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A new small-molecule Aurora B inhibitor shows antitumor activity in human cancer cell lines

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Abstract The Aurora kinase family, as a group of serine/threonine kinases regulating cell cycles, are frequently overexpressed or amplified in human tumors. Here, we showed that the small molecule S4 could inhibit Aurora kinase in both of biochemincal and cell-based levels. The Aurora B inhibition of S4 treatment inhibited the phosphorylation of Histone H3 at serine 10 in HeLa and SMMC7721 cells. Cell proliferation assay showed that inhibition of Aurora kinase led to reduced cancer cell growth. As assessed in colony formation experiment, S4 blocked the capability of the HeLa cells to develop colonies. Subsequently, S4 treatment blocked the mitotic G2/M-G1 phase progression which is characterized by the accumulations of cells with 4 N DNA content, induced a cell cycle arrest in a pseudo G1 phase and resulted in apoptotic cell death in a dose- and time-dependent manners. Taken together, this Aurora kinase inhibitor S4 induces growth inhibition of cancer cell line.

Keywords Aurora B · Small-molecule inhibitor · Histone H3 · IC50

Introduction

The Aurora kinase family is a family of serine/threonine protein kinases that regulate many essential mitotic processes during cell division. Members of the Aurora-kinase family have recently been regarded as promising and potential targets for cancer therapy.

Humans express three Aurora kinases: human Aurora A, Aurora B and Aurora C. Aurora-A localizes at the centrosome and is required for centrosome maturation and separation [1]. As a chromosome passenger protein, Aurora-B binds to three other chromosome passenger proteins INCENP, survivin and borealin and is required for correct chromosome alignment and segregation [2]. Aurora-B locates from the centrosome to the spindle midzone at the onset of anaphase, accumulates in the spindle midzone and then concentrates at the midbody during cytokinesis. [3]. Inhibition of Aurora B activity inhibits Histone H3 phosphorylation at Ser10, prevents correct chromosome alignment, inhibits cytokinesis, and results in cell death. Aurora C is highly expressed in testis and its function is less known. [4].

Currently chemotherapy drugs treatment is still a commonly used method for cancer therapy. For example, fluoropyrimidines, gemcitabine and topoisomerase inhibitors play their functions in the DNA replication process. Another type of chemotherapy drugs disrupt the microtubule cytoskeleton which is essential for the mitotic process and then cause death of proliferating cells [5]. However, in adult tissues, both of normal cells and tumor cells continuously proliferate. These cellular cytotoxic drugs indiscriminately target proliferating cells, damage both of normal and tumor tissues, and then cause toxicity to the patients. Some other proteins, playing important role in regulating cell cycle and highly overexpressed in specific tumor cell lines [6], would help to develop a new strategy of cancer therapy. Targeting these proteins, new generation of effective therapeutic drugs can inhibit tumor tissue specifically and have minimal toxicity to normal tissue.

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Aurora kinases have emerged as an exceptionally attractive target for anticancer drug discovery. In recent years, several small-molecule inhibitors targeting Aurora kinases have been developed. Our laboratory as early as 1998 independently cloned Aurora B gene and found out that it can be hyper expressed in a wide variety of neoplastic cells and carcinoma tissues. In 2006, with the establishment of a highly efficient and reliable kinase high-throughput screening model, we performed two rounds of selection to seventy thousand small molecule compounds at the National Center for Screening, and got dozens of small molecules lead compounds which can significantly inhibit the activity of Aurora B kinase. One of them is S4. In the present study, we evaluated the effects of a new small molecular S4, which is a new potential Aurora kinase inhibitor, on cell cycle progression, proliferation, apoptosis, colony formation of human cancer cell lines.

Material and method

Cell culture

HeLa & SMMC-7721cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) plus 10 % fetus bovine serum (FBS) (Invitrogen) at 37 °C and 5 % CO_2. S4 was dissolved in DMSO at 10 mM and stored at 4 °C.

