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含苯甲醚结构的 2,4,5,6-四取代嘧啶衍生物的合成及抗增殖活性研究

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摘要 为了寻找高效的新型抗肿瘤药物, 设计并合成了一系列含苯甲醚结构的 2,4,5,6-四取代嘧啶衍生物, 并采用噻唑蓝(MTT)法测定了目标化合物对 PC-3(人前列腺癌细胞)、MGC-803(人胃癌细胞)、MCF-7(人乳腺癌细胞)、HGC-27(人未分化胃癌细胞)四种人肿瘤细胞的抗增殖活性. 结果显示部分化合物表现出中度至强效的抗增殖活性, 其中 5-乙基-6-((4-甲氧基苯基)硫代)-N^4-(3,4,5-三甲氧基苯基)嘧啶-2,4-二胺(**14t**)对 HGC-27 细胞具有最好的抗增殖活性, IC_{50} 值为 (0.98±0.12) μmol•L^{-1}, 抗增殖活性明显优于阳性对照 5-氟尿嘧啶. 进一步抗肿瘤机制研究表明, 化合物 **14t** 可以显著抑制 HGC-27 细胞的克隆形成和迁移, 诱导细胞凋亡. 同时化合物 **14t** 可以下调抗凋亡蛋白 Bcl-2 的表达, 上调促凋亡蛋白 Bax 和 P53 的表达.

关键词 嘧啶; 苯甲醚; 合成; 抗增殖活性

Synthesis and Antiproliferative Activity of 2,4,5,6-Tetrasubstituted Pyrimidine Derivatives Containing Anisole

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Abstract In order to find efficient new anti-tumor drugs, a series of 2,4,5,6-tetrasubstituted pyrimidine derivatives containing anisole structure were designed, synthesized and evaluated for antiproliferative activity against four human cancer cell lines of PC-3, MGC-803, MCF-7 and HGC-27 using methyl thiazolyl tetrazolium (MTT) assay. Most of compounds displayed moderate to excellent antiproliferative activity against the four human tumor cell lines, among which the compound 5-ethyl-6-((4-methoxyphenyl)thio)-N^4-(3,4,5-trimethoxyphenyl)pyrimidine-2,4-diamine (**14t**) showed the best antiproliferative activity on HGC-27 cells, with IC_{50} value of (0.98±0.12) μmol•L^{-1}, the antiproliferative activity is significantly better than that of the positive control 5-fluorouracil. Further research on anti-tumor mechanism showed that compound **14t** could significantly inhibit the colony formation and migration of HGC-27 cells and induce apoptosis. At the same time, compound **14t** can down-regulate the expression of anti-apoptotic protein Bcl-2 and up-regulate the expression of pro-apoptotic proteins Bax and P53.

Keywords pyrimidine; anisole; synthesis; antiproliferative activity

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1 Introduction

Cancer ranks as a leading cause of death and a significant barrier to increasing life expectancy in every country of the world.[1] Over the next 20 years, the number of new cancer cases worldwide is expected to rise by about 50%.[2] Over the past few decades, it is obvious that cancer cells can obtain multidrug resistance to traditional anticancer drugs, resulting in recurrence of tumor.[3-4] Therefore, it is urgent to discover new effective anticancer drugs.

Compounds with pyrimidine structural units have a wide range of pharmacological activities, such as anti-tumor,[5-6] anti-viral,[7-8] anti-inflammatory[9-10] and antibacterial,[11-12] so they are still a very active field in the design and synthesis of new drug molecules. In recent years, many pyrimidine compounds have been approved by the US Food and Drug Administration (FDA) for clinical treatment of cancer, including capecitabine (**1**),[13] rociletinib (**2**),[14] osimertinib (**3**),[15-16] imatinib (**4**) (Figure 1),[17] *etc*. In addition, based on the small molecules GNE-6640 and GNE-6776 reported in the literature as the lead compounds,[18-19] we switched the pyridine ring to the pyrimidine ring based on the design strategy of scaffold hopping, retaining the amino structure and ethyl group that play an important role in antitumor activity.

Meanwhile, compounds **5**～**8**[20-23] have been reported to have superior anti-proliferative effects on tumor cells, and they all contain anisole groups (Figure 1). Therefore, inspired by the above research results, a series of 2,4,5,6-tetrasubstituted pyrimidine derivatives containing anisole groups were synthesized using the principle of molecular hybridization (Figure 2), and the antiproliferative activity of target compounds was evaluated in vitro by methyl thiazolyl tetrazolium (MTT) assay.

2 Results and discussion

2.1 Chemistry

The general synthetic route for the target compounds was described in Scheme 1. Commercially available diethyl

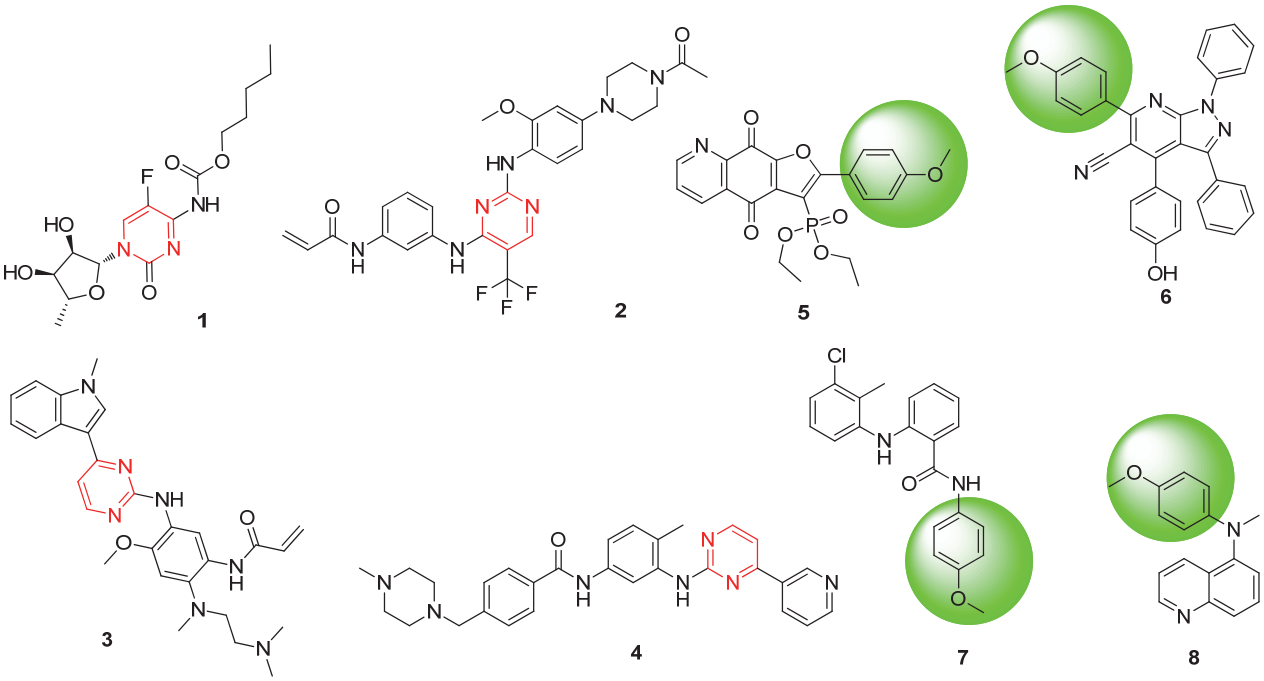


Figure 1 Structures of some pyrimidine derivatives and derivatives containing anisole

