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Antifolate and antiproliferative activity of 6,8,10-triazaspiro[4.5]deca-6,8-dienes and 1,3,5-triazaspiro[5.5]undeca-1,3-dienes

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ABSTRACT

Two series of triazaspiroalkanedienes, bearing a substituted phenoxy propyloxy side chain, were identified as potent mammalian DHFR inhibitors. One series has a 6,5-spiro bicyclic ring system and the other series has a 6,6-spiro bicyclic system. Both series were synthesized and tested for in vitro mammalian DHFR inhibitory activity and antiproliferative activity against A549 human lung-cancer cells. Compound **3c** showed the highest antiproliferative activity against A549 cells with an IC_{50} value of 27.1 nM. Rescue experiment confirmed its antifolate antiproliferative mechanism. The excellent antifolate and antiproliferative activity of selected analogues presented in this study warrants further investigation as potential leads in the anticancer drug discovery.

1. Introduction

Dihydrofolate reductase (DHFR), a ubiquitous enzyme in all eukaryotic and prokaryotic cells, is of pivotal importance in biochemistry and medicinal chemistry. DHFR catalyzes the biotransformation of dihydrofolate (DHF) to tetrahydrofolate (THF), using NADPH as a coenzyme. THF is required for the de novo synthesis of purines and thymidylate (TMP).[1] Therefore, inhibition of DHFR activity in the absence of salvage could shut off the supply of TMP for DNA biosynthesis, and eventually leads to cell death (thymineless death). This effect hence forms one of the important bases in cancer chemotherapy.[2]

While literally thousands of anticancer DHFR inhibitors were prepared over the years, most of these DHFR inhibitors are compounds possessing a nucleus that is made up from fused heterocyclic rings substituted with amino groups.[3–8] For instance, methotrexate (MTX, **1**) contains a 6,6-fused bicyclic ring system; and a new generation multi-targeted antifolate drug pemetrexed (PTX, Alimta®, **2**) contains a 6,5-fused bicyclic ring system, respectively (Fig. 1). In contrast, the inhibitory action of 6,6- or 6,5-spiro bicyclic ring systems against DHFR has not been extensively investigated. In our previous study, we described the discovery of compounds with 6,6- or 6,5-spiro bicyclic ring systems as potent DHFR inhibitors.[9] Among them, 7,9-diamino-10-(3′-phenoxypropyloxy)-6,8,10-triazaspiro[4.5]deca-6,8-diene hydrochloride (**3a**) and 2,4-diamino-5-(3′-phenoxypropyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (**4a**) were identified as key lead structures with excellent activity in DHFR inhibitory assay (IC_{50} = 2.3 nM and 6.9 nM, respectively) (Fig. 1).[9] Herein, in this paper we report the structure activity relationship (SAR) study of the leads with the objective of developing potent and selective antifolate anticancer agents.

The lead compounds **3a** and **4a** generally consist of three parts: a phenyl ring, a 1,3-dioxyalkane linker and a core moiety bearing a 1,3,5-triazine spiro cycloalkane ring. In the previous work, we have explored the linker length and spiro ring size variations.[9] Thus, in the present report we explored **3a** and **4a** analogues modified at 4 position of phenyl ring using a Craig plot guided optimization.[10] Analogues with a range of electronic properties, shapes and hydrophobicity were selected according to all four Craig plot quadrants. These compounds were synthesized and tested in vitro to demonstrate whether these properties could be a factor in affecting enzyme inhibition and antiproliferative activity against human cancer cells.

2. Results and discussion

2.1. Chemistry

Target compounds **3b–3i** and **4b–4j** (Table 1) were conveniently prepared via a Williamson type etherification sequence as de-

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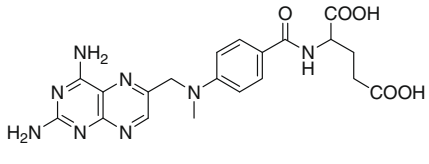
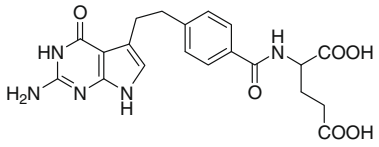
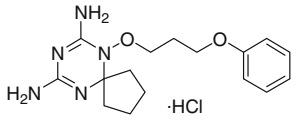


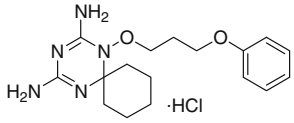
MTX, **1**



Pemetrexed, **2**



3a



4a

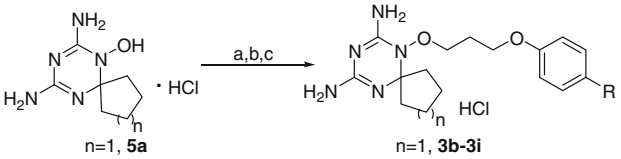
Figure 1. Structures of selected DHFR inhibitors.

Table 1
IC_{50} values of antifolate activity against DHFR and antiproliferative activity against A549

Compd	R	*n*	DHFR, IC_{50}[a] (nM)	A549, IC_{50}[b] (nM)
3a	H	1	2.3[c]	40.2 (2.1)
3b	F	1	10.1	58.8 (1.1)
3c	Cl	1	7.2	27.1 (0.8)
3d	NO_2	1	8.4	65.8 (3.2)
3e	Me	1	9.5	48.1 (2.2)
3f	*t*-Bu	1	17.0	159.9 (2.0)
3g	MeO	1	11.2	59.1 (4.0)
3h	CN	1	7.6	60.7 (3.1)
3i	CH_3CO	1	9.2	59.8 (1.2)
4a	H	2	6.9[c]	69.7 (3.0)
4b	F	2	15.4	59.2 (1.1)
4c	Cl	2	13.8	49.6 (4.1)
4d	NO_2	2	14.7	329.3 (12.5)
4e	Me	2	17.5	83.3 (1.7)
4f	*t*-Bu	2	21.5	495.6 (20.1)
4g	MeO	2	6.1	51.6 (3.2)
4h	CN	2	9.7	116.4 (7.1)
4i	CH_3CO	2	13.3	144.8 (5.6)
4j	SO_2NH_2	2	5.6	166.4 (11.2)
MTX			3.4	37.4 (2.1)