Cell-based determation of endogenous phospho-histone H3 (Ser10) level

HeLa cells were seeded into 6 cm dishes at the density of 2×10^5 cells per dish. Cells were then added with 0–20 μM S4 and only with DMSO as control. After 48 h, Cells were harvested with 1 × loading buffer. Then the proteins were separated using PAGE gel electrophoresis following wet electro-transfer onto nitrocellulose membrane. After 1 h blocking with 5 % milk, the membrane was incubated at 4 °C over night with rabbit anti-phospho-Histone H3 (Ser10) monoclonal antibody (9706, Cell Signaling), rabbit anti-Histone H3 polyclonal antibody (sc-8654, Santa Cruz), and mouse anti-β actin antibody (2146, Cell Signaling). After washed with 1X TBST for 4 times, the membrane was then incubated with HRP conjugated anti-mouse and anti-rabbit IgG antibody (Santa Cruz) at Room temperature for 1 h. After washing with 1X TBST for 4 times again, the membrane was developed with the ECL system (Santa Cruz).

Cell proliferation assay

Cells were plated into 96-well plate at the density of 1,000–5,000 cells per well and incubated at 37 °C in an atmosphere of 5 % CO2 and 95 % air overnight. Next day, cells were treated with series diluted concentration of S4 for 96 h. The number of viable cells was detected continuously every 24 h using CCK-8 kit (Dojindo, Japan). To avoid the effect of compounds and serum, complete medium containing S4 was replaced with fresh serum-free medium containing CCK-8 reagent. After incubation, absorbance was measured using microplate reader (model 550, Bio-Rad), 450/650 nm were used as measure/reference wavelength respectively.

Colony formation

HeLa cells were seeded into 60-mm plastic dish (Falcon) with approximate 300 cells/per dish and cultured over night. After the 24 h incubation, S4 was added at different concentrations (10–20 μM). After 96 h, medium containing S4 was replaced with fresh medium containing DMSO alone. After incubation for another 12 days, colonies were fixed with pre-cold methanol, stained with 5 % crystal violet, and counted.

Cell cycle analysis

HeLa cells were cultured with or without (only DMSO) various concentrations of S4 for 48 or 72 h. Then cells were harvested, washed with with PBS and fixed in 70 % ethanol for 24 h at 4 °C. Next day cell samples were labeled with 400 μl propidium iodide (50 μg/ml) plus RNase (1 mg/ml) for at least 10 min at 37 °C. Cell-cycle profiles were determined by flow cytometric analysis.

Apoptosis analysis

HeLa cells were treated with S4 for 48 and 72 h. Apoptosis was detected using an annexin V-PE apoptosis detection kit (BD). 2×10^5 Cells were harvested, washed twice with cold PBS and then resuspended in 100 μL 1 × binding buffer. 5 μL of Annexin V-PE and 5 μL of propidium iodide were added to the cell suspension. After 15 min in the dark, 400 μL 1 × binding buffer was added to each sampletube, apoptosis analysis were determined by flow cytometric analysis.

Cell synchronization

HeLa cells were synchronized to the beginning of S phase using a double thymidine block. After releasing from the block, cells were treated in the presence of 15 μM S4 or DMSO collected at the indicated time points and then labeled with propidium iodide as mentioned the cell cycle analysis. The DNA content of these cells were analyzed by flow cytometry.

Results

S4, a novel inhibitor of Aurora B

In order to prove the S4 can selectively inhibit kinase activity, we have made a kinase-S4 selective experiments of 20 kinases included Aurora A. The result of this experiment is that the IC50 of Aurora B is 9.85 μM, the IC50 of Aurora A is 69 μM which indicated that S4 almost have no inhibiting effect to Aurora A, and the IC50 of the other 18 kinases are all beyond 100 μM (Fig. 1c).

Histone H3 is a confessedly direct downstream substrate of Aurora-B kinase [7], and inhibition of Aurora B by the inhibitor treatment always decrease the phosphorylation level of Histone H3 at Ser10 dramatically. To determine whether S4 inhibits the Histone H3 phosphorylation at serine 10, 2×10^5 cells were seeded in 6-well plate. 24 h after seeding, S4 was added to the medium with series of concentrations and incubated at 37 °C for 48 h. Then cells were washed with PBS and lysed for following analysis. About 20 μg of total protein were used for western blot. Ser10-phosphorylated Histone H3 specific antibody and Histone H3 antibody were used to detect the phosphorylation level of Ser-10 in Histone H3 and total Histone H3. Compared with the untreated control cells, HeLa cell treated with 10 μM S4 showed significant decreases in Histone H3 phosphorylation. 20 μM S4 treatment showed a further reduction. However, total Histone H3 was not affected upon S4 treatment, demonstrating that the decreased PhH3 (Ser10) was due to the inhibition of phosphorylation but not due to the degradation or down-regulation of total Histone H3 proteins.

