Novel proapoptotic agent SM-1 enhances the inhibitory effect of 5-fluorouracil on colorectal cancer cells *in vitro* and *in vivo*

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Abstract. 5-Fluorouracil (5-FU) is one of the most important agents used to treat colorectal cancer. However, the therapeutic effect of 5-FU on colon cancer is limited. SM-1 is a novel type of proapoptotic agent that directly activates procaspase-3 to caspase-3, leading to apoptosis in human cancer cells. The aim of the present study was to evaluate the antitumor effects of 5-FU in combination with SM-1. The human colorectal cancer cell lines HCT116 and LoVo were cultured in the presence of SM-1 and 5-FU. The combination of SM-1 and 5-FU treatment exhibited increased proliferation inhibitory effects compared with 5-FU treatment alone in HCT116 and LoVo cells, as determined using an MTT assay. SM-1 significantly decreased the half-maximal inhibitory concentration of 5-FU from 8.07±0.49 to 2.55±0.41 μmol/l in HCT116 cells, and from 7.90±0.98 to 3.14±0.81 μmol/l in LoVo cells. Similarly, the apoptotic activity was increased to 47.95 and 35.19% in HCT116 and LoVo cells, respectively, as determined using Annexin V/propidium iodide staining and flow cytometry. The combination of SM-1 and 5-FU treatment led to significantly increased caspase-3 activity compared with either compound alone. The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis revealed the downregulation of B-cell lymphoma 2 and Survivin, and the upregulation of apoptosis regulator Bcl-2-associated X protein and cleaved poly (ADP-ribose) polymerase in HCT116 and LoVo cells. In addition, RT-qPCR identified downregulation of X-linked inhibitor of apoptosis protein mRNA. 5-FU and SM-1 treatment in combination increased tumor proliferation inhibition in HCT116 and LoVo xenograft mouse models of colorectal cancer, compared with SM-1 or 5-FU treatment alone. SM-1 significantly enhanced the antitumor activity of 5-FU in colorectal cancer. These improved effects were due to increased activity of the apoptotic signaling pathway.

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Introduction

Colorectal cancer is the fourth most common cause of cancer-associated mortality following lung, stomach and liver cancer (1,2). Treatment methods for colorectal cancer include chemotherapy, surgery and radiotherapy. Among the available chemotherapy drugs for treating colorectal cancer, 5-fluorouracil (5-FU) has been the first-line regimen for the treatment of colorectal cancer for a number of decades. In cancerous cells, 5-FU is metabolized into cytotoxic fluorodeoxyuridine monophosphate (3,4). However, the clinical benefit of 5-FU is limited because of resistance of colon tumor cells and adverse side effects (5,6). Previous studies are consistent with the concept that the combination therapies are able to improve the management of cancer and decrease systemic toxicity (7,8). Although colorectal cancer has been intensely researched, the problem of treatment failure remains a key obstacle in the improvement of overall patient survival rates, which remain low at ~50% at 5-year follow-up. Therefore, combination therapy, including molecular targeting agents and/or cytotoxic chemotherapy, may delay tumor progression and prolong survival time in colorectal cancer (9,10).

During apoptosis, procaspase-3 is activated to caspase-3, which initiates the apoptotic program (11,12). As procaspase-3 is overexpressed or exhibits increased expression in a variety of human tumors, drugs that direct active procaspase-3 are of interest as anticancer agents (13,14). First procaspase-activing compound (PAC-1) was the first procaspase-3 activator identified. SM-1, a novel PAC-1 derivative, directly activates procaspase-3 into caspase-3 (15). Our previous study demonstrated that SM-1 was able to induce cell apoptosis in various cancerous cells and *in vivo* murine tumor models (16). SM-1 and 5-FU exert their antitumor effects by distinct molecular mechanisms, suggesting the potential for synergistic effects in cancer treatment. In the present study, the combined effects of SM-1 and 5-FU in the treatment of colorectal cancer and the potential underlying molecular mechanisms were investigated.

Materials and methods

Cell culture. The human colorectal cancer cell lines HCT116 and LoVo were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in McCoy's 5A Modified Medium (HCT116) or F-12K medium (LoVo) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator containing 5% CO_2.

Cell proliferation assay. The effects of 5-FU and/or SM-1 on cell proliferation were determined using an MTT assay. HCT116 and LoVo cells ($5x10^3$ cells/well) were seeded in 96-well plates and incubated overnight at 37˚C, prior to exposure to 5-FU (KingYork Group Co., Ltd., Tianjin, China) (1.5625, 3.125, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 μmol/l), SM-1 (Xiangya Medical Research Institute, Changsha, China) (0.25, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 μmol/l) or 5-FU plus SM-1 at the same doses as single-agent treatments for 72 h at 37˚C. Control cells were processed identically except omitting the 5-FU or SM-1 treatment. Subsequently, 20 μl of MTT solution (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) (5 mg/ml) was added, and the cells were incubated at 37˚C for an additional 4 h. The culture medium was discarded and formazan crystals were dissolved in 200 μl DMSO (Sigma-Aldrich; Merck Millipore). The optical density (OD) of each well was measured at 570 nm using a microplate reader. The following formula was used: Cell proliferation inhibition rate=(1-OD of the experimental sample/OD of the control group) x100%.

