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Effects of Recombinant Circularly Permuted Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL) (Recombinant Mutant Human TRAIL) in Combination with 5-Fluorouracil in Human Colorectal Cancer Cell Lines HCT116 and SW480

Authors' Contribution:

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background:

Circularly permuted tumor necrosis factor-related apoptosis-inducing ligand, a mutant form of tumor necrosis factor-related apoptosis-inducing ligand, is an effective antitumor cytokine. However, its antitumor effect in colorectal cancer is unclear. This study assessed the antitumor effect of circularly permuted tumor necrosis factor-related apoptosis-inducing ligand alone or with 5-fluorouracil in colorectal cancer cells *in vitro* and explored the underlying mechanisms.

Material/Methods:

We used the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) assay to analyze cell proliferation inhibition. The apoptotic effects of circularly permuted tumor necrosis factor-related apoptosis-inducing ligand, 5-fluorouracil, or both in human colorectal cancer cells were evaluated using flow cytometry. Furthermore, the levels of apoptosis-related proteins were examined by Western blotting.

Results:

Compared to either agent alone, cotreatment with 5-fluorouracil and circularly permuted tumor necrosis factor-related apoptosis-inducing ligand showed obvious antitumor effects and induced significant apoptosis of colorectal cancer cells. 5-Fluorouracil enhanced circularly permuted tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis by increasing death receptor 4 and 5 levels in HCT116 cells, but only of death receptor 4 in SW480 cells. Moreover, 5-fluorouracil plus circularly permuted tumor necrosis factor-related apoptosis-inducing ligand increased apoptosis-related protein levels such as cleaved caspase-3, caspase-8, and poly-ADP-ribose polymerase and downregulated that of the survival protein B-cell lymphoma-extra-large. Pretreatment with the pan-caspase inhibitor, z-VAD-FMK, attenuated the caspase-dependent apoptosis induced by circularly permuted tumor necrosis factor-related apoptosis-inducing ligand alone or combined with 5-fluorouracil.

Conclusions:

Cotreatment with 5-fluorouracil and circularly permuted tumor necrosis factor-related apoptosis-inducing ligand showed enhanced antitumor effects on colorectal cancer cells.

MeSH Keywords:

Apoptosis • Colorectal Neoplasms • Fluorouracil • Receptors, Death Domain • TNF-Related Apoptosis-Inducing Ligand

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Background

Colorectal cancer (CRC) is the third and second most common cancer in men and women, respectively, with more than 1.3 million recorded cases worldwide in 2012. It accounts for approximately 700 000 deaths annually in both sexes globally [1]. Patients with CRC are generally treated with surgery, chemotherapy, or radiotherapy. 5-Fluorouracil (5-FU)-based systematic chemotherapy has for over several decades been the primary treatment for CRC, particularly for patients with the advanced form of the disease [2]. Since CRC involves multiple alternative genetic pathways, traditional methods of treatment have some limitations because of the lack of tumor selectivity. Tumor protein 53 (TP53) is the most frequently mutated gene in metastatic CRC (mCRC) [3,4], which can lead to 5-FU resistance that hinders its clinical use [5,6]. Furthermore, adverse effects can also restrict the clinical application of 5-FU [7,8]. In recent years, the development of molecularly targeted drugs has improved the outcome of patients with mCRC. However, the effect of molecular targeting therapy is affected by the status of certain gene. For example, patients with mCRC harboring RAS and BRAF mutations or both showed a poor response to molecular targeting therapy [9,10]. The 5-year survival rate for advanced CRC is still not promising [11]; therefore, the development of new effective therapeutic strategies is required.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand or Apo2 ligand (TRAIL/Apo2L) is a member of the TNF superfamily and a type II membrane-bound cytokine [12]. Since its discovery in 1995, TRAIL has gained intense interest as a promising agent for cancer therapy because of its remarkable ability to induce apoptosis of a variety of human tumor and transformed cells while sparing normal cells [13,14]. Five different receptors have been identified to interact with TRAIL in humans: death receptor 4 (DR4)/TRAIL receptor-1, DR5/TRAIL receptor-2, decoy receptor 1 (DcR1)/TRAIL receptor-3, DcR2/TRAIL receptor-4, and osteoprotegerin (OPG) [15,16]. TRAIL can bind to 3 decoy receptors (DcR1, DcR2, and DcR3) that have no functional death domain (DD) and therefore cannot induce apoptosis. On the contrary, TRAIL triggers the extrinsic apoptotic pathway by binding to and stimulating fully functional DR4 and DR5, which subsequently aggregate and recruit signaling proteins at the membrane through their DD. First, the Fas-associated DD (FADD) is recruited. Then, FADD recruits the initiator caspase-8 and -10 to form a death-inducing signaling complex (DISC), within which caspase-8 and -10 are activated [17]. Subsequently, activated caspase-8 and -10 cleave multiple intracellular substrates (such as the downstream effectors caspase-3, -6, and -7), which leads to apoptosis [18]. Activated caspase-8 can also cleave the pro-apoptotic Bcl-2 family protein BH3-interacting domain death agonist (Bid) to generate a truncated Bid (t-Bid) that can translocate to the mitochondria to transduce apoptotic signals and, eventually,

the intrinsic apoptotic pathway is activated [15,19]. This indicates that TRAIL is a potent cytokine that induces malignant tumor cell apoptosis. Moreover, TRAIL has a distinctive advantage over chemotherapy or radiotherapy in its ability to induce apoptosis regardless of the TP53 status. Furthermore, a study showed that the sensitivity of TRAIL was not related to the status of oncogenic KRAS and BRAF in CRC cell lines [20].

Therefore, TRAIL is considered one of the most promising candidates for the treatment of cancers, which has prompted extensive efforts to develop derivatives [21]. Recombinant mutant human TRAIL (rmhTRAIL) or circularly permuted TRAIL (CPT), a mutant form of the native human TRAIL with conservative sequences, has been developed by Beijing Sunbio Biotech. Compared to wild-type TRAIL, CPT has significantly improved in stability, solubility, and safety with prolonged antitumor activity [22]. Studies have shown that CPT significantly inhibits a variety of non-small cell lung cancer (NSCLC) cell lines and has no obvious toxic effect on normal human cells. A combination of CPT and cisplatin showed a synergistic inhibitory effect on NSCLC cells and had no toxicity to normal human cells [23]. Similar results were obtained from an *in vivo* experiment [24]. Furthermore, CPT has been studied extensively in multiple myeloma (MM), and the data show that in combination with other agents, it demonstrates promising antitumor activity against MM *in vitro* and *in vivo* [25–28].

However, no study of CPT alone or combined with other agents in treating CRC has been reported to date. Therefore, in the present study we investigated the antitumor effects of 5-FU and CPT as single agents or in combination in TRAIL-sensitive and -resistant human CRC cells *in vitro*. Furthermore, we examined whether 5-FU enhances CPT-induced apoptosis and explored the underlying molecular mechanisms.

