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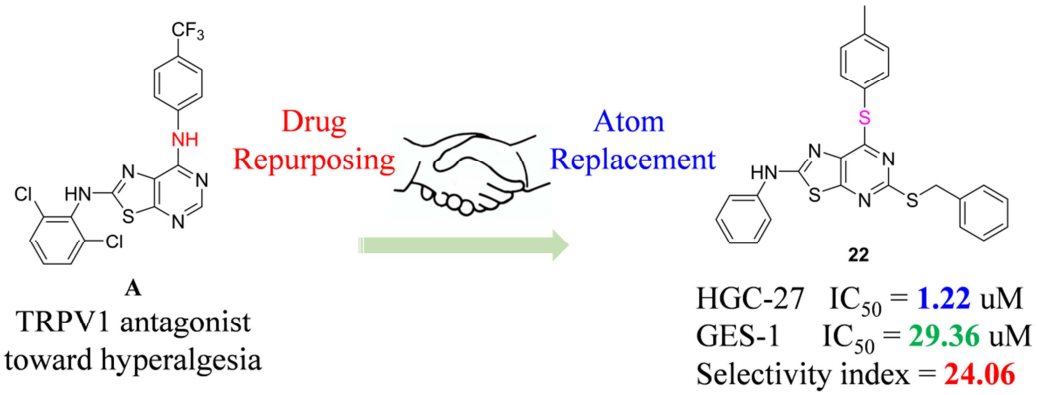
Graphic abstract



Design, synthesis and antiproliferative activity of thiazolo[5,4-d]pyrimidine derivatives through the atom replacement strategy

Zhong-Hua Li [1], Xue-Qi Liu [1], Peng-Fei Geng, Ji Zhang, Jin-Lian Ma, Bo Wang, Tao-Qian Zhao, Bing Zhao, Xin-Hui Zhang, Bin Yu, Hong-Min Liu**

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ABSTRACT:

A series of thiazolo[5,4-d]pyrimidine derivatives were designed through the atom replacement strategy based on biologically validated scaffolds and then evaluated for their antiproliferative activities on cancer cell lines. The structure-activity relationship studies were conducted, leading to the identification of compound **22**, which exhibited good antiproliferative activity against HGC-27 with an IC_{50} value of 1.22 μM and low toxicity against GES-1 cells. Mechanistic studies showed that compound **22** inhibited the colony formation and migration of HGC-27 as well as induced apoptosis. The western blot experiments proved that compound **22** up-regulated expression of Bax, down-regulated expression levels of Bcl-2 and cleaved caspased-3/9. These findings indicate that compound **22** may serve as a template for designing new agents for the treatment of human gastric cancers. The atom replacement strategy could be viable strategy for designing new anticancer drugs and may find its applications in drug design.

KEYWORDS: Thiazolo[5,4-d]pyrimidine, Antiproliferative activity, Apoptosis, Atom replacement

INTRODUCTION:

Drug repurposing (also termed as drug repositioning) has emerged as a valuable strategy for identifying new applications of old drugs [1, 2]. For example, thalidomide, as an anticonvulsive drug, is now used for the treatment of erythema nodosum leprosy and some cancers [3]. The antifungal drug itraconazole has advanced into several phase II clinical studies for the treatment of cancers [4]. Most recently, the Remya group designed a new series of 1-phenylpyrazole analogs as potent antitubercular agents based on the scaffold of anti-obesity drug rimonabant [5]. As part of our ongoing efforts toward identifying new anticancer agents [6-10], we screened our in-house small-molecule library against some cancer cell lines using the MTT (3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide) assay. Intriguingly, we found that compound **A** (Fig. 1) as a TRPV1 (vanilloid receptor 1) antagonist [11], showed acceptable antiproliferative activities against several cancer cell lines. Based on this scaffold, we have synthesized a series of amine substituted thiazole-pyrimidine derivatives and preliminarily evaluated their antiproliferative activities towards HGC-27 cell line[12]. Among them, compound **B** inhibited growth of HGC-27 cells with an IC_{50} value of about 20 μM. And compound **8** derived from compound **B** with the NH group replaced with the S atom (termed as the atom replacement strategy) showed selective inhibition toward gastric cancer cells HGC-27 and MGC-803 (IC_{50} = 8.87 and 9.33 μM, respectively). This finding promoted us to design new compounds targeting gastric cancer cells based on the scaffold of thiazolo[5,4-d]pyrimidine, finally leading to the identification of the most potent compound **22**, which inhibited growth of HGC-27 cell line with an IC_{50} values of 1.22 μM and exhibited around 24-fold selectivity between HGC-27 and normal gastric epithelial cell GES-1 (Fig. 1).