Hoechst staining. Hoechst 33342 staining was used to confirm the alterations in the nuclear morphology of HCT116 and LoVo cells following 5-FU and/or SM-1 treatment. Cells were cultured and treated as described above, prior to staining with 10 μg/ml Hoechst 33342 (Sigma-Aldrich; Merck Millipore) for 15 min at 37˚C. Stained cells were observed using an inverted fluorescence microscope at magnification, x400.

Flow cytometry. HCT116 and LoVo cells at $3x10^5$ cells/well were incubated in 6-well plates overnight at 37˚C, then treated with SM-1 or 5-FU or combinations of SM-1 and 5-FU for 72 h as aforementioned. Untreated HCT116 and LoVo cells served as the control. Cells were collected, incubated with Annexin V/propidium iodide (PI) (BioLegend, Inc., San Diego, CA, USA), and measured using a Guava EasyCyte 5HT flow cytometer (EMD Millipore, Billerica, MA, USA). The compound 5,5', 6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Beyotime Institute of Biotechnology, Haimen, China) was used to assay the change in mitochondrial membrane potential (MMP). Following treatment, cells were harvested and stained with JC-1 (0.5 μmol/l) at 37˚C for 20 min. The fluorescence intensity was measured using a Guava EasyCyte 5HT flow cytometer (EMD Millipore). Guava ExpressPro software (version 5.0, EMD Millipore) was used for sample analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HCT116 and LoVo cells were seeded into a 6-well plate ($2x10^6$cells/well) and incubated at 37˚C overnight, prior to treatment with SM-1, 5-FU or combinations of SM-1 with 5-FU for 72 h as described above. Total RNA was extracted using TRIzol reagent (CWBiotech, Shanghai, China). RNA (1 μg) was used to synthesize the first-stand cDNA using the PrimeScript RT reagent kit (TakaraBio, Inc., Otsu, Japan) following the manufacturer's protocol. qPCR was performed using the SYBR-Green qPCR mixture (TakaraBio, Inc.) following the manufacturer's protocol. The 20 μl reaction mixture contained 10 μl 2x SYBR-Green qPCR mixure, 0.5 μl of the forward and reverse primers each, 1 μl cDNA template and 8 μl RNase-free water. The PCR cycle at which amplification was detectable above a background threshold (threshold cycle, or Cq) was calculated using the maximum second derivative method with the MX3000P qPCR system (Agilent Technologies, Inc., Santa Clara, CA, USA) (17). All samples were run in triplicate in each experiment. Primer sequences used to amplify genes are presented in Table I.

Table I. Primer sequences.

Primer name	Sequence
Actin	F: 5'-AGCGGGAAATCGTGCGTG-3'
	R: 5'-CAGGGTACATGGTGGTGCC-3'
Survivin	F: 5'-TACGCCTGTAATACCAGCAC-3'
	R: 5'-TCTCCGCAGTTTCCTCAA-3'
XIAP	F: 5'-TGATCGTGCCTGGTCAGAAC-3'
	5'-CGCCTTAGCTGCTCTTCAGT-3'
PARP	F: 5'-CATCGAGGTGGCCTACAGTC-3'
	R: 5'-ACCCATCAGCAACTTAGCGG-3'
Bax	F: 5'-AAGCTGAGCGAGTGTCTCAAG-3'
	R:5'-CAAAGTAGAAAAGGGCGACAAC-3'
Bcl-2	F: 5'-GTTTGATTTCTCCTGGCTGTCTC-3'
	R: 5'-GAACCTTTTGCATATTTGTTTGG-3'

F, forward; R, reverse; XIAP, X-linked inhibitor of apoptosis protein; PARP, poly (ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

Western blot analysis. HCT116 and LoVo cells at ~$1x10^6$ cells/well were harvested following pretreatment with SM-1, 5-FU or combinations of SM-1 with 5-FU. Cells were incubated in lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C for 30 min. Lysates were centrifuged at 10,000 x g for 15 min at 4˚C. The supernatant obtained was quantified using the Bio-Rad Protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein (30 μg) was separated using SDS-PAGE (8, 10 or 12%) and transferred onto an Immobilon-FL polyvinylidene difluoride membrane (EMD Millipore). Then the blots were blocked in 5% milk in TBS-Tween-20 (TBST) for 1 h at room temperature and incubated at 4˚C overnight with the following antibodies: anti-caspase-3 (cat. no. 9662), anti-Survivin (cat. no. 2808), anti-B-cell lymphoma 2 (Bcl-2; cat. no. 15071), Bcl-2-associated X protein (Bax; cat. no. 5023), anti-poly (ADP-ribose) polymerase (PARP; cat. no. 9532) and anti-β-actin (cat. no. 94970) (all 1:1,000 dilution; Cell Signaling Technology, Inc.). Following this, blots were washed with TBST and incubated