[a] Values are means of three experiments.
[b] Values are means (SEM), $n = 3$.
[c] Ref. 9.

scribed in Scheme 1. The starting materials **5a** and **5b** were prepared according to our previous report.[9] 1-(*para*-Substituted phenyloxy)-3-bromoalkanes were synthesized via an alkylation of commercial available *para*-substituted-phenols with dibromopropane in the presence of potassium carbonate. The target compounds were then obtained from the above two fragments via a one-pot reaction, where **5a** or **5b** were deprotonated using sodium hydroxide; and the produced *N*-hydroxide anion underwent nucleophilic substitution to furnish the ether followed by an acidification to form target compounds in 44.3–71.4% yields.



n=1, **5a**
n=2, **5b**

n=1, **3b-3i**
n=2, **4b-4j**

Scheme 1. Synthesis of 7,9-diamino-10-(3′-aryloxypropyloxy)-6,8,10-triazaspiro[4.5]deca-6,8-diene hydrochlorides (**3b–3i**) and 2,4-diamino-5-(3′-phenoxypropyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochlorides (**4b–4j**). Reagents and conditions: (a) NaOH, methanol; (b) DMF, aryloxy propyl bromide rt; (c) HCl, pH 2.

2.2. Biological activity

2.2.1. DHFR inhibitory activity

The screening of the target compounds was carried out spectroscopically using an enzyme inhibitory assay against bovine liver DHFR, which was a type of commercial available model DHFRs from mammalian sources and showed relatively small differences to human DHFR.[11] The inhibitory activity of the tested compounds compared with that of MTX was expressed in terms of IC_{50} values tabulated in Table 1. Guided by the Craig plot, R = F, Cl, NO_2 ($+\pi$, $+\sigma$), R = OCH_3 ($-\pi$, $-\sigma$), R = CH_3 and *tert*-butyl ($+\pi$, $-\sigma$) and R = CN, SO_2NH_2 and $COCH_3$ ($-\pi$, $+\sigma$) were selected for this study.[10] As shown in Table 1, no increases in inhibitory activity were observed with all 4′-substituted compounds **3b–3i**. Among them, the bulky substituent introduced by *tert*-butyl group at 4′-position of phenyl ring led to a 10-fold loss of activity as compared to that of the non-substituted **3a**. Compounds with other substituents, no matter electron-withdrawing or electron-donating, did not exhibit better inhibitory activity as compared to the lead **3a**. This observation indicated that substitution at 4′-position on the phenyl ring may present negative steric hindrance on the interaction to the active site of the enzyme. Analogues of **4a** with different substitutions on the phenyl ring showed a slightly different activity pattern from the **3a** analogues. The electron-withdrawing and electron-donating substituents with $+\pi$ hydrophobicity exhibited decreased inhibitory activity; and **4f** with a bulky *tert*-butyl group was the least active compound with IC_{50} of 21.5 nM. Compounds with $-\pi$ hydrophobicity substituents showed a varied inhibitory activity. Compounds having an electro-donating substituent MeO or an electron-withdrawing substituent SO_2NH_2 were compatible with high inhibitory activity. Cyano-substituted compound **4h** showed decreased activity with the IC_{50} value up into 10–20 nM range.

2.2.2. Antiproliferative activity

The potent DHFR inhibitory activity of both series suggests that they may exhibit good antiproliferative activity against human cancer cells. To this end, the antiproliferative activity against A549, an estrogen receptor negative cell line akined to non-small cell lung carcinoma, was investigated in vitro using a semiautomated fluorometric microculture cytotoxicity assay (FMCA).[12,13] The IC_{50} values of **3a–3i** and **4a–4j** compared with that of MTX are being listed in Table 1. Both series showed potent activity against A459 cells with IC_{50} values ranged from 27.1 nM to 495.6 nM. 4′-Chloro substituted **3c** showed the best antiproliferative activity, and the activity was even better than the positive control, MTX. Compound **3f** with a bulky *tert*-butyl group led to a great decrease in the antiproliferative activity. Antiproliferative activity improvement in **4a** series occurred from the first quadrant of Craig's plot where the lipophilic and electron-withdrawing groups were present, such as Cl and F. One exception as shown in **4d**

exhibited decreased activity, and it may due to electronic property of the NO_2 group, which is highly electron-withdrawing and relatively bulky in size. Electron-donating methoxy group was observed to improve the inhibitory activity, while bulky electron-donating and lipophilic *t*-butyl group reduced the antiproliferative activity, suggesting an unfavorable effect of the bulky substitution. All the substitution in the fourth quadrant of Craig's plot led to a decrease in antiproliferative effects.

3. Salvage rescue effects

The antifolate mechanism of action with respect to the affected target enzymes can be identified by employing the salvage route for nucleotide synthesis in whole cells.[14] It was known as a rescue experiment in which the antifolate effect could be overridden by the introduction of the relevant metabolites. The nature of these relevant metabolites provides clues about the metabolic pathway affected by test compounds. Several of these affected enzymes are involved in nucleotide biosynthesis metabolic pathway, namely, DHFR, thymidylate synthase (TS), glycinamide ribonucleotide formyltransferase (GARFT) and aminoimidazole carboxamide ribonucleotide formyltransferase (AICARFT).[15] In cancer cells, for example, the cytotoxic effect due to the inhibition of TS can be alleviated by the supply of thymidine (TdR), which is metabolically converted to TdR monophosphate.[16] Likewise, the cytotoxic effect due to the inhibition of GARFT or AICARFT can be alleviated by the introduction of hypoxanthine (Hx), which is metabolized to inosine monophosphate, an end product of purine biosynthetic pathway.[17] DHFR inhibitors, which affect both TMP and purine nucleotide synthesis, required the combination of TdR and Hx for the revival of normal metabolism. In this study, rescue experiment of **3c**, the best inhibitor against A549 cells in both series, was carried out to investigate the end product protection profile and to gain further insight into the mechanism of action. In comparison, MTX (a primarily DHFR inhibitor),[18] and PTX (a multi-targeted antifolate against TS, DHFR, GARFT and AICARFT)[15] were involved in the investigation. During the rescue experiment, purine and TMP were depleted in the cancer cells in the presence of a DHFR inhibitor. If the main mechanism was due to the DHFR inhibition, cells supplied with exogenous Hx and TdR would be revived. If the mechanism other than the DHFR inhibition was involved, no complete reversal of growth would be observed. We hypothesized that **3c** may have the same mechanism of action to MTX, while it may exhibit a different mechanism from PTX.