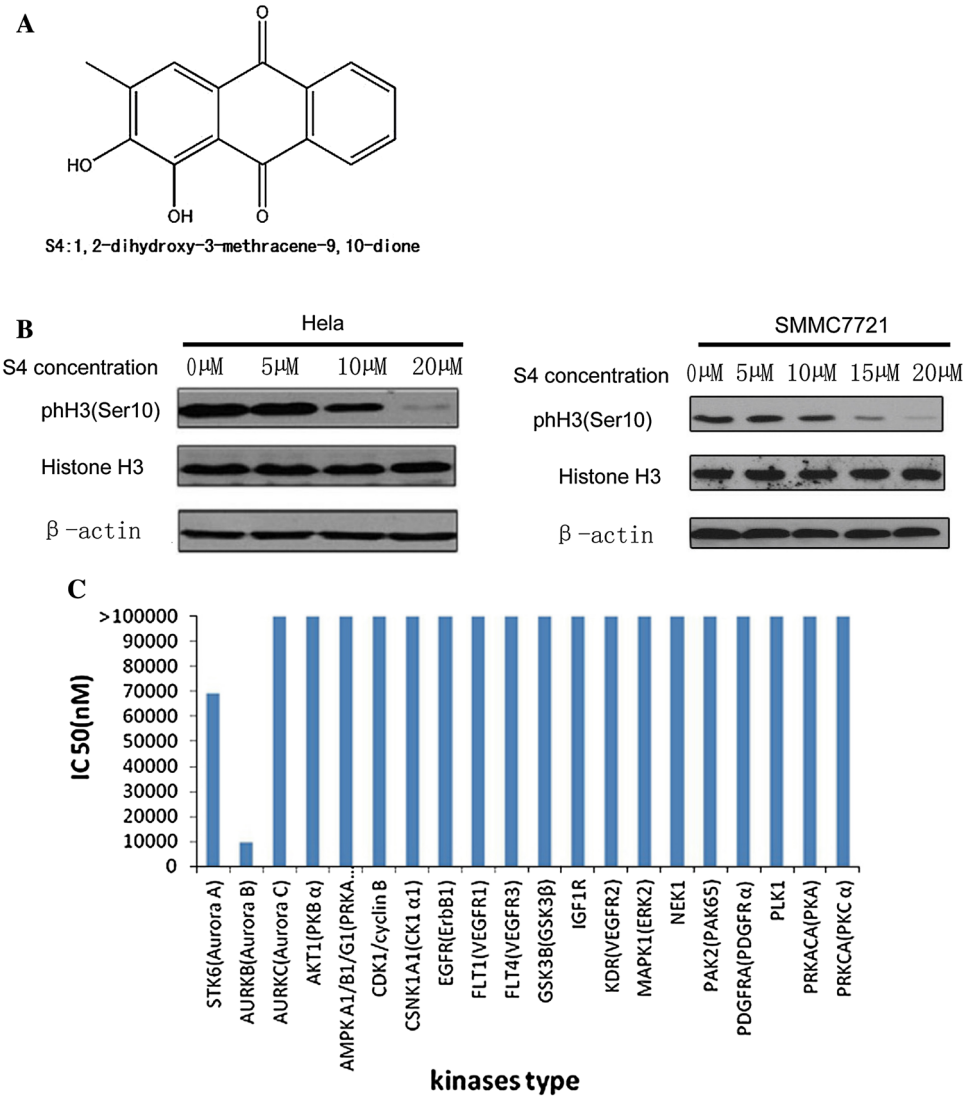

Fig. 1 Effect of S4 on phosphorylation of histon H3. **a** Chemical structure of S4(1,2-dihydroxy-3-methracene-9,10-dione). **b** HeLa cells and SMMC7721 cells were treated with various concentrations of S4. At 48 h, cells were harvested and proteins were extracted, and subjected to Western blot analysis. Histone H3 (Ser10) phosphorylation was downregulated in a concentration-dependent manner. Representative sample of three experiments with similar results. **c** Result of kinase-S4 selective experiments, indicated that S4 almost have no inhibiting effect to Aurora A and the other kinases

