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Thiosemicarbazone-based selective proliferation inactivators inhibit gastric cancer cell growth, invasion, and migration†

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A series of novel thiosemicarbazone derivatives were synthesized and evaluated for their antiproliferative activity against several selected tumor cell lines of different origins using the MTT assay. The preliminary results indicated that the MGC-803 cell line was remarkably sensitive to all the synthesized compounds. Among this series, compound **5n** showed the best inhibitory activity with an IC_{50} value of 0.93 μM (about 10-fold more potent than **3-AP**) against MGC-803. Further mechanism studies revealed that compound **5n** could obviously inhibit the proliferation of MGC-803 cells by inducing apoptosis and arresting the cell cycle at the S phase. Compound **5n** also showed marked inhibition of cell migration and invasion, without significant cytotoxicity against gastric epithelial immortalized GES-1 cells.

Introduction

Cancer, being one of the leading causes of death globally, poses a major socioeconomic hazard to humanity at large. Although there has been progress in the development of prevention and treatment of cancer, successful treatment of cancer remains a challenge. There is still an urgent need to search for some newer and safer anticancer agents that have a broader spectrum of cytotoxicity to cancer cells.

As reported, neoplastic cells have high requirements for metal due to their generally higher rates of proliferation than normal cells. Considering the crucial roles of these metals, development of novel metal chelators has become a promising anticancer strategy.[1] Many *in vitro* and *in vivo* studies and clinical trials have demonstrated that metal (Fe, Cu, and Zn) chelators are effective antiproliferative agents.[2]

Multi-functionalized thiosemicarbazones and their metal complexes have been a focus of chemists and biologists because of their wide range of therapeutic uses with antiviral, antiparasitic, antibacterial, anticonvulsant, and, most intriguingly, anticancer activity.[3–14] Encouraged by the modest results obtained with traditional metal chelators, a variety of novel thiosemicarbazone-based ligands with antiproliferative activity have been identified (Fig. 1), including **3-AP**,[15] Dp44mT,[16,17] Ap44mT,[18–21] and DpC.[22–24] In fact, **3-AP** has been investigated in over 20 phase I and phase II clinical trials in a variety of advanced cancers.[25–27] Unfortunately, **3-AP** has suffered multiple problems, including low efficacy in some tumor-types and serious side effects, which limit its clinical utility.[28–30] Therefore, highly selective chelators with strong toxicity toward cancer cells and less or no side effects on normal cells remain to be identified.

A key feature of progressive tumors is having the potential to invade neighboring tissue and to spread throughout the body and form metastatic lesions at distant sites.[31] In addition, there is increasing evidence that epithelial–mesenchymal transition (EMT) is a key step for cancer cell migration, invasion, and metastasis.[32] During the process of EMT, cells lose their epithelial characteristics such as epithelial morphology, cell polarity, and cell–cell contact, and gain mesenchymal properties such as fibroblastic morphology and increased migration and invasion, causing cancer cell metastasis.[33–36]

We have previously reported a novel series of pyrimidine-based hybrids with potent anticancer activity. One of the compounds, 6-LM (Fig. 1), a thiosemicarbazone-containing pyrimidine hybrid, showed marked inhibition of cell migration and invasion *in vitro* and *in vivo*.[37] To explore the mechanism of compound 6-LM regulated EMT more thoroughly, we designed a series of small molecules and evaluated their antitumor activity using thiosemicarbazone as a minimal scaffold.

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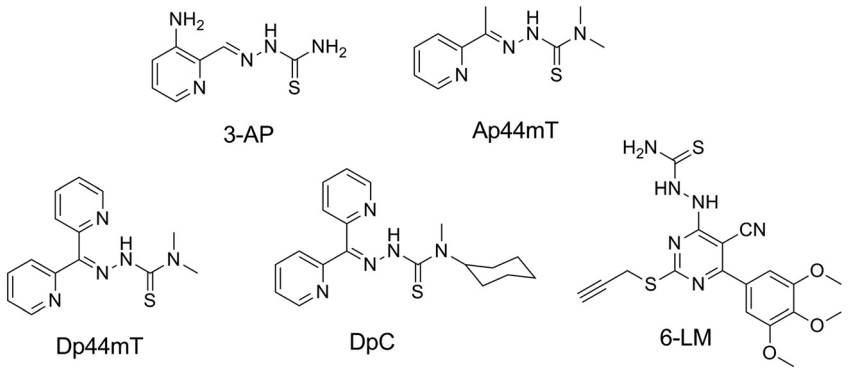


Fig. 1 Representative examples of thiosemicarbazone derivatives.