To compare the effects of the end product rescue and their inhibition, the cells were exposed to equitoxic levels, their ten times respective IC_{50} values. The concentrations used in this study were therefore selected as: 270 nM for **3c**, 370 nM for MTX, and 6.4 μM for PTX. The effect on the A549 cells growth inhibition in the present of 20 μM TdR and/or 100 μM Hx together with **3c** and controls for an exposure of 72 h was assessed using FMCA. As indicated in Table 2 and Figure 2, the relative growth control of **3c**, MTX and PTX at $10 \times IC_{50}$ were ranged from 11.7% to 15.2% without addition of rescue agents. The inhibition of **3c** on the growth of A549 cells

Table 2
Protection of A549 from antifolates growth inhibitory effects by 100 μM Hx, 20 μM TdR, or the combination of 100 μM Hx + 20 μM TdR

Compd	Relative growth[a] (% of the control)			
	w/o Rescue agent	Hx (100 μM)	TdR (20 μM)	Hx (100 μM) + TdR(20 μM)
3c	15.2 ± 1.1	14.3 ± 1.2	68.0 ± 0.8	90.0 ± 4.3
MTX	14.3 ± 3.0	16.0 ± 0.8	77.3 ± 11.2	96.6 ± 0.8
Pemetrexed	11.7 ± 0.5	12.6 ± 0.0	84.5 ± 2.3	99.3 ± 1.5

[a] Values are means ± SEM of three experiments.

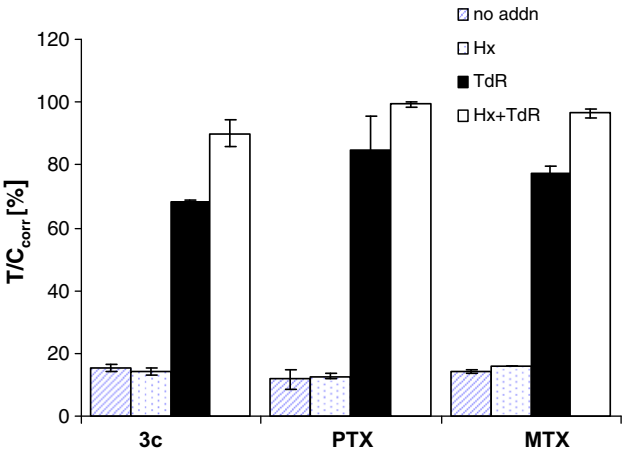


Figure 2. TdR and Hx rescue from antifolates growth inhibition in A549 Cells.

was 90.0% reversed by a combination of TdR and of Hx. Only partial protection was afforded with only TdR added, while addition of Hx alone was devoid of protection for **3c**. It would appear that in the absence of Hx the inhibition of purine synthesis is indirect and incomplete. These results were similar to those obtained for MTX and PTX, thus implicating DHFR as their primary intracellular target. It also suggested the action of DHFR inhibitors, including **3c**, MTX and PTX, has attributed mainly to the induction of a thymineless state in A549 cells. The protective degree for **3c** (90.0%) was slightly lower than that of MTX (96.6%) and PTX (99.3%), suggested that the effect of **3c** on cell growth might not be exclusively due to the purine and TMP depletion; and other mechanisms independent of antifolate pathways might involve in the cytotoxic effects. The concentrations of TdR and Hx in the current experiment were higher than what was normally found in normal human plasma; however, significant variations were found in different tissues and the concentrations in tumors may be greater due to the release of nucleosides from dead and dying cells. Moreover, tumor cells in situ would receive continuous supply of circulating salvageable nucleotides and bases rather than the finite supply, which was consumed during growth inhibition studies in vitro. Thus, nucleoside and base salvage may limit the efficacy of these antifolates in cancer patients. Compound **3c** seems to have another mechanism to partially block the rescue by TdR and Hx. The property may make **3c** potentially more efficacious compared to MTX and PTX.

4. Conclusion

In conclusion, a number of potent antifolates have been synthesized. All compounds from both series exhibited antiproliferative effects against A549 lung-cancer cells. Compound **3c** showed the highest antiproliferative activity against A549 cells with an IC_{50} value of 27.1 nM. Rescue experiment on **3c** demonstrated antifolate profile of antiproliferative effect on A549 cancer cells. Further results from our continuous efforts towards the identification of antifolates with an improved antiproliferative activity will be reported in due course.

5. Experimental section

5.1. Reagents and general method

All reactions were performed in oven-dried glassware with magnetic stirring. All reagents were purchased from Sigma–Aldrich

and used as received unless otherwise noted. TLC analysis was performed using Merck precoated Silica Gel 60 F_{254} plates. Reactions were monitored by TLC on silica gel, with detection by UV light (254 nm). Flash column chromatography was performed with 230–400 mesh silica gel. Melting points were determined on a Gallenkamp Melting Point Apparatus and were uncorrected. Infrared spectra (using potassium bromide discs) were recorded with a Jasco FT/IR-430 Fourier Transform Infrared Spectrometer and reported in reciprocal centimeters (cm^{-1}). The ^1H NMR spectra were recorded on a Bruker ACF 300 MHz NMR Spectrometer. The chemical shift (δ) values are expressed in parts per million (ppm) relative to tetramethylsilane as an internal standard: s = singlet, d = doublet, dd = double doublet, m = multiplet. Maximum UV absorption wavelengths of the compounds were determined on the UV-160A UV–vis recording spectrophotometer. Elemental analyses were performed using the Perkin–Elmer 2400 Elemental Analyzer Series II and all the values are within ±0.4% of calculated values.