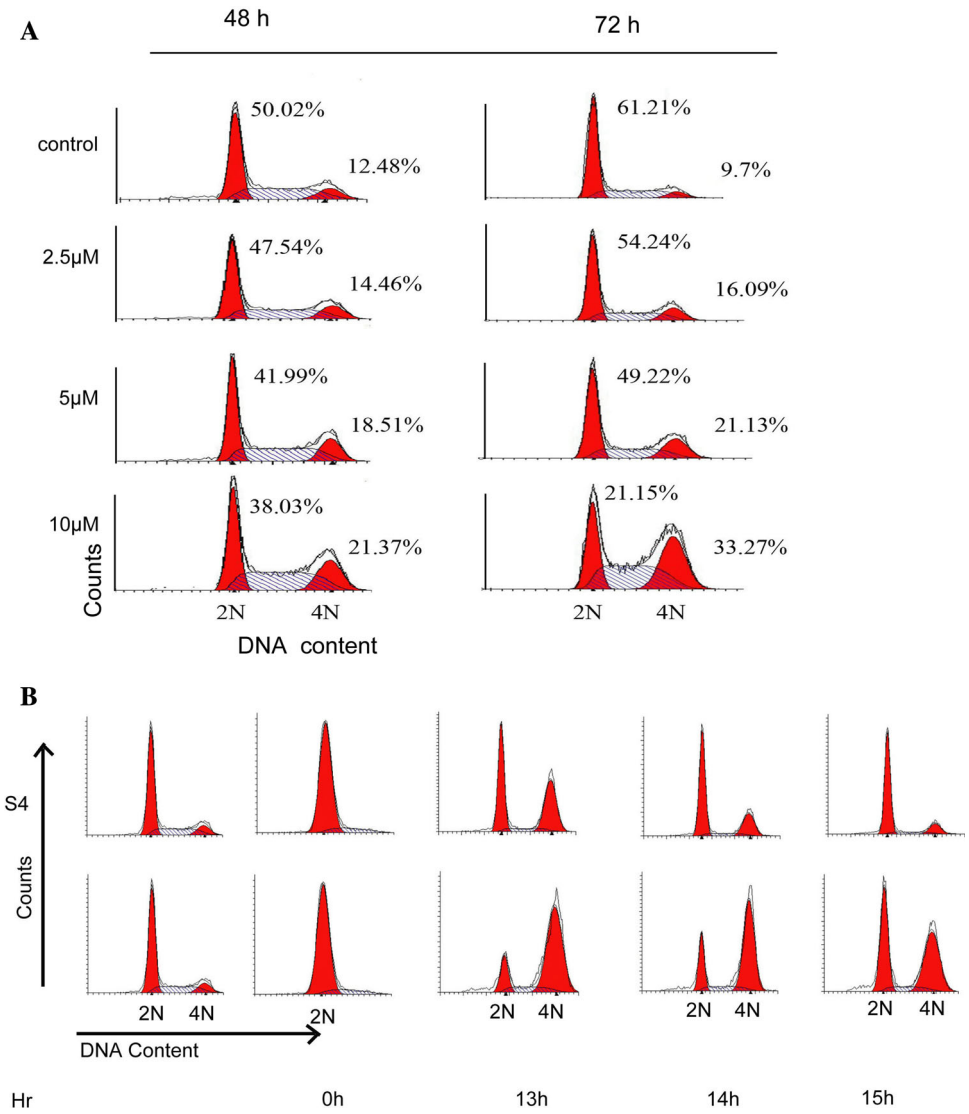

Fig. 2 Effects of S4 on cell cycle. **a** HeLa cells was seeded at the density of 0.5×10^5 cells/ml and preincubated for 48 and 72 h. After incubation, the cells were analyzed with a flow cytometry as described in materials and methods. **b** HeLa cells were synchronized in G1 by double thymidine treatment and were released from G1-S block in the presence of 15 μM of S4 or DMSO vehicle. DNA content of cells collected at the indicated time points was assessed by flow cytometry analysis of cells labeled with propidium iodone (PI)

To conclude, phosphorylation levels of pH3 (Ser10) in HeLa and SMMC7721 cells were decreased by S4 in a dose-dependent manner, indicating S4 can inhibits the activity of Aurora B kinase in these cells effectively.

Inhibition of Aurora activity with S4 causes cells to be arrested at G2-M

Aurora B kinase plays an important function in cytokinesis. Therefore, inhibition of Aurora kinases disturbs the progression of cells through normal mitosis and results in the accumulation of cells with 4N DNA [8].To test the possibility of S4 to affect cellular mitosis, as shown in Fig. 2a, HeLa cells were exposed to S4 for 48 and 72 h. Flow cytometry analysis were performed to find increased 4 N cells, which were considered to be in the G2/M phase. The proportion of HeLa cells in G2-M phase with S4 treatment at all concentrations was higher than that with control treatment ($P < 0.05$) significantly. Examination of HeLa cells with a longer treatment of S4 revealed that the S4 can induce the accumulation of cells at G2 M phase in a time-dependent manner. As shown in Fig. 2a 21.37 ± 0.74 and 33.27 ± 0.74 % of cells underwent G2-M accumulation upon treatment with 10 μM S4 for 48 and 72 h, respectively, compared with 12.48 ± 0.65 and 9.7 ± 0.73 (DMSO treatment).

