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Non-classical Antifolates, 5-(*N*-Phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines and 2,4-Diamino-6(5*H*)-oxopyrimidines, Synthesis and Antitumor Studies

Yen-Lin Huang,[a,b] Chyun-Feng Lin,[a,b] Yi-Jen Lee,[a] Wei-Wei Li,[c] Ting-Chou Chao,[c] Valeriy A. Bacherikov,[a] Kuo-Tung Chen,[b] Chin-Ming Chen[b] and Tsann-Long Su[a,*]

[a]*Laboratory of Bioorganic Chemistry, Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan*
[b]*Department of Medicinal Chemistry, College of Pharmacy, Taipei Medical University, Taipei, Taiwan*
[c]*Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA*

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Abstract—A series of non-classical antifolates, namely 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines (**25a**–**i**) and 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidines (**26a**,**b**,**c**,**f**,**h**,**i**) was synthesized and evaluated for their in vitro cytotoxicity. Reacting aniline derivatives with 1,4-dibromo-2-butanol gave 1-phenyl-3-pyrrolidinols (**19a**–**i**), which were oxidized to pyrrolidin-3-ones (**20a**–**i**). The Knoevenagel reaction of **20a**–**i** with malononitrile or ethyl cyanoacetate gave 3-(dicyanomethylene)- (**21a**–**i**) and 3-[cyano(ethoxycarbonyl)methylene]-pyrrolidines (**22a**,**b**,**c**,**f**,**h**,**i**), respectively, which were subsequently reduced to the corresponding 3-(dicyano)methyl- or 3-[cyano(ethoxycarbonyl)methyl)]pyrrolidines (**23a**–**i** and **24a**,**b**,**c**,**f**,**h**,**i**, respectively). Condensation of either **23a**–**i** or **24a**,**b**,**c**,**f**,**h**,**i** with guanidine afforded the target compounds. The cytotoxicity of these compounds was evaluated based on their ability to inhibit various human tumors (human colon adenocarcinoma COLO 205, lung carcinoma H23 and its adriamycin resistant cell line H23/0.3, T-cell leukemia MOLT-4, promyelocytic leukemia HL-60, and T-cell acute lymphocytic leukemia CCRF-CEM) cell growth in culture. These studies revealed that the 2,4,6-triaminopyrimidine derivatives were more cytotoxic than the 2,4-diamino-6(5*H*)-oxopyrimidine counter parts, in which the latter was inactive in all testing systems. The 2,4,6-triaminopyrimidine derivatives bearing halogen substituent on the phenyl ring (**25f**,**h**,**i**) were cytotoxic in all cultured leukemia cell growth. Among these compounds, 5-(4-fluoro and 4-chlorophenyl)-2,4,6-triaminopyrimidines (**25e** and **25h**, respectively) were more potent than methotrexate (MTX) in inhibiting of H23/0.3 cell growth. These compounds inhibit the folate metabolic pathways as indicated by tritium release from [5-^{3}H]deoxyuridine in MTX sensitive human fibrosarcoma HT-1080 cells. Dihydrofolate reductase is the major target for **25f**,**h**,**i**, as shown by leucovorin (LV) rescue of MTX cytotoxicity.

Introduction

Research and development of antifolates for cancer chemotherapy have been one of a major focus in anticancer drug design. The discovery of aminopterin (AP) and methotrexate (MTX) having a potent antitumor efficacy in cancer patients,[1] lead to intensive investigations of numerous classical antifolates carrying a *p*-aminobenzoyl-L-glutamic acid moiety.[2] The inhibition of two important enzymes, dihydrofolate reductase (DHFR) and thymidylate synthase (TS, both involved in the metabolic pathway of folic acid), is the main subject in the development of chemotherapeutic agents.[3] Unfortunately, several problems are associated with the clinical use of MTX such as: (1) high levels of toxicity to bone marrow and gastrointestinal mucosa,[4] (2) resistance to the drug accompanied by an elevation of levels of the target enzyme, DHFR,[5] and (3) the decrease in the transport of the drug.[6,7] The most common reason of intrinsic resistance is a defect in the membrane transport of MTX.[8,9] To minimize problems caused by MTX, various antifolates with modifications in the pteridine ring (such as deazaaminopterins), phenyl moiety and their tetrahydrofolate analogues were studied.[10] The opened B-ring compounds, such as *N*-[4-(2,4-diamino-6-oxo-1*H*,6*H*-pyrimidin-5-yl)butyl]benzoyl]-L-glutamic acid and its 2,4,6-triamino derivatives (**1**[11,12] and **2**,[13,14] respectively, Fig. 1) are synthesized for both in

*Corresponding author. Tel.: +886-2-2782-7685; fax: +886-2-2782-7685; e-mail: tlsu@ibms.sinica.edu.tw

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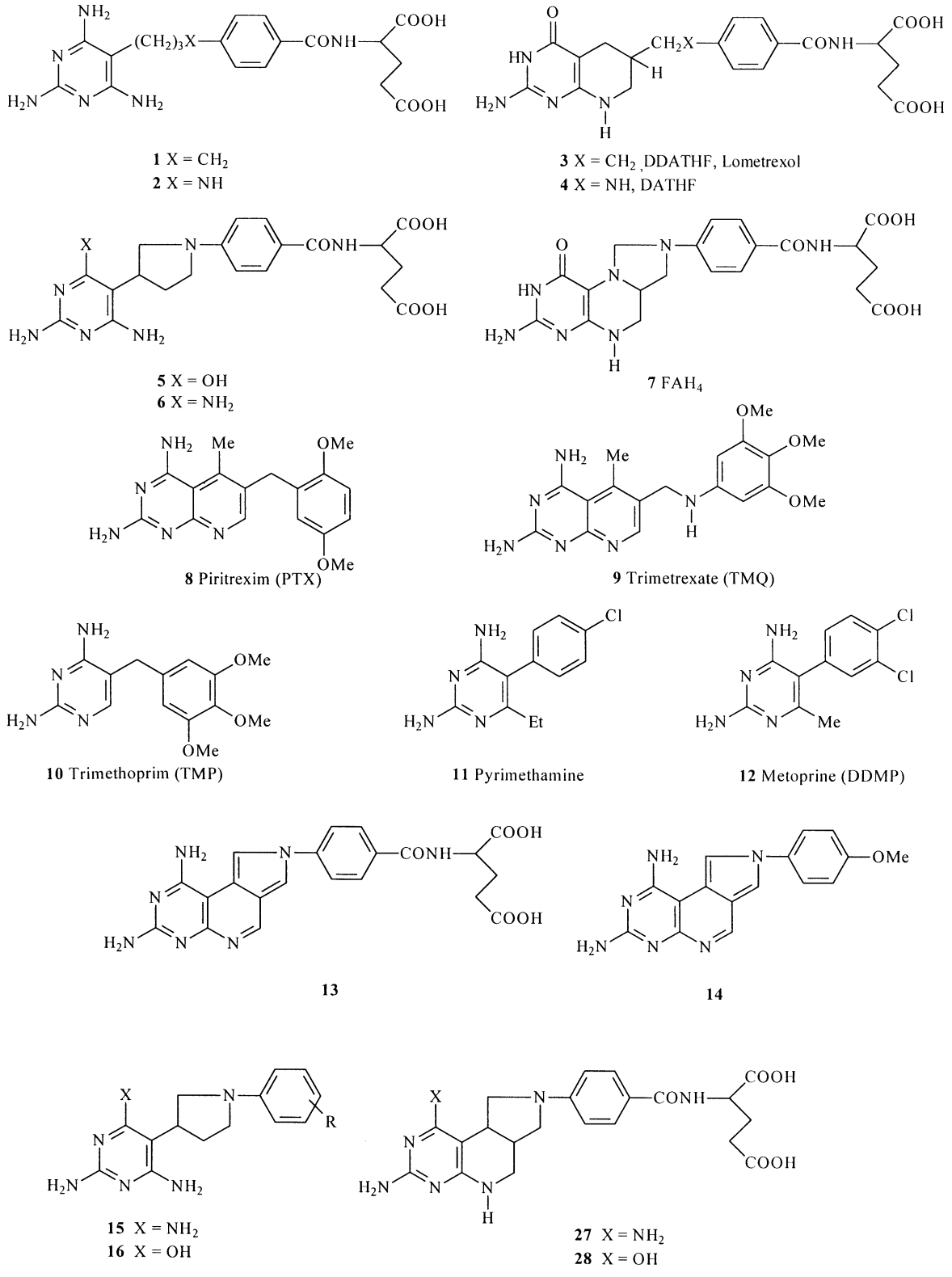


Figure 1. Structures of some antifolates.