5.2. General procedure for the synthesis of 3b–3i and 4b–4j series

1-Hydroxy-1,2-dihydro-1,3,5-triazine (1 g) was dissolved in methanol (20 mL). One molar equivalent of NaOH was added into the solution and the mixture was refluxed for 30 min. After being cooled down to room temperature, methanol was evaporated using vacuum. The dry white residue was then dissolved in DMF (5 mL), and 1.2 mol equiv of 3-aryloxypropyl bromide was added to this solution. The mixture was stirred at room temperature and TLC was used to monitor the reaction. After the reaction was completed, the solution was adjusted to pH 2 using concentrated HCl, followed by evaporating of DMF at room temperature. The residue was filtered and washed using water, followed by recrystallization in 90% EtOH. In this manner, following compounds were prepared.

5.2.1. 7,9-Diamino-10-(3′-(4″-fluoro-phenoxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3b)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-bromo-propyloxy)-4-fluoro-benzene (1.3 g, 5.5 mM), **3b** was obtained as a white solid (1.1 g) in 63.6% yield. Mp: 182–183 °C; ^1H NMR (DMSO-d_6): δ 1.51–1.73 (br m, 6H), 1.97 (br s, 2H), 2.15 (m, 2H, J = 5.6 Hz), 4.02–4.10 (m, 4H), 6.83–6.99 (m, 2H), 6.09–7.18 (m, 2H), 7.81 (br s, 1H, ex), 8.14 (s, 1H, ex), 8.66 (s, 1H, ex), 9.47 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.98, 157.61, 156.00, 155.71, 117.04, 116.73, 116.71, 116.59, 82.24, 75.20, 65.36, 34.34, 28.06, 23.20; ESI-MS: m/z = 336.2 $(M+1)^+$. Anal. Calcd for $C_{16}H_{22}FN_5O_2{\cdot}HCl$: C, 49.29; H, 6.46; N, 17.96. Found: C, 48.93; H, 5.99; N, 18.19.

5.2.2. 7,9-Diamino-10-(3′-(4″-chloro-phenoxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3c)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-bromo-propyloxy)-4-chloro-benzene (1.4 g, 5.5 mM), **3c** was obtained as a white solid (1.3 g) in 67.1% yield. Mp: 199–200 °C; ^1H NMR (DMSO-d_6): δ 1.53–1.74 (br m, 6H), 1.97 (br s, 2H), 2.15 (m, 2H, J = 5.7 Hz), 4.06 (m, 4H), 6.96–6.99 (d, 2H, J = 9.0 Hz), 7.32–7.35 (m, 2H, J = 9.0 Hz), 7.79 (br s, 1H, ex), 8.13 (s, 1H, ex), 8.66 (s, 1H, ex), 9.36 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.98, 158.22, 157.53, 130.30, 125.38, 117.18, 82.26, 75.16, 65.21, 34.35, 27.98, 23.26; ESI-MS: m/z = 352.2 $(M+1)^+$. Anal. Calcd for $C_{16}H_{22}ClN_5O_2{\cdot}1.7HCl$: C, 46.44; H, 5.77; N, 16.92. Found: C, 46.37; H, 5.57; N, 16.90.

5.2.3. 7,9-Diamino-10-(3′-(4″-nitro-phenoxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3d)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-Bromo-propyloxy)-4-nitro-benzene (1.4 g, 5.5 mM), **3d** was obtained as a white solid (1.2 g) in 62.6% yield. Mp: 210–211 °C; ^1H NMR (DMSO-d_6): δ 1.54–1.75 (br m, 6H), 1.97 (br s, 2H), 2.21 (m, 2H, J = 5.7 Hz), 4.10 (t, 2H, J = 5.7 Hz), 4.23 (t, 2H, J = 5.7 Hz), 7.00 (br s, 1H, ex) 7.16–7.19 (d, 2H, J = 9.0 Hz), 7.81 (br s, 1H, ex), 8.15 (s, 1H, ex), 8.20–8.25 (d, 2H, J = 9.0 Hz), 8.67 (s, 1H, ex), 9.22 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 164.71, 163.00, 157.52, 141.94, 126.96, 116.04, 82.28, 75.07, 66.13, 34.36, 27.85, 23.29; ESI-MS: m/z = 363.2 $(M+1)^+$. Anal. Calcd for $C_{16}H_{22}N_6O_2{\cdot}1.8HCl$: C, 44.90; H, 5.60; N, 19.63. Found: C, 44.82; H, 5.33; N, 19.39.

5.2.4. 7,9-Diamino-10-(3′-(4″-tolyloxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3e)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-bromo-propyloxy)-4-methyl-benzene (1.3 g, 5.5 mM), **3e** was obtained as a white solid (1.0 g) in 61.3% yield. Mp: 192–193 °C; ^1H NMR (DMSO-d_6): δ 1.54–1.73 (br m, 6H), 2.11 (br s, 2H), 2.13 (t, 2H, J = 6.0), 2.23 (s, 3H), 4.00–4.09 (m, 4H), 6.83 (d, 2H, J = 8.7 Hz), 7.09 (d, 2H, J = 8.7 Hz), 7.13 (br s, 1H, ex), 7.79 (br s, 1H, ex), 8.12 (s, 1H, ex), 8.65 (s, 1H, ex), 9.47 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.98, 157.63, 157.23, 130.87, 130.32, 115.23, 82.24, 75.29, 64.73, 34.36, 28.11, 23.20, 21.12; ESI-MS: m/z = 332.2 $(M+1)^+$. Anal. Calcd for $C_{16}H_{22}N_6O_2{\cdot}HCl$: C, 52.91; H, 7.31; N, 18.15. Found: C, 52.86; H, 7.42; N, 18.53.