Material and Methods

Cells and cell culture

Human colon cancer cell lines HCT116 and SW480 (gifts from Columbia University) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Hyclone, South Logan, UT, USA), and 100 IU/mL penicillin and 100 IU/mL streptomycin (both from Shen zhen Hua yao Nan fang Pharmaceutical Co., Ltd., Guangdong, China). The cells were cultured in an incubator maintained at 37°C with an atmosphere of 5% CO₂.

Chemicals and antibodies

Freeze-dried circularly permuted TRAIL (CPT) powder (a gift from Beijing Sunbio Biotech Co., Ltd., Beijing, China) was diluted in

distilled water to obtain a 1-mg/mL CPT solution, which was preserved at -80°C in aliquots and protected from light. The solution was diluted in RPMI-1640 to a working concentration prior to use. 5-Fluorouracil (5-FU) was purchased from Shanghai Xudong Haipu Pharmaceutical Co., Ltd. Mouse anti-human monoclonal antibodies (caspase-3 and caspase-8), rabbit anti-human cleaved caspase-3, cleaved caspase-8, caspase-9, poly-ADP ribose polymerase (PARP), DR5, X-linked inhibitor of apoptosis (XIAP), and DR4 antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Rabbit anti-human B-cell lymphoma-2 (Bcl-2)-associated X protein (BAX) and Bcl-extra-large (Bcl-XL) antibodies were obtained from Absin Bioscience Co., Ltd (Shanghai, China). z-VAD-FMK was ordered from ApexBio Technology LLC (Houston, TX, USA) and all other chemicals were of reagent grade and purchased from local sources.

Cell proliferation inhibition analysis

The cell proliferation inhibition rates were measured by the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) (Promega, Madison, WI, USA) assay [35]. Human CRC cell lines HCT116 and SW480 (5×10^3 cells/well) were seeded in 96-well plates and left to adhere for 24 h. Both cell lines were then treated with different concentrations of 5-FU for 48 h or with different concentrations of CPT for 12, 24, or 48 h. Thereafter, the medium was replaced with 100 μL of fresh medium; 20 μL of MTS (Promega, Madison, WI, USA) was added to the medium, followed by continued incubation at 37°C in a humidified 5% CO_2 atmosphere for 3 h. The optical density (OD) was read at 490 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Then, the half-maximal inhibitory concentration (IC_{50}) values for 5-FU and CPT were determined. The HCT116 and SW480 cells were subsequently treated with 5-FU (5 and 12.5 $\mu\text{g}/\text{mL}$, respectively) and different concentrations of CPT alone or in combination for 48 h. Subsequently, the cell inhibition rates were measured using the MTS assay as described above. The inhibition rates were calculated using the following formula: Cell inhibition rate (%) = $(1 - \text{OD of the experimental group} / \text{OD of the control group}) \times 100\%$. All experiments were performed in triplicate and repeated 3 times independently.

Flow cytometric analysis

HCT116 and SW480 cells were seeded in 6-well plates at a density of 5×10^5 cells/well with 2 mL of medium. After incubation for 24 h, the growth medium was replaced with fresh medium and the cells were treated with 5-FU and/or CPT for another 48 h. Then, both adherent and suspended cells were collected, washed with phosphate-buffered saline (PBS), and suspended in Annexin-binding buffer. Subsequently, the cells

were stained with Annexin V-FITC and propidium iodide (PI) together for 5 min at room temperature in the dark. Finally, the cell apoptosis was analyzed using a FACS-Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). All experiments were repeated at least 3 times.

To evaluate the effect of caspase inhibition on cell apoptosis, HCT116 and SW480 cells were pretreated with or without the pan-caspase inhibitor z-VAD-FMK at 20 μM for 1 h, followed by treatment with CPT or 5-FU plus CPT for 48 h. Then, the cells were harvested and processed as described above.

Western blot analysis

HCT116 and SW480 cells were incubated with 5-FU or CPT alone or in combination for 48 h. Then, the cells were washed with cold PBS and lysed on ice in radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, and 0.5% sodium deoxycholate). The cell lysate protein was quantified using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL, USA), and then 50 μg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Thereafter, the membranes were blocked with 5% non-fat milk in TBST at room temperature for 2 h, followed by incubation with primary antibodies at an appropriate concentration (1: 1000 dilution for DR4; DR5; caspase-3, caspase-8, and caspase-9, cleaved caspase-3 and caspase-8, and PARP; 1: 2000 for β -actin) overnight at 4°C . Subsequently, the membranes were washed with TBST 3 times and incubated with homologous horseradish peroxidase-labeled secondary antibodies (1: 5000; Proteintech Group, Chicago, IL, USA) at room temperature for 1.5 h. After washing with TBST 3 times, the immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Beyotime Institute of Biotechnology, Shanghai, China) and analyzed using a chemiluminescent imaging system (Tanon Science & Technology Co., Ltd., Shanghai, China). Finally, the bands were analyzed using the Image J image analysis system (National Institutes of Health, Bethesda, MD, USA). Each experiment was repeated 3 times.

Statistical analysis

The results are expressed as the mean \pm standard deviation (SD) values of triplicate experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) and Bonferroni's or Games-Howell's post hoc tests. Variable responses at different time points were analyzed using repeated measures ANOVA. A linear correlation was detected using Pearson correlation analysis using SPSS 21.0 software (IBM, Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

Results

Combination of 5-FU and CPT shows an obvious antitumor effect on human CRC cells by inhibiting cell proliferation.

HCT116 and SW480 CRC cells were treated with different concentrations of 5-FU for 48 h or treated with various concentrations of CPT for 12, 24, or 48 h. Subsequent determination of the cell proliferation inhibition rates revealed that 5-FU inhibited the growth of both HCT116 and SW480 cells in a dose-dependent manner ($p < 0.001$). The IC_{50} values of 5-FU for HCT116 and SW480 cells were 6.31 and 13.79 $\mu\text{g/mL}$, respectively (Figure 1A). As shown in Figure 1B, treatment with various concentrations of CPT significantly inhibited the proliferation of both cell lines. ANOVA of repeated measures indicated that the efficacy of CPT was dose- and time-dependent ($p < 0.001$). The IC_{50} values of CPT for HCT116 and SW480 cells at 48 h were 8.49 and 338.43 ng/mL , respectively.

To investigate the antitumor effects of 5-FU plus CPT, HCT116 and SW480 cells were incubated with 5-FU (5 and 12.5 $\mu\text{g/mL}$, respectively) and/or different concentrations of CPT for 48 h. The results showed that compared to either agent alone, cotreatment with 5-FU and CPT significantly increased the death of both cell lines (Figure 1C). Taken together, these results demonstrated that the combination of 5-FU and CPT had an enhanced antitumor effect.

Combination of 5-FU and CPT promotes the apoptosis of CRC cells.

