Low-dose 5-fluorouracil sensitizes HepG2 cells to TRAIL through TRAIL receptor DR5 and survivin-dependent mechanisms

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Keywords
5-FU; Apoptosis; Chemotherapy; Hepatocarcinoma; TRAIL

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Low-dose 5-fluorouracil sensitizes HepG2 cells to TRAIL through TRAIL receptor 1 DR5 and survivin-dependent mechanisms

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Each of the authors listed above denies conflicts of interest.

Running title: Low-dose 5-FU and TRAIL for HepG2
Abstract

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising candidate for cancer treatment due to its highly selective apoptosis-inducing action on tumor cells without harming normal cells. However, because of TRAIL resistance by some cancer cells, combined treatment with sensitizing agents is required to enhance the anticancer potential of TRAIL. In the present study, we investigated the sensitizing effect of 5-fluorouracil (5-FU) on TRAIL-induced apoptosis in TRAIL-resistant HepG2 hepatocarcinoma cells. The results show that 5-FU pretreatment could sensitize HepG2 cells to TRAIL-mediated apoptosis. The enhanced induction of cell death by the 5-FU/TRAIL combination was mediated by DR5 up-regulation and survivin down-regulation. Furthermore, this combination treatment significantly inhibited the growth of human xenografts in vivo. In conclusion, this study demonstrates that the combination of a sensitizing agent and TRAIL may be a novel and effective therapeutic regimen for treating human hepatocellular carcinoma (HCC).

Key words: 5-FU, apoptosis, chemotherapy, Hepatocarcinoma, TRAIL
Introduction

Liver cancer is a major cause of cancer death in men worldwide, and the leading cause of cancer death in many developing countries.\(^1\) Hepatocellular carcinoma (HCC) is the most common histological type (70% to 90%) of primary liver cancers.\(^2\) Only a minority of patients can be cured, while the majority of the patients suffer from recurrence within a few years.\(^3\) Therefore, development of new therapeutic strategies for HCC is required.

TNF-related apoptosis-inducing ligand (TRAIL), or Apo2L, is a protein ligand, that induces apoptosis. It binds to a receptor complex comprising two death receptors (DR), DR4 and DR5, and three decoy receptors.\(^4,5\) TRAIL interacts with DR to trigger the apoptotic signaling pathway. The activated receptor complex recruits Fas-associated death domain (FADD) and subsequently activates caspase-8.\(^6\) Since DR4 and DR5 death receptors are expressed at higher levels in tumor cells than in normal cells, TRAIL selectively kills the malignant cells by inducing apoptosis without damaging normal cells.\(^7,8\) Therefore, TRAIL is considered to be one of the most promising candidates for the treatment of cancers. This promise has prompted extensive efforts toward developing recombinant human TRAIL and agonistic antibodies against DR4 and DR5 as potential therapeutic agents.\(^9,10\) However, previous work indicated that some tumor cells, including HCC cells, are resistant to TRAIL-induced apoptosis.\(^11,12\) Therefore, developing approaches to overcome this resistance is essential for the success of this therapeutic strategy.

Several reports have shown that a variety of chemotherapeutic agents or natural products can sensitize TRAIL-resistant cells and result in an improved anti-tumor effect by TRAIL treatment.\(^13,14\) However, the mechanism of TRAIL-resistance by some tumor cells is not well understood and it may also be dependent on the cell background. Therefore, understanding the
mechanism of TRAIL resistance and chemotherapeutic sensitization is important for developing the most effective regimen for TRAIL-based therapy on a variety of tumors.

In this study, we confirmed that HepG2 hepatocellular carcinoma cells are resistant to TRAIL treatment, but they can be sensitized to TRAIL treatment by sub-toxic levels of 5-FU. We further investigated the mechanism underlying TRAIL resistance and the sensitizing effects of 5-FU. The results revealed that the HepG2 cells are resistant to TRAIL treatment due to a low expression of DR5 and over expression of survivin, a major anti-apoptotic protein, and 5-FU sensitizes these cells by inducing the expression of DR5 while suppressing the expression of survivin.
Results

Sensitization effects of 5-FU on TRAIL-induced apoptosis

We first evaluated the effect of 5-FU or TRAIL alone on HepG2 cell viability. Treatment of cells with 5-FU up to 100 µg/ml for 12 h modestly reduced cell viability in a dose-dependent manner, with 100 µg/ml 5-FU decreasing cell viability to 80% compared with the control group. TRAIL alone up to 800 ng/ml had no impact on HepG2 cell viability (Fig. 1A). Thus, 5-FU up to 100 µg/ml or TRAIL up to 800 ng/ml was considered as subtoxic. The selection of these concentrations as subtoxic is consistent with literature reports (Refs).