5.2.5. 7,9-Diamino-10-(3′-(4″-*tert*-butyl-phenoxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3f)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-bromo-propyloxy)-4-*tert*-butyl-benzene (1.5 g, 5.5 mM), **3f** was obtained as a white solid (1.2 g) in 62.7% yield. Mp: 197–198 °C; ^1H NMR (DMSO-d_6): δ 1.25 (s, 9H), 1.51–1.72 (br m, 6H), 1.96 (br s, 2H), 2.14 (m, 2H), 4.01–4.09 (m, 4H), 6.85 (d, 2H, J = 8.6 Hz), 7.13 (br s, 1H, ex), 7.29 (d, 2H, J = 8.6 Hz), 7.79 (br s, 1H, ex), 8.12 (s, 1H, ex), 8.65 (s, 1H, ex), 9.53 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.99, 157.65, 157.11, 143.87, 127.10, 114.90, 82.22, 75.33, 64.77, 34.79, 34.31, 32.38, 28.12, 23.09; ESI-MS: m/z = 374.3 $(M+1)^+$. Anal. Calcd for $C_{20}H_{31}N_5O_2{\cdot}HCl{\cdot}0.8H_2O$: C, 56.61; H, 7.98; N, 16.50. Found: C, 56.62; H, 7.85; N, 16.40.

5.2.6. 7,9-Diamino-10-(3′-(4″-methoxy-phenoxy)-propyloxy)-6,8,10-triaza-spiro[4.5]deca-6,8-diene (3g)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-(3-bromo-propyloxy)-4-methoxy-benzene (1.3 g, 5.5 mM), **3g** was obtained as a white solid (1.1 g) in 58.0% yield. Mp: 187–188 °C; ^1H NMR (DMSO-d_6): δ 1.54–1.73 (br m, 6H), 1.98 (br s, 2H), 2.12 (m, 2H, J = 5.6 Hz), 3.69 (s, 3H), 3.99 (t, 2H, J = 6.0 Hz), 4.07 (t, 2H, J = 6.0 Hz), 6.87 (s, 4H), 7.10 (br s, 1H, ex), 7.64 (br s, 1H, ex), 8.12 (s, 1H, ex), 8.65 (s, 1H, ex), 9.44 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.98, 157.62, 154.46, 153.33, 116.29, 115.65, 82.24, 75.31, 65.21, 56.40, 34.36, 28.16, 23.21; ESI-MS: m/z = 348.2 $(M+1)^+$. Anal. Calcd for $C_{17}H_{25}N_5O_3{\cdot}HCl{\cdot}0.8H_2O$: C, 51.27; H, 6.98; N, 17.58. Found: C, 51.22; H, 6.61; N, 17.61.

5.2.7. 4″-(3′-(7,9-Diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-yloxy)-propyloxy)-benzonitrile (3h)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 4-(3-bromo-propyloxy)-benzonitrile (1.3 g, 5.5 mM), **3h** was obtained as a white solid (1.2 g) in 66.6% yield. Mp: 202–203 °C; ^1H NMR (DMSO-d_6): δ 1.49–1.73

(br m, 6H), 1.95 (br s, 2H), 2.18 (m, 2H, J = 5.6 Hz), 2.52 (s, 3H), 4.05 (t, 2H, J = 6.0 Hz), 4.09 (t, 2H, J = 6.0 Hz), 7.13 (q, 2H, J = 7.1 Hz, J = 2.3 Hz), 7.79 (d, 2H, J = 7.1 Hz, J = 2.3 Hz), 8.13 (s, 1H, ex), 8.65 (s, 1H, ex), 9.53 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.96, 162.86, 157.65, 135.28, 120.15, 116.55, 103.98, 82.22, 75.06, 65.55, 34.31, 27.84, 23.16; ESI-MS: m/z = 343.2 $(M+1)^+$. Anal. Calcd for $C_{17}H_{26}N_6O_2{\cdot}HCl{\cdot}H_2O$: C, 51.45; H, 6.35; N, 21.18. Found: C, 51.42; H, 6.02; N, 21.25.

5.2.8. 1-(4″-(3′-(7,9-Diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-yloxy)-propyloxy)-phenyl)-ethanone (3i)

From 7,9-diamino-6,8,10-triaza-spiro[4.5]deca-7,9-dien-6-ol hydrochloride (1 g, 4.6 mM) and 1-[4-(3-bromo-propyloxy)-phenyl]-ethanone (1.4 g, 5.5 mM), **3i** was obtained as a white solid (1.3 g) in 66.9% yield. Mp: 194–195 °C; ^{1}H NMR (DMSO-d_6): δ 1.54–1.73 (br m, 6H), 1.97 (br s, 2H), 2.19 (m, 2H, J = 5.6 Hz), 2.52 (s, 3H), 4.09 (t, 2H, J = 6.0 Hz), 4.16 (t, 2H, J = 6.0 Hz), 7.05 (d, 2H, J = 5.8 Hz), 7.93 (d, 2H, J = 5.8 Hz), 8.14 (s, 1H, ex), 8.65 (s, 1H, ex), 9.36 (s, 1H, ex). ^{13}C NMR (DMSO-d_6): δ 197.36, 163.24, 162.98, 157.61, 131.55, 131.03, 115.26, 82.25, 75.13, 65.31, 34.35, 27.95, 27.47, 23.22; ESI-MS: m/z = 360.2 $(M+1)^+$. Anal. Calcd for $C_{18}H_{25}N_5O_3{\cdot}HCl{\cdot}H_2O$: C, 52.23; H, 6.82; N, 16.92. Found: C, 51.87; H, 6.47; N, 16.86.

