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Short communication

Design, synthesis and biological evaluation of substituted aminopyridazin-3(2*H*)-ones as G0/G1-phase arresting agents with apoptosis-inducing activities

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ABSTRACT

A series of aminopyridazin-3(2H)-one derivatives has been designed and synthesized. Their antiproliferative activities were evaluated against three human cancer cell lines (SH-SY5Y human neuroblastoma, K562 human myelogenous leukemia and AGS gastric cancer cell lines) using the MTT assay. The preliminary activity test displayed that compound **8a** exhibited comparable activities against all test cells with the positive control fluorouracil. Meanwhile compounds **8b**, **8e** and **9c-e** displayed selective antiproliferative activities for SH-SY5Y cells. Furthermore, compounds **8a-b** with low-micromole GI_{50} value for SH-SY5Y cells induced apoptosis with cell cycle arrest at G0/G1 phase in SH-SY5Y cells in a dose-dependent manner.

Cancer is one of the common malignant diseases that presents an increasingly serious threat to the health of everyone in the world [1]. Therefore, the continued efforts for new small-molecule anticancer agents remain critically important. It has been reported that phosphodiesterase 4 (PDE4), which specifically catalyzes the hydrolysis of cAMP, is ubiquitous in the body and has been proposed to play a role in growth and tumor promotion [2,3]. For example, the phosphodiesterase 3/4 inhibitor **1** (zardaverine) exhibits potent and selective antitumor activity against hepatocellular carcinoma (HCC) [2].

In addition, the pyridazinone framework has emerged as a promising and attractive scaffold in the development of potent antitumor agents [4–10]. For example, the c-Met inhibitors pyridazin-3(2*H*)-one derivatives (**2** [10] and **3** [6], Fig. 1) have been reported to show remarkable antitumor cytotoxicity; compound **4** shows remarkable activity against SR (leukemia) and NCI-H522 (non-small cell lung cancer) with a GI_{50} value of less than 0.1 μM [8]; and aminophthalazinone **5** displays wonderful antiproliferative activities via apoptosis of proliferating cells [9]. Furthermore, some pyridazinone derivatives displayed excellent antitumor activity toward neuroblastoma, such as c-Met kinase inhibitor **6** (EMD1214063) [11]. 1*H*-indeno [1,2-*d*]pyridazin-1-one **7** exhibits potent cancer cell growth inhibition activity against human neuroblastoma IMR-32 cell line with nanomole GI_{50} value [12].

To get new pyridazin-3(2*H*)-one derivatives as small-molecule anticancer agents with PDE4 inhibitory activities, we designed aminopyridazin-3(2*H*)-ones (compounds **8** and **9**) by a simple strategy, inducing a longer amide side-chain (Fig. 2). Longer amide side-chain, such as 2-(2-methoxyphenoxy)ethylamino group, frequently occur at many antitumor compounds with excellent anticancer activities. For example, *N*-hydroxycinnamamides **10** [13] and 6,6-diphenyl-1,4-dioxanes **11** [14] (Fig. 2) bearing 2-(2-methoxyphenoxy)ethylamino group show good antitumor activities; the tubulin inhibitor **12** [15] exhibits antiproliferative activities with nanomolar GI_{50} values; and chalcone **13** [16] demonstrates NF-κB inhibitory activity with a micromolar GI_{50} and potent cytotoxicity against lung cancer cells. Herein, we describe the synthesis of (2-(2-methoxyphenoxy)ethyl)aminopyridazin-3(2*H*)-one derivatives bearing substituted benzyl groups