Results and discussion

Chemistry

The general route for the synthesis of the target thiosemicarbazones is depicted in Scheme 1. Commercially available **1a–c** were reacted with CS_2 and hydrazine hydrate in the presence of triethylamine to form compounds **4a–c**, which were subjected to reaction with an appropriately substituted aldehyde to afford the target compounds **5a–q** with good yields.

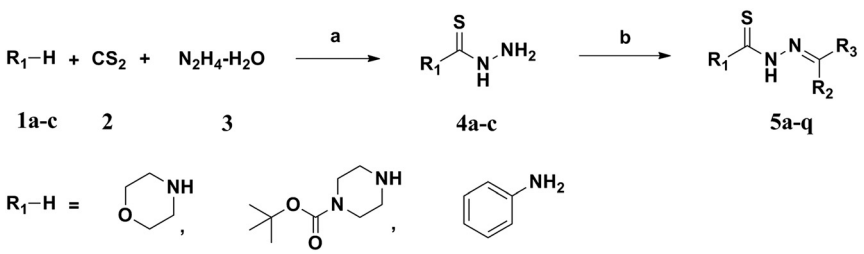
Evaluation of biological activity

Anti-proliferative activity. All the compounds synthesized in this study were examined for their anti-proliferative activity against three human cancer cell lines (commercially available from Cell Bank, Shanghai Institutes for Biological Sciences, Shanghai, China) such as MGC-803 (human gastric cancer cell line), SMMC-7721 (human liver cancer cell line), and MCF-7 (human breast cancer cell line). **3-AP** was chosen as a positive control. The results of preliminary evaluation are summarized in Table 1.

During the SAR studies, we found that all synthesized compounds showed a more potent inhibitory activity against MGC-803 than SMMC-7721 and MCF-7. This observation suggests that these compounds may act through targeting specific biological targets that are overexpressed in certain cells. In addition, a slight difference in inhibition of growth of MGC-803 cells was observed for compounds with different substitution patterns. A significant boost in potency came from the substitution with a methyl group in position R_2: compound **5p** was found to exhibit a single-digit micromolar inhibitory activity (IC_{50} = 3.06 μM). The same outcome was observed for **5k** (IC_{50} = 11.17 μM), an analog of compound **5a** (IC_{50} = 29.18 μM). Removing the hydroxyl group resulted in decreased potency (**5k** *vs.* **5j**), suggesting that the hydroxyl group is a net contributor to the *in vitro* potency of the molecule. In addition, it could be noticed that the 2-OH (**5k**) on the phenyl group (R_3) presented a more potent inhibitory effect than the 3-OH (**5l**) and 4-OH (**5m**) substitution. Also, the substitution of the phenyl ring with a chloro group in position 5 increased the potency (**5p** *vs.* **5k**, **5b** *vs.* **5a**). These results revealed that the position of the substituent on the phenyl group (R_3) has a remarkable effect on the inhibitory activity. In particular, compound **5n** showed the best inhibitory effect against MGC-803 with an IC_{50} value of 0.93 μM (10-fold more potent than **3-AP**).

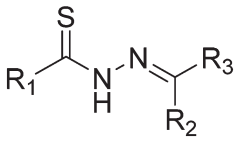
Because compound **5n** exhibits potent proliferative inhibitory activity *in vitro*, we further characterized the selectivity of this compound against GES-1 (normal human gastric epithelial cell line). As can be seen in Table 2, we found that compound **5n** did not show a significant inhibitory effect on GES-1, compared to the positive control, **3-AP**. Our findings indicate that compound **5n** has good selectivity between cancer and normal cells.

