JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Caspase-mediated pro-apoptotic interaction of panaxadiol and irinotecan in human colorectal cancer cells

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Keywords
apoptosis; cell cycle; human colorectal cancer; irinotecan; panaxadiol

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Received September 3, 2011
Accepted December 16, 2011

doi: 10.1111/j.2042-7158.2012.01463.x

Abstract

Objectives Panaxadiol is a purified sapogenin of ginseng saponins that exhibits anticancer activity. Irinotecan is a second-line anticancer drug, but clinical treatment with irinotecan is limited due to its side effects. In this study, we have investigated the possible synergistic anticancer effects of panaxadiol and irinotecan on human colorectal cancer cells and explored the potential role of apoptosis in their synergistic activity.

Key findings The combination of panaxadiol and irinotecan significantly enhanced antiproliferative effects in HCT-116 cells ($P < 0.05$). Cell cycle analysis demonstrated that combining irinotecan treatment with panaxadiol significantly increased the G1-phase fractions of cells, compared with irinotecan treatment alone. In apoptotic assays, the combination of panaxadiol and irinotecan significantly increased the percentage of apoptotic cells compared with irinotecan alone ($P < 0.01$). Increased activity of caspase-3 and caspase-9 was observed after treating with panaxadiol and irinotecan. The synergistic apoptotic effects were supported by docking analysis, which demonstrated that panaxadiol and irinotecan bound two different chains of the caspase-3 protein.

Conclusions Data from this study suggested that caspase-3- and caspase-9-mediated apoptosis may play an important role in the panaxadiol enhanced antiproliferative effects of irinotecan on human colorectal cancer cells.

Introduction

Colorectal cancer is the third most common malignancy in the United States and the second most frequent cause of cancer-related death.[1–2] Although curative surgical resection is possible in 70–80% of patients after diagnosis, the role of chemotherapeutic regimens is limited due to side effects and toxicity. In addition, almost half of patients will die from recurrence of the cancer.[3] Previous studies have shown that chemotherapy toxicity could be reduced by chemoadjuvant compounds that potentiate the tumoricidal effects of lower doses.[4–7] Cancer treatment with botanicals such as American ginseng has received increasing attention in recent years.[8–12] Natural products have also been a valuable source of new candidate compounds for therapy.[13–14] Panaxadiol, a pseudoaglycone of a diol-type triterpenoid with a dammarane skeleton (Figure 1), is an active anticancer compound found in steamed American ginseng.[15]

Irinotecan is one of the most common second-line chemotherapeutic agents used for colorectal cancer to control symptoms, maintain or improve quality of life, and prolong survival.[16] The potential risks of treatment-related mortality and morbidity, which consist mainly of neutropenia, nausea, vomiting, diarrhoea, and asthenia, must be considered when treating with irinotecan.[17] Thus, irinotecan is not usually considered a first-line medicine and is more frequently used in combination with 5-fluorouracil as a therapy for metastatic colorectal cancer.[16,18]

In this study, we have investigated the combination effects of panaxadiol and irinotecan on two human colorectal cancer cell lines, HCT-116 and SW-480. Panaxadiol significantly increased the antiproliferative effects of irinotecan. Furthermore, we observed the combined effect on cell apoptosis, cell cycle and caspase assays to elucidate the possible mechanism of panaxadiol and irinotecan combination effects on colorectal cancer cells. Docking was used to simulate the intermolecular interactions between these compounds and the caspase-3 protein. This study is the first to investigate the

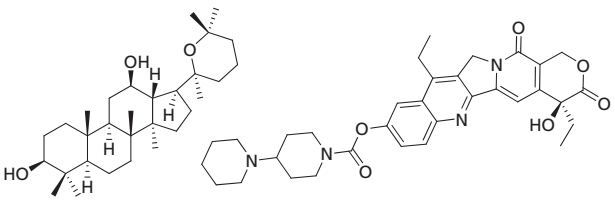

Figure 1 Chemical structures of panaxadiol and irinotecan.

combinatory anticancer effects of irinotecan and natural compounds from ginseng as an effective chemoadjuvant for colorectal cancer treatment.

