **15a**, **17a**, **18a**, **27a** and **28a**) have been designed and synthesized as antifolate antitumor agents. The anti-proliferative activities of these compounds against HL60, A549, H1299, Hela, HCT116 and HT29 tumor cells were evaluated. Most of the compounds exhibited micromolar anti-proliferative potencies. Compound **15a**, the most potent one, has GI_{50} value of 0.73, 1.72, and 8.92 μM against A549, H1299 and HL60 cells, respectively. The cell cycle distribution assay displayed that **15a** could increase the accumulation of G2/M-phase cells. **15a** showed low potency in induction of apoptosis. However, the inhibition of A549 cell colony formation was observed. These indicated that the tumor cell death relied on the irreversible effect of **15a** on clonogenicity and cell proliferation. The identification of targeted pathway of **15a** implied that the anti-proliferative potencies of **15a** probably act through dual inhibition of thymidylate synthase (TS) and dihydrofolate reductase (DHFR).

Keywords:

6-substituted pyrrolo[3,2-*d*]pyrimidines;
Anti-proliferation;
Structure–activity relationship;
Dihydrofolate reductase;
Thymidylate synthase;
G2/M-phase increased;
Colony formation inhibition;

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Introduction

Folate-dependent metabolic pathway, which is required in plenty of interrelated enzymatic reactions, has long been recognized as an attractive target of cancer chemotherapy[1, 2]. Among the enzymes involved in the folate-dependent metabolic pathway, dihydrofolate synthase (DHFR), thymidylate synthase (TS), glycinamide ribonucleotide formhyltransferase (GARFTase), 5-aminoimidazole-4-carboxamide and ribonucleotide formyltransferase (AICARFTase) are critical targets in the development of antifolate agents. DHFR reduces 7,8-dihydrofolic acid to tetrahydrofolic acid [3, 4], while TS catalyze the de novo synthesis of 2'-deoxythymidine-5'-monophosphate (dTMP) from 2'-deoxyuridine5'-monophosphate (dUMP) by transferring a methyl group from 5,10-methylenetetrahydrofolate to afford 7,8-dihydrofolate [5]. GARFTase or AICARFTase catalyze a formyl group transferring from 10-formyltetrahydrofolate to glycinamide ribonucleotide monophosphate (GARMP) or 5-aminoimidazole-4-carboxamide ribonucleotide monophosphate (AICARMP), which are involved in the de novo purine nucleotide biosynthesis [6]. The DHFR inhibitors (e.g., methotrexate (MTX) [7] and pralatrexate, (PDX) [8]) and TS inhibitors (e.g., pemetrexed (PMX) [9] and raltiterxed (RTX) [10]) play important roles in the clinical treatment of solid tumor currently. Furthermore, GARFTase inhibitors such as lometrexol (LMTX) [11], LY309887 [12] and AG2034 [13] are undergoing clinical trials. Furthermore, there are three major membrane transport systems, including the reduced folate carrier (RFC) [14], the proton-coupled folate transporters (PCFT) [15], and folate receptors (FRs) α and β [16], involving in the cellular uptake of (anti)folate. The latter two are highly expressed in human tumors [17]. Folate-based compounds with transport selectivity for FRs or PCFT over RFC are reported to possess improved tumor targeting properties these years [18-20].

Since the introduction of MTX in 1950s [21], plenty of compounds were synthesized and evaluated as antifolate anticancer candidates. However, most of them were failed due to dose-limiting toxicities and tumor resistance [18]. In the development of antifolate agents, discovery of novel antifolate agents with low dose-limiting toxicities and low tumor resistance remain to be an attractive research area [22, 23].

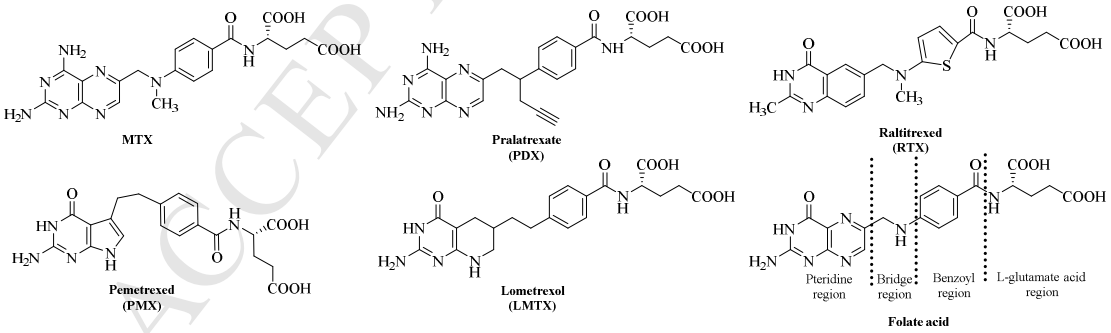
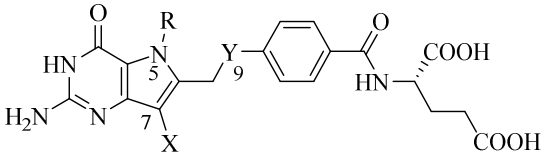


Fig. 1. Representative examples of classical antifolates and the structure of folic acid

The structure of folate acid could be divided in to four regions (**Fig. 1**), including the pteridine region, the bridge region [24], the benzoyl region and the L-glutamate acid region. The structure activity relationship (SAR) studies of the classical antifolates usually vary in these four regions. Our previous studies mainly focus on the varying in the pteridine and the bridge region [25-27]. Although the pteridine regions of the approved antifolate anticancer drugs are mostly 6-6 fused ring system, more and more antifolates with 6-5 fused scaffold, such as pyrrolo[2,3-*d*]pyrimidines, thieno[2,3-*d*]pyrimidine and furo[2,3-*d*]pyrimidine, are reported in recent years [18-20, 28-31].

However, there are limited reports on the antifolates with pyrrolo[3,2-*d*]pyrimidines scaffold. As a continued effort to discovery novel antifolates with multi-targeted antifolate potencies and improved dose-limiting toxicities, in this article, we report a series of 6-substituted 2-amino-4-oxo-pyrrolo[3,2-*d*]pyrimidine analogues varied at position 5, 7 and 9 as novel antifolate antitumor agents (**Fig. 2**).

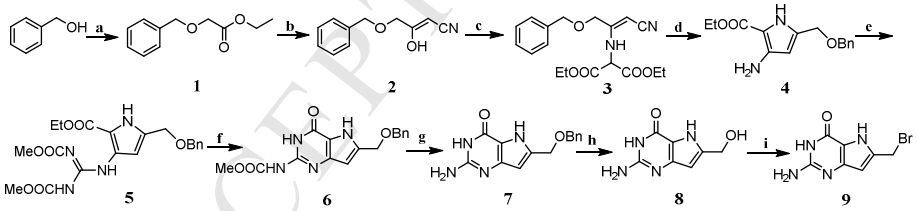


R=H, CH_3, $CH_2CH{=}CH_2$, $CH_2CHBrCH_2Br$
X=H, Br, I
Y=NH, NCH_3, NCH_2CH_3, $NCHC{\equiv}CH$, CH_2,

Fig. 2. The structures of target compounds

2. Chemistry

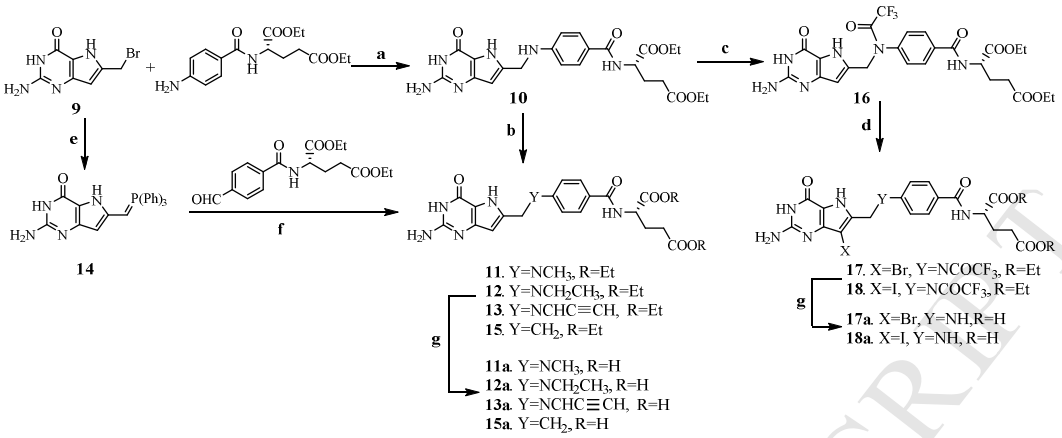
A key intermediate **9** was synthesized as shown in **Scheme 1**. The synthesis started from benzyl alcohol, which was condensed with ethyl bromoacetate to obtain compound **1**. Then **1** was reacted with acetonitrile lithium, which was prepared by adding acetonitrile to 2.5 M n-butyl lithium in THF dropwise, to afford **2**. Compound **2** was condensed with diethyl aminomalonate in the present of acetic acid to achieve **3**, which was further cyclized under basic condition to produce compound **4**. Thus, the pyrrole **4** was then subjected to condensation with 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea [32] with acetic acid as catalyst in MeOH to afford **5**. The self-condensation reaction of compound **5** could be processed in the present of sodium methoxide in MeOH to obtain **6**. Hydrolysis of the carbamate group with aqueous sodium hydroxide at 60 ºC afforded compound **7**. Then, the benzyl group was removed by hydrolysis in 6 N aqueous hydrochloric solution at 60 ºC to obtain **8**. Subsequently, the crucial intermediate compound **9** was achieved through bromination of **8** by phosphorus tribromide.



Scheme 1. Synthetic route to compounds **9.** Reagents and conditions: a) NaH, $BrCH_2COOEt$, rt; b) n-BuLi, CH3CN, -78 ºC to rt; c) diethyl aminomalonate, AcOH, rt; d) NaOEt, EtOH, rt; e) 1,3-bis(methoxycarbonyl)-2-methylthiopseudourea, AcOH, N_2, rt; f) NaOMe, MeOH, rt; g) i)1 N NaOH, 50 ºC; ii) AcOH, pH=6; h) 6 N HCl, 60 ºC; i) PBr_3, THF, reflux.

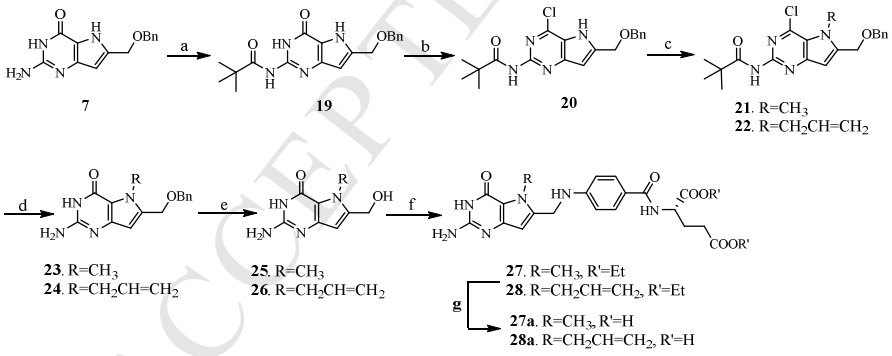
With compound **9** in hands, condensation reaction with diethyl (4-aminobenzoyl)-L-glutamate was first processed to afford **10**. The later one could be alkylated by several alkyl halides (methyl iodide, bromoethane or propargyl bromide) at N^9 position to obtain compounds **11**-**13**. Meanwhile, trifluoroacetylation of **10** could achieve compound **16**, which would be halogenated by NBS or I_2 at C^7 position to produce the 7-halogenated target compounds **17** and **18**. To obtain C-C bridge analogue **15**, phosphorus ylide (**14**) was prepared with compound **9**. Without further purification,

compound **14** was condensed with diethyl-N-[4-(formyl)benzoyl]-L-glutamate and hydrogenated subsequently to achieve compound **15**.



Scheme 2. Synthetic route to compounds **10**-**18**. Reagents and conditions: a) DMAC, 80 ºC; b) CH_3I, CH_3CH_2Br or propargyl bromide, K_2CO_3, anhydrous DMF, rt; c) $(CF_3CO)_2O$, DCM, rt; d) NBS or I_2, DCM, rt; e) $(C_6H_5)_3P$, anhydrous DMF, argon, 60 ºC, 2h, then NaOMe, rt, 1h; f) i) anhydrous DMF, 60 ºC, 24h; ii) H_2, Pd/C; g) 1 mol/L NaOH, r.t.

The N^5-substituted analogues were obtained through the synthetic route outlined in scheme 3. Acylation of compound **7** with pivaloyl chloride afforded 2-NH_2-protected compound **19**, which was proceeded chlorination with $POCl_3$. Alkylation of compound **20** by alkyl halides (methyl iodide or bromopropylene) afforded compounds **21** and **22**. Hydrolysis of **21** and **22** could obtain compounds **23** and **24**. The same method as preparation of compound **8** was used to remove the benzyl group to produce **25** and **26**. Bromonation of **25** and **26** and subsequently condensation with diethyl (4-aminobenzoyl)-L-glutamate respectively afforded compounds **27** and **28**.



Scheme 3. Synthetic route to compounds **19**-**27.** Reagents and conditions: a) PivCl, DCM, reflux, 5h; b) $POCl_3$, reflux, 10h; c) MeI or Allyl bromide, NaH; d) 2 mol/L NaOH, 1,4-dioxane, reflux; 6h; e) 6 mol/L HCl, reflux, 6h; f) i) PBr_3, THF; ii) diethyl (4-aminobenzoyl)-L-glutamate, DMAC, 80 ºC; g) 1 mol/L NaOH, r.t.

Hydrolysis of compounds **10**, **11**-**13**, **15**, **17**, **18**, **27**, and **28** could finally obtain our target compounds **10a**, **11a**-**13a**, **15a**, **17a**, **18a**, **27a**, and **28a** as carboxylic acid form [27].

