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Design, synthesis, docking studies and biological evaluation of novel dihydro-1,3,5-triazines as human DHFR inhibitors

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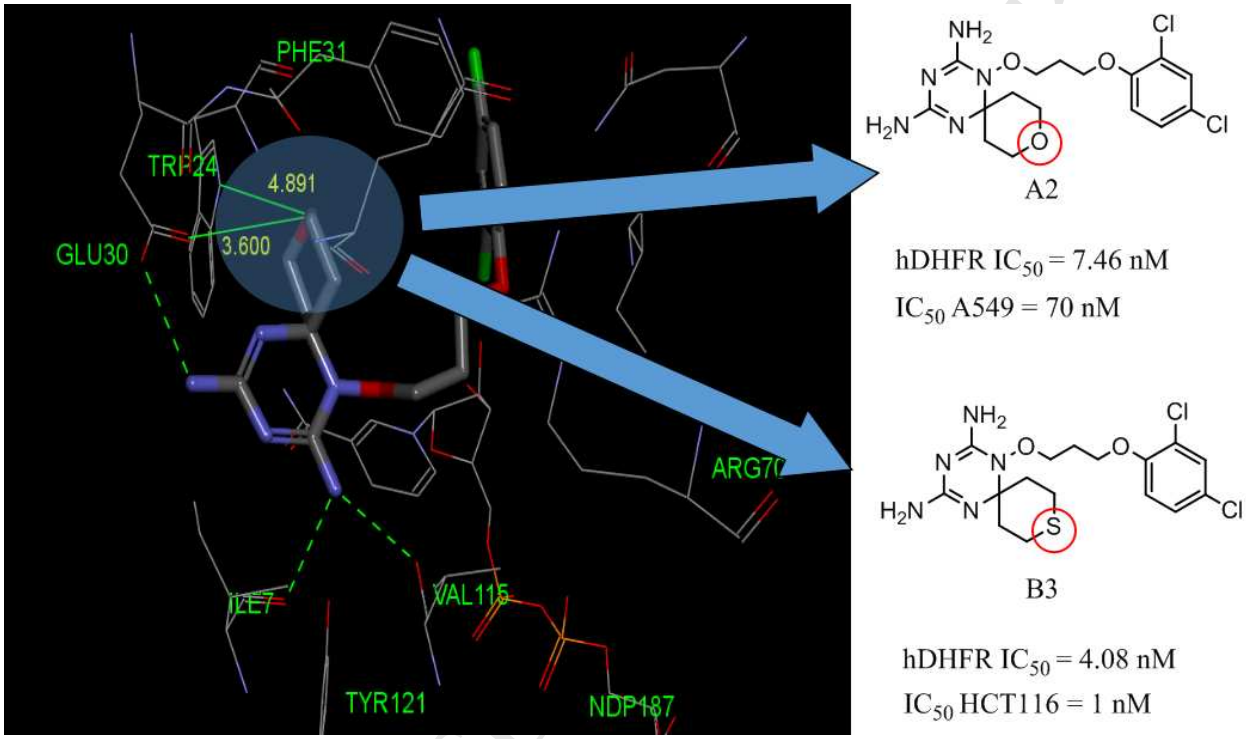
Graphical abstract



1. Introduction

Dihydrofolate reductase (DHFR) is a critical enzyme in folate metabolism. It converts dihydrofolate (DHF) to tetrahydrofolate (THF), which is essential for purine and thymidylate (TMP) synthesis in cell proliferation[1]. Depressing DHFR activity results in THF deficiency and disruption of subsequent DNA replication and resulting in cell death[2]. Therefore, human DHFR (hDHFR) has long been a vital target enzyme in the development of antitumor chemotherapeutic agents[3].

Several hDHFR inhibitors have been successfully used in clinical oncology such as methotrexate (MTX) and trimetrexate (TMQ). Analysis of the structure of these hDHFR inhibitors found that most of them contain a crucial nucleus: a planar bicyclic ring consisted of nitrogen-heterocyclics substituted with amino groups such as the diaminopteridine ring of MTX, the quinazoline ring of trimetrexate (TMQ), pyrrolo[2,3-d]pyrimidine ring of pemetrexed [4-11]. Therefore, they have similar binding mode with hDHFR, in which, e.g., N8 of the pteridine ring of MTX contacts with Glu30 and Trp24 through a water molecule, the 4-amino group forms hydrogen bonding with Ile7, Val115, Tyr121 and NADPH[12] (Figure 3, A).

In our previous studies, a series of 1,3,5-triazine compounds with spiro bicyclic ring were designed and synthesized, and their biological activities were evaluated[13-14]. The results showed that some compounds, e.g., compound M0 (fig. 1) showed potent anti-folate activity against mammalian DHFR and *in vitro* anti-tumor activities against human alveolar basal epithelial cell line (A549)[14], which is comparable to MTX. The results showed that 1,3,5-triazine with spiro bicyclic ring was a novel molecular scaffold for hDHFR inhibitors. This prompted us to undertake further investigation on new spiro-triazine derivatives with higher anticancer activity.

In this work, we analyzed the interactions of the planar bicyclic ring of hDHFR inhibitors with the residues in the hDHFR active site observed in several X-ray complexes (PDB ID: 1U72, 1DLS, 1KMS, 1OHK, 1S3U, 2W3B, 3NTZ) [15-22]. All showed an anti-folate binding mode in which heteroatom and amino groups contact with residues Ile7, Trp24, Glu30, Val115, Tyr121 and NADPH. Then, the molecular docking work of compound M0 was performed with the Flexible Docking program[24-25]. The molecular docking data suggested that we insert oxygen and sulfur atom separately into the spiro ring of compound M0 to create more favorable interactions. A novel series of 1,3,5-triazine derivatives bearing 9-oxaspiro or 9-thiaspiro were designed and synthesized, and their biological activities were evaluated.

2. Results and discussion

2.1 Molecular docking studies

The molecular docking study was performed by using Flexible Docking protocol[26] in Discovery Studio 3.0. All the X-ray complexes were extracted from the Brookhaven Protein Database (PDB http://www.rcsb.org/pdb). The structure of hDHFR derived from the complex 1U72[4] was prepared in Discovery Studio 3.0 by removal of the original ligand MTX and preservation of co-factor NADPH, and the hydrogen atoms and CHARMm force fields were then added. The entire hDHFR enzyme was defined as a receptor and the site sphere was selected based on the ligand binding location within a radius of 10.0 Å. Other parameters were set as default. For compound M0 and its derivatives, energy minimization used the 'Minimize Ligands' protocol.

After a superimposition work of the hDHFR proteins from X-ray complexes involving 1U72, 1DLS, 1KMS, 1OHK, 1S3U, 2W3B and 3NTZ (Figure 2), a significant conformational change in Glu31 was found seen in a ternary complex of the furopyrimidine derivative and hDHFR[23] (PDB ID: 3NXV) (Fig. 2). This allowed us to hypothesize that the volume of key active-site pocket could be further enlarged due to the shift of flexible residue Glu31. Thus, the residue Glu31 was identified and selected as the flexible amino acid residue for this docking. At first, a flexible docking of original ligand MTX was carried out to demonstrate the reliability of this model for hDHFR (RMSD to the X-ray < 1Å). Subsequently, compound M0 and its derivatives were added. After completing the molecular docking procedure, docking poses were scored and selected based on calculated -CDOCKER energy. We exhibited the docking pose of M0 with the best -CDOCKER energy in Figure 3B. Figures 2&3 were prepared by Discovery Studio 3.0.

In the docking results (Figure 3B), compound M0 generated the key hydrogen bonds with hDHFR in the catalytic domain. The 4-amino group of diaminotriazine ring formed hydrogen bonds with residues Ile7, Val115 and Tyr121; the 2-amino group of diaminotriazine ring made contacts with the residue Glu30, and the triazine ring appeared at the same position as diaminopteridine ring of MTX. The phenoxypropyl side chain reached the hydrophobic pocket. These interactions followed the rule of normal binding mode reported in X-ray complex 1U72. In contrast with the MTX-binding mode, the spiro-ring of M0 with the unique steric hindrance was placed at the new extended space caused by the shift in the flexible PHE31, closer to Glu30 and Trp24 than N8 of the pteridine ring of MTX. However, no possible hydrogen bond existed since the absence of electron-rich atoms on the spiro ring for M0. The docking result of the target compound A2 bearing 9-oxaspiro indicated a homologous binding pattern as that of M0 (Figure 4). The oxygen atom 3.60 Å from Glu 30 and 4.89 Å from Trp24, while the distances in MTX from 1U72 complex were 4.01 Å and 6.06 Å respectively. These docking results explain the similar binding mode in the bicyclic region from that of MTX and suggested that inserting oxygen or sulfur atom on spiro-ring might be a promising approache to enhance the binding affinity with hDHFR by generating stronger hydrogen bonds with Glu30 and Trp24 via water-mediated manner (or directly).

