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ORIGINAL ARTICLE

Panaxadiol, a purified ginseng component, enhances the anti-cancer effects of 5-fluorouracil in human colorectal cancer cells

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Abstract
Purpose Colorectal cancer is a major cause of morbidity and mortality for cancer worldwide. Although 5-fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents in first-line therapy for colorectal cancer, serious side effects limit its clinical usefulness. Panaxadiol (PD) is the purified sapogenin of ginseng saponins, which exhibit anti-tumor activity. In this study, we investigated the possible synergistic anti-cancer effects of PD and 5-FU on a human colorectal cancer cell line, HCT-116.
Methods Cell viability was evaluated by an MTS cell proliferation assay. Morphological observation was performed by crystal violet cell viability staining assay. Cell cycle distribution and apoptotic effects were analyzed by flow cytometry after staining with PI/RNase or Annexin V/PI.
Results Cell growth was markedly suppressed in HCT-116 cells treated by 5-FU (20–100 μM) for 24 or 48 h with time-dependent effects. The significant suppression on HCT-116 cell proliferation was observed after treatment with PD (25 μM) for 24 and 48 h. Panaxadiol (25 μM) markedly ($P < 0.05$) enhanced the anti-proliferative effects of 5-FU (5, 10, 20 μM) on HCT-116 cells compared to single treatment of 5-FU for 24 and 48 h. Flow cytometric analysis on DNA indicated that PD and 5-FU selectively arrested cell cycle progression in the G1 phase and S phase ($P < 0.01$), respectively, compared to the control condition. Combination use of 5-FU with PD significantly ($P < 0.001$) increased cell cycle arrest in the S phase compared to that treated by 5-FU alone. The combination of 5-FU and PD significantly enhanced the percentage of apoptotic cells when compared with the corresponding cell groups treated by 5-FU alone ($P < 0.001$).
Conclusions Panaxadiol enhanced the anti-cancer effects of 5-FU on human colorectal cancer cells through the regulation of cell cycle transition and the induction of apoptotic cells.

Keywords Panaxadiol · 5-Fluorouracil · HCT-116 human colorectal cancer cells · Anti-proliferation · Cell cycle · Apoptosis

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Introduction

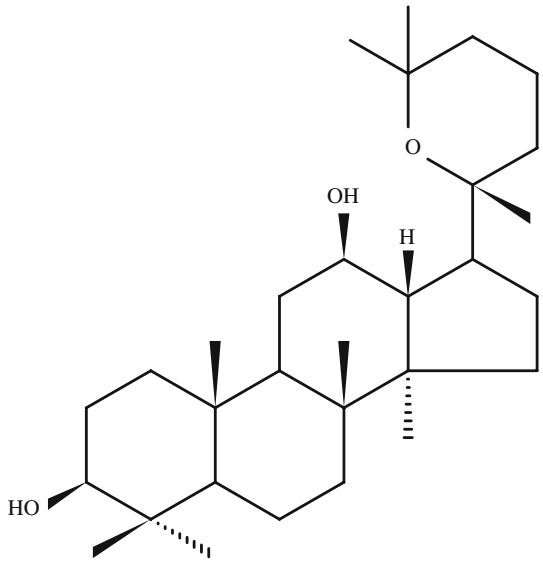
Colorectal cancer is the third most common noncutaneous malignancy in the United States and the second most frequent cause of cancer-related death [7, 32]. Although curative surgical resection is possible in 70–80% of patients after diagnosis, almost half of them will die from recurrence of cancer [33]. Recently, the median overall survival of patients with advanced colorectal cancer has been substantially extended from <9 months with no treatment to approximately 24 months when chemotherapeutic agents

are administered [5, 32]. Thus, chemotherapy prolongs survival.

5-Fluorouracil (5-FU) is one of the most widely used chemotherapeutic agents in first-line therapy for colorectal cancer [23, 26], and an overall survival benefit after fluorouracil-based chemotherapy has been firmly established [32]. Its clinical usefulness, however, is limited because of adverse effects such as nausea, fatigue, and a decline in the number of blood cells [4]. Cardiotoxicity is another well described and potentially lethal adverse effect of 5-FU [27]. For the treatment of metastatic colon cancer, higher doses of 5-FU produced more adverse effects but were no more effective than lower doses [18]. Therefore, if decreasing the dose of chemotherapy and increasing its anti-cancer effect could be accomplished by combining 5-FU with other agents, the patient may benefit.