Materials and Methods

Reagents and materials

All cell culture plasticware was obtained from Falcon Labware (Franklin Lakes, NJ, USA) and Techno Plastic Products (Trasadingen, Switzerland). Trypsin, McCoy's 5A and Leibovitz's L-15 media, and phosphate buffered saline (PBS) were obtained from Mediatech, Inc. (Herndon, VA, USA). Benzylpenicillin (penicillin G)/streptomycin and irinotecan were obtained from Sigma-Aldrich (St Louis, MO, USA). An MTS assay kit, CellTiter 96 Aqueous One Solution Cell Proliferation Assay, was obtained from Promega (Madison, WI, USA). An annexin V-FITC apoptosis detection kit was obtained from BD Biosciences (Rockville, MD, USA). PI/RNase staining buffer was obtained from BD Biosciences Pharmingen (San Diego, CA, USA). Cell Lysis Buffer was obtained from Cell Signaling Technology, Inc. (Boston, MA, USA). Caspase-3, 8 and 9 kits (with buffer and synthetic substrates DTT, DEVD-rNA, LEHD-rNA, IETD-rNA) were obtained from BioVison (Mountain View, CA, USA). Panaxadiol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Cell culture

The human colorectal cancer cell lines HCT-116 and SW-480 were obtained from American Type Culture Collection (ATCC). The young adult mouse colon (YAMC) cell line is a conditionally immortalized marina colon epithelial cell line isolated from the H-2Kb–tsA58 mouse (Immortomouse, Charles River Laboratories, Chicago, IL, USA). Cells were routinely grown in McCoy's 5A medium (for HCT-116) or Leibovitz's L-15 medium (for SW-480) or RPM 1640 medium (for YAMC), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (50 U/ml). For YAMC cells, 5 U/ml murine IFN -γ, 100 U/ml penicillin and streptomycin, 5 μg/ml insulin, and 5 μg/ml transferring were added. Cells were maintained in a tissue culture flask and kept in a humidified incubator (5% CO_2 in air at 37°C for HCT-116 and SW-480, 33°C for YAMC). The medium was changed every two to three days. When the cells reached 70–80% confluence they were trypsinized, harvested, and seeded into a new tissue culture flask.

Cell proliferation assay

The effect of panaxadiol and irinotecan on the proliferation of HCT-116 and SW-480 cell lines was determined using a modified trichrome stain (MTS) assay. Cancer 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 panaxadiol, irinotecan or both at various concentrations. All experiments were performed in triplicate. At the end of the sample exposure period, either 48 or 72 h, the 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 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 a percentage of control (vehicle set at 100%).

Cell cycle analysis

The cell cycle profile was assayed by flow cytometry after staining with PI/RNase, and the assay data from panaxadiol and irinotecan were compared. HCT-116 cells were seeded in 24-well tissue culture plates. On the second day, the medium was changed, and cells were treated with panaxadiol, irinotecan or both at different concentrations (panaxadiol 10 μM, irinotecan 1, 3, 5, 10, 20 or 30 μM). Cells were incubated for 48 h before harvesting. The cells were fixed gently with 80% ethanol before being placed in a freezer for 2 h. They were then treated with 0.25% Triton X-100 for 5 min in an ice bath. The cells were resuspended in 30 μl PBS containing 40 μg/ml propidium iodide (PI) and 0.1 mg/ml RNase. Cells were incubated in a dark room for 20 min at room temperature before cell cycle analysis with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and the FlowJo software (Ashland, OR, USA). For each measurement, at least 10 000 cells were counted.

Apoptotic analysis

HCT-116 cells were seeded in 24-well tissue culture plates. After 24 h, the medium was changed and panaxadiol, irinotecan or both drugs were added. After treatment for 48 h, cells floating in the medium were collected. The adherent cells were detached with 0.05% trypsin. Culture medium containing 10% FBS (and floating cells) was added to inactivate

trypsin. When gentle pipetting was completed, the cells were centrifuged for 5 min at 1500*g*. The supernatant was removed and cells were stained with annexin V-fluorescein isothiocyanate (FITC) and PI according to the manufacturer's instructions. Untreated cells were used as control for double staining. Immediately after staining the cells were analysed by a FACScan flow cytometer. For each measurement, at least 20 000 cells were counted.

Caspase assay

HCT-116 cells were seeded in six-well tissue culture plates. After 24 h the medium was changed and panaxadiol, irinotecan or both drugs were added. After treatment for 6, 12 and 24 h, the medium was removed. Cell Lysis Buffer 100 μl was added and cells were incubated on ice for 10 min. Cells were harvested and added with Lysis Buffer to a 1.5 ml tube, then centrifuged for 5 min at 1500*g*. Protein concentration was assayed with a Bio-Rad Protein Assay kit. Into each well of a 96-well plate, 50 μg protein was diluted by adding Cell Lysis Buffer to make a volume of 100 μl. 2X Reaction Buffer 50 μl (containing 10 mM dithiothreitol) was added to each well. DEVD-*ρ*NA 5 μl 4 mM (LEHD-*ρ*NA for caspase-9, IETD-*ρ*NA for caspase-8) substrate (200 μM final concentration) was added, and the plates were incubated at 37°C for 24 h. The sample plate was read at 400 or 405 nm with a microtitre plate reader.