2.2 Chemistry

Target compounds A1-A16 and B1-B12 were synthesized following the steps in Schemes 1. The 3-substituted phenoxypropyl bromides were obtained via alkylation of commercially available substituted-phenols with K_2CO_3 as a base and acetonitrile as a solvent. The synthesis of the required key intermediates 2,4-diamino-5-hydroxy-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrochloride (A0) followed the procedure reported in our previous publication[8,9]. Compound A0 was treated with NaOH and 3-substituted phenoxypropyl bromide in DMF to obtain the corresponding 2,4-diamino-5-(3'-(substituted phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A1-A16). 3-substituted phenoxypropyl bromides were treated with benzohydroxamic acid and sodium hydroxide in ethanol under reflux to get the corresponding N-benzoyl-protected substituted phenoxypropyloxy hydroxylamine hydrochlorides. Next, the hydrolysis was performed to synthesize substituted phenoxypropyloxy hydroxylamine hydrochlorides (C1-C12) by hydrochloric acid. Reaction of the substituted phenoxypropyloxy hydroxylamine hydrochlorides (C1-C12) and cyanoguanidine for 5 hours provided the biguanide hydrochlorides. They in reaction solution without isolation was directly subjected to HCl-catalyzed cyclocondensation with tetrahydro-4-thiopyrone in a one pot reaction. After 5 to 15 days, final desired 2,4-diamino-5-(3'-(substituted phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-dienes hydrochloride (B1-B12) were obtained.

2.3 hDHFR inhibitory activity

All target compounds were evaluated for the inhibitory activities against human DHFR by the reported procedure [14, 27, 28]. The results are shown in Table 1. In the inhibition assay, twenty compounds showed favorable inhibition at 5 μM, which is superior to both leading compound M0 and MTX. Thirteen compounds (A1-A10, B1-B3) with an inhibition ratio over 90% at 5 μM were chosen for the further evaluation of IC_{50} values, and the others (A11-A16, B4-B12) with inhibition ratios below 90% at 5 μM were arranged to test hDHFR inhibition ratio at a concentration of 50 μM. At 50 μM, nine compounds showed 88.2% to 94.4% inhibition ratio superior to the leading compound M0 (87.6% inhibition ratio); four compounds showed 89.8% to 93.8% inhibition superior to MTX (89.7% inhibition ratio).

The activities of thirteen target compounds (A1-A10, B1-B3) were reported as IC_{50} values. Eleven compounds among them showed the IC_{50} values of 46.62 to 3.72 nM, which is superior to M0 (IC_{50}=49.04 nM), and four (A2, A5, B1,and B3) showed favorable hDHFR inhibitory activities with IC_{50} value of 7.46 nM, 3.72 nM, 6.46 nM, 4.08 nM—superior to MTX (IC_{50}=6.67 nM). In comparison to M0 (IC_{50}=49.04 nM), 4-chloro substituted compound A6 (IC_{50}=33.56 nM) and B2 (IC_{50}=18.28 nM) bearing 9-oxaspiro ring or 9-thiaspiro ring exhibited increased activities showing that insertion of oxygen and sulfur atom into the spiro-ring could increase the activity. Furthermore, when comparing B2 (IC_{50}=18.28 nM), B1 (IC_{50}=6.46 nM), and B3 (IC_{50}=4.08 nM) with A6 (IC_{50}=33.56 nM), A9 (IC_{50}=32.49 nM), and A2 (IC_{50}=7.46 nM), respectively, it implied that compounds of the thiaspiro series were generally more active than compounds of the oxaspiro series.

2.4 *In vitro* antiproliferative activity

The anti-tumor potency was carried out by the MTT assay [29,30] for all the target compounds against three human tumor cell lines including human colorectal cancer cell line (HCT116), human alveolar basal epithelial cell line (A549), and human leukemia cell line (HL-60). Twenty-one compounds showed better activity against HCT116 cells with IC50 values ranging from 0.69 μM to 0.001 μM relative to the positive control MTX. Thirteen compounds showed better activity against A549 cells with IC_{50} values ranging from 0.24 μM to 0.001 μM relative to MTX. Twenty-two compounds showed activity against HL-60 cells with IC_{50} values ranged from 0.83 μM to 0.03 μM, which is superior to MTX. In addition, compounds A10-A16, B1-B12 were also evaluated anti-tumor activity against liver hepatocellular cell line (HepG2) and metastatic breast cancer cell line (MDA-MB-231). Eleven compounds showed activity against HepG2 cells with IC_{50} values ranged from 0.38 μM to 0.05 μM superior to that of MTX, and 20 compounds showed activity against MDA-MB-231 cells with IC_{50} values ranging from 3.57 μM to 0.001 μM, which is superior to MTX. Consistent with the hDHFR inhibitory activity data, there were improvements in the anti-tumor activity due to introducing oxygen and sulfur atom. The activity of the thiaspiro series surpassed that of the oxaspiro series.

2.5 *In vivo* anti-tumor activity

The *in vivo* anti-tumor efficacy of compound A2 was evaluated in male BALB/c nude mice bearing A549 cells, according to the published protocol[31,32]. The MTX (2 mg/kg) and 50 mg/kg compound A2 were injected i.p. 14 times over a 22 days. Body weights were recorded per day. The tumor sizes were measured using calipers, and tumor volumes were calculated by the formula $A \times B^2/2$ where A and B are the larger and smaller diameter of the tumor, respectively. Tumor volume (TV) was calculated by the following formula: TV $1/2 \times a \times b^2$. Relative tumor volume (RTV) was calculated by the following formula: RTV = Vt/Vo (Vo = TV at day 1). An evaluation index of the anti-tumor effect was tumor growth inhibition rate calculated by the following formula: Tumor growth inhibition rate (%) = $(1\text{-}RTV_{test} / RTV_{control}) \times 100\%$. Table 2 shows the relative tumor volume and tumor growth inhibition rate of different treatment groups. Compound A2 was identified to possess good *in vivo* anti-tumor effect with 36.80% tumor growth inhibition at day 22 (for MTX, 50.84%). There was no correlation between *in vitro* and *in vivo* activities, which was probably due to the pharmacokinetic properties.

Table 3 describes the change in body weight of the nude mice. There was a smaller decrease in mice weight in the A2-treated group than the MTX-treated group.

3. Conclusion

In this research, we describe a molecular docking study for compound M0 from our previous report. The results implied that the possible extended space that results from the shift of the flexible residue Phe31 might be favorable for the binding of the spiro-ring to the active site of hDHFR. A novel series of dihydro-1,3,5-triazine derivatives bearing a heteroatom spiro-ring were designed and synthesized on the basis of a hypothesis from molecular flexible docking. All compounds exhibited hDHFR inhibitory activity and anti-proliferative activity against tumor cell lines (HCT116, A549, HL-60, HepG2, and MDA-MB-231). Compounds A2, A5, B1, and B3 showed potent hDHFR inhibitory activity with IC50 values of 7.46 nM, 3.72 nM, 6.46 nM, and 4.08 nM, compared with reference drug MTX. 24 Compounds showed *in vitro* antiproliferative activity toward several tumor cell lines with IC_{50} values ranging from 0.79 to 0.001 μM—better than MTX. The further *in vivo* anti-tumor study showed that compound A2 could inhibit tumor growth in a nude mouse A549 model. The results showed that insertion of oxygen and sulfur atom to the spiro-ring could maintain or increase the hDHFR inhibition.