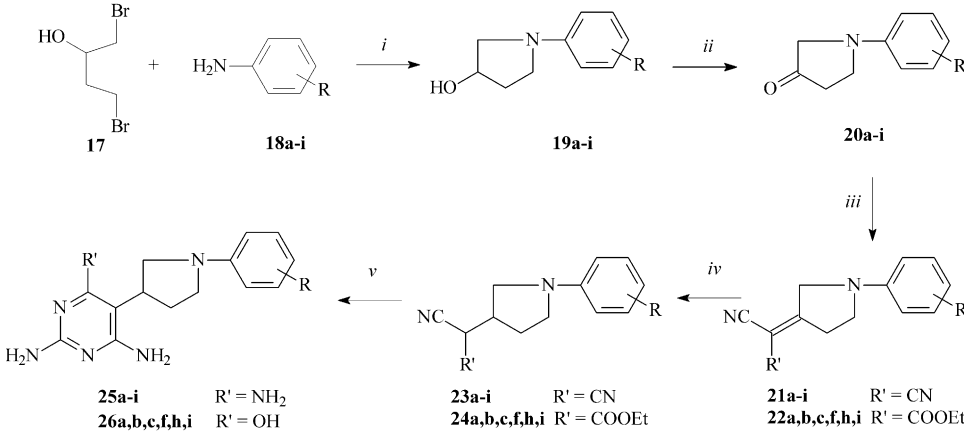
vitro and in vivo antitumor studies.[15] Compounds **1** and **2** are viewed as 7-desmethylene analogues of the potent purine biosynthesis inhibitors, DDATHF (**3**)[16] and DATHF (**4**).[17] Rosowsky et al.[18] synthesized *N*-[4-[3-(2,4-diamino-6(5*H*)-oxopymidin-5-yl)pyrrolidino]benzoyl]-L-glutamic acid (**5**) and its 1,2,6-triamino derivatives (**6**), which are 5-deaza-7-desmethylene analogues of 5,10-methylene-5,6,7,8-tetrahydrofolic acid (**7**). Compound **5** and **6** are good substrates for mouse liver folylpolyglutamate synthetase and the latter is a DHFR inhibitor, which inhibits the growth of culture tumor cells (SCC25 human squamous cell carcinoma).[18]

Numerous antifolates, not carrying the glutamate moiety (i.e., non-classical antifolates), have versatile biological activity. The non-classical antifolates are developed in an attempt to overcome the resistance caused by MTX. These compounds are envisioned to be more lipophilic, thus can enter into the cell via passive diffusion. Compounds belonging to non-classical antifolates are derivatives of pyrido[2,3-*d*]pyrimidine[19–22] [such as piritrexim (PTX, **8**)[19] and trimetrexate (TMQ, **9**)[22]], quinazoline,[23,24] and pyrimidine [such as trimethoprim (TMP, **10**),[25] pyrimethamine (PYR, **11**)[26] and metoprine (DDMP, **12**).[26] Although reports demonstrated TMP as a poor inhibitor of mammalian DHFR, it is approximately 10-fold more potent against *Pneumocystis carrinii* DHFR.[27] Similarly, PYR analogues with poor potency against *P. carrinii* DHFR does not show significant selectivity.[27] Unlike TMP and PYR, both (**8**) and (**9**) inhibit mammalian DHFR more potently than pathogen DHFRs. However, they do not offer any beneficial selectivity.[28] Therefore, many of these agents have been developed as potent antimicrobial and/or anticancer activities.[29–32] For example, PTX and TMQ are in clinical trials for treatment of AIDS-related opportunistic infections[33,34] as well as advanced bladder cancer.[35,36] Studies also show DDMP to be a potentially useful drug against brain tumors.[37]

Previously, we synthesized 1,3-diaminopyrrolo[3′,4′:4,5]pyrido[2,3-*d*]pyrimidine derivatives (**13** and **14**, Fig. 1). Compound **13** is an analogue of 5,10-methylene-FAH_4[36,37] and is 1/10 as potent as MTX against human leukemia HL-60 cell in culture. Compound **14** lacks the *p*-aminobenzoyl-L-glutamic acid moiety and is an intricate subject for biological evaluations because of its poor solubility. To continue our research on the development of non-classical antifolates as potential antitumor agents, we synthesized a series of 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triamniopyrimidines (**15**) and 2,4-diamino-5-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidine (**16**) derivatives, which can be considered as 5-deaza-7-desmethylene analogues of **14** as well as derivatives of **5** and **6** without the glutamate moiety. Our findings show that the halogen substituted derivatives of 2,4,6-triaminopyrimidine are cytotoxic to the cell growth of several of human leukemias in culture. These compounds inhibit the folate metabolic pathways as indicated by tritium release from [5-^{3}H]-deoxyuridine in MTX sensitive human fibrosarcoma HT-1080 cells. These studies have also revealed dihydrofolate reductase as the major target for **25f**,**h**,**i**, as shown by leucovorin (LV) rescue of MTX cytotoxicity.

Chemistry

The synthetic routes for the preparation of our compounds are based on Rosowsky's publication[18] and our previous report[37] with some modifications. In our previous study,[37] we synthesized 1-(4-alkyloxycarbonylphenyl)-3-pyrrolidinone by the condensation of 1,4-dibromo-2-butanol (**17**) with alkyl *p*-aminobenzoate, followed by the oxidation (DCC/pyridine/DMSO/TFA) of the product 1-(4-alkyloxycarbonylphenyl)-3-pyrrolidinol. Using the same procedure, treating **17** with a variety of substituted anilines (**18a**–**i**) in triethyl phosphate in the presence of potassium carbonate under reflux for 4–6 h afforded 1-phenyl-3-pyrrolidinols (**19a**–**i**) in low to moderate yields (16–62%), depending on the substituent in the phenyl ring (Scheme 1). Condensation of **17** with aniline (**18a**), 2-Cl-, 4-Cl-, or 4-Br-aniline (**18g**, **18h**, or **18i**, respectively) afforded 1-phenyl-3-pyrrolidinols (**19a**,**g**,**h**,**i**, respectively) in moderate yield, while reacting **17** with fluoro-substituted anilines (**18e**,**f**), anilines bearing a electron-withdrawing function (i.e., CN and NO_2, **18c**,**d**) or a electron-donating function (OMe, **18b**) gave a low yield of 1-phenyl-3-pyrrolidinols (**19b**–**d**). The original synthetic method for 1-phenyl-3-pyrrolidinone was reported by Taylor et al.,[38] who synthesized 1-[(4-*tert*-butoxycarbonyl)phenyl]-3-pyrrolidinone by the condensation of 3-pyrrolidinol with *tert*-butyl 4-fluorobenzoate, followed by oxidation. Our methodology for the synthesis of 1-phenyl-3-pyrrolidinols **19** has some advantage since various *N*-substituted 3-pyrrolidinols can be prepared under the facile reaction



Scheme 1. Synthesis of non-classical antifolates, 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines and 2,4-diamino-6(5*H*)-oxopyrimidines: (a) R = H; (b) R = 3-OMe; (c) R = 4-CN; (d) R = 4-NO_2; (e) R = 2-F; (f) R = 4-F; (g) R = 2-Cl; (h) R = 4-Cl; (i) R = 4-Br. Reagents and conditions: (i) $(EtO)_3PO/K_2CO_3/150\,^\circ C$; (ii) DCC/DMSO/pyridine/TFA, 0 °C; (iii) $CH_2(CN)_2$ or $NCCH_2COOEt$/DBU/benzene/0 °C to rt; (iv) $NaBH_3CN$/AcOH; (v) guanidine dicarbonate/K_2CO_3/DMF.

conditions by the reaction of substituted anilines with inexpensive **17**.

Using the Pfitzner–Moffatt[39] procedure, 3-pyrrolidinols **19a–i** were oxidized to give pyrrolidinones **20a–i** in moderate to good yield. The Knoevenagel reaction of pyrrolidin-3-ones **20a–i** with malononitrile or ethyl cyanoacetate afforded 3-(dicyanomethylene)pyrrolidines (**21a–i**) or 3-[cyano(ethoxycarbonyl)methylene]pyrrolidines (**22a**,**b**,**c**,**f**,**h**,**i**), respectively. In most cases, the reactions were carried out in dry ether in the presence of base catalyst (DBU) and the dried molecular sieves at 0 °C, and the reaction mixture was then gradually warmed up to room temperature and continuously stirred for 2–8 h. Compounds **21e** and **21g** were prepared by reacting **20e** and **20g**, respectively, with malononitrile in dry ethanol in the presence of KF. Products **21a**,**b**,**f**,**h**,**i** precipitated during the course of reaction and were isolated in a stable solid form. The crude products **21c**,**d**,**e**,**g** did not precipitate out and contained a small amount of the starting material along with by-products and were used in the next reaction. Since the desired product and the starting ketone have the same R_f value on the TLC (SiO_2) with a variety of solvent system, we were unsuccessful in isolating the product by column chromatography. In these cases, prolongation of the reaction time or addition of excess malononitrile usually resulted more decomposition. Similarly, reacting 3-pyrrolidinones **20a**,**b**,**c**,**f**,**h**,**i** with ethyl cyanoacetate using *N*,*N*-dimethylaminoethanol as base afforded **22a**,**b**,**c**,**f**,**h**,**i** in moderate to good yield. The olefin function of **21a–i** and **22a**,**b**,**c**,**f**,**h**,**i** was reduced by sodium cyanoborohydride in acetic acid to give 3-(dicyanomethyl)pyrrolidines (**23a–i**) and 3-[cyano(ethoxycarbonyl)methyl]pyrrolidines (**20a**,**b**,**c**,**f**,**h**,**i**), respectively. Both were then condensed with guanidine dicarbonate and furnished the desired racemic 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines (**25a–i**) and 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidines (**26a**,**b**,**c**,**f**,**h**,**i**), respectively, in a low to moderate yield. It should be noted, that our preliminary results revealed that 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidines (**26a**,**b**,**c**,**f**,**h**,**i**) were inactive in inhibiting various tumor cell growth in culture (see below) and compounds **20d**,**e**,**g** were difficult to handle because of their instability, therefore, we did not prepare **22d**,**e**,**g** for the synthesis of **26d**,**e**,**g**.