The schedule of treatment with 5-FU has been modulated with oxaliplatin, leucovorin, and irinotecan for advanced colorectal cancer [22, 24, 25]. Yet the efficacy of these combinations is controversial because they have not yet contributed to an improvement in overall patient survival, despite their advantages over 5-FU alone [13, 23]. 5-FU also has been combined with antioxidants [2] and non-steroidal anti-inflammatory drugs [17] in human colon cancer cell lines. Although the active components and compounds derived from natural herbal medicines have been widely investigated in vitro and in vivo, chemotherapy with 5-FU and herbal medicines has seldom been studied.

In previous studies in our laboratory, notoginseng extracts, which contain high amounts of ginsenosides, enhanced 5-FU-induced apoptotic cell death in human colorectal cancer cells [30, 31]. The mechanism responsible is difficult to elucidate because the extracts contain various kinds of components and the quality of the crude extracts varies. We applied a purified ginseng component, panaxadiol (PD), a pseudoaglycone of diol-type ginsenoside with a dammarane skeleton (Fig. 1) in this study because it represents a class of new emerging antitumor agents. It has a number of biological properties, such as anti-cancer [8, 21], anti-emetic [19], and radioprotective effects [14, 15]. Jin et al. [8, 9] described the molecular mechanism by which PD stimulates cell death in human hepatoma cells. Its efficacy and the molecular mechanism by which it affects colorectal cancer cells in combination with 5-FU remain unknown. We investigated the potential synergistic anti-cancer effects of PD and 5-FU in a human colorectal cancer cell line, HCT-116. This cell line has been a model for studies of the cell pathway of chemotherapy on cancer cells [3, 20]. Our goal was to describe the therapeutic mechanism on HCT-116 cells when 5-FU and PD are combined.



Panaxadiol (Molecular Weight: 460.73)

Fig. 1 Chemical structure of panaxadiol

Materials and methods

Materials

All cell culture plasticware were purchased from Falcon Labware (Franklin Lakes, NJ) and Techno Plastic Products (Trasadingen, Switzerland). Trypsin, McCoy's 5A medium, and phosphate buffered saline were obtained from Mediatech, Inc. (Herndon, VA). 5-Fluorouracil was obtained from American Pharmaceutical Partners Inc. (Schaumburg, IL). Penicillin G/streptomycin was obtained from Sigma (St. Louis, MO). An MTS assay kit (CellTiter 96 Aqueous One Solution Cell Proliferation Assay) was purchased from Promega (Madison, WI). An Annexin V-FITC Apoptosis Detection Kit was obtained from BD Biosciences (Rockville, MD). PI/RNase staining buffer was supplied from BD Biosciences Pharmingen (San Diego, CA). Panaxadiol was provided by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Cell culture

The human colorectal cancer cell line HCT-116 was purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C with McCoy's 5A medium with 10% fetal bovine serum and 50 IU penicillin G/streptomycin. Cells were grown in a 25-ml flask and were routinely subcultured using 0.05% trypsin-EDTA solution. Cells

were maintained at the culture conditions described above for all experiments.

Cell viability assay

The effect of tested samples on the viability of HCT-116 cells was determined by the MTS assay. The logarithmically growing HCT-116 cells were plated into a 96-well plate at a density of 1×10^4 cells/well. After seeding for 24 h, the cells were treated with one of six concentrations of 5-FU (5, 10, 20, 30, 50, or 100 μM); or one of three concentrations of PD (5, 15, or 25 μM); or both drugs. All experiments were performed in triplicate.

At the end of the sample exposure period, either 24 or 48 h, the spent medium of each well was discarded and 100 μl fresh medium and 20 μl CellTiter 96 aqueous solution were added. The plate was returned to the incubator where it remained for 1∼4 h in a humidified atmosphere at 37°C. Then 60 μl of medium from each well was transferred to an ELISA 96-well plate, and the absorbance of the formazan product was measured at 490 nm. The blank control was recorded by measuring the absorbance at 490 nm with wells containing medium mixed with CellTiter 96 aqueous solution but no cells. Results were expressed as percent of control (vehicle set at 100%).