5.2.9. 2,4-Diamino-5-(3′-(4″-fluorophenoxy)-propyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (4b)

From 5-hydroxy-1,3,5-triazaspiro[5.5]undeca-1,3-diene-2,4-diamine hydrochloride (1 g, 4.3 mM) and 3′-(*p*-fluorophenoxy) propyl bromide (1.2 g, 5.1 mM), **4b** was obtained as a white solid (1.0 g) in 62.1% yield. Mp: 211–212 °C; ^{1}H NMR (DMSO-d_6): δ 0.77 (br s, 1H), 1.48–1.69 (br d, 9H), 2.14 (br s, 2H), 4.04–4.07 (m, 4H), 6.93–6.99 (m, 2H), 7.09–7.16 (d, 2H), 7.40 (br s, 1H, ex), 7.81 (br s, 1H, ex), 8.08(s, 1H, ex), 8.60 (s, 1H, ex), 9.28 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.31, 157.16, 155.54, 117.04, 116.73, 116.60, 116.50, 75.15, 75.03, 65.10, 27.83, 24.56, 21.72; ESI-MS: m/z = 350.3 $(M+1)^+$. Anal. Calcd for $C_{17}H_{24}FN_5O_2{\cdot}HCl$: C, 52.92; H, 6.53; N, 18.15. Found: C, 52.75; H, 6.56; N, 18.06.

5.2.10. 2,4-Diamino-5-(3′-(4″-chlorophenoxy)-propyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (4c)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 3′-(*p*-chlorophenoxy) propyl bromide (1.2 g, 5.1 mM), **4c** was obtained as a white solid (1.1 g) in 64.8% yield. Mp: 219–220 °C; ^{1}H NMR (DMSO-d_6): δ 0.79 (br s, 1H), 1.48–1.68 (br d, 9H), 2.16 (br s, 2H), 4.08 (m, 4H), 6.99 (d, 2H, J = 8.6 Hz), 7.34 (d, 2H, J = 8.7 Hz), 7.37 (br s, 1H, ex), 7.81 (br s, 1H, ex), 8.08(s, 1H, ex), 8.60 (s, 1H, ex), 9.28 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.28, 158.12, 157.21, 130.31, 125.43, 117.10, 75.15, 75.01, 64.99, 27.79, 24.62, 21.74; ESI-MS: m/z = 366.1 $(M+1)^+$. Anal. Calcd for $C_{17}H_{24}ClN_5O_2{\cdot}HCl$: C, 50.75; H, 6.26; N, 17.41. Found: C, 50.49; H, 6.20; N, 17.57.

5.2.11. 2,4-Diamino-5-(3′-(4″-nitrophenoxy)-propyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (4d)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 1-(3-bromo-propyloxy)-4-nitrobenzene (1.3 g, 5.1 mM), **4d** was obtained as a yellowish solid (1.1 g) in 60.5% yield. Mp: 212–213 °C; ^{1}H NMR (DMSO-d_6): δ 0.79 (br s, 1H), 1.45–1.70 (br d, 9H), 2.22 (br s, 2H), 4.06 (br s, 2H), 4.25 (m, 2H, J = 6.0 Hz), 7.17 (br s, 1H, ex), 7.19 (d, 2H, J = 9.4 Hz), 7.83 (br s, 1H, ex), 8.11 (s, 1H, ex), 8.24 (d, 2H, J = 9.0 Hz), 8.62 (s, 1H, ex), 8.99 (s, 1H, ex). ^{13}C NMR (DMSO-d_6): δ 164.75, 162.15, 157.47, 141.94, 126.95, 116.08, 75.20, 74.96, 66.05, 27.79, 24.84, 21.81; ESI-MS: m/z = 377.2 $(M+1)^+$. Anal. Calcd for $C_{17}H_{24}N_6O_4{\cdot}1.6HCl$: C, 46.97; H, 5.93; N, 19.33. Found: C, 46.99; H, 5.78; N, 19.12.

5.2.12. 2,4-Diamino-5-(3′-(4″-tolyloxy)-propyloxy)-1,3,5-triaza-spiro[5.5]undeca-1,3-diene hydrochloride (4e)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 1-(3-bromo-propyloxy)-4-methyl-benzene (1.2 g, 5.1 mM), **4e** was obtained as a white solid (1.0 g) in 61.4% yield. Mp: 214–215 °C; ^{1}H NMR (DMSO-d_6): δ 0.80 (br s, 1H), 1.44–1.69 (br t, 9H), 2.14 (br s, 2H), 2.23 (s, 3H), 4.04 (m, 4H), 6.84 (d, 2H, J = 8.7 Hz), 7.09 (d, 2H, J = 8.3 Hz), 7.33 (br s, 1H, ex), 7.81 (br s, 1H, ex), 8.06 (s, 1H, ex), 8.59 (s, 1H, ex), 9.21 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.16, 157.54, 157.25, 130.86, 130.32, 115.21, 75.23, 64.63, 28.06, 24.84, 21.77, 21.11; ESI-MS: m/z = 346.3 $(M+1)^+$. Anal. Calcd for $C_{18}H_{27}N_5O_2{\cdot}HCl$: C, 56.61; H, 7.39; N, 18.34. Found: C, 56.47; H, 7.45; N, 18.60.

5.2.13. 2,4-Diamino-5-(3′-(4″-*tert*-butylphenoxy)-propyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (4f)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 1-(3-bromo-propyloxy)-4-*tert*-butyl-benzene (1.4 g, 5.1 mM), **4f** was obtained as a white solid (0.94 g) in 47.4% yield. Mp: 222–223 °C; ^{1}H NMR (DMSO-d_6): δ 0.74 (br s, 1H), 1.35 (s, 9H), 1.39–1.68 (br t, 9H), 2.15 (br s, 2H), 4.05 (t, 4H, J = 5.3 Hz, J = 5.7 Hz), 6.87 (d, 2H, J = 8.6 Hz), 7.14 (br s, 1H, ex), 7.29 (d, 2H, J = 8.7 Hz), 7.84 (br s, 1H, ex), 8.08(s, 1H, ex), 8.60 (s, 1H, ex), 8.88 (s, 1H, ex).; ^{13}C NMR (DMSO-d_6): δ 162.19, 157.37, 157.17, 143.86, 127.10, 114.85, 75.18, 75.06, 64.49, 34.78, 32.37, 28.06, 24.72, 21.78; ESI-MS: m/z = 388.3 $(M+1)^+$. Anal. Calcd for $C_{21}H_{33}N_5O_2{\cdot}2HCl$: C, 54.78; H, 7.66; N, 15.21. Found: C, 54.82; H, 7.46; N, 15.23.