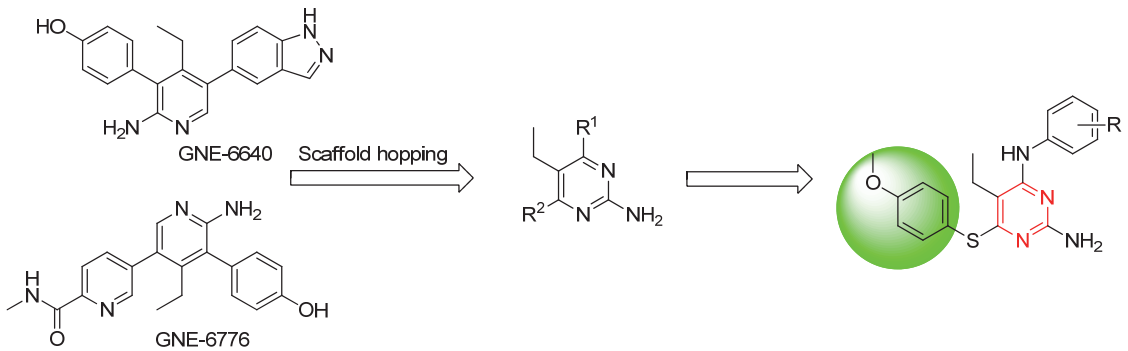


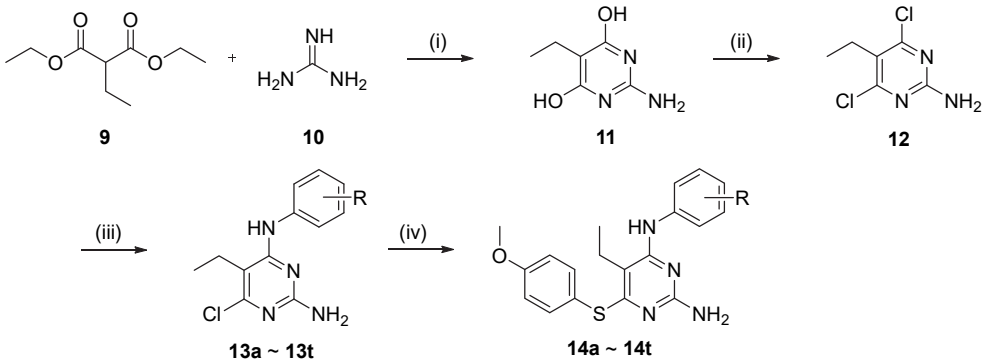
Figure 2 Design strategy for 2,4,5,6-tetrasubstituted pyrimidine derivatives containing anisole

ethylmalonate (**9**) and guanidine hydrochloride (**10**) were used as starting materials, anhydrous methanol was used as solvent, a certain amount of sodium methoxide was added for reflux reaction to obtain compound **11**. compound **11** was reacted with excess phosphorus oxychloride at 90 ℃ to obtain compound **12**. Compounds **13a**～**13t** were prepared by nucleophilic substitution reaction between compound **12** and different substituted anilines. Finally, compounds **13a**～**13t** were reacted with 4-methoxythiophenol in isopropanol using sodium *tert*-butoxide as a catalyst at 82 ℃ to prepare target compounds **14a**～**14t**. The structures of target compounds were characterized by ^{1}H NMR, ^{13}C NMR and HRMS.

2.2 Anti-proliferative activity

To explore the antiproliferative activity of target compounds, compounds **14a**～**14t** were evaluated against PC-3 (human prostate cancer cells), MGC-803 (human gastric cancer cells), MCF-7 (human breast cancer cells) and HGC-27 (human gastric carcinoma cell line) using MTT assay. 5-Fluorouracil (5-Fu) was used as a positive control. The results are summarized in Table 1.

Analysis of the data in Table 1 shows that most compounds exhibited moderate to good antiproliferative activity against the four selected cancer cell lines. To further elucidate the effect of different substituents on proliferative activity, the structure-activity relationship of the target



Reagents and conditions: (i) CH_3ONa, CH_3OH, 65 ℃, 8 h; (ii) $POCl_3$, 90 ℃, 5 h; (iii) different anilines, ethanol, 100 ℃, 4 h; (iv) isopropanol, sodium *tert*-butoxide, 82 ℃, 6 h

Scheme 1 Synthetic route of compounds **14a**～**14t**

Table 1 *In vitro* antiproliferative activity evaluation results of compounds **14a**～**14t**

Compd.	R	IC_{50}[a]/(μmol·L^{-1})			
		PC-3	MGC-803	MCF-7	HGC-27
14a	—	＞50	＞50	＞50	＞50
14b	2-F	15.70±1.19	25.36±0.76	27.67±1.44	30.96±1.49
14c	3-F	19.03±0.99	39.79±1.60	45.20±1.66	37.87±1.44
14d	4-F	20.67±1.03	44.66±1.65	＞50	35.73±1.55
14e	2-Cl	11.13±1.05	20.68±1.32	23.65±1.64	14.32±1.16
14f	3-Cl	＞50	24.89±1.40	41.63±1.62	＞50
14g	4-Cl	26.58±1.42	＞50	27.72±1.44	49.93±1.60
14h	2-Br	10.08±1.00	14.59±1.16	33.74±1.53	12.22±1.09
14i	3-Br	＞50	＞50	＞50	＞50
14j	4-Br	12.39±0.97	17.99±1.25	35.95±1.14	28.75±1.46
14k	2-CH_3	＞50	40.01±1.60	＞50	＞50
14l	3-CH_3	＞50	＞50	＞50	＞50
14m	4-CH_3	＞50	＞50	＞50	＞50
14n	2-OCH_3	5.22±0.72	10.06±1.00	7.79±0.89	5.15±0.71
14o	3-OCH_3	8.41±0.92	32.69±1.51	17.53±1.24	27.55±1.44
14p	4-OCH_3	10.70±1.03	18.18±1.26	15.36±1.19	11.75±1.07
14q	3,4-Cl_2	10.91±1.04	13.77±1.14	6.64±0.88	11.52±1.06
14r	2,4-Cl_2	＞50	＞50	42.73±1.63	＞50
14s	3-Cl-4-F	27.97±0.90	42.36±1.63	29.99±1.48	＞50
14t	3,4,5-$(OCH_3)_3$	5.59±0.75	2.41±0.38	5.94±0.77	0.98±0.12
5-Fu[b]	—	6.39±0.71	10.64±1.09	10.23±0.68	7.25±0.72