To confirm this observation of the S4-induced mitotic arrest, the double thymidine block were performed to synchronize HeLa cells at the G1/S border. At the time of release, the proportion of cells at G1/S phase was 63.11 ± 0.8–88.14 ± 0.88 %, indicating that HeLa cells are blocked at G1-S phase, as shown in Fig. 2b. Following the release, cells were treated with either 15 μM S4 or a corresponding concentration of DMSO. At the time point of 0 h

Table 1 IC50 values of S4 on cancer cell line and cell information

Cell line	Source	IC5O (μM)
HeLa	Human cervical cancer	7.7
SW620	Human colon cancer	9.1
SMMC7721	Human liver cancer	13.1
QGY	Human liver cancer	19.1
SW480	Human colon cancer	19.5
MCF-7	Human breast cancer	19.8
HCT116	Human colon cancer	22.8
MAD-MB-231	Human breast cancer	26.7
A375	Human malignant Melanoma	38.6
HepG2	Human liver cancer	55
Hep3B	Human liver cancer	>100
SK-Hep1	Human liver cancer	>100
HT-29	Human colon cancer	>100
PANC	Human pancreatic cancer	>100

after the release, the S4-treated HeLa cells entered G1-S phase, just as the control cells (not exposed to S4). However, at the time point of 13 h after the release, approximately 72.91 ± 1.59 % of S4-treated HeLa cells remain temporarily at G2-M phase, while only 42.67 ± 1.77 % of the untreated cells are at G2-M phase ($P < 0.01$; Fig. 2b); similar results were obtained at 14 and 15 h time points after the release, suggesting that S4 blocked G2/M → G1 phase progression.

S4 inihibits the proliferation of HeLa cancer cell lines

To test whether Aurora kinase inhibition would lead to cell death, we examined the anticancer effects of S4 on several human cancer cell lines. S4 was serially diluted and added into the cells in 96-well plate. After 96 h incubation, culture of cells treated with various concentrations of S4 showed a inhibition of cell growth in a dose-dependent manner. The half-maximal inhibitory concentration (IC50) values are shown in Table 1. Of the several cell lines analysed, HeLa cell line was the most sensitive cancer cell line to S4 treatment.

In addition to test the effects of S4 on aggressive potential, we studied the consequences colony formation of HeLa cells. In these experiments, the cells were cultured with or without S4 for 12 days. As shown in Fig. 3, the treatment with 0 μM S4 on HeLa cells produced 215 ± 15 number of colonies. Fewer colonies were produced in the 10 μM S4-treated HeLa cells, 113 ± 38, and the smallest number of colonies, 18 ± 6, was produced in the treatment with high concentration (20 μM) of S4. Exposure to 10 μM S4 inhibited colony formation by nearly 50 %, while exposure to 20 μM drug inhibited colony formation by nearly 90 %.