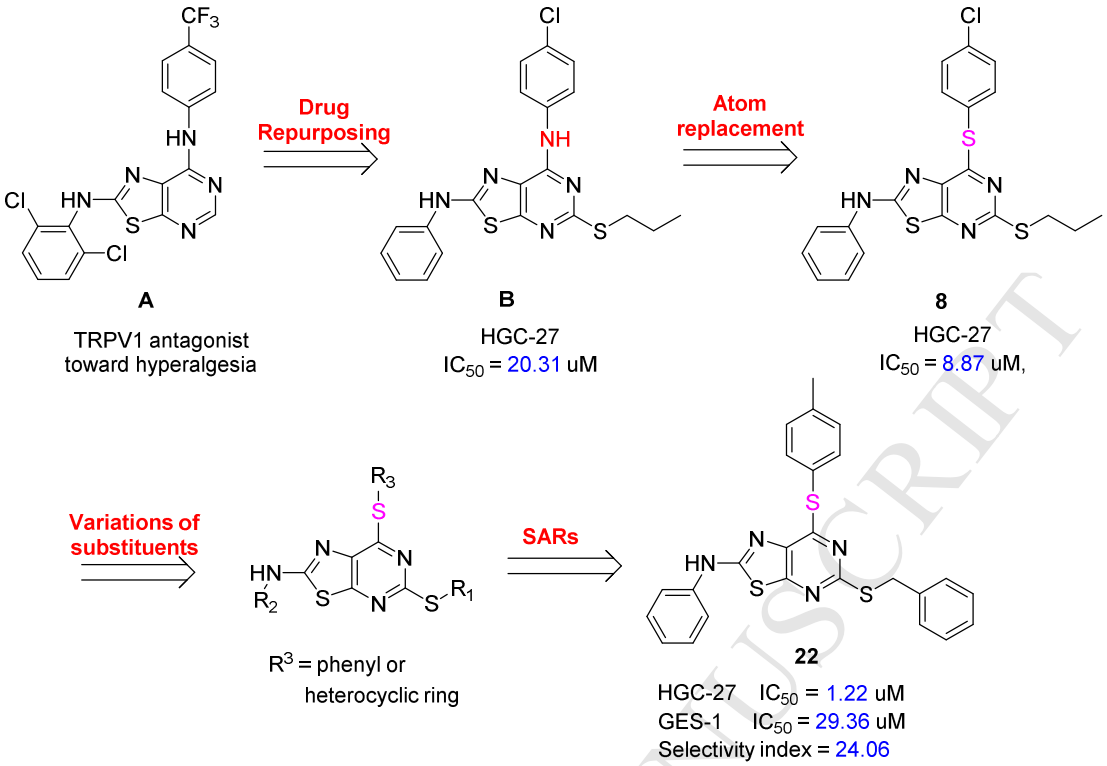
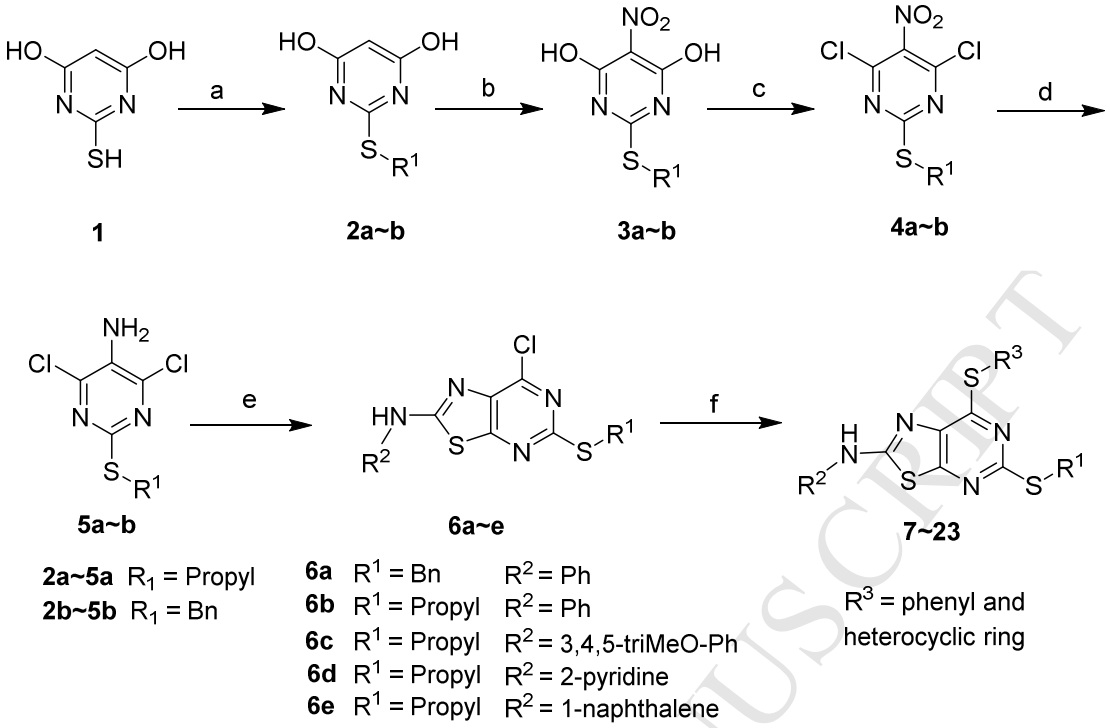


Fig. 1. Design of new compounds targeting HGC-27 cells based on the drug repurposing and atom replacement strategies.

2. Results and discussion

2.1 Chemistry

The general synthetic route was illustrated in Scheme 1. The intermediate derivatives **5a~b** were synthesized following the previously reported procedure [10]. Then isothiocyanate analogues reacted with **5a~b** in the presence of cesium carbonate in acetonitrile to give the key active intermediates **6a~e** [13]. The target compounds **7-23** were readily obtained through the NaI-catalyzed nucleophilic substitution reactions between mercapto analogues and compounds **6a-e** under alkaline conditions.



Scheme 1. Synthesis of thiazolo[5,4-d]pyrimidine derivatives. Reagent and conditions: (a) Alkyl bromide, TEA, methanol, reflux, 2h; (b) Fuming nitric acid, AcOH, 25~45 ^{o}C, 1 h; (c) $POCl_3$, DMA, reflux, 2 h; (d) Fe, AcOH, methanol, rt~reflux; (e) R^2SCN, Cs_2CO_3, acetonitrile, rt, overnight; (f) TEA, NaI, mercapto analogues, isopropanol, reflux, 6 h.

2.2 Evaluation of biological activity

2.2.1 Antiproliferative activity

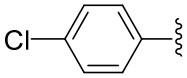
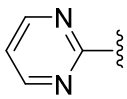
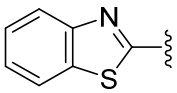
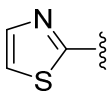
All target compounds were evaluated for their antiproliferative activities against human gastric cancer lines (MGC-803 and HGC-27), human breast cancer cell line (MCF-7) and human esophageal cancer cell line (EC-109) using the MTT assay, and 5-fluorouracil (5-FU) was employed as the reference drug. The preliminary antiproliferative results are summarized in Table 1.