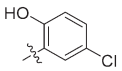
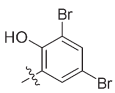
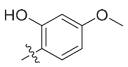
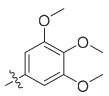
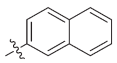
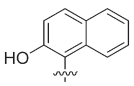
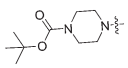
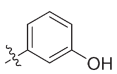
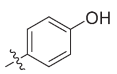
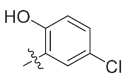
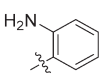
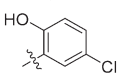
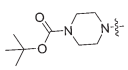
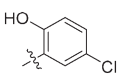
Among those compounds, we chose compound **5n**, exhibiting the most potent activity against MGC-803 with an IC_{50} value of 0.93 ± 0.02 μmol L^{-1}, to further investigate its inhibitory effect on MGC-803. As shown in Fig. 2A, the



Scheme 1 Reagents and conditions: a: (i) methanol, triethylamine, rt, 1 h; (ii) hydrazine hydrate 80%, reflux, 3 h; b: methanol, appropriate aldehyde, reflux, 4 h.

Table 1 Inhibitory results of the novel thiosemicarbazone derivatives against three cancer cell lines



Comp	R_1	R_2	R_3	IC_{50}[a] (μM) MGC-803	SMMC-7721	MCF-7
5a		H		29.18 ± 1.46	37.28 ± 1.57	>64
5b		H		16.31 ± 1.21	35.32 ± 1.54	>64
5c		H		20.38 ± 1.46	32.62 ± 1.51	>64
5d		H		56.05 ± 1.74	38.26 ± 1.58	>64
5e		H		>64	>64	>64
5f		H		32.59 ± 1.51	>64	>64
5g		H		>64	44.88 ± 1.65	>64
5h		H		10.17 ± 1.00	27.34 ± 1.43	41.92 ± 1.62
5i		H		2.42 ± 0.38	34.06 ± 1.53	12.38 ± 1.09
5j		CH_3		32.61 ± 1.10	>64	44.64 ± 1.65
5k		CH_3		11.17 ± 0.85	>64	49.23 ± 1.69
5l		CH_3		>64	>64	>64
5m		CH_3		>64	>64	>64
5n		CH_3		0.93 ± 0.02	19.18 ± 1.28	9.99 ± 1.00
5o		CH_3		>64	>64	>64
5p		CH_3		3.06 ± 0.48	>64	29.15 ± 1.46
5q		CH_3		58.37 ± 1.76	>64	>64
3-AP	—	—	—	9.68 ± 0.93	42.81 ± 1.63	18.85 ± 1.27

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

antiproliferative activity of compound **5n** against MGC-803 is presented in a dose-dependent and time-dependent manner with IC_{50} values of >64, 9.22 ± 0.96, and 0.93 ± 0.02 μmol L^{-1} at 24 h, 48 h, and 72 h, respectively. The colony formation assay shown in Fig. 2B also demonstrated that compound **5n** could inhibit the proliferation of MGC-803.

Table 2 Inhibitory results of compound **5n** and **3-AP** against the normal gastric epithelial cell line GES-1 and the gastric cancer cell line MGC-803

Comp	IC_{50}[a] (μM)	
	GES-1	MGC-803
5n	27.93 ± 1.254	0.93 ± 0.02
3-AP	5.40 ± 0.73	9.68 ± 0.93

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

To better understand the anti-proliferative activity of compound **5n** against MGC-803, cell-cycle analysis was performed. After treatment with compound **5n** for 48 h, it was observed that the percentage of cells in the S phase was 28.4%, 32.82%, 46.07%, and 51.85% at different concentrations of 0, 0.1, 0.25, and 0.5 μmol L^{-1}, respectively, which demonstrated that compound **5n** caused an obvious S-phase arrest pattern against MGC-803 in a dose-dependent manner.