We found that 5-FU plus CPT significantly increased cell death. To investigate whether the cell death was apoptotic, we performed flow cytometric analysis. HCT116 and SW480 cells were incubated with 5-FU or CPT or 5-FU plus CPT for 48 h. 5-FU was used at a fixed concentration of 5 and 12.5 $\mu\text{g/mL}$ for HCT116 and SW480 cells, respectively. The concentration of CPT used for HCT116 cells was 5 or 10 ng/mL while that for SW480 cells was 300 or 1000 ng/mL . We found that 5-FU and CPT could induce cell apoptosis when used singly (Figure 2A). We also found that the apoptosis rates were higher in the CPT group than in the control and 5-FU groups for both the HCT116 and SW480 cell lines ($p < 0.01$). More importantly, cotreatment with 5-FU and CPT significantly increased the apoptosis of both HCT116 and SW480 cells compared to that achieved by treatment with either agent singly for 48 h ($p < 0.01$). Furthermore, the apoptosis rates in the high-dose CPT group were significantly higher than those in the other groups were ($p < 0.05$) (Figure 2B). In summary, these results were consistent with the MTS assay results and indicated that 5-FU could potentiate CPT-mediated cell death by inducing cell apoptosis.

Combination of 5-FU and CPT enhances the activation of caspase-3, caspase-8, and PARP. To investigate the possible mechanisms involved in the apoptosis, we performed Western blotting. The results showed that CPT alone activated caspase-3, caspase-8, and PARP in both HCT116 and SW480 CRC cells.

Furthermore, the levels of cleaved caspase-3 and caspase-8 significantly increased following treatment with 5-FU plus CPT. PARP, which was considered a target of caspase-3, was also cleaved in both cell lines after incubation with 5-FU plus CPT (Figure 3A, 3B). Caspase-9, another member of the caspase family, was also detected. We found that the pro-form of caspase-9 decreased in both cell lines after treatment with 5-FU plus CPT, but the cleaved form of caspase-9 was not observed (Figure 3A, 3B). The results demonstrate that the apoptosis of CRC cells induced by 5-FU plus CPT is caspase-dependent.

z-VAD-FMK inhibits caspase-dependent apoptosis induced by 5-FU plus CPT.

Flow cytometric analysis and Western blotting showed that 5-FU plus CPT promoted caspase-dependent apoptosis of both HCT116 and SW480 CRC cells. To further demonstrate whether the enhanced apoptosis was caspase-dependent, HCT116 and SW480 cells were treated with or without the pan-caspase inhibitor z-VAD-FMK (20 μM) 1 h prior to incubation with CPT or 5-FU plus CPT for 48 h. The results of the flow cytometric assay showed that z-VAD-FMK significantly inhibited the apoptosis induced by CPT or 5-FU plus CPT (16.3 \pm 0.4% vs. 59.9 \pm 0.9% and 33.6 \pm 2.4% vs. 90.8 \pm 0.8% in HCT116 cells; 18.9 \pm 0.5% vs. 43.7 \pm 0.2% and 29.7 \pm 0.2 vs. 69.6 \pm 0.9% in SW480 cells) (Figure 4A). These results indicate that the enhanced apoptosis by 5-FU plus CPT was caspase-dependent.

Western blot analysis showed that the levels of cleaved caspase-3, caspase-8, and PARP significantly increased in the CPT and 5-FU plus CPT groups compared to the corresponding levels in the control and 5-FU groups. When the cells were pre-treated with the pan-caspase inhibitor z-VAD-FMK, the activation of caspase-3, caspase-8, and PARP was abrogated in both the CPT and 5-FU plus CPT groups (Figure 4B, 4C). These findings were in accordance with the results of the cytometric analysis. Taken together, these results suggest that the apoptosis induced by a cotreatment with 5-FU and CPT was mediated by caspase activation. The data also indicate that activation of the caspase pathway is one of the mechanisms by which 5-FU enhances CPT-mediated apoptosis of CRC cells.

5-FU increases the expression of death receptors (DRs) in CRC cells.

DRs play a key role in TRAIL-mediated apoptosis [15]. To determine whether 5-FU modulates the expression of DR4 and DR5 in HCT116 and SW480 CRC cells, Western blot analysis was performed. HCT116 and SW480 cells were treated with 5-FU at concentrations lower than the respective IC_{50} values for 48 h. As shown in Figure 5, 5-FU elevated the expression of DR4 in both cell lines, but DR5 expression increased only in the HCT116 cells and not in the SW480 cells. Thus, these findings indicate that 5-FU-mediated DRs upregulation was involved in the antitumor effect of 5-FU plus CPT in CRC cells.