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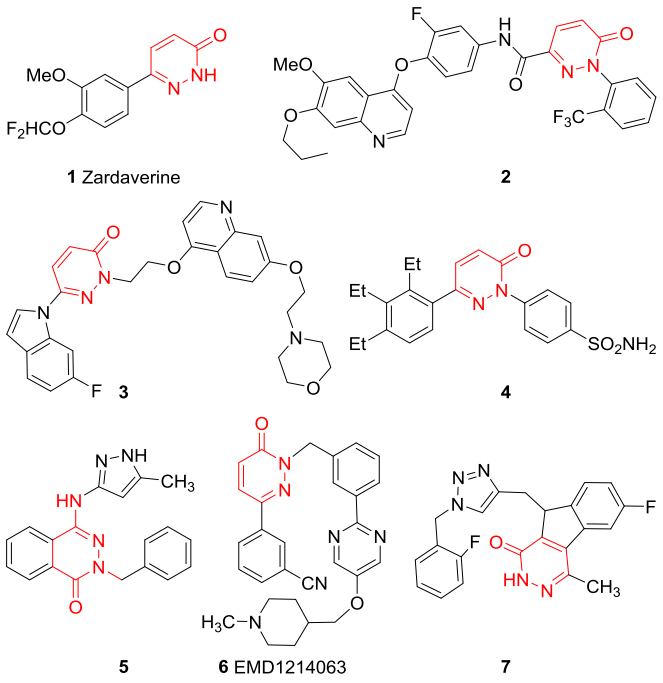
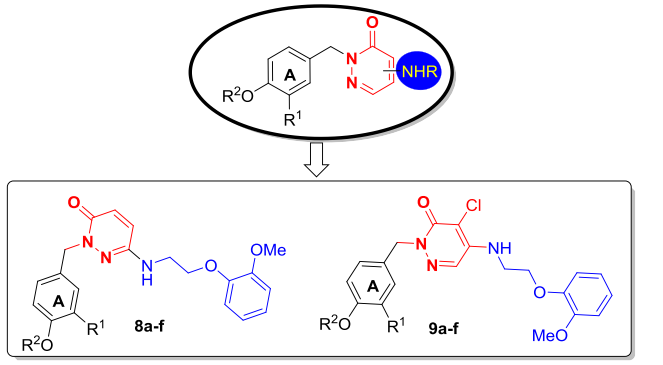


Fig. 1. Pyridazinone derivatives with antitumor activities.



8a R^1 = cyclopentyloxy, R^2 = CHF_2
8b R^1 = cyclopropylmethyloxy, R^2 = CHF_2
8c R^1 = MeO, R^2 = Me
8d R^1 = cyclopentyloxy, R^2 = Me
8e R^1 = cyclopropylmethyloxy, R^2 = Me
8f R^1 = Br, R^2 = Me

9a R^1 = cyclopentyloxy, R^2 = CHF_2
9b R^1 = cyclopropylmethyloxy, R^2 = CHF_2
9c R^1 = MeO, R^2 = Me
9d R^1 = cyclopentyloxy, R^2 = Me
9e R^1 = cyclopropylmethyloxy, R^2 = Me
9f R^1 = 3-chlorophenyl, R^2 = Me

Fig. 2. Design of aminopyridazin-3(2H)-one derivatives as potential antitumor agents.

as potential antitumor agents (Scheme 1). The preliminary evaluation of antiproliferative activity against three human cancer cell lines (K562, SH-SY5Y, and AGS), PDE4 inhibitory activities (the core catalytic domains of human PDE4), cell cycle analysis and apoptosis assay of the synthesized compounds were also performed. (See Fig. 3).

The route adopted for the preparation of (2-(2-methoxyphenoxy) ethyl)aminopyridazin-3(2*H*)-one derivatives is depicted in Scheme 1. As shown in Scheme 1, 3-alkoxy-4-difluoromethoxybenzyl alcohols **16** were obtained by the reduction of 3-alkoxy-4-difluoromethoxybenzaldehydes **15**, which were prepared from 3,4-dihydroxybenzaldehyde using our prior synthetic methodology [17]. Compounds **16** were brominated using PBr_3 as a brominating reagent, whereas compounds **14** were brominated using NBS as a brominating reagent. All the bromides were used in all subsequent reactions without further purification, owing to their instability. Compounds **8a-f** and **9a-f** were synthesized by the nucleophilic substitution of the bromides with the corresponding pyridazin-3(2*H*)-ones. Pyridazin-3(2*H*)-ones **18a** (R = H) and **18b** (R = Cl) were synthesized from 3,6-dichloropyridazine and 4,5-dichloropyridazin-3(2*H*)-one, respectively [18].