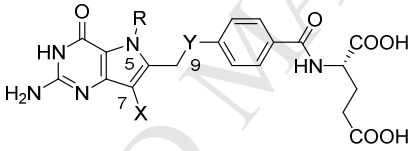
3. Biological evaluation and discussion

3.1. Anti-proliferative effects of target compounds

The anti-proliferative effects of **10a**, **11a-13a**, **15a**, **17a**, **18a**, **27a**, and **28a** were evaluated toward HL60, A549, H1299, Hela, HCT116 and HT29 cell lines using MTS method. The growth inhibitory effects (GI_{50}) results are illustrated in Table 1 and compared with MTX.

The biological data in Table 1 established that compounds**15a**, **27a** and **28a** showed moderate to good inhibitory effect against most of the cell lines. **15a** was the best, obviously. Although most of the compounds exhibited micromolar anti-proliferative potencies toward cancer cell lines, some structure activity relationships about this kind of compounds could be summarized as follow. Firstly, the inhibitory activities of **10a-13a** indicated that introducing substitution to the bridge region could significantly diminish the inhibitory effects against the cancer cell lines. Secondly, introduction of bromine or iodine at position 7 of **10a** (afforded **17a** and **18a)** would also decrease their anti-proliferative effects. Thirdly, the inhibitory activities of **27a** and **28a** showed that substituents at position 5 of **10a** would not reduce the inhibitory effects. In turn, they could moderately increase the inhibitory activities against HL60, A549 and H1299 cell lines. Finally, **15a** established much better anti-proliferative effects than **10a** against these tumor cell lines, especially for HL60, A549 and H1299 cell lines, although their only difference is position 9. In brief, we could conclude that position 7 and 9 of this kind of compounds play important roles in the anti-proliferative effects and introduction of certain substituents on position 5 was conducive to the inhibitory effects.

Table 1. The anti-proliferative effects of the target compounds



10a, 11a-13a, 15a, 17a, 18a, 27a and 28a

Cpd	X	Y	R	HL60	A549	H1299	Hela	HCT116	HT29
10a	H	NH	H	47.42±6.94	79.03±7.30	73.07±4.01	85.14±3.21	>100	>100
11a	H	NCH_3	H	>100	>100	>100	>100	>100	>100
12a	H	NCH_2CH_3	H	>100	>100	>100	>100	>100	>100
13a	H	$NCH_2C\equiv CH$	H	>100	>100	>100	>100	>100	>100
15a	H	CH_2	H	8.92±1.60	0.73±0.16	1.72±0.67	73.08±3.46	54.61±8.66	99.14±4.90
17a	Br	NH	H	40.06±9.38	83.03±8.46	>100	>100	>100	>100
18a	I	NH	H	54.20±3.64	>100	>100	>100	>100	>100
27a	H	NH	CH_3	13.51±5.68	46.01±5.99	51.33±5.71	94.37±5.28	>100	>100
28a	H	NH	$CH_2CH=CH_2$	15.45±1.93	57.27±17.70	68.39±16.86	82.72±4.24	>100	98.55±10.84
MTX				0.038±0.001	0.014±0.002	0.072±0.002	>100	0.13±0.02	>100

3.2. Inhibitory activities toward DHFR

To identify the targeted enzyme of these compounds, first of all, compounds **10a**, **11a-13a**, **15a**, **17a**, **18a**, **27a** and **28a** were evaluated as inhibitors of human DHFR. The inhibitory ratio at 1 μM,

10 μM and 100 μM are showed in Figure 3. The DHFR inhibition results established that most of the compounds showed inhibitory potencies in some degree toward DHFR. **15a**, which has a inhibitory ratio of 66.7% at 100 μM, was the best one among these compounds. As **15a** displayed poor inhibitory potencies at low concentration, considering of its high anti-proliferative potency against tumor cells, it seemed probably that DHFR was not the main targeted enzyme of **15a**.

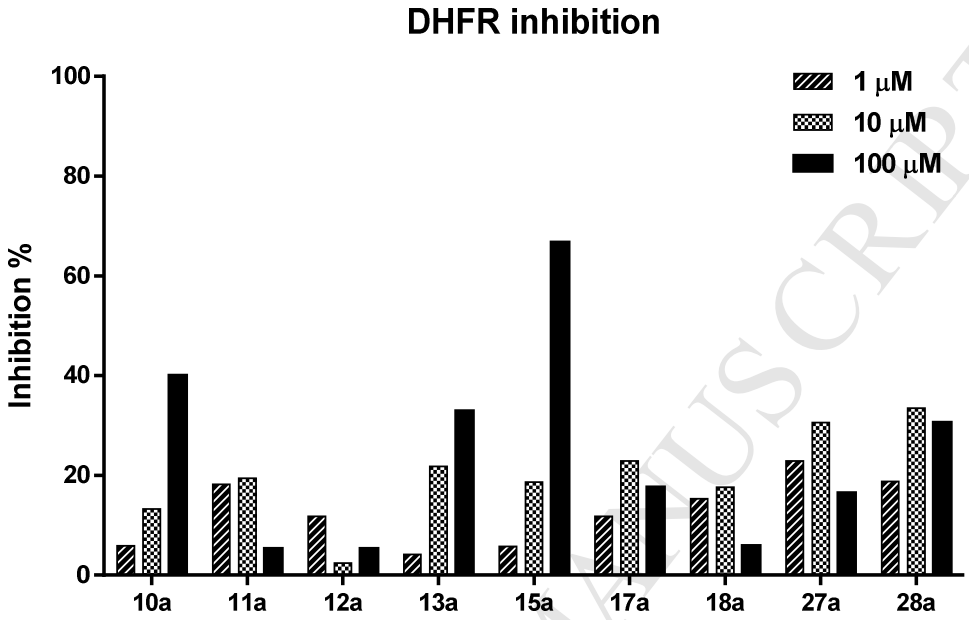


Fig. 3. The inhibitory activities toward DHFR

3.3. Identification of the targeted enzyme of 6-Substituted Pyrrolo [3,2-*d*] Pyrimidines antifolates

As the substrates for folate-dependent enzymes, including DHFR, TS, GARFTase and AICARFTase, share a similar scaffold, antifolate anticancer drugs always exhibited multi-targeted effects currently.

To determine the targeted pathway of compound **15a**, the nucleoside protection experiments were conducted to distinguish de novo purine nucleotide from thymidylate biosynthesis employing adenosine (60 μM) and thymidine (10 μM), respectively [33, 34]. As there are two folate-dependent enzymes (GARFTase and AICARFTase) involving in de novo purine nucleotide biosynthesis [20], in order to further identify the targets, HL60 cells were treated in presence of AICA (320 μM), which is the substrate for AICARFTase, thus bypassing the step catalyzed by GARFTase.

The nucleoside/AICA protection results for compound **15a** were showed in Fig. 4. For **15a**, single addition of excess adenosine of AICA was slightly protective, while single addition of excess thymidine was protective to some extent. These results implied that compound **15a** was probably an inhibitor of thymidylate synthase (TS). Based on the above results and the inhibitory activities toward DHFR, the anti-proliferative potency of **15a** probably act through dual inhibition of TS and DHFR.

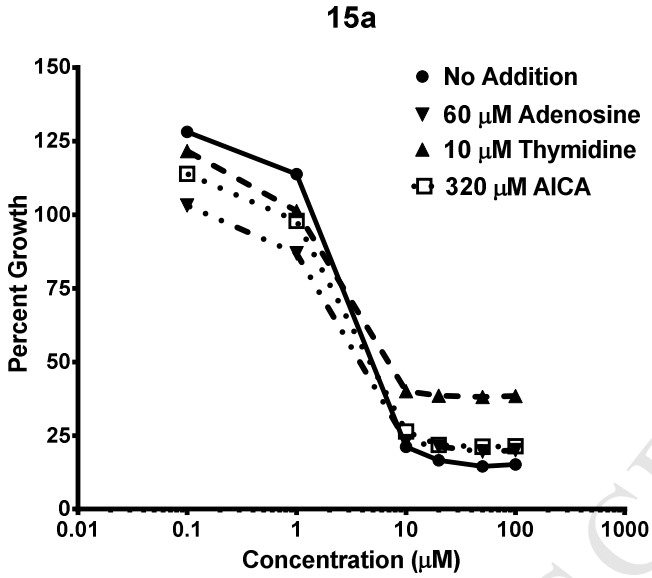


Fig. 4. Cell proliferation assays with protection by nucleosides including thymidine and adenosine and by 5-aminoimidazole-4-carboxamide (AICA) to identify intracellular targets of compound **15a**. To identify the targeted pathways and the folate-dependent intracellular enzymes in HL60 cells treated with compound **15a** (0.1-100 μM), cell proliferation assays were performed in the presence of 10 μM thymidine, 60 μM adenosine, or 320 μM AICA. The results were normalized to those for untreated cells (no drug).

3.4. Effect of compound 15a on cell cycle distribution in A549 cells

In order to verify the effect of compound **15a** on the cell cycle distribution, A549 cells were treated with compound **15a** (1μM and 10μM) and MTX (1μM) for 72 h, along with an untreated control. Cells were washed with PBS, fixed with ice-cold 70% ethanol overnight, stained with propidium iodide (PI), and analyzed for cell cycle distribution by flow cytometry. As shown in **Fig. 5**, MTX induced a significant G1-phase arrest relative to the untreated control. Beyond our expectation, **15a** displayed a different effect on the cell cycle distribution. The percent of G2/M-phase cells increased to 15.0% and 19.4% for **15a** (1 μM) and **15a** (10 μM) from 7.8% (untreated control). These results indicated that **15a** could effectively induce G_2/M-phase arrest.

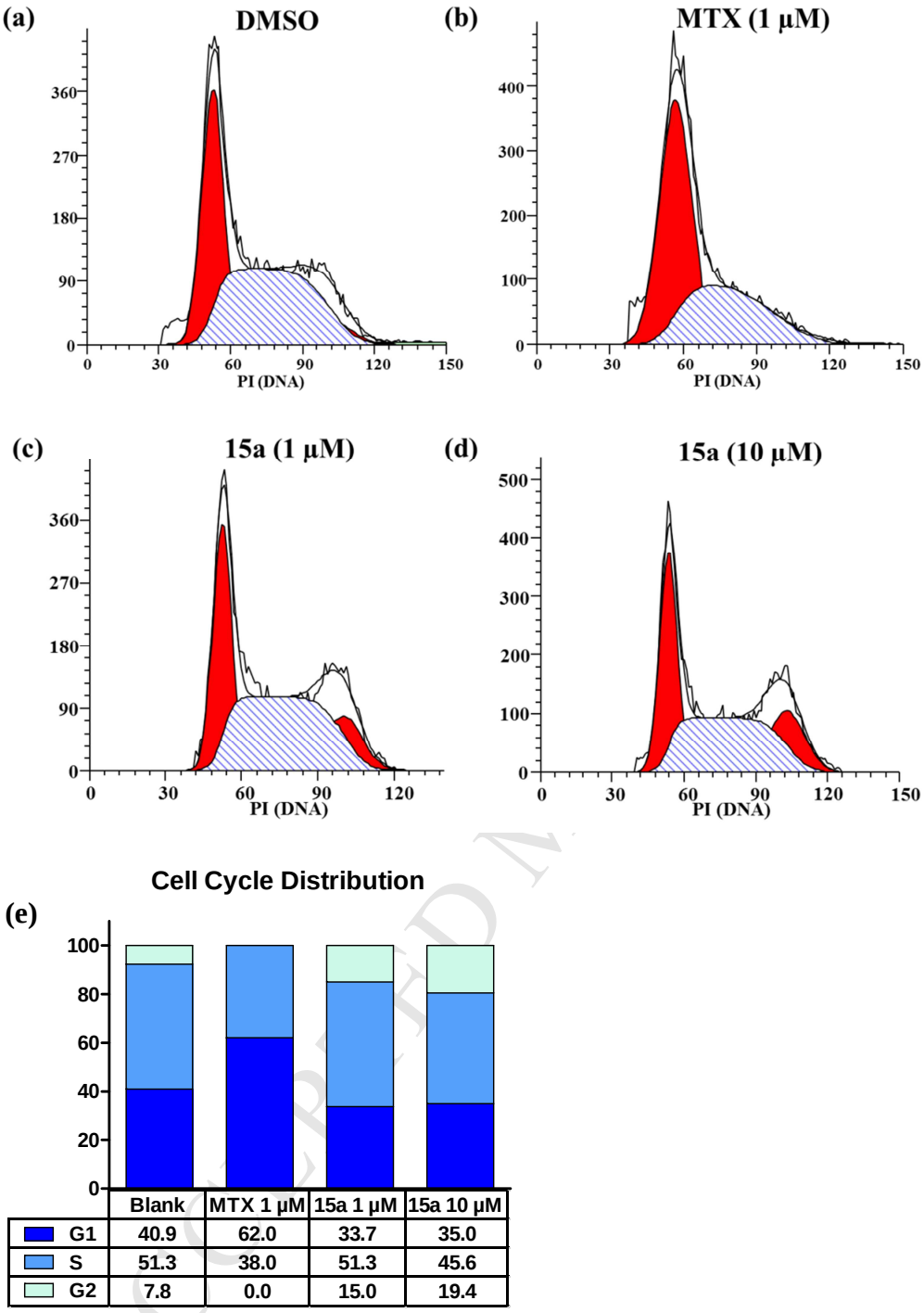
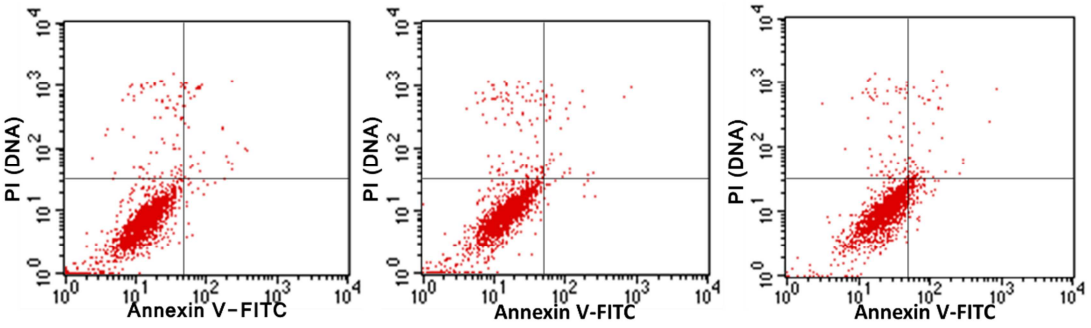


Fig. 5. Cell cycle assay. A549 cells were treated with 1 µM MTX, 1 µM **15a** or 10µM **15a** for 72 h, washed, fixed, and stained with PI. Cell cycle distribution was detected by flow cytometry. The results are compared with those treated with DMSO instead of drug. a) DMSO; b) MTX (1µM); c) **15a** (1 µM); d) **15a** (10 µM); e) The percentage of cells in different phase of cell cycle (G1, S, G2/M).