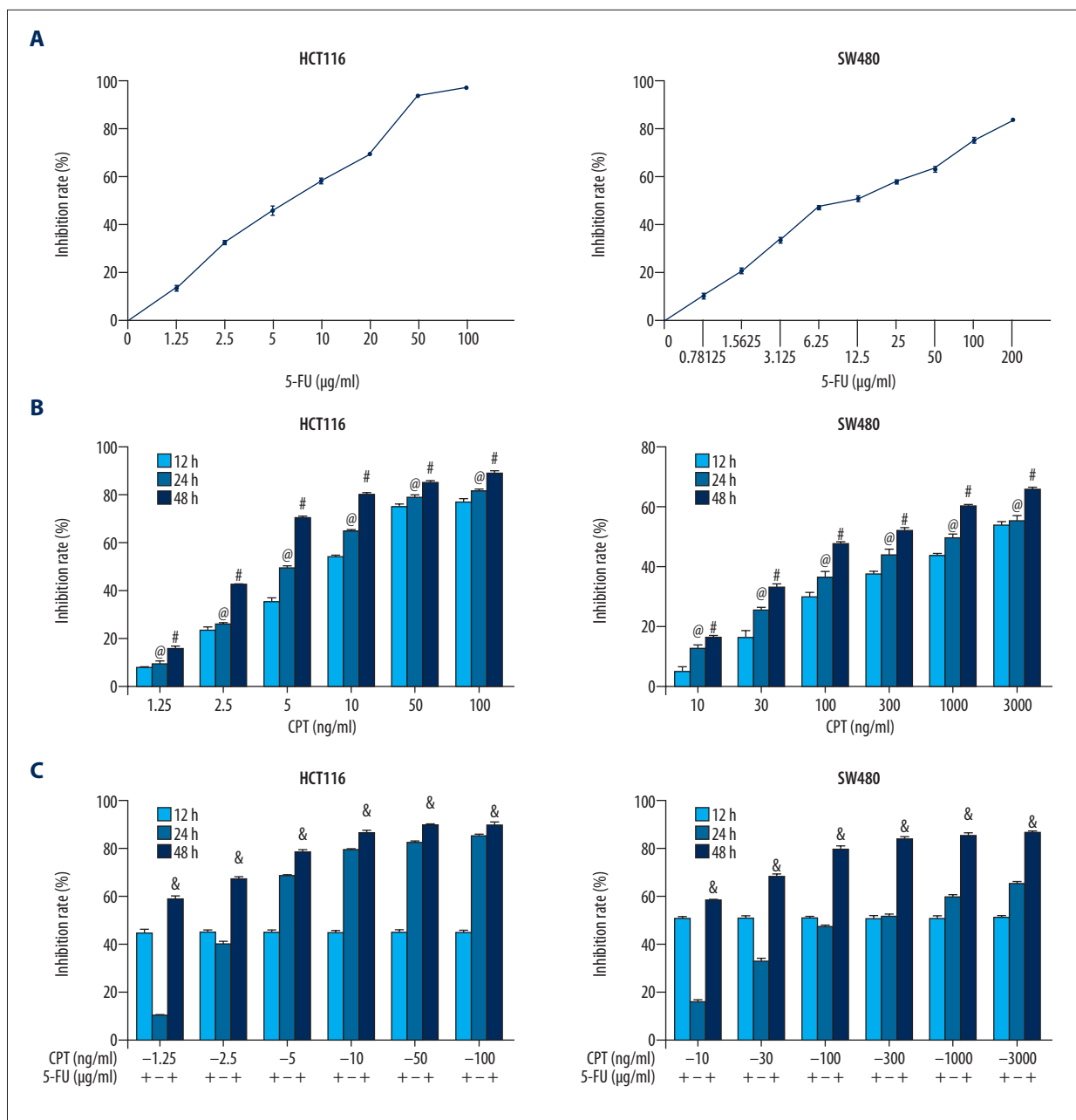


Figure 1. 5-FU and CPT alone or in combination inhibits the proliferation of colorectal cancer cells. **(A)** CRC HCT116 and SW480 cell lines were treated with the indicated concentrations of 5-FU for 48 h, and then the MTS assay was performed to evaluate cell proliferation inhibition rates. There was a significantly positive correlation between 5-FU concentrations and cell inhibition rates in both HCT116 cells ($r=0.992$, $p<0.001$) and SW480 cells ($r=0.993$, $p<0.001$). **(B)** HCT116 and SW480 cells were treated with various doses of CPT as indicated for 12, 24, or 48 h, followed by the MTS assay. CPT inhibited CRC HCT116 and SW480 cells in a dose- and time-dependent manner. **(C)** HCT116 and SW480 cells were treated with 5-FU (5 and 12.5 µg/mL, respectively) combined with different concentrations of CPT for 48 h and cell proliferation was measured using MTS assay. Cotreatment with 5-FU and CPT significantly increased cell death of both cell lines. Data are means \pm SD of results of 3 independent experiments, @ $p<0.001$ versus cells treated for 12 h; # $p<0.001$ versus cells treated for 12 or 24 h; & $p<0.001$ versus the 5-FU or CPT group. 5-FU – 5-fluorouracil; CPT – circularly permuted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); MTS – (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); SD – standard deviation; CRC – colorectal cancer; µg – microgram.