Anti-migration and anti-invasion assays. As described in the introduction, the migration and invasion ability of tumor cells is an intractable problem in cancer therapy, while EMT is tightly associated with the migration and invasion of tumor cells. Excitingly, compound **5n** could potently inhibit this transition. As shown in Fig. 3, wound healing, transwell and matrigel-coated transwell assays all demonstrated that compound **5n** could inhibit the migration and invasion ability of MGC-803 in a dose-dependent manner.

We also examined the expression of some typical proteins of the EMT process. The western blotting assay showed that compound **5n** could up-regulate the expression of the epithelial cell biomarker, E-cadherin, while the mesenchymal cell biomarker, N-cadherin, is down-regulated. These data indicated that compound **5n** may impair the migration and invasion ability of MGC-803 cells through inhibiting the EMT process.

Cell apoptosis analysis. Inducing the apoptosis of tumor cells is considered a major way to defeat cancer. Due to its potent activity against MGC-803 cells, compound **5n** was chosen to evaluate its influence on the apoptosis of MGC-803 cells. We observed the morphology of MGC-803 under the treatment of compound **5n** at different concentrations (0, 0.1, 0.25, and 0.5 μmol L^{-1}) for 48 h, and the changes in the morphology were observed by fluorescence microscopy using Hoechst 33258 staining. As shown in Fig. 4A, with increasing compound concentration, the typical apoptotic markers, including cell rounding, chromatin shrinkage and apoptotic bodies, become more and more frequently compared with the control. Meanwhile, the data obtained using