[a] Antitumor activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cells proliferation by 50% (IC_{50}). [b] Positive control.

compounds was discussed next. The unsubstituted compound **14a** had poor activity against the four cancer cell lines and was basically inactive. When different electrical substituents were introduced on aniline, the antiproliferative activity of most compounds was improved to a certain extent, indicating that different substituents on aniline had certain effects on antiproliferative activity. Comparing compounds **14b**～**14p**, when substituted by the same substituent, whether it was an electron-donating group or an electron-withdrawing group, the antiproliferative activity of the *ortho*-position was always better than that of the *meta*-position and the *para*-position. When both were electron-withdrawing groups, the activity decreases with the enhancement of electron-withdrawing ability. When the electron-withdrawing group was replaced by an electron-donating group such as methyl, the antiproliferative activity was lost, but with the continuous enhancement of electron donating ability, such as methoxy group, the activity will be greatly improved. If substituted with a polysubstituted methoxy group, such as compound **14t**, the antiproliferative activity was the best in this series, the IC_{50} of **14t** on PC-3, MGC-803, MCF-7 and HGC-27 cells were (5.59±0.75), (2.41±0.38), (5.94± 0.77) and (0.98±0.12) μmol/L, respectively, which were better than the positive control drug of 5-fluorouracil. Among them, **14t** had the strongest inhibitory effect on HGC-27 cells. Therefore, in order to study the mechanism of action of the target compounds, compound **14t** and HGC-27 cells were selected for further study.

To investigate the toxicity of the target compounds to normal cells, compound **14t** which had best antiproliferative activity was selected to further explore its cytotoxicity against GES-1 (normal human gastric epithelial cell line) and HEEC (human normal esophageal cells). As shown in Table 2, the results indicated that compounds **14t** had weak or no cytotoxicity against GES-1 and HEEC, which was significantly lower than the positive control 5-Fu.

2.3 Compound **14t** suppressed the proliferation of HGC-27 cells

We first assessed the effect of compound **14t** on tumor cell proliferative capacity and invasiveness using colony formation assay. As shown in Figure 3, compared with the blank group, with the increase of the concentration of compound **14t**, the number of HGC-27 cell clones decreased, and the colonies became smaller, especially when the compound concentration was 0.5 and 1.0 μmol/L, it could significantly inhibit cell clones, the inhibition rates were 63% and 92%. respectively. Since migration is an important feature of metastatic cancer,[24] the effect of compound **14t** on HGC-27 cell migration was investigated by cell scratch experiments. As shown in Figure 4, when the drug concentrations of compound **14t** were 0.5 and 1 μmol/L, respectively, compared with the blank control group, the cell migration rate decreased with the prolongation of the action time. Meanwhile, compound **14t** significantly inhibited cell scratch healing in a concentration-dependent manner.

Table 2 *In vitro* antiproliferative activity of compound **14t** against human normal cells

Compd.	IC_{50}/(μmol·L^{-1})	
	GES-1	HEEC
14t	43.98±1.64	＞100
5-Fu	13.30±1.12	＞100

2.4 Compound **14t** induced HGC-27 cells apoptosis

Apoptosis is an important process in tumor growth.[25] It is generally believed that the occurrence and development of tumor are not only the result of uncontrolled cell proliferation and abnormal cell differentiation, but also related to the imbalance of tumor cell apoptosis. Therefore, it was observed by DAPI staining whether compound **14t** had an effect on nuclear morphology. The cells were treated with different concentrations of compound **14t**, as shown in Figure 5, compared with the blank control group, the nuclei of the cells shrank and ruptured, indicating that the compound **14t** could induce cell apoptosis. To further explore the effect of compound **14t** on apoptosis, the treated HGC-27 cells were analyzed by flow cytometry. As shown in Figure 6, compared with the blank control group, with the prolongation of the action time, the percentage of apoptosis

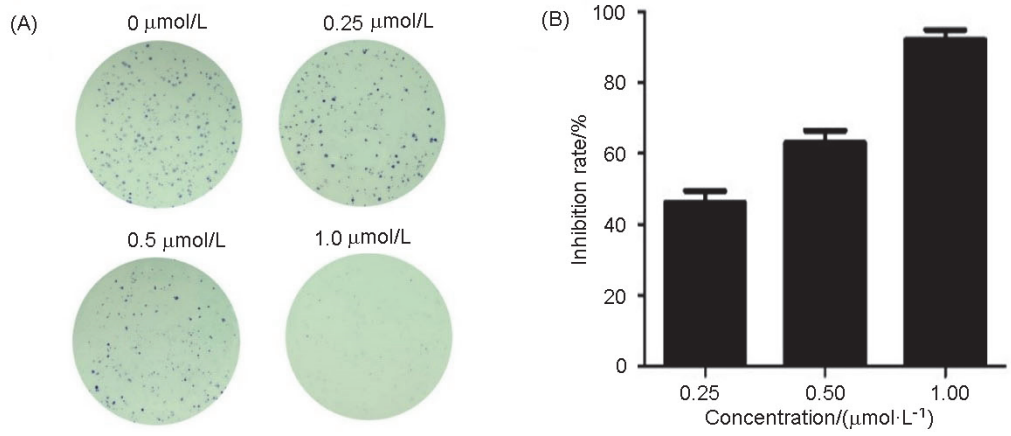


Figure 3 Effect of compound **14t** on colony formation of HGC-27 cells

(A) HGC-27 Cell colonies treated with compound **14t** at different concentrations; (B) Quantitative analysis of colony formation inhibition rate

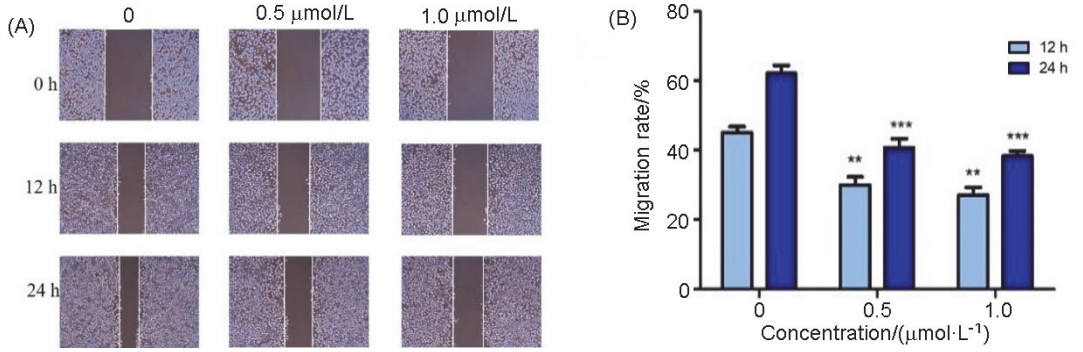


Figure 4 *In vitro* wound healing assay on HGC-27 cells

(A) Phase contrast images were obtained by the treatment of compound **14t** at indicated concentrations for 0, 12 and 24 h; (B) Quantitative analysis of cell migration rate. Compared with the control group, **P*<0.05, ***P*<0.01, ****P*<0.001