The structure of all compounds were determined and confirmed by IR and ^{1}H NMR spectroscopy methods (Tables 1 and 2, respectively). The ^{1}H NMR spectra of 3-substituted-*N*-phenylpyrrolidine derivatives reported previously[18] were described without any assignments of the chemical shift of each proton on the pyrrolidine ring. Protons on the pyrrolidine ring appeared as multispin systems and were difficult to assign from one-dimensional spectra because of overlapping complexities. In the present study we have analyzed the ^{1}H NMR and COSY spectra of the newly synthesized compounds and assigned the chemical shifts of protons of pyrrolidine ring, where orientation of substituents is more important in a stereochemical point of view. The parameters of ^{1}H NMR spectra of the synthesized compounds are shown in Table 2. Generally, the protons at C-4 appeared in higher field in the area of δ 1.88–2.60, while the protons at C-2 and C-5 appeared in the region of δ 3.0–3.75 and C-3 protons are showed at δ 2.95–4.40. The chemical shifts of these protons are affected by the substituent at C-3, especially for those compounds with pyrimidine moiety at C-3.

CHN analysis of newly synthesized compounds

Compd	Formula	Anal. calcd			Found		
		C (%)	H (%)	N (%)	C (%)	H (%)	N (%)
19a	$C_{10}H_{13}NO$	73.59	8.03	8.58	73.60	8.05	8.52
19b	$C_{11}H_{15}NO_2$:	68.37	7.82	7.25	68.21	7.75	7.32
19c	$C_{11}H_{12}N_2O$	70.19	6.43	14.88	70.16	6.45	14.84
19d	$C_{10}H_{12}N_2O_3$	57.68	5.81	13.45	57.55	5.76	13.24
19e	$C_{10}H_{12}FNO$	66.28	6.67	7.73	66.12	6.50	7.61
19f	$C_{10}H_{12}FNO$	66.28	6.67	7.73	65.98	6.72	7.66
19g	$C_{10}H_{12}ClNO$	60.76	6.12	7.09	60.58	6.05	7.15
19h	$C_{10}H_{12}ClNO\cdot 5/8H_2O$	57.49	6.39	6.70	57.34	6.31	6.22
19i	$C_{10}H_{12}BrNO$	49.61	5.00	33.01	49.75	5.10	33.10
20a	$C_{10}H_{11}NO$	74.51	6.88	8.69	74.71	6.72	8.58
20b	$C_{11}H_{13}NO_2$	69.09	6.85	7.33	69.00	7.10	7.02
20c	$C_{11}H_{10}N_2O\cdot 1/5H_2O$	6.95	5.52	14.76	7.02	5.31	14.56
20d	$C_{10}H_{10}N_2O_3$	58.25	4.89	13.59	58.50	4.79	13.24
20e	$C_{10}H_{10}FNO$	67.03	5.63	7.82	66.92	5.45	7.67
20f	$C_{10}H_{10}FNO$	67.03	5.63	7.82	66.87	5.65	7.77
20g	$C_{10}H_{10}ClNO$	61.39	5.15	7.16	61.21	5.06	7.22
20h	$C_{10}H_{10}ClNO$	61.39	5.15	7.16	61.53	5.13	7.00
20i	$C_{10}H_{10}BrNO$	50.02	4.20	5.83	49.88	4.23	5.82
21a	$C_{13}H_{11}N_3$	74.62	5.30	20.08	74.56	5.54	19.90
21b	$C_{14}H_{13}N_3O$	70.27	5.48	17.56	70.10	5.42	17.38
21f	$C_{13}H_{10}FN_3$	68.71	4.44	18.49	68.61	4.68	18.33
21h	$C_{13}H_{10}ClN_3$	64.64	4.14	17.24	64.81	4.36	17.56
21i	$C_{13}H_{10}BrN_3$	54.19	3.50	14.58	54.02	3.65	14.35
22a	$C_{15}H_{16}N_2O_2$	70.29	6.29	10.93	70.10	6.16	10.79
22b	$C_{16}H_{18}N_2O_2$	71.11	6.71	10.36	71.02	6.65	10.22
22c	$C_{16}H_{15}N_3O_2$	68.31	5.37	14.94	68.21	5.19	14.85
22f	$C_{15}H_{15}FN_2O_2$	65.68	5.51	10.21	65.51	5.58	10.33
22h	$C_{15}H_{15}ClN_2O_2$	61.96	5.20	9.64	61.77	5.46	9.63
22i	$C_{15}H_{15}BrN_2O_2$	53.75	4.51	8.36	53.67	4.52	8.23
23a	$C_{13}H_{13}N_3\cdot 1/10H_2O$	73.28	6.24	19.72	73.48	6.27	19.80
23b	$C_{14}H_{15}N_3O$	69.69	6.27	17.42	69.53	6.18	17.33
23c	$C_{14}H_{12}N_4\cdot 1/5H_2O$	70.10	5.21	23.36	71.24	5.27	23.18
23d	$C_{13}H_{12}N_4O_2$	60.93	4.72	21.86	60.81	4.65	21.71
23e	$C_{13}H_{12}FN_3$	68.11	5.28	18.33	67.95	5.44	18.66
23f	$C_{13}H_{12}FN_3$	68.11	5.28	18.33	68.09	5.34	18.46
23g	$C_{13}H_{12}ClN_3\cdot 7/4H_2O$	48.53	4.87	13.06	48.45	4.68	12.83
23h	$C_{13}H_{12}ClN_3\cdot 1/5H_2O$	62.62	5.01	16.86	63.83	5.25	16.73
23i	$C_{13}H_{12}BrN_3$	53.81	4.17	14.48	53.70	4.06	14.36
24a	$C_{15}H_{18}N_2O_2$	69.74	7.02	10.85	69.55	7.20	10.66
24b	$C_{16}H_{20}N_2O_3$	66.65	6.99	9.72	66.45	6.75	9.61
24c	$C_{16}H_{17}N_3O_2$	67.83	6.05	14.83	17.56	5.83	14.66
24f	$C_{15}H_{17}FN_2O_2$	65.20	6.20	10.14	65.03	6.32	10.30.
24h	$C_{15}H_{17}ClN_2O_2$	61.54	5.85	9.57	61.33	5.70	9.45
24i	$C_{15}H_{17}BrN_2O_2$	53.42	5.08	8.31	53.35	5.15	8.17

25a	$C_{14}H_{18}N_6$	62.20	6.71	31.09	61.99	6.88	31.23
25b	$C_{15}H_{20}N_6O\cdot 1/2H_2O$	58.23	6.84	27.17	58.36	6.78	27.32
25c	$C_{15}H_{17}N_7$	61.00	5.80	33.20	60.84	5.98	33.39
25d	$C_{14}H_{17}N_7O_2$	53.32	5.43	31.09	53.54	5.66	31.40
25e	$C_{14}H_{17}FN_6$	58.32	5.94	29.15	58.33	5.84	29.23
25f	$C_{14}H_{17}FN_6$	58.32	5.94	29.15	58.02	6.05	29.33
25g	$C_{14}H_{17}ClN_6$	55.17	5.62	27.58	54.92	5.73	27.40
25h	$C_{14}H_{17}ClN_6$	55.17	5.62	27.58	54.96	5.60	27.35
25i	$C_{14}H_{17}BrN_6$	48.15	4.91	24.07	48.45	5.01	23.83
26a	$C_{14}H_{17}N_5O$	58.12	6.62	24.21	58.02	6.75	24.43
26b	$C_{15}H_{19}\cdot N_5O_2\cdot H_2O$	56.41	6.63	21.93	56.66	6.62	21.75
26c	$C_{15}H_{16}N_6O\cdot H_2O$	57.31	5.77	26.74	57.11	5.66	26.88
26f	$C_{14}H_{16}FN_5O\cdot H_2O$	54.71	5.80	22.79	54.65	6.05	22.58
26h	$C_{14}H_{16}ClN_5O$	54.99	5.27	22.91	54.57	5.32	22.88
26i	$C_{14}H_{16}BrN_5O$	48.01	4.60	20.00	47.90	4.43	20.25