Docking analysis

The caspase-3 protein (Caspase-X, PDB code: 3KJF) was selected for docking analysis. The Surflex-Dock program was used to determine the binding sites of panaxadiol and irinotecan to caspase-3. The binding affinities of these compounds to different binding sites were determined, along with the locations of the hydrogen bonds formed.

Statistical analysis

The data are presented as mean ± standard error (SE). A one-way analysis of variance and the Student's *t*-test were used to determine whether the results had statistical significance. The level of statistical significance was set at $P < 0.05$.

Results

Antiproliferative effects of panaxadiol and irinotecan on two human colon cancer cell lines

After treatment with panaxadiol, irinotecan or both drugs for 48 h, the proliferation of HCT-116 and SW-480 cells was slightly suppressed dose-dependently in each case. Concentrations of irinotecan at 1, 3, 5, 10, 20 μM and/or panaxadiol at 10 μM (Figure 2) were tested. Panaxadiol 10 μM exerted approximately a 19.1 and 10.7% antiproliferative effect on HCT-116 and SW-480 cells, respectively. The half maximal inhibitory concentration (IC50) of irinotecan on HCT-116 was at a concentration of 12.1 μM; however, after the addition of panaxadiol, the IC50 dropped to a concentration of 4.4 μM. In SW-480 cells, the IC50 of irinotecan was at 11.8 μM, and after the addition of panaxadiol the IC50 dropped to 5.0 μM.

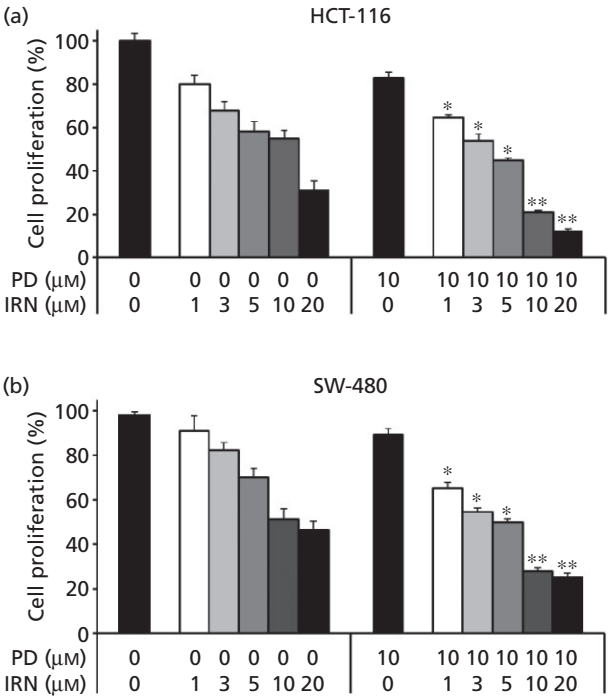

Figure 2 Antiproliferative effects of irinotecan and panaxadiol on human colorectal cancer cells. HCT-116 (a) and SW-480 (b) cells were treated with panaxadiol (PD; 10 μM) and/or irinotecan (IRN: 1, 3, 5, 10, or 20 μM) for 48 h. Data are presented as mean ± SE. $*P < 0.05$, $**P < 0.01$, compared with corresponding groups of irinotecan only.

In addition, we selected the YAMC epithelium cell line to evaluate the effect of panaxadiol and irinotecan on normal intestinal epithelial cells. We observed that at the concentration of panaxadiol 10 μM combined with irinotecan 20 μM, the proliferation was 83.5% in YAMC cells. However, the proliferation was only 12.7% in HCT-116 cells. Thus, compared with the significant antiproliferative effect of panaxadiol and irinotecan on colorectal cancer cells, YAMC cells were not obviously affected.