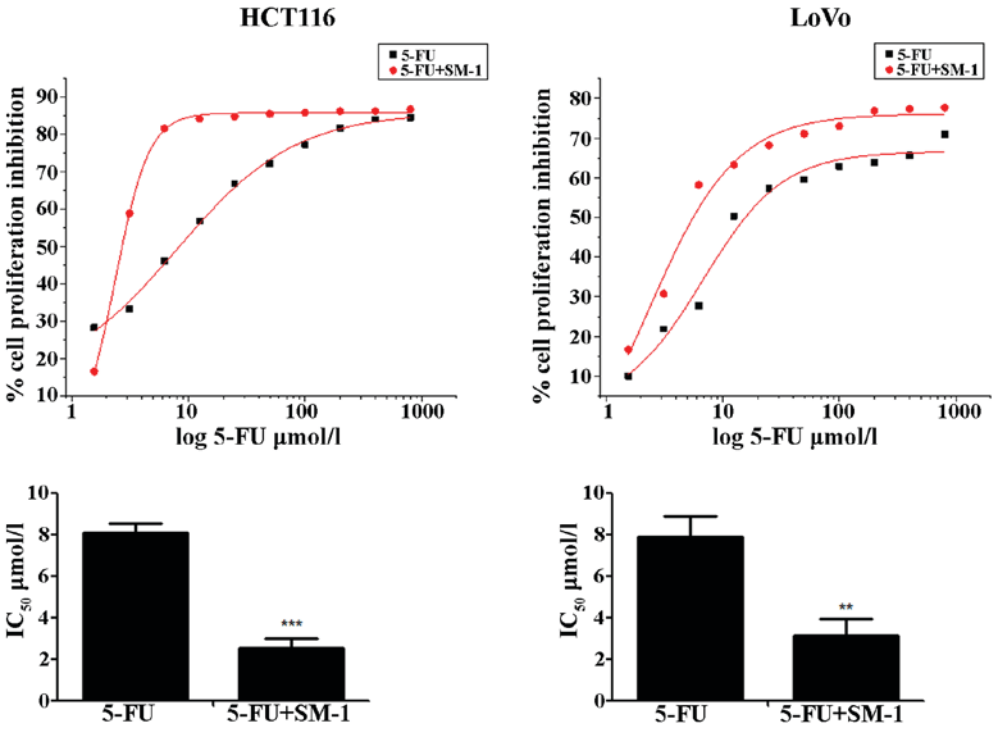

Figure 1. SM-1 significantly enhances the anti-proliferative effect of 5-FU in HCT116 and LoVo cells. Cell activity was analyzed using an MTT assay 72 h subsequent to 5-FU and/or SM-1 treatment. IC_{50} values were calculated using a non-linear regression model. Cotreatment with SM-1 and 5-FU led significant antitumor activities in HCT116 and LoVo cells. $^{**}P<0.01$; $^{***}P<0.001$. 5-FU, 5-fluorouracil; IC_{50}, half-maximal inhibitory concentration.

with a horseradish peroxidase-conjugated goat anti-rabbit (cat. no. 7074; dilution, 1:10,000; Cell Signaling Technology, Inc.) or goat anti-mouse (cat. no. 7076; dilution, 1:10,000; Cell Signaling Technology, Inc.) secondary antibody for 1 h at room temperature, followed by an additional three washes with TBST. The immunoreactive bands were visualized using ECL Western Blot kit (ComWin Biotech, Beijing, China). The experiment was repeated three times and similar results were obtained.

Mouse xenograft models and histology. The present study was approved by the Ethics Committee of the Beijing Medical Experimental Animal Care Commission (Beijing, China). Female athymic *nu/nu* mice, between 3 and 4 weeks old, weighing between 18 and 20 g, were purchased from Vital River Laboratories Co., Ltd. (Beijing, China). Mice were kept under conditions of constant temperature (21-23˚C) and humidity (40-60%) with a 12 h light/dark cycle. Mice were allowed free access to an irradiated standard rodent diet and sterilized water. To generate tumors, viable HCT116 cells ($5x10^6$ cells/mouse) and LoVo cells ($5x10^6$ cells/mouse) were subcutaneously injected into the right flanks of the mice. Vernier calipers were used to measure tumor dimensions, and tumor volume was calculated as 0.5 x length x $width^2$. When the tumor volume reached ~100 mm^3, 32 mice were divided into four groups at random, with each group containing 8 mice: i) Control group treated with saline alone; ii) SM-1 group in which the drug was given at 50 mg/kg/day via oral gavage (13); iii) 5-FU group in which the drug was administered intraperitoneally at 30 mg/kg/3 days (13); and iv) combination group of SM-1 and 5-FU at the same dose and schedule as the single-agent groups. Tumor size and body weight were measured every 3 days. At the conclusion of the experiment, mice were sacrificed and tumors were excised. Tumor specimens were stained with hematoxylin and eosin (H&E) for histological evaluation. Slides were scanned using a Pannoramic MIDI scanner and analyzed using Pannoramic ViewerRTM software (version 15.3) (both 3DHistech, Ltd., Budapest, Hungary).