Biological Results and Discussion

Table 3 shows the cytotoxicity of a series of 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidines (**25a–i**) and 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidines (**26a**,**b**,**c**,**f**,**h**,**i**) in inhibiting human colon adenocarcinoma (COLO 205), lung carcinoma (H23) and its adriamycin resistant cell line (H23/0.3), T-cell leukemia (MOLT-4), promyelocytic leukemia (HL-60), human T-cell acute lymphocytic leukemia (CCRF-CEM) and human lung carcinoma (A549) cell growth in culture. MTX was used for comparison. The results demonstrated that in the series of 2,4,6-triaminopyrimidine derivatives (**25a–i**), the halogen substituted substances (i.e., **25e–i**) exhibited potent cytotoxicity; compounds **25e** and **25h** are more potent than MTX (inactive at concentrations up to 100 μM) in inhibiting of H23/0.3 cell growth with IC_{50} values of 3.47 and 3.01 μM, respectively; **25f**, **25h**, and **25i** inhibited MOLT-4 cell growth in culture with higher affinity than other derivatives with IC_{50} values of 3.70, 1.88 and 2.48 μM, respectively. Among halogen substituted compounds, 4-bromophenyl derivative **25i** displayed potent cytotoxicity in inhibiting cell growth of cultured human leukemias (MOLT-4, HL-60 and CCRF-CEM) and human lung carcinoma (A549) with IC_{50} values of 2.48, 1.58, 1.80 and 1.07 μM, respectively. In the same series of derivatives, compounds with electron-withdrawing function [CN (**25c**) and NO_2 (**25d**)] on the phenyl ring were inactive. These studies also reveal that phenyl- (**25a**) or 3-methoxyphenyl- (**25b**) derivatives have marginal cytotoxicity in inhibiting MOLT-4 cell growth in culture only. However, all 2,6-diamino-4(3*H*)-oxopyrimidine derivatives (**26a**,**b**,**c**,**f**,**h**,**i**) are inactive at concentrations up to 100 μM in all testing systems. The results demonstrated that 2,4,6-triaminopyrimidine derivatives are more cytotoxic than 2,4-diamino-6(5*H*)-oxopyrimidine derivatives. Although some 2,4,6-triaminopyrimidine derivatives (i.e., **25g** and **25i**) are better inhibitors of COLO 205, H23 and H23/0.3 cell growth in culture than MTX, these compounds are less active than MTX in human leukemia cell growth in culture.

Previously, DeGraw et al.[40] reported that 5,10-methylenetetrahydro-8,10-dideazaaminopterin (**27**, Fig. 1) was a weak inhibitor to both L1210 murine leukemia DHFR and L1210 growth in culture. Compound **27** was only 1/100 as potent as MTX in inhibiting L1210 cell growth in culture. Our previous report also showed that compound **13** was one tenth less active than MTX against human leukemia HL-60 cell growth in culture. Gangjee et al.[41] synthesized the tetrahydro analogue of **13**, 5,10-methylenetetrahydro-5-deazafolic acid (**28**), which was inactive in cultured lymphoma cell growth or TS. The inactivity of **28** was attributed to its lack of flexibility, thus leading to its inability to bind to TS. The 7-desmethylene-5,10-methylenetetrahydro-5-deazafolic acid (**5**), on the other hand, did not display any activity against TS or glycinamide ribotide formyltransferase, two other key enzymes of folate-mediated one-carbon metabolism.[23] However, the triamino derivative **6** was moderately active as an inhibitor of cultured tumor cell growth (SCC25 human squamous cell carcinoma), but was a good substrate for folylpolyglutamate synthetase. These reports clearly demonstrated that derivatives of aminopterin or 2,4,6-triaminopyrimidine were more potent than the corresponding folic acid analogues or 2,6-diamino-4-hydroxypyrimidine derivatives, respectively. Similar results were observed in our present studies.

In situ [5-^{3}H]-deoxyuridine tritium release assays[42,43] were used to evaluate the effect of compounds **25f**,**h**,**i** inhibition on the folate metabolic pathways in MTX-sensitive human fibrosarcoma HT-1080 cells. Table 4 shows MTX and **25f**,**h**,**i** at concentrations of 0.1–1.0 μM inhibiting the pathway involving TS in HT-1080 cell lines. The result revealed that the fluoro-substituted compound, **25f**, was as potent as MTX, while the chloro- and bromo-substituted derivatives, **25h** and **25i**, were less potent than MTX on the inhibition at 0.1 μM.

Leucovorin (*N*-5-formyltetrahydrofolate) (LV) is widely used to selectively rescue the serious toxic side effects of MTX and TMQ.[44–46] In particular, LV appears to protect hemopoitic progenitors against damages by MTX or TMQ. Therefore, we studied the effects of LV on HT-1080 cell growth inhibition by MTX and **25f**,**h**,**i** at various concentrations in the presence or absence of 10 μM LV after a 24 h exposure (Fig. 2A–D). The results showed that MTX was more cytotoxic than **25f**,**h**,**i** on inhibiting HT-1080 cell growth in culture (Table 5). In the presence of LV, 3–54% of HT-1080 cells were rescued by MTX + LV. A partial rescue of HT-1080 cell growth was observed when the cells were treated with either **25h** (3–23% rescue) or **25i** (1–5% rescue) simultaneously, at high concentration (10 μM). However, no effect of LV on the rescue of HT-1080 cells growth at high concentration of **25h** and **25i**. While, up to 56% of HT-1080 cell growth was rescued by LV + **25f**.

In conclusion, we have synthesized a series of non-classical antifolates, 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidine (**25a–i**) and 2,4-diamino-(*N*-phenylpyrrolidin-3-yl)-6(5H)-oxopyrimidine (**26a**,**b**,**c**,**f**,**h**,**i**) derivatives. The cytotoxicity of these compounds was evaluated upon their ability to inhibit of various human tumor cell

Table 1. Yields and physical data of the synthesized compounds

Compd	Yield (%)	mp (°C)	IR KBr (cm^{-1})[a]	Formula[b]
19a	62	95–96		$C_{10}H_{13}NO$
19b	39	Syrup		$C_{11}H_{15}NO_2$
19c	19	164–165		$C_{11}H_{12}N_2O$
19d	16	188–189		$C_{10}H_{12}N_2O_3$
19e	25	Syrup		$C_{10}H_{12}FNO$
19f	23	70–71		$C_{10}H_{12}FNO$
19g	52	Syrup		$C_{10}H_{12}ClNO$
19h	23	106–107		$C_{10}H_{12}ClNO{\cdot}5/8H_2O$
19i	54	Syrup		$C_{10}H_{12}BrNO$
20a	79	89–90	1755 (C=O)	$C_{10}H_{11}NO$
20b	49	108–109	1753 (C=O)	$C_{11}H_{13}NO_2$
20c	49	170–171	2213 (CN), 1756 (C=O)	$C_{11}H_{10}N_2O{\cdot}1/5H_2O$
20d	51	187–188	1763 (C=O)	$C_{10}H_{10}N_2O_3$
20e	55	Syrup	1763 (C=O)	$C_{10}H_{10}FNO$
20f	79	88–89	1763 (C=O)	$C_{10}H_{10}FNO$
20g	69	Syrup	1763 (C=O)	$C_{10}H_{10}ClNO$
20h	45	128–129	1754 (C=O)	$C_{10}H_{10}ClNO$
20i	66	141–142	1757 (C=O)	$C_{10}H_{10}BrNO$
21a	72	176–177	2239 (CN)	$C_{13}H_{11}N_3$
21b	27	151–152	2234 (CN)	$C_{14}H_{13}N_3O$
21f	72	176–177	2239 (CN)	$C_{13}H_{10}FN_3$
21h	65	178–179	2230 (CN)	$C_{13}H_{10}ClN_3$
21i	61	185–186	2236 (CN)	$C_{13}H_{10}BrN_3$
22a	34	176–177	2239 (CN)	$C_{15}H_{16}N_2O_2$
22b	74	102–103	2230 (CN)	$C_{16}H_{18}N_2O_2$
22c	75	163–163	2215 (CN)	$C_{16}H_{15}N_3O_2$
22f	63	96–97	2233 (CN)	$C_{15}H_{15}FN_2O_2$
22h	80	39–140	2258 (CN)	$C_{15}H_{15}ClN_2O_2$
22i	91	159–160	2230 (CN)	$C_{15}H_{15}BrN_2O_2$
23a	60	137–138	2256 (CN)	$C_{13}H_{13}N_3{\cdot}1/10H_2O$
23b	96	Syrup		$C_{14}H_{15}N_3O$
23c	31	168–169	2220 (CN)	$C_{14}H_{12}N_4{\cdot}1/5H_2O$
23d	22	134–135	2252 (CN)	$C_{13}H_{12}N_4O_2$
23e	22	Syrup		$C_{13}H_{12}FN_3$
23f	56	137–138	2256 (CN)	$C_{13}H_{12}FN_3$
23g	33	Syrup	2210 (CN)	$C_{13}H_{12}ClN_3$
23h	81	124–125	2250 (CN)	$C_{13}H_{12}ClN_3{\cdot}1/5H_2O$
23i	83	156–157	2263 (CN)	$C_{13}H_{12}BrN_3$
24a	48	Syrup	2256 (CN)	$C_{15}H_{18}N_2O_2$
24b	89	Syrup	2256 (CN)	$C_{16}H_{20}N_2O_3$
24c	80	Syrup	2250 (CN)	$C_{16}H_{17}N_3O_2$
24f	67	Syrup	2256 (CN)	$C_{15}H_{17}FN_2O_2$
24h	82	Syrup	2250 (CN)	$C_{15}H_{17}ClN_2O_2$
24i	80	Syrup	2256 (CN)	$C_{15}H_{17}BrN_2O_2$
25a	48	289–290		$C_{14}H_{18}N_6$
25b	34	219–220		$C_{15}H_{20}N_6O{\cdot}1/2H_2O$
25c	7	158–159	2210 (CN)	$C_{15}H_{17}N_7$
25d	12	225–226		$C_{14}H_{17}N_7O_2$
25e	8	223–224		$C_{14}H_{17}FN_6$
25f	56	289–291		$C_{14}H_1FN_6$
25g	7	185–186		$C_{14}H_{17}ClN_6$
25h	43	250–251		$C_{14}H_{17}ClN_6$
25i	38	257–258		$C_{14}H_{17}BrN_6$
26a	56	289–290		$C_{14}H_{17}N_5O$
26b	30	222–223		$C_{15}H_{19}O_2N_5\ H_2O$
26c	15	250–251		$C_{15}H_{16}N_6OH_2O$
26f	26	285–286		$C_{14}H_{16}FN_5OH_2O$
26h	15	228–229		$C_{14}H_{16}ClN_5O$
26i	33	259–260		$C_{14}H_{16}BrN_5O$

