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Novel small molecules as apoptosis inducers: Synthesis, preliminary structure–activity relationships, and in vitro biological evaluation

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ABSTRACT

Inducing apoptosis is a promising therapeutic approach to overcome cancer. In this study, 30 compounds were synthesized and evaluated for their antiproliferative activity against three tumor cell lines in vitro: A875, H460 and Hela cancer cells by the MTT assay. The most potent analogue **7a**, a novel compound was first reported by our group, inhibited the proliferation of A875 cells with an IC_{50} value of 98 nM. Flow cytometry analysis and morphological analysis suggested that compound **7a** had potential anticancer efficacy via G_2/M cell cycle arrest, which could be attributed to its proliferation and apoptosis, and also in a concentration-dependent manner. The SAR analysis indicated that the substituents R^2 played a crucial role in the antiproliferation activity.

Chemotherapy is one of the most common treatment options for cancer patients, especially for unresectable patients.[1–3] However, conventional chemotherapy drugs have not shown better improvement in extension and quality of patients' life mainly because of their poor inhibitor potency or the emergence of drug resistance after chemotherapeutic more than six months.[4,5] The discovery and development of the novel cancer inhibitors with higher potency anticancer activity are strongly demanded at the present time. All of the cancer cells have the same property that they proliferate rapidly whatever they are diverse and heterogeneous.[6] The programmed cell death, also known as apoptosis is a complex mechanism by which cell undergo its own destruction to control the process of cell proliferation.[7,8] Apoptosis plays an important role in tissue homoeostasis and prevention of tumor cell proliferation,[9] as a defense mechanism apoptosis remove unwanted and potentially dangerous cells including tumor cells.[10,11] Its deregulation is widely believed to be involved in the pathogenesis of cancer. Certain drugs and agents have been identified treatment of the cancers depending on triggering the apoptotic pathway, then inducing the apoptosis process in tumor cells.[12–14] Thus, development of drugs that can effectively trigger the apoptotic process and evaluation the apoptosis inducing ability have been receiving considerable attention.

Our project is to develop novel small molecule anticancer drugs that could induce apoptosis. In a previous cell-based screening study of anticancer drugs, we found a hit compound **1** (Fig. 1) which exhibited moderate inhibition against the human melanoma cell line A875 (IC_{50} = 18.6 μM), human lung adenocarcinoma cell line H460 (IC_{50} = 16.8 μM) and the human cervical carcinoma cell line Hela (IC_{50} = 25.8 μM) in the MTT assay.[15] The purpose here is to carry out a structural modification to optimize the activity of compound **1**, a series of novel compounds **6a–7r** were synthesized,[16,17] and their anticancer activity and apoptosis ability were tested in vitro.

Figure 1 illustrates three regions of interest for SAR evaluation of screening hit compound **1**. The synthetic strategies adopted for the synthesis of compound **1** and other compounds **6a**–**7r** are depicted in Scheme 1 and 2. The starting materials **3a–h** were prepared from commercially available benzylamine, pyridinylamin derivatives **2a–h**, and 2-chloroacetyl chloride at room temperature. In the next step, **3a–h** were condensed with 4-aminobenzenethiol or 5-amino-1,3,4-thiadiazole-2-thiol in THF with refluxing, affording the amines, **4a–d** and **5a**, **5b**, **5e–5h**. Then, target compounds, **1**, **6a–k** and **7a–r** were obtained, respectively, by the reaction with corresponding acyl chloride in the presence of potassium carbonate, at room temperature, in dichloromethane.[18] The yield of all reactions was satisfactory. All the products were characterized by ^{1}H NMR, ^{13}C NMR and ESI-MS analysis.