Crystal violet staining assay

Cells were seeded in 24-well plates (1×10^5 cells/well) and treated under the same conditions as described above for the cell viability assay. After 48 h of drug exposure or control conditions, the medium was removed and the cells were washed and stained with 0.2% crystal violet in 10% phosphate-buffered formaldehyde for 2 min. The staining solution was removed and the cells were washed twice with PBS. The remaining cells adhering to the wells were observed under the microscope and photographed.

Cell cycle analysis

HCT-116 cells were plated at a density of 2×10^5 cells onto 24-well tissue culture plates. The medium was replaced 24 h after seeding with fresh medium containing PD 25 μM; 5-FU of either 5, 10, or 20 μM; or both.

To analyze the cell cycle distribution, cells were trypsinized after 48 h of exposure to these samples, fixed with 80% ethanol, and stored at −20°C until analysis. Cells were suspended in PI/RNase staining buffer solution, and cell cycle analysis was performed using a flow cytometer (Bectom Dickinson, Mountain View, CA) and the FloJo software (Ashland, OR).

Apoptosis analysis

For apoptosis detection, floating cells in the medium and adherent cells were collected after 48 h of treatment. Using an Annexin V-FITC Apoptosis Detection Kit, cells were stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions. Untreated cells were used as the control for double staining. Cells were analyzed immediately. For each measurement, at least 20,000 cells were counted.

Statistical analysis

Experimental results represent triplicate determinations for each treatment group and are presented as means ± SEM. When one-way ANOVA showed significant differences between groups, Tukey's post hoc test was used to determine the specific pairs of groups between which statistically significant differences were found. $P < 0.05$ was the accepted level for statistical significance.

Results

Anti-proliferation effects of 5-FU and panaxadiol on HCT-116 cells

After treatment with 5-FU for 24 or 48 h, the proliferation of HCT-116 cells was markedly suppressed time-dependently. Dose dependence was observed at concentrations between 10 and 30 μM at each time point. Approximately 75% of proliferation of HCT-116 cells treated by 5-FU at the concentration of 50 and 100 μM was maintained at 24 h and approximately 48% at 48 h (Fig. 2a). Proliferation did not decrease further compared to groups treated by 30 μM 5-FU.

Significant suppression of proliferation of HCT-116 cells was observed after the treatment with PD 25 μM for 24 and 48 h. Anti-proliferation effects appeared to be dose-dependent at the two time points (Fig. 2b).

Based on the above results, we selected concentrations of 5-FU from 5 to 20 μM, which was in the dose-sensitive range, and of 25 μM of PD.

The influence of PD on 5-FU-induced anti-proliferation in HCT-116 cells is shown in Fig. 3. The suppression effect of treatment with 5-FU and PD combined was significantly stronger ($P < 0.05$) than that with 5-FU alone with the same treatment time and concentration of 5-FU. With longer incubation time, the co-treatment reduced the proliferation of HCT-116 cells more. Co-treatment with 25 μM PD agonized the effects of 5-FU, suggesting that PD may improve the anti-cancer effects of 5-FU on HCT-116 cells.