4. Experimental section

4.1 General

All target compounds were characterized on the basis of ^{1}H NMR and ^{13}C NMR spectroscopic data (Bruker Avance III-400 MHz and 600 MHz, respectively). Chemical shifts are reported in ppm using tetramethylsilane (TMS) as an internal standard. the peak patterns were described as: (br) broad, (s) singlet, (d) doublet, (t) triplet, (q) quartet, and (m) multiplet. Mass spectra were recorded with a Q-TOF mass spectrometer using electrospray ionization (ESI). A.R. grade solvents were directly used and further purification or degas of the solvents was not required. HPLC analysis was done on Agilent HPLC system (model: 1260) equipped with a DAD detector using Agilent Eclipse Plus C18 (5um, 4.6×150 mm) column using ACN: 0.1% phosphoric acid aqueous solution mobile phase by gradient elution at flow rate of 1 ml/min.

4.2 General Procedure for the Synthesis of A1-A16 series

2,4-Diamino-5-hydroxy-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrochloride (0.5g, 0.002 mol) was dissolved in 20 ml of methanol, 1 molar equivalent of NaOH was added into the solution , and the mixture was refluxed for 30 min. After being cooled to room temperature, the solvent was evaporated using vacuum, the dry white precipitate was obtained. The precipitate was dissolved in 5 ml of DMF. 1.2 molar equivalent of 3-aryloxypropyl bromide was added. The mixture was stirred at room temperature and TLC was used to monitor the reaction. The solution was adjusted to pH 1 using concentrated HBr, when reaction was completed. Evaporate the solution at room temperature to remove DMF. The residue was filtered , followed by recrystallization in 90% EtOH. Compounds A1-A16 were prepared in this method.

4.2.1 2,4-Diamino-5-(3'-phenoxypropyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A1)

White solid yield: 36.5%, mp: 206-208 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69-1.66(d,2H, J=12.8), 2.03(br, s, 2H), 2.18-2.14(dd, 2H, J=6), 3.59-3.54(t, 2H, J=11.6), 3.78-3.74(dd, 2H, J_1=4.8, J_2=12), 4.11-4.06(dd, 4H, J_1=6, J_2=12), 6.97-6.93(m, 3H), 7.31-7.28(dd, 2H, J_1=7.2, J_2=8.8), 7.81(s, br, 1H, ex), 8.09(s, 1H, ex), 8.66(s, 1H, ex), 9.50(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO) δ 161.60, 157.17, 134.59, 130.20, 129.39, 128.86, 79.61, 72.27, 62.71, 33.38, 32.08; HRMS calcd. for $C_{16}H_{23}N_5O_3$ [M-HBr+H]$^+$: 334.1874. found: 334.1871.

4.2.2 2,4-Diamino-5-(3'-(2",4"-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A2)

White solid yield: 32.1%, mp: 220-222 °C; HPLC: 98.69% (t_R=18.189 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.68-1.65 (d, 2H, J=12.8), 2.00(br, s, 2H), 2.22-2.19(t, 2H, J=6), 3.60-3.55(t, 2H, J=12), 3.75-3.71(dd, 2H, J_1=4.4, J_2=12), 4.10-4.07(t, 2H, J=6.4), 4.20-4.17(t, 2H, J=6), 7.21-7.19(d, 1H, J=8.8), 7.38-7.35(dd, 1H J_1=2.4, J_2=8.8), 7.55-7.54(d, 1H, J=2.4), 7.83(s, br, 1H, ex), 8.11(s, 1H, ex), 8.66(s, 1H, ex), 9.37(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.24, 156.60, 152.85, 129.35, 128.29, 124.76, 122.56, 115.35, 74.34, 71.76, 65.505, 62.38, 31.47, 33.60, 30.47, 26.97; HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_3$ [M-HBr+H]$^+$: 402.1094. found: 402.1100.

4.2.3 2,4-Diamino-5-(3'-(4"-fluoro-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A3)

White solid yield: 25.8%, mp: 210-214 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.67-1.63(d, 2H, J=12.8), 2.01(br, s, 2H), 2.51-2.49(m, 2H, J=3.6), 3.44-3.38(t, 2H), 3.78-3.72(dd, 2H, J_1=4.8, J_2=12), 4.06-4.01(dd, 4H, J_1=6.4, J_2=12), 6.94-6.91(m, 2H), 7.09-7.04(m, 2H, J=2.4), 7.78(s, br, 1H, ex), 8.10(s, 1H, ex), 8.65(s, 1H, ex), 9.13(s, br, 1H, ex); HRMS calcd. for $C_{16}H_{22}FN_5O_3$ [M-HBr+H]$^+$: 352.1779. found: 352.1784.

4.2.4 2,4-Diamino-5-(3'-(4"-methoxy-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A4)

White solid yield: 48.5%, mp: 217-219 °C;HPLC: 99.78% (t_R=7.026 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69-1.66(d, 2H, J=12.8), 2.0w(br, s, 2H), 2.16-2.11(t, 2H, J=6), 3.55-3.49(t, 2H, J=11.8), 3.80-3.76(dd, 2H, J_1=4.8, J_2=12), 3.69(s, 3H), 4.07-4.00(m, 4H), 6.87-6.82(m, 4H), 7.85(s, br, 1H, ex), 8.09(s, 1H, ex), 8.67(s, 1H, ex), 9.11(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.60, 157.17, 134.59, 130.20, 129.39, 128.86, 79.61, 72.27, 62.71, 33.38, 32.08; HRMS calcd. for $C_{17}H_{25}N_5O_4$ [M-HBr+H]$^+$: 364.1979. found: 364.1985.

4.2.5 2,4-Diamino-5-(3'-(4"-tert-butyl-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A5)

White solid yield: 65.8%, mp: 216-218 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.25(s,9H), 1.69-1.66(d,2H, J=12.8), 2.02(br, s, 2H), 2.16-2.13(t, 2H, J=6), 3.52-3.46(t, 2H, J=12), 3.79-3.75(dd, 2H, J_1=4.4, J_2=12), 4.07-4.04(t, 4H, J=6), 6.91-6.88(d, 2H, J=9.2), 7.33-7.31(d, 2H, J=8.8), 7.85(s, br, 1H, ex), 8.10(s, 1H, ex), 8.67(s, 1H, ex), 8.92(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.60, 157.17, 134.59, 130.20, 129.39, 128.86, 79.61, 72.27, 62.71, 33.38, 32.08; HRMS calcd. for $C_{20}H_{31}N_5O_3$ [M-HBr+H]$^+$: 390.2500. found: 390.2499.

4.2.6 2,4-Diamino-5-(3'-(4"-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A6)

White solid yield: 23.9%, mp: 217-219 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.68-1.65(d,2H, J=12.8), 2.00(br, s, 2H), 2.15-2.12(t, 2H, J=6), 3.47-3.41(t, 2H, J=12), 3.78-3.74(dd, 2H, J_1=4.8, J_2=12), 4.08-4.04(dd, 4H, J_1=6, J_2=12), 6.91-6.88(d, 2H, J=8.8), 7.31-7.29(d, 2H, J=8), 7.80(s, br, 1H, ex), 8.11(s, 1H, ex), 8.65(s, 1H, ex), 8.90(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.43, 157.60, 156.94, 129.74, 124.81, 116.77, 74.95, 72.26, 64.83, 62.65, 31.98, 27.35; HRMS calcd. for $C_{16}H_{22}ClN_5O_3$ [M-HBr+H]$^+$: 368.1484. found: 368.1485.

4.2.7 2,4-Diamino-5-(3'-(4"-methyl-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A7)

White solid yield: 62.1%, mp: 221-222 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.67(d, 2H, J=12), 2.03(br, s, 2H), 2.16-2.13(t, 2H, J=6), 2.23(s,3H), 3.52-3.46(t, 2H, J=12), 3.80-3.76(dd, 2H,J_1=4.4, J_2=12), 4.08-4.03(dd, 4H, J_1=6, J_2=12), 6.82-6.80(d, 2H, J=8.8), 7.08-7.05(d, 2H, J=8.4), 7.85(s, br, 1H, ex), 8.10(s, 1H, ex), 8.68(s, 1H, ex), 9.05(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.49, 156.96, 156.61, 130.31, 129.79, 114.81, 75.12, 72.22, 64.39, 62.68, 31.75, 27.50, 20.55; HRMS calcd. for $C_{17}H_{25}N_5O_3$ [M-HBr+H]$^+$: 348.2030. found: 348.2037.

