

Chemotherapy and TRAIL-mediated colon cancer cell death: the roles of p53, TRAIL receptors, and c-FLIP

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Abstract

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has recently attracted attention as a potential therapeutic agent in the treatment of cancer. We assessed the roles of p53, TRAIL receptors, and cellular Fas-associated death domain–like interleukin-1 β -converting enzyme inhibitory protein (c-FLIP) in regulating the cytotoxic effects of recombinant TRAIL (rTRAIL) alone and in combination with chemotherapy [5-fluorouracil (5-FU), oxaliplatin, and irinotecan] in a panel of colon cancer cell lines. Using clonogenic survival and flow cytometric analyses, we showed that chemotherapy sensitized p53 wild-type, mutant, and null cell lines to TRAIL-mediated apoptosis. Although chemotherapy treatment did not modulate mRNA or cell surface expression of the TRAIL receptors death receptor 4, death receptor 5, decoy receptor 1, or decoy receptor 2, it was found to down-regulate expression of the caspase-8 inhibitor, c-FLIP. Stable overexpression of the long c-FLIP splice form but not the short form was found to inhibit chemotherapy/rTRAIL–induced apoptosis. Furthermore, siRNA-mediated down-regulation of c-FLIP, particularly the long form, was found to sensitize colon cancer cells to rTRAIL-induced apoptosis. In addition, treatment of a 5-FU-resistant cell line with 5-FU down-regulated c-FLIP expression and sensitized the chemotherapy-resistant cell line to rTRAIL. We conclude that TRAIL-targeted therapies may be used to enhance conventional chemotherapy

regimens in colon cancer regardless of tumor p53 status. Furthermore, inhibition of c-FLIP may be a vital accessory strategy for the optimal use of TRAIL-targeted therapies. [Mol Cancer Ther 2005;4(12):2026–36]

Introduction

Colorectal cancer remains to be one of the most predominant causes of cancer-related death in the western world. Although the combinations of 5-fluorouracil (5-FU) and oxaliplatin (FOLFOX) or irinotecan (CPT-11; FOLFIRI) have improved response rates to chemotherapy in advanced colorectal cancer (1), resistance to chemotherapy remains a major problem in the treatment of this disease and new approaches are urgently required. Recently, the use of death receptor ligands as novel therapeutic agents has come under scrutiny (2). Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2 ligand) was first identified in 1995 due to its sequence homology with other tumor necrosis factor family members (3). TRAIL is a type II membrane protein that can be proteolytically cleaved to a soluble molecule that forms a homotrimeric complex. TRAIL mRNA is abundantly expressed in most normal tissues. However, recombinant soluble human TRAIL (rTRAIL) seems to selectively induce apoptosis in transformed cells but not in most normal cell lines (4). Although the use of the Fas (CD95) death receptor ligand as a systemic therapeutic agent seems to be restricted due to severe liver toxicity, there is growing interest in targeting TRAIL as a therapeutic strategy, as TRAIL has been shown to exert antitumor effects without resulting in serious side effects in several xenograft studies (5–8). Various TRAIL-targeted therapies are currently in clinical trials.

TRAIL has been shown to bind to five type I transmembrane receptors: death receptor 4 (DR4/TRAIL-R1), death receptor 5 (DR5/TRAIL-R2/KILLER/TRICK2), decoy receptor 1 (DcR1/TRAIL-R3/TRID), decoy receptor 2 (DcR2/TRAIL-R4/TRUND), and osteoprotegerin (9). Of these, only DR4 and DR5 contain a functional cytoplasmic death domain that can transmit an intracellular death signal on ligand binding (10, 11). DcR1 and DcR2, which lack or have a truncated death domain, respectively, are termed decoy receptors. They are unable to induce cell death and are thought to block the apoptotic effect of TRAIL and, in DcR2, transduce antiapoptotic signals (12, 13). Binding of TRAIL to either DR4 or DR5 leads to receptor oligomerization and clustering of their intracellular death domains. The adaptor molecule Fas-associated death domain binds to these death domains and, through its NH₂-terminal death effector domain, recruits and promotes the proteolytic

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activation of caspase-8 (Fas-associated death domain-like interleukin-1 β -converting enzyme; refs. 4, 14). Activated caspase-8 in turn activates executioner caspases, such as caspase-3 and caspase-7, leading to cleavage of apoptotic substrates and cell death (15).