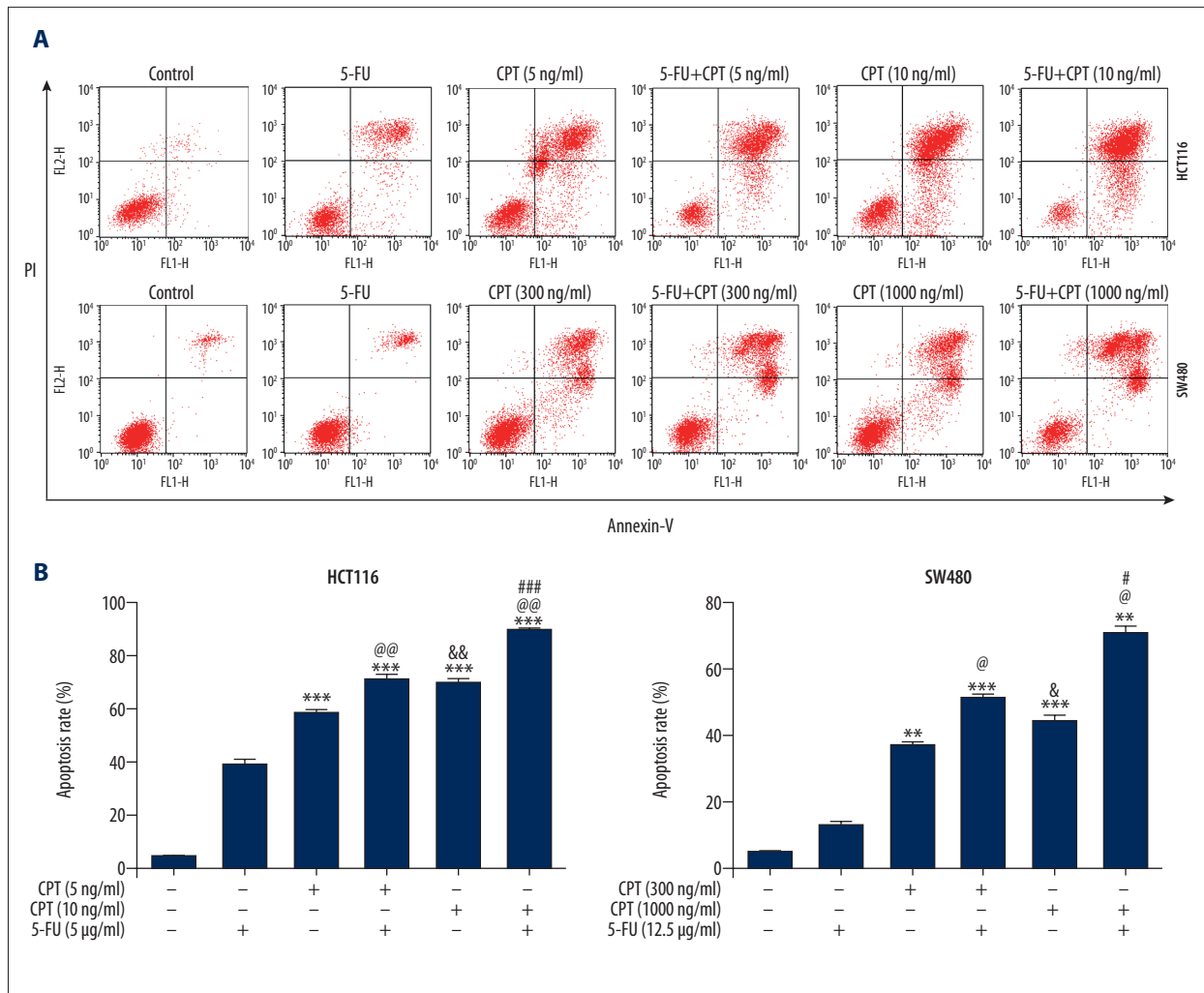


Figure 2. 5-FU promotes the apoptosis induced by CPT in colorectal cancer (CRC) HCT116 and SW480 cells. **(A)** HCT116 and SW480 CRC cells were treated with 5-FU and CPT alone or in combination for 48 h. Then, the cells were stained with Annexin V-FITC and PI to analyze apoptotic cells using flow cytometry. **(B)** Histogram shows the apoptosis rates of HCT116 and SW480 cells after treatment for 48 h. The apoptosis rates of HCT116 cells were 5.0±0.3%, 39.7±1.2%, 58.3±0.9%, 71.4±1.0%, 69.7±1.2%, and 89.8±0.2%, respectively, in control, 5-FU, 5 ng/mL CPT, 5-FU + 5 ng/mL CPT, 10 ng/mL CPT, and 5-FU + 10 ng/mL CPT groups. The apoptosis rates of SW480 cells were 5.2±0.2%, 13.4±0.6%, 36.9±1.0%, 51.5±0.5%, 44.5±1.0%, and 70.4±2.1%, respectively, in control, 5-FU, 300 ng/mL CPT, 5-FU + 300 ng/mL CPT, 1000 ng/mL CPT, and 5-FU + 1000 ng/mL CPT groups. Data are means ±SD of results of 3 experiments, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control and 5-FU groups; @ $p < 0.01$ and @@ $p < 0.001$ versus the CPT groups; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ versus the combined groups with low-dose CPT; and & $p < 0.01$ and && $p < 0.001$ versus cells treated with low-dose CPT alone. 5-FU – 5-fluorouracil; CPT – circularly permuted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); FITC – fluorescein isothiocyanate; PI – propidium iodide; SD – standard deviation; CRC – colorectal cancer.

Combination of 5-FU and CPT downregulates the expression of survival protein Bcl-XL. Since DRs upregulation is involved in the antitumor effect of 5-FU plus CPT, the involvement of other mechanisms should be investigated. Therefore, we investigated whether apoptosis-associated proteins were changed by treatment with 5-FU plus CPT. Western blot analysis showed that Bcl-XL expression significantly decreased after treatment with 5-FU plus CPT in both the HCT116 and SW480 cell lines (Figure 6A, 6B). However, the expression of BAX, a

pro-apoptotic protein belonging to the Bcl-2 family, was unchanged. Further, no obvious changes were noted in the expression of XIAP, a member of the IAP family. The ratio of BAX/Bcl-XL, which is considered critical for apoptosis induction [43], was markedly increased after cotreatment compared with that after treatment with either agent singly ($p < 0.01$) (Figure 6A, 6B). Therefore, the results suggest that the downregulation of Bcl-XL may be one of the mechanisms by which 5-FU enhanced CPT-induced apoptosis of CRC cells.

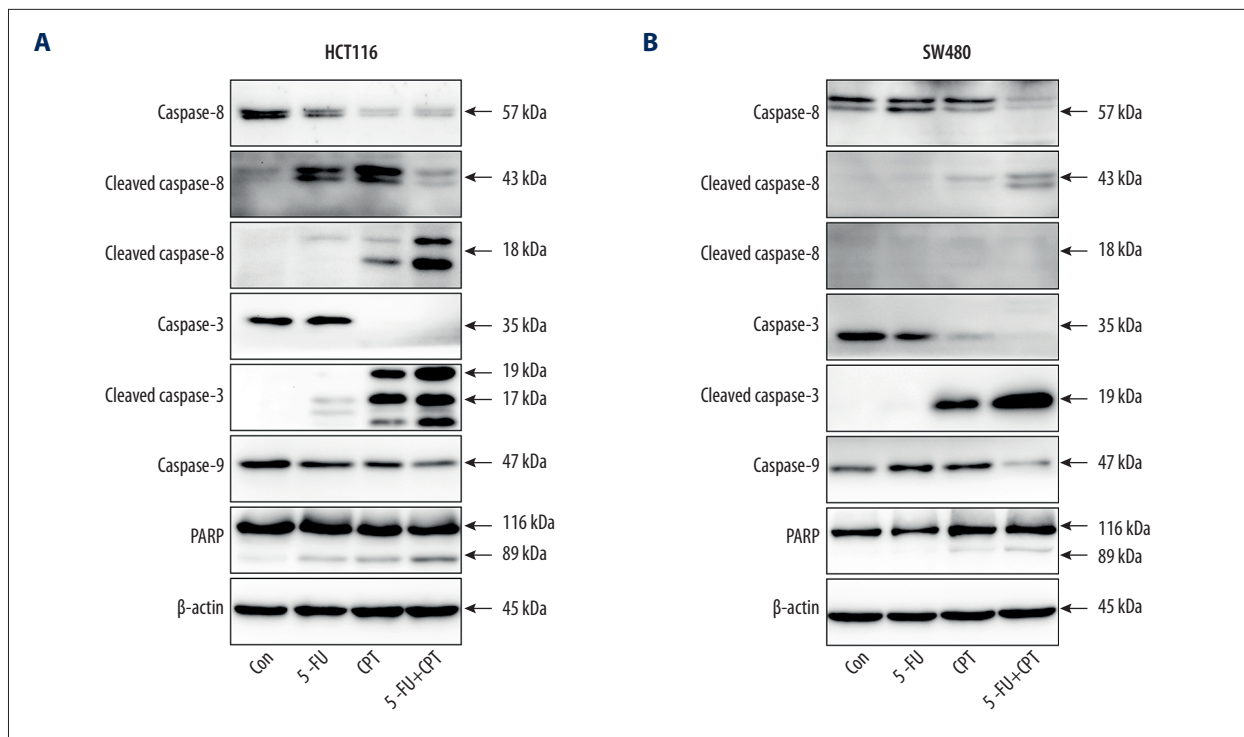


Figure 3. Combination of 5-FU and CPT triggers apoptosis through caspase-dependent pathways in colorectal cancer (CRC). CRC HCT116 (A) and SW480 (B) cells were treated with 5-FU (5 and 12.5 $\mu\text{g}/\text{mL}$, respectively) and/or CPT (10 and 1000 ng/mL , respectively) for 48 h. Then, the expression of caspase-3, caspase-8, caspase-9, and PARP as well as of cleaved caspase-3, caspase-8, and PARP was detected using Western blot analysis. β -Actin protein was used as the internal control. Each band represents 3 experiments. 5-FU – 5-fluorouracil; CPT – circularly permuted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); PARP – poly (ADP-ribose) polymerase; CRC – colorectal cancer.

Discussion

TRAIL is a promising antitumor cytokine based on its unique features of inducing apoptotic cell death of a variety of cancer or transformed cells, but not most normal cells. However, numerous cancer cell types are resistant to TRAIL-induced apoptosis, including CRC cells. Fortunately, it has been demonstrated that many commonly used chemotherapeutic drugs can overcome TRAIL resistance in human cancer cells and show a synergistic effect in tumor xenografts [29]. 5-fluorouracil (5-FU) is the first-line drug used for the treatment of CRC. Furthermore, a series of studies has shown that 5FU could promote TRAIL-induced apoptosis of a variety of cancer cell lines *in vitro* and *in vivo* [30–34].