4.2.8 2,4-Diamino-5-(3'-(2",4",5"-trichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A8)

White solid yield: 45.2%, mp: 215-217 °C; HPLC: 98.93% (t_R=15.967 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69-1.66(d, 2H.J=12.8), 2.01(br, s, 2H), 2.22-2.19(t, 2H, J=6.4), 3.53-3.46(t, 2H, J=12), 3.79-3.75(dd, 2H, J_1=4.8, J_2=11.8), 4.09-4.06(t, 2H, J=6.4), 4.27-4.22(t, 2H, J=6), 7.49(s, 1H), 7.79(s, 1H), 6.91(s, br, 1H, ex), 8.12(s, 1H, ex), 8.68(s, 1H, ex), 9.03(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.54, 156.88, 153.61, 131.08, 130.98, 123.30, 121.63, 115.95, 74.56, 72.15, 66.35, 62.76, 32.01, 27.17; HRMS calcd. for $C_{16}H_{20}Cl_3N_5O_3$ [M-HBr+H]$^+$: 436.0704, found: 436.0708.

4.2.9 2,4-Diamino-5-(3'-(4"-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A9)

White solid yield: 31.6%, mp: 215-216 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69-1.66(d, 2H, J=12.8), 2.01(br, s, 2H), 2.17-2.12(t, 2H, J=6), 3.54-3.48(t, 2H, J=12), 3.79-3.75(dd, 2H, J_1=4.4,J_2=12), 4.09-4.04(m, 4H), 6.92-6.89(d, 2H, J=8.8), 7.43-7.41(d, 2H, J=8.4), 7.95(s, br, 1H, ex), 8.09(s, 1H, ex), 8.66(s, 1H, ex), 9.21(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.47, 158.05, 157.03, 132.63, 117.28, 112.56, 74.95, 72.23, 64.78, 62.68, 31.84, 27.34; HRMS calcd. for $C_{16}H_{22}BrN_5O_3$ [M-HBr+H]$^+$: 412.0979. found: 412.0976

4.2.10 2,4-Diamino-5-(3'-(3",4"-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A10)

White solid yield: 52.3%, mp: 218-220 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.67(d, 2H, J=12.8), 2.01(br, s, 2H), 2.17-2.14(t, 2H, J=6), 3.54-3.48(t, 2H, J=12), 3.80-3.76(dd, 2H, J_1=4.8, J_2=12), 4.07-4.04(t, 2H, J=6), 4.14-4.11(t, 2H, J=6), 7.00-6.97(dd, 1H, J_1=2.8, J_2=9.2), 7.25-7.24 (d, 1H, J=2.8), 7.53-7.51(m, 1H), 7.98(s, br, 1H, ex), 8.11(s, 1H, ex), 8.68(s, 1H, ex), 9.08(s, 1H, ex); HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_3$ [M-HBr+H]$^+$: 402.1094. found: 402.1101.

4.2.11 2,4-Diamino-5-(3'-(2",3"-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A11)

White solid yield: 30.7%, mp: 208-210 °C (dec) ; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.67-1.64 (d, 2H, J=12.4), 2.00(br, s, 2H), 2.23-2.20(t, 2H, J=6), 3.61-3.58(m, 2H), 3.73-3.69(dd, 2H, J_1=4.8, J_2=12), 4.11-4.08(t, 2H, J=6), 4.23-4.20(t, 2H, J=6), 7.23-7.17(m, 2H), 7.35-7.31(t, 2H, J=8), 7.81(s, br, 1H, ex), 8.10(s, 1H, ex), 8.65(s, 1H, ex), 9.65(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6) : δ 161.60, 157.17, 134.59, 130.20, 129.39, 128.86, 79.61, 72.27, 62.71, 33.38, 32.08; HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_3$ [M-HBr+H]$^+$: 402.1094. found: 402.1100.

4.2.12 2,4-Diamino-5-(3'-(3",5"-dimethyl-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A12)

White solid yield: 52.0%, mp: 203-205 °C; HPLC: 99.27% (t_R=6.101 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.67 (d, 2H, J=12.8), 2.04-1.99(m, 2H), 2.13-2.16(m, 2H), 2.23(s, 6H), 3.56-3.50(t, 2H, J=12), 3.81-3.77(dd,2H, J_1=4.4, J_2=12), 4.08-4.03(dd, 4H, J_1=6, J_2=10.8), 6.69-6.58(d,3H,J=8.4), 7.83(s, br, 1H, ex), 8.11(s, 1H, ex), 8.66(s, 1H, ex), 9.36(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.56, 158.76, 156.96, 139.09, 122.81, 112.73, 75.18, 72.15, 64.23, 62.74, 56.49, 31.76, 27.50, 21.52, 19.02; HRMS calcd. for $C_{18}H_{27}N_5O_3$ [M-HBr+H]$^+$: 362.2187. found: 362.2193.

4.2.13 2,4-Diamino-5-(3'-(2''-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A13)

White solid yield: 37.7%, mp: 207-209 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.69-1.66(d, 2H, J=12.4), 2.01(br, s, 2H), 2.22-2.17(dd,2H, J_1=6,J $_2$=12), 3.49-3.43(t, 2H, J=12), 3.76-3.72(dd,2H, J_1=4.8, J_2=12), 4.12-4.09(t, 2H, J=6), 4.19-4.16(t,2 H, J=6), 6.98-6.94(m, 1H), 7.18-7.16(dd, 1H, J_1=1.6, J_2=8), 7.33-7.28(m, 1H), 7.43-7.40(dd, 1H, J_1=1.6, J_2=8), 8.13(s, 1H, ex), 8.67(s, 1H, ex), 9.96(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.61, 156.87, 154.02, 130.38, 128.90, 122.14, 121.80, 114.44, 74.65, 72.03, 65.18, 62.75, 31.75, 27.38; HRMS calcd. for $C_{16}H_{22}ClN_5O_3$ [M-HBr+H]$^+$: 368.1484. found: 368.1484.

4.2.14 2,4-Diamino-5-(3'-(3''-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A14)

White solid yield: 41.6%, mp: 209-211 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.67(d,2H, J=13.2), 2.01(br, s, 2H), 2.17-2.14(t, 2H, J=6), 3.54-3.48(t, 2H, J=12), 3.80-3.76(dd,2H,J_1=4.8,J_2=12), 4.08-4.04(t,2H,J=6), 4.13-4.10(t,2H,J=6), 7.03-6.92(m,3H), 7.33-7.29(t,1H,J=8), 8.11(s, 1H, ex), 8.68(s, 1H, ex), 9.05(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.61, 159.73, 156.87, 134.24, 131.40, 121.16, 115.05, 114.08, 74.98, 72.05, 64.91, 62.79, 31.78, 27.30; HRMS calcd. for $C_{16}H_{22}ClN_5O_3$ [M-HBr+H]$^+$: 368.1484. found: 368.1487.

4.2.15 2,4-Diamino-5-(3'-(2''-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A15)

White solid yield: 38.0%, mp: 215-216 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.60(d,2H, J=12.8), 2.01(br, s, 2H), 2.23-2.19(dd, 2H, J_1=6, J_2=12), 3.47-3.41(t, 2H, J=12), 3.75-3.71(dd, 2H,J_1=4.8, J_2=12), 4.13-4.10(t, 2H, J=6), 4.19-4.16(t, 2H, J=6), 6.92-6.89(m, 1H), 7.15-7.13(d, 1H, J=8.4), 7.37-7.33(m, 1H), 8.13(s, 1H, ex), 8.67(s, 1H, ex), 8.89(s, br, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.64, 156.85, 154.86, 133.41, 129.59, 122.66, 114.28, 111.45, 74.63, 71.99, 65.15, 62.76, 31.91, 27.39; HRMS calcd. for $C_{16}H_{22}BrN_5O_3$ [M-HBr+H]$^+$: 412.0979. found: 412.0983.

4.2.16 2,4-Diamino-5-(3'-(3''-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-oxa-spiro[5.5]undeca-1,3-diene hydrobromide (A16)

White solid yield: 56.6%, mp: 210-212 °C; HPLC: 98.54% (t_R=11.398 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.70-1.67(d, 2H, J=12.8), 2.02(br, s, 2H), 2.19-2.13(t, 2H, J=6), 3.53-3.47(t, 2H, J=12), 3.80-3.77(dd, 2H, J_1=4.4, J_2=12), 4.13-4.04(m, 4H), 7.00-6.96(dd, 1H, J_1=2.4, J_2=8), 7.17-7.12(m, 2H), 7.27-7.23(m, 1H), 8.10(s, 1H, ex), 8.68(s, 1H, ex), 9.02(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.59, 159.77, 156.82, 131.73, 124.08, 122.60, 117.89, 114.46, 74.93, 72.17, 64.94, 62.83, 31.58, 27.32; HRMS calcd. for $C_{16}H_{22}BrN_5O_3$ [M-HBr+H]$^+$: 412.0979. found: 412.0982.