[a]Only the peak for CN group was shown.
[b]Except when noted, the compounds gave elemental analyses for C, H, and N with ±0.4% of the theoretical values.

growth in culture. The structure–activity relationship studies reveal that 2,4,6-triaminopyrimidines are more active than the 2,4-diamino-6(5*H*)-oxopyrimidines counter parts. The latter are inactive in all testing systems. Among these compounds, the halogen substituted derivatives of 2,4,6-triaminopyrimidine (**25f**,**h**,**i**) exhibit potent cytotoxicity, especially, in inhibiting human leukemia (MOLT-4, HL-60, and CCRF-CEM), even though they are less cytotoxic than MTX. The present studies also demonstrate that **25f**,**h**,**i** inhibit the folate metabolic pathways in HT-1080 cell lines. The fluoro-substituted derivative (**25f**) is as potent as MTX. Study on the inhibition of HT-1080 cell growth in culture demonstrates that **25f**,**h**,**i** are less cytotoxic than MTX. At high concentration HT-1080 cell growth is partially rescued in the presence of LV (10 μM) when the cells were

Table 2. Parameters of ^{1}H NMR spectra of the synthesized compounds[a]

Compd	2-CH_2	3-CH	4-CH_2	5-CH_2	ArH	Others
19a	3.05 (1H, d, 10.0), 3.38 (1H, dd, 4.9, 10.0)	4.39 (1H, m)	1.88 and 2.02 (each 1H, m)	3.25 and 3.31 (each 1H, m)	6.49 (1H, d, 8.2), 6.56 (1H, t, 7.2), 7.14 (1H, t, 7.8)	4.94 (1H, *s*, OH)
19b	3.03 (1H, d, 10.0 Hz), 3.37 (1H, dd, 4.9, 10.0)	4.38 (1H, m)	1.89 and 2.02 (each 1H, m)	3.23 and 3.28 (each 1H, m)	6.00 (1H, s), 6.10 (1H, dd, 1.3, 8.0), 6.17 (1H, dd, 1.3, 6.5), 7.03 (1H, t, 8.0)	3.69 (3H, s, CH_3), 5.01 (1H, br s, OH)
19c	3.15 (1H, d, 10.0 Hz), 3.43 (1H, dd, 4.6, 10.8)	4.42 (1H, m)	1.94 and 2.03 (each 1H, m)	3.29 and 3.39 (each 1H, m)	6.58 (2H, d, 8.7), 7.51 (2H, d, 8.7)	5.00 (1H, s, OH)
19d	3.24 (1H, d, 11.0 Hz), 3.37 (1H, m)	4.43 (1H, m)	1.94 and 2.04 (each 1H, m)	3.46 (2H, m)	6.61 (2H, d, 9.0), 8.05 (2H, d, 9.0)	5.10 (1H, br s, OH)
19e	3.17 (1H, d, 10.1), 3.56 (1H, dd, 5.0, 10.1)	4.36 (1H, m)	1.85 and 1.99 (each 1H, m)	3.45 (2H, m)	6.67 (2H, m), 7.02 (2H, m)	4.95 (1H, s, OH)
19f	3.10 (1H, d, 10.0) 3.37 (1H, dd, 4.9, 10)	4.38 (1H, m)	1.88 and 2.03 (each 1H, m)	3.22 and 3.29 (each 1H, m)	6.46 (2H, m), 6.99 (2H, m)	4.93 (1H, s, OH)
19g	3.11 (1H, dd, 2.5, 10.1), 3.65 (1H, dd, 5.1, 10.1)	4.34 (1H, m)	1.83 and 1.98 (each 1H, m)	3.24 and 3.49 (each 1H, m)	6.77 (1H, m), 6.90 (1H, m), 7.16 (1H, m), 7.27 (1H, dd, 1.3, 5.4)	4.96 (1H, s, OH)
19h	3.04 (1H, d, 10.2), 3.37 (1H, dd, 4.9, 10.2)	4.40 (1H, m)	1.89 and 2.03 (each 1H, m)	3.20 and 3.32 (each 1H, m)	6.49 (2H, d, 8.5), 7.16 (2H, d, 8.5)	4.97 (1H, s, OH)
19i	3.05 (1H, dd, 2.4, 10.0), 3.37 (1H, dd, 4.9, 10.2)	4.40 (1H, m)	1.91 and 2.03 (each 1H, m)	3.20 and 3.33 (each 1H, m)	6.45 (2H, d, 8.9), 7.25 (2H,d, 8.9)	4.91 (1H, s, OH)
20a	3.66 (2H, s)		2.73 (2H, t, 7.2)	3.64 (2H, t, 7.3)	6.61 (2H, m), 7.01 (2H, m)	
20b	3.69 (2H, s)		2.71 (2H, t, 7.3)	3.67 (2H, t, 7.3)	6.20 (1H, d, 1.5), 6.28 (1H, dd, 1.5, 8.0), 6.39 (1H, dd, 1.5, 6.5), 7.20 (1H, dd, 6.5, 8.0)	3.80 (3H, s, Me)
20c	3.76 (2H, s)		2.79 (2H, t, 7.5)	3.79 (2H, t, 7.5)	6.62 (2H, d, 8.7), 7.53 (2H, d, 8.7)	
20d	3.82 (2H, s)		2.83 (2H, t, 8.1)	3.84 (2H, t, 8.1)	6.59 (2H, d, 9.2), 8.18 (2H, d, 9.2)	
20e	3.78 (2H, s)		2.65 (2H, t, 7.2)	3.69 (2H, t, 7.2)	6.80 (2H, m), 7.06 (2H, m)	
20f	3.66 (2H, s)		2.73 (2H, t, 7.2)	3.64 (2H, t, 7.2)	6.61 (2H, m), 7.01 (2H, m)	
20g	3.73 (2H, s)		2 .48 (2H, t, 7.4)	3.59 (2H, t, 7.4)	6.64 (1H, m), 6.98 (1H, m), 7.11 (1H, m), 7.48 (1H, m)	
20h	3.69 (2H, s)		2.75 (2H, t, 7.4)	3.66 (2H, t, 7.4)	6.59 (2H, d, 8.7), 7.25 (2H, d, 8.7)	
20i	3.65 (2H, s)		2.73 (2H, t, 7.4)	3.69 (2H, t, 7.4)	6.52 (2H, d, 8.9), 7.36 (2H, d, 8.9)	
21a	4.37 (2H, s)		3.27 (2H, t, 6.8)	3.61 (2H, t, 6.8)	6.69 (2H, m), 6.89 (1H, m. ArH), 7.32 (2H, m)	
21b	4.40 (2H, s)		3.27 (2H, t, 6.8)	3.65 (2H, t, 6.8)	6.22 (1H, s), 6.30 (1H, dd, 8.0), 6.45 (1H, dd, 8.0), 7.22 (1H, t, 8.0)	3.82 (3H, s, Me)
21f	4.37 (2H, s)		3.27 (2H, t, 6.8)	3.61 (2H, t, 6.8)	6.62 (2H, m), 7.02 (2H, m)	
21h	4.38 (2H, s)		3.29 (2H, t, 6.7)	3.63 (2H, t, 6.7)	6.60 (2H, d, 8.7), 7.25 (2H, d, 8.7).	
21i	4.37 (2H, s)		3.28 (2H, t, 6.8)	3.62 (2H, t, 6.8)	6.54 (2H, d, 8.9), 7.38 (2H, d, 8.9)	
22a	4.58 (2H, s)		3.26 (2H, t, 7.1)	3.54 (2H, t, 7.1)	6.72 (2H, m), 6.83 (1H, m), 7.29 (2H, m)	1.38 (3H, t. 7.1, Me), 4.33 (2H, q, 7.1, CH_2

(*continued on next page*)

Table 2 (*continued*)