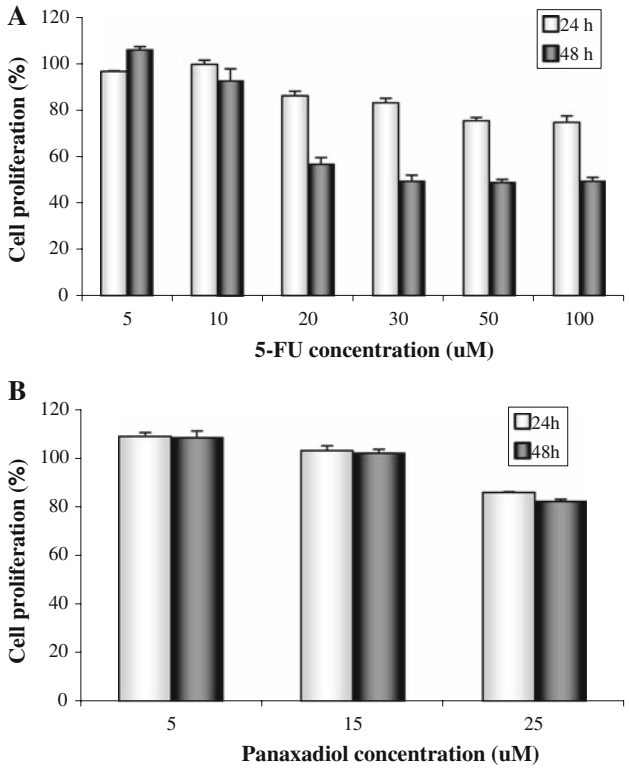


Fig. 2 The effects of 5-fluorouracil (5-FU) and panaxadiol on the proliferation of the HCT-116 cells. HCT-116 cells were incubated with varying concentrations of 5-FU (5, 10, 20, 30, 50, or 100 μM) and panaxadiol (5, 15, or 25 μM) for 24 and 48 h. **a** Percentage change of proliferation from control with 5-FU. **b** Percentage change with panaxadiol. Data are presented as the mean ± standard error of mean of triplicate experiments

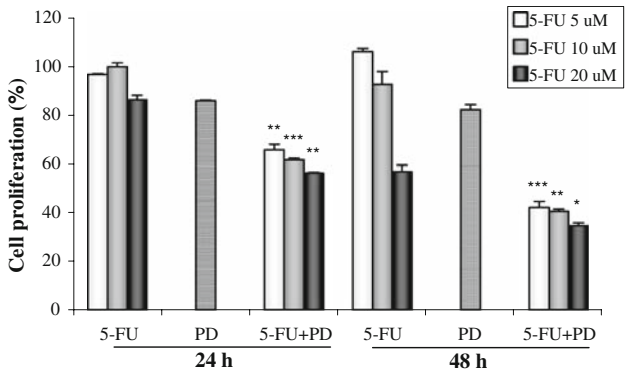


Fig. 3 The effects of panaxadiol on 5-fluorouracil (5-FU)-induced anti-proliferation. The combined effects of 5-FU at varying concentrations (5, 10, 20 μM) with panaxadiol (25 μM) on the proliferation of HCT-116 cells were compared with that of single use of 5-FU. Data are presented as the mean ± standard error of mean of triplicate experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, versus corresponding 5-FU groups

Morphological observation was conducted after crystal violet staining. Figure 4 shows the morphological characteristics after treatment for 48 h. In the control group, cells were smaller and compacted, and staining was even. In the 5-FU treatment groups, especially at higher concentrations, the number of dead cells was greater, and the cell confluence was markedly lower than in the control group. A similar result was observed in the group treated with PD 25 μM, and some cells contained small vacuoles. Cell confluence in the groups treated with 5-FU and PD was much lower than in the control group or the groups treated by 5-FU alone, and small vacuoles also appeared in the cells. Combined use of 5-FU and PD inhibited the growth of HCT-116 human colorectal cancer cells more effectively than did 5-FU alone or PD alone.

Effects of 5-FU and panaxadiol on cell cycle distribution in HCT-116 cells

To examine whether proliferation in treated cells decreased because of cell cycle arrest at a specific phase or the induction of apoptosis, we used flow cytometry for analysis.

As shown in Fig. 5b, flow cytometric analysis on DNA indicated that treatment with 5-FU (5–20 μM) for 48 h markedly ($P < 0.01$) induced the S-phase arrest of the cell cycle in a dose-dependent manner. In the group treated with 25 μM PD for 48 h, the G1 phase was arrested significantly ($P < 0.001$) compared with the control group. Following the exposure to 5-FU at 5, 10 or 20 μM, plus 25 μM PD for 48 h, the percentage of HCT-116 cells at the S phase of the cell cycle increased by 34, 33, and 40%, respectively, compared with the corresponding groups treated by 5-FU only (Fig. 5c, $P < 0.001$). The percentage of cells in the G1 and G2/M phases was significantly reduced in co-treatment groups. The changes in cell cycle distribution in groups treated for 24 h was similar (data not shown).