As shown in Table 1 and 29 analogs of compound **1** were synthesized to survey the SAR of Region 1, Region 2 and Region 3 by evaluating the cell growth inhibitory activity (IC_{50}) on the three human cell lines A875, H460 and HepG2 in the MTT assay. Most compounds were able to increase the inhibitory activities against the three human cell lines, some compounds were better than

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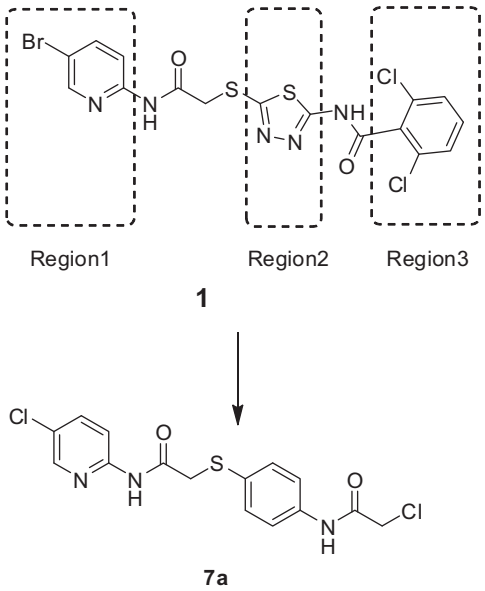


Figure 1. Selected regions of interest for SAR evaluation of screening hit compound **1** and the structural formula of compound **7a**.

cisplatin, while six compounds had no activity against the three human cell lines. The most potent analog **7a** significantly inhibited the proliferation of the human melanoma cell line A875 at low nanomolar concentration (IC_{50} = 98 nM).

As illustrated in Scheme 2, **6a**–**6k**. The Region 2 was 1,3,4-thiadiazole, we started our SAR studies at the Region 1 and Region 3. Introduction of a 2,6-position of the phenyl ring with the electron-withdrawing chlorine group (**1**) was well tolerated, whereas the 2-methyloxyphenyl analog (**6a**) led to almost total loss of activity. Meanwhile the R^1 was replaced by 5-bromo-2-pyridinyl. However, replacing the R^3 with electron-donating 2-methyloxyphenyl resulted in the loss of activity with the introduction of 5-methyl-2-pyridinyl (**6b**), 5-chloro-2-pyridinyl (**6c**), 2-chloro-4-methyl-3-pyridinyl (**6d**) in the R^1 group. This suggested that the electron-withdrawing group on the phenyl ring increased the anticancer activity in contrast of electron-donating group.

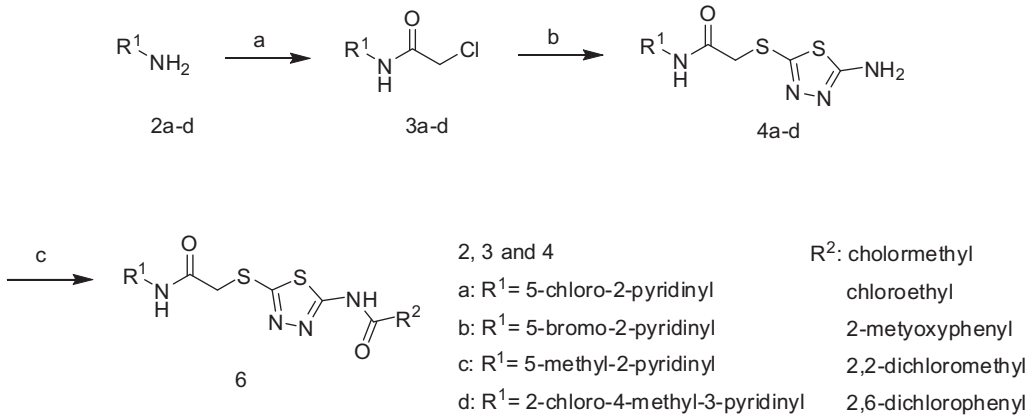
In another variation, we replaced the substituted R^2 with chloromethyl, 2-chloroethyl, 3-chloropropyl and dichloromethyl groups at R^2, affording **6e–6j** and **6k**. As shown in Table 1, **6e**–**6j**, especially **6h** (IC_{50} = 0.43 μM) had considerably better activity than compound **1**, indicating chloromethyl and 2-chloroethyl might be a good substitution for modification compared with 3-chloropropyl (**6k**) that almost loss of activity. Interestingly, when the R^2 was replaced by chloromethyl, the groups including 5-chloro-2-pyridinyl (**6e**), 2-chloro-4-methyl-3-pyridinyl (**6i**), 5-methyl-2-pyridinyl (**6j**), in R^1 did not result in the loss of activity, these results showed no clear SAR trends for Region 1. This observation confirmed further that chloromethyl and 2-chloroethyl played an important role in increasing the anticancer activity.