To determine if 5-FU at subtoxic concentrations can sensitize HepG2 cells to TRAIL-induced cytotoxicity, cells were pretreated with 5-FU at concentrations up to 100 µg/ml, and then treated with TRAIL. Treatment by 5-FU alone induced only limited growth inhibition (<20%) over 12 h, consistent with the results in Fig. 1A. However, addition of TRAIL to cells pretreated with 5-FU significantly reduced cell viability in a dose-dependent manner, suggesting a chemosensitization effect of 5-FU on TRAIL (Fig. 1B). This was also confirmed by a reduction of the IC50 of 5-FU from 323.5±18.2 to 42.1±1.6 µg/ml. ComboSyn software was used to further analyze the synergistic effects between 5-FU and TRAIL.17 (Table 1). The results showed that low-dose 5-FU (50 µg/ml) has the smallest CI value and therefore the strongest synergy with TRAIL. For this reason we chose 50 µg/ml of 5-FU for further experiments.

DAPI staining was performed to determine if the reduced cell viability was due to apoptosis. Blue fluorescence with consistent intensity indicated even distribution of chromatin within the nuclei in untreated cells as expected, whereas nucleolar condensation and chromatin condensation were observed in treated cells, indicating early apoptotic events in these cells.
We observed that the apoptotic events were more evident in the 5-FU/TRAIL group than in the 5-FU or TRAIL alone groups.

We then determined whether the 5-FU/TRAIL treatment caused reduced cell viability through the induction of apoptosis. This was done by flow cytometry, which determines the percentage of early apoptotic cells (annexin V+/PI- cells). The apoptotic rates in the TRAIL group and the 5-FU group were 5.1% and 7.2%, respectively, whereas it was 42.8% in the combined group (Fig. 1D). These results indicate that combined treatment with 5-FU and TRAIL effectively induces apoptosis in HepG2 cells.

The involvement of DR and the mitochondrial apoptotic pathways

To determine whether 5-FU/TRAIL mediated cell death by activating caspases, we examined the effect of z-VAD-fmk, a broad spectrum caspases inhibitor, on 5-FU/TRAIL-induced cell death. As shown in Fig. 2A, z-VAD-fmk significantly blocked cell death induced by the combined treatment, indicating that 5-FU/TRAIL induced cell death via a caspase-dependent pathway.

To further confirm that 5-FU/TRAIL treatment indeed activates the apoptotic pathway, the activity of caspase-3/7, the principal effector caspases in apoptosis, in HepG2 cells under various treatments was determined. Caspase-3/7 activity increased 9-fold after addition of TRAIL to cells pretreated with 5-FU in HepG2 cells, while treatment with TRAIL alone or 5-FU alone resulted in increases of less than 2-fold (Fig. 2B). To confirm that the increase in Caspase-3/7 activity was due to the activation of these enzymes, we determined the cleavage of pro-caspase 3 directly by Western blotting. Consistent with the results of the colorimetric
caspase activity assay, addition of TRAIL to cells pretreated with 5-FU significantly induced the decrease of pro-caspase-3 and the release of an 18-kD fragment, indicating procaspase-3 cleavage and activation (Fig. 2C). We also investigated the effect of 5-FU and TRAIL on PARP [poly (ADP-ribose) polymerase] cleavage, which is considered as a hallmark of apoptosis. As shown in Fig. 2C, addition of TRAIL to cells pretreated with 5-FU led to a significant increase of PARP cleavage, while TRAIL or 5-FU alone did not.

We then investigated the involvement of mitochondrial and DR pathways in 5-FU/TRAIL-induced apoptosis by evaluating the activity of caspase-8 (Fig. 2D) and caspase-9 (Fig. 2E), respectively. Caspase 8 is activated by external death signals mediated by the cell surface death receptors and caspase 9 is intrinsically activated by the apoptosome, which is formed when cytochrome C is released from the mitochondria. 5-FU or TRAIL alone had minimal effect on caspase-8 or caspase-9 activity. In contrast, the combination treatment induced a marked increase of caspase-8 activity (5-fold) and an even more pronounced increase in caspase-9 activity (12-fold). These results suggest that both mitochondrial and DR pathways are involved in apoptotic induction by 5-FU/TRAIL.