Circularly permuted TRAIL (CPT) is a novel derivative of wild-type TRAIL and preclinical studies have showed that it is a potent tumor-killing biologic agent. However, CPT alone or in combination with 5-FU in the treatment of CRC has not been reported. Therefore, in the present study, we investigated the antitumor effect of CPT and 5-FU alone or in combination in human CRC cell lines for the first time. Our results showed that cotreatment with 5-FU and CPT had an enhanced antitumor effect on both TRAIL-sensitive and -resistant HCT116

and SW480 CRC cell lines, respectively. Moreover, CPT inhibited cell proliferation in a dose- and time-dependent manner. The IC_{50} of CPT indicated that HCT116 CRC cells were sensitive to CPT while SW480 cells were resistant. Our results were in line with previously reported results [20]. We also demonstrated that 5-FU combined with CPT promoted the apoptosis of not only TRAIL-sensitive HCT116 CRC cells but also that of TRAIL-resistant SW480 CRC cells. Interestingly, cells apoptosis induced by CPT or 5-FU plus CPT was blocked after caspase inhibition, suggesting that the CRC cells apoptosis was caspase-dependent. In addition, several molecular mechanisms underlying the enhanced antitumor effect of 5-FU plus CPT were explored. Our findings indicated that CPT or combined treatment with 5-FU and CPT increased cell apoptosis via activation of caspase-3, caspase-8, and PARP. Furthermore, the enhanced antitumor effects of 5-FU plus CPT were mediated by downregulation of the antiapoptotic protein Bcl-XL and up-regulation of DRs expression. Therefore, our results suggest that CPT as a single agent or in combination with 5-FU may be an effective therapeutic strategy for patients with CRC, especially for patients with drug-resistant CRC.

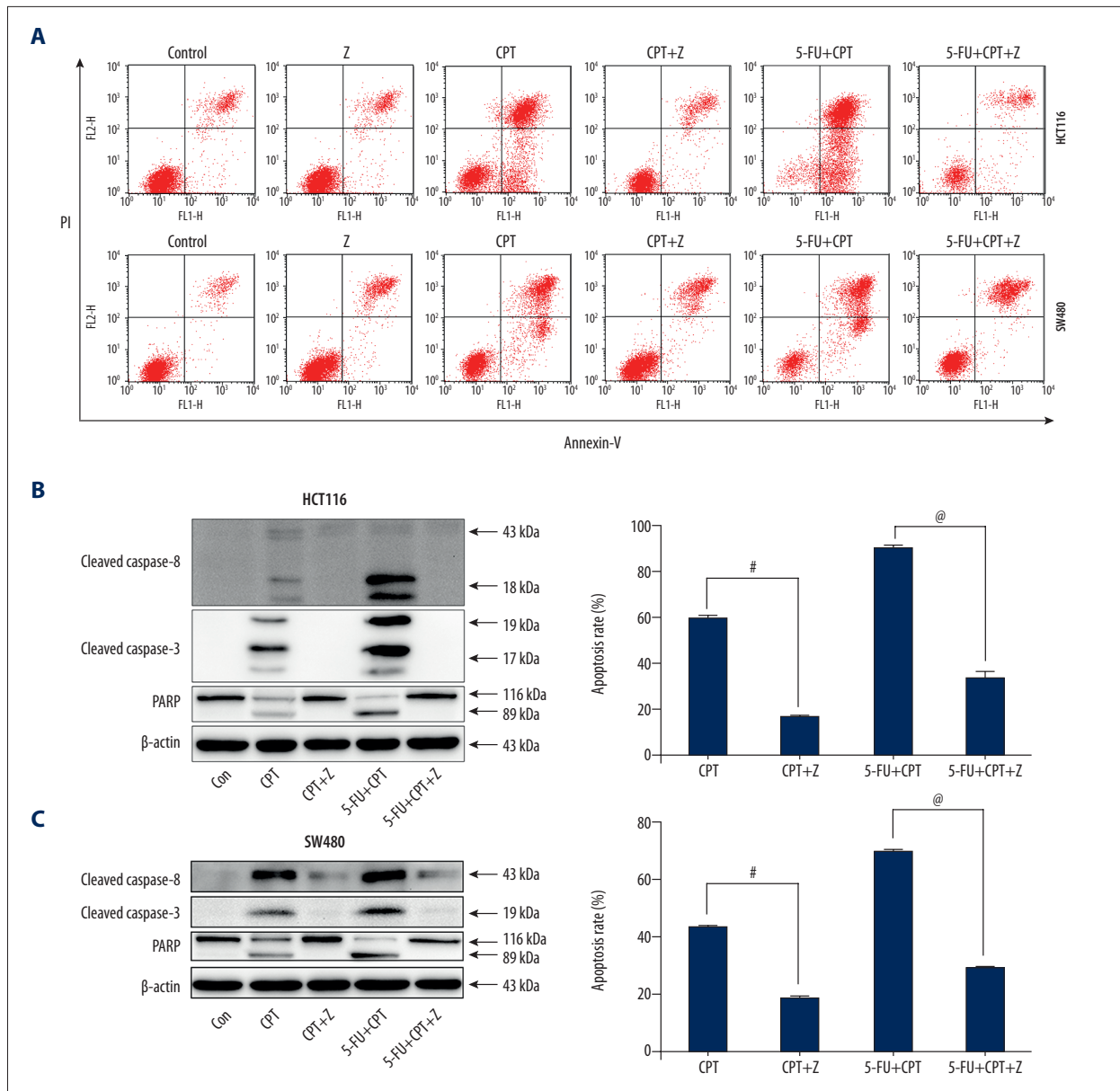


Figure 4. The pan-caspase inhibitor z-VAD-FMK blocks caspase-dependent apoptosis induced by 5-FU plus CPT in colorectal cancer (CRC). **(A)** CRC HCT116 and SW480 cell lines were pretreated with or without the pan-caspase inhibitor z-VAD-FMK (20 μM) for 1 h and then incubated with CPT (10 and 1000 ng/mL, respectively) or 5-FU (5 and 12.5 μg/mL, respectively) plus CPT for 48 h. Then, apoptosis was measured using flow cytometry using Annexin V-FITC and PI staining. **(B)** and **(C)** HCT116 and SW480 cells were treated as described above. The expression of cleaved caspase-3, caspase-8, and PARP was determined using Western blotting. β-Actin protein was the internal control. Each band represents 3 experiments. Histogram shows the apoptosis rates of CRC cells treated with CPT alone or with in the presence or absence of z-VAD-FMK. Data are means ±SD of results of 3 experiments, # $p < 0.001$ versus the CPT group; @ $p < 0.001$ versus the 5-FU plus CPT group. 5-FU – 5-fluorouracil; CPT – circularly permuted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); PARP – poly (ADP-ribose) polymerase; FITC – fluorescein isothiocyanate; PI – propidium iodide; SD – standard difference; CRC – colorectal cancer.