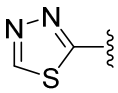
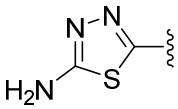
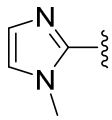
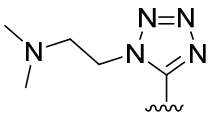
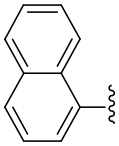
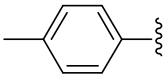
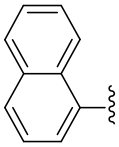
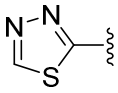
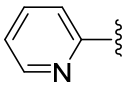
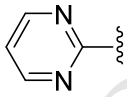
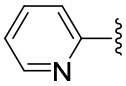
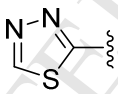
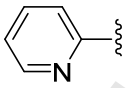
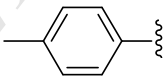
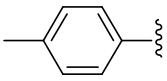
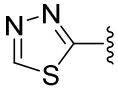
Generally, the synthesized compounds showed moderate to good antiproliferative activities against the tested cancer cell lines. As for EC-109 cells, most of the compounds showed weak inhibition (IC_{50} > 20 μM). It should be noted that compound **22** inhibited growth of EC-109 cells potently with an IC_{50} value of 2.69 μM, significantly much more potent than compound **7** (IC_{50} = 25.03 μM) and 5-FU (IC_{50} =

5.01 μM), while compound **23** was about 4-fold less potent (IC_{50} = 10.55 μM) than compound **22**, highlighting the importance of substituents attached to the thiazolo[5,4-d]pyrimidine scaffold for the activity. For MCF-7 cells, a similar trend was observed. Only compounds **12** and **22** inhibited the growth of MCF-7 cells with the IC_{50} values less than 10 μM, comparable to that of 5-FU.

Interestingly, this series of compounds exhibited improved potency against human gastric cancer lines MGC-803 and HGC-27 and certain selectivity to HGC-27 over MGC-803. For MGC-803 cells, some compounds showed comparable activity with 5-FU. Compound **7** showed the best inhibitory effect against MGC-803 cells with an IC_{50} value of 4.73 μM. Of note, this series of compounds displayed improved inhibition against HGC-27 cells, compounds **7**, **11**, **14**, and **22** inhibited HGC-27 cells potently with the IC_{50} values less than 5 μM. Among these compounds, compound **22** showed the best inhibition (IC_{50} = 1.22 μM), about 9-fold more potent than 5-FU. Besides, compound **22** also showed good inhibition against EC-109 cells with an IC_{50} values of 2.69 μM.

Table 1. Antiproliferative activities of thiazolo[5,4-d]pyrimidine derivatives against four cancer cell lines.

NO.	R^1	R^2	R^3	IC_{50} (μM)[a]			
				MGC-803	HGC-27	MCF-7	EC-109
7	Propyl	Ph		4.73±0.67	4.65±1.02	>64	25.03±1.59
8	Propyl	Ph		9.33±1.97	8.87±0.94	44.23±2.88	19.55±2.69
9	Propyl	Ph		9.82±0.99	9.55±2.13	13.22±2.45	49.55±3.20
10	Propyl	Ph		14.16±2.15	12.21±1.94	>64	24.32±1.55
11	Propyl	Ph		23.41±3.36	2.21±0.34	>64	>64

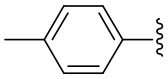
12	Propyl	Ph		10.66±1.02	5.07±0.70	8.55±2.65	>64
13	Propyl	Ph		12.85±1.10	>64	>64	>64
14	Propyl	Ph		30.02±2.47	4.03±0.60	>64	>64
15	Propyl	Ph		18.53±1.26	6.51±0.81	14.51±1.63	>64
16	Propyl			46.21±5.23	>64	>64	>64
17	Propyl			>64	22.65±1.35	>64	>64
18	Propyl			11.22±1.87	12.19±1.08	>64	>64
19	Propyl			10.22±2.36	18.19±1.26	11.58±2.00	>64
20	Propyl			>64	7.28±0.86	34.25±3.35	>64
21	Propyl	3,4,5-triMeO-Ph		>64	6.03±0.78	>64	23.87±0.99
22	Bn	Ph		12.21±1.91	1.22±0.18	8.55±0.55	2.69±0.64
23	Bn	Ph		>64	18.88±1.27	38.66±3.99	10.55±2.11
5-FU	-	-	-	6.89±1.33	11.02±2.28	7.21±0.91	5.01±0.72

[a] Inhibitory activity was assayed by exposure for 72 h to substance and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means ± SDs of three independent experiments.

The excellent antiproliferative activity of compound **22** promoted us to explore its

underlying toxicity against normal cells. Compound **22** was then subjected to the antiproliferative evaluation toward normal human gastric epithelialcells GES-1 by the MTT assay. As shown in Table 2, compound **22** inhibited GES-1 potently (IC_{50} = 29.36 μM), showing 24-fold selectivity between HGC-27 and GES-1. In contrast, 5-FU displayed no selectivity between HGC-27 and GES-1 with the IC_{50} values of 11.02 and 8.59 μM, respectively. These findings indicate that compound **22** may serve as a template for designing new agents for the treatment of human gastric cancers. The atom replacement strategy could be viable for designing new anticancer drugs.

Table 2. Inhibitory results of compound **22** against normal cells GES-1.

Comp	R^1	R^2	R^3N	IC_{50} (μM)[a]		SI[b]
				HGC-27	GES-1	
22	Bn	Ph		1.22±0.18	29.36±3.69	24.06
5-FU	-	-	-	11.02±2.28	8.59±1.38	0.78

[a] Inhibitory activity was assayed by exposure for 72 h to substance and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means ± SDs of three independent experiments.

[b]The selectivity index (SI) was calculated as IC_{50} (GES-1) / IC_{50} (HGC-27).

2.2.2 Clone assay and cell migration

The excellent antiproliferative activity of compound **22** against HGC-27 promoted us to investigate the effect on colony formation and cell migration. The clone formation of cancer cells represents an indirect estimation of neoplastic transformation [14]. As shown in Fig. 2A and 2B, treatment of HGC-27 cells with compound **22** led to fewer and smaller colonies compared to the control with an inhibition rate of 79% at 4 μM. In addition, the cell migration ability was also initially evaluated by the wound healing assay. As shown in Fig. 2C, treatment of HGC-27 cells with compound **22** at indicated concentration markedly suppressed the wound healing in a concentration-dependent manner.