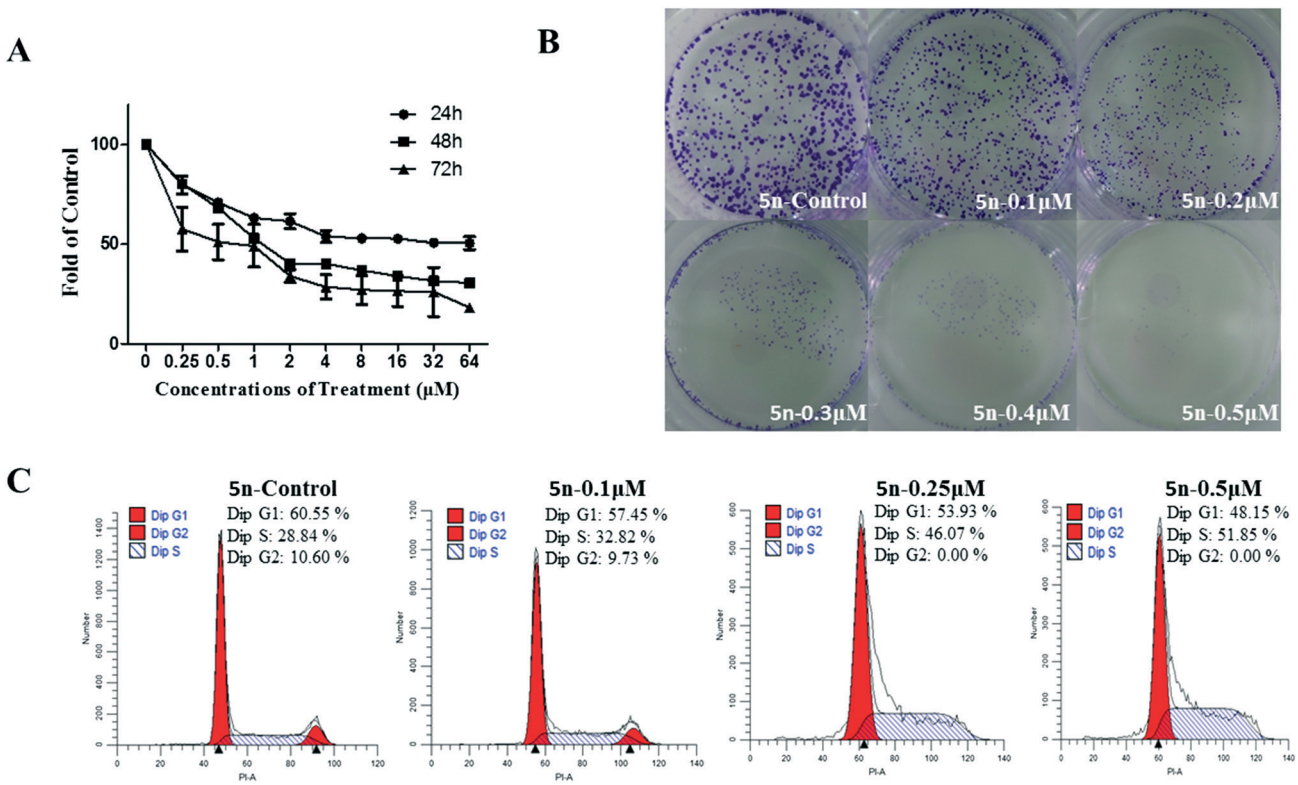


Fig. 2 Effects of compound **5n** on the proliferation of the human gastric cancer cell line MGC-803. (A) MGC-803 were treated with compound **5n** at different concentrations for 24, 48, and 72 h, respectively, then the MTT assay was used to evaluate its effect on cell viability; (B) the colony formation after treatment with compound **5n** at different concentrations for 7 days; (C) effect of compound **5n** on the cell cycle distribution of MGC-803. Cells were treated with different concentrations of compound **5n** for 48 h. Then, the cells were fixed and stained with PI to analyze their DNA content by flow cytometry. The experiments were performed three times, and a representative experiment is shown.