After identifying chloromethyl and 2-chloroethyl were the potent substituent, we tried to replace Region 2 with phenyl ring. As shown in Table 1, among **7a–7v** we found the most potent anticancer activity compound **7a** that moderate inhibitory activity was more than 200-fold improvement against the human melanoma cell line A875 (IC_{50} = 98 nM), compared with cisplatin as a positive control (IC_{50} = 22.4 μM). We next turned to examining the SAR at Region 1 and Region 3. The Region 3 was replaced with chloromethyl, the anticancer activity was slightly fluctuate with the replacement of 5-chloro-2-pyridinyl (**7a**), 5-bromo-2-pyridinyl (**7b**), 4-chloro-2-pyridinyl (**7c**) and decreased with the replacement of 3-chlorobenzyl (**7d**), 4-chlorobenzyl (**7e**), 2-pyridinyl (**7f**) in R^1 group. The results suggested 2-pyridinyl ring with electron-withdrawing chloro or bromo at the 4-position would increase the anticancer activity. However, the potent activity was decreased and even more loss with increasing of alky chain in R^2 (**7g–7k**). Introduction of 1.1-dimethylethyl (**7l**, **7o**), trihloromethyl (**7m**), dichloromethyl (**7n**) instead of chloromethyl in R^2 group resulted in the loss of activity, then R^2 was replaced with ethenyl (**7p**, **7q**, **7r**), IC_{50} values were between 14 and 24 μM against A875 cells. This observation confirmed further that chloromethyl could play an important role in the anticancer activities.

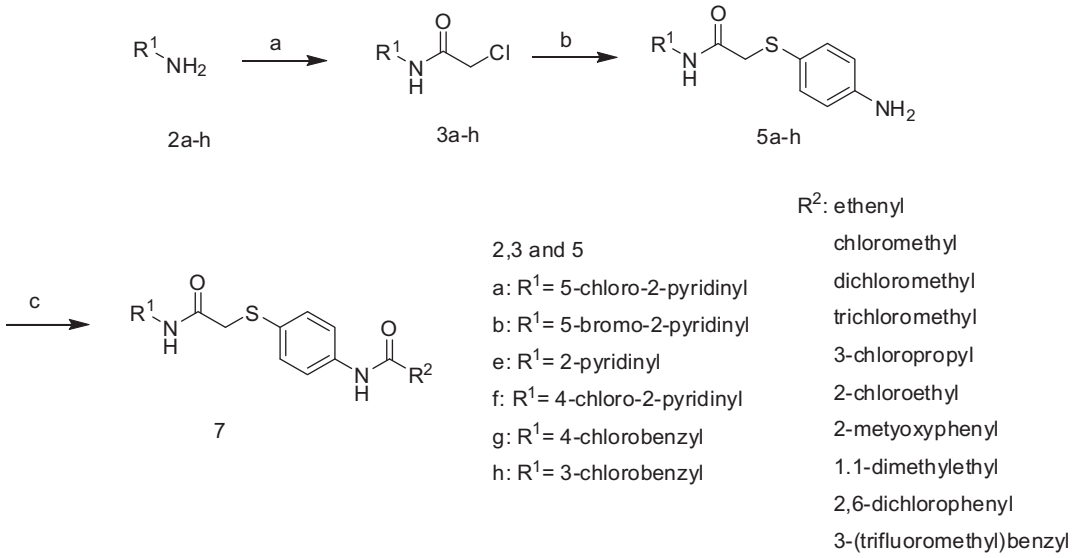
Through the structure–activity relationship study, we found that chloromethyl could significantly improve the antitumor activity of this series analogs in vitro. Region 3 seemed to play a more important role in increasing the activities than Region 1 and Region 2. This observation provided us with important information for further molecular structure modification.