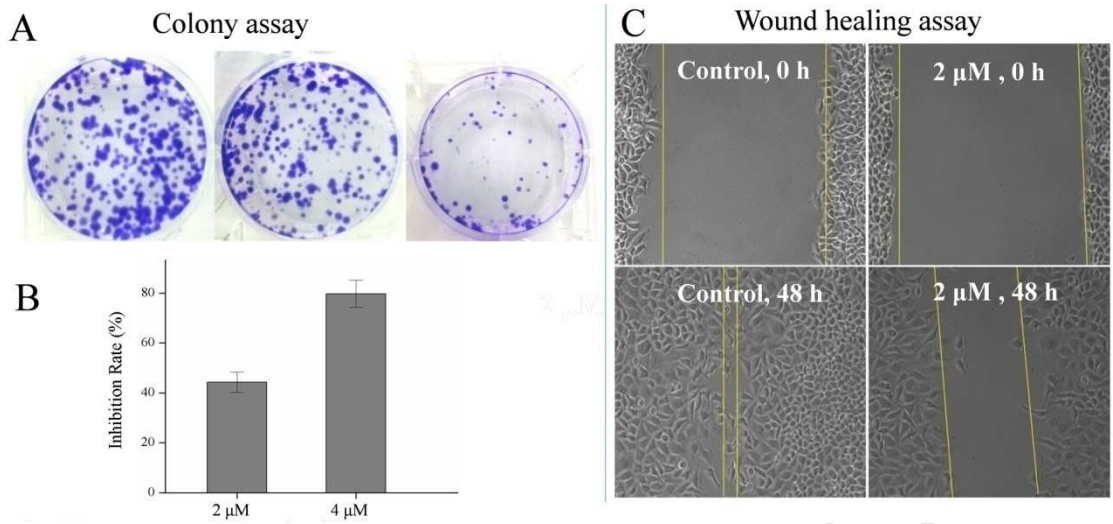


Fig. 2. (A) Representative images of HGC-27 cells colonies after treatment with various concentrations for 9 days; (B) Quantitative analysis of the colony formation inhibition rate; (C) Wound healing assay.

2.2.3 Cell apoptosis and possible mechanism involved

Apoptosis is typically characterized by distinctive cell morphological changes [15]. Compound **22** was chosen for evaluating its ability of inducing apoptosis. Hochest 33258 staining was performed to investigate the effect on morphological changes of HGC-27 cells [16]. After 24 h incubation with compound **22** at indicated concentrations, characteristic apoptotic morphological changes were observed, especially at high concentrations, including cell rounding, chromatin shrinkage and formation of apoptotic bodies (Fig. 3A). To further explore the effect of compound **22** on cell apoptosis, the apoptotic analysis was also performed with Annexin V-FITC/PI double staining and analyzed with high content [17]. Treatment of HGC-27 cells with compound **22** induced apoptosis in a concentration-dependent manner (Fig. 3B), the percentage of apoptotic cells was 10.01%, 31.8%, and 67.9%, respectively, compared to the control (1.8%) (Fig. 3C).

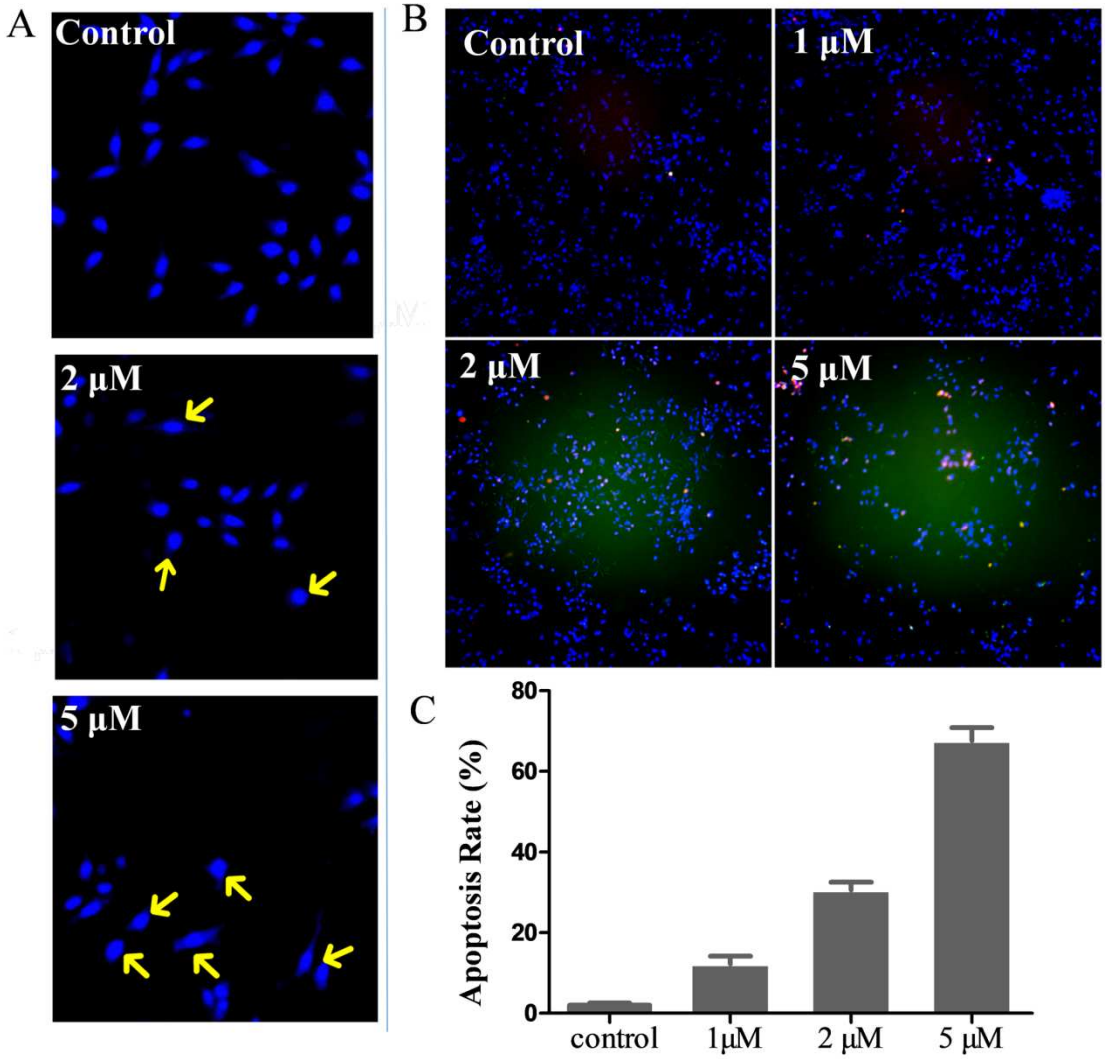


Fig. 3. Compound **22** induced apoptosis of HGC-27 cells. (A) Apoptosis analysis with Hoechst-33258 staining; (B, C) Quantitative analysis of apoptotic cells using Annexin V-FITC/PI double staining and high content analysis. Data are the mean ± SD. All experiments were carried out at least three times.