Several observations suggest that intracellular molecules inhibit the TRAIL signaling pathway. Firstly, TRAIL, DR4, and DR5 are expressed at significant levels in a wide range of normal tissues that are not sensitive to TRAIL-mediated apoptosis. In addition, expression of DcR1 and DcR2 does not correlate with TRAIL resistance in several *in vitro* tumor models (16, 17), whereas protein synthesis inhibitors have been shown to sensitize cancer cells to TRAIL-induced apoptosis (17–19). The cellular Fas-associated death domain-like interleukin-1 β -converting enzyme inhibitory protein (c-FLIP/Casper/I-Fas-associated death domain-like interleukin-1 β -converting enzyme/FLAME-1/CASH/CLARP/MRIT) has been reported to inhibit death receptor-mediated signaling (20). c-FLIP shows a high level of homology to caspase-8 and caspase-10 but has no protease activity. It inhibits death receptor-mediated apoptosis by binding to Fas-associated death domain and inhibiting caspase-8 activation at the death-inducing signaling complex (20). Of note, c-FLIP overexpression has been observed in some cancers, including colon cancer (21), and c-FLIP protein levels have been shown to correlate with TRAIL resistance in some tumor types (16, 17, 22, 23). Although c-FLIP is expressed as multiple splice variants, two main forms are expressed at the protein level: c-FLIP short form (c-FLIP_S), which is 28 kDa in size and contains two death effector domains, and c-FLIP long form (c-FLIP_L), which is 55 kDa in size and has two death effector domains and an inactive caspase-like domain (20). Whereas c-FLIP_S completely inhibits procaspase-8 activation at the death-inducing signaling complex, c-FLIP_L permits partial cleavage of procaspase-8 to an intermediate p41/p43 form but prevents further processing of procaspase-8 to its active p18/p10 subunits.

It has been shown that TRAIL-induced apoptosis can be enhanced by chemotherapy in several *in vitro* and xenograft models of cancer (24–28), an effect reported to be mediated through increased DR4 and DR5 expression (25, 28). In the present study, we have assessed the combined effect of rTRAIL and chemotherapy (5-FU, oxaliplatin, and CPT-11) in a panel of colon cancer cell lines. We found that rTRAIL enhanced apoptosis in response to chemotherapy in all cell lines examined, including a chemotherapy-resistant cell line. Furthermore, this effect was not dependent on functional p53 expression as it was observed in p53 wild-type (WT), mutant, and null cell lines. In addition, the enhanced rTRAIL sensitivity was not due to increased DR4 or DR5 or decreased DcR1 or DcR2 membrane expression following chemotherapeutic drug treatment. In contrast, we found that c-FLIP, particularly c-FLIP_L, plays a critical role in regulating TRAIL sensitivity in colon cancer cells following chemotherapy.

Materials and Methods

Materials

rTRAIL (Calbiochem, Darmstadt, Germany) was resuspended in PBS/0.1% bovine serum albumin as a 20 ng/mL stock solution and stored at -70°C .

Cell Culture

All cells were maintained in 5% CO₂ at 37°C. HCT116 p53 WT (p53^{+/+}) and p53 null (p53^{-/-}) isogenic human colon cancer cells were kindly provided by Prof. Bert Vogelstein (Johns Hopkins University, Baltimore, MD) and maintained in McCoy's 5A medium. 5-FU-resistant HCT116 cells were generated and maintained as described previously (29). H630 and HT29 cells (obtained from the National Cancer Institute, Bethesda, MD) and LoVo cells (kindly provided by AstraZeneca, Macclesfield, United Kingdom) were maintained in DMEM. All growth medium was supplemented with 10% dialyzed FCS, 2 mmol/L L-glutamine, and 50 $\mu\text{g}/\text{mL}$ penicillin/streptomycin. Additionally, all media, with the exception of that used for LoVo cells, were supplemented with 1 mmol/L sodium pyruvate; all media and supplements were from Invitrogen Life Technologies Corp. (Paisley, United Kingdom).

Generation of c-FLIP-Overexpressing Cell Lines

c-FLIP_L and c-FLIP_S coding regions were PCR amplified and ligated into the pcDNA/V5-His TOPO vector (Invitrogen Life Technologies) according to the manufacturer's instructions. p53 WT HCT116 cells were cotransfected with 10 μg of each c-FLIP expression construct and 1 μg of a construct expressing a puromycin resistance gene (pIRES-puro3, Clontech, BD Biosciences, Oxford, United Kingdom) using the Genejuice transfection reagent (Merck Biosciences, Darmstadt, Germany). Transfected HCT116 cells were maintained in medium supplemented with 1 $\mu\text{g}/\text{mL}$ puromycin (Invitrogen Life Technologies). Stable overexpression of c-FLIP was assessed by Western blot analysis.

siRNA Transfections

siRNAs were designed using the Invitrogen RNA interference designer tool¹ to down-regulate both c-FLIP splice variants or to specifically down-regulate the long form or the short form. The sequences used were as follows: splice variant AAGCAGTCTGTTC AAGGAGCA, long form AAGGAACAGCTTGGCGCTCAA, short form AACATGGAAGCTGCCTCTACTT, and control siRNA AATTCTCCGAACGTGTCACGT. siRNA transfections were done on subconfluent cells incubated in Opti-MEM using the Oligofectamine reagent (both from Invitrogen Life Technologies) according to the manufacturer's instructions.

