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Synthesis and biological evaluation of novel pyrimidine-benzimidazol hybrids as potential anticancer agents

Kun-Peng Shao[a], Xu-Yao Zhang[a], Peng-Ju Chen, Deng-Qi Xue, Peng He, Li-Ying Ma, Jia-Xin Zheng, Qiu-Rong Zhang[*], Hong-Min Liu[*]

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Abstract: A series of pyrimidine-benzimidazol hybrids was synthesized and evaluated for anticancer activity on four human cancer cell lines including MCF-7, MGC-803, EC-9706 and SMMC-7721. Some of the synthesized compounds exhibited moderate to potent activity against MGC-803 and MCF-7. Among them, compounds **5a-b** and **6a-b** showed most effective activity. Compounds **5b** and **6b** were more cytotoxic than 5-fluorouracil against all tested four human cancer cell lines, with IC_{50} values ranging from 2.03 to 10.55 μM and 1.06 to 12.89 μM, respectively. Flow cytometry analysis demonstrated that treatment of MGC-803 with **6b** led to cell cycle arrest at G2/M phase accompanied by an increase in apoptotic cell death.

Key words: pyrimidine; benzimidazole; anticancer; cell cycle arrest ; apoptosis.

Cancer is a leading cause of death worldwide, accounting for 8.7 million deaths (around 14% of all deaths) in 2012. The pharmacological fight against cancer has made significant progress in the last twenty years. In 2012 (October 1, 2011–September 30, 2012) 35 novel drugs were approved by the FDA, among which, 10 (28.57%) were used for the treatment of cancer.[1] Although many chemotherapeutic agents have been developed to treat different kinds of cancer effectively, some side effects could happen simultaneously. Therefore novel molecules to fight this disease are still urgently needed.[2,3]

Pyrimidine is found as a core structure in a large variety of compounds that exhibit important biological activity, and several member of this class has earned valued places in chemotherapy as effective agents, like 5-fluorouracil, erlotinib, gefitinib and caneratinib.[4] Many papers have reported that pyrimidine derivatives showed impressive anticancer activity.[5-13] On the other hand, benzimidazole being an isostere of purine nucleosides and an important scaffold in various biologically active molecules is widely explored for development of anticancer agents. Several promising antitumor active agents were found to contain the benzimidazole ring system. They were found to exert their antitumor activity by acting mainly as antiangiogenic agents,[14–15] alkylating agents[16–18] and topoisomerases inhibitors.[19–21]

In 2010, Heba T. Abdel-Mohsenreported that benzimidazole-pyrimidine conjugates exhibited high antitumor activity.[22] Inspired by the literature and in continuation of our previous work,[23] we herein reported the synthesis of novel pyrimidine-benzimidazole hybrids and their anticancer activity. The anticancer activity evaluation results revealed that the pyrimidine-benzimidazole hybrids exhibited potent anticancer activity.