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

After staining with PI, the assay data from cells treated with panaxadiol, irinotecan or both drugs at various concentrations were compared. As shown in Figure 3, compared with control (44.0% in G1 phase, 26.0% in S phase, and 30.0% in

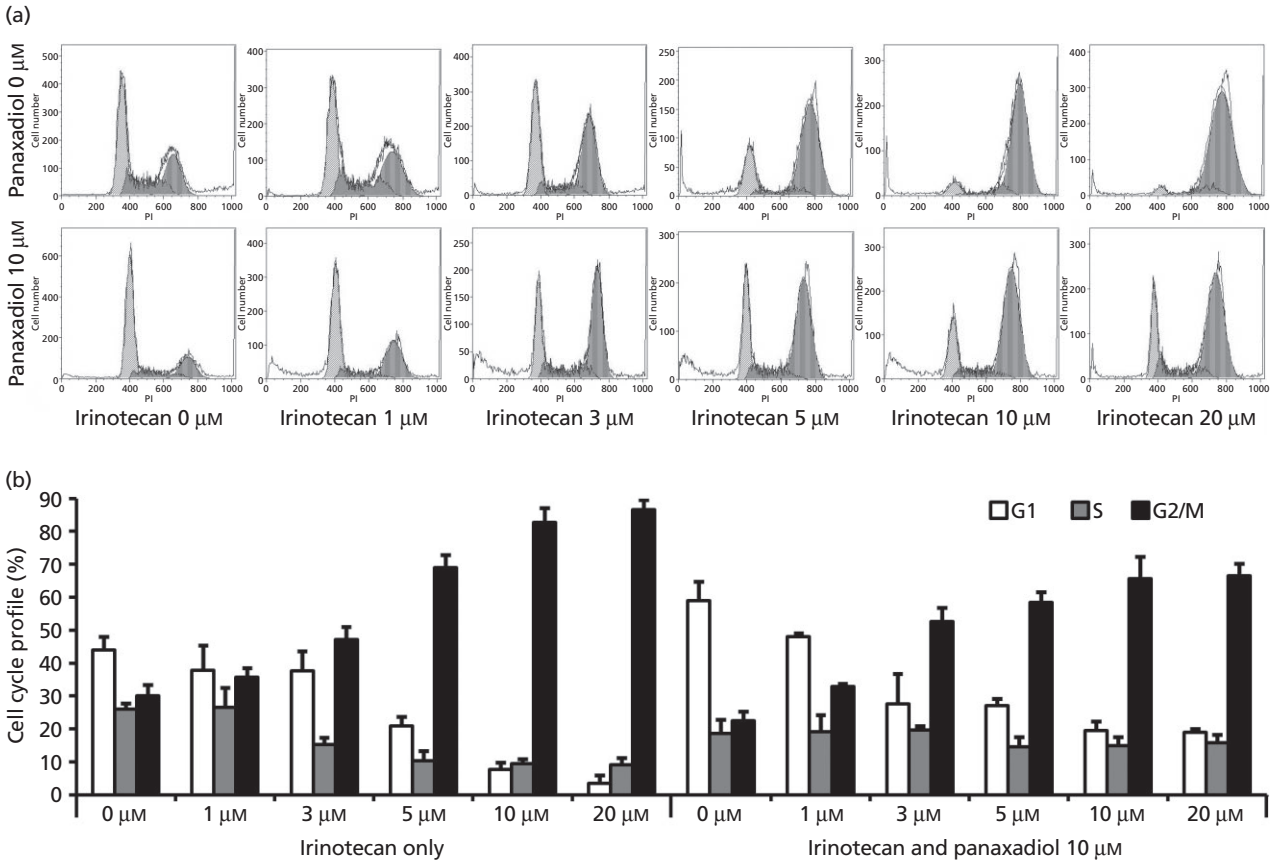

Figure 3 Effects of irinotecan and panaxadiol on HCT-116 cell cycle. HCT-116 cells were treated with panaxadiol (10 μM) and/or irinotecan (1, 3, 5, 10, or 20 μM) for 48 h. Cell cycle profile was determined using flow cytometry after staining with PI/RNase. (a) Representative histograms of cell cycle distribution. (b) Percentage of each cell cycle phase with various treatments or with control. Data are presented as the mean ± SE of triplicate experiments.

G2/M phase), treatment with irinotecan gradually changed the cell cycle profile of HCT-116 cells. After treatment with irinotecan at 20 μM, the distribution was 3.4% in G1 phase, 9.2% in S phase, and 87.4% in G2/M phase. At panaxadiol 10 μM, the fractions of cells were 58.9% (G1 phase), 18.7% (S phase) and 22.4% (G2/M phase). These data showed that the G1 phase was slightly arrested. After treatment with 20 μM irinotecan plus panaxadiol 10 μM, the cell cycle profile was 19.3% (G1 phase), 16.0% (S phase) and 64.6% (G2/M phase). Notably, irinotecan arrested the G2/M phase significantly, while combining irinotecan with panaxadiol increased the G1 phase and decreased the G2/M phase, when compared with irinotecan treatment alone. These data suggested that panaxadiol induced cell cycle arrest at the G1 phase, and irinotecan induced arrest at the G2/M phase. The combination of irinotecan with panaxadiol increased G1 and G2/M phase arrest, different from that induced by irinotecan or panaxadiol alone.