Next, the Western blot analysis was performed to examine the expression of apoptosis-related proteins. As shown in Fig. 4A, treatment of HGC-27 cells with compound **22** resulted in an increased expression of Bax in a concentration-dependent manner (Fig. 4B). Bax was able to activate the caspases, and promoted the release of cytochrome c and other pro-apoptotic factors from the mitochodria [18]. Meanwhile, the expression levels of anti-apoptotic protein Bcl-2 decreased accordingly (Fig. 4C). As shown in Fig. 4D and 4E, treatment of HGC-27 cells with compound **22**

concentration-dependently activatedcaspase-3 and caspase-9. These results indicated that compound **22** may induce HGC-27 apoptosis through the intrinsic apoptotic pathway.

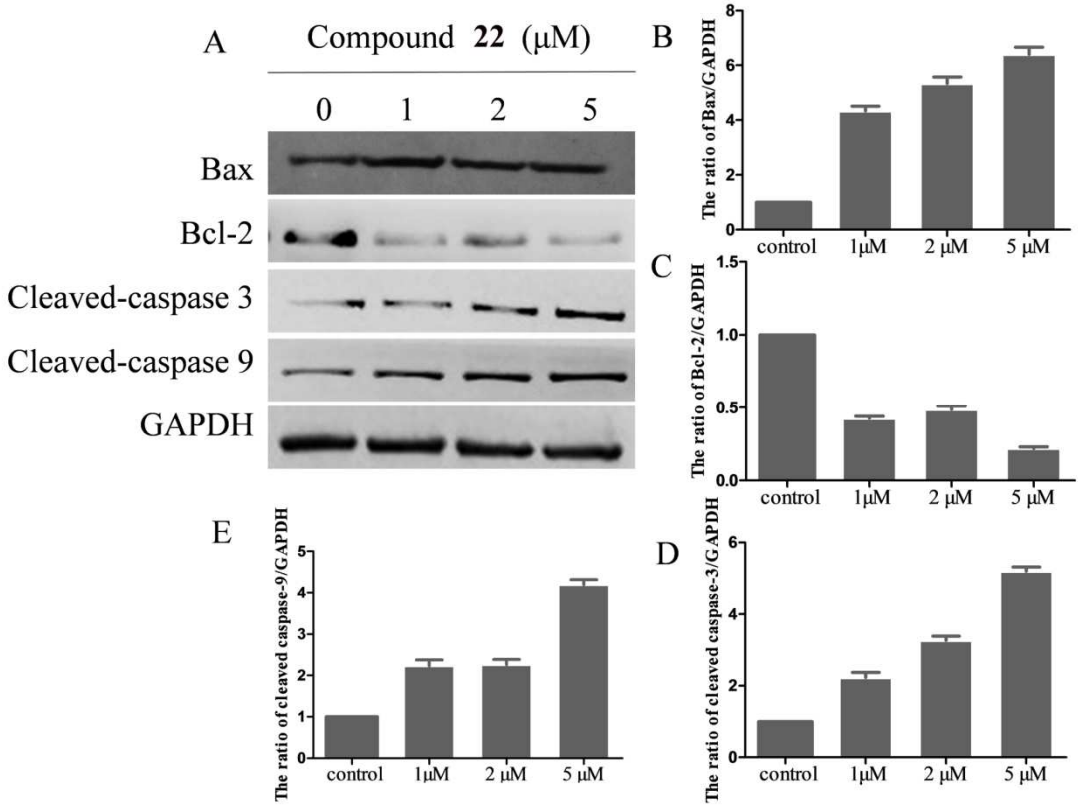


Fig. 4. Expression changes of apoptosis-related proteins induced by compound **22**. (**A**) Compound **22** induced expression changes of Bax, Bcl-2 and caspase family members in HGC-27 cells; (**B-E**) Statistical analysis of expression levels of Bax, Bcl-2 and cleaved caspase -3/-9.

3. Conclusions

In summary, a new series of thiazolo[5,4-d]pyrimidine derivatives were designed based on previously identified scaffolds through the atom replacement strategy and then evaluated for their antiproliferative activity. Among them, compound **22** exhibited good inhibition against HGC-27 and was less toxic to GES-1, indicating a good selectivity to cancer cells over normal cells. Further mechanistic investigations showed that compound **22** can inhibit the cell colony formation and migration, induce apoptosis through the intrinsic apoptotic pathway. These findings indicate that compound **22** may serve as a template for designing new agents for the treatment of

human gastric cancers. The atom replacement strategy could be viable for designing new anticancer drugs.

4. Experimental section

4.1 General

Reagents and solvents were purchased from commercial sources and were used without further purification. Melting points were determined on an X-5 micromelting apparatus and are uncorrected. ^{1}HNMR and ^{13}CNMR spectra were recorded on a Bruker 400 MHz and 100 MHz spectrometer respectively. High resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionizaton (ESI).

4.2 General procedure for the synthesis of compounds 7-23

The mixture of **6b** (1 eq), appropriate thiophenol or mercapto heterocyclic analogues (1.5 eq), triethylamine (2 eq) and catalytic amount of sodium iodide was refluxed in isopropanol for 6 h and monitored by thin layer chromatography (TLC) (petroleum (PE) / ethyl acetate (EA) = 4:1 ~ 1:1, v/v). After the completion of the reaction, the reaction mixture was cooled to room temperature and diluted with ethyl acetate, then washed with water for three times. The organic phase was dried with anhydride sodium sulfate and concentrated under reduced vacuum. The residue was purified by flash column chromatography (PE/EA = 4:1 ~ 1:1) to give the target products.

*4.2.1 N-phenyl-5-(propylthio)-7-(p-tolylthio)thiazolo[5,4-d]pyrimidin-2-amine (**7**)*

White solid, Mp 203~204 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.11 (br, 1H), 7.76-7.79 (m, 2H), 7.50-7.52 (m, 2H), 7.38-7.42 (m, 2H), 7.31-7.33 (m, 2H), 7.07-7.11 (m, 1H), 2.64-2.68 (t, J = 7.2 Hz, 2H), 2.37 (s, 3H), 1.32-1.37 (m, 2H), 0.73-0.77 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.0, 160.2, 158.8, 155.7, 139.9, 139.3, 136.7, 135.6, 130.0, 129.9, 129.1, 128.1, 123.5, 122.9, 118.2, 32.3, 22.3, 20.9, 13.0. HR-MS (ESI): Calcd. $C_{21}H_{20}N_4S_3$, $[M+H]^+$m/z: 425.0928, found: 425.0929.