Western Blot Analysis

Cells were washed in PBS and protein extraction was carried out using egg lysis buffer [20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA (pH 8.0), 1% Triton X-100, and 0.1% SDS] and standard methodology. Proteins (30 μg) were separated by SDS-PAGE and transferred to a Hybond P membrane (Bio-Rad, Ivy sur

¹ <https://rnaidesigner.invitrogen.com/rnaiexpress/>

Seine, France) by electroblotting. After blocking nonspecific binding sites for 1 hour at room temperature using 5% nonfat milk in PBS-0.3% Tween 20, membranes were incubated with specific primary monoclonal antibodies [poly(ADP-ribose) polymerase (PharMingen, BD Biosciences, Oxford, United Kingdom) and c-FLIP and caspase-8 (Alexis Corp., Lausen, Switzerland)] overnight at 4°C. Membranes were then washed thrice with PBS-0.3% Tween 20 and incubated for a further 2 hours with horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham, Little Chalfont, United Kingdom). Membranes were washed thrice with PBS-0.3% Tween 20 and bands were visualized using the SuperSignal chemiluminescent detection system (Pierce, Rockford, IL). Equal loading was assessed using a β -tubulin mouse monoclonal primary antibody (Sigma-Aldrich, Dorset, United Kingdom).

Quantitative Reverse Transcription-PCR

HCT116 cells were seeded on P90 plates at 5×10^5 per P90. Twenty-four hours later, cells were treated with 5-FU (5.0 $\mu\text{mol/L}$), oxaliplatin (0.5 $\mu\text{mol/L}$), or CPT-11 (5.0 $\mu\text{mol/L}$) for 24, 48, or 72 hours. Total RNA was extracted using the RNA STAT-60 reagent (Tel-Test, Friendswood, TX), and purified RNA (1 μg) was reverse transcribed using the Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Gene expression was assessed by quantitative PCR using an Opticon II instrument (MJ Research, Waltham, MA) in conjunction with the DYNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's instructions. The primer sequences used were as follows (5'-3'): DR4 forward CATCGGCTCAGGTTGTGGA and reverse TGCCGGTCCCAGCAGACA, DR5 forward CTGCTGTTGGTCTCAGCTGA and reverse TGCCGGTCCCAGCAGACA, and 18S forward CATTGCTATTGCGCCGCTA and reverse CGACGGTATCTGATCGTCT. The following thermal cycling protocol was used for each primer set: an initial denaturation of 94°C for 15 minutes followed by 40 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 1 minute. Analysis of 18S rRNA was used as a loading control.

Flow Cytometric Analysis

Cells were seeded on six-well plates at 2×10^5 per well. For the analysis of DNA content/apoptosis, cells were treated with rTRAIL alone for 24 hours or the indicated doses of chemotherapeutic agent for 24 hours followed by rTRAIL for an additional 24 hours. Cells were harvested and nuclei were stained with propidium iodide. For the analysis of cell surface receptor expression, cells were treated with the indicated doses of chemotherapeutic agent for 48 hours. Cells were then harvested, washed in buffer (PBS with 0.1% sodium azide and 0.2% bovine serum albumin), and stained with phycoerythrin-conjugated DR4, DR5, DcR1, and DcR2 monoclonal antibody (eBiosciences, San Diego, CA) according to the manufacturer's instructions. Nonspecific staining was assessed using a phycoerythrin-conjugated isotype control antibody (eBiosciences). All analysis was carried out on an EPICS XL flow cytometer (Coulter, Miami, FL) using CellQuest software (PharMingen BD Biosciences).

Clonogenic Survival Assay

To determine the effects of rTRAIL and chemotherapeutic agents on cell proliferation, cells were seeded on 24-well plates at a density of 750 per well. The following day, cells were treated with 5-FU, oxaliplatin, or CPT-11 and 24 hours later with rTRAIL (5 ng/mL). After 72 hours, the growth medium was replaced with fresh medium and cells were allowed to grow for a further 3 to 5 days. Colonies were visualized by crystal violet staining. All cell viability assays were carried out at least thrice.