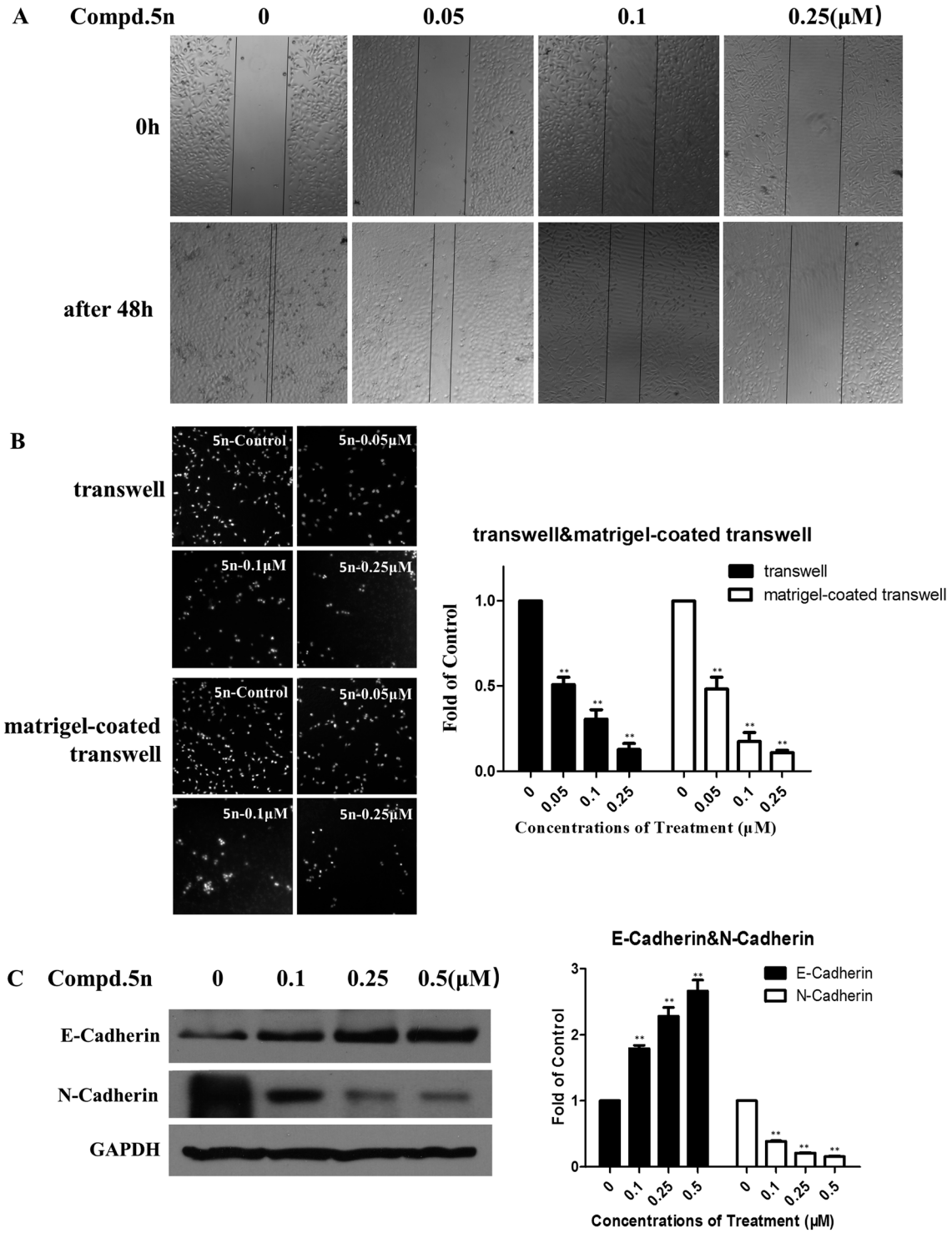


Fig. 3 Compound **5n** blocked the migration and invasion of MGC-803. (A) Anti-migration analysis performed using the wound healing assay after treatment of MGC-803 cells with compound **5n** for 48 h; (B) anti-migration and anti-invasion analyses performed using transwell and matrigel-coated transwell assays, respectively, after treatment of MGC-803 cells with compound **5n** for 48 h; (C) expression of E-cadherin and N-cadherin in MGC-803 cells after treatment with compound **5n** for 48 h. $^{**}P < 0.01$ was considered statistically highly significant. Data are presented as mean ± SD. All experiments were carried out at least three times.

Annexin V-FITC/PI double staining implied that after 48 h of treatment with compound **5n**, the total percentage of early apoptosis and late apoptosis increased up to 10.9%, 16.4%, and 32.3% compared to the control (3.7%), which was presented in a concentration-dependent manner (Fig. 4B).

To further clarify the mechanism of the apoptosis induced by compound **5n**, we investigated the key proteins in the