5-FU upregulates DR5 as an important part of the sensitizing effect

TRAIL triggers apoptosis upon binding to DR4 or DR5 receptors. One reason for the resistance of some cancer cells to TRAIL-induced apoptosis may be the lack of such receptors. In this case, sensitization may involve up-regulation of the expression of these receptors. To test this possibility, we investigated the effect of 5-FU on the expression of both DR4 and DR5. We first examined whether 5-FU treatment for 12 h would upregulate DR4 and DR5 expression
levels. 5-FU at sub-toxic levels induced DR5 expression in HepG2 cells, but had no detectable effect on the DR4 protein level (Fig. 3A). To further assess the roles of DR5 and DR4 in 5-FU/TRAIL-mediated apoptosis, we evaluated the impact of DR4/Fc or DR5/Fc antagonists on the viability of 5-FU/TRAIL-treated cells. The reduction in cell viability induced by 5-FU/TRAIL was totally reversed by DR5/Fc inhibition, but not by DR4/Fc (Fig. 3B). These data suggest that activation of DR5 is principally involved in the sensitization effect of 5-FU to TRAIL-induced cell death.

5-FU downregulates survivin, another element of 5-FU sensitization

Survivin acts as an inhibitor of caspase-9, and blocks apoptosis via cooperative interactions with other partners, such as Smac/DIABLO. As the data in Fig. 2E indicated caspase-9 activation in 5-FU/TRAIL-treated cells, we then determined by immunoblotting whether survivin expression level was affected by 5-FU treatment. As shown in Fig. 4A, treatment with 5-FU for 12 h reduced the expression of survivin in a dose dependent manner. Exposure to 5-FU at 50 µg/ml reduced the survivin protein level by 50% in HepG2 cells. This observation raised the possibility that survivin down-regulation may be part of the sensitizing mechanism by 5-FU.

To test this possibility, a small molecule inhibitor of survivin expression, YM-155, was used to evaluate whether it is sufficient to reproduce the sensitization effect of 5-FU to TRAIL-induced apoptosis. As shown in Fig. 4B, survivin protein expression decreased markedly after cells were incubated with 200 nM of YM-155 for 24 h. Next, we examined the cytotoxic effects of the combination of YM-155 (200 nM) and TRAIL in HepG2 cells, which is similar to our
experiments with 5-FU. Cell viability was determined using the MTT assay after the YM-155 pretreated cells were treated with TRAIL (Fig. 4C). YM-155 alone or TRAIL alone did not reduce cell viability significantly while together they reduced cell viability to less than 60% compared to untreated cells. It is noteworthy that the combination of YM-155 and TRAIL was not as effective as that of 5-FU and TRAIL in reducing cell viability, but selective suppression of survivin is almost sufficient to reproduce the 5-FU-induced sensitization to TRAIL. This analysis suggests that the observed TRAIL-sensitization effect of 5-FU was partially due to its down-regulation of survivin. Although we cannot exclude other possible mechanisms, the results presented above demonstrate that the sensitizing effect of 5-FU on TRAIL-induced apoptosis in HepG2 cells is achieved by both up-regulating DR5 and down-regulating survivin.

**Effects of the combination of 5-FU and TRAIL on the growth of HepG2 xenografts**

A xenograft model was established to further explore the potential of 5-FU/TRAIL treatment for hepatocarcinoma. As shown in Fig. 5A, statistically significant antitumor efficacy was observed for the combination treatment. The tumor size in the 5-FU/TRAIL group (429 mm³, \( P < 0.001 \)) was significantly smaller than that of the control group (1214 mm³), the TRAIL alone (5 mg/kg) group (1151 mm³), or the 5-FU alone (5 mg/kg) group (826 mm³). As shown in Fig. 5B, the inhibitory rates of TRAIL alone and 5-FU alone were 5.2% and 19.3%, respectively. In the 5-FU/TRAIL group, the inhibition rate reached 64.6%. Confocal laser scanning microscopy images of TUNEL stained tumor tissue specimens of the mice treated with 5-FU/TRAIL exhibited significant green fluorescence, indicating significant induction of apoptosis, whereas the tissue specimens of mice untreated or treated with TRAIL or 5-FU alone showed no green
fluorescence (Fig. 5C). These data indicate that the combination of low-dose of 5-FU and TRAIL is capable of inhibiting growth of HepG2 cells \textit{in vivo}.