Results

TRAIL Receptor Expression and Sensitivity to rTRAIL in HCT116 Colon Cancer Cells

The cell surface expression of DR4, DR5, DcR1, and DcR2 was assessed in HCT116 p53^{+/+} and p53^{-/-} colon cancer cell lines by flow cytometry (Fig. 1A). Whereas DR5 was highly expressed in both cell lines (84.7% and 86.2% receptor-specific fluorescence compared with isotype control), DR4 and the two TRAIL decoy receptors, DcR1 and DcR2, were not significantly expressed on the cell surface of these cells. Both HCT116 p53^{+/+} and p53^{-/-} cells were found to be relatively sensitive to rTRAIL by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay with the p53 WT cell line being the more sensitive (Table 1). The HCT116 p53^{+/+} cell line was also found to be more sensitive to rTRAIL by flow cytometry, with a more potent induction of apoptosis observed in this line compared with its p53-null counterpart (Fig. 1B). Furthermore, rTRAIL induced poly(ADP-ribose) polymerase cleavage more potently and at lower concentrations in the p53 WT cell line (Fig. 1C), again indicating that these cells were the more sensitive to rTRAIL.

Chemotherapy Enhances rTRAIL-Induced Apoptosis

To assess the combined effect of rTRAIL and each chemotherapeutic agent on cell viability in the HCT116 colon cancer cells, we initially used clonogenic survival assays. Colony formation in both p53 WT and null HCT116 cell lines was found to be clearly inhibited to a greater extent in wells cotreated with rTRAIL and chemotherapy than in wells treated with each agent alone, particularly in the p53 WT setting (Fig. 2A). Our results indicate that combined chemotherapy/rTRAIL treatment results in additive or superadditive inhibition of colony formation in both p53 WT and null HCT116 cell lines. Similar results were obtained using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assays (data not shown). The effect of rTRAIL and chemotherapy cotreatment on apoptosis in the HCT116 cells was assessed by flow cytometric analysis (Fig. 2B). In the HCT116 p53^{+/+} and p53^{-/-} cells, treatment with rTRAIL in combination with each of the three chemotherapies resulted in a greater than additive increase in the percentage of cells undergoing apoptosis than treatment with each agent individually. However, the absolute levels of apoptosis following chemotherapy/rTRAIL cotreatment were lower in the p53-null cell line compared

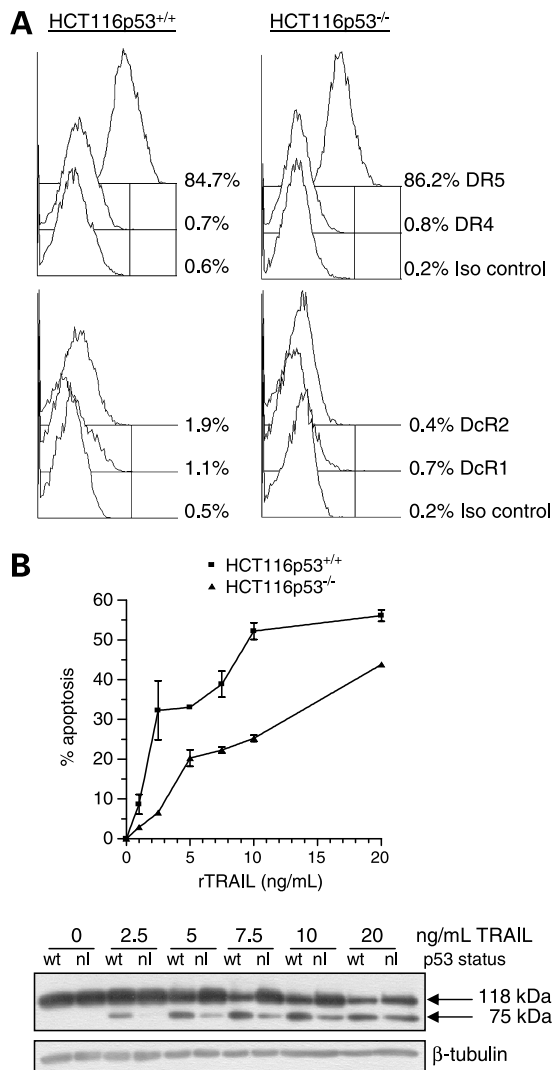


Figure 1. TRAIL receptor expression and rTRAIL sensitivity in p53 WT and null HCT116 colon cancer cell lines. **A**, DR4, DR5, DcR1, and DcR2 membrane expression was assessed by flow cytometry using receptor-specific phycoerythrin-conjugated monoclonal antibodies. Expression was compared with a nonspecific isotype-matched control antibody (*Iso control*). **B**, rTRAIL-induced apoptosis in HCT116 p53^{+/+} (*wt*) and p53^{-/-} (*nl*) cells as measured by flow cytometry. The DNA content of propidium iodide-stained cells was analyzed 24 h after treatment with rTRAIL and apoptosis was measured as the percentage of cells in the sub-G₀-G₁ fraction. Western blot analysis of poly(ADP-ribose) polymerase (PARP) cleavage in rTRAIL-treated HCT116 p53^{+/+} and p53^{-/-} cells.

with the p53 WT cell line. Collectively, these results indicate that 5-FU, oxaliplatin, and CPT-11 enhance TRAIL-induced cell death in both p53 WT and null HCT116 cell lines.