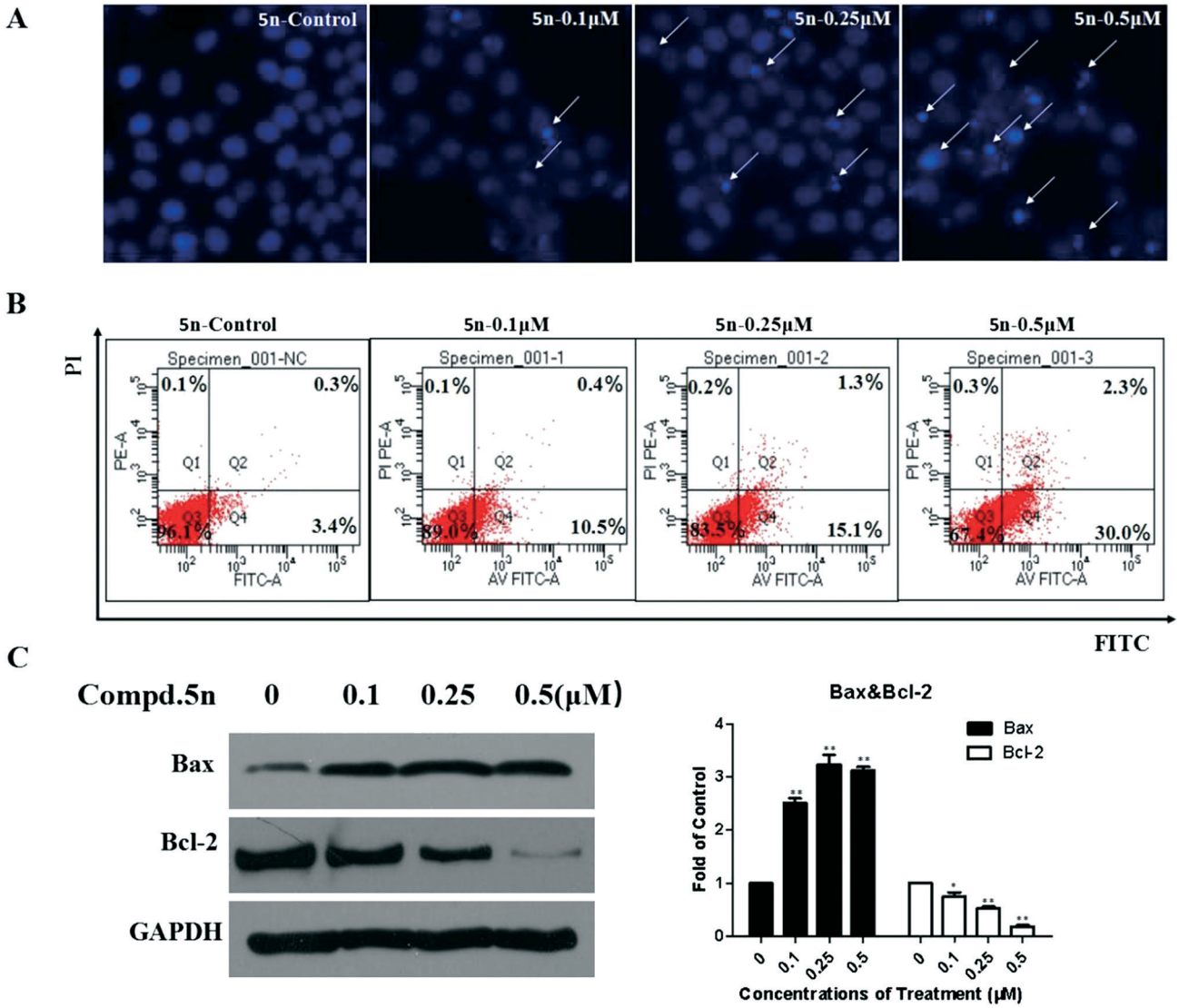


Fig. 4 Compound **5n** induced apoptosis in MGC-803. (A) Apoptosis analysis using Hoechst-33258 staining after treatment of MGC-803 cells with compound **5n** for 48 h; (B) quantitative analysis of apoptotic cells using Annexin V-FITC/PI double staining and flow-cytometry calculation; quadrant Q2 represents live cells, quadrant Q3 is for early/primary apoptotic cells, and quadrant Q4 is for late/secondary apoptotic cells, while quadrant Q1 represents cells damaged during the procedure; (C) expression of Bax and Bcl-2 in MGC-803 cells after treatment with compound **5n** for 48 h. $^{**}P < 0.01$ was considered statistically highly significant. Data are presented as mean ± SD. All experiments were carried out at least three times.

mitochondria-related apoptotic pathway. As shown in Fig. 4C, the pro-apoptotic protein, Bax, was up-regulated after the treatment with compound **5n**, while the anti-apoptotic protein, Bcl-2, was down-regulated, which were all presented in a concentration-dependent manner. These findings indicated that compound **5n** may be involved in mitochondria-related apoptosis.

Conclusions

In summary, we have synthesized and identified a series of thiosemicarbazone-based analogues as potent and selective antiproliferative agents to modulate cancer cell growth, migration, and invasion. The structure–activity relationship study showed that compound **5n** had the best inhibitory activity with an IC_{50} value of 0.93 μM (about 10-fold more potent than **3-AP**) against MGC-803 without significant cytotoxicity against gastric epithelial immortalized GES-1 cells. Further mechanism studies revealed that compound **5n** regulates the proliferation activity of MGC-803 cells by inducing apoptosis, arresting the cell cycle at the S phase, and inhibiting the EMT. Further SAR and mechanistic studies on the selectivity toward MGC-803 cells are ongoing in our laboratories and will be reported in due course.

Conflicts of interest

The authors declare no competing interests.

Acknowledgements

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