Statistical analysis. SPSS (version 13.0; SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analysis. Results are presented as the mean ± standard deviation. Statistical intergroup differences were analyzed using one-way analysis of variance followed by Bonferroni's post hoc test. Differences between two groups were evaluated using a two-tailed Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SM-1 significantly enhances the anti-proliferative effect of 5-FU in colorectal cancer cells. HCT116 and LoVo cells were exposed to increasing concentrations of SM-1 and/or 5-FU for 72 h, and proliferation inhibition rates were analyzed using an MTT assay. 5-FU inhibited the proliferation of HCT116 and LoVo cells in a concentration-dependent manner. The half-maximal inhibitory concentration (IC_{50}) values were 8.07±0.49 and 7.90±0.98 μmol/l for HCT116 and LoVo cells, respectively. When cells were cotreated with 5-FU and SM-1 simultaneously, SM-1 significantly enhanced the anti-proliferative activity of 5-FU and decreased the IC_{50} values to 2.55±0.41 and 3.14±0.81 μmol/l in HCT116 and LoVo cells, respectively ($P<0.001$ and $P<0.01$, respectively; Fig. 1).

SM-1 and 5-FU cotreatment induces apoptosis in HCT116 and LoVo cells. Combination treatment with SM-1 and 5-FU led to the appearance of a number of apoptotic biomarkers. During apoptosis, cells are unable to modulate phospholipid distribution in the cell membrane, and phosphatidylserine is exposed to the outer membrane of cells, as assessed using an Annexin V/PI co-staining assay (18). HCT116 and LoVo cells were incubated with SM-1 and/or 5-FU. SM-1 markedly increased the proportion of apoptotic cells from 7.27 to 21.75% in HCT116 cells, and from 3.07 to 17.05% in LoVo cells. Similarly, 5-FU increased the apoptotic rate from 7.27 to 19.10% in HCT116 cells and from 3.07 to 12.88% in LoVo cells. SM-1 cotreatment with 5-FU led to markedly increased proapoptotic effects and an increased proportion of apoptotic cells to 47.95% in HCT116 and 35.19% in LoVo cells (Fig. 2A). Condensation of chromatin is another apoptotic hallmark (19). Consistent with the flow cytometry data, only the combination of SM-1 and 5-FU exhibited significant morphological alterations in Hoechst 33342-stained HCT116 and LoVo cells, including condensed chromatin and formation of apoptotic bodies (Fig. 2B). A JC-1 assay was used to detect mitochondrial outer membrane permeabilization. The decreased fluorescence of JC-1 aggregates indicates a loss of MMP and primarily appears in the early phase of mitochondrial apoptosis (20). Significantly decreased fluorescence intensity was identified in HCT116 and LoVo cells cotreated with SM-1 and 5-FU, which implied that the combined treatment mediated the loss of MMP and induced apoptosis (Fig. 2C).

Expression of apoptosis-associated genes in HCT-116 and LoVo cells. qPCR was used to detect expression of Bax, Bcl-2, Survivin, X-linked inhibitor of apoptosis protein (XIAP) and PARP mRNA following cotreatment with SM-1 and 5-FU (Fig. 3). In HCT-116 and LoVo cells, significantly increased expression levels of Bax and PARP were observed following cotreatment with 5-FU and SM-1 compared with 5-FU or SM-1 alone (Fig. 3). Compared with 5-FU alone, cotreatment with SM-1 significantly decreased the mRNA expression levels of Bcl-2, Survivin and XIAP (Fig. 3).

Detection of apoptosis-associated proteins by western blotting. As aforementioned, SM-1 induces apoptosis by targeting procaspase-3 and allowing it to autoactivate. Therefore, the effects of SM-1 and 5-FU in combination on the level of caspase-3 were investigated. HCT116 and LoVo cells were pretreated for 72 h with SM-1 (1 μmol/l), 5-FU (8 μmol/l) or a combination of SM-1 and 5-FU (1 and 8 μmol/l, respectively), and expression of caspase-3 was measured by western blotting. Minimal activation of caspase-3 was observed when cells were treated with SM-1 and 5-FU alone. However, when SM-1 and 5-FU were used in combination, markedly increased levels of cleaved caspase-3 were observed (Fig. 4).