Apoptosis of HCT-116 cells

To observe the induction of apoptosis, HCT-116 cells exposed to the treatment for 48 h were stained with Annexin V/PI and analyzed by flow cytometry. The cytogram in Fig. 6a shows that incubation with 5-FU at 5 or 10 μM for 48 h did not alter the number of apoptotic cells, which was essentially the same as in the control group. With 5-FU 20 μM, the percentage of apoptosis increased to 10.1% (Fig. 6b, $P < 0.001$) and with PD 25 μM apoptosis increased to 9.7% ($P < 0.001$) compared to that of control (5.05%). The combination of 5-FU and PD significantly enhanced the percentage of apoptotic cells, (both those in early and late apoptosis) when compared with the corresponding cell groups treated by 5-FU only (Fig. 6b, $P < 0.001$). The presence of PD strengthened the 5-FU-induced apoptosis in HCT-116 cells.

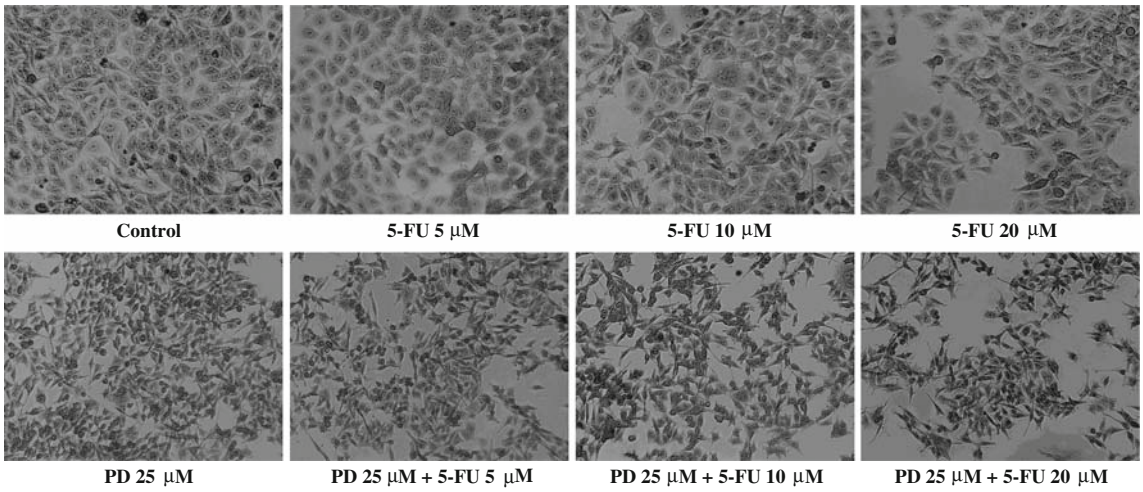


Fig. 4 Crystal violet staining assay on HCT-116 cells. The number of cells following treatment with the combination of 5-fluorouracil (5-FU) and panaxadiol decreased markedly and cell aspects were different from those treated with 5-FU alone. The microscope photo in each condition is representative of three independent experiments in which four fields were randomly captured for each group

Discussion

Previous studies have shown that both *Panax notoginseng* and American ginseng possess anti-proliferative effects on human colorectal cancer cells in vitro [28, 31]. *Panax notoginseng* root extract enhanced the actions of the chemotherapeutic agent, 5-FU, on human colorectal cancer cells [31]. The active constituents in *P. notoginseng* and American ginseng are the ginsenosides, Rb1, Rg1, and Rg3 [28, 29, 31]. Panaxadiol, the sapogenin of ginseng saponins, has been studied for the inhibition of human cancer cell lines by regulating cyclin A-cdk2 activity [8], but the effects of combination therapy with 5-FU on colorectal cancer cells remains unknown.

In this study, we evaluated the combined anti-tumor activity of 5-FU with PD on human colorectal cancer cells, HCT-116. The present study showed that the inhibitive effect on cell proliferation of 5-FU did not increase continuously (Fig. 2a) when the treatment concentration of 5-FU was increased. This result was consistent with that found in a previous clinical study [18] and in an in vitro study [30]. In our study, after HCT-116 cells were co-treated with 5-FU and PD for 24 or 48 h, cell proliferation was nearly 60 and 40%, respectively, results lower than those observed 5-FU alone at corresponding concentrations and times. Results were also lower than those after treatment with the highest dose (100 μM) of 5-FU for 24 h (74.6%) or 48 h (49.5%), suggesting that PD significantly boosts the anti-proliferative effect of 5-FU on HCT-116 cells and may reduce the dose of 5-FU needed to achieve the desired effects. Although 5-FU is cytotoxic to primary cells [1], the synergistic effect of PD on 5-FU-induced anti-proliferation of cancer cells may make it possible to reduce the dose of 5-FU in combination therapy and thereby decrease the dose-related toxicity caused by 5-FU.