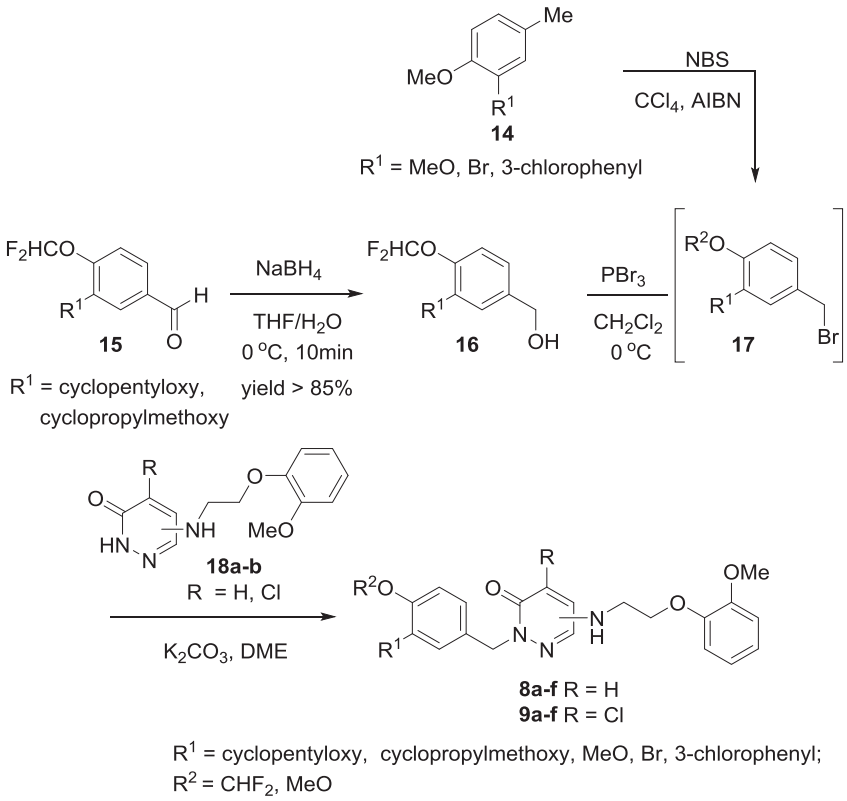
The antiproliferative activities of **8a-f** and **9a-f** were assessed against three human cancer cell lines, including human neuroblastoma (SH-SY5Y), human myelogenous leukemia (K562), and gastric cancer (AGS), using the MTT method [19]. Fluorouracil (5-FU), which is one of the most effective anticancer agents, was included in the experiments as a reference cytotoxic compound for the three cell lines. The results were expressed as growth inhibitory concentration (GI_{50}) values, which represent the compound concentrations required to produce 50% growth inhibition of cell growth after 48 h of incubation compared with untreated controls (Table 1).

As shown in Table 1, compounds **8a-f** and **9a-f** displayed different antiproliferative activities against different cancer cell lines, with the GI_{50} values ranging from 6.3 to >100 μM. For compounds **8a-f** bearing a 2-(2-methoxyphenoxy)ethylamino moiety at the 6 position of the pyridazin-3(2*H*)-one ring, their antiproliferative activities toward different cancer cell lines vary significantly. Compounds **8c** and **8f** were inactive in all test cells. Compound **8d** exhibited no activity toward SH-SY5Y and AGS cells, but exhibits moderate inhibitory activity against K562. Furthermore, compounds **8a**, **8b**, and **8e** showed moderate to good antiproliferative activity against the tested cancer cell lines and were more active toward SH-SY5Y cells. Of these, compound **8b** exhibited the best antiproliferative activity against SH-SY5Y cells with a GI_{50} value of 6.3 μM, which was higher than that of 5-FU (GI_{50} = 11.9 μM). Compounds **8a** and **8e** exhibited slightly decreased inhibitory activity against SH-SY5Y cells with the GI_{50} values of 9.3 and 10.5 μM, respectively, which were comparable to that of 5-FU.