4 \textbf{Discussion}

Some cancer cells are sensitive to TRAIL-induced apoptosis, while normal cells and some other cancer cells are resistant to this treatment. The reason for the resistance is cell background-dependent, and not fully understood. The resistance to TRAIL treatment can be overcome by various chemotherapeutic drugs in some cases. In this study, we investigated if cells that are resistant to TRAIL treatment can be sensitized by chemotherapeutic drugs. If so, then the combination of chemo- and TRAIL therapy may be an effective treatment strategy for all cancers regardless of their original sensitivity to TRAIL.

For this study, we chose HepG2 cells that are generally considered to be resistant to TRAIL treatment.\textsuperscript{15,21} We demonstrated that treatments with subtoxic concentrations of 5-FU and TRAIL are synergistic, and investigated the mechanisms of the synergy. The results showed that pretreatment with 5-FU made HepG2 hepatocarcinoma cells sensitive to TRAIL-induced apoptosis. The data also showed that caspase inhibition could block apoptosis induced by 5-FU and TRAIL, suggesting that caspase pathway activation contributes to the induction of apoptosis by TRAIL, which is a fundamental component of the response.\textsuperscript{16} Moreover, the activation of caspase-8 and caspase-9 by 5-FU/TRAIL treatment indicated that both intrinsic and extrinsic pathways are involved in the process. We further demonstrated that 5-FU induced an increase in the expression of DR5 and down-regulated the expression of survivin and both contributed to the enhanced sensitivity of HepG2 cells to TRAIL. These findings indicate that HepG2 cells are
capable of magnifying apoptotic signaling initiated by death receptor via the recruitment of the intrinsic apoptotic pathway, as reported in other cell types.\textsuperscript{22,23}

Although a promising therapeutic strategy for treating cancers, TRAIL-based anti-cancer therapy has achieved only low to moderate success in clinical trials due to inherited or acquired resistance to TRAIL by advanced cancers.\textsuperscript{24,25} It has been reported that resistance to TRAIL can occur at different levels in the TRAIL-mediated signaling pathway, such as defects of death receptors, over-expression of survival proteins, or low expression levels of proapoptotic proteins.\textsuperscript{9,26} Among them, decreased expression of TRAIL receptors and/or elevated expression of anti-apoptotic proteins are the main factors that block TRAIL-signaling and cause HCC cells to be resistant to TRAIL.\textsuperscript{27}

There are five TRAIL receptors that have been reported to interact with TRAIL. Among them, DR4 and DR5 are considered as the death-inducing receptors because they contain functional cytoplasmic signaling domains, the death domain motifs, which are required for triggering the extrinsic apoptosis signaling pathway.\textsuperscript{4}

Previous studies have demonstrated that chemotherapeutic drugs could increase DR4 or DR5 expression in cancer cells.\textsuperscript{7,21} The upregulation of death receptors enhances cell death when these chemotherapeutic drugs are combined with TRAIL. Our results showed that 5-FU upregulated DR5, but not DR4 expression in HepG2 cells. This was consistent with the findings presented by Wen et al that Ara C, etoposide, or doxorubicin sensitized TRAIL-induced apoptosis by upregulation of DR5 but not DR4.\textsuperscript{28} The important role of DR5 in TRAIL-induced apoptosis is further confirmed by the experiment in which a human recombinant DR5-specific chimera antibody strongly reduced cell viability in cells pretreated with 5-FU, while DR4 chimera
antibody did not. These results indicate that DR5 plays a key role in the sensitizing effect of 5-FU on TRAIL-induced cytotoxicity.

As a member of the inhibitors of apoptosis protein (IAP) family, survivin is able to block apoptosis by caspase-9 inhibition. It has been reported that survivin is overexpressed in most types of malignant tumor, whereas it is low in most terminally differentiated tissues. In the present study, we demonstrated that 5-FU treatment significantly decreased survivin expression in HepG2 cells, which is consistent with the sensitization effect to TRAIL. Furthermore, inhibiting survivin expression alone by YM-155 was sufficient to sensitize HepG2 cells to TRAIL treatment. These results are consistent with a previous report that survivin downregulation by an antisense reagent induces apoptosis in HepG2 cells. They are also consistent with the observation that 5-FU/TRAIL treatment resulted in a greater activation of caspase-9 than caspase-8, suggesting that the suppression of survivin expression was partly responsible for caspase-9 activation. In combination, the results in this study demonstrate that 5-FU both upregulates the extrinsic reception to TRAIL and downregulates survivin to amplify the intrinsic response to the TRAIL signal. The combined effects of 5-FU on both the extrinsic and intrinsic apoptotic pathways make HepG2 cells sensitive to TRAIL-induced apoptosis.