In the apoptotic analysis by flow cytometry, the percentage of apoptotic cells increased more than 10% in the co-treatment groups compared with groups treated with 5-FU only, regardless of the concentration applied. The value of the apoptotic percentage in co-treatment groups was the total of the percentages in groups treated by 5-FU only plus the percentage in the group treated by PD, suggesting a potential synergistic interaction on the induction of apoptosis at the concentrations tested in this study.

Cell cycle progression, a series of events in a eukaryotic cell leading to its replication, is halted at the transition from the G1 to the S-phase or from the G2 to the M-phase after DNA damage [6]. 5-FU, a pyrimidine analog, is generally believed to induce G1-S-phase arrest via inhibition of thymidylate synthase, a key enzyme in DNA synthesis [34]. Our study also illustrated the specific arrest on the S phase by 5-FU and demonstrated that PD suppressed cell growth in HCT-116 cells by selectively arresting the cell cycle at the G1 phase. This result supports earlier observations that PD induces cell cycle arrest at the G1/S phase in human HeLa and SK-HEP1 cancer cell lines [8]. According to current data, both 5-FU and PD arrest the majority of cells in the G1-S phase of the cell cycle, thereby preventing entry into the G2/M phase. We showed that 5-FU and PD selectively arrest the S phase and G1 phase in HCT-116 cells.

Co-treatment with 5-FU and PD increased cell accumulation at the S phase, indicating that PD has the additive or synergistic effect on 5-FU-stimulated arrest at the S-phase in cell cycle progression. Panaxadiol derived from ginseng, may heighten the arrest of colon cancer cells in the S-phase induced by 5-FU.