The effects of SM-1 and 5-FU in combination on other proapoptotic and antiapoptotic proteins, including Bcl-2, Bax, Survivin and PARP were also investigated. The levels of Survivin and Bcl-2 were decreased markedly in HCT116 and LoVo cells following cotreatment with SM-1 and 5-FU, whereas low or no expression of these proteins was observed with SM-1 or 5-FU treatment alone at the same concentrations evaluated (Fig. 4). Similarly, cotreatment of HCT116 and LoVo cells with SM-1 and 5-FU resulted in marked increases in Bax and PARP expression compared with incubation with SM-1 or 5-FU alone. These results indicated that a combination of SM-1 and 5-FU induces apoptosis of colorectal cancer cells via Bcl-2, Survivin and PARP.

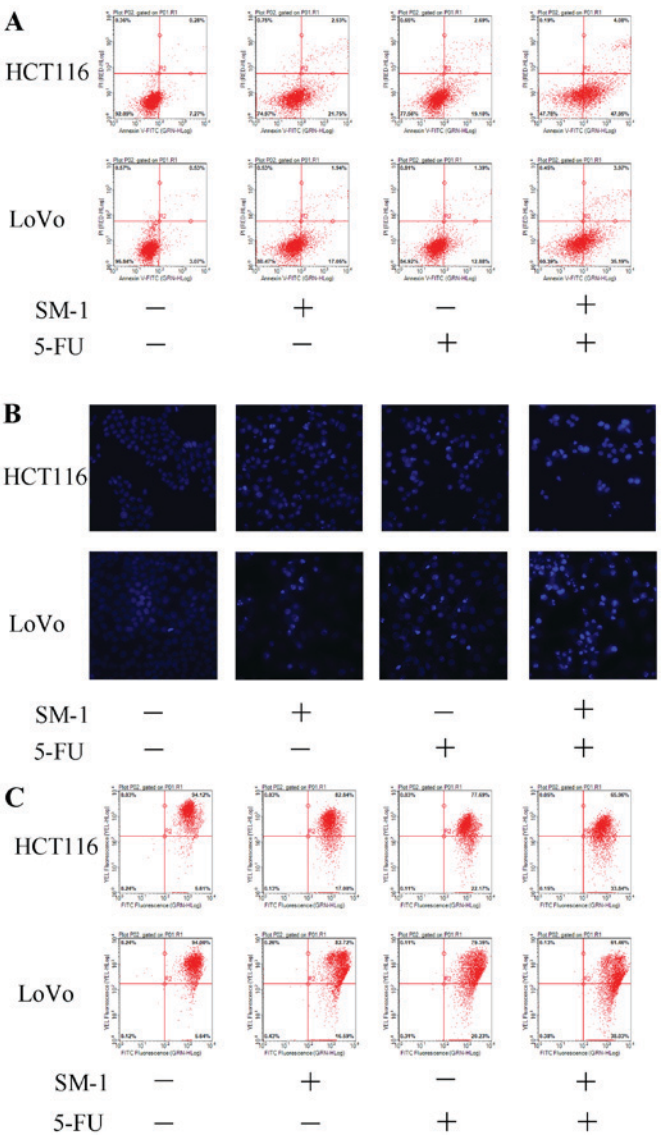

Figure 2. SM-1 and 5-FU combination treatment induces apoptosis in HCT116 and LoVo cells. (A) HCT116 and LoVo cells were incubated with SM-1 (1 μmol/l), 5-FU (8 μmol/l) or a combination of SM-1 and 5-FU (1 and 8 μmol/l, respectively) for 72 h. Phosphatidylserine exposure was measured by annexin V/PI co-staining. The combination group demonstrated a marked increase in apoptosis. (B) HCT116 and LoVo cells were incubated with SM-1 (1 μmol/), 5-FU (8 μmol/l) or a combination of SM-1 and 5-FU (1 and 8 μmol/l, respectively) for 72 h, and cells were observed using fluorescence microscopy (magnification, x400). (C) HCT116 and LoVo cells were treated with SM-1 (1 μmol/l), 5-FU (8 μmol/l) or a combination of SM-1 and 5-FU (1 and 8 μmol/l, respectively) for 72 h, prior to incubation with 5,5', 6,6'-tetrachloro-1,1', 3,3'-tetraethylbenzimidazolylcarbocyanine iodide for 20 min. Mitochondrial membrane depolarization was measured using flow cytometry. 5-FU, 5-fluorouracil; PI, propidium iodide; FITC, fluorescein isothiocyanate.