Apoptotic effects of panaxadiol and irinotecan on HCT-116 cells

To further characterize the potential mechanisms of the anticancer effect of these two medicines, we performed an apoptotic assay by flow cytometry after staining with annexin V and PI. Compared with the control it was shown that panaxadiol slightly induced apoptosis in HCT-116 cells. At concentrations of 1–20 μM, irinotecan gradually induced apoptosis. However, the combination with panaxadiol was more potent in inducing apoptosis than irinotecan alone. After treatment for 48 h, the percentage of apoptotic cells induced by panaxadiol 10 μM was 14.8%. The percentage of apoptotic cells induced by irinotecan at concentrations of 1–20 μM increased from 20.9% to 50.5%. After combination with panaxadiol, the percentage range was 29.2–66.1%, compared with control at 5.0% (Figure 4). These results suggested that the antiproliferative effect of irinotecan plus panaxadiol could be mediated by the induction of apoptosis.

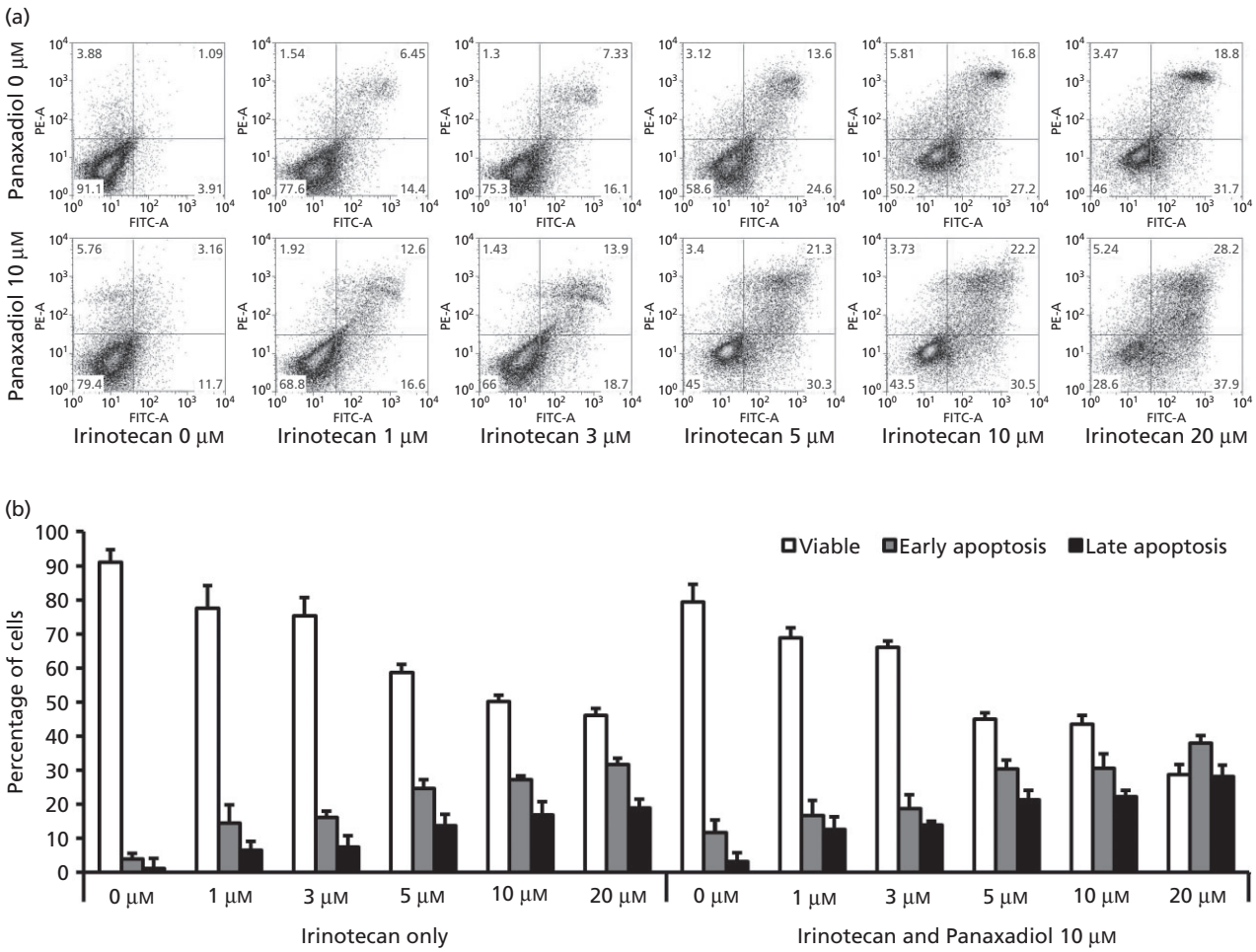

Figure 4 Effects of irinotecan and panaxadiol on HCT-116 cell apoptosis. HCT-116 cells were treated with panaxadiol (10 μM) and/or irinotecan (1, 3, 5, 10, or 20 μM) for 48 h. Apoptosis was quantified using flow cytometry after staining with annexin V/PI. (a) Representative scatter plots of PI (y-axis) vs annexin V (x-axis). (b) Percentage of viable, early apoptotic and late apoptotic cells. Data are presented as the mean ± SE of triplicate experiments.

Caspase assay

Caspase-3 and caspase-9 are two key proteins of the caspase family of proteases, which are highly conserved in multicellular organisms and function as central regulators of apoptosis. They have been identified as playing a key role in the progression of apoptosis.