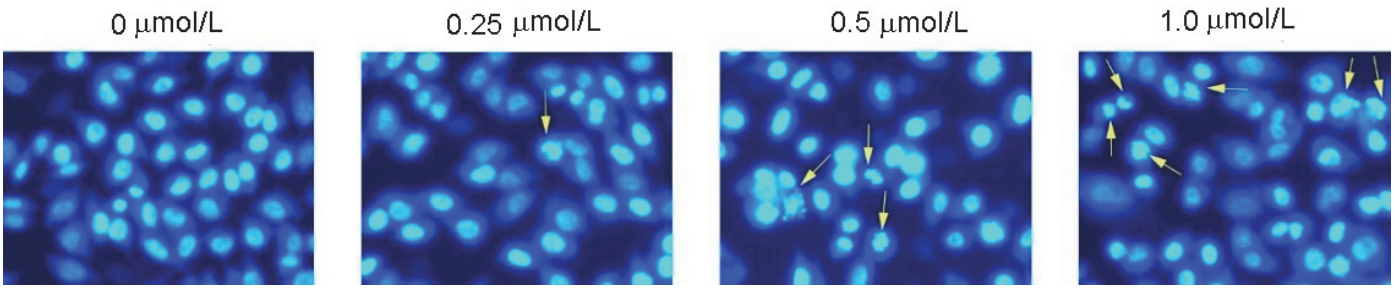


Figure 5 Effect of compound **14t** on nuclear morphology of HGC-27 cells

The arrows in the figure indicate the cells whose nuclei have shrunk or broken

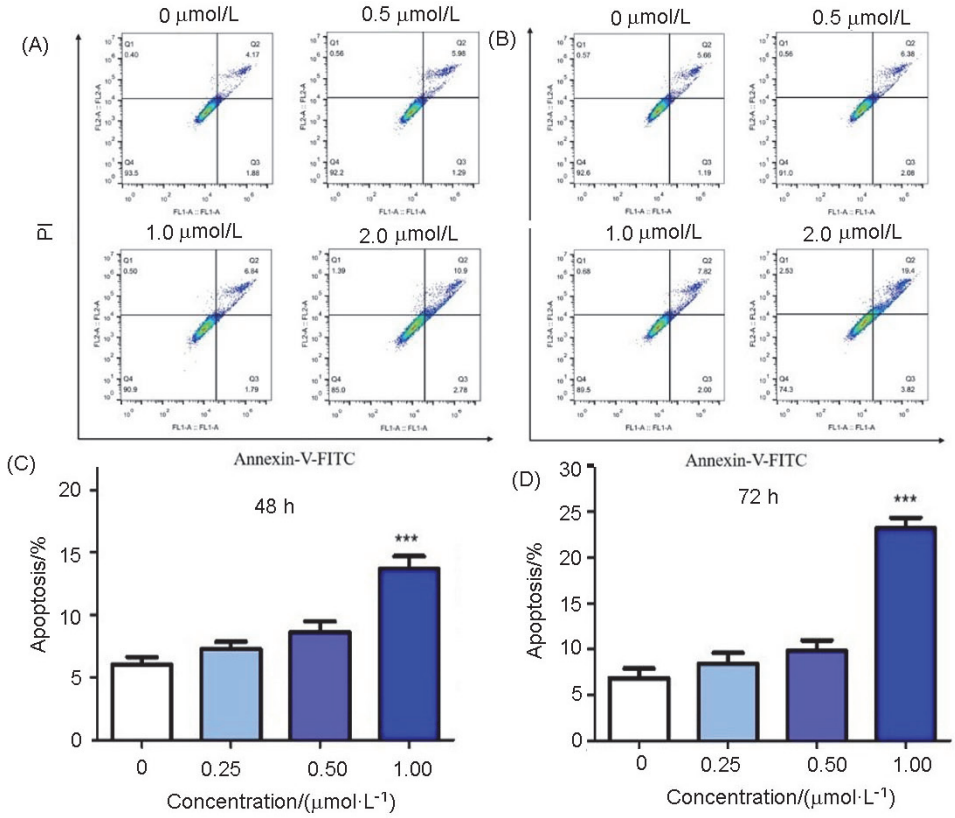


Figure 6 Effect of compound **14t** on apoptosis of HGC-27 cells

(A) Annexin V-FITC/PI detected apoptosis of HGC-27 cells treated with different concentrations of compound **14t** for 48 h; (B) annexin V-FITC/PI detected apoptosis of HGC-27 cells treated with different concentrations of compound **14t** for 72 h; (C) quantitative analysis of apoptosis cells of HGC-27 for 48 h; (D) quantitative analysis of apoptosis cells of HGC-27 for 72 h (Compared with the control group, ****P*<0.001)

of cells increased significantly. Moreover, compound **14t** significantly induced apoptosis in a concentration-dependent manner. In order to further study the mechanism of the anti-proliferative activity of compound **14t** on HGC-27 cells, the effect on the expression of apoptosis-related proteins was analyzed by western blotting. As shown in Figure 7, compared with the blank control, after HGC-27 cells were treated with different concentrations of compound **14t**, the expression of anti-apoptotic protein Bcl-2 was significantly decreased and the expression of pro-apoptotic protein Bax and tumor suppressor protein P53 were upregulated. These data demonstrate that compound **14t** can induce changes in intracellular apoptosis-related proteins in a concentration-dependent manner.

3 Conclusions

In conclusion, a series of 2,4,5,6-tetrasubstituted pyrimidine derivatives containing anisole structure were designed, synthesized and evaluated for antiproliferative activity against four human cancer cell lines. The cytotoxicity experiment results showed that compound **14t** had the best antiproliferative activity on HGC-27 cells. Further mechanism research showed that compound **14t** significantly inhibited the colony formation and migration of HGC-27 cells. In addition, DAPI staining, apoptosis, western blotting experiments showed that compound **14t** might induced apoptosis of HGC-27 cells through the activation of intrinsic apoptotic pathway. From the above results, compound **14t** could be a potential antitumor agent.