*4.2.2 7-((4-Chlorophenyl)thio)-N-phenyl-5-(propylthio)thiazolo[5,4-d]pyrimidin-2-amine (**8**)*

White solid, Mp 217~219 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.04 (s, 1H), 7.74-7.77 (m, 2H), 7.65-7.68 (m, 2H), 7.57-7.59 (m, 2H), 7.39-7.43 (m, 2H), 7.08-7.12 (m, 1H), 2.66-2.69 (t, *J* = 7.2 Hz, 2H), 1.33-1.39 (m, 2H), 0.76-0.80 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.1, 160.5, 158.9, 154.7, 139.7, 137.4, 136.8, 134.7, 129.3, 129.1, 126.2, 123.0, 118.2, 32.4, 22.2, 13.1. HR-MS (ESI): Calcd. $C_{20}H_{17}ClN_4S_3$, $[M+H]^+$m/z: 445.0382, found: 445.0388.

*4.2.3 N-phenyl-5-(propylthio)-7-(pyrimidin-2-ylthio)thiazolo[5,4-d]pyrimidin-2-amine (**9**)*

Pale yellow solid, Mp 88~91 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.93 (s, 1H), 8.75-8.77 (m, 2H), 7.37-7.41 (m, 3H), 7.24-7.28 (m, 2H), 7.02-7.06 (m, 1H), 3.00-3.03 (t, *J* = 7.2 Hz, 2H), 1.61-1.66 (m, 2H), 0.92-0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.5, 163.1, 162.2, 158.4, 158.3, 150.8, 140.7, 139.4, 128.9, 123.1, 119.4, 118.1, 32.5, 22.0, 13.2. HR-MS (ESI): Calcd. $C_{18}H_{16}N_6S_3$, $[M+Na]^+$m/z: 435.0496, found: 435.0502.

*4.2.4 7-(Benzo[d]thiazol-2-ylthio)-N-phenyl-5-(propylthio)thiazolo[5,4-d]pyrimidin-2-amine (**10**)*

White solid, Mp 156~158 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.44 (s, 1H), 8.19 (m, 1H), 8.08 (m, 1H), 7.75 (m, 2H,), 7.50-7.60 (m, 2H), 7.35-7.39 (m, 2H), 7.07-7.11 (m, 1H), 2.85-2.89 (t, *J* = 7.4 Hz, 2H), 1.42-1.48 (m, 2H), 0.70-0.74 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, DMSO) δ 162.3, 161.8, 159.4, 157.4, 151.9, 150.3, 139.6, 137.4, 136.7, 129.1, 126.6, 125.8, 123.2, 122.6, 122.0, 118.4, 32.4, 22.1, 12.9. HR-MS (ESI): Calcd. $C_{21}H_{17}N_5S_4$, $[M+Na]^+$m/z: 490.0264, found: 490.0266.

*4.2.5 N-phenyl-5-(propylthio)-7-(thiazol-2-ylthio)thiazolo[5,4-d]pyrimidin-2-amine (**11**)*

Pale yellow solid, Mp 185~187 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (m, 1H), 7.74 (br, 1H), 7.61 (m, 1H), 7.48-7.50 (m, 2H), 7.38-7.42 (m, 2H), 7.17-7.21 (m, 1H), 2.86-2.90 (t, *J* = 7.3 Hz, 2H), 1.56-1.61 (m, 2H), 0.92-0.96 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.0, 161.2, 160.9, 154.7, 153.6, 143.5, 138.6, 137.0, 129.7, 125.1, 124.3, 120.3, 33.3, 22.6, 13.5. HR-MS (ESI): Calcd. $C_{17}H_{15}N_5S_4$, $[M+H]^+$m/z: 418.0289, found: 418.0286.

*4.2.6 7-((1,3,4-thiadiazol-2-yl)thio)-N-phenyl-5-(propylthio)thiazolo[5,4-d]pyrimidin-2-amine (**12**)*

Pale yellow solid, Mp 229~231 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.07 (s, 1H), 9.88 (s, 1H), 7.72-7.74 (m, 2H), 7.40-7.44 (m, 2H), 7.10-7.14 (m, 1H), 2.94-2.97 (t, *J* = 7.2 Hz, 2H), 1.54-1.59 (m, 2H), 0.90-0.94 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.4, 161.4, 159.6, 157.9, 156.8, 149.2, 139.5, 137.2, 129.2, 123.3, 118.4, 32.5, 22.0, 13.2. HR-MS (ESI): Calcd. $C_{16}H_{14}N_6S_4$, $[M+Na]^+$m/z: 441.0060, found: 441.0060.

*4.2.7 7-((5-Amino-1,3,4-thiadiazol-2-yl)thio)-N-phenyl-5-(propylthio)thiazolo[5,4-d] pyrimidin-2-amine (**13**)*

Off-white solid, Mp 273~275 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.41 (s, 1H), 7.80 (m, 2H), 7.74-7.77 (s, 2H), 7.40-7.44 (m, 2H), 7.08-7.12 (m, 1H), 2.91-2.95 (t, *J* = 7.2 Hz, 2H), 1.53-1.58 (m, 2H), 0.91-0.94 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.2, 162.3, 161.3, 159.4, 152.1, 140.7, 139.7, 137.0, 129.1, 123.1, 118.3, 32.6, 22.3, 13.1. HR-MS (ESI): Calcd. $C_{16}H_{15}N_7S_4$, $[M+Na]^+$m/z: 456.0169, found: 456.0173.

*4.2.8 7-((1-Methyl-1H-imidazol-2-yl)thio)-N-phenyl-5-(propylthio)thiazolo[5,4-d] pyrimidin-2-amine (**14**)*

Pale yellow solid, Mp 230~231 °C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.00 (s, 1H), 7.74-7.76 (m, 2H), 7.59 (m, 1H), 7.41-7.45 (m, 2H), 7.17 (m, 1H), 7.10-7.14 (m, 1H), 3.65 (s, 3H), 2.68-2.71 (t, *J* = 7.2 Hz, 2H), 1.41-1.46 (m, 2H), 0.85-0.89 (t, *J* = 7.3 Hz,

3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.4, 160.9, 159.3, 153.9, 139.6, 137.0, 132.1, 129.9, 129.2, 125.7, 123.2, 118.4, 33.5, 32.4, 22.1, 13.2. HR-MS (ESI): Calcd. $C_{18}H_{18}N_6S_3$, $[M+H]^+$m/z: 415.0833, found: 415.0834.