SM-1 combined with 5-FU inhibits tumor proliferation in vivo. To evaluate the antitumor potential of SM-1 combined with 5-FU *in vivo*, the ability of SM-1 and/or 5-FU to inhibit tumor proliferation in HCT116 and LoVo xenograft models

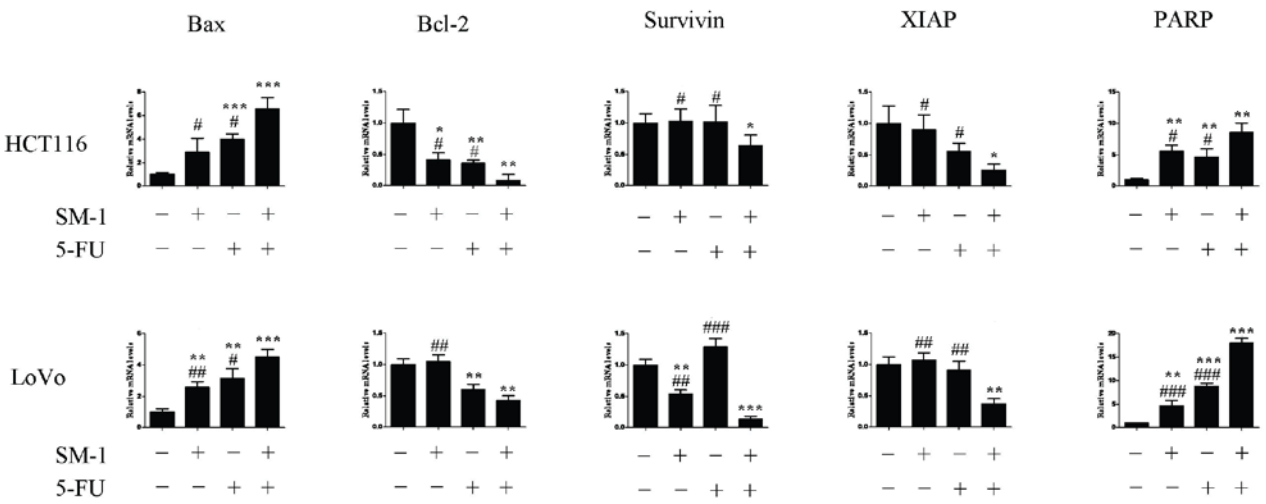

Figure 3. Effects of SM-1 and 5-FU cotreatment on XIAP, Survivin, Bax, Bcl-2 and PARP mRNA levels were measured using quantitative polymerase chain reaction. Bax and PARP levels were significantly increased when treated with SM-1 and 5-FU in combination compared with the untreated control, or SM-1 or 5-FU alone. XIAP, Survivin and Bcl-2 levels were significantly decreased when treated with SM-1 and 5-FU in combination compared with the untreated control, or SM-1 or 5-FU alone. *P<0.05, **P<0.01, ***P<0.001 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. SM-1 and 5-FU combination group. 5-FU, 5-fluorouracil; XIAP, X-linked inhibitor of apoptosis protein; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly (ADP-ribose) polymerase.

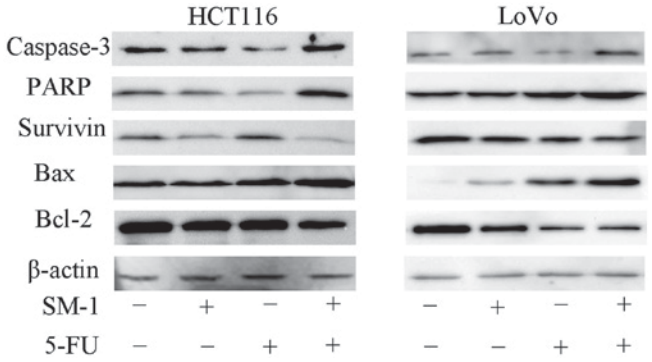

Figure 4. Western blot analysis of the apoptotic molecules caspase-3, Bcl-2, Bax, Survivin and PARP in HCT116 and LoVo cells incubated with SM-1 and/or 5-FU. β-actin was used as a loading control. For HCT116 and LoVo cells, caspase-3 levels were markedly increased following treatment with SM-1 and 5-FU in combination. In addition, cotreatment with SM-1 and 5-FU markedly increased the levels of Bax and PARP, and markedly decreased the levels of Bcl-2 and Survivin. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; PARP, poly (ADP-ribose); 5-FU, 5-fluorouracil.

was examined. For the HCT116 xenograft model, all treatments were able to markedly decrease tumor cell proliferation compared with the control. The tumor suppression rates for SM-1, 5-FU and the combination of SM-1 and 5-FU were 41.69, 38.81 and 70.42%, respectively. Furthermore, mice cotreated with SM-1 and 5-FU exhibited the most increased inhibition of tumor cell proliferation, compared with SM-1 alone (P<0.01), 5-FU alone (P<0.05) and control (P<0.001) groups. In the LoVo xenograft model, 25 days following the start of treatment, the combination treatment group demonstrated a statistically significant decrease in tumor cell proliferation compared with SM-1 alone (P<0.05), 5-FU alone (P<0.01) and control (P<0.001) treatment groups. The inhibition rates of tumor cell proliferation were 38.09, 39.64 and 78.07%, respectively (Fig. 5A). However, no significant difference in tumor volume between the SM-1, 5-FU group and control groups was identified. Neither significant weight loss nor mortality was observed in any of the groups during the course of the experiment (Fig. 5B).