To further study the antiproliferative, **7a** and **7b** were selected for evaluation of their inhibitory activities against a panel of different types of human cancer cell lines: colon cancer cell lines HCT-116 and HT29, lung cancer cell lines A549 and H460, liver cancer cell lines PC-3, Bel-7402, SMMC-7721and HepG2, pancreatic cancer cell line BxPC-3, cervical cancer cell line Hela, melanoma cancer cell line A875. As shown in Table 2, compounds **7a** and **7b** exhibited good anticancer activities against the different types of human cancer cell lines in vitro, with IC_{50} value ranging from 0.098 to 2.5 μM. This broad-spectrum antiproliferative activity is encouraging for the development of new anticancer drugs.

At the beginning of design these compounds, our project was to develop new anticancer drugs that could induce apoptosis. Therefore, we evaluated the apoptosis inducing ability of these

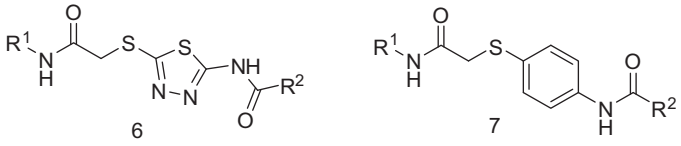


Scheme 1. Reagents and conditions: (a) 2-chloroacetyl chloride, CH_2Cl_2, K_2CO_3, rt, 4 h; (b) 5-amino-1,3,4-thiadiazole-2-thiol, THF, K_2CO_3, reflux, 5 h; (c) CH_2Cl_2, K_2CO_3, rt, 4 h.



Scheme 2. Reagents and conditions: (a) 2-chloroacetyl chloride, CH_2Cl_2, K_2CO_3, rt, 4 h; (b) 4-aminothiophenol, THF, K_2CO_3, reflux, 5 h; (c) CH_2Cl_2, K_2CO_3, rt, 4 h.

Table 1
In vitro activities of compound **6a–7r**



Compound	R^1	R^2	IC_{50}[a] (μM)		
			A875	H460	Hela
6a	5-Bromo-2-pyridinyl	2-Methoxyphenyl	>80	>80	>80
6b	5-Methyl-2-pyridinyl	2-Methoxyphenyl	>80	>80	>80
6c	5-Chloro-2-pyridinyl	2-Methoxyphenyl	>80	>80	>80
6d	2-Chloro-4-methyl-3-pyridinyl	2-Methoxyphenyl	>80	>80	>80
6e	5-Chloro-2-pyridinyl	Chloromethyl	8.6	5.7	3.6
6f	5-Bromo-2-pyridinyl	Chloromethyl	13.9	7.5	3.4
6g	5-Bromo-2-pyridinyl	Dichloromethyl	6.5	5.3	15.2
6h	5-Bromo-2-pyridinyl	2-Chloroethyl	0.43	0.56	5.3
6i	2-Chloro-4-methyl-3-pyridinyl	Chloromethyl	34.7	59.6	70.5
6j	5-Methyl-2-pyridinyl	Chloromethyl	18.3	15.4	10.9
6k	5-Chloro-2-pyridinyl	3-Chloropropyl	>80	55.2	>80
7a	5-Chloro-2-pyridinyl	Chloromethyl	0.098	0.42	2.5
7b	5-Bromo-2-pyridinyl	Chloromethyl	0.29	0.53	2.1
7c	4-Chloro-2-pyridinyl	Chloromethyl	0.85	5.6	2.8
7d	3-Chlorobenzyl	Chloromethyl	7.8	33.4	>80
7e	4-Chlorobenzyl	Chloromethyl	8.9	23.1	>80
7f	2-Pyridinyl	Chloromethyl	17.2	11.9	7.8
7g	4-Chloro-2-pyridinyl	2-Chloroethyl	12.3	10.9	21.7
7h	5-Bromo-2-pyridinyl	2-Chloroethyl	24.3	1.6	15.6
7i	2-Pyridinyl	2-Chloroethyl	29.5	65.4	70.4
7j	4-Chlorobenzyl	2-Chloroethyl	31.4	60.8	>80
7k	5-Chloro-2-pyridinyl	3-Chloropropyl	>80	>80	>80
7l	5-Chloro-2-pyridinyl	1.1-Dimethylethyl	>80	67.2	>80
7m	5-Chloro-2-pyridinyl	Trichloromethyl	>80	>80	>80
7n	5-Bromo-2-pyridinyl	Dichloromethyl	56.8	47.8	>80
7o	4-Chloro-2-pyridinyl	1.1-Dimethylethyl	>80	35.3	>80
7p	4-Chloro-2-pyridinyl	Ethenyl	14.7	19.7	25.2
7q	2-Pyridinyl	Ethenyl	23.4	76.8	>80
7r	3-Chlorobenzyl	Ethenyl	21.1	74.5	>80