By contrast, most of the compounds **9a-f**, which bear a 2-(2-methoxyphenoxy)ethylamino moiety at the 5 position of the pyridazin-3(2*H*)-one ring showed remarkably decreased inhibitory activity against the test cells. Compounds **9a** and **9f** were inactive against all the test cells, while compound **9b** exhibited weak inhibitory activity against K562. However, compounds **9c-e** showed selective inhibitory activities for SH-SY5Y with moderate GI_{50} value.

These results indicated that most of these compounds exhibited weak (or even zero) to moderate activity against the tested cell lines. However, compounds **8a**, **8b**, and **8e** were more active against SH-SY5Y cells, exhibiting good inhibitory activity. Moreover, compound **8b** with low-micromolar GI_{50} value displayed selectivity for SH-SY5Y cells over other two test cell lines. These findings provide useful information regarding the structural requirements for better potency and will help in designing more potent small molecules that selectively target SH-SY5Y cells.

Several studies have indicated that inhibition of PDE4 reduces proliferation, inhibits brain tumor cell growth [20,21], and causes selective apoptosis of malignant cells without affecting normal healthy cells [22]. Thus, the inhibitory activities (Table 1) of compounds **8a-f** and **9a-f** were evaluated against PDE4 according to reported protocols [17,23] using rolipram as a positive control (see Fig. S1 in supporting information). All compounds were tested at nine concentrations (10^{-8}–10^{-4} M) and their IC_{50} values were determined by the nonlinear regression analysis of their inhibition curves. As shown in Table 1, most compounds exhibited moderate PDE4 inhibition activity. Among these compounds, compounds **8a-**



Scheme 1. Synthetic route for aminopyridazin-3(2*H*)-one derivatives **8a-f** and **9a-f**.

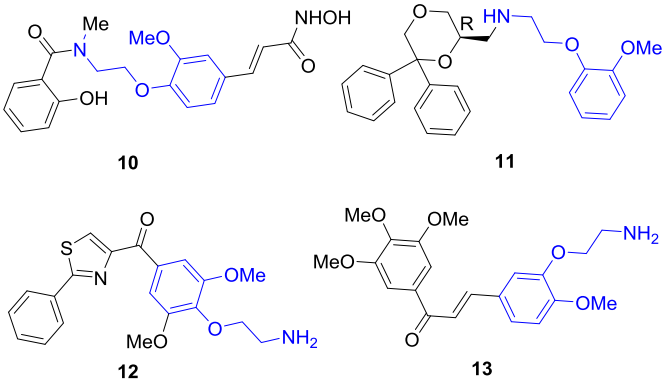
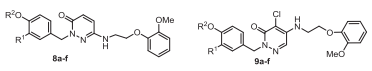


Fig. 3. Anticancer agents bearing the 2-(2-methoxyphenoxy)ethylamino moiety.

b with the best antitumor activities against SH-SY5Y cells displayed best PDE4 inhibition activities.

To study the effect of the synthesized compounds on cell cycle progression, the flow-activated cell sorting analysis was performed. The most promising compounds **8a** and **8b** were tested in SH-SY5Y cells. After treating the SH-SY5Y cells with compounds **8a** and **8b** at 5, 10, and 20 μM for 48 h, the cells were fixed and stained with propidium iodide (PI) for flow cytometry analysis. As shown in Fig. 4, the cells treated with compounds **8a** and **8b** were arrested at the G0/G1 phase, exhibiting an increase in the percentage of cells at the G0/G1 phase with a concurrent reduction in the percentage of cells at the S phases. The percentages of cells at the G0/G1 phase increased to 63.55% and 62.61% by **8a** and **8b**, respectively, at high concentration (20 μM) from 47.67% for the control group. It has

Table 1
Antiproliferative activities (GI_{50}, μM)[a] and PDE4 inhibitory activities (IC_{50}, μM)[a] of the aminopyridazin-3(2*H*)-one derivatives.