Compd	2-CH_2	3-CH	4-CH_2	5-CH_2	ArH	Others
22b	4.56 (2H, s)		3.24 (2H, t, 7.0)	3.52 (2H, t, 7.0)	6.24 (1H, s), 6.32 (1H, m), 6.38 (1H, m), 7.18 (1H, m)	1.37 (3H, t, 7.2, Me), 3.80 (3H, s, Me), 4.31 (2H, q, 7.2, CH_2)
22c	4.44 (2H, s)		3.53 (2H, t, 7.0)	3.66 (2H, t, 7.0)	6.62 (2H, d, 8.4), 7.51 (2H, d, 8.4)	1.34 (3H, t, 7.0, Me), 4.29 (2H, q, 7.0, Me)
22f	4.34 (2H, s)		3.47 (2H, t, 6.2)	3.54 (2H, t, 6.2)	6.64 (2H, m), 7.00 (2H, m)	1.37 (3H, t, 7.0,Me), 4.31 (2H, q, 7.0, Me)
22h	4.34 (2H, s)		3.47 (2H, t, 7.2)	3.55 (2H, t, 7.2),	6.59 (2H, d, 8.8), 7.22 (2H, d, 8.8)	1.35 (3H, t, 7.0, Me), 4.30 (2H, q, 7.0, CH_2)
22i	4.39 (2H, s)		3.48 (2H, t, 6.2)	3.53 (2H, t, 6.2)	6.56 (2H, d, 8.9), 7.40 (2H, d, 8.9)	1.35 (3H, t, 7.0, Me), 4.35 (2H, q, 7.0, CH_2)
23a	3.34 (1H, dd, 9.8, 6.0), 3.59 (1H, dd, 7.2, 9.8)	2.97 (1H, m)	2.10 and 2.40 (each 1H, m)	3.36 and 3.51 (each 1H, m)	6.59 (1H, d, 8.2), 6.77, (1H, t, 7.8), 7.26, (1H,t, 7.9)	3.75 (1H, d, 8.1, CH)
23b	3.38 (1H, m), 3.60 (1H, dd, 7.1, 9.8)	2.99 (1H, m)	2.10 and 2.40 (each 1H, m)	3.40 and 3.53 (each 1H, m)	6.13 (1H, s), 6.21 (1H, m), 6.34 (1H, m), 7.16 (1H, m)	3.79 (3H, s, Me), 3.82 (1H, d, 7.5, CH)
23c	3.38 (1H, dd, 6.9, 10.0), 3.71 (1H, dd, 7.5, 10.0)	3.05 (1H, m)	2.16 and 2.48 (each 1H, m)	3.58 and 3.68 (each 1H, m)	6.54 (2H, d, 8.7), 7.48 (2H, d, 8.7)	3.86 (1H, d, 7.2, CH)
23d	3.35–3.68 (2H, m)	2.98 (1H, m)	2.11 and 2.48 (each 1H, m)	3.35–3.68 (2H, m)	6.69 (2H, d, 9.1), 8.03 (2H, d, 9.1)	3.87 (1H, d, 8.4, CH)
23e	3.41–3.60 (2H, m)	2.98 (1H, m)	2.07 and 2.39 (each 1H, m)	3.41–3.60 (2H, m)	6.71 (2H, m), 6.80 (1H, m), 7.20 (2H, m)	3.87 (1H, d, 8.4, CH)
23f	3.32–3.51 (1H, m), 3.57 (1H, dd, 7.2, 9.5)	3.02 (1H, m)	2.12 and 2.43 (each 1H, m)	3.32–3.51 (2H, m)	6.52 (2H, m), 6.97 (2H, m)	3.83 (1H, d, 7.3, CH)
23g	3.34–3.59 (4H, m, 2-CH_2 and 5-CH_2)	3.03 (1H, m)	2.12 and 2.44 (each 1H, m),	—	6.71 (2H, m), 6.80 (2H, m)	3.83 (1H, d, 8.4, CH)
23h	3.32–3.41 (2H, m, 2-CH and 5-CH), 3.60 (1H, dd, 7.7, 9.0, 2-CH)	3.03 (1H, m)	2.13 and 2.44 (each 1H, m)	3.51 (1H, m, 5-CH)	6.50 (2H, d, 8.4), 7.20 (2H, d, 8.4)	3.83 (1H, d, 7.9, CH)
t23i	3.32 (1H, dd, 5.9, 9.8), 3.59 (1H, dd, 7.2, 9.9)	3.02 (1H, m)	2.13 and 2.43 (each 1H, m)	3.38 and 3.50 (each 1H, m)	6.45 (2H, d, 8.8), 7.33 (2H, d, 8.8)	3.84 (1H, d, 8.0, CH)
24a	3.27–3.54 (4H, m, 2-CH_2 and 5-CH_2)	2.92 (1H, m)	2.01 and 2.28 (each 1H, m)		6.53 (2H, m), 6.68 (1H, m), 7.20 (2H, m)	1.31 (3H, t, 7.0, Me), 3.58 (1H, t, 7.6, CH), 4.26 (2H, q, 7.2, CH_2)
24b	3.33–3.56 (4H, m, 2-CH_2 and 5-CH_2)	2.95 (1H, m)	2.00 and 2.28 (each 1H, m)		6.10 (1H, m), 6.18 (1H, m), 6.29 (1H, d, 8.0), 7.14 (1H, t, 8.1)	1.33 (3H, t, 7.2, Me), 3.60 (1H, d, 8.0, CH), 3.79 (3H, s, CH_3), 4.29 (2H, q, 7.2, CH_2)
24c	3.24–3.66 (4H, m, 2-CH_2 and 5-CH_2)	3.01 (1H, m)	2.05 and 2.38 (each 1H, m)		6.52 (2H, d, 8.6), 7.46 (2H, d, 8.6)	1.34 (3H, t, 7.2, Me), 3.65 (1H, d, 7.5, CH), 4.31 (2H, q, 7.2, CH_2)
24f	3.30–3.55 (4H, m, 2-CH_2 and 5-CH_2)		2.04 and 2.30 (each 1H, m)		6.47 (2H, m), 6.94 (2H, m)	1.33 (3H, t, 7.0, Me), 3.63 (1H, d, 7.0, CH), 4.29 (2H, q, 7.0, CH_2)
24h	3.27–3.55 (4H, m, 2-CH_2 and 5-CH_2)	2.97 (1H, m)	2.02 and 2.31 (each 1H, m)		6.44 (2H, d, 8.6), 7.16 (2H, d, 8.6)	1.33 (3H, t, 7.0, Me), 3.61 (1H, d, 7.4, CH), 4.28 (2H, q, 7.0, CH_2)
24i	3.17 (1H, dd, 7.5, 9.6), 3.50 (1H, m)	2.96 (1H, m)	2.06 and 2.35 (each 1H, m)	3.31 (1H, t, 7.8) 3.40 (1H, dt, 5.4, 8.9)	6.42 (2H, d, 8.0), 7.29 (2H, d, 8.0)	1.33 (3H, t, 7.1, Me), 3.59 (1H, d, 7.6, CH), 4.29 (2H, q, 7.1, CH_2)
25a	3.12 (1H, t, 9.5), 3.49 (1H, dd, 6.5, 9.5)	3.65 (1H, m)	2.11 (2H, m)	3.01 and 3.65 (each 1H, m,)	6.67 (3H, m), 7.19 (2H, m)	5.27 (2H, s, NH_2), 5.56 (4H, s, 2×NH_2)

(*continued on next page*)

Table 2 (*continued*)

Compd	2-CH_2	3-CH	4-CH_2	5-CH_2	ArH	Others
25b	3.13 and 3.47 (each 1H, m)	3.48 (1H, m)	2.10 (2H, m)	3.02 and 3.63 (each 1H, m)	6.19 (1H, s), 6.27 (2H, m), 7.07 (1H, m)	3.72 (3H, s, Me), 5.19 (2H, s, NH_2), 5.47 (4H, s, 2×NH_2)
25c	3.37 (1H, m, 2′-CH), 3.54 (2H, m, 2′-CH, 5′-CH)	3.63 (1H, m)	2.13 and 2.35 (each 1H, m)	3.26 (1H, m, 5′-CH)	6.69 (2H, d, 8.6), 7.55 (2H, d, 8.6)	7.21 (2H, br s, NH_2), 8.99 (2H, br s, NH_2), 9.04 (2H, br s, NH_2)
25d	2.82 and 3.34 (each 1H, m)	3.59 (1H, m)	2.04 and 2.17 (each 1H, m)	2.76 and 3.58 (each 1H, m)	6.59 (2H, d, 8.7), 8.05 (2H, d, 8.7)	5.22 (2H, br s, NH_2), 5.51 (4H, br s, 2×NH_2)
25e	3.08 and 3.60 (each 1H, m)	3.45 (1H, m)	1.97 and 2.12 (each 1H, m)	2.99 and 3.67 (each 1H, m)	6.87 (1H, m), 6.95 (1H, m), 7.09 (2H, m)	5.50 (2H, br s, NH_2), 5.86 (4H, s, 2×NH_2)
25f	3.08 (1H, t, 9.6), 3.46 (1H, dd, 6.0, 9.6)	3.64 (1H, m)	2.06 and 2.14 (each 1H, m)	2.99 and 3.67 (each 1H, m)	6.70 (2H, m), 7.03 (2H, m)	5.16 (2H, br s, NH_2), 5.52 (4H, br s, 2×NH_2)
25g	3.09 and 3.49 (each 1H, m)	3.64 (1H, m)	1.95 and 2.16 (each 1H, m)	2.89 and 3.63 (each 1H, m)	7.03 (1H, m, 1H), 7.21 (1H, m), 7.28 (1H, m), 7.40 (1H, m)	5.52 (2H, br s, NH_2), 6.01 (4H, br s, 2×NH_2)
25h	3.14 and 3.44 (each 1H, m)	3.59 (1H, m)	2.08 and 2.19 (each 1H, m)	3.05 and 3.61 (each 1H, m)	6.65 (2H, d, 8.8), 7.21 (2H, d, 8.8)	5.21 (2H, s, NH_2), 5.47 (4H, s, 2×NH_2)
25i	3.16 (1H, t, 9.8), 3.46 (1H, t, 8.8)	3.59 (1H, m)	2.05 and 2.19 (each 1H, m)	3.07 and 3.63 (each 1H, m)	6.62 (2H, d, 8.8), 7.32 (2H, d, 8.8)	5.19 (2H, br s, NH_2), 5.45 (4H, br s, 2×NH_2)
26a	3.14 and 3.53 (each 1H, m)	3.37 (2H, m, 3′-CH and 5′-CH)	1.85 and 2.59 (each 1H, m)	3.18 (1H, m, 5′-CH)	6.50 (2H, d, 8.6), 6.55 (1H, t, 7.3), 7.14 (2H, t, 7.3)	6.03 (2H, br s, NH_2), 6.91 (2H, br s, NH_2)
26b	3.19 (1H, m), 3.51 (1H, t, 8.4)	3.35 (1H, m)	1.81 and 2.58 (each 1H, m)	3.21 and 3.30 (each 1H, m)	6.01 (1H, s), 6.13 (2H, m), 7.02 (1H, m)	3.69 (3H, s, Me), 5.82 (2H, s, NH_2), 5.94 (2H, s, NH_2), 9.76 (1H, br s, OH)
26c	3.17 (1H, m), 3.64 (1H, t, 9.1)	3.44 (1H, m)	1.85 and 2.64 (each 1H, m)	3.26 and 3.47 (each 1H, m)	6.56 (2H, d, 8.7), 7.48 (2H, d, 8.7)	5.86 (2H, br s, NH_2), 5.96 (2H, s, NH_2), 9.79 (1H, br s, OH)
26f	3.18 (1H, m), 3.52 (1H, t, 8.7)	3.35 (2H, m, 3′-CH and 5′-CH)	1.89 and 2.58 (each 1H, m)	3.21 (1H, m, 5′-CH)	6.51 (2H, m), 7.00 (2H, m)	5.85 (2H, s, NH_2), 5.99 (2H, s, NH_2), 9.78 (1H, br s, OH)
26h	3.14 (1H, m), 3.51 (1H, t, 8.6)	3.35 (2H, m, 3′-CH and 5′-CH)	1.88 and 2.49 (each 1H, m)	3.18 (1H, m, 5′-CH)	6.51(2H, d, 8.8), 7.16 (2H, d, 8.8)	5.87 (2H, s, NH_2), 5.98 (2H, s, NH_2)
26i	3.12 (1H, m), 3.52 (1H, t, 9.0)	3.36 (2H, m, 3′-CH and 5′-CH)	1.83 and 2.60 (each 1H, m)		6.48 (2H, d, 8.8), 7.26 (2H, d, 8.8)	5.84 (2H, s, NH_2), 5.96 (2H, br s, NH_2), 9.78 (1H, br s, OH)