4 Experimental section

4.1 Materials

Reagents and solvents were purchased from commercial sources and were used without further purification. Column chromatography was carried out on 200-300 mesh silica gel (Qingdao Haiyang Chemical, China). Reactions were monitored on thin-layer chromatography (TLC) on 0.25 mm silica gel plates (GF254). Melting points were determined on an X-5 Micro Melting Point Apparatus. ^{1}H NMR and ^{13}C NMR spectra were measured on a Bruker 400 and 101 MHz spectrometer, respectively. High-resolution mass spectra (HRMS) of all derivatives were recorded on a Waters Micro-mass Q-T of Micro-mass spectrometer by electrospray ionization (ESI). 2-Amino-5-ethylpyrimidine-4,6-diol (**11**), 4,6-dichloro-5-ethylpyrimidin-2-amine (**12**) and compounds **13a**～**13t** were synthesized according to the literatures[26-28]

4.2 Chemistry

4.2.1 General procedure for synthesis of target compounds **14a**～**14t**

Compounds **13a**～**13t** (1.21 mmol) were dissolved in an appropriate amount of isopropanol, followed by sodium *tert*-butoxide (1.33 mmol), 4-methoxythiophenol (1.33 mmol), and the reaction was carried out at 82 ℃. The reaction was monitored by TLC. After the reaction was completed, an appropriate amount of water was added to the system, extracted three times with ethyl acetate, the organic layers were combined, dried over anhydrous magnesium

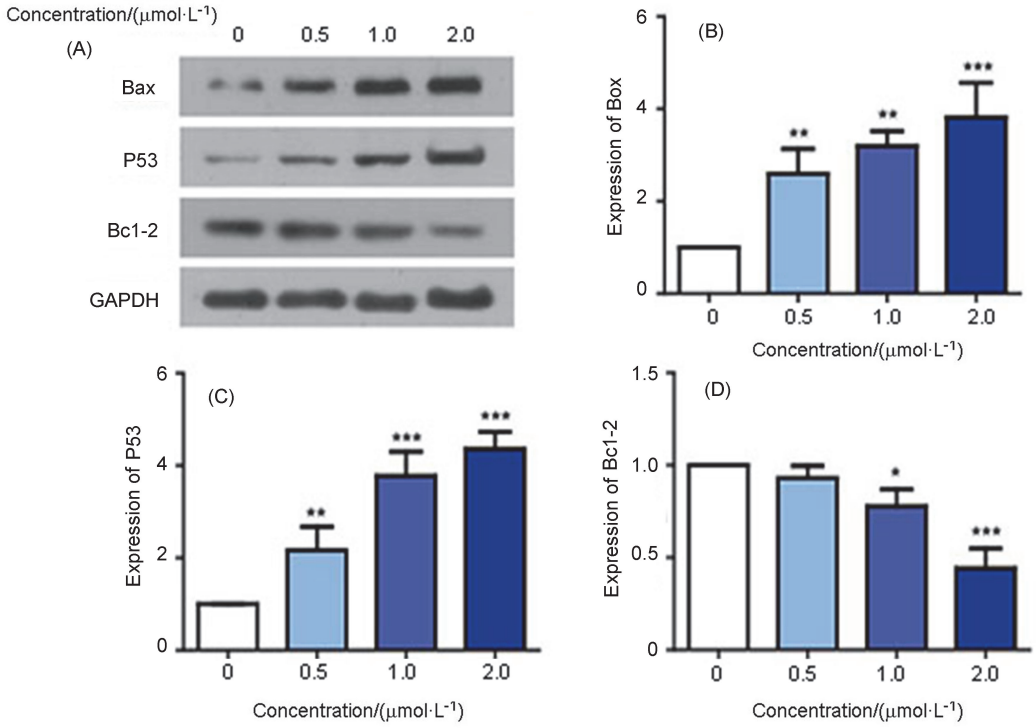


Figure 7 Effect of compound **14t** on apoptosis-related proteins

(A) Changes in protein expression after treatment with different concentrations of compound **14t**. GAPDH is the reference protein. (B, C, and D) quantitative analysis diagrams of each protein. Compared with the control group, *P<0.05, **P<0.01, ***P<0.001

sulfate, concentrated under reduced pressure, fried the samples, the target compounds **14a**～**14t** were isolated and purified by column chromatography.

5-Ethyl-6-((4-methoxyphenyl)thio)-N^4-phenylpyrimidine-2,4-diamine (**14a**): White solid, yield 61.16%. m.p. 176.5～177.4 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.03 (s, 1H), 7.73～7.69 (m, 2H), 7.40 (d, J=8.7 Hz, 2H), 7.25 (dd, J=8.5, 7.3 Hz, 2H), 6.99～6.94 (m, 3H), 5.76 (s, 2H), 3.78 (s, 3H), 2.71 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.1, 160.0, 159.5, 157.8, 140.5, 136.4, 128.1, 121.9, 121.5, 120.5, 114.4, 105.4, 55.2, 18.2, 13.3; HRMS (ESI) calcd for $C_{19}H_{21}N_4OS$ [M+H]$^+$ 353.1436, found 353.1435.

5-Ethyl-N^4-(2-fluorophenyl)-6-((4-methoxyphenyl)thio)-pyrimidine-2,4-diamine (**14b**): White solid, yield 72.47%. m.p. 132.2～132.4 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.92 (d, J=3.5 Hz, 1H), 7.60～7.52 (m, 1H), 7.40 (dt, J=8.8, 2.4 Hz, 2H), 7.23～7.11 (m, 3H), 6.98～6.93 (m, 2H), 5.65 (d, J=3.3 Hz, 2H), 3.78 (s, 3H), 2.68 (q, J=7.5, 7.1 Hz, 2H), 1.18～1.11 (m, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.0, 160.2, 159.5, 158.5, 157.5 (d, J=246.3 Hz), 136.4, 127.8 (d, J=11.6 Hz), 127.6, 125.5 (d, J=7.5 Hz), 124.0 (d, J=3.5 Hz), 120.5, 115.5 (d, J=20.2 Hz), 114.4, 105.1, 55.2, 18.4, 13.0; HRMS (ESI) calcd for $C_{19}H_{20}FN_4OS$ [M+H]$^+$ 371.1342, found 371.1343.

5-Ethyl-N^4-(3-fluorophenyl)-6-((4-methoxyphenyl)-thio) pyrimidine-2,4-diamine (**14c**): White solid, yield 69.59%. m.p. 156.0～156.6 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.16 (s, 1H), 7.80 (dt, J=12.5, 2.3 Hz, 1H), 7.54 (ddd, J=8.3, 2.0, 0.9 Hz, 1H), 7.43～7.38 (m, 2H), 7.29～7.22 (m, 1H), 6.99～6.94 (m, 2H), 6.78～6.72 (m, 1H), 5.91 (s, 2H), 3.78 (s, 3H), 2.71 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 170.0, 168.5 (d, J=240.6 Hz), 165.2, 164.8, 162.7, 147.8 (d, J=11.4 Hz), 141.7, 134.7 (d, J=9.8 Hz), 125.6, 121.8, 119.7, 113.3 (d, J=21.3 Hz), 112.9 (d, J=26.3 Hz), 110.8, 60.4, 23.4, 18.5; HRMS (ESI) calcd for $C_{19}H_{20}FN_4OS$ [M+H]$^+$ 371.1342, found 371.1341.