3.5. Apoptosis Analysis

An additional experiment were performed with A549 cells treated with compound **15a** to assess its effect on induction of apoptosis. As shown in **Fig. 6**, the fraction of cells in early apoptosis were

0.39%, 4.40% and 0.32% for **15a** (1 µM), **15a** (10 µM) and untreated control. The fraction of cells in late apoptosis were 1.07%, 3.21% and 1.24% for **15a** (1 µM), **15a** (10 µM) and untreated control. These results indicated that compound **15a** could induce cell apoptosis to some extent at higher concentration. However, the low ratio of apoptosis implied that the anti-proliferative effect of compound **15a** was largely independence of apoptosis.



	Untreated control	**15a** 1 µM	**15a** 10 µM
UL	3.58%	4.46%	3.56%
UR	1.24%	1.07%	3.21%
LL	94.86%	94.08%	88.02%
LR	0.32%	0.39%	4.40%

Fig. 6. Analysis of apoptosis by Annexin V-FITC/PI staining and flow cytometry is shown for A549 cells treated with **15a** 1 µM or 10 µM for 72 h along with untreated control. UL: dead cells; UR: late apoptosis cells; LL: viable cells; LR: early apoptosis cells.

3.6 Colony Formation Inhibition Assay

In the colony formation inhibition assay, A549 tumor cells were treated with compound **15a** in various concentrations (1-20 µM) for 48 h, then washed and incubated in absence of drug. Colonies were enumerated after 8 days (**Fig 7**). These results exhibited that **15a** could totally inhibit colony formation at highest drug concentration and the effect of **15a** on clonogenicity were irreversible, which indicated that compound **15a** was cytotoxic, but not cytostatic.

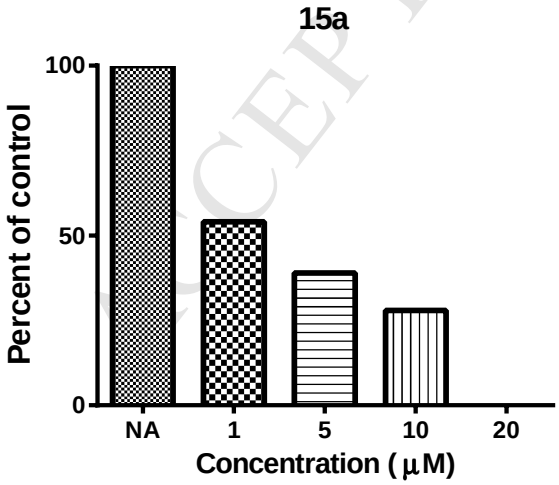


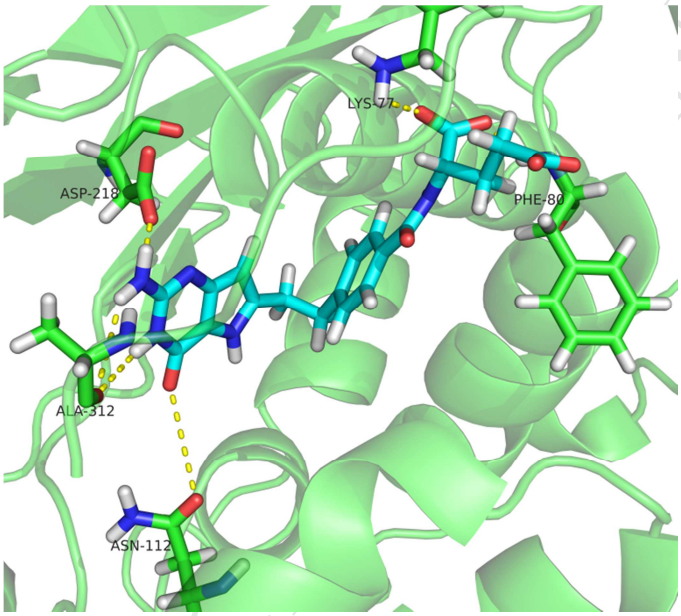
Fig. 7. Colony formation assay. A549 cells were plated in six-well plate (100 cells per well) and treated with compound **15a** (1-20 µM) for 48h, after which time the drug was removed and colonies were allowed to grow in drug-free medium over 8 days.

4. Molecular modeling studies

On the basis of DHFR inhibition and the cellular metabolic data, compound **15a** displayed dual inhibitory potencies toward thymidylate synthase (TS) and DHFR in tumor cells, molecular modeling studies were performed using Discovery Studio 2.5.

Figure **8** shows the docked pose of compound **15a** (cyan) and the overlay of the docked pose of compound **15a** (cyan) with the crystal structure of PMX (purple) in the human TS (PDB ID 1JU6) active site [35]. The binding site for the folate cofactor moiety consists of three parts: the pteridine binding site, the benzoylglutamate region, and the bridge region. The docked pose shows the pyrrolo[3,2-*d*]pyrimidine scaffold of **15a** form a H-bond network with Asp218, Ala312 and Asn112 in the pteridine binding site. The H-bond between 4-oxo moiety of **15a** and Asn112 was an extra H-bond compared to that of PMX. The glutamate tail of **15a** forms two hydrogen bond with Lys77 and Phe80 in the benzoylglutamate region. The binding pose of PMX indicate that the pyrrolo[2,3-*d*]pyrimidine scaffold of PMX and benzoylglutamate tail form a tortuous 'L' structure through the bridge region. **15a** established a similar binding pose to that of PMX. The modeling study imply that **15a** should bind and inhibit the thymidylate synthase (TS), which is aligned with the results of our cellular metabolic assays.

a)



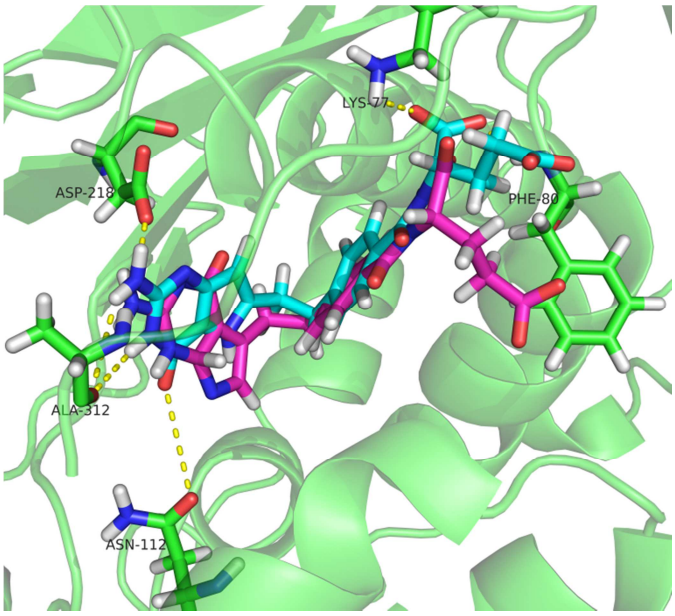
b) 

Fig. 8. a) Docked pose of compound **15a** (cyan) in the human TS (PDB ID 1JU6) active site; b) Overlay of the docked pose of compound **15a** (cyan) with the crystal structure of PMX (purple) in the human TS (PDB ID 1JU6) active site.

Figure **9** shows the docked pose of compound **15a** (cyan) and the overlay of the docked pose of **15a** (cyan) with the crystal structure of MTX (purple) in the human DHFR (PDB ID 1U72) active site [36]. The docked pose displays that the pyrrolo[3,2-*d*]pyrimidine scaffold of **15a** form only two hydrogen bonds with Glu30 residue through 3-NH and 4-oxo. In contrast, there are three hydrogen bonds for MTX in the corresponding binding site. The benzoylglutamate tail of **15a** form four hydrogen bonds with Gln35, Arg70 and Asn64 residues, which are same as that of MTX. The overlay of the docked pose of **15a** with the crystal structure of MTX indicated that **15a** established a very similar binding mode with that of MTX in the DHFR active site, especially for the benzoylglutamate tail. The modeling results imply that **15a** could be an inhibitor of DHFR, but should be less potent than MTX.

The above modeling results suggest that **15a** should be a dual inhibitor of TS and DHFR, which is consistent with the results of cellular metabolic assays and DHFR inhibition assay.

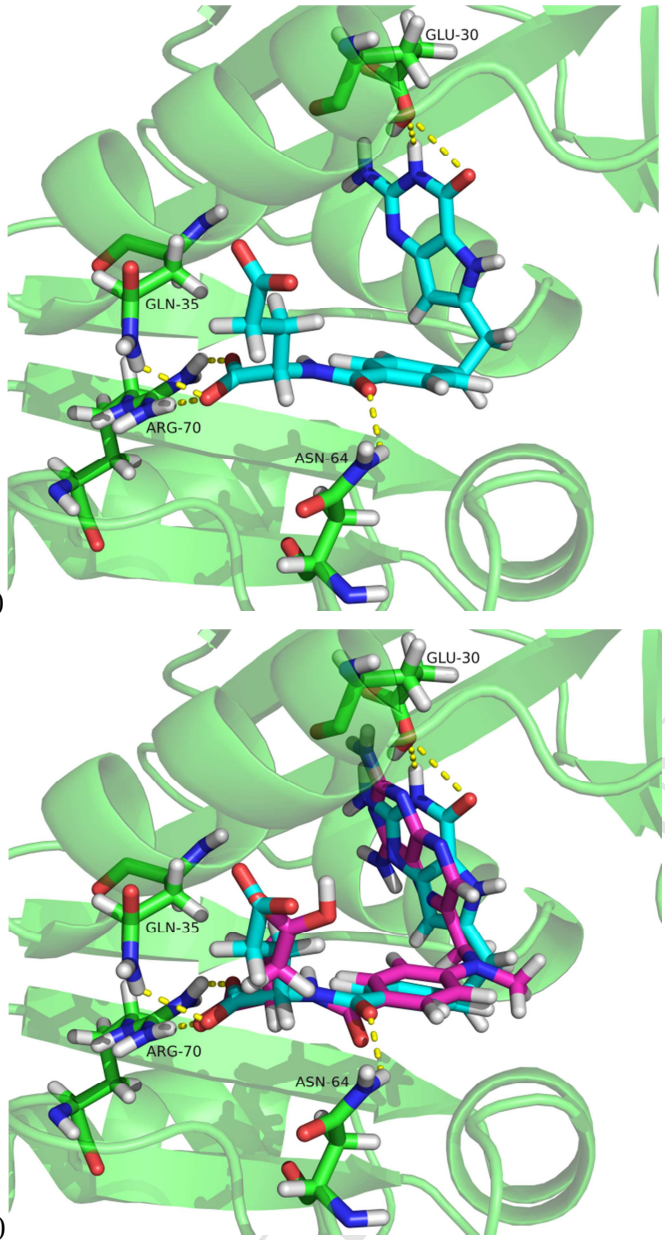


Fig. 9. a) Docked pose of compound **15a** (cyan) in the human DHFR (PDB ID 1U72) active site; b) Overlay of the docked pose of compound **15a** (cyan) with the crystal structure of MTX (purple) in the human DHFR (PDB ID 1U72) active site.

5. Conclusion

In a continued effort to discovery of novel antifolate anticancer candidates, in this study, a series of novel 6-substituted pyrrolo [3,2-*d*] pyrimidine analogues **10a**, **11a**-**13a**, **15a**, **17a**, **18a**, **27a**, and **28a** were designed and synthesized as potential antifolate agents. The biological activities of these compounds toward HL60, A549, H1299, Hela, HCT116, and HT29 cell lines were evaluated. Most of the compounds exhibited micromolar anti-proliferative potencies against most of these tumor cell lines. The structure activity relationships about this kind of compounds were summarized. Compound **15a**, which has GI_{50} of 0.73, 1.72 and 8.92 μM against A549, H1299 and HL60 tumor cells respectively, was the most potent one of this series. **15a** could significantly affect the cell cycle distribution of tumor cells and induce G2/M-phase arrest to some extent. The apoptosis assay and colony formation inhibition assay demonstrated that the tumor cell death relied on the irreversible effect of **15a** on clonogenicity. The identification of targeted enzymes of **15a** implied that the anti-proliferative activities of **15a** probably act through dual inhibition of TS and DHFR. This conclusion was supported by the molecular modeling studies. These results make **15a** as a good lead compound as multi-targeted antifolate agents for further study.

6. Experimental Section

6.1. Chemistry

6.1.1. General information

Reagents and solvents were purchased from common commercial suppliers and were used without further purification. Organic solvents were dried according to standard procedures. Melting points were determined with a SGW® X4 apparatus. Mass spectra were recorded on MDS SCIEX QSTAR system. ^{1}H-and ^{13}C NMR spectra were recorded with a Varian INOVA-400 or INOVA-600 with $CDCl_3$ or DMSO-d_6 as solvent. Tetramethylsilane was used as an internal standard to express the chemical shift in ppm (parts per million): s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; m, multiplet; and bs, broad singlet. Thin-layer chromatography (TLC) with a fluorescent indicator were utilized to monitor the reaction progress, and the spots were visualized under 254 and 365 nm illumination.