*4.2.9 7-((1-(2-(Dimethylamino)ethyl)-1H-tetrazol-5-yl)thio)-N-phenyl-5-(propylthio) thiazolo[5,4-d]pyrimidin-2-amine (**15**)*

Yellow solid, Mp 187~188 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.19 (br, 1H), 7.71-7.74 (m, 2H), 7.41-7.45 (m, 2H), 7.11-7.15 (m, 1H), 4.52-4.55 (t, *J* = 6.1 Hz, 2H), 2.65-2.71 (m, 4H), 2.10 (s, 6H), 1.38-1.43 (m, 2H), 0.84-0.88 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.3, 161.9, 159.7, 149.8, 146.6, 139.5, 137.6, 129.2, 123.4, 118.5, 57.5, 46.0, 44.9, 32.3, 22.0, 13.0. HR-MS (ESI): Calcd. $C_{19}H_{23}N_9S_3$, $[M+H]^+$m/z: 474.1317, found: 474.1315.

*4.2.10 N-(naphthalen-1-yl)-5-(propylthio)-7-(p-tolylthio)thiazolo[5,4-d]pyrimidin-2-amine (**16**)*

Pale yellow solid, Mp 208~210 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.92 (s, 1H), 8.24-8.27 (m, 1H), 8.17 (m, 1H), 8.00 (m, 1H), 7.82-7.85 (m, 1H), 7.60-7.63 (m, 3H), 7.50 (m, 2H), 7.32 (m, 2H), 2.62-2.66 (t, *J* = 7.2 Hz, 2H), 2.37 (s, 3H), 1.30-1.36 (m, 2H), 0.72-0.76 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.6, 162.3, 160.7, 156.1, 139.9, 137.2, 136.2, 135.5, 134.4, 130.4, 128.9, 127.4, 127.0, 126.8, 126.4, 126.0, 123.9, 122.6, 120.1, 32.8, 22.8, 21.4, 13.5. HR-MS (ESI): Calcd. $C_{25}H_{22}N_4S_3$, $[M+H]^+$m/z: 475.1085, found: 475.1082.

*4.2.11 7-((1,3,4-Thiadiazol-2-yl)thio)-N-(naphthalen-1-yl)-5-(propylthio)thiazolo [5,4-d]pyrimidin-2-amine (**17**)*

Pale yellow solid, Mp 160~162 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.16 (s, 1H), 9.89 (s, 1H), 8.25 (m, 1H), 8.11-8.14 (m, 1H), 8.00-8.03 (m, 1H), 7.86 (m, 1H), 7.59-7.63 (m, 3H), 2.92-2.96 (t, *J* = 7.2 Hz, 2H), 1.52-1.57 (m, 2H), 0.89-0.92 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.6, 162.1, 158.6, 157.2, 149.6,

137.7, 135.2, 134.4, 128.9, 127.4, 127.0, 126.9, 126.4, 122.6, 120.4, 33.0, 22.6, 13.6. HR-MS (ESI): Calcd. $C_{20}H_{16}N_6S_4$, $[M+Na]^+$m/z: 491.0217, found: 491.0217.

*4.2.12 5-(Propylthio)-N-(pyridin-2-yl)-7-(pyrimidin-2-ylthio)thiazolo[5,4-d]pyrimidin-2-amine (**18**)*

Pale yellow solid, Mp 225~228 ^oC. ^1H NMR (400 MHz, DMSO-d_6) δ 12.18 (s, 1H), 8.75 (m, 2H), 8.38 (m, 1H), 7.78-7.83 (m, 1H), 7.43 (m, 1H), 7.07-7.13 (m, 2H), 2.93-2.97 (t, *J* = 7.2 Hz, 2H), 1.54-1.62 (m, 2H), 0.88-0.92 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 167.7, 164.1, 162.7, 159.1, 157.4, 151.6, 151.0, 146.9, 139.3, 138.6, 120.0, 118.4, 112.0, 33.0, 22.6, 13.7. HR-MS (ESI): Calcd. $C_{17}H_{15}N_7S_3$, $[M+Na]^+$m/z: 436.0449, found: 436.0450.

*4.2.13 7-((1,3,4-Thiadiazol-2-yl)thio)-5-(propylthio)-N-(pyridin-2-yl)thiazolo[5,4-d]pyrimidin-2-amine (**19**)*

Yellow solid, Mp 249~250 ^oC. ^1H NMR (400 MHz, DMSO-d_6) δ 12.30 (s, 1H), 9.91 (s, 1H), 8.38 (m, 1H), 7.80-7.84 (m, 1H), 7.15 (m, 1H), 7.07-7.10 (m, 1H), 2.96-3.00 (t, *J* = 7.2 Hz, 2H), 1.55-1.61 (m, 2H), 0.91-0.95 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.8, 162.5, 158.6, 158.1, 157.1, 150.9, 146.9, 139.3, 135.0, 118.4, 112.1, 33.0, 22.6, 13.7. HR-MS (ESI): Calcd. $C_{15}H_{13}N_7S_4$, $[M+Na]^+$m/z: 442.0013, found: 442.0016.

*4.2.14 5-(Propylthio)-N-(pyridin-2-yl)-7-(p-tolylthio)thiazolo[5,4-d]pyrimidin-2-amine (**20**)*

White solid, Mp 242~244 ^oC. ^1H NMR (400 MHz, DMSO-d_6) δ 12.17 (s, 1H), 8.37-8.39 (m, 1H), 7.79-7.83 (m, 1H), 7.52 (m, 2H), 7.33 (m, 2H), 7.15 (m, 1H), 7.05-7.08 (m, 1H), 2.64-2.68 (t, *J* = 7.2 Hz, 2H), 2.38 (s, 3H), 1.30-1.39 (m, 2H), 0.73-0.77 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.6, 161.2, 157.3, 157.1, 151.2, 147.0, 140.0, 139.2, 136.3, 134.6, 130.4, 123.8, 118.1, 111.9, 32.8, 22.8, 21.4, 13.5. HR-MS (ESI): Calcd. $C_{20}H_{19}N_5S_3$, $[M+Na]^+$m/z: 448.0700, found: 448.0703.