5-Ethyl-N^4-(4-fluorophenyl)-6-((4-methoxyphenyl)thio)-pyrimidine-2,4-diamine (**14d**): Yellow solid, yield 64.48%. m.p. 163.4～163.9 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.09 (s, 1H), 7.72～7.67 (m, 2H), 7.41～7.37 (m, 2H), 7.08 (t, J=8.9 Hz, 2H), 6.98～6.94 (m, 2H), 5.75 (s, 2H), 3.78 (s, 3H), 2.69 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.1, 160.0, 159.5, 158.7 (d, J=239.6 Hz), 157.8, 136.8 (d, J=2.5 Hz), 136.4, 123.5 (d, J=7.6 Hz), 120.5, 114.6 (d, J=22.0 Hz), 114.4, 105.2, 55.2, 18.2, 13.2; HRMS (ESI) calcd for $C_{19}H_{20}FN_4OS$ [M+H]$^+$ 371.1342, found 371.1341.

N^4-(2-Chlorophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14e**): White solid, yield 60.58%. m.p. 132.0～133.0 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.82 (d, J=8.9 Hz, 2H), 7.48 (d, J=8.0 Hz, 1H), 7.40 (d, J=8.3 Hz, 2H), 7.31 (t, J=7.8 Hz, 1H), 7.14 (t, J=7.7 Hz, 1H), 6.96 (d, J=8.3 Hz, 2H), 5.72 (s, 2H), 3.78 (s, 3H), 2.67 (q, J=7.4 Hz, 2H), 1.17 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.1, 160.2, 159.5, 158.2, 136.9, 136.4, 129.2, 128.1, 127.3, 127.2, 125.3, 120.4, 114.5, 105.0, 55.2, 18.5, 12.9; HRMS (ESI) calcd for $C_{19}H_{20}ClN_4OS$ [M + H]$^+$ 387.1046, found 387.1046.

N^4-(3-Chlorophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14f**): White solid, yield 69.42%. m.p. 165.3～166.2 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.16 (s, 1H), 7.84～7.77 (m, 2H), 7.43～7.37 (m, 2H), 7.26 (t, J=8.1 Hz, 1H), 7.01～6.94 (m, 3H), 5.89 (s, 2H), 3.78 (s, 3H), 2.70 (q, J=7.0 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.8, 159.9, 159.6, 157.4, 142.2, 136.5, 132.5, 129.6, 121.3, 120.3, 120.2, 119.4, 114.5, 105.6, 55.2, 18.1, 13.3; HRMS (ESI) calcd for $C_{19}H_{20}ClN_4OS$ [M + H]$^+$ 387.1046, found 387.1044.

N^4-(4-Chlorophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14g**): White solid, yield 73.56%. m.p. 154.5～155.3 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.15 (s, 1H), 7.81～7.75 (m, 2H), 7.42～7.36 (m, 2H), 7.30～7.25 (m, 2H), 6.99～6.93 (m, 2H), 5.83 (s, 2H), 3.78 (s, 3H), 2.69 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.5, 159.9, 159.5, 157.5, 139.6, 136.5, 127.8, 125.4, 122.9, 120.4, 114.5, 105.4, 55.2, 18.2, 13.3; HRMS (ESI) calcd for $C_{19}H_{20}ClN_4OS$ [M+H]$^+$ 387.1046, found 387.1046.

N^4-(2-Bromophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14h**): Yellow solid, yield 68.47%. m.p. 135.3～136.1 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.85～7.81 (m, 1H), 7.77 (s, 1H), 7.64 (d, J=8.0 Hz, 1H), 7.40 (d, J=8.3 Hz, 2H), 7.35 (t, J=7.8 Hz, 1H), 7.10～7.04 (m, 1H), 6.96 (d, J=8.4 Hz, 2H), 5.72 (s, 2H), 3.78 (s, 3H), 2.67 (q, J=7.4 Hz, 2H), 1.19 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.1, 160.2, 159.5, 158.2, 138.1, 136.4, 132.3, 127.9, 127.3, 125.8, 120.3, 119.2, 114.5, 104.9, 55.2, 18.5, 12.9; HRMS (ESI) calcd for $C_{19}H_{20}BrN_4OS$ [M + H]$^+$ 431.0541, found 431.0539.

N^4-(3-Bromophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14i**): Yellow solid, yield 73.58%. m.p. 193.9～194.5 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.15 (s, 1H), 7.93～7.87 (m, 2H), 7.42～7.38 (m, 2H), 7.20 (t, J=8.0 Hz, 1H), 7.12 (ddd, J=7.9, 2.0, 1.1 Hz, 1H), 6.99～6.93 (m, 2H), 5.88 (s, 2H), 3.78 (s, 3H), 2.70 (q, J=7.2 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.8, 159.9, 159.6, 157.4, 142.4, 136.5, 130.0, 124.2, 123.1, 121.0, 120.3, 119.8, 114.5, 105.6, 55.2, 18.1, 13.3; HRMS (ESI) calcd for $C_{19}H_{20}BrN_4OS$ [M+H]$^+$ 431.0541, found 431.0539.

N^4-(4-Bromophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14j**): White solid, yield 75.41%. m.p. 143.7～143.9 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.15 (s, 1H), 7.74～7.70 (m, 2H), 7.41 (d, J=2.7 Hz, 2H), 7.38 (d, J=2.6 Hz, 2H), 6.98～6.94 (m, 2H), 5.83 (s, 2H), 3.78 (s, 3H), 2.69 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.6, 159.9, 159.5, 157.5, 140.0, 136.5, 130.7, 123.3, 120.4, 114.5, 113.4, 105.5, 55.2, 18.2, 13.3; HRMS (ESI)

calcd for $C_{19}H_{20}BrN_4OS$ $[M+H]^+$ 431.0541, found 431.0538.

5-Ethyl-6-((4-methoxyphenyl)thio)-N^4-(*o*-tolyl)pyrimidine-2,4-diamine (**14k**): Yellow solid, yield 63.15%. m.p. 164.8～165.7 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.82 (s, 1H), 7.41～7.36 (m, 2H), 7.25 (d, J=7.8 Hz, 1H), 7.21 (d, J=7.5 Hz, 1H), 7.18～7.12 (m, 1H), 7.08 (dt, J=8.4, 4.2 Hz, 1H), 6.97～6.91 (m, 2H), 5.52 (s, 2H), 3.77 (d, J=1.8 Hz, 3H), 2.67 (q, J=7.4 Hz, 2H), 2.16 (s, 3H), 1.15 (d, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 163.2, 160.3, 159.4, 159.0, 138.4, 136.3, 134.3, 130.1, 127.2, 125.8, 125.0, 120.8, 114.4, 104.8, 55.2, 18.5, 18.2, 13.0; HRMS (ESI) calcd for $C_{20}H_{23}N_4OS$ $[M+H]^+$ 367.1593, found 367.1588.