We also investigated the antitumor activity of 5-FU combined with TRAIL in nude mice implanted with HepG2 cells. The data showed that the antitumor effects of TRAIL could be enhanced significantly by low-dose 5-FU. These results demonstrate the in vivo therapeutic efficacy of the combination of 5-FU and TRAIL.

In summary, we have demonstrated that low-dose 5-FU significantly enhances the antitumor effect of TRAIL on HepG2 cells both in vitro and in vivo. The enhancement was
mediated by DR5 upregulation and survivin suppression. Our data suggest that the combination of low-dose 5-FU with TRAIL can offer an effective treatment strategy for HCC. Moreover, this combination could circumvent the limited efficiency of single-agent chemotherapy and simultaneously overcome TRAIL resistance and the side effects of 5-FU as reported elsewhere in the treatment of HCC.

Materials and methods

Reagents

5-FU was purchased from Sigma Chemicals (F6627, USA) and dissolved in dimethyl sulfoxide (DMSO) to a stock solution of 50 mg/ml. The stock solution was diluted to 5 mg/ml with phosphate buffered saline (PBS). Dulbecco's modified Eagle's medium (DMEM) (High Glucose) (BC001), fetal bovine serum (FBS) (AZ001), trypsin-EDTA (BS7002) and penicillin/streptomycin (BS7011) were purchased from Sangon Biotechnology (China). YM-155 was purchased from Selleck Chemicals (S1130, USA). Pan-caspase inhibitor z-VAD-fmk (FMK001) and human recombinant DR4/Fc (6949-DR) and DR5/Fc (631-T2) chimera proteins were obtained from R&D Systems (USA). Antibodies against caspase-3 (cs9662), cleaved PARP (cs5625) and DR5 (cs3696) were purchased from Cell Signaling (USA) and those against DR4 (sc6823), survivin (sc10811) and β-actin (sc47778) were obtained from Santa Cruz Biotechnology (USA). Horseradish peroxidase-conjugated goat anti-rabbit (111-035-045) and goat anti-mouse (115-035-062) secondary antibodies were obtained from Jackson ImmunoResearch (USA). All other chemicals were purchased from Sigma.

Cell culture
The human hepatocellular carcinoma HepG2 cell line was obtained from the Chinese Academy of Sciences Institute of Cell Resource Center (Shanghai, China) and maintained in DMEM (High Glucose) with 10% FBS and penicillin/streptomycin. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Cell viability analysis

Cell viability was assessed by 3-(4,5-Dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Briefly, cells were seeded in 96-well plate overnight, and then treated with 5-FU (0-200 μg/ml) and/or TRAIL (0-800 ng/ml). These concentrations were experimentally determined to be subtoxic, and were used in this study. At the end of the treatment, 20 μL MTT solution (5 mg/ml) was added to each well and incubated at 37°C for an additional 4 h. Then 150 μL of DMSO was added to each well after the medium was removed and mixed by agitation at room temperature for 2 min. The absorbance of each well was measured at 570 nm by a microplate reader (Eon, Bio-Tek Instrument Inc., USA) and the cell viability (%) was calculated. The synergy between the effects of 5-FU and TRAIL was evaluated using the CompuSyn software (version 3.0.1; ComboSyn, Inc.).

Observation of apoptotic morphological change

Cells were seeded in a 12-well plate and incubated for 24h, then treated with 5-FU for 12 h followed by TRAIL for 6 h. The morphology of the cells was monitored under an inverted light microscope. Cells were stained with fluorochrome dye 4’,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescence microscope (Olympus IX51, Japan) with a peak excitation wavelength of 340 nm.

Analysis of apoptosis
Apoptotic cell death was quantified by flow cytometry using annexin V and Propidium iodide (PI) double staining kit from Sangon Biotechnology according to the manufacturer's protocol. Cells were incubated with 5-FU and/or TRAIL, harvested and resuspended in 1× binding buffer and stained with annexin V and PI for 15 min at room temperature. Apoptotic cell distribution was evaluated by Flow Cytometer (FC500, Beckman Coulter, USA). Cells that were annexin V+/PI- were considered as apoptotic cells.