Compd.	R^1	R^2	K562	SY5Y	AGS	PDE4 inhibitory activities
8a	cyclopentyloxy	CHF_2	9.1 ± 1.1	9.3 ± 1.2	23.6 ± 1.1	12.2 ± 0.3
8b	cyclopropylmethoxy	CHF_2	60.9 ± 1.2	6.3 ± 1.1	24.4 ± 1.0	15.3 ± 0.7
8c	methoxy	CH_3	>100	>100	>100	66.9 ± 2.2
8d	cyclopentyloxy	CH_3	28.0 ± 1.3	>100	>100	>100
8e	cyclopropylmethoxy	CH_3	16.6 ± 1.2	10.5 ± 1.2	45.8 ± 1.1	>100
8f	Br	CH_3	>100	>100	>100	44.0 ± 1.9
9a	cyclopentyloxy	CHF_2	>100	>100	>100	39.1 ± 2.3
9b	cyclopropylmethoxy	CHF_2	67.6 ± 1.1	>100	>100	>100
9c	methoxy	CH_3	>100	33.1 ± 1.1	>100	73.0 ± 2.8
9d	cyclopentyloxy	CH_3	>100	23.3 ± 1.2	>100	21.5 ± 1.7
9e	cyclopropylmethoxy	CH_3	>100	37.9 ± 1.1	>100	45.2 ± 2.4
9f	3-chlorophenyl	CH_3	>100	>100	>100	>100
5-FU			18.5 ± 1.1	11.9 ± 1.1	24.6 ± 1.0	

[a] Data are expressed as means ± SDs (standard deviations) from three independent experiments.