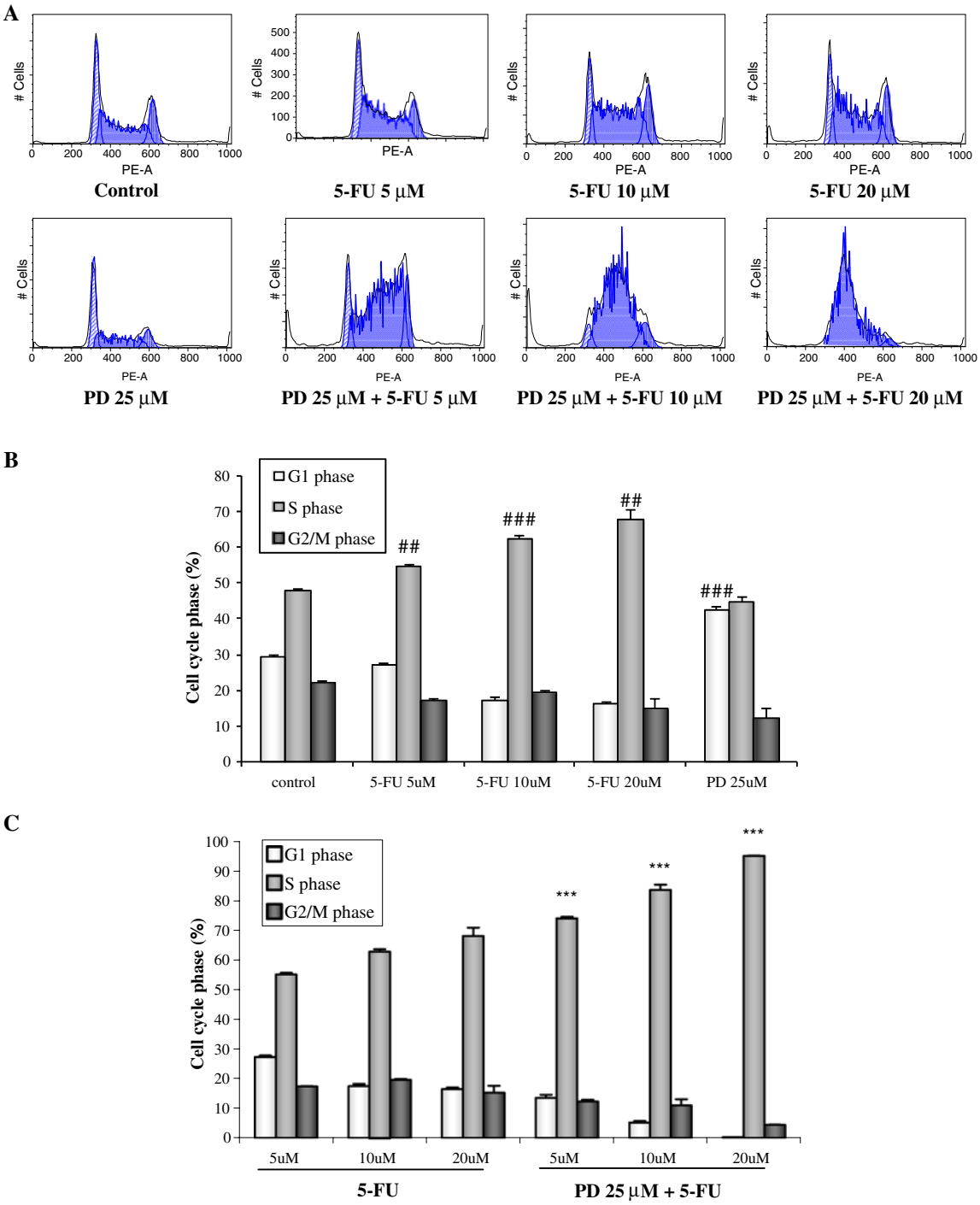


Fig. 5 The effects of panaxadiol on 5-fluorouracil (5-FU)-induced cell cycle arrest. HCT-116 human colorectal cancer cells were treated with 5-FU at various concentrations (5, 10, 20 μM) with the absence or presence of panaxadiol (25 μM) for 48 h, The cell cycle was assessed using PI/RNase staining and flow cytometric analysis. **a** The representative picture in each experimental group. **b**, **c** The percentages of each cell cycle phase with various treatment or with control. Data are presented as the mean ± standard error of mean of triplicate experiments. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus control; $^{***}P < 0.001$, vs. corresponding 5-FU groups

Cyclin A, a member of cyclins which are a family of proteins involved in the progress of cells through the cell cycle, binds to cdk2 and is required for the cell to progress through the S phase [17]. Previous in vitro study in mammalian cells revealed that PD selectively inhibits cyclin A-associated cdk2 activity by elevating p21WAF1/CIP1 protein levels [8]. These data may partially explain the observed synergistic effect of PD on the cell accumulation