6.1.2. Synthesis of Ethyl 2-(benzyloxy)acetate (1)

To a 250 mL round-bottomed flask was added PhMe (100 mL) and NaH (4.0 g, purity 60%, 0.1 mol). The mixture was cooled in an ice bath. Phemethylol (10 mL, 0.097 mol) was then added dropwise. Once the reaction reached completion, a solution of ethyl bromoacetate (10.7 mL, 0.097 mmol) in PhMe was added and the mixture was stirred at room temperature for 24 h. The reaction mixture was poured into ice water (200 mL) and neutralized with dilute hydrochloric acid. Then, the mixture was extracted by ethyl acetate (3 × 100 mL), and the organic phase was washed with brine (200 mL), dried with Na_2SO_4 and concentrated. The crude product was purified by silica gel chromatography using *n*-hexane-ethyl acetate (30:1, v/v) to yield compound **1** (12.3 g, 65.6%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.31 (t, J = 6.8 Hz, 3H, CH_3), 4.12 (s, 2H, CH_2CO), 4.26 (q, J = 6.8 Hz, 2H, CH_2CH_3), 4.67 (s, 2H, $PhCH_2$), 7.36-7.42 (m, 5 H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.2, 60.9, 67.3, 73.3, 128.0, 128.1, 128.5, 137.2, 170.4. ESI-TOF-MS: $[M+H]^+$ m/z: 195.2, $[M+Na]^+$ m/z: 217.2.

6.1.3. Synthesis of 4-(Benzyloxy)-3-oxobutanenitrile (2)

To a 150 mL round-bottomed flask was added 50 mL THF. The flask was cooled to -78 ºC, and 2.2 N *n*-BuLi (4.7 mL, 10.3 mmol) and acetonitrile (0.55 mL, 10.6 mmol) were added sequentially. After stirring at -78 ºC for 1 h, 50 mL ethyl 2-(benzyloxy)acetate (compound **1**, 2.0 g, 10.3 mmol) in THF was added. The solution was stirred at -78 ºC for 0.5 h and then warmed to room temperature. The mixture was poured into ice water (200 mL) and neutralized with dilute hydrochloric acid. Then, the mixture was extracted with ethyl acetate (3×100 mL), and the organic phase was washed with brine (200 mL), dried with Na_2SO_4 and concentrated. The crude product was then purified by silica gel chromatography using *n*-hexane–ethyl acetate (3:1, v/v) to give compound **2** (1.61 g, 82.6%) as colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.69 (s, 2H, $=CH_2CN$), 4.15 (s, 2H, CH_2), 4.62 (s, 2H, $PhCH_2$), 7.35-7.42 (m, 5H, Ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 29.7, 73.9, 74.1, 112.4, 128.1, 128.5, 128.8, 136.3, 196.8.

6.1.4. *Diethyl (Z)-2-((3-(benzyloxy)-1-cyanoprop-1-en-2-yl)amino)malonate* (3)

Compound **2** (22.0 g, 116 mmol) was dissolved in a mixture of MeOH and acetic acid (1:1, 50 mL), and diethyl aminomalonate hydrochloride (25 g, 118 mmol) were added sequentially. After stirring at room temperature overnight, the reaction mixture was poured into water (100 mL). Then the mixture was neutralized with 1 N NaOH solution and extracted by ethyl acetate (3×50 mL), washed with brine (100 mL), dried with Na_2SO_4. The solvent was evaporated under vacuum to obtain compound **3** (36g, 89.4%)as viscous oil. ^{1}H NMR (400 MHz,$CDCl_3$)δ： 1.30 (t, J = 7.2 Hz, 6H, 2 × CH_3), 4.28~4.30 (q, J = 7.2 Hz, 4H, 2 × CH_2), 4.43 (d, J = 8.0 Hz, 1H, C*H*NH), 4.57 (s, 1H, =CHCN), 4.67 (s, 4H, CH_2OCH_2), 7.36~7.37 (m, 5H, ph); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.0, 59.1, 63.1, 65.3, 67.2, 73.2, 118.7, 127.0, 127.6, 128.5, 140.9, 158.2, 165.5; ESI-TOF-MS: $[M+H]^+$ m/z: 347.18, $[M+Na]^+$ m/z: 369.16.

6.1.5. ***Ethyl 3-amino-5-(benzyloxymethyl)-1H-pyrrole-2-carboxylate (4)***

173 mg (7.52 mmol) Na was added to anhydrous EtOH (20 mL) to form a solution of NaOEt in ethanol. Then a solution of 2.06 g (7.51 mmol) compound **3** in ethanol (10 mL) was dropwise added to the NaOEt solution. The reaction mixture was stirred for 6 h at room temperature. The solvent was evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel with *n*-hexane–ethyl acetate (3:1, v/v) as the eluent to yield **4** (1.18 g, 57.3%) as an off-white solid. mp 86-87 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.33 (t, J = 6.8 Hz, 3H, CH_3), 4.26-4.31 (m, 2H, OC*H*$_2$CH$_3$), 4.40 (s, 2H, C*H*$_2$OBn), 4.49 (s, 2H, PhC*H*$_2$), 5.63 (s, 1H, 4-CH), 7.29-7.34 (m, 5H, Ph); ^{13}C NMR (100 MHz,$CDCl_3$) δ: 14.7, 59.4, 64.6, 72.1, 98.9, 110.0, 127.9, 127.9, 128.5, 130.9, 133.6, 137.6; ESI-TOF-MS: $[M+H]^+$m/z: 275.15.

6.1.6. ***Ethyl (Z)-5-(benzyloxymethyl)-3-(2,3-bis(methoxycarbonyl)guanidino)-1H-pyrrole-2-carboxylate (5)***

The pyrrole **4** (1.03 g, 3.76 mmol) was dissolved in anhydrous MeOH (20 mL), and 1,3-bis(methoxycarbonyl)-2-methyl-2-thiopseudourea (0.81 g, 3.93 mmol) and AcOH (10 mL) were added sequentially. The mixture was stirred at room temperature overnight and became a thick paste. Then, the mixture was filtered to give compound **5** (1.24, 76.4%) as a white solid. mp 155-157 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.38 (t, J = 6.8 Hz, 3H, CH_3), 3.76 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 4.42 (q, J = 6.8 Hz, 2H, OCH_2), 4.53-4.54 (d, 4H, CH_2OCH_2), 7.14 (s, 1H, 4-CH), 7.26~7.36 (m, 5H, ph), 8.84 (bs, 1H, CONH), 11.50 (bs, 1H, 1-NH), 11.82 (s, 1H, N^1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 14.6, 52.7, 53.5, 60.5, 64.7, 72.6, 104.1, 128.0, 128.0, 128.5, 132.5, 137.4, 152.6, 153.8, 160.7, 164.3; ESI-TOF-MS: $[M+H]^+$ m/z： 433.2476, $[M+Na]^+$ m/z： 455.2319.

6.1.7. Methyl (6-benzyloxymethyl-4-oxo-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-2-yl)carbamate (6)

1.27 g compound **5** was dissolved in anhydrous MeOH, then 0.925g (17.1 mmol) NaOMe was added in batches. The mixture was stirred at room temperature for 2 h. The mixture was neutralized with AcOH, and the solid was collected by filtration and washed thoroughly with water. After drying, **6** (0.81 g, 83.9%) was obtained as an off-white solid. mp 220–221 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.73 (s, 3H, CH_3), 4.53 (s, 2H, 8-CH_2), 4.55 (s, 2H, OCH_2), 6.23 (s, 1H, 7-CH), 7.27-7.36 (m, 5H, Ph), 11.14 (bs, 2H, N^3H, CONH), 12.11 (s, 1H, N^5H); ^{13}C NMR

(100 MHz, DMSO-d_6) δ: 53.2, 64.9, 71.9, 102.0, 114.9, 127.9, 128.1, 128.7, 138.6, 139.4, 144.4, 152.9, 156.0. ESI-TOF-MS: $[M+H]^+$ m/z: 328.17, $[M+Na]^+$ m/z: 351.16.

6.1.8. 2-Amino-6-benzyloxymethyl-3,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (7)

To a 50 mL round-bottomed flask was added **6** (0.84 g, 2.54 mmol) suspended in 1 N NaOH (10 mL). The reaction mixture was heated at 50 °C for 6 h. The resulting solution was cooled in an ice bath and neutralized with AcOH. The precipitated solid was collected by filtration washed with water, and dried in vacuo to afford **7** (0.68 g, 99.0%) as a white solid. mp 231-233 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.49 (s, 4H, CH_2OCH_2), 5.97 (s, 1H, 7-CH), 6.17 (s, 2H, 2-NH_2), 7.28-7.35 (m, 5H, Ph), 10.94 (bs, 1H, N^3H), 11.58 (s, 1H, N^5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 65.0, 71.7, 100.7, 113.1, 127.9, 128.0, 128.7, 138.2, 138.7, 151.4, 155.1. ESI-TOF-MS: $[M+H]^+$ m/z: 271.17, $[M+Na]^+$ m/z: 293.14.

6.1.9. 2-Amino-6-hydroxymethyl-3,5-dihydro-4*H*-pyrrolo[3,2-*d*]pyrimidin-4-one (8)

Compound **7** (80 mg, 0.296 mmol) was suspended in 5 N HCl (10 mL). The mixture was stirred at 60 °C for 6 h under an atmosphere of argon. After TLC showed the disappearance of starting material, the reaction mixture was filtered and neutralized with aqueous NaOH. Further purification by the silica gel chromatography using dichloromethane–methanol (5:1, v/v) yielded **8** (45 mg, 84.4%) as a white solid. mp > 300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.42 (s, 2H, 8-CH_2), 5.18 (s, 1H, OH), 5.86 (s, 1H, 7-CH), 6.09 (s, 2H, 2-NH_2), 10.82 (bs, 1H, N^3H), 11.30 (s, 1H, N^5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 57.0, 98.8, 112.3, 142.7, 151.2, 151.3, 154.7. ESI-TOF-MS: $[M+H]^+$ m/z: 181.07.

6.1.10. 2-Amino-6-(bromomethyl)-1,5-dihydro-4H-pyrrolo[3,2-d]pyrimidin-4-one (9)

0.20 g (1.11 mmol) **8** was suspended in THF (5 mL), and then 0.03 mL PBr_3 was added. The mixture was refluxed for 4 h at an atmosphere of argon. After cooled to room temperature, 100 mL diethyl ether was added to the mixture. The precipitate was collected by filtration and washed with diethyl ether (3×100 mL) to afford **9** (0.28 g) as a crude yellow solid which was used without further purification.

6.1.11. Diethyl 4-((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl) methylamino) benzoyl-L-glutamate (10)

0.32 g (1.32 mmol) **9** and 0.40 g (1.24 mmol) diethyl (4-aminobenzoyl)-L-glutamate was dissolved in anhydrous DMAC (5 mL). The mixture was heated at 60 °C for 6 h under an atmosphere of argon. TLC showed the disappearance of **9** and formation of one major spot. The reaction mixture was added CH_2Cl_2 (50 mL), extracted with H_2O (10 mL × 3) and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded on a silica gel column and chromatographed with CH_2Cl_2/MeOH (9:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford 369 mg (57.9%) of **10** as a light-yellow solid; mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.14-1.19 (m, 6H, 2 × CH_3), 1.97-2.08 (m, 2H, C$H_2$$CH_2$CO), 2.42 (t, 2H, CH_2O, J = 6.8 Hz), 4.01-4.09 (m, 4H, 2 × OCH_2), 4.35~4.36 (m, 3H, 8-CH_2, NCHCO), 5.97 (s, 1H, 7-CH), 6.67 (d, J = 8.4 Hz, 2H, ph), 6.95 (m, 1H, N^9H), 7.11 (bs, 2H, 2-NH_2), 7.69 (d, J = 8.4 Hz, 2H, ph), 8.42 (d, J = 7.2 Hz, 1H, CONH), 11.89 (s, 1H, N^5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.5, 26.2, 30.7, 48.9, 52.3, 60.3, 60.8, 98.0, 111.7, 121.3, 129.4,

141.1, 151.5, 151.6, 153.5, 167.0, 172.5, 172.7; ESI-TOF-MS: $[M+H]^+$ m/z: 485.22; HRMS: $[M+H]^+$ m/z: 485.21435, $C_{23}H_{29}N_6O_6$ Exact Mass: 485.21431.

6.1.12. General procedure for preparation of substituted diethyl 4-((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl) methyl) amino) benzoyl-L-glutamate (11-13)

To a solution of **10** (100 mg, 0.21 mmol) in anhydrous DMF (5 mL) was added an 1.1 equivalent (0.23 mmol) of required halohydrocarbon (methyl iodide, ethyl bromide or propargyl bromide) and K_2CO_3 (30 mg, 0.21 mmol). The reaction mixture was stirred under argon at room temperature for different times as reported below. TLC showed the disappearance of **10** and formation of one major spot. The reaction mixture was added CH_2Cl_2 (50 mL), extracted with H_2O (10 mL × 3) and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded on a silica gel column and chromatographed with CH_2Cl_2/MeOH (9:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford **11-13**.

6.1.13. Diethyl 4-(((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl) methyl)(methyl)amino) benzoyl-L-glutamate (11)

This compound was obtained at 85.0 % yield starting from **10** (100 mg, 0.21 mmol) and methyl iodide (32.4 mg, 0.23 mmol) after 24 h; mp 136-137 ºC; ^{1}H NMR (600 MHz, CD_3OD) δ: 1.21 (t, J = 7.1 Hz, 3H, CH_3), 1.26 (t, J = 7.1 Hz, 3H, CH_3), 2.05-2.12 (m, 1H, CH_aH$_b$CH$_2$CO), 2.21-2.29 (m, 1H, CH$_a$$H_bCH_2$CO), 2.46 (t, J = 7.3 Hz, 2H, CH_2CO), 3,12 (s, 3H, NCH_3), 4.10 (q, J = 7.1 Hz, 2H, OCH_2), 4.19 (q, J = 7.1 Hz, 2H, OCH_2), 4.60-4.63 (m, 1H, NCHCO), 4.67 (s, 2H, 8-CH_2), 5.89 (s, 1H, 7-CH), 6.83 (d, J = 9.0 Hz, 2H, ph), 7.74 (d, J = 9.0 Hz, 2H, ph); ^{13}C NMR (150 MHz, CD_3OD) δ: 13.2, 14.5, 18.4, 27.5, 31.6, 39.1, 43.8, 50.6, 53.8, 55.9, 58.3, 61.7, 62.4, 99.5, 112.7, 122.3, 130.1, 141.5, 153.4, 170.3, 173.6, 174.6. ESI-TOF-MS: $[M-H]^+$ m/z: 497.21; HRMS: $[M-H]^+$ m/z: 497.2143, $C_{24}H_{29}N_6O_6$ Exact Mass: 497.2149.