5-Ethyl-6-((4-methoxyphenyl)thio)-N^4-(*m*-tolyl)pyrimidine-2,4-diamine (**14l**): Yellow solid, yield 62.58%. m.p. 186.7～187.5 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.94 (s, 1H), 7.55 (d, J=8.3 Hz, 1H), 7.50 (s, 1H), 7.42～7.36 (m, 2H), 7.13 (t, J=7.8 Hz, 1H), 6.99～6.93 (m, 2H), 6.78 (d, J=7.5 Hz, 1H), 5.76 (s, 2H), 3.78 (s, 3H), 2.70 (q, J=7.4 Hz, 2H), 2.28 (s, 3H), 1.10 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.0, 160.0, 159.5, 157.8, 140.4, 137.1, 136.4, 127.9, 122.7, 122.0, 120.6, 118.7, 114.4, 105.4, 55.2, 21.1, 18.2, 13.3; HRMS (ESI) calcd for $C_{20}H_{23}N_4OS$ $[M+H]^+$ 367.1593, found 367.1586.

5-Ethyl-6-((4-methoxyphenyl)thio)-N^4-(*p*-tolyl)-pyrimidine-2,4-diamine (**14m**): White solid, yield 64.41%. m.p. 170.6～171.9 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.97 (s, 1H), 7.55 (d, J=8.1 Hz, 2H), 7.39 (d, J=8.4 Hz, 2H), 7.06 (d, J=8.1 Hz, 2H), 6.95 (d, J=8.3 Hz, 2H), 5.70 (s, 2H), 3.77 (s, 3H), 2.69 (q, J=7.3 Hz, 2H), 2.25 (s, 3H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 163.8, 160.0, 159.4, 158.0, 137.9, 136.3, 130.9, 128.5, 121.9, 120.7, 114.4, 105.3, 55.2, 20.4, 18.2, 13.3; HRMS (ESI) calcd for $C_{20}H_{23}N_4OS$ $[M+H]^+$ 367.1593, found 367.1586.

5-Ethyl-N^4-(2-methoxyphenyl)-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14n**): Brown solid, yield 64.75%. m.p. 168.3～168.8 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.28 (dd, J=8.0, 1.7 Hz, 1H), 7.40 (d, J=9.0 Hz, 3H), 7.05～6.98 (m, 2H), 6.96 (d, J=8.5 Hz, 2H), 6.94～6.87 (m, 1H), 5.87 (s, 2H), 3.86 (s, 3H), 3.78 (d, J=1.5 Hz, 3H), 2.63 (q, J=7.5 Hz, 2H), 1.16 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 163.7, 160.2, 159.5, 157.6, 149.4, 136.4, 129.0, 122.6, 121.5, 120.4, 120.4, 114.5, 110.7, 105.0, 56.0, 55.2, 18.7, 12.5; HRMS (ESI) calcd for $C_{20}H_{23}N_4O_2S$ $[M+H]^+$ 383.1542, found 383.1535.

5-Ethyl-N^4-(3-methoxyphenyl)-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14o**): Brown solid, yield 65.83%. m.p. 126.5～126.8 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.99 (s, 1H), 7.42～7.37 (m, 3H), 7.32 (d, J=8.2 Hz, 1H), 7.14 (t, J=8.2 Hz, 1H), 6.96 (d, J=8.6 Hz, 2H), 6.53 (dd, J=8.3, 2.3 Hz, 1H), 5.80 (s, 2H), 3.78 (d, J=1.6 Hz, 3H), 3.74 (s, 3H), 2.70 (q, J=7.4 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.2, 160.0, 159.5, 159.2, 157.7, 141.7, 136.4, 128.7, 120.5, 114.4, 113.5, 107.7, 106.7, 105.5, 55.2, 54.9, 18.2, 13.3; HRMS (ESI) calcd for $C_{20}H_{23}N_4O_2S$ $[M+H]^+$ 383.1542, found 383.1535.

5-Ethyl-N^4-(4-methoxyphenyl)-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14p**): Yellow solid, yield 71.35%. m.p. 135.8～136.1 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.95 (s, 1H), 7.56～7.49 (m, 2H), 7.41～7.35 (m, 2H), 6.98～6.92 (m, 2H), 6.87～6.80 (m, 2H), 5.63 (s, 2H), 3.77 (d, J=1.8 Hz, 3H), 3.72 (d, J=1.8 Hz, 3H), 2.67 (q, J=7.4 Hz, 2H), 1.11 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 163.5, 160.1, 159.4, 158.2, 154.8, 136.3, 133.4, 123.8, 120.8, 114.4, 113.3, 105.1, 55.2, 18.5, 18.2, 13.2; HRMS (ESI) calcd for $C_{20}H_{23}N_4O_2S$ $[M+H]^+$ 383.1542, found 383.1536.

N^4-(3,4-Dichlorophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14q**): White solid, yield 78.43%. m.p. 156.8～157.3 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.26 (s, 1H), 8.07 (d, J=2.4 Hz, 1H), 7.86 (dd, J=9.0, 2.3 Hz, 1H), 7.45 (d, J=8.9 Hz, 1H), 7.40 (d, J=8.3 Hz, 2H), 6.97 (d, J=8.3 Hz, 2H), 5.95 (s, 2H), 3.79 (s, 3H), 2.69 (q, J=7.3 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 165.1, 159.9, 159.6, 157.2, 140.9, 136.6, 130.3, 129.7, 122.9, 121.9, 120.9, 120.1, 114.5, 105.6, 55.2, 18.1, 13.3; HRMS (ESI) calcd for $C_{19}H_{19}Cl_2N_4OS$ $[M+H]^+$ 421.0657, found 421.0651.

N^4-(2,4-Dichlorophenyl)-5-ethyl-6-((4-methoxyphenyl)-thio)pyrimidine-2,4-diamine (**14r**): White solid, yield 74.47%. m.p. 170.3～171.0 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.89 (s, 1H), 7.77 (d, J=8.7 Hz, 1H), 7.64 (d, J=2.3 Hz, 1H), 7.38 (t, J=9.7 Hz, 3H), 6.96 (d, J=8.3 Hz, 2H), 5.74 (s, 2H), 3.78 (s, 3H), 2.66 (q, J=7.4 Hz, 2H), 1.16 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.4, 160.1, 159.5, 158.1, 136.5, 136.2, 129.5, 128.7, 128.6, 127.3, 120.2, 114.5, 105.0, 55.2, 18.4, 12.9; HRMS (ESI) calcd for $C_{19}H_{19}Cl_2N_4OS$ $[M+H]^+$ 421.0657, found 421.0650.