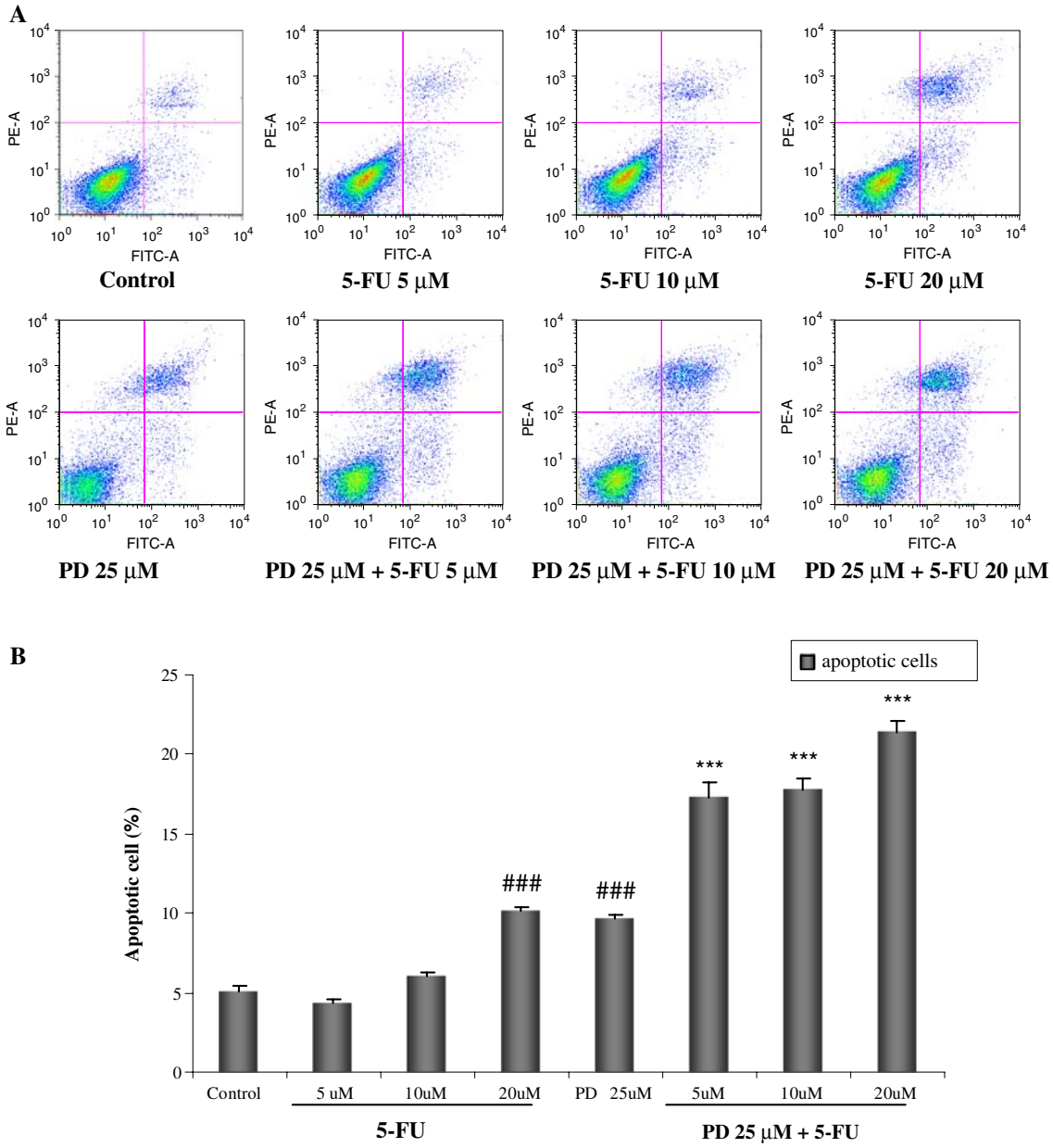


Fig. 6 The effects of panaxadiol on 5-fluorouracil (5-FU)-induced apoptosis. HCT-116 human colorectal cancer cells were treated by 5-FU at various concentrations (5, 10, 20 μM) with the absence or presence of PD (25 μM) for 48 h. Apoptosis was quantified using Annexin V/PI staining followed by flow cytometric analysis. **a** The representative picture in each experimental group. **b** The percentage of apoptoic cells. Data are presented as the mean ± standard error of mean of triplicate experiments. $^{###}P < 0.001$, versus control; $^{***}P < 0.001$, versus corresponding 5-FU groups

at the S phase induced by 5-FU, the cytotoxic effect of which is well documented to be dependent on p53 pathway [34].

When other drugs are combined with 5-FU, as pretreatment or in simultaneous combination with 5-FU, antagonistic or additive cytotoxic effects have been produced [10–12, 16] via different mechanisms. Our study clarified that 5-FU and PD suppress cell cycle progression by selectively arresting the S-phase and G1-phase. Thus, when 5-FU is used in combination with PD to enhance the therapeutic response, an important consideration is the schedule by which the drugs are administered. We evaluated the anticancer efficacy in response to the simultaneous treatment of 5-FU with PD only. The issue, whether treatment sequencing will affect the outcome of combination therapy has not been studied to obtain an optimal combination schedule.

In summary, a beneficial effect is achieved with the combination of 5-FU and PD chemotherapy on human colorectal cancer cells in vitro. The enhancement of S-phase arrest and the increased susceptibility to apoptosis are the synergistic

effects of PD on 5-FU. The issue whether this combination will similarly affect other human cancer cells remains to be determined.

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