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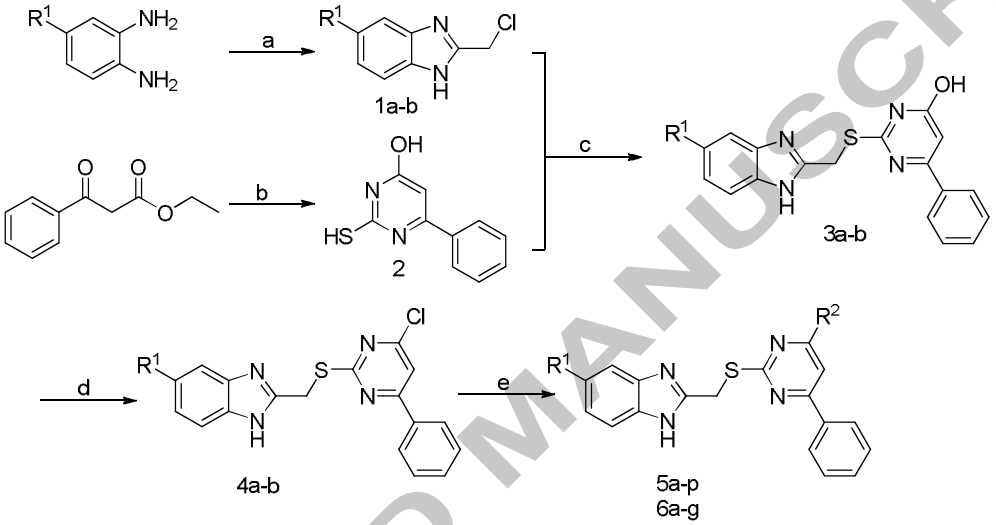
The synthetic strategy to prepare the target compounds is depicted in scheme 1. The key intermediates, 2-(chloromethyl)-1*H*-benzo[*d*]imidazole **1a** and 5-chloro-2-(chloromethyl)-1*H*-benzo[*d*]imidazole **1b** were synthesized from commercially available benzene-1,2-diamine or 4-chlorobenzene-1,2-diamine as previously reported.[24] 2-mercapto-6-phenyl- pyrimidin-4-ol (**2**) was synthesized from the reaction of ethyl benzoylacetate with thiourea according to the reported procedure.[25] Compounds **3a** and **3b** were acquired from the reactions of compound **2** with compounds **1a** and **1b**, which were further reacted with $POCl_3$ to yield compounds **4a** and **4b**. These highly activated intermediates were then reacted with different substituents to obtain compounds **5a-p** and **6a-g**. Finally, all the structures of **5a-p** and **6a-g** were fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS.[26]



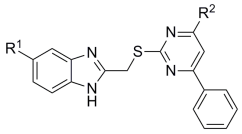
Scheme 1. Reagents and conditions: (a) chloroacetyl chloride, 5N HCl, reflux, 4 h (85% yield); (b) thiourea, KOH, EtOH, reflux, 10 h (82% yield); (c) KOH, H_2O-acetone (2:1), 55-60℃, 30 min (87-92% yield); (d) $POCl_3$, 90℃, 20 min(89-91% yield); (e) EtOH, reflux, 10 h (65-92% yield).

All the newly synthesized compounds were investigated for their in vitro anticancer activity on four human cancer cell lines including MCF-7 (human breast cancer cell line), MGC-803 (human gastric cancer cell line), EC-9706 (human esophageal cancer cell line) and SMMC-7721 (human liver cancer cell line) using MTT assay method. The IC_{50}(μM) values (concentrationrequired to achieve 50% inhibition of the tumor growth) of the tested compounds and the standard are listed in Table 1. The antitumor drug discovery screen has been designed to distinguish between broad-spectrum antitumor compounds and tumor selective agents.

In present study, the active analogs showed a distinctive potential pattern of selectivity as well as broad-spectrum anti-tumor activity. With regard to selectivity against individual cell lines, many of the compounds showed effectiveness against cell line human breast cancer MCF-7 and human gastric cancer MGC-803 with IC_{50} values ranging from 1.40 to 10.58 μM and 1.06 to 9.70 μM respectively comparative to 5-Fu IC_{50} (7.12 and 3.45μM). EC-9706 human esophageal cancer cell line proved to be sensitive toward compounds **5a-c**, **5e**, **6a-b** and **6e** with IC_{50} concentration range of 2.79-9.48 μM comparative to 5-Fu IC_{50} (8.07μM). Regarding SMMC-7721 human liver cancer cell line, higher sensitivity was observed with compounds **5b**, **6b** and **6c** with IC_{50} concentration range of 10.55-14.08 μM comparative to 5-Fu IC_{50} (15.08 μM). With regard to broad-spectrum antitumor activity, compounds **5b** and **6b** showed IC_{50} concentration range of

1.06-12.89 μM against the four cell lines. On the other hand compounds **5a**, **5c**, **5e**, **6a** and **6e** showed higher cytotoxic activity against MCF-7, MGC-803 and EC-9706 with low activity against SMMC-7721. Moreover compounds **5f-g**, **5l-n** and **6f-g** proved to be ineffective against the four cell lines.

Table 1. Inhibitory activity of pyrimidine derivatives against four human cancer cell lines.