[a]Chemical shifts in ppm (δ), followed by integration values, multiplicity and coupling constants in Hz (in parentheses), in some cases, the location of the proton is indicated. When the signals appeared superimposed with other signals a range is indicated, followed by the total number of H and their location (in parentheses).

Table 3. Inhibition of human tumor cell growth by 5-(*N*-phenylpyrrololidin-3-yl)pyrimidine derivatives

Compd	R^1	R^2	Inhibitory concentration (IC_{50}, μM)							LV rescue
			COLO 205	H23	H23/0.3	MOLT-4	HL-60	CCRF-CEM	A549	
25a	H	NH_2	>100	>100	>100	21.5	ND	ND	ND	ND
25b	3-OMe	NH_2	>100	90.0	>100	11.8	ND	ND	ND	ND
25c	4-CN	NH_2	>100	>100	>100	>100	ND	ND	ND	ND
25d	4-NO_2	NH_2	>100	>100	>100	>100	ND	ND	ND	ND
25e	2-F	NH_2	>100	>100	3.47	18.9	ND	ND	ND	ND
25f	4-F	NH_2	>100	>100	>100	3.70	8.59	18.6	7.28	++++
25g	2-Cl	NH_2	79.4	49.5	80.3	12.3	ND	ND	ND	ND
25h	4-Cl	NH_2	>100	44.1	3.01	1.88	4.62	12.3	5.93	++
25i	4-Br	NH_2	23.3	55.5	53.9	2.48	1.58	1.80	1.07	++
26a	H	OH	>100	>100	>100	>100	ND	ND	ND	ND
26b	4-CN	OH	>100	>100	>100	>100	ND	ND	ND	ND
26c	4-NO_2	OH	>100	>100	>100	>100	ND	ND	ND	ND
26f	4-F	OH	>100	>100	>100	>100	ND	ND	ND	ND
26h	4-Cl	OH	>100	>100	>100	72.6	ND	ND	ND	ND
26i	4-Br	OH	>100	>100	>100	>100	ND	ND	ND	ND
MTX			>100	>100	>100	0.0817	0.058	0.022	0.083	+++++

Table 4. Inhibition of in situ folate metabolic pathway (tritium release) by compound **25f**, **25h**, and **25i** in human fibrosarcoma HT-1080 cells

Cell	Tritium release (% of control)							
	MTX		**25f**		**25h**		**25i**	
	0.1 (μM)	1.0	0.1 (μM)	1.0	0.1 (μM)	1.0	0.1 (μM)	1.0
HT-1080	78	56	80	63	65	70	65	44

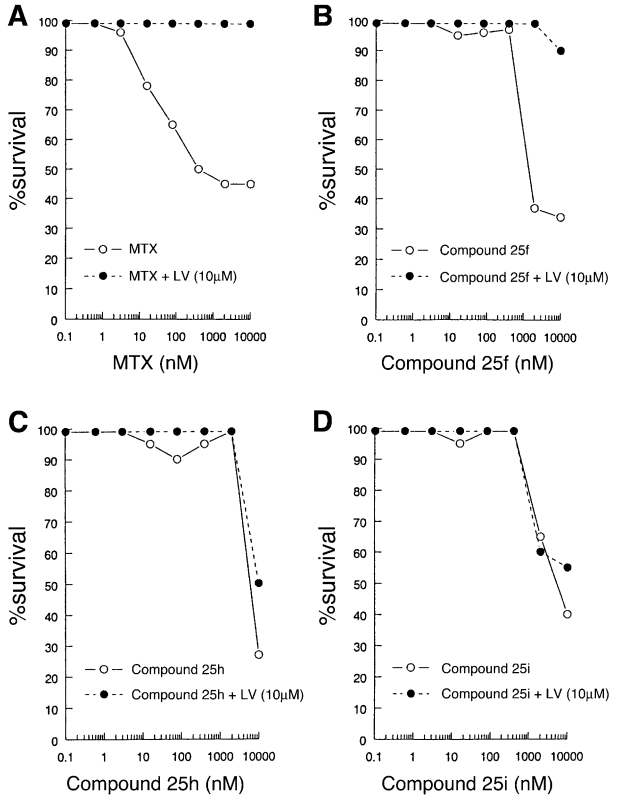


Figure 2. Inhibition effects of MTX, compounds **25f**, **25h**, and **25i** and in the presence of LV (10 μM) against human fibrosarcoma HT-11 cell growth in culture after 24 h exposure.

Table 5. The effects of leucovorin on human fibrosarcoma HT-1080 cell growth inhibition by MTX and compounds **25f**, **25h** and **25i**

Compd	LV	IC_{50} (μM)	% Rescue in survival in the presence of LV (10 μM) at 0.00001–10 μM of antifolate
MTX	–	1.14	
	+	>>>10	3–54
25f	–	2.45	
	+	>>>10	2–56
25h	–	8.80	
	+	>10.0	3–23
25i	–	2.45	
	+	>>>10	1–15

treated with **25h** or **25i** simultaneously. However, the HT-1080 cell growth was rescued when cells were treated with **25f** + LV. The results indirectly prove that **25f**,**h**,**i** inhibit the DHFR activity in HT-1080 cell lines. These studies demonstrate that the target compounds are different from classical folate antagonists and may be involved in the reduced folate transport system.[47,48] Further antitumor properties of these compounds need to be investigated.

Experimental

Column chromatography was performed with silica gel G60 (70–230 mesh, ASTM; Merck) as the stationary phase, while thin-layer chromatography was carried out on silica gel G60 F254 (Merck) using short-wavelength UV light for visualization. Melting points were determined on a Fargo melting point apparatus and were uncorrected. Elemental analyses were performed on a Heraeus CHN-O Rapid instrument and the results (for C, H, and N), indicated in Table 1, were within ±0.4% of the calculated values. The physical properties and the yield of each compound were also listed in Table 1. The ^{1}H NMR spectra of all compounds were recorded on a Brucker AMX-400 spectrometer (400 MHz) with Me_4Si as the internal standard. The COSY experiments were recorded using standard pulse sequences in phase-sensitive mode. Chemical shifts were reported in ppm (δ),

and the signals were described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet) and br (broad). Values reported for coupling constant are first-order. IR spectra were measured on Infrared spectrophotometer, Model 500, Buck Scientific Inc. The IR and ^{1}H NMR spectra of each compound are listed in Tables 1 and 2, respectively. The representative method for the synthesis of each series of compounds are described as follows:

Derivatives of 1-phenyl-3-pyrrolidinol (19a–i). The general procedure used was similar to that reported previously.[37] The procedure for the preparation of 1-phenyl-3-pyrrolidinol (**19a**) is representative. 1,4-Dibromo-2-butanol (**17**, 35.0 g, 0.16 mol) was added dropwise to a blend of aniline (**18a**, 14.5 g, 0.16 mol), K_2CO_3 (65.0 g, 0.48 mol) and triethyl phosphate (150 mL) under nitrogen at room temperature and then a reaction mixture was heated at 150 °C (oil bath temperature) for 6 h. After cooling, the mixture was filtered and the solid cake was washed with ethyl acetate (200 mL). The combined filtrate and washings were evaporated in vacuo and the residue was diluted with water (600 mL) and extracted several times with ethyl acetate. The combined organic extracts were washed with water, dried over Na_2SO_4 and evaporated in vacuo to dryness. The dark residue was chromatographed on a silica gel column (5×30 cm) using hexane/EtOAc (20:1 v/v) as the eluent. The major UV-absorbing fraction was concentrated to afford **19a**, 15.7 g (62%). Compounds **19b–i** were prepared using the same procedure as **19a**.