6.1.14. Diethyl 4-(((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl) methyl)(ethyl)amino) benzoyl-L-glutamate (12)

This compound was obtained at 78.0 % yield starting from **10** (100 mg, 0.21 mmol) and ethyl bromide (25.0 mg, 0.23 mmol) after 48 h; mp 148-149 ºC; ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.11 (t, J = 7.0 Hz, 3H, N^9-CH_2CH_3), 1.16 (t, J = 7.1 Hz, 3H, CH_3), 1.18 (t, J = 7.1 Hz, 3H, CH_3), 1.93-2.02(m, 1H, CH_aH$_b$CH$_2$CO), 2.07-2.11 (m, 1H, CH$_a$$H_bCH_2$CO), 2.41 (t, J = 7.6 Hz, 2H, CH_2CO), 3.53 (q, J = 7.0 Hz, 2H, N^9-CH_2), 4.02-4.07 (m, 2H, OCH_2), 4.07-4.13 (m, 2H, OCH_2), 4.35–4.44 (m, 1H, NCHCO), 4.53 (s, 2H, 8-CH_2), 5.73 (s, 1H, 7-CH), 5.83 (s, 2H, 2-NH_2), 6.74 (d, J = 9.0 Hz, 2H, ph), 7.71 (d, J = 9.0 Hz, 2H, ph), 8.28 (d, J = 7.5 Hz, 1H, CONH), 11.48 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 12.0, 14.1, 25.8, 30. 2, 44.7, 46.8, 51.8, 59.9, 60.4, 98.8, 110.8, 111.9, 120.3, 129.0, 138.9, 149.9, 150.8, 154.0, 166.5, 172.1, 172.3; ESI-TOF-MS: $[M+H]^+$ m/z: 513.34; HRMS: $[M+H]^+$ m/z: 513.2455, $C_{25}H_{33}N_6O_6$ Exact Mass: 513.2462.

6.1.15. Diethyl 4-(((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl) methyl)(propargyl)amino) benzoyl-L-glutamate (13)

This compound was obtained at 78.0 % yield starting from **10** (100 mg, 0.21 mmol) and propargyl bromide (27.0 mg, 0.23 mmol) after 48 h; mp 148-149 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ:

1.13-1.19 (m, 6H, 2 × CH_3), 1.92-2.01(m, 1H, C*H*$_a$H$_b$CH$_2$CO), 2.06 (dd, *J* = 13.5, 5.4 Hz, 1H, CH$_a$*H*$_b$CH$_2$CO), 2.40 (t, *J* = 7.5 Hz, 2H, CH_2CO), 3.24 (s, 1H, ≡CH), 4.01-4.05 (m, 2H, OCH_2), 4.06-4.10 (m, 2H, OCH_2), 4.32 and 4.33 (2 × S, 2H, N^9-CH_2), 4.35-4.38 (m, 1H, NCHCO), 4.83 (s, 2H, 8-CH_2), 5.90 (s, 1H, 7-CH), 6.63 (d, *J* = 8.7 Hz, 2H, ph), 7.64 (d, *J* = 8.7 Hz, 2H, ph), 8.23 (d, *J* = 7.5 Hz, 1H, CONH), 9.75 (s, 1H, N^3H), 11.17 (bs, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 14.0, 22.0, 25.7, 28.9, 30.1, 31.2, 33.6, 59.8, 60.3, 74.0, 111.2, 112.3, 120.7, 128.8, 129.0, 150.0, 150.9, 166.4, 172.0, 172.1, 182.6; ESI-TOF-MS: $[M+H]^+$ m/z: 523.33.

6.1.16. Diethyl (4-(N-((2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)-2,2,2-trifluoroacetamido)benzoyl)-L-glutamate (16)

To a solution of **10** (110 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (5 mL) was added an excess (2 mL) of trifluoroacetic anhydride (TFAA). The reaction mixture was stirred under argon at room temperature overnight. TLC showed the disappearance of **10** and formation of one major spot. The reaction solution was washed with 5% $NaHCO_3$ solution, extracted with H_2O (10 mL × 3), and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded onto a silica gel column and chromatographed with CH_2Cl_2/MeOH (15:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford 123 mg (93.3%) of **16** as light-yellow solid; mp 129-130 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.13-1.20 (m, 6H, 2 × CH_3), 1.98-2.12 (m, 2H, C*H*$_2$CH$_2$CO), 2.42-2.45 (t, *J* = 7.4 Hz, 2H, CH_2CO), 4.00-4.11 (m, 4H, 2 × OCH_2), 4.38-4.45 (m, H, NCHCO), 4.95 (s, 2H, 8-CH_2), 5.62-5.87 (m, 3H, 2-NH_2, 7-CH), 7.36-7.38 (d, *J* = 8.3 Hz, 2H, ph), 7.85-7.87 (d, *J* = 8.3 Hz, 2H, ph), 8.82-8.84 (d, 1H, CONH), 10.51 (bs, 1H, N^3H), 11.58 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 14.6, 26.0, 31.2, 49.5, 53.2, 59.6, 60.5, 98.4, 111.9, 116.0, 121.8, 128.8, 141.7, 152.1, 152.6, 154.3, 155.8, 167.4, 172.1, 172.4; HRMS: $[M+H]^+$ m/z: 581.19773; $C_{25}H_{28}F_3N_6O_7$ Exact Mass: 581.19661.

6.1.17. General procedure for preparation of 7-halogenated diethyl (4-(N-((2-amino -4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)-2,2,2-trifluoroacetamido)benzoyl)-L-glutamates (17, 18)

To a solution of **16** (100 mg, 0.17 mmol) in anhydrous CH_2Cl_2 (10 mL) was added 1.1 equivalent of required halogenating reagent (NBS or I_2). The reaction mixture was stirred under argon at room temperature overnight. After the disappearance of **16** as indicated by TLC, the reaction solution was washed with 5% $NaHSO_3$ solution to remove the excess halogen. The resulting mixture was extracted with H_2O (10 mL × 3) and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded onto a silica gel column and chromatographed with CH_2Cl_2/MeOH (15:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford **17** or **18**.

6.1.17. Diethyl (4-(N-((2-amino-7-bromo-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)-2,2,2-trifluoroacetamido)benzoyl)-L-glutamates (17)

This compound was obtained at 89.0 % yield starting from **16** and N-bromosuccinimide (NBS); mp 149-150 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.15 (t, *J*=7.1 Hz, 3H, CH_3), 1.19 (t, *J* =7.1 Hz, 3H, CH_3), 1.97-2.02 (m, 1H, C*H*$_a$H$_b$CH$_2$CO), 2.07-2.11 (m, 1H, CH$_a$*H*$_b$CH$_2$CO), 2.43-2.45 (m, 2H,

CH_2CO), 4.03 (q, *J* = 7.1 Hz, 2H, OCH_2), 4.09-4.12 (m, 2H, OCH_2), 4.41 (dt, *J* = 9.5, 7.3 Hz, 1H, NCHCO), 6.12 (s, 2H, 8-CH_2) 7.35 (d, *J* = 8.2 Hz, 2H, ph), 7.84 (d, *J* = 8.4 Hz, 2H, ph), 8.81 (d, *J* = 7.3 Hz, 1H, CONH), 10.63 (bs, 1H, N^3H), 12.10 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) *δ*: 14.0, 25.5, 30.1, 45.4, 52.0, 59.8, 60.5, 112.2, 128.2, 128.4, 130.9, 134.1, 139.9, 151.3, 153.5, 165.7, 171.5, 172.1; ESI-TOF-MS: $[M-H]^-$ m/z: 657.31 and 659.30; HRMS: $[M-H]^-$ m/z: 657.0907 and 659.0894, $C_{25}H_{25}BrF_3N_6O_7$ Exact Mass: 657.0920.

6.1.18. Diethyl (4-(N-((2-amino-7-bromo-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)-2,2,2-trifluoroacetamido)benzoyl)-L-glutamates (18)

This compound was obtained at 85.0 % yield starting from **16** and I_2; mp 140-141 °C. ^{1}H NMR (600 MHz, DMSO-d_6) *δ*: 1.14 (t, *J* = 7.1 Hz, 3H, CH_3), 1.17 (t, *J* = 7.1 Hz, 3H, CH_3), 1.91-2.03 (m, 1H, CH_aH$_b$CH$_2$CO), 2.05-2.11 (m, 1H, CH$_a$$H_bCH_2$CO), 2.41-2.44 (m, 2H, CH_2CO), 4.02 (q, *J* = 7.1 Hz, 2H, OCH_2), 4.10 (q, *J* = 7.1 Hz, 2H, OCH_2), 4.37-4.41 (m, NCHCO, 1H), 6.02 (s, 2H, 8-CH_2), 7.33 (d, *J* = 8.3 Hz, 2H, ph), 7.81 (d, *J* = 8.6 Hz, 2H, ph), 8.78 (d, *J* = 7.4 Hz, 1H, CONH), 10.51 (s, 1H, N^3H), 12.09 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) *δ*: 13.9, 25.5, 30.0, 46.8, 52.0, 59.8, 60.5, 113.0, 115.1, 117.0, 128.1, 128.5, 133.8, 134.1, 139.8, 147.6, 151.1, 153.6, 165.6, 171.5, 172.1; ESI-TOF-MS: $[M-H]^-$ m/z: 705.23; HRMS: $[M-H]^-$ m/z: 705.0765 $C_{25}H_{25}F_3IN_6O_7$ Exact Mass: 705.0781.

6.1.19. Diethyl (4-(2-(2-amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)ethyl)benzoyl)glutamate (15)

To a solution of **9** (90 mg, 0.37 mmol) in 15 mL anhydrous DMF was added 118 mg (0.45 mmol) dry triphenylphosphine and heated at 60 ºC for 2 h under argon. TLC in $CHCl_3$-CH_3OH (9:1) indicated the disappearance of **9**. The reaction mixture was cooled to room temperature and was added 60 mg (1.11 mmol) NaOMe and 127 mg (0.38 mmol) diethyl *N*-[4-(formyl)benzoyl]-L-glutamate in sequence, the solution was heated at 60 ºC for another 60 h under argon. After evaporation of solvent, water (30 mL) was added to the gummy residue. The pH of the suspension was adjusted to 7 by 2 M acetic acid and the resulting solution was extracted with dichloromethane (50 mL × 3). The organic layer was concentrated to a small volume, and was chromatographed on silica gel and eluted with CH_2Cl_2/CH_3OH (9:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, 50 mg of critical intermediate was afford as a yellow solid and was dissolved in 10 mL of anhydrous ethanol. After addition of 5 mg Pd/C, the resulting suspension was hydrogenated (0.4 MPa) for 12 h. The result mixture was filtered through celite and the filtrate was evaporated to dryness under reduced pressure. The residue was loaded onto a silica gel column and chromatographed with CH_2Cl_2/MeOH (15:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford 20 mg (11.1% of three steps) of **15** as a yellow solid. mp 156-157 °C. ^{1}H NMR (400 MHz, DMSO-d_6) *δ*: 1.14-1.20 (m, 6H, 2 × CH_3), 1.98-2.02 (m, 1H, CH_aH$_b$CH$_2$CO), 2.08-2.11 (m, 1H, CH$_a$$H_bCH_2$CO), 2.41-2.45 (t, *J* = 7.4 Hz, 2H, CH_2CO), 2.87-2.90 (t, *J* = 7.8 Hz, 2H, 8-CH_2), 2.98-3.01 (t, *J* = 7.8 Hz, 2H, 9-CH_2), 4.02-4.13 (m, 4H, 2 × OCH_2), 4.39-4.45 (m, 1H, NCHCO), 5.71-5.73 (m, 3H: 2-NH_2, 7-CH), 7.30-7.32 (d, *J* = 8.1 Hz, 2H, ph), 7.78-7.80 (d, *J*=8.1 Hz, 2H, ph), 8.63-8.65 (d, *J* = 7.4 Hz, 1H, CONH), 10.31 (bs, 1H, N^3H), 11.28 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) *δ*: 14.5, 26.2, 29.6, 30.6, 34.9, 52.4, 60.4, 61.0, 99.2, 112.0, 127.9, 128.6, 131.9, 134.5, 141.7,

145.5, 149.5, 151.0, 167.0, 172.2, 172.6. ESI-TOF-MS: $[M+H]^+$ m/z: 484.36; HRMS : $[M+H]^-$ m/z: 484.2197 $C_{24}H_{30}N_5O_6$ Exact Mass: 484.2196.

6.1.20. N-(6-((benzyloxy)methyl)-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-2-yl)pivalamide (19)

To a solution of **7** (200 mg, 0.74 mmol) in 20 mL CH_2Cl_2 was added 182 µl (1.48 mmol) pivaloyl chloride, 9 mg (0.07 mmol) DMAP and 216 µl (1.55 mmol) triethylamine. The resulting mixture was reflux for 5h. TLC showed the disappearance of **7** and formation of one major spot. The reaction mixture was extracted with H_2O (10 mL × 3) and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded onto a silica gel column and chromatographed with PE/EtOAc (1:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford 257 mg (98.0%) of **19** as light yellow solid. mp 170-171 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.25 (s, 9H, 3 × CH_3), 4.54-4.57 (s, 4H, CH_2OCH_2), 6.26 (s, 1H, 7-CH), 7.27-7.37 (m, 5H, ph), 10.80 (s, 1H, N^3H), 12.01 (s, 1H, N^5H), 12.16 (s, 1H, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 26.9, 64.9, 72.0, 102.1, 115.2, 127.9, 128.1, 128.7, 138.6, 139.5, 144.7 145.4, 152.5, 181.1.