Apoptosis, which is a type of programmed cell death, is mediated by 2 major pathways: the extrinsic and the intrinsic pathways [35]. Caspases play a crucial role in these 2 apoptotic pathways. The extrinsic apoptotic pathway is initiated by the binding of death ligands to cell-surface DRs [36].

Activation of caspase-8 is the initial step and plays a critical role in TRAIL-induced apoptosis and the caspase cascade. Caspase-3 is the terminal executor of apoptosis. A variety of chemotherapeutic agents have been reported to induce apoptotic cell death through the caspase cascade when combined

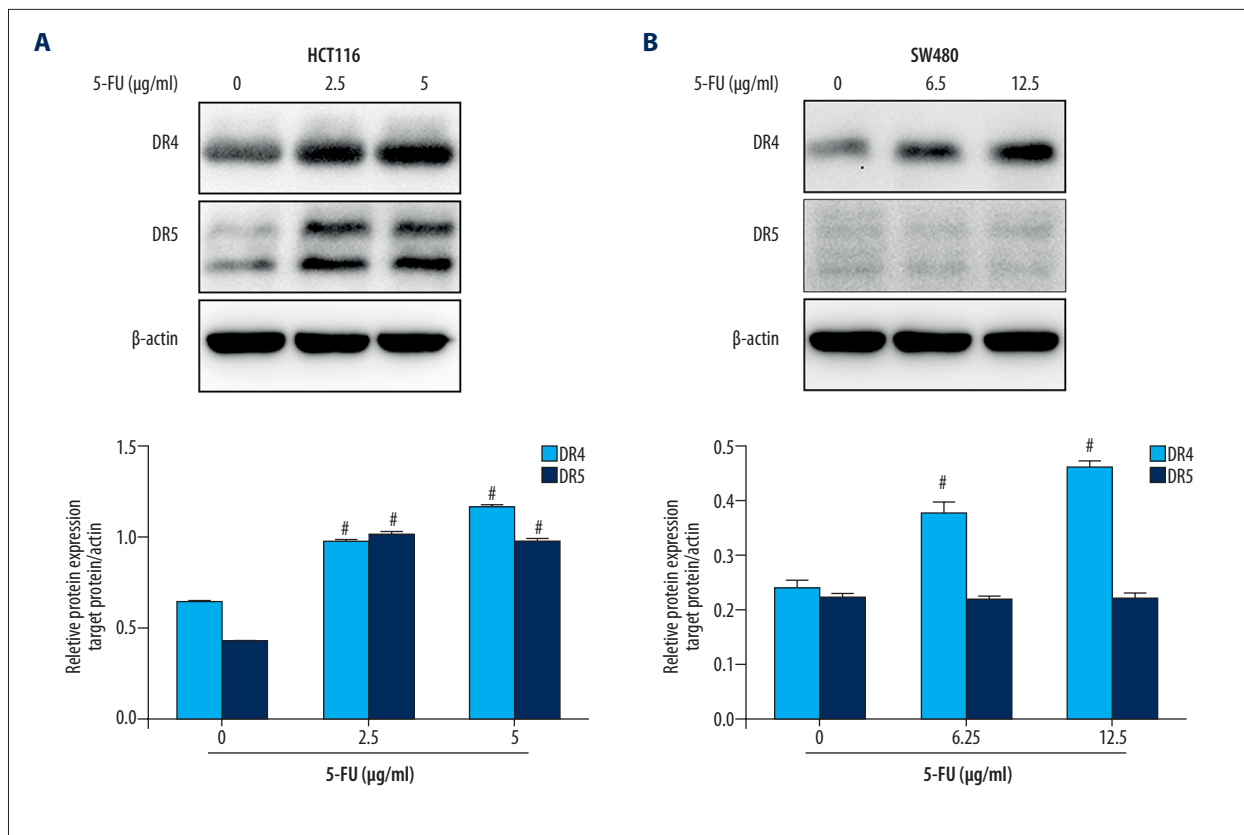


Figure 5. 5-FU upregulates the expression of DR4 and DR5 in HCT116 cells but only that of DR4 in SW480 cells. (A) HCT116 and (B) SW480 CRC cells were treated with the indicated concentrations of 5-FU for 48 h. Then, Western blot analysis was performed to detect expression of DR4 and DR5. β -Actin protein was the internal control. Each band represents 3 experiments. Relative intensity was analyzed using Image J software. Data are means \pm SD of results of 3 independent experiments; [#] $p < 0.001$ versus the control group. 5-FU – 5-fluorouracil; DR – death receptor; SD – standard deviation; CRC – colorectal cancer.

with TRAIL [37,38]. Furthermore, studies have shown that 5-FU in combination with TRAIL activates caspase-3, -8, and -9 to promote apoptosis of CRC and other cancer cells [30]. To understand whether the caspases cascade plays the same key role in apoptosis induced by 5-FU plus CPT, we assessed caspases expression levels in apoptotic pathways using Western blot analysis. The results showed that levels of cleaved caspase-3 and -8 significantly increased in both TRAIL-sensitive HCT116 and TRAIL-resistant SW480 CRC cells treated with CPT alone or in combination with 5-FU. PARP, which has been identified as the target protein of caspase-3, was also cleaved in both cell lines after incubation with CPT or 5-FU plus CPT. Moreover, the expression of the pro-forms of caspase-3, caspase-8, and PARP decreased evidently, especially in the combination group. Caspase-9, another member of the caspase family, showed a significant decline in expression after treatment with 5-FU plus CPT. Although we failed to detect cleaved caspase-9, it is possible that its cleavage occurred at an undetectable level that was sufficient to activate downstream apoptotic signals, which eventually led to apoptosis. The cleavage of caspase-3, caspase-8, and PARP was abrogated when CRC

cells were pretreated with the pan-caspase inhibitor z-VAD-FMK. Our data indicate that the caspases cascade was crucial in the CPT- or 5-FU plus CPT-induced apoptosis of CRC cells. Specifically, the extrinsic apoptotic pathway was involved in the mechanisms underlying the enhanced antitumor effects achieved by 5-FU plus CPT in CRC cells.