To further examine the role of p53 in TRAIL-mediated apoptosis, we extended our study to a wider panel of colon cancer cell lines. We analyzed cell surface TRAIL receptor expression in H630 cells that contain a deletion in exon 10 of the *p53* gene, HT29 cells that contain the "hotspot" p53 R273H mutation, and LoVo cells that express p53 WT

Table 1. rTRAIL IC₅₀ (72 hours) in a panel of colorectal cancer cell lines as measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell line	IC ₅₀ rTRAIL (ng/mL)
HCT116 p53 ^{+/+}	12
HCT116 p53 ^{-/-}	25
H630	29
HT29	>200
LoVo	>200

(Fig. 3A). Similar to the HCT116 cell lines, the H630 cells were found to express a relatively high level of DR5 (97.3% receptor-specific fluorescence compared with the isotype control). Furthermore, they also expressed cell surface DR4 (53.8%) and DcR1 (24.2%) but not DcR2. A relatively low level of DR4 (13.8%) and a high level of DR5 (84.8%) expression was detected on the surface of the HT29 cell line. However, these cells did not express significant levels of DcR1 or DcR2. Compared with the other colon cancer cell lines, the LoVo cell line was found to express a lower level of DR5 (33.5%). The LoVo cells also did not express DR4, DcR1, or DcR2. The H630 cell line was found to have a similar sensitivity to rTRAIL as the HCT116^{-/-} cell line, with an IC₅₀ (72 hours) dose of 29 ng/mL; however, the HT29 and LoVo cell lines were found to be highly resistant to rTRAIL with IC₅₀ (72 hours) doses of >200 ng/mL (Table 1). In the H630 and HT29 cell lines, 5-FU, oxaliplatin,

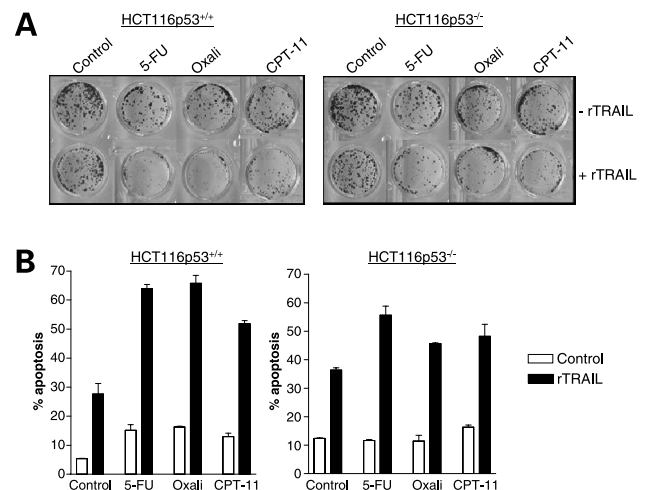


Figure 2. Chemotherapy enhances rTRAIL sensitivity in p53 WT and null HCT116 colon cancer cell lines. **A**, clonogenic cell survival following drug treatment in the HCT116 p53^{+/+} and p53^{-/-} cell lines visualized by crystal violet staining. Cells were treated with 5 μmol/L 5-FU, 1 μmol/L oxaliplatin (*Oxali*), or 5 μmol/L CPT-11 and 5 ng/mL rTRAIL for 72 h, after which time the growth medium was changed and colonies were allowed to grow for a further 4 to 5 d. **B**, effect of rTRAIL (5 ng/mL) on chemotherapy-induced apoptosis in HCT116 p53^{+/+} and p53^{-/-} cells as measured by propidium iodide staining and flow cytometry. Cells were pretreated with 5 μmol/L 5-FU, 1 μmol/L oxaliplatin, or 5 μmol/L CPT-11 for 24 h followed by rTRAIL for a further 24 h. For both assays, results are representative of at least three separate experiments.

and CPT-11 were each found to cause a greater than additive increase in rTRAIL-induced apoptosis, whereas the effects of chemotherapy on rTRAIL-induced apoptosis were more additive in the LoVo cell line (Fig. 3B). These results show that chemotherapy-mediated enhancement of rTRAIL-induced apoptosis occurs in a range of colon cancer cell lines, including p53 null and mutant cell lines. Furthermore, colon cancer cell lines, such as the HT29, which are relatively resistant to rTRAIL as a single agent, can be sensitized to rTRAIL-mediated apoptosis by treatment with chemotherapy.