[19–21] To further characterize the potential mechanism of the anticancer effect of irinotecan and panaxadiol, we performed a caspase-3/8/9 assay. Compared with the control, we tested the increase in activity of caspase-3 and 9 in HCT-116 cells in response to panaxadiol 10 μM, irinotecan 10 μM, and the combination of the two. For caspase-8, the activity was stable with panaxadiol, irinotecan or a combination, and only increased by 2.2% with the combination at 24 h. As shown in Figure 5, at 6 h the activity of caspase-3 was increased approximately 26.8% with panaxadiol only, 55.2% with irinotecan only, 70.7% with the combination; at 12 h the activity was increased approximately 79.3% with panaxadiol only, 64.9% with irinotecan only and 102.4% with the combination; and after 24 h the activity was increased approximately 53.6% with panaxadiol only, 105.9% with irinotecan only, and approximately 155.7% with the combination. With caspase-9, there was no obvious activity change with panaxadiol treatment only; after 6 h there was an approximate 76.9% increase with irinotecan only, and a 109.6% increase with the combination of panaxadiol and irinotecan; at 12 h, there was an approximate 33.1% increase with irinotecan only, and 65.1% with combination; and after 24 h there was an approximate 74.7% increase with irinotecan only and a 113.1% increase with the combination.

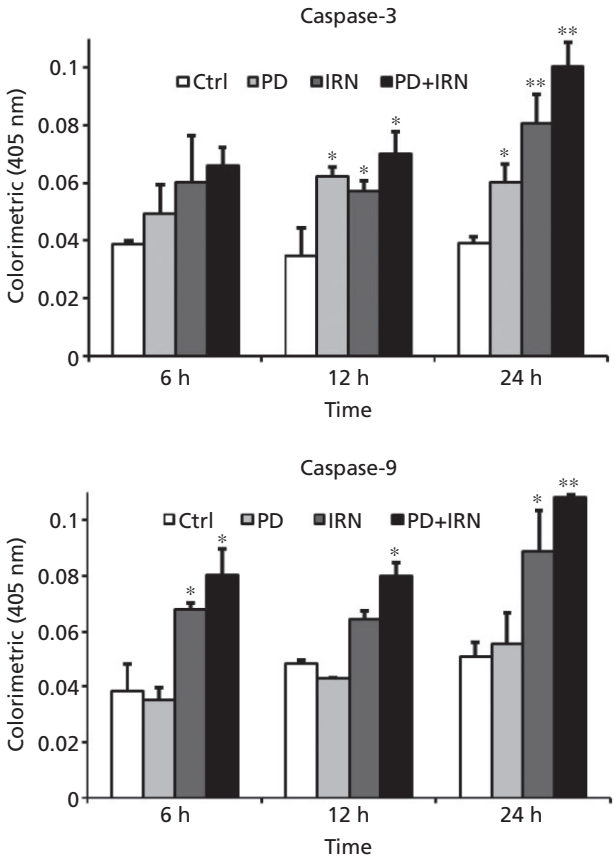

Figure 5 Effect of panaxadiol and irinotecan on caspase-3/9 activity in HCT-116 cells. HCT-116 cells were treated with panaxadiol (PD; 10 μM) and/or irinotecan (IRN; 10 μM) for 6, 10 or 24 h. Caspase activity was measured by caspase-3, 9/CPP 32 Colorimetric Assay kits. Data are presented as mean ± SE. $*P < 0.05$, $**P < 0.01$, vs corresponding control groups.