5.2.14. 2,4-Diamino-5-(3′-(4″-methoxyphenoxy)-propyloxy)-1,3,5-triazaspiro[5.5]undeca-1,3-diene hydrochloride (4g)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 3′-(*p*-methoxyphenoxy) propyl bromide (1.3 g, 5.1 mM), **4g** was obtained as a white solid (0.93 g) in 51.3% yield. Mp: 202–203 °C; ^{1}H NMR (DMSO-d_6): δ 0.80 (br s, 1H), 1.45–1.70 (br t, 9H), 2.13 (br s, 2H), 3.69 (s, 3H), 3.99–4.07 (m, 4H), 6.84–6.91 (m, 4H), 7.12 (br s, 1H, ex), 7.84 (br s, 1H, ex), 8.08(s, 1H, ex), 8.61 (s, 1H, ex), 8.95 (s, 1H, ex); ^{13}C NMR (DMSO-d_6): δ 162.37, 157.45, 154.49, 153.38, 116.25, 115.68, 75.20, 75.10, 66.95, 56.43, 28.11, 24.82, 21.83; ESI-MS: m/z = 362.3 $(M+1)^+$. Anal. Calcd for $C_{18}H_{27}N_5O_2{\cdot}HCl{\cdot}1.2H_2O$: C, 51.53; H, 7.30; N, 16.69. Found: C, 51.54; H, 6.97; N, 16.47.

5.2.15. 4″-(3′-(2,4-Diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-yloxy)-propyloxy)-benzonitrile hydrochloride (4h)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and *p*-(3-bromo-propyloxy)-benzonitrile (1.2 g, 5.1 mM), **4h** was obtained as a white solid (0.91 g) in 53.4% yield. Mp: 221–222 °C; ^{1}H NMR (DMSO-d_6): δ 0.76 (br s, 1H), 1.48–1.69 (br d, 9H), 2.20 (br s, 2H), 4.05 (br s, 2H), 4.19 (t, 2H, J = 5.6 Hz, J = 6.0 Hz), 7.15 (d, 2H, J = 9.0 Hz), 7.44 (br s, 1H, ex), 7.79 (d, 2H, J = 9.1 Hz), 7.79 (br s, 1H, ex), 8.09(s, 1H, ex), 8.60 (s, 1H, ex), 9.32 (s, 1H, ex). ^{13}C NMR (DMSO-d_6): δ 162.89, 162.09, 157.61, 135.26, 120.15, 116.57, 103.95, 75.26, 74.91, 65.45, 27.80, 24.90, 21.75; ESI-MS: m/z = 357.2 $(M+1)^+$. Anal. Calcd for $C_{18}H_{24}N_6O_2{\cdot}HCl{\cdot}0.2H_2O$: C, 54.53; H, 6.46; N, 21.20. Found: C, 54.60; H, 6.42; N, 21.02.

5.2.16. 1-(4″-(3′-(2,4-Diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-yloxy)-propyloxy)-phenyl)-ethanone hydrochloride (4i)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 1-[4-(3-bromo-propyloxy)-phenyl]-ethanone (1.3 g, 5.1 mM), **4i** was obtained as a white solid (1.2 g) in 67.1% yield. Mp: 206–207 °C; ^{1}H NMR (DMSO-d_6): δ 0.77 (br s, 1H), 1.47–1.69 (br t, 9H), 2.19 (br s, 2H), 2.54 (s, 3H),

4.05 (t, 2H, J = 5.6 Hz, J = 4.5 Hz), 4.19 (t, 2H, J = 5.6 Hz, J = 6.0 Hz), 7.07 (d, 2H, J = 8.6 Hz), 7.33 (br s, 1H, ex), 7.80 (br s, 1H, ex), 7.94 (d, 2H, J = 8.7 Hz), 8.09(s, 1H, ex), 8.60 (s, 1H, ex), 8.85 (s, 1H, ex), 9.21 (s, 1H, ex); ESI-MS: m/z = 374.2 $(M+1)^+$; ^{13}C NMR (DMSO-d_6): δ 197.37, 163.28, 162.15, 157.58, 139.68, 131.55, 131.04, 115.36, 75.24, 75.03, 65.24, 34.63, 27.47, 24.88, 21.78. Anal. Calcd for $C_{19}H_{27}N_5O_3{\cdot}HCl{\cdot}H_2O$: C, 53.78; H, 7.03; N, 16.50. Found: C, 53.81; H, 6.88; N, 16.62.

5.2.17. 4″-(3′-(2,4-Diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-yloxy)-propyloxy)-benzenesulfonamide hydrochloride (4j)

From 2,4-diamino-1,3,5-triaza-spiro[5.5]undeca-2,4-dien-1-ol hydrochloride (1 g, 4.3 mM) and 4-(3-bromo-propyloxy)-benzenesulfonamide (1.5 g, 5.1 mM), **4j** was obtained as a white solid (0.96 g) in 44.3% yield. Mp: 198–199 °C; ^1H NMR (DMSO-d_6): δ 0.79–0.83 (br d, 1H, J = 12.1 Hz), 1.40–1.72 (br t, 9H), 2.20 (br s, 2H), 4.07 (br s, 2H), 4.16–4.20 (t, 2H, J = 6.0 Hz), 7.00 (br s, 1H, ex), 7.11–7.14 (d, 2H, J = 9.0 Hz), 7.23 (s, 2H), 7.76–7.79 (d, 2H, J = 8.6 Hz), 7.80 (br s, 1H, ex), 8.12(s, 1H, ex), 8.70 (br s, 2H, ex); ^{13}C NMR (DMSO-d_6): δ 162.20, 161.78, 157.32, 137.34, 128.74, 115.47, 75.18, 75.07, 65.32, 27.85, 24.77, 21.83; ESI-MS: m/z = 410.6 $(M+1)^+$. Anal. Calcd for $C_{17}H_{26}N_6O_4S{\cdot}2HCl{\cdot}H_2O$: C, 40.72; H, 6.03; N, 16.76. Found: C, 40.64; H, 5.78; N, 16.66.