Enhancement of Chemotherapy-Induced Apoptosis by rTRAIL Is Not Due to Altered TRAIL Receptor Expression

Expression of DR4 and DR5 mRNA was assessed in the HCT116 cell lines following chemotherapy treatment (Fig. 4A). 5-FU treatment was found to induce a very modest (~2-fold) increase in DR4 expression in both p53 WT and null cell lines after 48 hours, whereas expression returned to baseline levels at 72 hours. A similar pattern of up-regulation was observed for DR5 expression in the p53 WT cells only. No significant induction of either DR4 or DR5 mRNA expression was observed following oxaliplatin treatment in either cell line. Furthermore, CPT-11 treatment had little effect (<2-fold change) on either DR4 or DR5 mRNA expression in either cell line. Each of the cell lines was also examined for changes in levels of cell surface DR4,

DR5, DcR1, and DcR2 following chemotherapy. Interestingly, no significant changes in cell surface expression of either DR4 or DR5 or the decoy receptors were detected in any of the cell lines following drug treatment (Fig. 4B; data not shown). These results indicate that the increased sensitivity to rTRAIL following chemotherapy treatment was not due to increased DR4 or DR5 mRNA expression, nor was it due to increased DR4 or DR5 cell surface expression or to decreased DcR1 or DcR2 cell surface expression.

Chemotherapy Down-Regulates c-FLIP Expression in Colon Cancer Cells

As chemotherapy-mediated sensitization of our colon cancer cells to rTRAIL-induced apoptosis did not seem to be facilitated by modulation of TRAIL receptor membrane expression, we turned our attention to expression of procaspase-8 and c-FLIP. The rTRAIL-sensitive H630 cell line expressed ~2-fold higher levels of procaspase-8; however, similar levels of expression were observed in the rTRAIL-sensitive HCT116 cell lines and the rTRAIL-resistant LoVo and HT29 lines (Fig. 5A). Of note, the expression of c-FLIP_S was highest in the two rTRAIL-resistant cell lines HT29 and LoVo, whereas expression of c-FLIP_L and c-FLIP_S was lowest in the HCT116 p53^{+/+} cell line, which was the most sensitive to rTRAIL (Fig. 5A; Table 1). These results suggested that c-FLIP expression may play a role in determining sensitivity to rTRAIL in