Molecular docking of panaxadiol and irinotecan to the caspase-3 protein

It has been reported that caspase-3 plays a key role in apoptosis and influences the progression of the cell death program. In our study, the combination of panaxadiol and irinotecan displayed a significant pro-apoptotic effect. We selected caspase-3 as our target protein for docking analysis. Figure 5 shows the interactions between panaxadiol, irinotecan, and caspase-3, and that panaxadiol and irinotecan bound to different binding sites of caspase-3 (Figure 6). It can be seen that irinotecan lies in the left chain (chain A) of the protein while panaxadiol lies in the right chain (chain B). The docking results (Figure 6) showed that irinotecan formed six hydrogen bonds with Arg 207, Tyr 204 and His 121, while panaxadiol formed a hydrogen bond with Tyr 62. These results were in agreement with the results of our caspase assay.

Discussion

Ginseng, including Asian ginseng and American ginseng, occupies a prominent position on the list of the best-selling natural products in the world. Ginseng has been reported to have a wide range of pharmacological effects, including anticancer activity.[22–24] Compared with unsteamed white ginseng, steamed or red ginseng has significantly increased anticancer activity.[25–27]

Using two commonly used colorectal cancer cell lines, HCT-116 and SW-480, we evaluated the interaction of panaxadiol and irinotecan in the inhibition of cancer cell activity. After co-treatment with 10 μM panaxadiol plus 1, 3, 5, 10 or 20 μM irinotecan for 48 h, the IC50 value of HCT-116 was determined to be 4.4 μM, much lower than that of irinotecan alone (12.1 μM); in SW-480 cells the IC50 value dropped from 11.8 to 5.0 μM. This suggested that panaxadiol significantly boosted the antiproliferative effect of irinotecan on HCT-116 and SW-480 cells and may have reduced the dose of irinotecan needed to achieve the desired effects.

To explore the synergistic antiproliferative effects of panaxadiol and irinotecan, we evaluated the effects of the two compounds on cancer cell cycle regulation. Using the HCT-116 cell line, the cell cycle profile was assayed by flow cytometry after staining with PI. If the DNA is damaged, the cell cycle is halted at the transition from G1 to S phase, or at the transition from G2 to M phase.[28] We found that panaxadiol and irinotecan significantly increased the fractions of cells in G1 and G2/M phases, but decreased the percentage in S phase, suggesting that the combination of panaxadiol and irinotecan damaged cell DNA and inhibited cancer cell growth.

To further characterize the potential mechanism of the anticancer effects of panaxadiol plus irinotecan, we performed an apoptotic assay using flow cytometry. Results showed that the percentage of apoptotic cells induced by the combination was increased compared with that induced by panaxadiol or irinotecan only. This suggested a potential synergistic interaction of the two compounds in the induction of apoptosis.

Apoptosis is a highly regulatory process of programmed cell death, in which the caspase protease family is considered to be a key factor.[21,29] Many reports show that caspase-9 is the initiator associated with the intrinsic pathway of apoptosis. Once activated, caspase-9 cleaves and activates a variety of downstream effectors such as caspase-3 etc., which target main regulatory and structural proteins for proteolysis to promote apoptosis.[30] To confirm whether the apoptotic effects induced by irinotecan plus panaxadiol were mediated by the caspase family, we performed a caspase colorimetric assay. The activity of caspases 3, 8, and 9 was tested-in HCT-116 cells after exposure to panaxadiol, irinotecan, or the combination treatment at 6, 12 and 24 h. The results indicated