[a] The antiproliferation activities of compounds **6a–7r** in vitro were determined by the MTT assay on A875, H460, Hela cell lines, IC_{50} was average of three determinations and deviation from the average was <5% of the average value.

compounds. Finally, compound **7a** was selected as a lead for further study because of its best potency against A875 cancer cells in vitro.

To further evaluate the antiproliferative mechanism of compound **7a**, flow cytometric analysis was used to quantitatively assess and measure the apoptotic cells (sub-G1 cells) and the cell

cycle after PI-stained.[19] As shown in Figure 2A and B, The percentage of apoptotic cells were 3.67%, 25.45%, 42.88% for 24 h and 11.17%, 41.59%, 61.59% for 48 h, respectively, after treatment with 0.38, 0.75 and 1.5 μM **7a**. The results suggested that **7a** inhibited the proliferation of A875 cancer cells by inducing apoptosis in a concentration-dependent manner. In addition, a significant accumulation of cells in the G_2/M and S phase, accompanied by a decrease in G_0/G_1 phase was observed after treatment with compound **7a** in A875 cancer cells, (Fig. 2C). The percentage of cells in the G_0/G_1 phase was 78.18% for the control, and 60.01%, 48.94% and 30.52% for 48 h, respectively, after treatment with 0.38, 0.75 and 1.5 μM compound **7a**. The results suggested that compound

Table 2
The antiproliferation activities of compounds **7a**, **7b** and cisplatin against various cancer cell lines

Compound	IC_{50}[a] (μM)									
	HT29	Hela	Bel-7402	SMMC-7721	HepG2	A549	HCT-116	A875	H460	BxPC-3
7a	8.7	2.5	0.45	0.35	3.6	0.59	0.61	0.098	0.42	0.19
7b	13.7	2.1	0.67	0.43	5.8	6.2	1.2	0.29	0.53	0.28
Cisplatin	28.9	19.8	17.2	12.6	23.1	14.5	15.6	22.4	8.7	13.2

[a] Values were average of three determinations and deviation from the average is <5% of the average value.