The tumor tissues were further analyzed using H&E staining. As presented in Fig. 5C, the tissue from the control group exhibited compact tumor cells and a limited number of cells exhibiting small hyperchromic fragmented nuclei. In the 5-FU or SM-1 experimental groups, numerous gaps between tumor cells were observed. In the 5-FU and SM-1 experimental group, the tumor tissue exhibited increased damage compared with that in the 5-FU or SM-1 group; in addition, the cellular arrangement was disordered and karyopyknosis was observed. As a result, the combination group was identified to ameliorate the severity of tumor.

Discussion

As a first-line chemotherapeutic drug, 5-FU is widely used in clinical treatment of colon cancer (2,3). However, tumor cells have demonstrated resistance to 5-FU (4). Combined chemotherapy has been considered as an alternative treatment strategy, providing the potential for enhanced efficacy (21,22). Despite these improvements, drug resistance remains and novel combined treatment strategies are urgently required. Previous studies have indicated that abnormal apoptosis may be involved in drug resistance to 5-FU (2), including mutation of Bcl-2 or p53 proteins (23,24). Therefore, the combination of 5-FU and drugs that induce apoptosis are frequently used in the treatment of colorectal cancer (25). SM-1 has exhibited antitumor and proapoptotic effects (16), therefore, in the present study, the antitumor effects of a combination of 5-FU and SM-1 were investigated.

An MTT assay demonstrated that the IC_{50} values in HCT-116 and LoVo cells were significantly decreased following cotreatment with 5-FU and SM-1, compared with treatment using 5-FU alone. Therefore, a combination of 5-FU