N^4-(3-Chloro-4-fluorophenyl)-5-ethyl-6-((4-methoxyphenyl)thio)pyrimidine-2,4-diamine (**14s**): Yellow solid, yield 62.42%. m.p. 185.2～186.2 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.17 (s, 1H), 7.96 (dd, J=6.9, 2.5 Hz, 1H), 7.77 (dt, J=7.5, 3.3 Hz, 1H), 7.39 (d, J=8.3 Hz, 2H), 7.27 (t, J=9.1 Hz, 1H), 6.96 (d, J=8.3 Hz, 2H), 5.88 (s, 2H), 3.78 (s, 3H), 2.68 (q, J=7.4 Hz, 2H), 1.11 (t, J=7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.7, 159.9, 159.6, 157.4, 153.6 (d, J=242.1 Hz), 137.9 (d, J=2.8 Hz), 136.5, 122.4, 121.4 (d, J=6.6 Hz), 120.3, 118.5 (d, J=18.2 Hz), 116.1 (d, J=21.4 Hz), 114.5, 105.3, 55.2, 18.1, 13.3; HRMS (ESI) calcd for $C_{19}H_{19}ClFN_4OS$ $[M+H]^+$ 405.0952, found 405.0947.

5-Ethyl-6-((4-methoxyphenyl)thio)-N^4-(3,4,5-trimethoxyphenyl)pyrimidine-2,4-diamine (**14t**): Brown solid, yield 58.43%. m.p. 165.7～166.7 ℃; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.91 (s, 1H), 7.39 (d, J=8.3 Hz, 2H), 7.15 (s, 2H), 6.96 (d, J=8.3 Hz, 2H), 5.83 (s, 2H), 3.78 (s, 3H), 3.76 (s, 6H), 3.61 (s, 3H), 2.69 (q, J=7.4 Hz, 2H), 1.11 (t, J=7.4 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ: 164.1, 159.9, 159.5, 157.6, 152.2, 136.5, 136.4, 132.4, 120.5, 114.5,

105.3, 98.9, 60.0, 55.7, 55.2, 18.2, 13.3; HRMS (ESI) calcd for $C_{22}H_{27}N_4O_4S$ $[M+H]^+$ 443.1753, found 443.1747.

4.3 Antiproliferative activity evaluation

4.3.1 MTT assay

Cells in logarithmic growth phase were seeded in 96-well plates at 2000～3000 cells per well. After the cells were incubated for 24 h, 200 μL of different concentrations of drug-containing medium were added to each well, and 9 concentrations were set for each compound. Each concentration was 3 replicate wells, and a positive control group and a blank control group were set separately, and were incubated in a constant temperature incubator at 37 ℃ for 72 h. 20 μL of MTT solution with a concentration of 0.5 mg/mL was added to each well in the dark, incubated at 37 ℃ for 4 h in the dark. The culture medium was discarded, 150 μL of DMSO was add to each well, and then the solution was shaken horizontally on a shaker for 10 min. After the formazan was fully dissolved, the absorbance was measured at a wavelength of 490 nm with a microplate reader. IC_{50} values were calculated for each compound using SPSS Statistics.

4.3.2 Colony formation assay

HGC-27 cells in logarithmic growth phase were seeded in 6-well plates at 500 cells/well, and after culturing for 24 h, drug-containing medium with concentrations of 0, 0.25, 0.50, and 1.00 μmol/L was added, respectively. Continue to culture for 7～10 d at 37 ℃ and 5% CO_2, and terminate the culture when visible and appropriate cell colonies appear at the bottom of the 6-well plate. Cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS, and stained with 0.1% crystal violet for 30 min, then washed with water until there was no obvious purple color between the cell colonies. After the plate is naturally dried, images were photographed. Morphological analysis was performed through microscopy (Leica EZ4W, China). The experiment was repeated three times.

4.3.3 Scratch assay

Take HGC-27 cells in logarithmic growth phase and inoculate them in a 6-well plate. After the confluence rate reaches 100%. A cross was drawn at the bottom with a 200 μL pipette tip, and the exfoliated cells were washed off with PBS. Serum-free complete medium containing drug was added at concentrations of 0, 0.5, and 1.0 μmol/L, respectively, and photos were taken at the same scratch position under a microscope at 0, 12, and 24 h. Photoshop and Illustartor software were used to process Images.

4.3.4 Cell morphology analysis and DAPI staining

The HGC-27 cells in logarithmic growth phase were taken and inoculated in 6-well plates at 1×10^5 cells/well after counting. After cells adhered, cells were treated with different concentrations of compound **14t** (0, 0.25, 0.50, 1.00 μmol/L) for 48 h, incubated with DAPI staining solution. Three fields of each sample were selected and photographed with a fluorescence microscope (Nikon Eclipse Ti-S, Nikon Ltd, Japan), and the images were processed with Photoshop and Illustartor software.

4.3.5 Analysis of apoptosis by flow cytometry

Apoptosis of HGC-27 cells was collected and measured with the Annexin V-FITC/PI Apoptosis Detection Kit (Keygen Biotech, China), according to the manufacturer's instructions. HGC-27 cells in logarithmic growth phase were seeded in 6-well plates at 1×10^5 cells/well. After the cells adhered, different concentrations (0, 0.5, 1.0, 2.0 μmol/L) of compound **14t** were added to treat them for 48 and 72 h. Cells were incubated with Annexin V-FITC/PI staining solution, and cell apoptosis was detected by flow cytometry.

4.3.6 Western blot analysis

HGC-27 cells were treated with compound **14t** at different concentrations (0, 0.5, 1.0, 2.0 μmol/L). The intracellular proteins were extracted the RIPA lysate buffer (Beyotime Biotechnology) and protein contents were determined by the BCA protein quantification kit (Beyotime Biotechnology). Proteins were separated by the SDS-PAGE according to the molecular weight. Blots were blocked at room temperature for 2 h in 5% fat-free milk in PBS-Tween-20 (0.05%). The relevant primary anti-bodies (dilution 1∶1000) were incubated for overnight at 4 ℃ and then washed with PBS-Tween-20 (0.05%). The membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (dilution 1∶5000) and then washed with PBS-Tween-20 (0.05%). Blots exposure was performed with an ECL chemiluminescence solution. All bands were analyzed by Image J software. All the above experimental results were repeated three times.

Supporting Information The ^{1}H NMR, ^{13}C NMR and HRMS of compounds **14a**～**14t**. The Supporting Information is available free of charge via the Internet at http://sioc-journal.cn/.

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