**In vitro caspase activity assay**

Enzymatic activities of caspase-3/7, caspase-8 and caspase-9 were determined by colorimetric assays. Cells were lysed in the lysis buffer, the lysates were centrifuged at 14,000 rpm for 20 min, and the supernatants were collected. Assay buffer and colorimetric caspase-specific substrates (Ac-DEVD-pNA for caspase-3/7, Ac-IETD-pNA for caspase-8, and Ac-LEHD-pNA for caspase-9) (APT165, APT129 and APT172, Millipore, USA) were added to each sample and incubated at 37°C for 2 h in the dark. Caspase activity was determined by measuring the relative absorbance at 405 nm using a microplate reader.

**Western blotting**

After treatment, cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 8.0, with 150 mM NaCl, 0.1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (P8340, Sigma). Lysates were centrifuged at 14,000 rpm for 20 min. Protein concentration was measured with BCA protein assay kit (#500-0201, Bio-Rad, USA). Then equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were detected using specific antibodies for caspase-3, PARP, survivin, DR4 and DR5 followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at 37°C. Finally, the protein
signals were visualized by enhanced chemiluminescence advance detection kit (34080, Thermo). The bands were quantified by densitometric analysis with Quantity One 4.5.0 software (Bio-Rad). β-actin was used as a loading control for the western blotting assay.

Effects of TRAIL and 5-FU on HepG2 xenograft in nude mice

Five-week-old male nude mice (BALB/c-nc/nu) were purchased from Vital River Laboratories (Beijing, China). All animal procedures were approved by the Animal Care and Scientific Committee of Shanxi Medical University. Nude mice were inoculated subcutaneously with injections of $1 \times 10^7$ HepG2 cells/mouse at the right axilla. Twelve days after inoculation, 20 mice with tumor xenografts of approximately 75 mm$^3$ in size were chosen and randomly divided into 4 groups (with 5 mice/group). Intraperitoneal (i.p.) injection of PBS was used as control. The mice of the TRAIL alone group and the 5-FU alone group were injected i.p. with TRAIL (5 mg/kg) and intravenously (i.v.) with 5-FU (5 mg/kg), respectively. The mice in the combination group were injected i.v. with 5-FU (5 mg/kg) followed by i.p. injection of TRAIL (5 mg/kg) six hours later. All injections were repeated 2 days later. Consecutive injections of 5-FU and TRAIL were given a total of 10 times. The first day of administration was considered as D$_0$. At D$_{21}$, all animals were sacrificed and the tumor xenografts were removed and measured.

Tumor sizes were determined using slide calipers and tumor volume (TV) was calculated using the formula: $TV (\text{mm}^3) = (\text{width}^2 \times \text{length})/2$. Inhibition ratio was calculated using the formula: Tumor inhibition ratio = $(1 - \text{average tumor weights in treated group}/\text{average tumor weights in control}) \times 100\%$.

Apoptosis detection
Excised tumor tissues were fixed in formalin, embedded in paraffin, and sectioned at 3-µm thickness. Apoptosis was assessed by the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the In Situ Detection Kit (Boster Biotech Ltd., Wuhan, China) according to the manufacturer's protocol. Apoptotic cells were observed under a light microscope and a confocal laser scanning microscope (Olympus, Japan).

**Statistical analysis**

Statistical analysis was performed using Student's t-test. Results are shown as the mean ± standard deviation (SD) and P value <0.05 is considered statistically significant. IC50 was calculated using Gradpad Prism software (6.0).

**Conflicts of interest**

None.

**Acknowledgments**

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**References**


Figure legends

Fig.1. 5-FU sensitizes HepG2 cells to TRAIL-mediated apoptosis. (A) Effects of 5-FU or TRAIL on HepG2 cell viability. Cells were treated with various concentrations of 5-FU (0-200 µg/ml) or TRAIL (0-800 ng/ml) for 12 h and cell viability was measured by MTT assay (n = 6 per group). *P< 0.05 compared with the control (PBS). (B) Effects of 5-FU and/or TRAIL on cell viability. Cells were treated with 5-FU (0-100µg/ml) for 12 h followed by TRAIL (100 ng/ml) for 6 h (n = 6 per group). *P< 0.05, **P< 0.001 compared with the control. (C) Nucleolus morphological changes observed by DAPI staining. (D) Effect of 5-FU on TRAIL-induced apoptosis. Cells were treated with 5-FU (50 µg/ml) for 12 h followed by TRAIL (100 ng/ml) for 6 h and apoptosis was measured by flow cytometric analysis after double-staining with annexin V-FITC/PI. Representative results are shown in the upper panel. The lower panel is the bar graph of early
apoptotic cells (%). The values represent mean ± SD (n=3). Statistical significance: *P< 0.05

compared with the control, TRAIL and 5-FU groups.