Derivatives of 1-phenyl-3-pyrrolidinone (20a–i). All compounds in this group were prepared by following the procedure described previously.[37] The procedure for the preparation of 1-phenyl-3-pyrrolidinone (**20a**) is representative. Trifluoroacetic acid (3.7 mL, 48 mmol) was added dropwise to a mixture of **19a** (15.6 g, 95 mmol) in dry benzene (600 mL) containing *N*,*N*-dicyclohexylcarbodimide (DCC, 39.3 g, 190 mmol), anhydrous DMSO (7.0 mL) and anhydrous pyridine (8 mL) with vigorous stirring under nitrogen at 0 C for 30 min. The mixture was stirred at room temperature for 24 h. After evaporation of benzene under reduced pressure a solid was removed by filtration and washed thoroughly with ethyl acetate (300 mL). The combined filtrate and washings were washed with H_2O followed with 5% copper(II)-sulfate aqueous solution to remove pyridine. It was then dried over Na_2SO_4, filtered and evaporated in vacuo to dryness. The residue was crystallized from MeOH/$CHCl_3$ to afford white needle crystals **20a**: 17.1 g (79%). Compounds **20a–i** were prepared using the same procedure as for the synthesis of **20a**.

Derivatives of 3-(dicyanomethylene)-1-phenylpyrrolidine (21a–i) and 3-[cyano(ethoxycarbonyl)methylene]-1-phenylpyrrolidine (22a,b,c,f,h,i). All compounds in these two groups were prepared by following the procedure described previously,[23,37] with some modifications. Derivatives of 3-(dicyanomethylene)-1-phenylpyrrolidine (**21a–i**) were prepared by the reaction of 1-phenylpyrrolidinones with malononitrile in dry ether in the presence of DBU (for compounds **21a**,**b**,**c**,**f**,**h**,**i**) or in dry EtOH in the presence of KF (for compounds **21e**,**g**). Products **21a**,**b**,**f**,**h**,**I**, precipitated during the course of the reaction were isolated in a stable solid form. The crude products **21c**,**d**, which did not precipitate out and contained a small amount of starting material along with by-products, were used directly for the next reaction. 3-[Cyano(ethoxycarbonyl)methylene]-1-phenylpyrrolidine derivatives (**22a**,**b**,**c**,**f**,**h**,**i**) were synthesized by reacting **20a–i** with ethyl cyanoacetate in dry ether in the presence of *N*,*N*-dimethylethanolamine. The procedure for the preparation of 3-(dicyanomethylene)-1-phenylpyrrolidine (**21a**) is representative. Ten drops of DBU were added slowly to a mixture of **20a** (7.08 g, 44.0 mmol) and malononitrile (8.81 g, 88.0 mmol) in dry ether (20 mL) with vigorous stirring at 0 °C. After 10 min, the reaction mixture was stirred at room temperature for an additional 8 h and the resulting solid product **21a** was collected by filtration and recrystallized from MeOH/$CHCl_3$ (3.28 g; 72%) as light green crystals. Following the same procedure as that for the synthesis of **21a**, the compounds **21b–i** and **22a**,**b**,**c**,**f**,**h**,**i** were prepared.

Derivatives of 3-(dicyanomethyl)-1-phenylpyrrolidine (23a–i) and 3-[cyano(ethoxycarbonyl)methyl]-1-phenylpyrrolidine (24a,b,c,g,h,i). All compounds of these two groups were prepared by the reduction either **21a–i** or **22a**,**b**,**c**,**g**,**h**,**i** with sodium cyanoborohydride in acetic acid. Products, **23b**,**e**,**g** and **24a**,**b**,**c**,**f**,**h**,**i**, were isolated as syrup by liquid column chromatography (SiO_2, hexane/EtOAc, 8:1 v/v). Compounds **23a**,**c**,**d**,**f**,**h**,**i** were isolated as crystals by direct crystallization after filtration and removal of solvent from the reaction mixture. The procedure for the preparation of 3-(dicyanomethyl)-1-phenylpyrrolidine (**23a**) is representative. Sodium cyanoborohydride (0.66 g, 10.5 mmol) was added portionwise to a suspension of **21a** (2.20 g, 10.5 mmol) in acetic acid (5.0 mL) at room temperature. After stirring for 30 min, the clear solution was evaporated in vacuo to dryness and co-evaporated several times with toluene. The residue was dissolved in EtOAc (100 mL), washed with water (20 mL×2), dried over Na_2SO_4, and evaporated in vacuo to dryness. The residue was crystallized from EtOH to give **23a**: (1.32 g; 60%). By following the same procedure as that for the synthesis of **23a**, compounds **23b–i** and **24a**,**b**,**c**,**g**,**h**,**i** were prepared. The yields of compounds **23d**,**e**,**g** were calculated based on **20d**,**e**,**g**, respectively.

Derivatives of 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidine (25a–i) and 2,4-diamino-5-(*N*-phenylpyrrolidin-3-yl)-6(5*H*)-oxopyrimidine (26a,b,c,g,h,i). All compounds of these two groups were prepared by following the procedure as described previously.[27,37] The procedure for the preparation of 5-(*N*-phenylpyrrolidin-3-yl)-2,4,6-triaminopyrimidine (**25a**) is representative. A mixture of **23a** (1.20 g, 5.68 mmol), guanidine carbonate (1.10 g, 5.68 mmol) in diglyme (10 mL) was heated at 140 °C (oil bath temperature) with vigorous stirring for 1 h. After cooling, the reaction mixture was triturated with hexane (5×10 mL) to remove diglyme. The gummy residue was crystallized from MeOH to give **25a**, 0.73 g (48%). Using the same procedure as for the synthesis of **25a**, the compounds **25b–i** and **26a**,**b**,**c**,**g**,**h**,**i** were prepared.

Biological assays

Cytotoxicity assays. XTT-tetrazolium assay determined the effects of the compounds on cell growth were determined in all human tumor cells, namely human colon adenocarcinoma (COLO 205), lung carcinoma (H23) and its adriamycin resistant cell line (H23/0.3), T-cell leukemia (MOLT-4), promyelocytic leukemia (HL-60), human T-cell acute lymphocytic leukemia (CCRF-CEM), and human lung carcinoma (A549) in a 72 h incubation, as described by Scundieo et al.[49] After treatment with phenazine methosulfate-XTT solution at 37 °C for 6 h, absorbance at 450 and 630 nm was detected with a microplate reader (EL 340, Bio-Tek Instruments Inc., Winooski, VT, USA). Six to seven concentrations of each compound were used. The IC_{50} and dose–effect relationships of the compounds for antitumor activity were calculated by a median-effect plot,[50,51] using a computer program on an IBM-PC workstation.[51]

Tritium release assay. The whole cell in situ folate metabolic pathway inhibition assay,[42] as modified by Rodenhuis et al.[43] was used to measure the effect of **25f**,**h**,**i** with or without leucovorin (LV) in HT-1080 cells. Cells were exposed to various concentrations of **25f**,**h**,**i** in the presence or absence of 10 μM LV either for 3 h or for 4 h incubation in drug-free medium. 2′-[5-^{3}H]-Deoxyuridine was then added at a final concentration of 2 μCi/mL and its conversion to thymidylate was determined by ^{3}H release at 0, 15, 30, and 45 min. At these times, a 100 μL aliquot of cells was placed in 200 μL of 4% (w/w) trichloroacetic acid with 15% charcoal to stop the reaction. The mixture was centrifuged for 5 min, and the supernatant (100 μL) was added to 5 mL of Ecolume (ICN) scintillation fluid and counted in a scintillation counter (Beckman Model 5801). A blank consisting of medium without cells and drugs was used for background subtraction. The results of the scintillation counts were analyzed by calculating the slope of the ^{3}H release using linear regression. Drug inhibition was expressed as a percentage of the slope of untreated control cells in assay.

Leucovorin rescue studies.[46] Exponentially growing a MTX-sensitive human fibrosarcoma cell line (HT-1080) (5×10^5 cells/mL) were exposed to various concentrations of MTX and **25f**,**h**,**i** for 24 h in the presence or absence of 10 μM LV. After the medium was removed, the cells were washed twice with cold phosphate buffered saline (PBS), and fresh drug-free medium was added. Growth was followed for 94 h and cells were counted using a Model ZB Coulter Counter. The percentage of growth inhibition and IC_{50} values were determined from the growth inhibition data.[52]

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