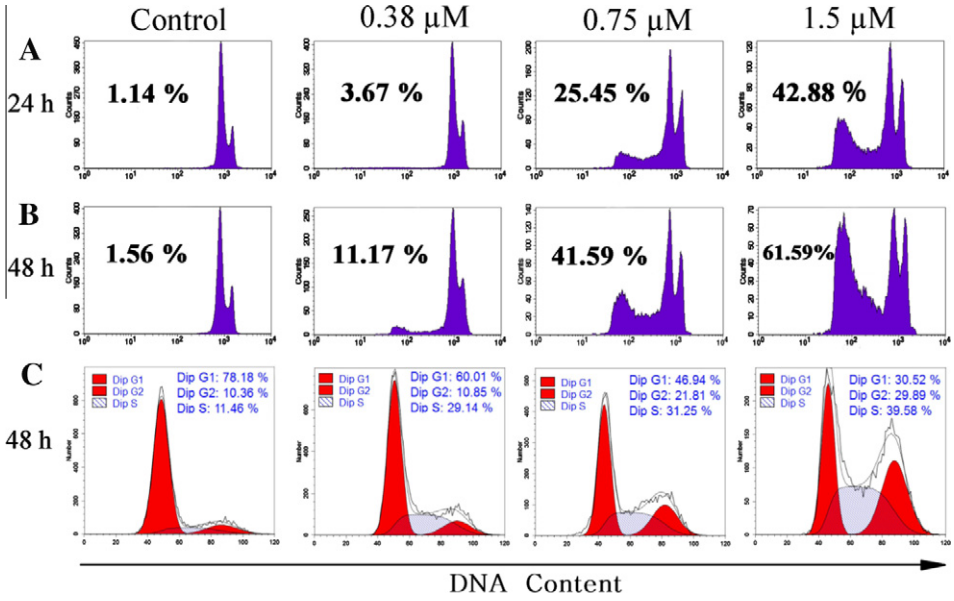


Figure 2. Effect of **7a** on the induction of apoptosis and cell cycle distribution: (A) DNA fluorescence histograms of PI-stained A875 cells. Cells were treated with 0.38 μM, 0.75 μM, 1.5 μM for 24 h; (B) DNA fluorescence histograms of PI-stained A875 cells. Cells were treated with 0.38 μM, 0.75 μM, 1.5 μM for 48 h. The cells in the sub-G1 phase were considered as apoptotic cells; (C) effect of cell cycle distribution with 0.38 μM, 0.75 μM, 1.5 μM in A875 cells for 48 h.

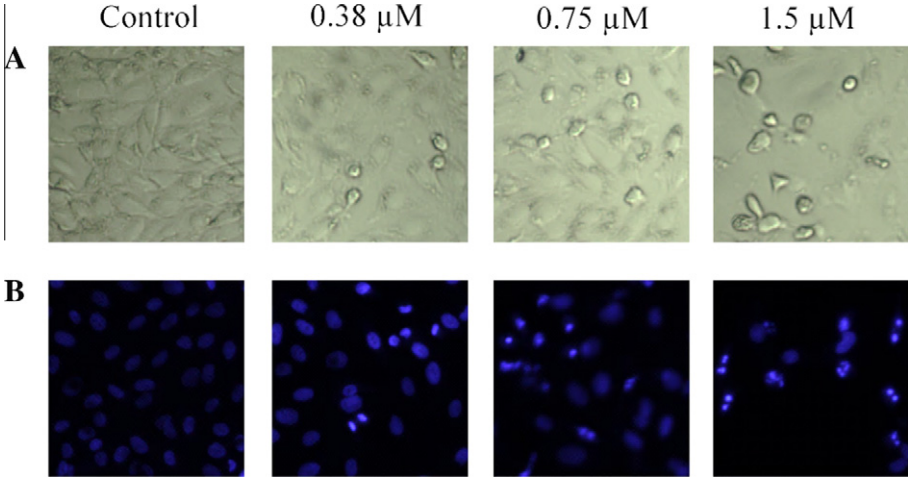


Figure 3. Effect of **7a** on cell morphology: (A) Bright-field microscopy images and (B) fluorescence microscopic appearance of Hoechst 33342 staining nuclei of A875 cancer cells, after incubation with **7a** for 36 h at varying concentrations: 0.38 μM, 0.75 μM, 1.5 μM. Apoptosis cells were observed in treated cells containing condensed and fragmented fluorescent nuclei.

7a could remarkably arrested G_2/M and S phase and induced apoptosis at low micromolar concentrations in vitro.