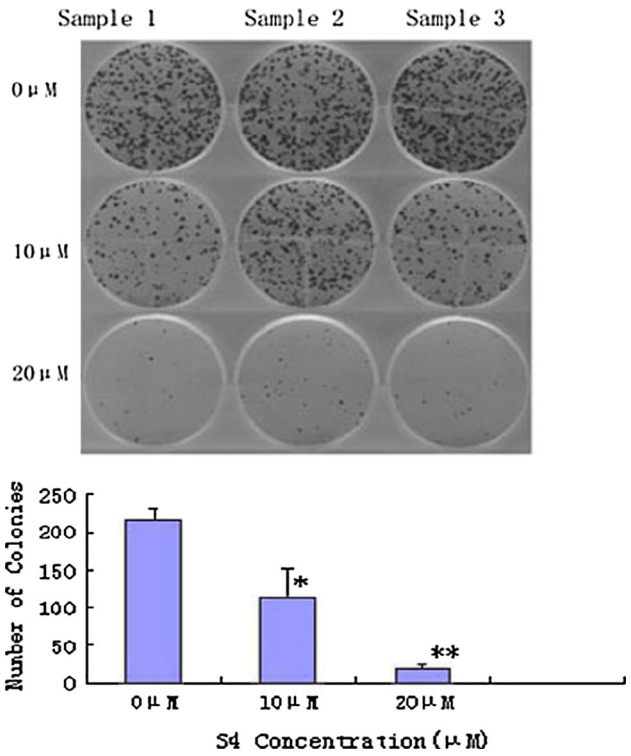

Fig. 3 Colony formation of S4 on HeLa. S4 showed inhibition on colony-forming abilities of HeLa cancer cell line in dose–response relationship. "*Sample1, Sample 2, Sample 3*" means three independent experiments (*$p < 0.05$, **$p < 0.01$)

S4 induces apoptotic cell death in concentration and time dependent manners

Since the above findings showed S4 has significant cytotoxic effection HeLa cells, FACS analysis was performed to evaluate whether the cells underwent apoptosis. As shown in Fig. 4, The percentage of sub-G1 cell population which is with less than 2 N DNA content, was quantified as a measure of apoptosis. The percentage of sub-G1 cells was found increased under S4 treatment. S4 induced concentration- and time-dependent apoptosis in HeLa cells. For example, exposure to either 10 or 20 μmol S4 induced either 11.37 or 26.86 % of HeLa cells, respectively, to become apoptotic at 48 h. Longer exposition (72 h) to S4 resulted in a significant increase of sub-G1 cells, which was more evident than 48 h treatment.

Annexin V staining was performed to detect the ability of S4 to induce the accumulation of annexin V-positive cells. Apoptotic cells were assessed by measuring annexin V staining in HeLa cells under S4 treatment. Apoptosis of HeLa cells were induced by the exposure to S4 (5–20 μM) for 48 or 72 h induced in dose- and time-dependent manners. As shown in Fig. 5, with the increasing duration of exposure and concentration to S4, percentages of cells in both early (lower right