4.3 General Procedure for the Synthesis of B1-B12 series

The mixture of substituted phenoxypropyloxy hydroxylamine hydrochlorides (0.005 mol), dicyandiamide (0.4 g, 0.005 mol) and EtOH (25 ml) was refluxed for 5h, follwed by being cooled to room temperature. To a suspension of the resulting biguanide hydrochlorides in absolute EtOH (25 ml) was added conc. HCl (0.15 ml), was stirred at room temperature for 4 days to 15 days. After evaporation of the solvent, the residue was triturated with acetone. The solid was collected by filtration and washed again with ether and dried under oven. All samples were recrystallized from ethanol-water before analysis.

4.3.1 2,4-Diamino-5-(3'-(4''-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B1)

White solid yield: 52.9%, mp: 212-214 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.03-2.00(m, 4H), 2.17-2.14(t, 2H, J=6), 2.77-2.67(m, 2H), 2.88(br, s, 2H), 4.10-4.00(dd, 4H, J_1=6, J_2=12.8), 6.95-6.89(dd, 2H, J_1=4.4, J_2=14.4), 7.46-7.41(t, 2H, J=8.8), 7.88(s, br, 1H, ex), 8.08(s, 1H, ex), 8.66(s, 1H, ex), 9.14(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.28, 158.08, 156.86, 132.63, 117.30, 112.57, 74.87, 73.80, 64.70, 27.36, 23.61; HRMS calcd. for $C_{16}H_{22}BrN_5O_2S$ [M-HCl+H]$^+$: 428.0750. found: 428.0745

4.3.2 2,4-Diamino-5-(3'-(4''-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B2)

White solid yield: 25.5%, mp: 226-228 °C (dec); HPLC: 99.93% (t_R=11.658 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06(s, 4H), 2.16-2.13(t, 2H, J=6), 2.52-2.50(m, 2H), 2.83(br, s, 2H), 4.09-4.04(m, 4H), 7.00-6.96(m, 2H), 7.33-7.29(m, 2H), 8.07(s, 1H, ex), 8.62(s, 1H, ex), 8.94(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.25, 157.64, 156.92, 129.72, 124.86, 116.76, 74.86, 73.86, 64.75, 27.38, 23.62; HRMS calcd. for $C_{16}H_{22}ClN_5O_2S$ [M-HCl+H]$^+$: 384.1255. found: 384.1256.

4.3.3 2,4-Diamino-5-(3'-(2'',4''-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B3)

White solid yield: 45.3%, mp: 210-212 °C (dec); HPLC: 99.81% (t_R=12.855 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.05(s, 4H), 2.20-2.19(t, 2H, J=6), 2.49-2.44(m, 2H), 2.89(br, s, 2H), 4.11-4.08(t, 2H, J=6), 4.20-4.17(t, 2H, J=6), 7.21-7.19(d, 1H, J=8.8), 7.39-7.36(dd,1H, J_1=2.4, J_2=8.8), 7.56-7.55(d, 1H, J=2.4), 7.82(s, br, 1H, ex), 8.10(s, 1H, ex), 8.66(s, 1H, ex), 9.23(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.28, 156.85, 153.21, 129.71, 128.69, 125.07, 122.81, 115.59, 74.40, 73.83, 65.58, 27.30, 23.63; HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_2S$ [M-HCl+H]$^+$: 418.0866. found: 418.0871.

4.3.4 2,4-Diamino-5-(3'-(4''-methoxy-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B4)

White solid yield: 58.6%, mp: 220-222 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07(s, 4H), 2.15-2.12(t, 2H, J=6), 2.55-2.50(m, 2H), 2.87(br, s, 2H), 3.69(s,3H), 4.09-4.02(m,4H), 6.91-6.85(m,4H), 8.07(s, 1H, ex), 8.67(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.26, 156.93, 153.97, 152.76, 115.96, 115.11, 75.04, 73.86, 64.87, 55.87, 27.59, 23.61; HRMS calcd. for $C_{17}H_{25}N_5O_3S$ [M-HCl+H]$^+$: 380.1751. found: 380.1750.

4.3.5 2,4-Diamino-5-(3'-(4''-tert-butyl-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B5)

White solid yield: 57.3%, mp: 232-234 °C; HPLC: 99.71% (t_R=14.986 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.25(s, 9H), 2.05(s, 4H), 2.17-2.13(t, 2H, J=6), 2.50-2.45(m, 2H), 2.88(br, s, 2H), 4.09-4.06(t, 4H, J=6), 6.89-6.86(d, 2H, J=8.8), 7.30-7.28(t, 2H, J=8.8), 8.07(s, 1H, ex), 8.68(s, 1H, ex), 9.18(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.27, 156.91, 156.54, 143.33, 126.55, 114.49, 74.93, 73.82, 64.25, 34.23, 31.82, 27.55, 23.57; HRMS calcd. for $C_{20}H_{31}N_5O_2S$ [M-HCl+H]$^+$: 406.2271. found: 406.2274.

4.3.6 2,4-Diamino-5-(3'-(2''-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B6)

White solid yield: 25.5%, mp: 226-228 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.05(s, 4H), 2.22-2.19(t, 2H, J=6), 2.45-2.42(d, 2H, J=13.6), 2.91(br, s, 2H), 4.20-4.10(m, 4H), 6.98-6.94(m, 1H), 7.18-7.16(m, 1H), 7.32-7.29(m, 1H), 7.43-7.40(m, 1H), 7.84(s, br, 1H, ex), 8.10(s, 1H, ex), 8.65(s, 1H, ex), 9.32(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.28, 156.89, 154.04, 130.35, 128.90, 122.13, 121.80, 114.44, 74.48, 73.83, 65.08, 27.42, 23.60; HRMS calcd. for $C_{16}H_{22}ClN_5O_2S$ [M-HCl+H]$^+$: 384.1255. found: 384.1259.

4.3.7 2,4-Diamino-5-(3'-(3''-chrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B7)

White solid yield: 30.8%, mp: 209-211 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07(s, 4H), 2.18-2.15(t, 2H, J=6), 2.50-2.47(d, 2H, J=12), 2.93(br, s, 2H), 4.14-4.06(m, 4H), 7.04-6.93(m, 3H), 7.33-7.29(t, 1H, J=8), 7.85(s, br, 1H, ex), 8.08(s, 1H, ex), 8.66(s, 1H, ex), 9.27(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.26, 159.75, 156.91, 134.25, 131.37, 121.14, 115.06, 114.11, 74.81, 73.85, 64.80, 27.34, 23.62; HRMS calcd. for $C_{16}H_{22}ClN_5O_2S$ [M-HCl+H]$^+$: 384.1255. found: 384.1261.

4.3.8 2,4-Diamino-5-(3'-(2''-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B8)

White solid yield: 41.6%, mp: 214-216 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06(s, 4H), 2.22-2.19(t, 2H, J=6), 2.46-2.42(d, 2H, J=14), 2.89(br, s, 2H), 4.20-4.12(m, 4H), 6.93-6.89(m, 1H), 7.16-7.14(d, 1H, J=8.4), 7.37-7.33(m, 1H), 7.59-7.57(m, 1H), 7.86(s, br, 1H, ex), 8.12(s, 1H, ex), 8.66(s, 1H, ex), 9.22(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.27, 156.90, 154.87, 133.37, 129.57, 122.64, 114.29, 111.46, 74.45, 73.83, 65.06, 27.44, 23.60; HRMS calcd. for $C_{16}H_{22}BrN_5O_2S$ [M-HCl+H]$^+$: 428.0750. found: 428.0747.