6.1.21. N-(6-((benzyloxy)methyl)-4-chloro-5H-pyrrolo[3,2-d]pyrimidin-2-yl)pivalamide (20)

A suspension of **19** (257 mg, 0.73 mmol) in 10 mL $POCl_3$ was reflux for 10 h. After evaporation of $POCl_3$, the residue was added 10 mL of cold water to remove the excess $POCl_3$. The pH was adjusted to 7 by aqueous ammonia. The resulting solution was extracted with dichloromethane (50 mL × 3) and dried over Na_2SO_4. The organic layer was concentrated to a small volume, and was chromatographed on silica gel and eluted with PE/EtOAc (1:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, 243 mg (89.9%) of **20** was obtained as a white solid. mp 178-179 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.33 (s, 9H, 3 × CH_3), 4.60 (s, 2H, 8-CH_2), 4.75 (s, 2H, phCH_2), 6.45 (s, 1H, 7-CH), 7.31-7.37 (m, 5H, ph), 8.13 (s, 1H, CONH), 9.60 (s, 1H, N^5H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.5, 40.1, 64.9, 73.1, 101.2, 122.2, 128.1, 128.2, 128.6, 137.0, 142.1, 145.5, 150.3, 152.3, 175.8.

6.1.22. N-(6-((benzyloxy)methyl)-4-chloro-5-methyl-5H-pyrrolo[3,2-d]pyrimidin-2-yl)pivalamide (21)

To as solution of **20** (75 mg, 0.20 mmol) in 2 mL of anhydrous DMF was added 9 mg (0.38 mmol) NaH in batches. The suspension was stirred for 10 min and was dropwise added 13 µl (0.26 mmol) of methyl iodide in 1 mL of anhydrous DMF. The resulting solution was continued to stir for 6 h. TLC indicated the disappearance of **20**. The reaction mixture was added 10 mL of H_2O and extracted with dichloromethane (20 mL × 3) and dried over Na_2SO_4. The organic layer was concentrated to a small volume, and was chromatographed on silica gel and eluted with PE/EtOAc (2:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, 70 mg (89.9%) of **21** was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.34 (s, 9H, 3 × CH_3), 4.05 (s, 3H, N^5-CH_3), 4.55 (s, 2H, 8-CH_2), 4.67 (s, 2H, phCH_2), 6.62 (s, 1H, 7-CH), 7.31-7.36 (m, 5H, ph), 8.08 (s, 1H, CONH); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.5, 32.9, 40.1, 63.4, 72.3, 103.9, 123.0, 128.0, 128.1, 128.6, 137.0, 142.2,145.6, 149.9, 152.1, 175.6.

6.1.23. N-(5-Allyl-6-(benzyloxymethyl)-4-chloro-5H-pyrrolo[3,2-d]pyrimidin-2-yl)pivalamide (22)

86 mg (97.1%) of **22** was obtained as a white solid through a similar procedure as **21** using 80 mg (0.22 mmol) of **20** and 20 µl (0.24 mmol) allyl bromide as starting material. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 1.34 (s, 9H, 3 × CH_3), 4.54 (s, 2H, 8-CH_2), 4.63-4.64 (m, 3H, phCH_2, =CH_aH_b), 5.09-5.14 (m, 3H, N^5-CH_2, =CH_aH_b), 5.92-6.01 (m, 1H, CH=CH_2), 6.69 (s, 1H, 7-CH), 7.32-7.39 (m, 5H, ph), 8.09 (s, 1H, CONH); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 27.5, 40.1, 47.2, 67.3, 72.3, 104.4, 116.1, 122.5, 128.1, 128.1, 128.6, 133.8, 137.0, 142.1, 145.7, 150.1, 152.4, 175.6.

6.1.24. 5-Methyl-2-amino-6-(hydroxymethyl)-1H-pyrrolo[3,2-d]pyrimidin-4(5H)-one (23)

To a solution of **21** (48 mg, 0.124 mmol) in 1,4-Dioxane (5 mL) was added 10 mL of 2 N NaOH. The resulting solution was reflux for 6 h. TLC indicated the disappearance of **21**. After cooled to room temperature, the reaction mixture was neutralized by acetic acid and extracted with dichloromethane (20 mL × 3) and dried over Na_2SO_4. The organic layer was concentrated to a small volume, and was chromatographed on silica gel and eluted with CH_2Cl_2/MeOH (12:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, 30 mg (84.9%) of **23** was obtained as a white solid. mp 249-250 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.87 (s, 3H, CH_3), 4.50 (s, 2H, 8-CH_2), 4.54 (s, 2H, phCH_2), 5.81 (bs, 2H, 2-NH_2), 5.97 (s, 1H, 7-CH), 7.28-7.38 (m, 5H, ph), 10.53 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 32.1, 63.4, 71.7, 101.7, 113.7, 125.3, 128.0, 128.1, 128.7, 138.5, 138.7, 151.1, 155.4; HRMS: $[M+H]^+$ m/z: 285.13414, $C_{15}H_{17}N_4O_2$ Exact Mass: 285.13460.

6.1.25. 5-Allyl-2-amino-6-(hydroxymethyl)-1H-pyrrolo[3,2-d]pyrimidin-4(5H)-one (24)

80 mg (70.9%) of **24** was obtained as a white solid through a similar procedure as **23** using 150 mg (0.36 mmol) of **22** as starting material. mp 232-233 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.50 (s, 4H, CH_2OCH_2), 4.74 (d, J = 17.2 Hz, 1H, =CH_aH_b), 4.94 (s × 2, 2H, N^5-CH_2), 5.01 (d, J = 10.4 Hz, 1H, =CH_aH_b), 5.86 (bs, 2H, 2-NH_2), 5.89-5.97 (m, 1H, CH=), 6.02 (s, 1H, 7-CH), 7.30~7.38 (m, 5H, ph), 10.56 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 47.1, 63.3, 71.8, 102.2, 113.2, 115.8, 128.0, 128.2, 128.7, 135.9, 138.4, 138.5, 151.3, 155.1; HRMS: $[M+H]^+$m/z: 311.14991, $C_{17}H_{19}N_4O_2$ Exact Mass: 311.15025.

6.1.26. 5-Methyl-2-amino-6-(hydroxymethyl)-1H-pyrrolo[3,2-d]pyrimidin-4(5H)-one (25)

82 mg (80.0%) of **25** was obtained as a light yellow solid through a similar procedure as **8** using 150 mg (0.528 mmol) of **23** as starting material. mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.86 (s, 3H, N^5-CH_3), 4.47 (s, 2H, CH_2OH), 5.86 (bs, 3H, 2-NH_2, 7-CH), 10.69 (s, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 32.0, 55.4, 99.6, 113.0, 143.0, 145.6, 151.1, 155.5.

6.1.27. 5-Allyl-2-amino-6-(hydroxymethyl)-1H-pyrrolo[3,2-d]pyrimidin-4(5H)-one (26)

80 mg (56.4%) of **26** was obtained as a light yellow solid through a similar procedure as **8** using 200 mg (0.645 mmol) of **24** as starting material. mp > 300 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.44 (s, 2H, CH_2OH), 4.76 (d, J = 17.2 Hz, 1H, =CH_aH_b), 4.96 (s × 2, 2H, N^5-CH_2), 5.04 (d, J = 10.0 Hz, 1H, =CH_aH_b), 5.26 (bs, 1H, OH), 5.89-6.00 (m, 4H, 2-NH_2, 7-CH, CH=), 10.56 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 41.0, 55.5, 100.1, 112.5, 115. 7, 136.0, 143.1, 151.3, 155.2.

6.1.28. Diethyl 4-((5-methyl-2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin -6-yl)methylamino) benzoyl-L-glutamate (27)

50 mg (0.53 mmol) **25** was suspended in anhydrous THF (10 mL), and then 0.2 mL (2.11 mmol) PBr_3 was added. The mixture was refluxed for 4 h at an atmosphere of argon. After cooled to room temperature, 100 mL diethyl ether was added to the mixture. The precipitate was collected by filtration and washed with diethyl ether (3×100 mL) to afford a critical bromide intermediate (60 mg) as a crude yellow solid which was used without further purification. 45 mg (0.18 mmol) of above bromide and 70 mg (0.22 mmol) diethyl (4-aminobenzoyl)-L-glutamate was dissolved in anhydrous DMAC (10 mL). The resulting solution was added 45 mg (0.54 mmol) $NaHCO_3$ and heated at 60 °C under argon for 8 h. TLC showed the disappearance of the bromide and and formation of a new major spot. After cooled to room temperature, the reaction mixture was added H_2O (30 mL), extracted with CH_2Cl_2 (50 mL × 3) and dried over Na_2SO_4. After evaporation of solvent, the residue was loaded on a silica gel column and chromatographed with CH_2Cl_2/MeOH (15:1, v/v), and the desired fraction were pooled. After evaporation of the solvent, the residue was dried to afford 60 mg (68.5%) of **27** as a light-yellow solid. mp 236-237 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.15-1.20 (m, 6H, 2 × CH_3), 1.95-2.10 (m, 2H, C$H_2$$CH_2$CO), 2.42 (t, J = 7.4 Hz, 2H, CH_2CO), 3.90 (s, 3H, N^5-CH_3), 4.02-4.12 (m, 4H, 2 × OCH_2), 4.34-4.41 (m, 3H, 8-CH_2, NCHCO), 5.79 (bs, 2H, 2-NH_2), 5.89 (s, 1H, 7-CH), 6.65-6.70 (m, 3H, ph, N^9H), 7.66-7.68 (d, J = 8.5 Hz, 2H, ph), 8.25 (d, J = 7.2 Hz, 1H, CONH), 10.44 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.5, 26.3, 30.7, 32.0, 39.1, 52.3, 60.3, 60.8, 100.3, 111.6, 113.0, 121.4, 129.4, 140.5, 151.1, 151.5, 167.0, 172.6, 172.7; HRMS: $[M+H]^+$ m/z: 499.23030, $C_{24}H_{31}N_6O_6$ Exact Mass: 499.22996.

6.1.29. Diethyl 4-((5-allyl-2-amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin -6-yl)methylamino) benzoyl-L-glutamate (28)

32 mg (78.3%) of **28** was obtained as a light yellow solid through a similar procedure as **27** using 22 mg (0.08 mmol) of **26** as starting material. mp 183-184 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.14-1.20 (m, 6H, 2 × CH_3), 1.94-2.10 (m, 2H, C$H_2$$CH_2$CO), 2.39-2.43 (t, J = 7.4 Hz, 2H, CH_2CO), 4.02-4.11 (m, 4H, 2 × OCH_2), 4.30-4.41 (m, 3H, 8-CH_2, NCHCO), 4.78 (d, J = 7.4 Hz, 1H, =C$H_a$$H_b$), 5.02 (bs, 2H, N^5-CH_2), 5.08 (d, J = 10.4 Hz, 1H, =$CH_a$$H_b$), 5.83 (bs, 2H, 2-$NH_2$), 5.90 (s, 1H, 7-CH), 5.94-6.04 (m, 1H, CH=), 6.64 (d, J=8.5 Hz, 2H, ph), 6.70 (t, J = 5.4 Hz, 1H, N^9H), 7.65-7.67 (d, J = 8.5 Hz, 2H, ph),8.25 (d, J = 7.2 Hz, 1H, CONH), 10.46 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 14.5, 26.3, 30.7, 47.0, 50.9, 52.3, 60.3, 60.8, 111.6, 112.6, 115.8, 121.4, 129.4, 135.8, 140.6, 151.3, 151.5, 167.0, 172.6, 172.7; HRMS: $[M+H]^+$ m/z: 525.24555, $C_{26}H_{33}N_6O_6$ Exact Mass: 525.24561.

6.1.30.General procedure for preparation of 10a, 11a-13a, 15a, 17a, 18a, 27a, and 28a

A suspension of 20 mg **10**, **11-13**, **15**, **17**, **18**, **27** or **28** in THF (2 mL) was added 1 mol/L NaOH (1 mL), and the mixture was stirred at room temperature for 8 h. TLC showed the disappearance of the starting material. After evaporation of the solvent, the remaining aqueous solution was acidified carefully with 0.5 mol/L HCl, and the solid was collected by filtration, washed with water, and dried *in vacuo* to give **10a**, **11a-13a**, **15a**, **17a**, **18a**, **27a** or **28a** as pale yellow solid. The data are reported as follows.

6.1.31. 4-((2-Amino-4-oxo-4,5-dihydro-1H-pyrrolo[3,2-d]pyrimidin-6-yl)methylamino) benzoyl-L-glutamate (10a)

10a was obtained as a light yellow solid at 65.3% yield using 90 mg (0.19 mmol) of **10** as starting material. mp 232-233 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.92-1.97 (m, 1H, CH_aHbCH$_2$CO), 2.04-2.11 (m, 1H, CH$_a$$H_bCH_2$CO), 2.31-2.43 (m, 2H, CH$_2$CO), 4.30-4.35 (m, 3H, 8-CH$_2$, NCHCO), 5.88 (s, 1H, 7-CH), 6.01 (bs, 2H, 2-NH$_2$), 6.56 (m, 1H, N^9H), 6.64 (d, J=8.4 Hz, 2H, ph), 7.66 (d, J = 8.4 Hz, 2H, ph), 8.13 (d, J = 8.4 Hz, 1H, CONH), 11.48 (s, 1H, N^5H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 26.1, 30.5, 51.3, 51.8, 98.8, 111.2, 111.8, 121.2, 128.9, 139.8, 150.8, 151.0, 154.0, 166.5, 173.9, 174.0; HRMS: [M+H]$^+$ m/z: 429.15161, $C_{19}H_{21}N_6O_6$ Exact Mass: 429.15171.

6.1.32. (4-(((2-Amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)(methyl)amino)benzoyl)glutamic acid (11a)

11a was obtained as a light yellow solid at 60.5% yield using 20 mg (0.04 mmol) of **11** as starting material. mp>300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.92-1.96 (m,1H, CH_aH$_b$CH$_2$CO), 2.27 (dd, J = 14.7, 8.0 Hz, 1H, CH$_a$$H_bCH_2$CO), 2.31-2.35 (m, 2H, CH$_2$CO), 3.05 (s, 3H, N^9-CH$_3$), 4.32 (dd, J = 14.7, 8.0 Hz, 1H, NCHCO), 4.56 (s, 2H, 8-CH$_2$), 5.74 (s, 2H, 2-NH$_2$), 6.79 (d, J = 9.0 Hz, 2H, ph), 7.70 (d, J = 9.0 H, 2H, ph), 8.04 (s, 1H, CONH), 10.31 (bs, 2H, COOH), 11.43 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 22.1, 26.8, 29.0, 31.3, 48.8, 52.0, 99.0, 111.2, 111.9, 121.2, 128.6, 138.0, 150.9, 165.8, 173.7, 174.1; HRMS: [M-H]$^+$ m/z: 441.1520, $C_{20}H_{21}N_6O_6$ Exact Mass: 441.1523.