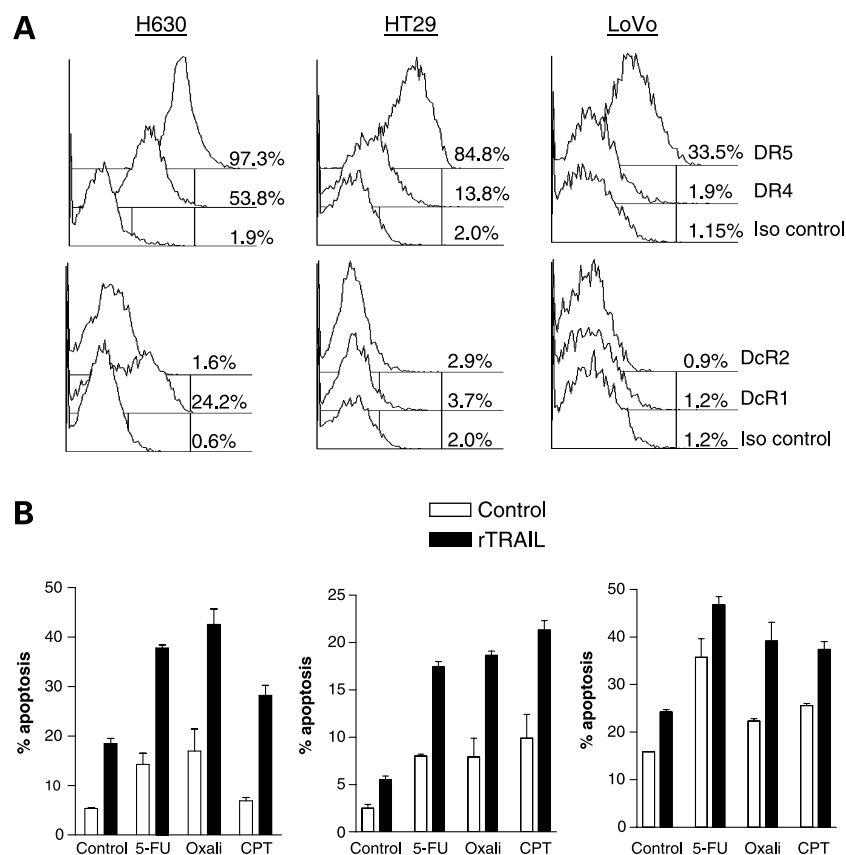
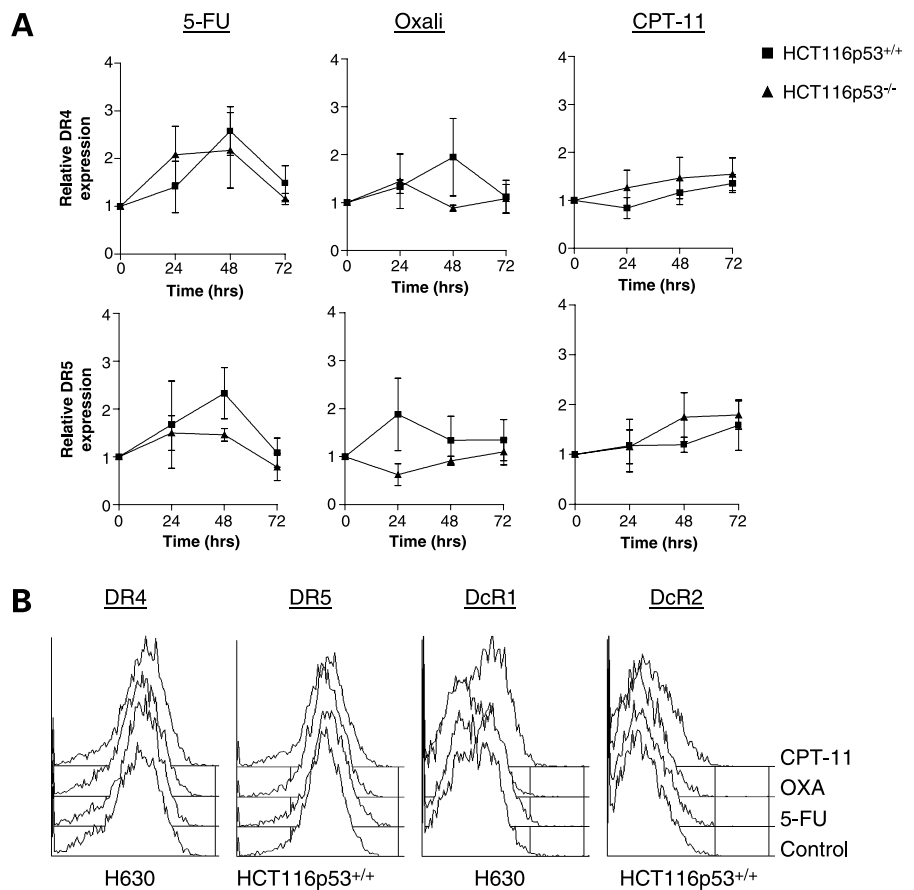


Figure 3. TRAIL receptor expression and sensitivity to rTRAIL and chemotherapy in p53 WT (LoVo) and mutant (H630 and HT29) colon cancer cell lines. **A**, analysis of constitutive DR4, DR5, DcR1, and DcR2 membrane expression in H630, HT29, and LoVo cells as measured by flow cytometric analysis of phycoerythrin-stained cells. **B**, apoptosis induced by rTRAIL in combination with chemotherapy in H630, HT29, and LoVo cells. Cells were treated with IC₅₀ (72 h) doses of each chemotherapeutic agent for 24 h and then with 10 ng/mL rTRAIL for a further 24 h. The sub-G₀-G₁ population was quantified by flow cytometric analysis of propidium iodide-stained cells. Representative of at least three separate experiments.

Figure 4. DR4 and DR5 gene expression and TRAIL receptor cell surface expression in chemotherapy-treated colon cancer cell lines. **A**, relative DR4 and DR5 mRNA expression in HCT116 p53^{+/+} and HCT116 p53^{-/-} cells was assessed by real-time reverse transcription-PCR following treatment with 5 μ mol/L 5-FU, 1 μ mol/L oxaliplatin, or 5 μ mol/L CPT-11 for 24, 48, and 72 h. **B**, TRAIL receptor cell surface expression in colon cancer cells following treatment with 5 μ mol/L 5-FU, 1 μ mol/L oxaliplatin (OXA), or 5 μ mol/L CPT-11. Cells were treated with an IC₅₀ dose of each cytotoxic for 48 h, and DR4/DR5, DcR1, and DcR2 membrane expression was assessed in each cell line by flow cytometry. No changes in receptor expression were detected in any cell line on comparison with untreated cells (Control). For each receptor, results from a representative cell line with detectable basal levels of receptor expression are shown.