4.3.9 2,4-Diamino-5-(3'-(3''-bromo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B9)

White solid yield: 22.5%, mp: 210-212 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07(s, 4H), 2.17-2.14(t, 2H, J=6), 2.50-2.47(d, 2H, J=14), 2.91-2.93(d, 2H, J=5.6), 4.14-4.06(m, 4H), 7.00-6.97(dd, 1H, J_1=2.4, J_2=8), 7.18-7.12(m, 2H), 7.27-7.23(t, 1H, J=8), 7.84(s, br, 1H, ex), 8.08(s, 1H, ex), 8.66(s, 1H, ex), 9.25(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.25, 159.79, 156.92, 131.70, 124.07, 122.61, 117.92, 114.47, 74.81, 73.86, 64.82, 27.35, 23.63; HRMS calcd. for $C_{16}H_{22}BrN_5O_2S$ [M-HCl+H]$^+$: 428.0750. found: 428.0744.

4.3.10 2,4-Diamino-5-(3'-(2'',3''-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B10)

White solid yield: 50.3%, mp: 223-225 °C (dec); HPLC: 100.00% (t_R=12.457 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.04(s, 4H), 2.23(br, s, 2H), 2.42-2.40(d, 2H, J=8), 2.92(br, s, 2H), 4.13-4.10(t, 2H, J=8), 4.23-4.21(t, 2H, J=4), 7.24-7.18(m, 2H), 7.37-7.32(t, 1H, J=8), 7.82(s, br, 1H, ex), 8.17(s, 1H, ex), 8.71(s, 1H, ex), 9.40(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.26, 156.87, 155.63, 132.72, 129.13, 122.64, 120.50, 112.87, 74.38, 73.83, 65.79, 27.32, 23.61; HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_2S$ [M-HCl+H]$^+$: 418.0866. found: 418.0869.

4.3.11 2,4-Diamino-5-(3'-(3'',4''-dichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B11)

White solid yield: 38.7%, mp: 229-231 °C; HPLC: 99.28% (t_R=13.119 min); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.06(s, 4H), 2.16(br, s, 2H), 2.51-2.50(m, 2H), 2.94(br, s, 2H), 4.14-4.11(m, 4H), 7.02-6.99(dd, 1H, J_1=4, J_2=8), 7.28-7.27(d, 1H, J=4), 7.54-7.52(d, 1H, J=8), 7.82(s, br, 1H, ex), 8.13(s, 1H, ex), 8.70(s, 1H, ex), 9.44(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.26, 158.30, 156.89, 132.11, 131.46, 123.03, 116.95, 116.02, 74.73, 73.85, 65.29, 27.25, 23.64; HRMS calcd. for $C_{16}H_{21}Cl_2N_5O_2S$ [M-HCl+H]$^+$: 418.0866. found: 418.0866.

4.3.12 2,4-Diamino-5-(3'-(2'',4'',5''-trichrolo-phenoxy)propyloxy)-1,3,5-triaza-9-thia-spiro[5.5]undeca-1,3-diene hydrochloride (B12)

White solid yield: 23.9%, mp: 220-222 °C (dec); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.07(s, 4H), 2.24(br, s, 2H), 2.52-2.47(m, 2H), 2.96(br, s, 2H), 4.17-4.13(m, 4H), 7.37(s, 1H), 7.67(s, 1H), 7.87(s, br, 1H, ex), 8.12(s, 1H, ex), 8.67(s, 1H, ex),

9.17(s, 1H, ex); ^{13}C NMR (151 MHz, DMSO-d_6): δ 161.28, 156.84, 153.65, 130.99, 123.33, 121.62, 115.98, 74.29, 73.84, 69.49, 66.18, 33.53, 29.16, 27.17, 23.67; HRMS calcd. for $C_{16}H_{20}Cl_3N_5O_2S$ [M-HCl+H]$^+$: 452.0476. found: 452.0482.

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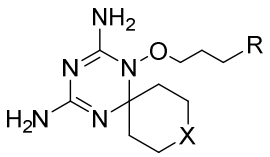
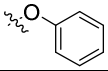
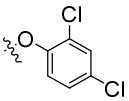
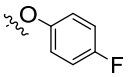
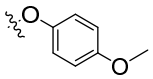
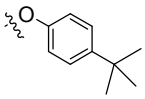
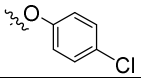
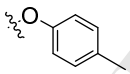
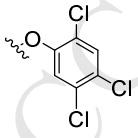
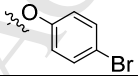
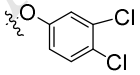
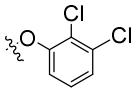
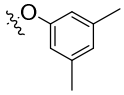
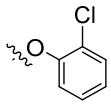
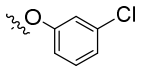
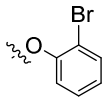
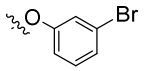
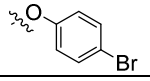
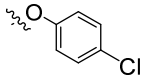
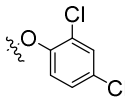
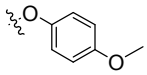
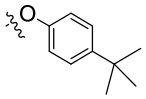
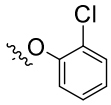
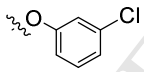
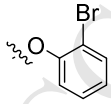
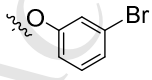
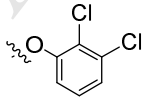
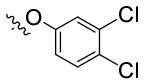
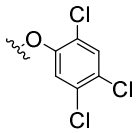
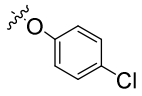
Table 1 *in vitro* hDHFR inhibitory activity and *in vitro* anti-proliferative activity



No	X	R	hDHFR Inhibition (%)		hDHFR IC50[a] (nM)	Anti-proliferative activity IC_{50}(μ M)				
			at 50μM	at 5μM		HCT116	A549	HL-60	HepG2	MDA-MB -231
A1	O		-[b]	>90	86.11	6. 01	1. 08	2. 46	-	-
A2	O		-	>90	7.46	0. 88	0. 07	0. 33	-	-
A3	O		-	>90	46.62	3. 28	1. 06	2. 38	-	-
A4	O		-	>90	11.07	1. 5	0. 66	1. 67	-	-
A5	O		-	>90	3.72	1. 61	0. 5	0. 87	-	-
A6	O		-	>90	33.56	0. 69	0. 36	0. 33	-	-
A7	O		-	>90	56.75	2. 39	1. 06	2. 58	-	-
A8	O		-	>90	36.01	2. 75	0. 68	2. 36	-	-
A9	O		-	>90	32.49	0. 32	0. 28	0. 31	-	-
A10	O		-	>90	8.23	0. 09	0. 79	1. 24	0. 38	0. 59
A11	O		89.8	81.7	-	0. 005	0. 1	0. 44	0. 33	0. 14

A12	O		88.6	84.3	-	0.19	0.71	0.72	0.68	3.57
A13	O		88.2	83.3	-	0.07	2.7	0.66	1.75	1.43
A14	O		85.8	83.2	-	0.12	0.15	0.52	0.15	1.02
A15	O		85.0	83.8	-	0.03	0.16	0.61	0.86	0.91
A16	O		85.8	86.7	-	0.04	0.16	0.56	0.32	0.4
B1	S		-	>90	6.46	0.02	0.74	0.35	1.4	0.44
B2	S		-	>90	18.28	0.01	0.58	0.43	1.1	0.35
B3	S		-	>90	4.08	0.001	0.21	0.33	1.38	0.06
B4	S		86.6	81.3	-	0.07	0.24	0.5	0.88	0.35
B5	S		86.6	85.3	-	0.001	0.001	0.28	0.07	0.001
B6	S		89.6	86.3	-	0.001	0.08	0.39	0.08	0.08
B7	S		88.8	88.3	-	0.001	0.06	0.26	0.09	0.13
B8	S		89.6	88.7	-	0.001	0.04	0.18	0.09	0.01
B9	S		86.5	84.2	-	0.001	0.05	0.2	0.09	0.06
B10	S		89.8	83.8	-	0.001	0.001	0.03	0.05	0.001
B11	S		89.8	88.4	-	0.001	0.001	0.14	0.07	0.01

B12	S		94.4	84.4	-	0.001	>100	0.73	>100	0.11
M0	C		87.6	86.4	49.04	0.38	0.99	0.58	2.33	1.21
MTX			89.7	86.4	6.67	0.75	0.25	1.09	0.41	9.49
Doxorubicin			-		-	0.31	0.12	-	0.02	0.72

(a) Values are means of three experiments. (b)The symbol '-' indicates 'not tested.'