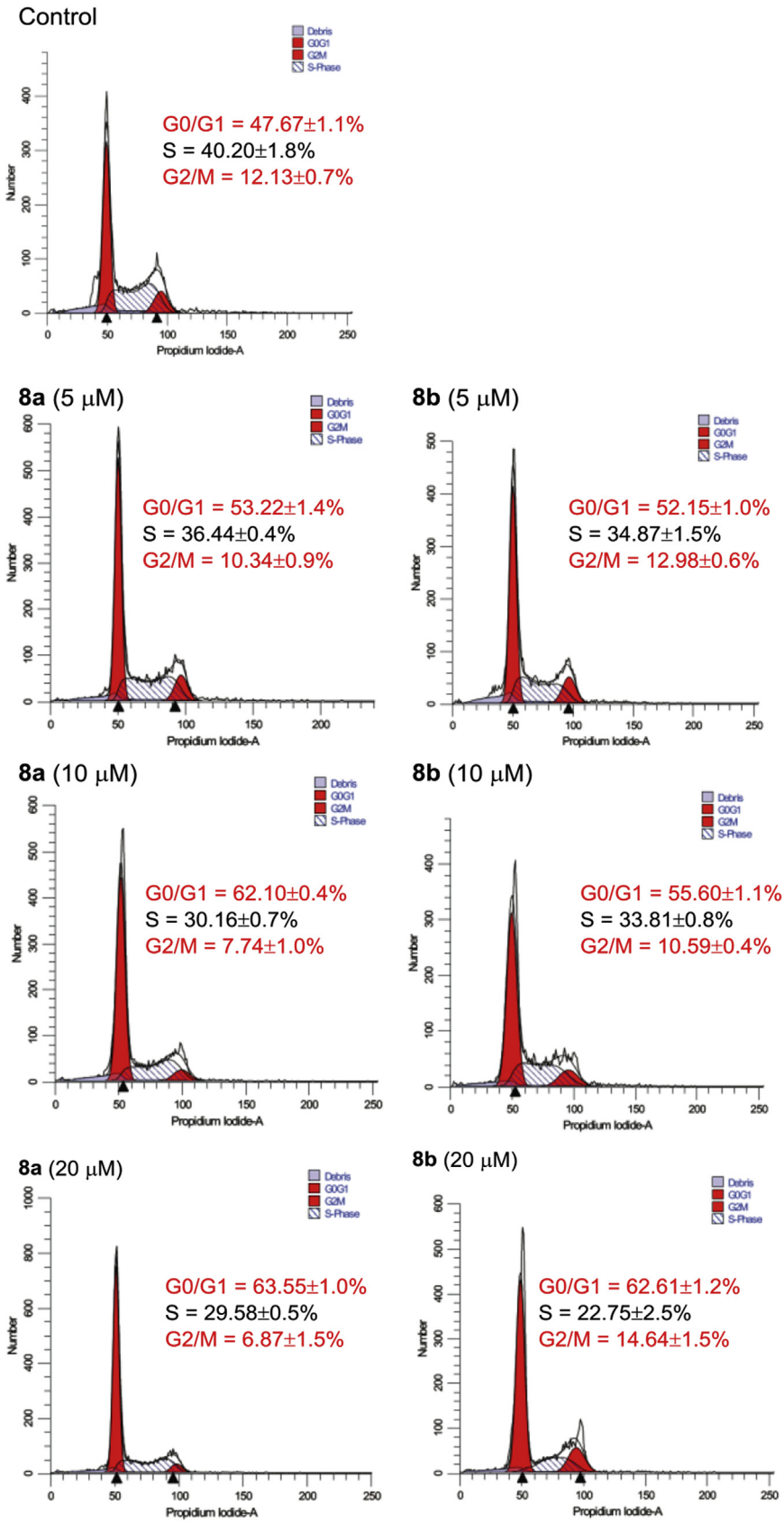


Fig. 4. Cell cycle distribution of SH-SY5Y cell lines after treatment with compounds **8a** and **8b** at different concentration. It determined by flow cytometry analysis after 48 h co-culture using DNA intercalating dye, propidium iodide (PI). DMSO was used as a control. Data are expressed as means ± SDs (standard deviations) from at least two independent experiments.

been reported that the phosphodiesterase 3/4 inhibitor zardaverine with potent antitumor activity induced G0/G1 phase cell cycle arrest of HCC cells [2]. Therefore, compounds **8a** and **8b** with moderate PDE4 inhibitory activities (12.2 and 15.3 μM respectively) may be have similar mechanism to zardaverine.

Further evaluation of the apoptotic effect of compounds **8a** and **8b** was performed using an Annexin V-FITC/PI (AV/PI) dual staining assay to examine the occurrence of phosphatidylserine externalization and to investigate whether it is due to physiological apoptosis or nonspecific necrosis. SH-SY5Y cells were treated with compounds **8a** and **8b** at 10 and 20 μM for 48 h to examine the apoptotic effect. As shown in Fig. 5, compounds **8a** and **8b** induced apoptosis of SH-SY5Y cells after 48 h co-culturing, and the percentages of late apoptosis by compounds **8a** and **8b** exhibited concentration dependence, increasing to 27.36% and 14.78%, respectively, from 4.89% for the control group. In addition, the percentage of apoptosis by **8a** is higher than that of **8b**.

In conclusion, a series of 2-aminopyridazin-3(2*H*)-ones bearing a 2-methoxyphenoxyethyl moiety (**8a-f** and **9a-f**) was synthesized and fully characterized. MTT assays results showed that most of the compounds were either poorly active or inactive against K562 and AGS cells. However, compound **8a** exhibited comparable activities against all test cells with the positive control fluorouracil, while compounds **8b**, **8e** and **9c-e** displayed selective antiproliferative