Compound	R^1	R^2	$IC_{50}(\mu M)$[a]			
			MCF-7	MGC-803	EC-9706	SMMC-7721
3a	H	OH-	23.89	20.82	35.43	56.12
3b	Cl	OH-	23.12	19.35	32.01	53.62
4a	H	Cl-	10.25	9.70	24.19	30.16
4b	Cl	Cl-	7.12	8.16	23.45	28.02
5a	H	4-CH_3-C_6H_5-NH-	1.43	1.33	3.33	20.50
5b	H	4-CH_3O-C_6H_5-NH-	2.90	2.03	5.83	10.55
5c	H	4-F-C_6H_5-NH-	4.24	2.30	8.54	22.58
5d	H	4-Cl-C_6H_5-NH-	12.27	3.24	22.74	22.30
5e	H	3-CF_3-C_6H_5-NH-	7.64	3.84	9.48	28.55
5f	H	2-CH_3O-C_6H_5-NH-	>100	45.82	>100	>100
5g	H	4-CH_3CH_2OCO-C_6H_5-NH-	>100	>100	>100	>100
5h	H	C_6H_5-NH-	5.61	6.12	10.09	31.52
5i	H	3-CH_3-C_6H_5-NH-	35.94	33.25	39.78	57.27
5j	H	4-butyl-C_6H_5-NH-	17.69	15.17	21.56	45.02
5k	H	4-isopropyl-C_6H_5-NH-	23.72	20.06	51.05	67.13
5l	H	2-F-C_6H_5-NH-	8.60	7.26	15.79	39.16
5m	H	3-Cl-C_6H_5-NH-	19.64	16.73	20.13	31.74
5n	H	4-NO_2-C_6H_5-NH-	>100	>100	>100	>100
5o	H	3-NO_2-C_6H_5-NH-	>100	>100	>100	>100
5p	H	4-CH_3CO-C_6H_5-NH-	>100	>100	>100	>100
6a	Cl	4-CH_3-C_6H_5-NH-	1.40	1.07	2.79	19.28
6b	Cl	4-CH_3O-C_6H_5-NH-	1.95	1.06	3.68	12.89
6c	Cl	4-F-C_6H_5-NH-	6.37	4.30	21.75	14.08
6d	Cl	4-Cl-C_6H_5-NH-	10.58	4.41	15.58	20.59
6e	Cl	3-CF_3-C_6H_5-NH-	4.78	2.63	5.85	25.59
6f	Cl	2-CH_3O-C_6H_5-NH-	>100	53.12	>100	>100
6g	Cl	4-CH_3CH_2OCO-C_6H_5-NH-	>100	>100	>100	>100
5-Fu[b]			7.12	3.45	8.07	15.08

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

[b] Positive control.

Some structure-activity relationships could be observed, mainly related to the influence of different substituents on phenyl ring and an Cl at benzimidazole ring. From the recorded IC_{50}

values of series-I (compounds **5a-p**) it was observed that different substituents on phenyl ring exemplified varying degrees of inhibition against the four cell lines. The comparison of the activities of compouns **5a-b**, **5h** and **5j-k**, compounds **5a-b** are the most active members with IC_{50} values of 1.33-20.50 μM, suggesting that the introduction of small electron-donating groups $-CH_3$ or $–OCH_3$ at para-position of phenyl ring contributed to anticancer activities and big electron-donating groups $–CH_2CH_2CH_2CH_3$ or $–CH(CH_3)_2$ showed a decrease in the antitumor properties when compared with the compound **5h**. On the other hand, introduction of electron-drawing group F at para-position afforded **5c** with a increase in the antitumor activity against the four cell lines, but introduction of Cl afforded **5d** with a decrease in the antitumor activity against MCF-7 and EC-9706 and a increase against MGC-803and SMMC-7721. The comparison of the activities of the pair of compounds **5a/5i**, **5b/5f**, **5c/5l** and **5d/5m** the first of each pair-component with a substituent at para-position and the second with the corresponding substituent at meta-position or ortho-position, showed that substituents at para-position were better. Analogues **5g** and **5n-p** with meta-directing groups $-O_2CCH_2CH_3$, $-OCCH_3$ and $-NO_2$ as para- or meta-substitutions showed no cytotoxicity against all tested cell lines, however, compound **5e** with $-CF_3$ at meta-position exhibited high antitumor activity. The introduction of a Cl at benzimidazole ring have some influence on the activity. We compared the activity of seven pairs of compounds (**5a/6a**; **5b/6b**; **5c/6c**; **5d/6d** ; **5e/6e**; **5f/6f** and **5g/6g**). This change could cause a increase (**6a** and **6e**) or decrease (**6c**) in the antitumor activity but could not determine the activities fundamentally.