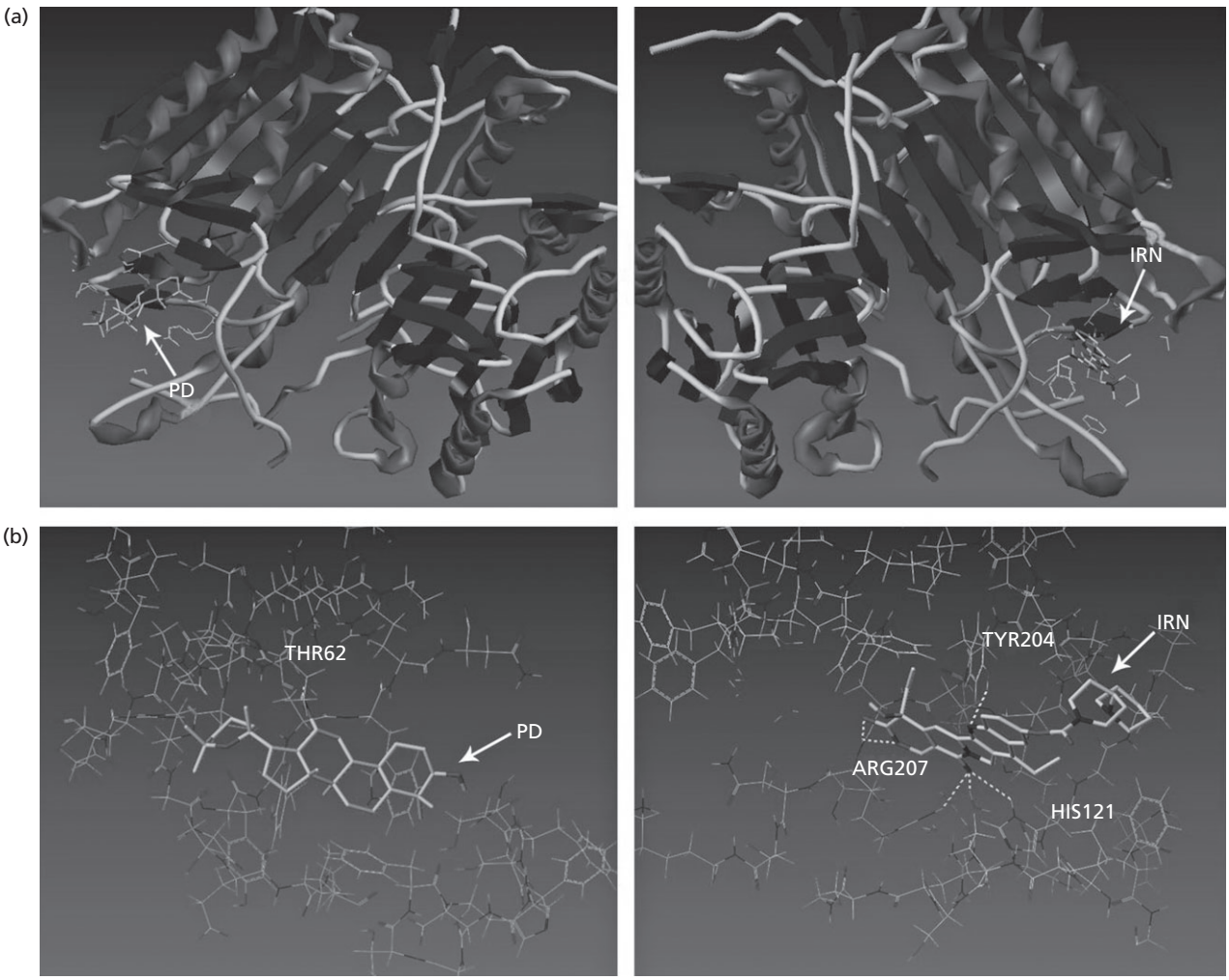

Figure 6 Docking simulation of irinotecan and panaxadiol on the caspase-3 protein. Panaxadiol (PD) and irinotecan (IRN) bind to distinct binding sites on the protein. A surface and ribbon view is shown above (a), while an insight ligand-site model is shown below (b).

that the activity of caspase-3 and caspase-9 was obviously increased in a time-dependent manner (Figure 5); however, caspase-8 did not show a significant change. Meanwhile, in a docking simulation, panaxadiol and irinotecan got a higher score in the interaction with caspase-3 or caspase-9 than with caspase-8. Therefore, we hypothesized that irinotecan combined with panaxadiol treatment might have induced HCT-116 cell apoptosis mainly by upregulating the activity of caspase-3 and caspase-9.

Caspase-3 plays a key role in the progression of the cell death program. In our docking analysis, panaxadiol and irinotecan were found to bind to two different sites on caspase-3 (Figure 6). Irinotecan showed significant binding affinity with caspase-3. The binding of both compounds to caspase-3 further provides a synergistic mechanism for their pro-apoptotic effects.

Conclusions

A synergistic anticancer effect of panaxadiol and irinotecan was observed in human colorectal cancer cells. This has been the first time that the antiproliferative activity of a ginseng product was evaluated in combination with a semisynthetic camptothecin derivative and second-line antitumour medicine. This observed synergistic effect might have been mediated through the induction of apoptosis. Our study also suggested that the anticancer activity of irinotecan may be enhanced by other natural products. Combined treatment with natural products could significantly reduce the dose of irinotecan needed to achieve the desired treatment effect and decrease the rate of side effects. This is an important preliminary step in the development of an effective chemoadjuvant for colorectal cancer treatment.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported in part by the NIH/NCCAM grants P01 AT004418 and K01 AT005362.

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