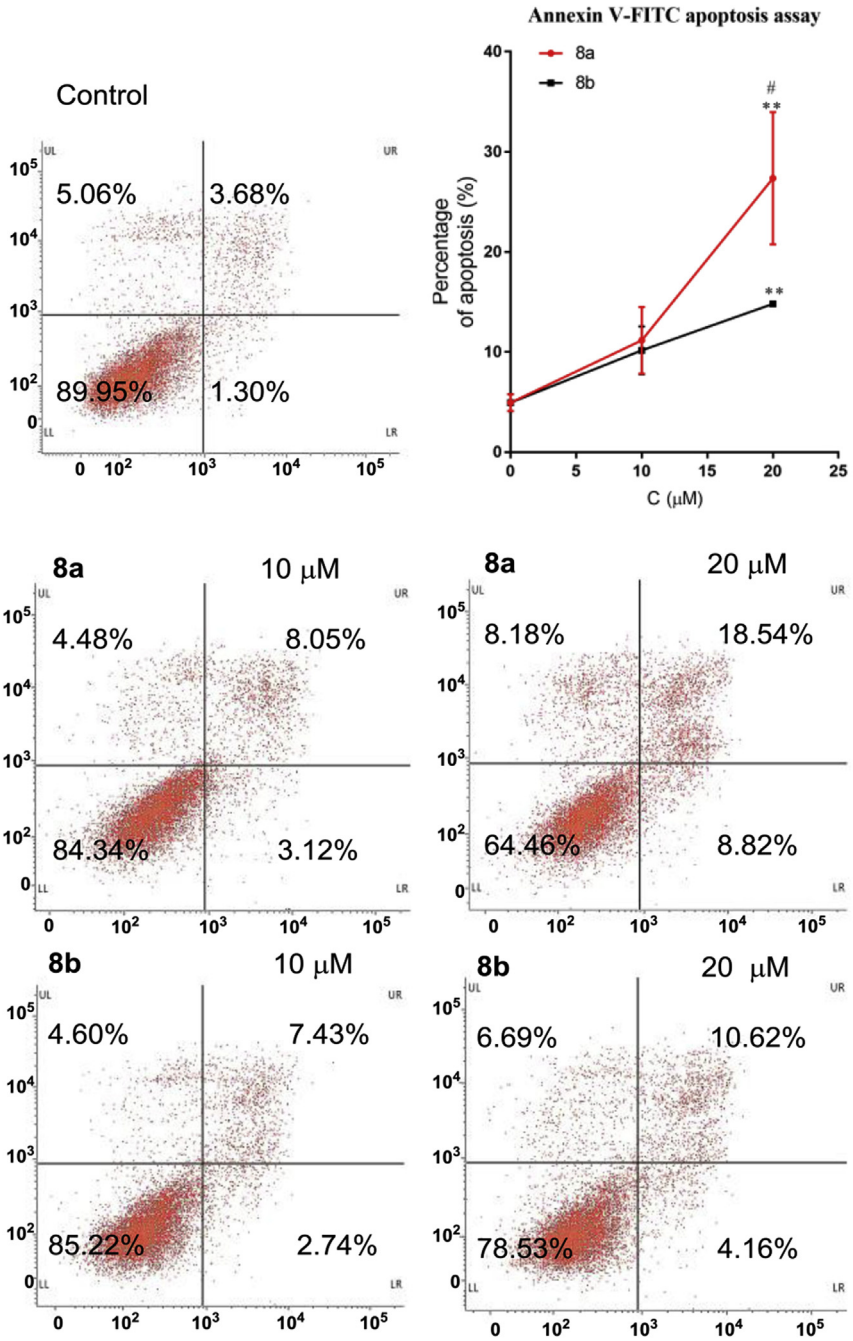


Fig. 5. Annexin V-FITC/propidium iodide analysis on apoptosis of SH-SY5Y after 48 h co-culture with compounds **8a** and **8b**. The four quadrants identified as: LL, live; LR, early apoptotic; UR, late apoptotic; and UL, necrotic. DMSO was used as a control. The values are mean of three independent experiments. *P < 0.05, **P < 0.01 versus control group. #P < 0.05 versus 10 μM groups.

activities for SH-SY5Y cells. Among these compounds **8a**, **8b**, and **8e** displayed more sensitive toward SH-SY5Y cells with low-micromole GI_{50} value (9.3, 6.3, and 10.5 μM, respectively), which were higher than that of the 5-FU control (GI_{50} = 11.9 μM). Furthermore, compounds **8a** and **8b** induced cell cycle arrest at the G0/G1 phase and apoptosis in human SH-SY5Y cells in a dose-dependent manner. Owing to the best selectivity antiproliferative activity of compound **8b** toward SH-SY5Y cells, compound **8b** could be identified as a lead compound that merits further optimization and development as an anticancer candidate against SH-SY5Y cells.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.09.077.

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