Furthermore, fluorescence microscopic examination of Hoechst 33342-stained cell confirmed the inducing apoptosis effect of **7a**.[20] The apoptotic cells are often characterized by its morphological, including cell shrinkage, membrane blabbing, chromatin condensation and DNA fragmentation.[21] When treated with **7a** for 36 h, A875 cancer cells shrank and rounded up, these phenomena became more significant with the **7a** concentration increasing. In contrast, the untreated A875 cells retained their normal size and shape, kept proliferating with time and became over-crowded by 36 h (Fig. 3A). Morphological changes were also characteristic of apoptosis after Hoechst 33342 staining. Apoptosis cells, bright blue fluorescent and condensed nuclei, were observed in a large number of treated cells in A875, and the change was concentration-dependent, whereas there was no significant apoptosis in untreated A875 cells that showed blue, diffusely stained intact nuclei (Fig. 3B). These results indicated that **7a** induced apoptosis in A875 cancer cells.

In conclusion, we have described the optimization of the inducing apoptosis compound **1**, 29 analogs were synthesized and evaluated the antiproliferative activity in vitro. The SAR analysis indicated that the substituents in R^2 played a crucial role in the antiproliferation activity, especially chloromethyl that could significantly increase antiproliferation ability. The most potent analog **7a** displayed very good inhibitory activity against a panel of different types of human cancer cell lines with IC_{50} values in the low micromolar range and exhibited an IC_{50} value of 98 nM compared with cisplatin as a positive control, with an IC_{50} value of 22.4 μM against A875 cells. The results of flow cytometry analysis and morphological analysis indicated that compound **7a** have potential anticancer efficacy via G_2/M of A875 cell cycle arrest, which could be attributed to its proliferation and apoptosis, and also in a concentration-dependent manner.

Our results will provide useful information for the design of novel compounds with better potency as antitumor agents. Further study on the antitumor activities in vivo and exact biological mechanism is underway

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.076.

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15. Briefly, cells (3×10^3/well) were seeded in 96-well plates and cultured for 24 h, followed by compounds treatment for 48 h. A volume of 20 μl of 5 mg/ml MTT was added per well and incubated for another 2 h at 37 °C, then the supernatant fluid was removed and DMSO was added 150 μl/well for 15–20 min. The absorptions (OD) were measured at 570 nm with SpectraMAX M5 microplate spectrophotometer (Molecular Devices). The effect of compounds on tumor cells viability was expressed by IC_{50} of each cell line.
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18. Typical procedure for the synthesis of **1a**–**1e** and **1g**–**1r**. *Compound **7a** was prepared as follows*: To a stirred solution of **5a** (15 mmol) in 50 mL tetrahydrofuran was added potassium carbonate (30 mmol) and stirred at room temperature for 5 min and then cooled to 0–5 °C. 2-Chloroacetyl chloride (22.5 mmol) was added dropwise at 0–5 °C and after addition the reaction mixture was allowed to warm to room temperature and then stirred for 4 h until TLC analysis indicated the complete disappearance of starting material. The reaction was quenched by addition of 20 mL distilled water, addition dichloromethane was added and the dichloromethane layer after extraction(50 mL × 2) was separated, dried (Na_2SO_4) and concentrated in vacuo to afford crud product. The crud product was purified by column chromatography on silica gel using petroleum ether/ethyl acetate (1/4) as eluent, the yield was 86%.
19. Briefly, cells were seeded in 6-well plates at the density of (1×10^5/well) and cultured for 24 h, followed by **7a** treatment for another 24 h. At the indicated intervals, both attached cells and floating cells were harvested, sediments were resuspended in 1 mL hypotonic fluorochrome solution containing 50 μg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100, and then analyzed by flow cytometer (ESP Elite, Beckman-Coulter, Miami, FL). Apoptotic cells appeared in the cell cycle distribution as cells with a DNA content of less than that of G1 cells and were estimated with Listmode software.
20. Briefly, cells (1×10^5/well) were seeded in 6-well plates and cultured for 24 h, followed by **7a** treatment for another 24 h. After rinsing with PBS, the cells were fixed using 75% of ethanol. The morphological change of cell was examined by inverted microscope, then the cells were stained with Hoechst 33342 (2 μg/mL, in PBS) and analyzed under fluorescence microscope (Zeiss, Axiovert 200, Germany) to identify the apoptotic cells.
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