6.1.33. (4-(((2-amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)(ethyl)amino)benzoyl)glutamic acid (12a)

12a was obtained as a light yellow solid at 70.5% yield using 40 mg (0.08 mmol) of **12** as starting material. mp>300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.10 (t, J = 7.0 Hz, 3H, CH$_3$), 1.91-1.94 (m, 2H, CH_2CH$_2$CO), 2.21-2.34 (m, 2H, CH$_2$CO), 3.51 (q, J = 7.0 Hz, 2H, N^5-CH$_2$), 4.29 (dd, J = 14.4, 7.2 Hz, 1H, NCHCO), 4.51 (s, 2H, 8-CH$_2$), 5.71 (s, 1H, 7-CH), 5.82 (s, 2H, 2-NH$_2$), 6.73 (d, J = 8.8 Hz, 2H, ph), 7.67 (d, J = 8.8 Hz, 2H, ph), 7.93 (d, J = 6.3 Hz, 1H, CONH), 11.44 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 11.9, 27.2, 31.6, 44.6, 46.7, 52.1, 98.8, 110.8, 111.9, 120.9, 128.5, 138.7, 149.7, 150.7, 165.5, 173.7, 174.3; ESI: [M+H]$^+$ m/z: 513.34; HRMS: [M-H]$^+$ m/z: 455.1675, $C_{21}H_{23}N_6O_6$ Exact Mass: 455.1670.

6.1.34. (4-(((2-Amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)(prop-2-yn-1-yl)amino)benzoyl)glutamic acid (13a)

13a was obtained as a light yellow solid at 57.5% yield using 20 mg (0.04 mmol) of **13** as starting material. mp>300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.89-1.94 (m, 1H, CH_aH$_b$CH$_2$CO), 2.02-2.07 (m, 1H, CH$_a$$H_bCH_2$CO), 2.32 (t, J = 7.6 Hz, 2H, CH$_2$CO), 3.29 (m, 1H, ≡CH), 4.30-4.34 (m, 1H, NCHCO), 4.80 (s, 2H, 8-CH$_2$), 5.85 (s, 1H, 7-CH), 6.63 (d, J=8.8 Hz, 2H, ph), 7.64 (d, J = 8.8 Hz, 2H, ph), 8.11 (d, J = 7.8 Hz, 1H, CONH), 11.45 (bs, 1H, N^5H); ^{13}C NMR (150

MHz, DMSO-d_6) δ: 22.1, 26.0, 29.0, 29.8, 30.4, 31.3, 51.7, 111.2, 121.2, 128.8, 150.2, 151.0, 166.4, 173.7, 173.9, 179.4; HRMS: $[M+H]^+$ m/z: 467.1678, $C_{22}H_{23}N_6O_6$ Exact Mass: 467.1679.

6.1.35. (4-(2-(2-Amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)ethyl)benzoyl)glutamic acid (15a)

15a was obtained as a light yellow solid at 55.0% yield using 10 mg (0.02 mmol) of **15** as starting material. mp 254-255 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.84 (bs, 1H, CH_aH$_b$CH$_2$CO), 1.93 (bs, 1H, CH$_a$$H_bCH_2$CO), 2.14-2.17 (m, 1H, C$H_aH_b$CO), 2.28 (bs, 1H, CH$_a$$H_b$CO), 2.87 (t, J = 7.8 Hz, 2H, 8-CH$_2$), 2.97 (t, J = 7.8 Hz, 2H, 9-CH$_2$), 4.09-4.32 (m, 1H, NCHCO), 5.72 (s, 1H, 7-CH), 5.75 (s, 2H, 2-NH$_2$), 7.29 (d, J = 8.0 Hz, 2H, ph), 7.74 (d, J = 8.0 Hz, 2H, ph), 8.08 (s, 1H, CONH), 10.33(s, 1H, N^3H), 11.25 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 29.1, 29.9, 34.5, 53.1, 98.8, 111.4, 126.9, 128.1, 131.4, 132.4, 141.2, 144.3, 147.4, 150.5, 164.7, 173.5; HRMS: $[M-H]^-$m/z: 426.1413, $C_{20}H_{20}N_5O_6$ Exact Mass: 426.1414.

6.1.36. (4-(((2-Amino-7-bromo-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)amino)benzoyl)glutamic acid (17a)

17a was obtained as a light yellow solid at 67.0% yield using 30 mg (0.05 mmol) of **17** as starting material. mp >300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.91-1.92 (m, 2H, CH_2CH$_2$CO), 2.23-2.26 (m, 1H, CH_aH$_b$CO), 2.31-2.35 (m, 1H, CH$_a$$H_b$CO), 4.27 (d, J = 5.6 Hz, 1H, NCHCO), 6.08 (s, 2H, 8-CH$_2$), 6.65 (d, J = 8.8 Hz, 2H, ph), 7.62 (d, J = 8.8 Hz, 2H, ph), 7.92 (s, 1H, CONH), 10.56 (s, 1H, N^3H), 11.92 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 38.6, 51.3, 88.0, 111.1, 111.5, 121.6, 128.5, 135.7, 144.4, 150.4, 151.3, 153.6, 165.6, 173.6, 174.2; HRMS: $[M-H]^-$ m/z: 505.0457, 507.0456, $C_{19}H_{18}BrN_6O_6$ Exact Mass: 505.0471.

6.1.37. (4-(((2-Amino-4-hydroxy-7-iodo-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)amino)benzoyl)glutamic acid (18a)

18a was obtained as a light yellow solid at 66.3% yield using 20 mg (0.03 mmol) of **18** as starting material. mp >300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ: 1.85-1.92 (m, 2H, CH_2CH$_2$CO), 2.24-2.16 (m, 1H, CH_aH$_b$CO), 2.26-2.31 (m, 1H, CH$_a$$H_b$CO), 4.22 (q, J = 7.0 Hz, 1H, NCHCO), 4.26 (d, J = 5.6 Hz, 2H), 6.05 (s, 2H, 8-CH$_2$), 6.63 (d, J = 8.7 Hz, 2H, ph), 7.59 (d, J = 8.7 Hz, 2H, ph), 7.79 (d, J = 6.5 Hz, 1H, CONH), 10.60 (s, 1H, N^3H), 11.94 (s, 1H, N^5H); ^{13}C NMR (150 MHz, DMSO-d_6) δ: 27.9, 30.0, 32.1, 52.7, 58.5, 111.4, 112.4, 121.9, 128.5, 138.9, 148.0, 150.5, 151.3, 153.7, 165.4, 173.8,174.8; HRMS: $[M-H]^-$ m/z: 553.0326, $C_{19}H_{18}IN_6O_6$ Exact Mass: 553.0333.

6.1.38. (4-(((2-Amino-4-hydroxy-5-methyl-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)amino)benzoyl)glutamic acid (27a)

27a was obtained as a light yellow solid at 78.9% yield using 50 mg (0.10 mmol) of **27** as starting material. mp 239-240 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.90-2.04 (m, 2H, CH_2CH$_2$CO), 2.31 (t, J = 3.2 Hz, 2H, CH$_2$CO), 3.89 (s, 3H, N^5-CH$_3$), 4.33-4.34 (m, 3H, 8-CH$_2$, NCHCO), 5.86 (bs, 2H, 2-NH$_2$), 5.89 (s, 1H, 7-CH), 6.62 (t, J = 5.4 Hz, 1H, N^9H), 6.68 (d, J = 8.4 Hz, 2H, ph), 7.76 (d, J = 8.4 Hz, 2H, ph), 8.05 (d, J = 6.8 Hz, 1H, CONH); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.9,

30.3, 38.5, 40.0, 46.4, 51.6, 111.0, 111.8, 112.3, 115.3, 121.1, 128.8, 135.1, 150.6, 150.8, 166.3, 173.7, 173.8; HRMS: $[M+H]^+$ m/z: 443.16744, $C_{20}H_{23}N_6O_6$ Exact Mass: 443.16736.

6.1.39.

(4-(((5-Allyl-2-amino-4-hydroxy-5H-pyrrolo[3,2-d]pyrimidin-6-yl)methyl)amino)benzoyl)-L-glutamic acid (28a)

28a was obtained as a light yellow solid at 78.9% yield using 20 mg (0.04 mmol) of **28** as starting material. mp 242-243 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.91 (bs, 2H, CH_2CO), 2.19-2.22 (m, 2H, CH_2CH_2CO), 4.19-4.28 (m, 3H, NCHCO, 8-CH_2), 4.78 (d, J = 17.2 Hz, 1H, =CH_aH$_b$), 5.01-5.09 (m, 3H, N^5-CH_2, =C$H_a$$H_b$), 5.89 (s, 1H, 7-CH), 5.94-6.01 (m, 1H, CH=), 6.11 (bs, 2H, 2-NH_2), 6.62-6.66 (m, 3H, N^9H, ph), 7.60 (d, J = 7.9 Hz, 2H, ph), 7.766 (s, 1H, CONH), 11.153 (bs, 1H, N^3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 22.0, 24.4, 28.4, 28.6, 28.9, 31.2, 31.5, 33.6, 38.6, 51.7, 111.0, 112.5, 120.8, 121.1, 128.5, 128.7, 140.0, 140.4, 150.5, 150.9, 166.2, 173.7,173.9; HRMS: $[M+H]^+$ m/z: 469.18335, $C_{22}H_{25}N_6O_6$ Exact Mass: 469.18301.

6.2. Biological evaluation

6.2.1. In vitro human cancer growth inhibition

Human promyelocytic leukemic cell line (HL-60), human lung adenocarcinoma epithelial cell line (A549), human lung adenocarcinoma cell line (H1299), human cervix adenocarcinoma cell line (Hela) and human colon adenocarcinoma cell line (HT29) were grown and maintained in RPMI-1640 or DMEM medium supplemented with 10% fetal bovine serum, penicillin (100 units mL^{-1}), streptomycin concentration at 37 ºC in a humidified incubators in an atmosphere of 5% CO_2. Exponentially growing cancer cells were used to perform the experiments. The cell lines were assessed by trypsinizing and seeding 5×10^3 cells per well into 96-well plates. Cells were grown for 12 h and then treated with compounds at concentration ranging from 10 nM to 100 µM and incubated for 48 h in 200 µL media. MTS reagent (20 µL) in serum-free medium was added to each well and incubated further for 3 h. O. D. value at 490 nm was measured using a Benchmark Plus Microplate Reader (Bio-Rad). Three individual experiments were performed to obtain mean cell viability. Cells treated with 0.1% DMSO were used as a control, while MTX were used as a positive control.

6.2.2. Dihydrofolate Reductase (DHFR) Assay.

The recombinant human (rh) DHFR was purchased from Sigma-Aldrich (Shanghai, China). The inhibitory activities of the target compounds against rhDHFR enzymes were assayed in a solution containing 0.1 mM dihydrofolate, 0.3 mM NADPH, and 100 mM KCl at pH 7.5 and 32 ˚C. The reaction was initiated with an amount of enzyme yielding a change in O.D. value at 340 nm measured by micro-plate spectrophotometer (BioRad).

6.2.3. Identification of the targeted pathway

To identify the targeted pathways/folate-dependent enzymes (i.e., TS, GARFTase or AICARFTase) of these pyrrolo[3,2-*d*]pyrimidine analogues, proliferation assays were tested by co-incubation with adenosine (60 µM), thymidine (10 µM), or 5-aminoimidazole-4-carboxamide hydrochloride

(320 µM) and the results compared to those of incubations in parallel without nucleoside/AICA or drug additions.

6.2.4. Cell Cycle Assay

A549 cells were treated with compound **15a** (1 µM and 10 µM) or MTX (1 µM) for 72 h in RPMI1640 medium/10% dFBS, 5% penicillin-streptomycin, and 2 nM LCV. Cells were washed twice with ice-cold PBS and fixed in 70% ethanol (4 ºC, overnight), then stained by re-suspension in 0.5 mL of PBS containing 50 µg/mL PI and 100 µg/mL RNase type I-A (Sigma Aldrich) at least 20 min at room temperature in the dark prior to analysis. The cells were detected by flow cytometry using the BD FACSCalibur flow cytometer for determining the percentage of cells in each phase of the cell cycle. In each experiment, 1×10^4 cells were assessed for cell cycle distribution. All data were analyzed with ModFitLT V3.0 software (BD Biosciences).

6.2.5. Apoptosis Analysis

A549 cells were treated with **15a** (1 µM and 10 µM) for 72 h in RPMI media complete with 10% DFBS, 5% penicillin-streptomycin, and 2 nM LCV. Cells were harvested by trypsinization and washed twice with cold PBS. Cells were assayed for apoptosis by flow cytometry using the BD FACSCalibur flow cytometer (BD Biosciences). Apoptosis was measured using the annexin V-FITC/PI kit, as specified by the manufacturer (BD Biosciences), with a minimum of 1×10^4 cells analyzed for early apoptosis (annexin-V-$FITC_{high}$/PI_{low}; LR) and late apoptosis/necrosis (annexin-V-$FITC_{high}$/PI_{high}; UR); viable cells are shown as LL (annexin-V-$FITC_{low}$/7-AAD_{low}).

6.2.6. Colony Formation Assay

Colony formation assays were performed with compound **15a** to testify a cytotoxic response. A549 cells (~100 cells) were plated in six-well plate in RPMI media complete with 10% DFBS, 5% penicillin-streptomycin, and 2 nM LCV for 24 h incubation period at 37 ºC in the presence of 5% CO_2. Concentration from 0 to 20 μM of compound **15a** were added for an additional 48 h, After which the media was aspirated and replaced with new RPMI media complete with 10% DFBS, 5% penicillin-streptomycin, and 2 nM LCV for 8 days. After that, the cells were washed twice with room temperature PBS and fixed with methanol for 30 min. Then, the cells were stained by Giemsa stain for another 30 min. After removing the stain and washing twice with water, the stained colonies were counted.