Table 2. The anticancer effect of compound A2 in an established nude mice A549 model.

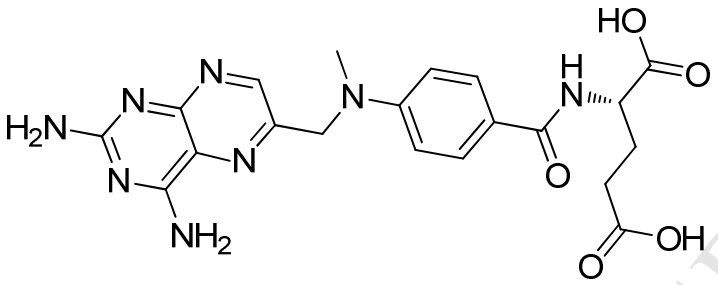
Group	Dose (mg/kg)	Treatment regimen	RTV, $\bar{X}$ ±SD d1	d4	d8	d11	d15	d18	d22
Control			1.00±0.00	1.76±0.46	2.46±0.46	3.19±0.54	4.28±0.64	5.90±0.94	7.77±1.20
MTX	2	ip×14	1.00±0.00	1.33±0.11* (24.65%)[a]	1.65±0.23** (32.69%)	1.94±0.23** (29.19%)	2.36±0.36** (44.97%)	2.91±0.42** (50.65%)	3.82±0.60** (50.84%)
A2	50	ip×14	1.00±0.00	1.45±0.18 (17.58%)	2.10±0.36 (14.50%)	2.67±0.46 (16.34%)	3.22±0.54** (24.73%)	3.97±0.75** (32.81%)	4.91±0.66** (36.80%)

***P<0.05 versus control，**P<0.01 versus control, (a)value in the () was tumor growth inhibition rate**

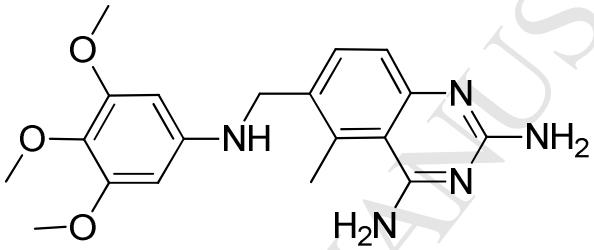
Table 3. The change in body weight of the nude mice

Group	Gose (mg/kg)	Treatment regimen	Body weight (g), $\bar{X}$ ±SD d1	d4	d8	d11	d15	d18	d22
Control			24.73±1.33	24.84±1.22	25.19±1.38	24.99±0.97	25.41±1.04	25.66±1.16	25.59±1.16
MTX	2	ip×14	25.28±0.97	25.47±0.99	24.88±0.82	23.98±0.58*	21.85±2.23**	21.62±2.87**	21.40±2.79**
A2	50	ip×14	23.65±1.01	23.53±1.23	23.17±1.14*	22.97±2.28*	23.63±2.13	23.65±1.98*	23.47±1.96*

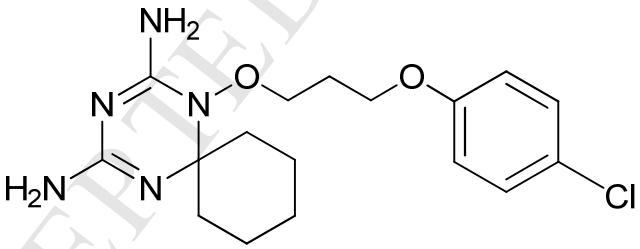
***P<0.05 versus control，**P<0.01 versus control,**



MTX



TMQ



M0

Figure 1. Structures of reported DHFR inhibitors.

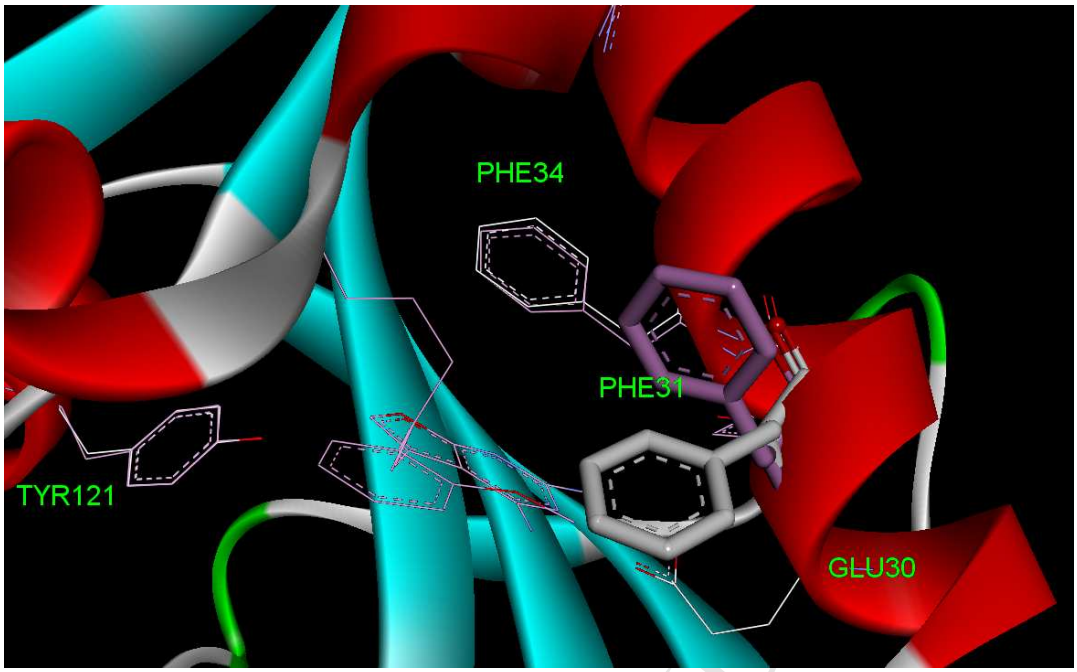


Figure 2. The conformations of PHE31 in 1U72 (white) and in 3NXV (purple).