Since the expression of caspase-9 was decreased in both HCT116 and SW480 CRC cells treated by 5-FU combined with CPT, the intrinsic or mitochondrial pathways might be involved in the apoptosis induced by this combination. The mitochondrial apoptotic pathway can be regulated by the Bcl-2 superfamily proteins, such as the pro-apoptotic proteins BAX and Bid and the antiapoptotic proteins Bcl-XL, Bcl-2, and myeloid cell leukemia 1 (Mcl-1) [39,40]. Previous studies demonstrated that downregulation of Bcl-2 and/or Bcl-xL or upregulation of BAX could sensitize CRC cells to TRAIL-induced apoptosis [41,42]. In the present study, our results indicated that the expression of Bcl-XL in both HCT116 and SW480 CRC cell lines decreased after treatment with 5-FU plus CPT. Surprisingly, the expression of BAX was unchanged when the cells were treated with

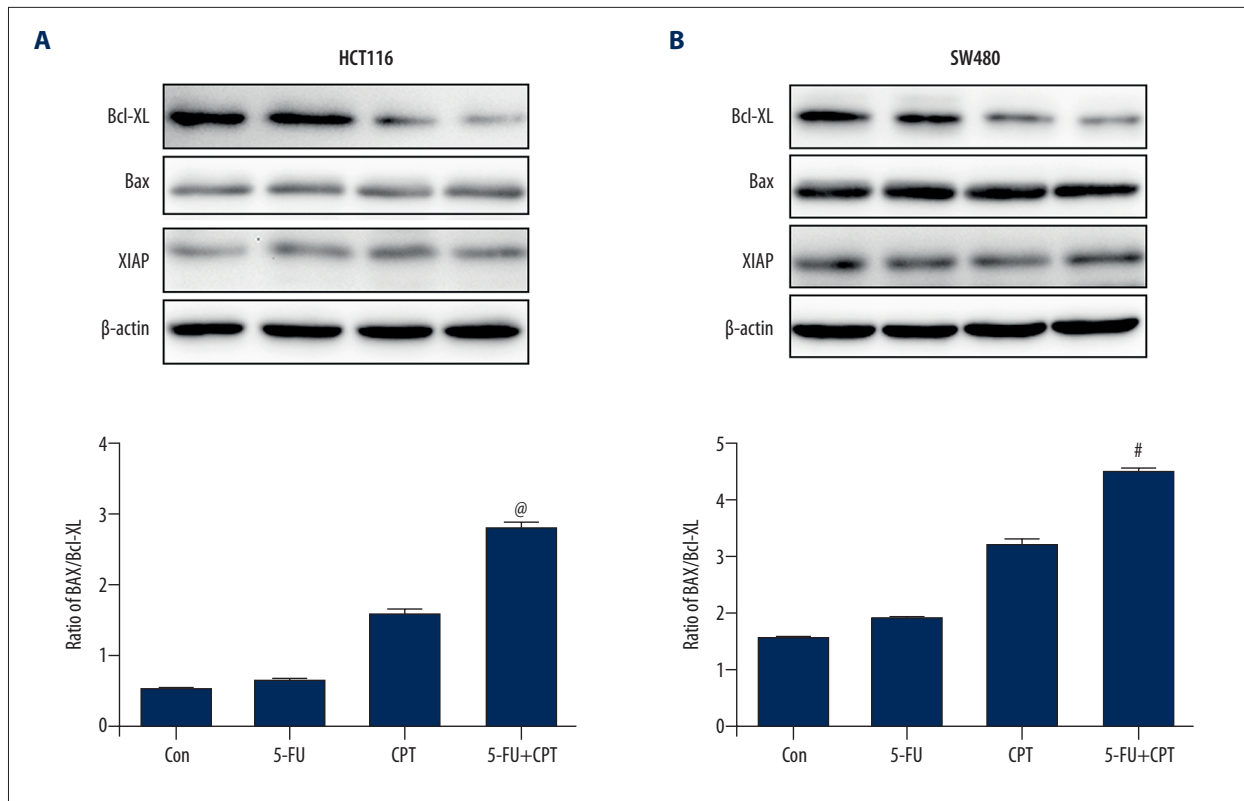


Figure 6. Combination treatment of 5-FU and CPT downregulates expression of the survival protein Bcl-XL. CRC (A) HCT116 and (B) SW480 cell lines were treated with 5-FU (5 and 12.5 µg/mL, respectively), CPT (10 and 1000 ng/mL, respectively), or both for 48 h. Then, Western blot analysis was performed using the indicated antibodies (Bcl-XL, BAX, and XIAP). β-Actin protein was used as the internal control. Each band represents 3 experiments. Histogram shows the ratio of BAX/Bcl-XL analyzed using Image J software; Data are means ±SD of results of 3 independent experiments; [@] $p < 0.01$ versus the control, 5-FU, or CPT group; [#] $p < 0.001$ versus the control, 5-FU, or CPT group. 5-FU – 5-fluorouracil; CPT – circularly permuted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL); XIAP – X-linked inhibitor of apoptosis; BAX – B-cell lymphoma (Bcl)-2-associated X; Bcl-XL – B-cell lymphoma extra-large; SD – standard deviation; CRC – colorectal cancer.

5-FU plus CPT. However, the BAX/Bcl-XL ratio, which was considered critical for apoptosis induction, was increased by co-treatment [43]. Additionally, studies have demonstrated that BAX expression is essential to TRAIL-induced CRC cell death by reducing the expression of Bcl-xL [44]. Our results showed that BAX was expressed normally in both TRAIL-sensitive HCT116 and TRAIL-resistant SW480 CRC cells. This was consistent with the above findings. XIAP, a member of the IAP family, has been shown to inhibit caspase activity and then inhibit apoptosis [45]. We examined its expression and the results showed no change in either cell line. These results suggest that downregulation of Bcl-XL is one of the mechanisms by which 5-FU enhances CPT-induced apoptosis in CRC cells. Moreover, the apoptosis induced by 5-FU plus CPT was likely enhanced through the mitochondrial pathway.

Previous studies showed that 5-FU enhances TRAIL-induced apoptosis by upregulating DR4, DR5, or both in a variety of cancer cells, including CRC cells [30,32,34,38]. Our present results indicated that 5-FU markedly upregulated the expression

of DR4 and DR5 in HCT116 cells, but only that of DR4 expression in SW480 cells. The findings were in agreement with those of another study showing that 5-FU increased DR4 and DR5 expression in HCT116 cells after treatment for 48 h [46]. Furthermore, Robyn et al. demonstrated that high levels of DR4 in SW480 cells contributed to their sensitivity to TRAIL [47]. This was supported by our results showing that increased DR4 expression enhanced sensitivity of SW480 cells to CPT. The results of the present study indicate that increased expression of DRs is a molecular mechanism involved in the enhanced antitumor effects of 5-FU plus CPT in CRC cells.

Although CPT alone or CPT in combination with 5-FU showed an effective antitumor effect and some mechanisms were explored, there is still some deficiencies and room for further elucidation. This was a preliminary study in a limited number of CRC cell lines. In addition, it would be necessary to further study the mechanisms mediating CPT resistance in CRC cells. For example, the antiapoptotic proteins cellular FADD-like interleukin (IL)-1β-converting enzyme (FLICE)-inhibitory protein

(c-FLIP), survivin, and MCL-1 may be expressed at a high level in CRC cells, which confers resistance to CPT. Moreover, the results of *in vitro* experiments need to be corroborated *in vivo*.

Conclusions

The results of the present study show that CPT alone inhibited proliferation and induced caspase-dependent apoptosis of both

TRAIL-sensitive and -resistant human CRC cells. Furthermore, the antitumor effects were enhanced when 5-FU was combined with CPT, and the enhanced antitumor effects of this combination were mediated by downregulation of the anti-apoptotic protein Bcl-XL and upregulation of DRs expression.

Conflicts of interest

None.

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