Because the compounds we synthesized have very similar structures to the prior work cited in reference 22，a comparison was made between the two series of compounds. Two compounds (**5A** and **5B**, Fig. 1) were synthesized having similar structure to compounds **5a** and **5b** according to the reference 22, and two human cancer cell lines were choosen. The IC_{50}(μM) values are listed in Table 2. The comparison of the activities of compouns **5a-b** and **5A-B**, compounds **5A-B** have better anticancer activities, suggesting that the introduction of a CN at pyrimidine ring contributed to anticancer activities.

Table 2. Inhibitory activity of compounds **5A** and **5B** against MCF-7and MGC-803.

Compound	IC_{50}(μM)	
	MCF-7	MGC-803
5A	1.17	1.14
5B	2.56	1.73

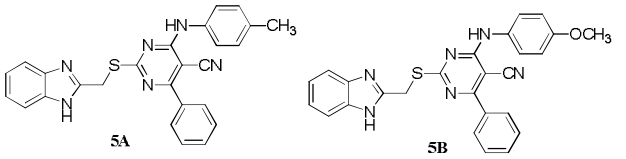


Fig. 1. Structures of compounds **5A** and **5B**

Compound **6b** had a remarkable broad spectrum activity against all tested human cancer cell lines and highest cytotoxic activity against MGC-803, and it was chosen to be further investigated regarding its mechanism of action. In order to better characterize the mode of cell death induced by compound **6b**, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and annexin-V-FITC in MGC-803 cells. After treatment with compound **6b** at various concentrations (0, 0.4, 0.8, 1.6 μmol/L) for 12 h, it was observed that the apoptosis rates were 4.8%, 15.9%, 21.4%, and 33.1%, respectively (Fig. 2 A), whereas after treatment with compound **6b** (0, 0.4, 0.8, 1.6 μmol/L) for 24 h, the the apoptosis rates were 5.2%, 25.4%, 49.2%, and 61.8%, respectively (Fig. 2 B). The results showed that **6b** markedly increased the cellular apoptosis in a concentration- and time-dependent manner.

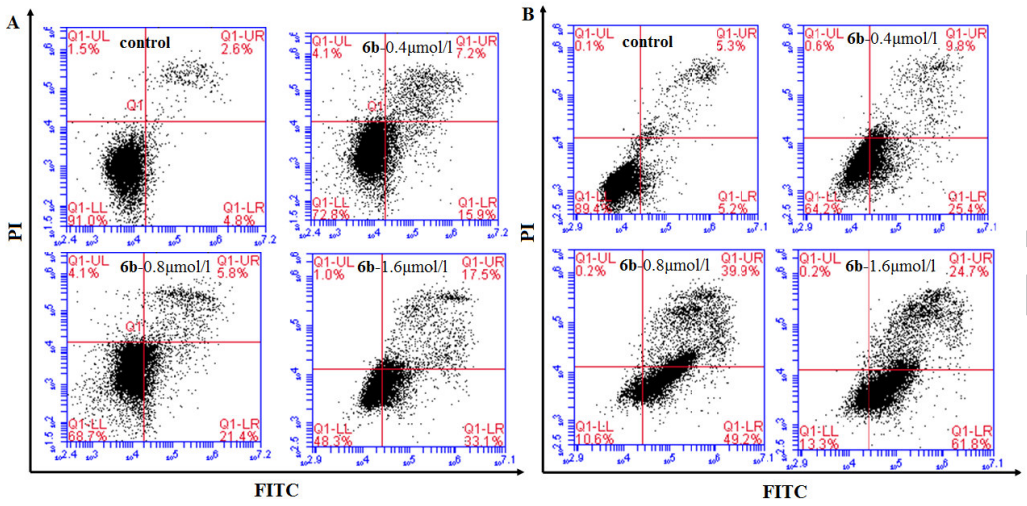


Fig. 2. Apoptosis effect on human MGC-803 cell line induced by compound **6b**. Apoptotic cells were detected with Annexin V/PI double staining after incubation with compounds **6b** (0, 0.4, 0.8, 1.6 μmol/L) for for 12 h or 24 h. The lower left quadrant represents live cells, the lower right quadrant is for early/primary apoptotic cells, upper right quadrant is for late/secondary apoptotic cells, while the upper left quadrant represents cells damaged during the procedure. (A) incubated for 12 h; (B) incubated for 24 h. The experiments were performed three times, and a representative experiment is shown.