5.3. DHFR enzyme assay

The assay was performed at 37 °C in a Hewlett–Packard 8453 Diode Array UV–vis Spectrophotometer with HP 89090A Peltier Temperature Controller at a detection wavelength of 340 nm and full scale deflection of 4.0 absorbance units. The assay was carried out over 6 min with absorbance readings taken every 5 s. A graph of absorbance readings versus time was plotted and the gradient over the linear range from 60 to 300 s was taken to be the rate of the reaction. The assay was conducted by mixing the appropriate volumes of phosphate buffer, NADPH, DHF, bovine liver DHFR and inhibitor in the cuvette. The rate of consumption of NADPH at 340 nm during the conversion of dihydrofolic acid to tetrahydrofolic acid was monitored. The reduction in absorbance over 6 min in the absence of the inhibitor was taken as the control. Total inhibition of DHFR was indicated by an insignificant change in the rate of consumption of NADPH and no inhibition would be seen when the rate of consumption of NADPH was equal to that of the control. The percentage activity of each inhibitor was calculated by the following formulae:

$$\text{(i)}\quad \text{Activity} = \frac{\text{Slope of inhibited enzyme}}{\text{Slope of uninhibited enzyme}} \times 100\%$$

$$\text{(ii)}\quad \text{Inhibition} = 100\% - \text{Activity}$$

A graph of percentage inhibition against the logarithmic concentration (μM) was plotted for each pot and the IC_{50} value was taken to be the concentration (μM) at which 50% inhibition was observed. All the experiment was carried out in triplicate.

5.4. Antiproliferative assay

5.4.1. Cells and cell culture

A549 cells were kindly supplied by Collaboration Labs in Department of Pharmacy, NUS. A549 cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum under the same condition. The cell lines were passaged two times weekly after previous treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (0.02%). Cell viability of the stock cultures used for subsequent experiments was always above 95% as assessed by trypan blue solution.

5.4.2. Drugs and reagents

Stock solution were prepared by dissolving compounds using 5% DMSO in phosphate-buffered saline (PBS) at a concentration of 1.0 mmol/L and stored at −20 °C. MTX was obtained from Department of Pharmacy, National University Hospital (NUH) as solution of 25 mg/2 mL. PTX was kindly supplied by Pharmacy of Oncology and Hematology department of NUH. The test compounds were diluted to the working concentration with culture medium before use. A stock solution of fluorescein diacetate (FDA) was prepared in 10 mg/mL concentration in DMSO, kept frozen (−20 °C) and protected from light.

5.4.3. Fluorometric microculture cytotoxicity assay (FMCA)

The FMCA is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate (FDA) to fluorescein by cells with intact plasma membranes and has been described in detail previously. The drug cytotoxicity and cell proliferation were determined using the previously described FMCA[12], exponential cell growth is guaranteed during the whole time of incubation. Briefly, by utilization of 96-well microtiter plates, 200 μL of a cell suspension were plated into each well (A549, 1000 cells per well). The plates were incubated at 37 °C for 24 h in a humidified atmosphere (5.0%, CO_2) to allow the cells to adhere to the bottom. The medium of the plates was then replaced with 200 μL of medium containing the test compounds. Twelve wells without compounds were served as controls and twelve wells containing culture medium only served as blanks. After an incubation period of 72 h, the medium was removed by multichannel pipette. After one wash with PBS, 200 μL/well PBS containing FDA (2 μg/mL) was added. Subsequently, the plates were incubated for 45 min at 37 °C and the fluorescence generated from each well was then read at an excitation wavelength of 485 nm and an emission wavelength of 535 nm by a scanning fluorometer. In a preliminary study, the fluorescence was found to be proportional to the number of viable cells in the well. Quality criteria for a technically successful assay included a proportion of 70% cells in control wells after 72 h of incubation, a fluorescence signal in control wells of greater than or equal to five times the mean blank value, and a mean coefficient of variation in control wells of <30%. The results are presented as survival index (SI), defined as fluorescence in test wells/fluorescence in control wells (blank values subtracted) × 100. Thus, a low numerical value indicates high sensitivity to the cytotoxic effect of the drug. IC_{50} value was defined as the concentration giving a SI 50% of the control SI. Compounds were tested in triplicate and the results are expressed as means ± SEM for the data combined from separate experiments.

5.5. Rescue experiment

FMCA assays were carried out using the method stated above. Investigations of the effect of rescue agents were conducted using a fixed concentration of antifolates and new synthesized compound **3c** at the 10 × IC_{50} concentration determined as above for A549. Cells in 200 μL of medium were seeded in each well of 96-well plates. After 24 h of incubation, the medium was replaced with medium containing 10 × IC_{50} concentration of compounds in the absence of protection, or with the same concentration of drug plus 20 μM TdR, 100 μM Hx, 20 μM TdR and 100 μM Hx in the medium. Twelve wells were used for each test solution and for the control, which contained the corresponding amount of solvents for dissolving compounds. After the incubation of 72 h, the medium was removed with multichannel pipette. After one wash with PBS, 200 μL/well PBS containing FDA (2 μg/mL) was added. Subsequently, the plates were incubated for 45 min at 37 °C and the fluorescence generated from each well was then read at an excitation wavelength of 485 nm and an emission wavelength of

535 nm by a scanning fluorometer. The effectiveness of the compounds at $10 \times IC_{50}$ is expressed as corrected $\% T/C_{corr}$ or $\% \tau$ values according to the following equations: where T (test) and C (control) are the fluorescences in test wells and control wells, and C_0 the fluorescences of the cells measured immediately before treatment.

$$\text{Antiproliferative effects}: T/C_{corr}(\%) = (T - C_0)/(C - C_0) \times 100$$

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