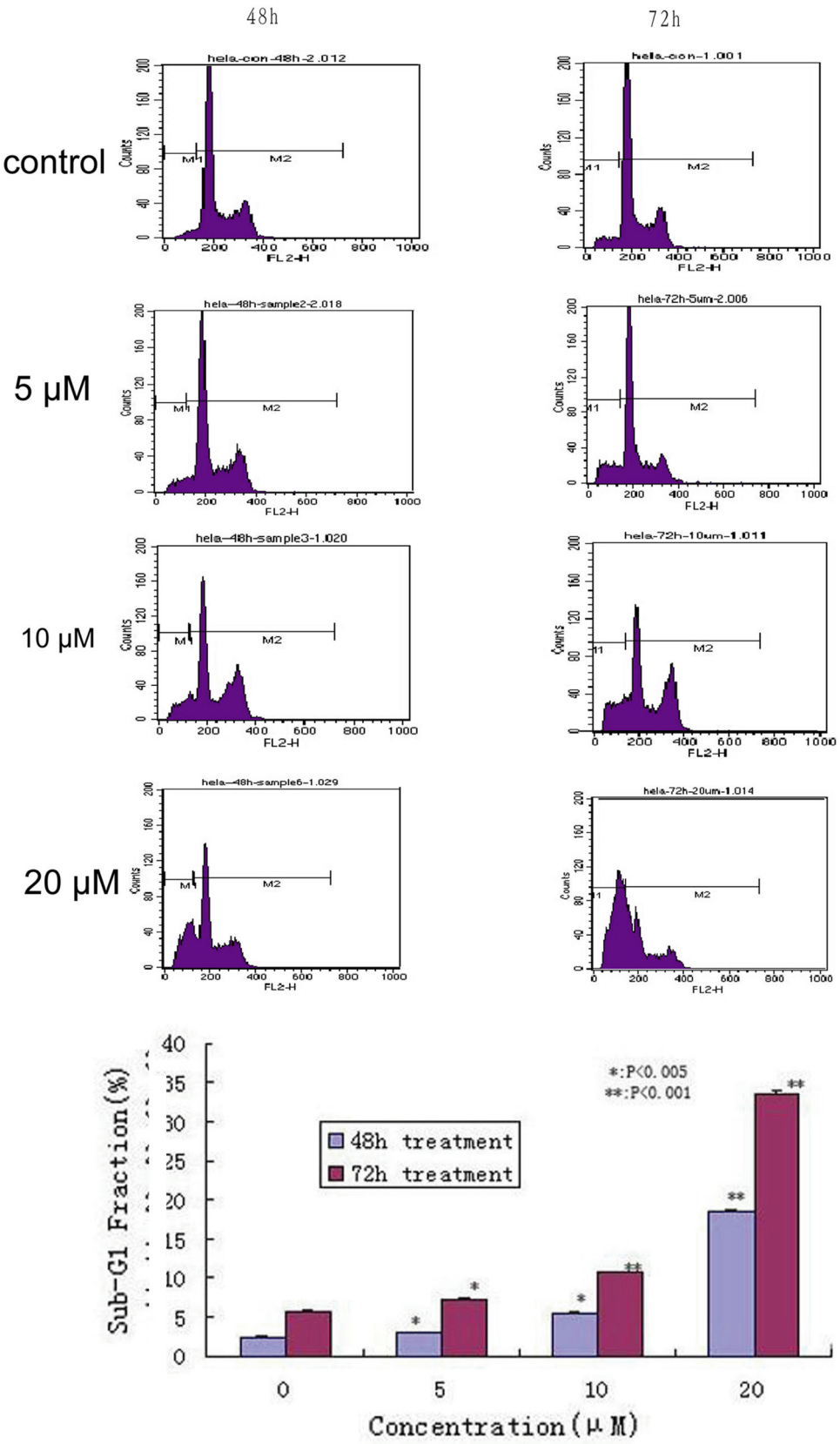

Fig. 4 Effect of the S4 on HeLa cell apoptosis. HeLa cells (0.5×10^5 cells/ml) were plated in 6-well plates and cultured with various concentrations of S4 (5–20μM). After 48 and 72 h, cells were harvested and SubG1 was analyzed by FACScan. Data reported were representative of one out of three similar experiments