Many anticancer drugs interact with cells leading to cell growth arrest. In order to explore the relationship between the high anticancer effects and cell cycle, the effects of different concentrations of compound **6b** on cell cycle progression were examined with MGC-803 cell line. After treatment with compound **6b** at various concentrations (0, 0.4, 0.8, 1.6 μmol/L) for 12 h, it was observed that the percentage of cells in G2/M phase were 16.46%, 23.38%, 47.79%, and 63.10%, respectively (Fig. 3 A), whereas after treatment of compound **6b** (0, 0.4, 0.8,1.6 μmol/L L) for 24 h, the percentage of cells in G2/M phase were 12.89%, 27.43%, 68.97%, and 73.70%, respectively (Fig. 3 B). The results suggested that **6b** caused a clear G2/M arrest pattern in a concentration- and time-dependent manner, with a concomitant decrease of cells in other phases of the cell cycle.

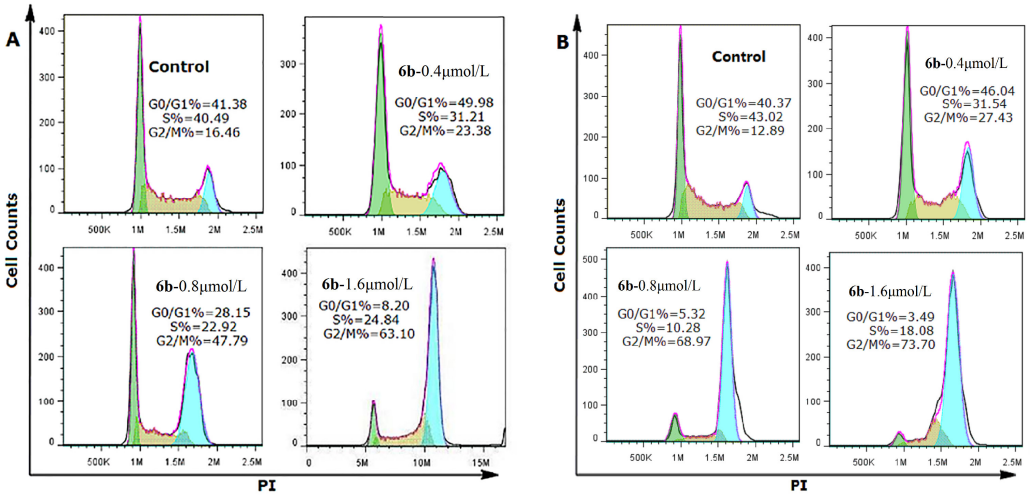


Fig. 3. Effect of compound **6b** on the cell cycle distribution of MGC-803 cells. Cells were treated with different

concentrations (0, 0.4, 0.8, 1.6 μmol/L) for 12 h or 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. (A) incubated for 12 h; (B) incubated for 24 h. The experiments were performed three times, and a representative experiment is shown.

In conclusion, A novel series of pyrimidine-benzimidazol hybrids were synthesized and evaluated their anticancer activity in vitro against four human cancer cell lines (MCF-7,MGC-803, EC-9706 and SMMC-7721). Many of the synthesized compounds exhibited very high anti-tumor activity. The nature of the substitutions on phenyl ring influenced the antitumor activities remarkably. Compounds **5b** and **6b** were more cytotoxic than 5-fluorouracil against all tested four human cancer cell lines. The results of apoptosis assay and cell cycle analysis demonstrated that **6b** clearly inhibits the proliferation of MGC-803 cancer cells by inducing apoptosis and arresting the cell cycle at G2/M phase. The pyrimidine-benzimidazole conjugates have simple structures and are easy to synthesize. These compounds are currently being evaluated for their in vivo efficacy in animal models. These findings have encouraged us to continue the development and testing of novel pyrimidine-benzimidazole conjugates to conduct further studies to investigate the structure-activity relationship and elucidate the detailed pharmacological mechanism(s).