*4.2.15 5-(Propylthio)-7-(p-tolylthio)-N-(3,4,5-trimethoxyphenyl)thiazolo[5,4-d] pyrimidin-2-amine (**21**)*

White solid, Mp 188~190 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.96 (s, 1H), 7.50 (m, 2H), 7.32 (m, 2H), 7.22 (s, 2H), 3.84 (s, 6H), 3.66 (s, 3H), 2.62-2.65 (t, J = 7.2 Hz, 2H), 2.38 (s, 3H), 1.30-1.35 (m, 2H), 0.72-0.75 (t, J = 7.3 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.6, 160.4, 159.2, 156.2, 153.4, 139.9, 137.0, 136.4, 136.3, 133.6, 130.4, 123.8, 96.7, 60.6, 56.2, 32.8, 22.8, 21.4, 13.5. HR-MS (ESI): Calcd. $C_{24}H_{26}N_4O_3S_3$, $[M+Na]^+$m/z: 537.1065, found: 537.1064.

*4.2.16 5-(Benzylthio)-N-phenyl-7-(p-tolylthio)thiazolo[5,4-d]pyrimidin-2-amine (**22**)*

White solid, Mp 190~193 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.21 (s, 1H), 7.78 (m, 2H), 7.54 (m, 2H), 7.38-7.42 (m, 2H), 7.28 (m, 2H), 7.23 (m, 3H), 7.07-7.11 (m, 1H), 7.03 (m, 2H), 4.04 (s, 2H), 2.28 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.9, 160.8, 159.4, 156.1, 140.3, 140.0, 138.3, 137.4, 136.0, 130.4, 129.6, 129.1, 128.7, 127.4, 124.0, 123.5, 118.7, 34.8, 21.3. HR-MS (ESI): Calcd. $C_{25}H_{20}N_4S_3$, $[M+Na]^+$m/z: 495.0748, found: 495.0749.

*4.2.17 7-((1,3,4-Thiadiazol-2-yl)thio)-5-(benzylthio)-N-phenylthiazolo[5,4-d]pyrimi din-2-amine (**23**)*

Yellow solid, Mp 246~248 ^{o}C. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.38 (s, 1H), 9.83 (s, 1H), 7.76 (m, 2H), 7.40-7.44 (m, 2H), 7.25-7.35 (m, 5H), 7.10-7.14 (m, 1H), 4.32 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.2, 162.0, 160.3, 158.3, 157.5, 149.5, 140.0, 138.0, 137.5, 129.7, 129.4, 128.9, 127.7, 123.9, 118.9, 35.3. HR-MS (ESI): Calcd. $C_{20}H_{14}N_6S_4$, $[M+Na]^+$m/z: 489.0060, found: 489.0059.

4.3 Antiproliferative activity assays

Exponentially growing cells were seeded into 96-well plates at a concentration of 3,000 cells per well. After 24 h of incubation, the culture medium was removed and fresh medium containing various concentrations of the candidate compounds was

added to each well. The cells were then incubated for 72 h, thereafter MTT assays were performed and cell viability was assessed at 570 nm by a microplate reader (Biotech, Shanghai, China).

4.4 Clonogenicity assay

HGC-27 cells (1000 cells/well) were seeded in a 6-well plate and incubated for 24 h, then the media were replaced with fresh mediacontaining different concentrations of compound **22**. After 9 days of treatment, the cells were washed twice with PBS, fixed with 4% paraformaldehyde, and colonies were visualized using 0.1% crystal violet staining. The cells were imaged, and the number of colonies were quantified by Image J software (Developed by National Institutes of Health). A group of >10 cells was defined as one colony. Inhibition rate = (1-number oftreatment/number of control) * 100%. All experiments were performed in triplicate.

4.5 Hoechst 33258 staining

HGC-27 cells were seeded into a 6-well plate (2×10^5/well) and incubated overnight for adherent and treated with compound **22** at different concentration for 24 h, and underwent Hoechst 33258 staining for 30 min in the dark. The cells were observed under a Nikon Eclipse TE 2000-S fluorescence microscope (Nikon, Japan).

4.6 Wound healing assay

HGC-27 cells were placed in a 24-well plate, and the cell surface was scratched using a 10 μL pipet tip. Then the cells were treated with compound **22** with different concentrations followed by indicated time incubation and photographed on an inverted microsope.

4.7 Cell apoptosis assay

HGC-27 cells were seeded into a 6-well plate (2×10^5/well) and incubated for 24 h. Then the cells were treated with different concentrations of the tested compound **22** for 24 h. Thereafter, the cells were collected and the Annexin-V-FITC/PI apoptosis kit

(Biovision) was used according to the manufacturer's protocol. The cells were analyzed by high content screening system (ArrayScan XTI, Thermo Fisher Scientific, MA).

4.8 Western blot analysis

HGC-27 cells were treated with different concentrations of compound **22** for 24 h, the cells were collected, lysed in RIPA buffer contained a protease inhibitor cocktail for 30 min, followed by centrifugation at 12,000 rpm for 10 min at 4 ^{o}C. After the collection of supernatant, the protein concentration was detected using a bicinchonininc acid assay kit (Beyotmie Biotechnology, Haimen, China). After added with loading buffer, cell lyses were boiled for 10 min at 100 ^{o}C for SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose (NC) membranes. Then the membranes were blocked with 5% skim milk at room temperature for 2 h, and then incubated overnight at 4 ^{o}C with primary antibodies. After washing the membrane with the secondary antibody (1: 5000) at room temperature for 2 h. Finally, the blots were washed in TBST/TBS. The antibody-reactive were revealed by enhanced chemiluminescence (ECL) and exposed on Kodak radiographic film.

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Highlights

- The thiazolo[5,4-d]pyrimidine derivatives showed moderate to good growth inhibition against the tested cancer cells.
- Among them, compound **22** exerted the most antiproliferative activity against HGC-27 cell line.
- Compound **22** inhibited the cell colony formation and migration of HGC-27.
- Compound **22** induced the apoptosis of HGC-27 cells, and led to the expression changes of key proteins related to apoptosis.
- The atom replacement strategy would be viable for designing new anticancer agents.