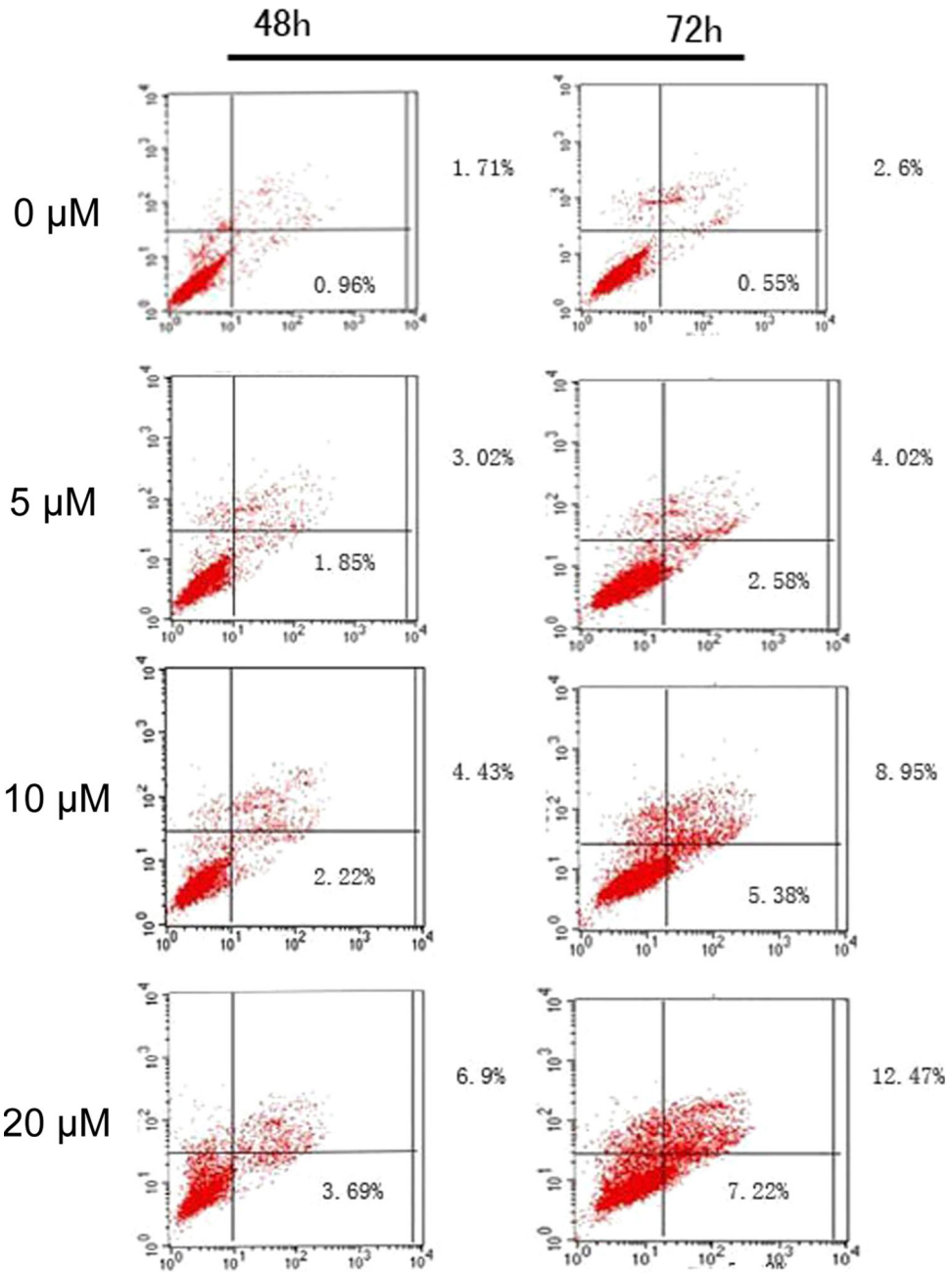

Fig. 5 Fluorescence-activated cell sorting analysis for the Annexin V confirms that S4 treatment induces cell death. HeLa cells (1×10^5 cells/ml) were plated in 6-well paltes and cultured with various concentrations of S4 (5–20 μM). After 48 or 72 h, cells were harvested, and Annexin V (*x-axis*) binding and propidium iodide (PI, *y-axis*) staining was analyzed by flow cytometry. *Lower left* quadrants show viable cells. *Lower right* quadrants show early apoptotic cells. *Upper right* quadrants show nonviable, late apoptotic cells and *upper left* shows necrotic cells. The numeric results represent the mean of triplicate plates, and a representative experiment is shown

panel) and late (upper right panel) apoptosis increased. After 48 h of treatment, there were 4.87, 6.65 and 10.59 % apoptotic cells at the concentration of 5, 10 and 20 μM respectively, compared to 2.67 % in the untreated control cells. After 72 h of treatment, there were 6.6, 14.33, and 19.69 % apoptotic cells at the concentration of 5, 10 and 20 μM respectively, compared to 3.15 % in the untreated control cells. These results indicate that S4 induces apoptotic cell death in concentration and time dependent manners.

Discussion

Mitosis is an extraordinarily complex and precise biological process. Errors in mitosis can induce genomic instability and then lead to tumorigenes typically. As key mitotic regulators, Members of the Aurora kinase family have been frequently found to be over-expressed in many types of human tumors. Perturbation of Aurora kinase's expression or function might tightly accociate with cancer.

Since Aurora kinase are thought to represent promising targets for anticancer drug development, small molecule inhibitors targeting Aurora kinase are regarded as new promising generation of cancer therapy drugs. Among them, the first generation of Aurora kinase inhibitors, ZM447439 [9], Hesperadin [3] and VX-680/MK-0457 [10] were identified or designed with different strategies. These three small molecule inhibitors inhibit the catalytic activity of the enzyme by competing with ATP [5]. All of them indicated typical penotypes of inhibiting Aurora kinase, such as inhibit the Histone H3 (Ser10) phosphorylation of and induce proliferation inhibition and apoptosis. Although Aurora-A was found to be tightly accociated with human cancer and attracted most of the attention so far, Aurora-B might be more suitable and promising as an anticancer drug target. Because inhibition of Aurora-B results in a catastrophic mitosis and then leads to cell death rapidly [11].

By means of western blotting experiments, we showed that the administration of S4 to HeLa and SMMC7721 cells decreased the phosphorylation level of Histone H3 at Ser10 dramatically and evaluated the effects of this new inhibitor targeting Aurora B kinase in vitro. We demonstrated that S4 treatment to these cells arrested proliferation in time- and dose-dependent manners and prevented the capability of the HeLa cells from developing colonies. S4 treatment blocked the G2/M → G1 phase progression characterized by the accumulation of cells with 4 N DNA content, arrested the cell cycle in a pseudo sub-G1 phase, and led to apoptotic cell death.

In conclusion, by inhibiting endogenous Aurora B kinase activity effectively, S4 induced the proliferation inhibition of human cancer cells.

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