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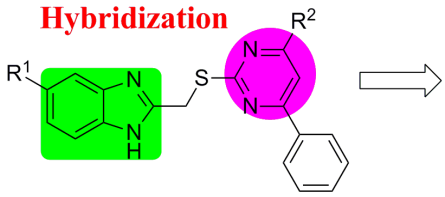
26 *Selected spectroscopic data*. **5a**: ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 10.08 (s, 1H), 7.80 (d, J = 6.9 Hz, 2H), 7.75 (dd, J_1 = 6.1, J_2 = 3.1 Hz, 2H), 7.49 (dd, J_1 = 6.2 Hz, J_2 = 3.2 Hz, 2H), 7.47–7.39 (m, 5H), 7.09 (d, J = 8.1 Hz, 2H), 7.03 (s, 1H), 4.94 (s, 2H), 2.26 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.2, 161.8, 161.4, 152.4, 136.9, 136.5, 131.8, 131.0, 129.7, 129.3, 126.8, 125.9, 120.9, 114.3, 26.8, 20.9; HR-MS (ESI) Calcd for $C_{25}H_{21}N_5S$ $[M+H]^+$: 424.1596, found: 424.1584. **5b**: ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 10.23 (s, 1H), 7.79 (t, J = 6.3 Hz, 2H), 7.76 (dd, J_1 = 6.1 Hz, J_2 =3.1 Hz, 2H), 7.50 (dd, J_1 = 6.1 Hz, J_2 = 3.1 Hz, 2H), 7.48 – 7.39 (m, 5H), 7.00 (s, 1H), 6.87 (d, J = 8.4 Hz, 2H), 4.95 (s, 2H), 3.73 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 167.9, 161.4, 152.3, 136.2, 131.2, 129.3, 126.9, 126.2, 122.8, 114.5, 114.3, 55.7, 26.7; HR-MS (ESI) Calcd for $C_{25}H_{21}N_5OS$ $[M+H]^+$: 440.1545, found: 440.1543. **6a**: ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.90 (s, 1H), 7.81 (m, 3H), 7.73 (d, J = 8.7 Hz, 1H), 7.48 (m, 4H), 7.40 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.4 Hz, 2H), 6.97 (s, 1H), 4.90 (s, 2H), 2.26 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 168.0, 161.4, 153.8, 136.4, 132.5, 131.1, 130.6, 130.4, 129.7, 129.3, 126.9, 126.4, 121.0, 115.9, 114.1, 26.9, 26.0; HR-MS (ESI) Calcd for $C_{25}H_{20}ClN_5S$ $[M+H]^+$: 458.1206, found: 458.1205. **6b**: ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 10.35 (s, 1H), 7.79 (m 4H), 7.48 (m 6H), 7.02 (s, 1H), 6.87 (s, 2H), 4.95 (s, 2H), 3.73 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6, δ, ppm): 167.9, 161.4, 153.8, 136.2, 132.3, 131.1, 130.4, 129.3, 126.9, 126.5, 123.0, 115.9, 114.5, 114.1, 55.7, 26.8; HR-MS (ESI) Calcd for $C_{25}H_{20}ClN_50S$ $[M+H]^+$: 474.1155, found: 474.1153.

Graphical abstract



Apoptosis

Cell cycle arrest

5b R^1=H, R^2=4-CH_3O-C_6H_5-NH-

6b R^1=Cl, R^2=4-CH_3O-C_6H_5-NH-

broad-spectrum antitumor activity

Compounds **5b** and **6b** were more cytotoxic than 5-fluorouracil against all tested four human cancer cell lines, with IC_{50} values ranging from 2.03 to 10.55 μM and 1.06 to 12.89 μM, respectively. Flow cytometry analysis demonstrated that treatment of MGC-803 with **6b** led to cell cycle arrest at G2/M phase accompanied by an increase in apoptotic cell death.