6.3. Molecular Modeling and Computational Studies.

The X-ray crystal structures of human TS at 2.89 Å resolution (PDB ID 1JU6) and DHFR at 1.9 Å resolution (PDB ID 1U72) were downloaded from the Protein Data Bank. Docking studies were performed using Accelrys Discovery Studio client 2.5 (DS 2.5) software. The protonation state of the proteins and the ligands were calculated using the default settings. Water molecules in the active site were removed. The active site was defined by a sphere of 9.0 Å from the native ligand in the crystal structure. Molecules used for the docking experiments were prepared by DS 2.5. The CDocker protocol was used to score the docked poses. The docked poses were further visualized and processed by PyMOL 1.6.x.

Acknowledgments

The authors thank the Peking University Medical and Health Analysis Center for their help on the cell cycle assay and apoptosis analysis. This work was supported by the National Natural Science Foundation of China (21302007).

Reference:

(1) M. Visentin, R. Zhao, I. D. Goldman, The Antifolates. Hematol. Oncol. Clin. North Am. 26 (2012) 629−648.

(2) N. Gonen, Y. G. Assaraf, Antifolates in Cancer Therapy: Structure, Activity and Mechanisms of Drug Resistance. Drug Resist. Updates 15 (2012) 183−210.

(3) J.R. Schnell, H.J. Dyson, P.E. Wright, Structure, dynamics and catalytic function of dihydrofolate reductase. Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 119-140.

(4) F.H. Leite, P.B. Santiago, T.Q. Froes, S.F.J. Da, S.S. Da, R.M. Ximenes, A.R.D. Faria, D.J. Brondani, F.C.D.A. Julianna, S.C. Marcelo, Structure-guided discovery of thiazolidine-2,4-dione derivatives as a novel class of leishmania major pteridine reductase 1 inhibitors. Eur. J. Med. Chem. 123 (2016) 639-648.

(5) D.V. Santi, P.V. Danenberg, Folates in Pyrimidine Nucleotide Biosynthesis. In *Folates and Pterins*; R. L. Blakley, S. J. Benkovik, Eds. John Wiley and Sons: New York, Vol. 1 (1984), pp 345-398.

(6) S. Mitchell-Ryan, Y. Wang, S. Raghavan, M. P. Ravindra, E. Hales, S. Orr, C. Cherian, Z. Hou, L. H. Matherly, A. Gangjee, Discovery of 5-Substituted Pyrrolo[2,3-D]Pyrimidine Antifolates as Dual-Acting Inhibitors of Glycinamide Ribonucleotide Formyltransferase and 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase in De Novo Purine Nucleotide Biosynthesis: Implications of Inhibiting 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase to Ampk Activation and Antitumor Activity, J. Med. Chem. 56 (2013) 10016-10032.

(7) Y.G. Assaraf, Molecular basis of antifolate resistance, Cancer Metastasis Rev. 26 (2007) 153-181.

(8) S.M. Malik, K. Liu, X. Qiang, R. Sridhara, S. Tang, W.D. McGuinn Jr., S.L. Verbois, A. Marathe, G.M. Williams, J. Bullock, C. Tornoe, S.C. Lin, T. Ocheltree, M. Vialpando, A. Kacuba, R. Justice, R. Pazdur, Folotyn (pralatrexate injection) for the treatment of patients with relapsed or refractory peripheral T-cell lymphoma: U.S. Food and drug administration drug approval summary, Clin. Cancer Res. 16 (2010) 4921-4927.

(9) A.R. Hanauske, V. Chen, P. Paoletti, C. Niyikiza, Pemetrexed Disodium: A Novel Antifolate Clinically Active against Multiple Solid Tumors, The Oncologist 6 (2001) 363-373.

(10) V.F. Surmont, J.P. van Meerbeeck, Raltitrexed in mesothelioma, Expert Rev. Anticancer Ther. 11 (2011) 1481-1490.

(11) J.D. Roberts, E.A. Poplin, M.B. Tombes, B. Kyle, D.V. Spicer, S. Grant, T. Synold, R. Moran, Weekly lometrexol with daily oral folic acid is appropriate for phase II evaluation. Cancer Chemother. Pharmacol. 45.2 (2000) 103-110.

(12) D.R. Budman, R. Johnson, B. Barile, R.R. Bowsher, V. Vinciguerra, S.L. Allen, J. Kolitz, C.S. Ernest, W. Kreis, P. Zervos, J. Walling, Phase I and Pharmacokinetic Study of LY309887: A Specific Inhibitor of Purine Biosynthesis. Cancer Chemother. Pharmacol. 47 (2001) 525−553.

(13) T.J. Boritzki, C.A. Barlett, C. Zhang, E.F. Howland, S.A. Margosiak, C.L. Palmer, W.H. Romines, R.C. Jackson, AG2034: a novel inhibitor of glycinamide ribonucleotide

formyltransferase[J]. Investigational New Drugs, 14.3 (1996) 295-303.
(14) L.H. Matherly, Z. Hou, Y. Deng, Human reduced folate carrier: translation of basic biology to cancer etiology and therapy, Cancer Metastasis Rev. 26 (2007) 111-128.
(15) R. Zhao, I.D. Goldman, The proton-coupled folate transporter: physiological and pharmacological roles, Curr. Opin. Pharmacol. 13 (2013) 875-880.
(16) H. Elnakat, M. Ratnam, Distribution, functionality and gene regulation of folate receptor isoforms: Implications in targeted therapy. Adv. Drug Delivery Rev. 56 (2004) 1067−1084.
(17) Y.G. Assaraf, C.P. Leamon, J.A. Reddy, The folate receptor as a rational therapeutic target for personalized cancer treatment, Drug Resist. Updat. 17 (2014) 89-95.
(18) Y. Wang, S. Mitchell-Ryan, S. Raghavan, C. George, S. Orr, Z. Hou, L.H. Matherly, A. Gangjee, Novel 5-Substituted Pyrrolo[2,3-*d*]Pyrimidines as Dual Inhibitors of Glycinamide Ribonucleotide Formyltransferase and 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase and as Potential Antitumor Agents, J. Med. Chem., 58 (2015) 1479-1493.
(19) L.K. Golani, C. George, S. Zhao, S. Raghavan, S. Orr, A. Wallace, M.R. Wilson, Z. Hou, L.H. Matherly, A. Gangjee, Structure-Activity Profiles of Novel 6-Substituted Pyrrolo[2,3-*d*]Pyrimidine Thienoyl Antifolates with Modified Amino Acids for Cellular Uptake by Folate Receptors Alpha and Beta and the Proton-Coupled Folate Transporter, J. Med. Chem. 57 (2014) 8152-8166.
(20) S. Mitchell-Ryan, Y. Wang, S. Raghavan, M.P. Ravindra, E. Hales, S. Orr, C. Cherian, Z. Hou, L.H. Matherly, A. Gangjee, Discovery of 5-Substituted Pyrrolo[2,3-*d*]Pyrimidine Antifolates as Dual-Acting Inhibitors of Glycinamide Ribonucleotide Formyltransferase and 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase in De Novo Purine Nucleotide Biosynthesis: Implications of Inhibiting 5-Aminoimidazole-4-Carboxamide Ribonucleotide Formyltransferase to Ampk Activation and Antitumor Activity, J. Med. Chem. 56 (2013) 10016-10032.
(21) A.S. Rao, S.R. Tapale, A Study on Dihydrofolate Reductase and Its Inhibitors: A Review, Int. J. Pharm. Sci. Res. 4 (2013) 2535-2547.
(22) A.C. Anderson, D.L. Wright, Antifolate Agents: A Patent Review (2010-2013), Expert Opin. Ther. Patents 24 (2014) 687-697.
(23) I. Szabó, E. Orbán, G. Schlosser, F. Hudecz, Z. Bánóczi, Cell-penetrating conjugates of pentaglutamylated methotrexate as potential anticancer drugs against resistant tumor cells, Eur. J. Med. Chem. 115 (2016) 361-368.
(24) Y. Deng, Y. Wang, C. Cherian, Z. Hou, S.A. Buck, L.H. Matherly, Aleem Gangjee, Synthesis and Discovery of High Affinity Folate Receptor-Specific Glycinamide Ribonucleotide Formyltransferase Inhibitors with Antitumor Activity, J. Med. Chem. 51 (2008) 5052-5063.
(25) Z. Zhang, J. Wu, F. Ran, Y. Guo, R. Tian, S. Zhou, X. Wang, Z. Liu, L. Zhang, J. Cui, J. Liu, Novel 8-Deaza-5,6,7,8-Tetrahydroaminopterin Derivatives as Dihydrofolate Inhibitor: Design, Synthesis and Antifolate Activity, Eur. J. Med. Chem. 44 (2009) 764-771.
(26) Z. Zhang, C. Tian, S. Zhou, W. Wang, Y. Guo, J. Xia, Z. Liu, B. Wang, X. Wang, B.T. Golding, R.J. Griff, Y. Du, J. Liu, Mechanism-Based Design, Synthesis and Biological Studies of N^5-Substituted Tetrahydrofolate Analogs as Inhibitors of Cobalamin-Dependent Methionine Synthase and Potential Anticancer Agents, Eur. J. Med. Chem. 58 (2012) 228-236.
(27) C. Tian, Z. Zhang, S. Zhou, M. Yuan, X. Wang, J. Liu, Synthesis, Antifolate and Anticancer Activities of N^5-Substituted 8,10-Dideazatetrahydrofolate Analogues, Chem. Biol. Drug Des. 87 (2016) 444-454.

(28) L. Wang, A. Wallace, S. Raghavan, S. M. Deis, M. R. Wilson, S. Yang, L. Polin, K. White, J. Kushner, S. Orr, C. George, C. O'Connor, Z. Hou, S. Mitchell-Ryan, C.E. Dann, 3rd, L.H. Matherly, A. Gangjee, 6-Substituted Pyrrolo[2,3-D]Pyrimidine Thienoyl Regioisomers as Targeted Antifolates for Folate Receptor Alpha and the Proton-Coupled Folate Transporter in Human Tumors, J. Med. Chem. 58 (2015), 6938-6959.

(29) L.K. Golani, A. Wallace-Povirk, S.M. Deis, J. Wong, J. Ke, X. Gu, S. Raghavan, M.R. Wilson, X. Li, L. Polin, P.W. de Waal, K. White, J. Kushner, C. O'Connor, Z. Hou, H.E. Xu, K. Melcher, C.E. Dann, 3rd, L.H. Matherly, A. Gangjee, Tumor Targeting with Novel 6-Substituted Pyrrolo [2,3-*d*] Pyrimidine Antifolates with Heteroatom Bridge Substitutions Via Cellular Uptake by Folate Receptor Alpha and the Proton-Coupled Folate Transporter and Inhibition of De Novo Purine Nucleotide Biosynthesis, J. Med. Chem. 59 (2016) 7856-7876.

(30) Y. Deng, X. Zhou, S.K. Desmoulin, J. Wu, C. Cherian, Z. Hou, L.H. Matherly, Aleem Gangjee, Synthesis and Biological Activity of a Novel Series of 6-Substituted Thieno[2,3-*d*]Pyrimidine Antifolate Inhibitors of Purine Biosynthesis with Selectivity for High Affinity Folate Receptors over the Reduced Folate Carrier and Proton-Coupled Folate Transporter for Cellular Entry, J. Med. Chem. 52 (2009) 2940-2951.

(31) Y. Liu, M. Li, H. Zhang, J. Yuan, C. Zhang, K. Zhang, H. Guo, L. Zhao, Y. Du, L. Wang, L. Ren, Design, synthesis and biological evaluation of 6-substituted pyrrolo[2,3-d]pyrimidines as dual inhibitors of ts and aicarftase and as potential antitumor agents. Eur. J. Med. Chem. 115 (2016) 245-256.

(32) A. Gangjee, W. Li, J. Yang, R.L. Kisliuk, Design, Synthesis, and Biological Evaluation of Classical and Nonclassical 2-Amino-4-Oxo-5-Substituted-6-Methylpyrrolo[3,2-*d*]Pyrimidines as Dual Thymidylate Synthase and Dihydrofolate Reductase Inhibitors, J. Med. Chem. 51 (2008), 68-76.

(33) L. Wang, C. Cherian, S.K. Desmoulin, L.H. Matherly, A. Gangjee, Synthesis and Biological Activity of 6-Substituted Pyrrolo[2,3-*d*]pyrimidine Thienoyl Regioisomers as Inhibitors of De Novo Purine Biosynthesis with Selectivity for Cellular Uptake by High Affinity Folate Receptors and the Proton-Coupled Folate Transporter over the Reduced Folate Carrier. J. Med. Chem. 55 (2012) 1758−1770.

(34) Y. Liu, C. Zhang, H. Zhang, M. Li, J. Yuan, Y. Zhang, J. Zhou, H. Guo, L. Zhao, Y. Du, L. Wang, L. Ren, Synthesis and Antitumor Activity of a Novel Series of 6-Substituted Pyrrolo[2,3-D]Pyrimidines as Potential Nonclassical Antifolates Targeting Both Thymidylate and Purine Nucleotide Biosynthesis, Eur. J. Med. Chem. 93 (2015), 142-155.

(35) P.H. Sayre, J.S. Finer-Moore, T.A. Fritz, D. Biermann, S.B. Gates, W.C. MacKellar, V.F. Patel, R.M. Stroud, "Multi-targeted antifolates aimed at avoiding drug resistance form covalent closed inhibitory complexes with human and Escherichia coli thymidylate synthases." Journal of Molecular Biology 313.4 (2001) 813-829.

(36) V. Cody, J.R. Luft, W. Pangborn, Understanding the role of Leu22 variants in methotrexate resistance: comparison of wild-type and Leu22Arg variant mouse and human dihydrofolate reductase ternary crystal complexes with methotrexate and NADPH. Acta Crystallogr. 61. 2 (2005) 147-155.

Highlights:

1. A series of 6 Substituted pyrrolo[3,2-*d*]pyrimidine analogues were synthesized as antifolate agents.
2. The *in vitro* anti-proliferative effect of these compounds on tumor cells were evaluated.
3. The anti-proliferative effect of the most potent compound (**15a**) acted through dual inhibition of TS and DHFR.
4. The effects of **15a** on cell cycle distribution, induction of apoptosis and colony formation inhibition were tested.
5. Molecular docking of **15a** was carried out against TS and DHFR.