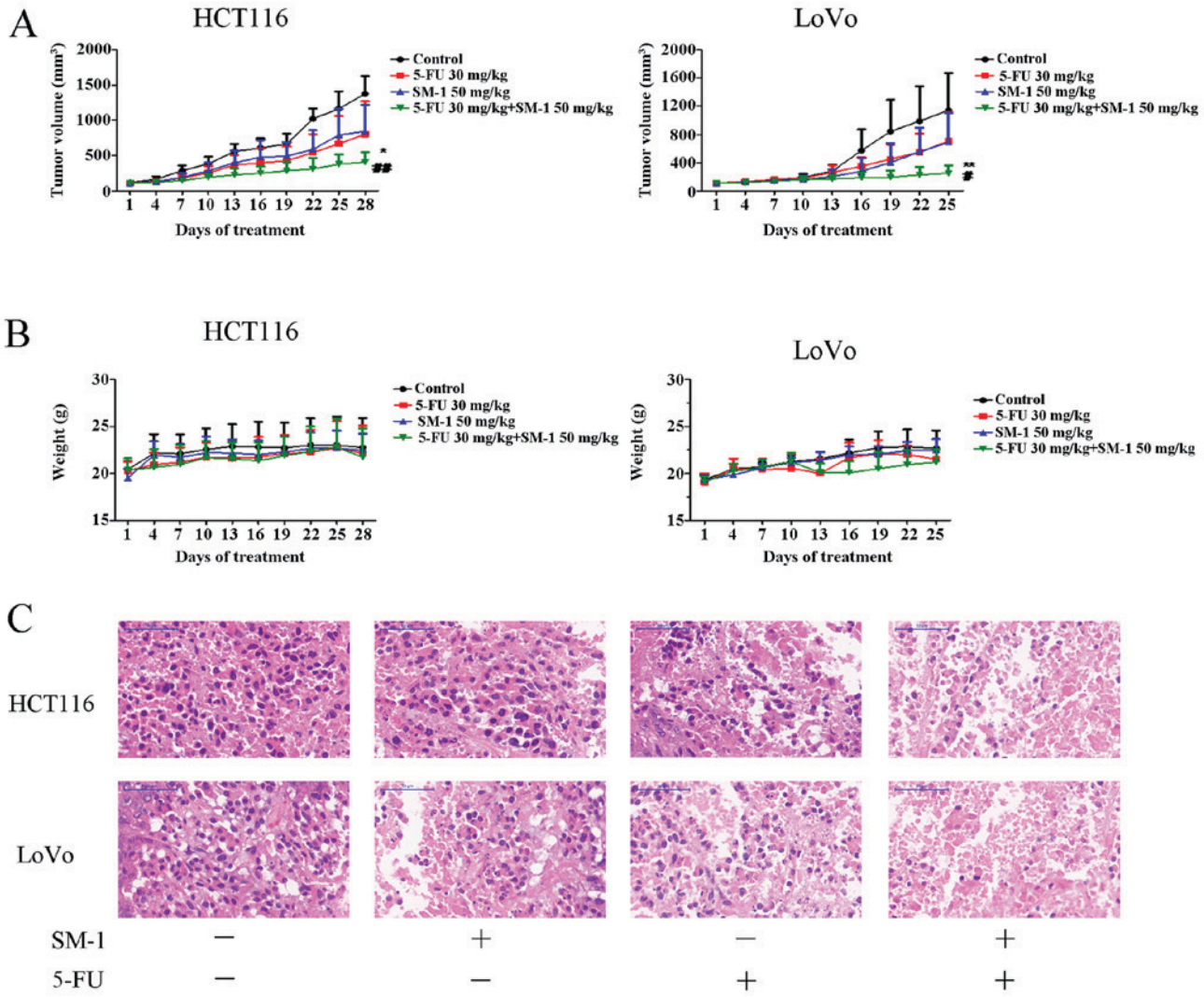

Figure 5. SM-1 and 5-FU treatment in combination significantly decreases tumor proliferation in HCT116- or LoVo-bearing mice. (A) HCT116 and LoVo cells were injected subcutaneously into the right flanks of each mouse. Once the tumors reached 100 mm^3, SM-1 was administered orally at 50 mg/kg/day, 5-FU was administered at 30 mg/kg/3 days intraperitoneally. The combination treatment group received SM-1 and 5-FU at the same doses and schedules as the single-agent groups. Tumor volumes were significantly decreased in mice receiving SM-1 and 5-FU in combination $^{*}P<0.05$, $^{**}P<0.01$ vs. 5-FU group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. SM-1 group. (B) The body weight of HCT116 or LoVo xenograft nude mice following treatment with SM-1, 5-FU or SM-1 and 5-FU in combination. (C) The microstructures of the tumors were observed using hematoxylin and eosin staining. Scale bar, 50 μm. 5-FU, 5-fluorouracil.

and SM-1 treatment may decrease the dose of 5-FU required to achieve maximal antitumor efficacy, without additional side effects.

The failure of 5-FU therapy in colorectal cancer was partly because of dysfunctional apoptosis (3). Therefore, it was investigated in the present study whether apoptosis was involved in the synergistic combination of 5-FU and SM-1. Hoechst 33342 staining identified that increased amounts of condensed chromatin and cell debris were observed in 5-FU- and SM-1-cotreated HCT-116 and LoVo cells compared with HCT-116 and LoVo cells treated with 5-FU and SM-1 alone, indicating that apoptosis was enhanced in HCT-116 and LoVo cells. Following combined treatment with SM-1 and 5-FU, the apoptotic rates in HCT-116 and LoVo cells were 47.95 and 35.19%, respectively, which were significantly increased compared with those following single treatment, as determined using Annexin V/PI treatment and flow cytometry. It was also determined whether SM-1 and 5-FU were able to trigger mitochondrial disorders in a synergistic manner. The results demonstrated that SM-1 and 5-FU markedly induced a loss of MMP, suggesting that mitochondrial depolarization may be triggered. These results suggested that 5-FU and SM-1 in combination was able to effectively induce the apoptosis of HCT-116 and LoVo cells.

Caspase-3 is the key point in the apoptotic signaling pathway, being the intersection of the external and internal apoptotic signaling pathways (26,27). The small molecule SM-1 directly activates procaspase-3 into caspase-3; therefore, the effect of SM-1 in combination with 5-FU on caspase-3 was investigated. Results revealed that SM-1 and 5-FU each led to minor activation of procaspase-3 in both cell lines. However, cotreatment with SM-1 and 5-FU markedly upregulated the level of caspase-3. Expression levels of upstream and downstream critical apoptotic indicators, including Bax, Bcl-2, Survivin and PARP, were investigated. Notably, cotreatment markedly upregulated Bax and cleaved PARP protein levels, and also decreased Bcl-2 and Survivin protein levels in HCT116 and LoVo cells. These results suggested that the

caspase-dependent apoptosis pathway was able to be activated and enhanced.

The *in vitro* results were confirmed *in vivo* using colorectal cancer xenograft models. Compared with the control group, 5-FU or SM-1 treatment alone has specific tumor inhibition effects. However, 5-FU and SM-1 treatment in combination exhibited increased antitumor activity compared with treatment using either 5-FU or SM-1 alone.

Combination treatment of SM-1 with 5-FU was able to enhance the antitumor activity of 5-FU *in vitro* and *in vivo*. These enhanced effects were due to activation of the caspase-dependent apoptosis signaling pathway. Therefore, combination treatment with SM-1 and 5-FU is a potential therapy for colorectal cancer.

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