Fig.2. Caspases is involved in 5-FU/TRAIL combination treatment-induced apoptosis in HepG2 cells. (A) Cells were pretreated with 20 μM pan-caspase inhibitor (z-VAD-fmk) for 30 min prior to the combined treatment of 5-FU (50 μg/ml) and TRAIL (100 ng/ml). Cell viability was evaluated by MTT assay. Results are presented as bar graphs from three independent experiments. Statistical significance: #P< 0.001. (B) The effects of combined treatment on the activity of executioner caspases were determined by a colorimetric assay. Cells were treated with 5-FU (50 μg/ml) for 12 h followed by TRAIL (100 ng/ml) for 6 h before measuring caspase activities. Data are presented as mean ± SD (n=6). Statistical significance: #P< 0.001 compared with the control, TRAIL and 5-FU groups. (C) The effects of combined treatment on caspase-3 and PARP cleavage were examined by Western blot analysis. Cells were treated as in (B) and cell lysates were separated by SDS-PAGE. β-actin was used as loading control. (D,E) Cells were treated as in (B). Caspase-8 (D) and -9 (E) were then evaluated. Data are presented as mean ± SD (n=6). Statistical significance : #P< 0.001 versus control, TRAIL and 5-FU groups.

Fig.3. Up-regulation of DR5 by 5-FU is essential for 5-FU/TRAIL-induced apoptosis. (A) Representative western blot after 12 h of treatment with 25 or 50 μg/ml of 5-FU and the DR4 and DR5 expression levels were quantified. Data are expressed as mean ± SD (n = 3 per group). *P< 0.05. (B) DR5/Fc chimera protein blocked the enhanced cell death by 5-FU/TRAIL combination. Cells were treated with 5-FU (50 μg/ml) and DR4/Fc (1μg/ml) or DR5/Fc (1μg/ml)
for 12 h followed by TRAIL (200 ng/ml) for 6 h. Cell viability was measured by MTT assay. *P<0.05.

Fig. 4. Survivin inhibition contributes to TRAIL-mediated apoptosis. (A) Representative western blot after 12 h of treatment with 25 or 50 μg/ml of 5-FU. Data are presented as mean ± SD (n = 3 per group). β-Actin was used as an internal standard. *P<0.05, #P<0.001 compared with the control group. (B) Representative western blot after 24 h treatment with 200 nM of YM-155 (n = 3 per group). #P<0.001 compared with control group. (C) Cells were treated with 100 ng/ml of TRAIL for 6 h after exposure to 200 nM of YM-155 for 24 h (n = 4 per group). Cell viability was measured by MTT assay. *P<0.05 compared with the control, TRAIL and YM-155 groups.

Fig. 5. Inhibitory effects of 5-FU and TRAIL on HepG2 xenografts in nude mice. (A) Tumor volume (cm³). #P<0.001 compared with control (PBS); (B) Inhibition rates of tumor. *P<0.05, #P<0.001; (C) TUNEL staining. Nuclei are stained blue and apoptotic cells green (×400).
(A) 5-FU (μg/ml) 0 25 50

DR5 → 48 kDa

DR4 → 47 kDa

β-actin →

(B) Relative change/control

0 25 50

5-FU (μg/ml)

Cell viability (%)

TRAIL + 5-FU - + + + + -

DR4/Fc (1 μg/ml) - - + - + + +

DR5/Fc (1 μg/ml) - - - + + + + 
Table 1 Combination Index (CI) values of drug combination in HepG2 cells

<table>
<thead>
<tr>
<th>5-FU (µg/ml)</th>
<th>TRAIL (ng/ml)</th>
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<th>CI</th>
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<td>42</td>
<td>0.10097</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>35</td>
<td>0.13661</td>
</tr>
</tbody>
</table>

Note: HepG2 cells were treated with the combination of 5-FU and TRAIL at dose indicated and cell viability was determined. CompuSyn software was used to analyze the data and calculate the CI value. The smaller CI value indicates stronger synergy.