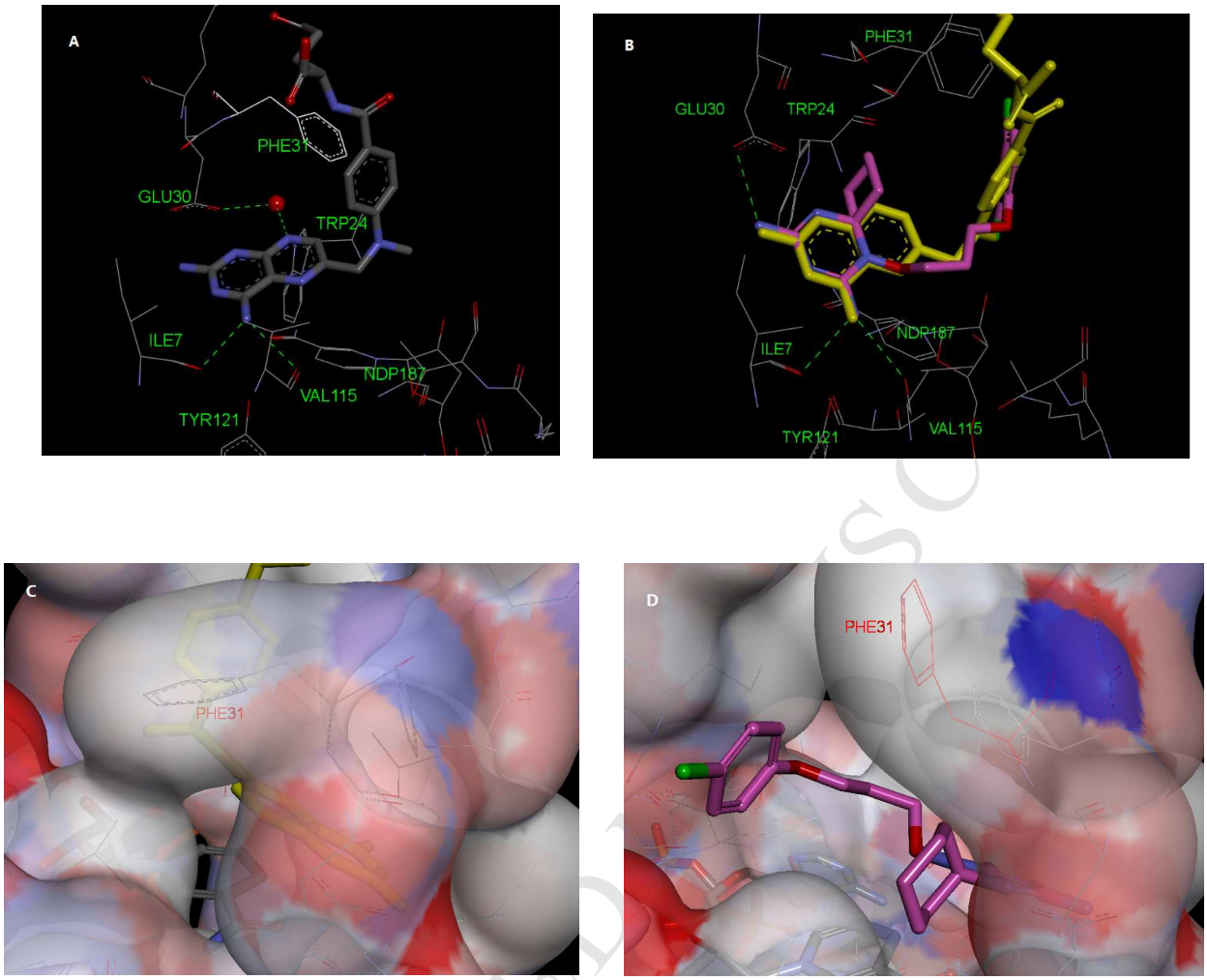


Figure 3. (A)The binding mode of MTX (grey) with hDHFR (PDB ID: 1U72), (B)The docking pose of compound M0 (purple) with hDHFR compared with the binding mode of MTX (yellow) in X-ray (PDB ID: 1U72), (C) Molecular surface of active-site in MTX (yellow) in X-ray, (D) Molecular surface of active-site in M0 (purple)-hDHFR docking result

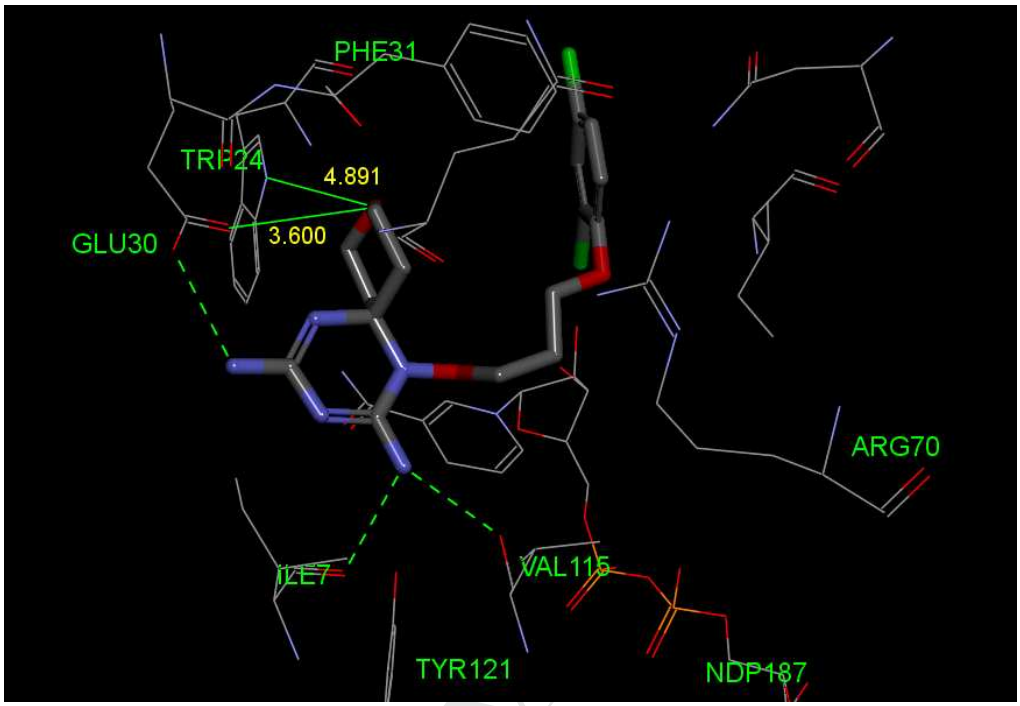
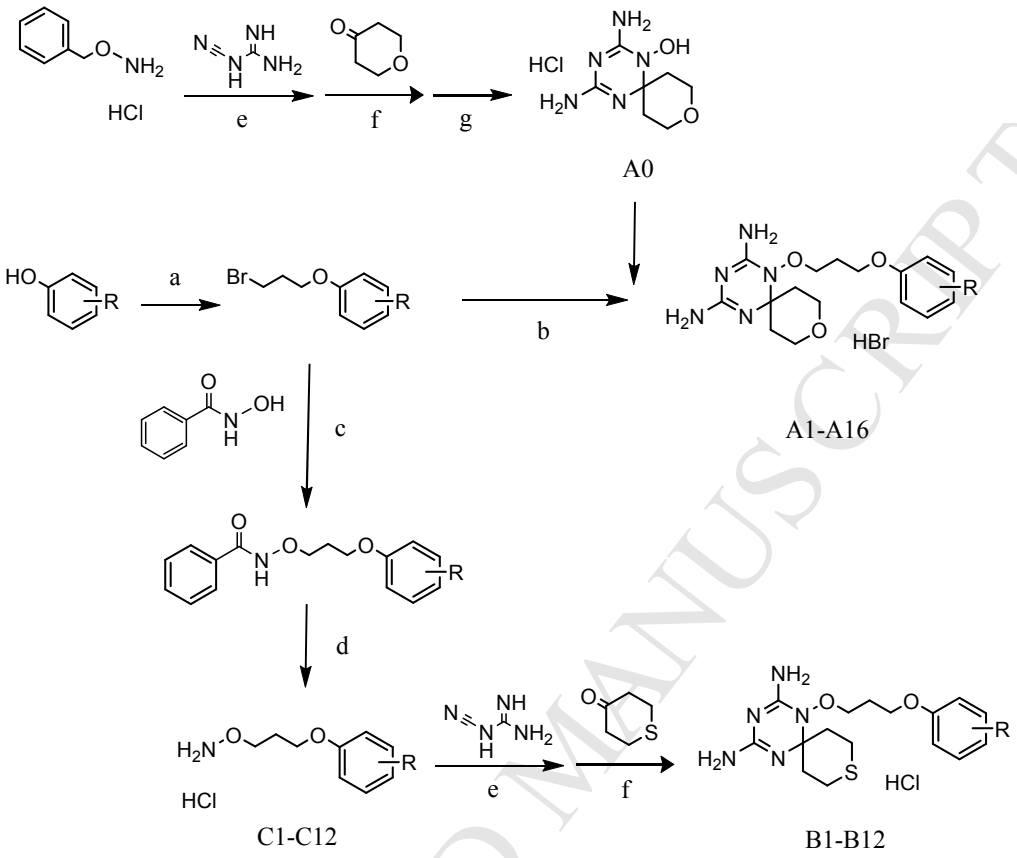


Figure 4. The docking result of compound A2 with hDHFR (1U72)



Scheme 1. Synthesis of A1-A16 and B1-B12. Reagents and conditions: (a) dibromoalkane, K_2CO_3, CH_3CN, reflux, 8 h; (b) NaOH, DMF, 8-24 h; (c) NaOH, EtOH, reflex 10 h; (d) conc. HCl, EtOH, reflex 5 h; (e) EtOH, reflex 5-10h; (f) conc. HCl, ethanol, rt. ; (g) 10% Pb/C, 90% EtOH, 1MPa, rt.

Highlights

1. A novel series of dihydro-1,3,5-triazine derivatives bearing a heteroatom spi ro-ring were designed and synthesized.

2. Docking studies showed the binding mode of triazaspirodiene derivatives to inhibit hDHFR.

3 Compound A2, A5, B1, and B3 had potent inhibitory activites against hDHF R, which was superior to MTX and the leading compound.

4. 24 Compounds showed anti-tumor activities toward several tumor cell lines with IC_{50} values ranging from 0.79 to 0.001 μM, which was superior to MT X.