colon cancer cells, so we next assessed the effect of chemotherapy treatment on the expression of c-FLIP_L and c-FLIP_S in each cell line (Fig. 5B). In HCT116 p53^{+/+} cells, treatment with 5-FU, oxaliplatin, and CPT-11 resulted in a ~2- to 3-fold decrease in c-FLIP_L expression (Fig. 5B). All three chemotherapies also down-regulated c-FLIP_S expression in HCT116 p53^{+/+} cells by at least 3-fold. Treatment with 5-FU, but not oxaliplatin and CPT-11, down-regulated c-FLIP_L by ~2-fold in the p53-null HCT116 cell line; however, all three chemotherapies significantly down-regulated c-FLIP_S expression (by ~2–3-fold) in this cell line. Similar results were obtained in the other cell lines, as treatment with each chemotherapy resulted in down-regulation of at least one splice form of c-FLIP. These results suggested that down-regulation of c-FLIP expression in response to chemotherapy may play a role in enhancing sensitivity of colon cancer cells to rTRAIL.

c-FLIP_L Overexpression Abolishes the rTRAIL Enhancement of Chemotherapy-Induced Apoptosis in Colon Cancer Cells

In light of the effects of chemotherapy on c-FLIP expression in the colon cancer cell lines, we created HCT116 p53^{+/+} cell models that stably overexpressed either c-FLIP_L or c-FLIP_S. Following the stable transfection procedure, c-FLIP expression was quantified by Western blot and one c-FLIP_L-overexpressing clone (HFL17) and

one c-FLIP_S-overexpressing clone (HFS19) were selected for subsequent analysis (Fig. 6A). c-FLIP_L overexpression (by ~4-fold) was found to inhibit rTRAIL-mediated apoptosis and abolish the enhanced apoptotic effect observed on dual rTRAIL and cytotoxic drug treatment (Fig. 6B). Somewhat surprisingly, overexpression of c-FLIP_S (by ~7-fold) failed to inhibit rTRAIL-induced cell death and did not inhibit apoptosis in response to cotreatment with rTRAIL and chemotherapy (Fig. 6B). Similar results were obtained with other c-FLIP-overexpressing clones (data not shown). Thus, c-FLIP_L seems to be a key regulator of apoptosis induced by rTRAIL alone and in combination with chemotherapy treatment.

Down-Regulation of c-FLIP Sensitizes Colon Cancer Cells to rTRAIL-Induced Apoptosis

In addition to assessing the effect of c-FLIP overexpression on TRAIL sensitivity, we used siRNA methodology to down-regulate c-FLIP expression. A siRNA targeted against both c-FLIP splice forms and siRNAs specific for either c-FLIP_L or c-FLIP_S were designed and transfected into each of the cell lines. c-FLIP_L and/or c-FLIP_S expression was successfully down-regulated in HCT116 p53^{+/+}, HCT116 p53^{-/-}, H630, and LoVo cells using these siRNAs (Fig. 7A; data not shown). However, siRNA transfection of HT29 cells was unsuccessful. Simultaneous down-regulation of both c-FLIP_L and c-FLIP_S (splice variant

siRNA) or c-FLIP_L alone was found to efficiently sensitize HCT116 p53^{+/+} cells to rTRAIL-induced apoptosis as assessed by poly(ADP-ribose) polymerase cleavage (Fig. 7B) and flow cytometry (Fig. 7C). Both splice variant and long form siRNAs also sensitized the p53-null HCT116 cell line to rTRAIL. Specific down-regulation of c-FLIP_S also sensitized both HCT116 cell lines to rTRAIL but to a lesser extent than in cells transfected with dual-targeted or c-FLIP_L-specific siRNAs (Fig. 7B and C). Both splice variant and long form siRNAs were also found to potently sensitize H630 cells to rTRAIL-induced apoptosis, whereas the short form siRNA also sensitized these cells to rTRAIL-induced apoptosis but again to a lesser extent (Fig. 7C). In the LoVo cells, specific down-regulation of c-FLIP_L but not c-FLIP_S sensitized the cells to rTRAIL (Fig. 7C). These results would suggest that c-FLIP, particularly c-FLIP_L, is a key regulator of TRAIL-mediated apoptosis in colon cancer cell lines and that down-regulation of c-FLIP in response to chemotherapy is at least partially responsible for the enhanced sensitivity of chemotherapy-treated colon cancer cells to rTRAIL.

5-FU Sensitizes a 5-FU-Resistant Cell Line to rTRAIL

We previously generated a 5-FU-resistant HCT116 p53^{+/+} cell line that is ~3-fold more resistant to 5-FU than the parental cell line (29). We found that this cell line expressed

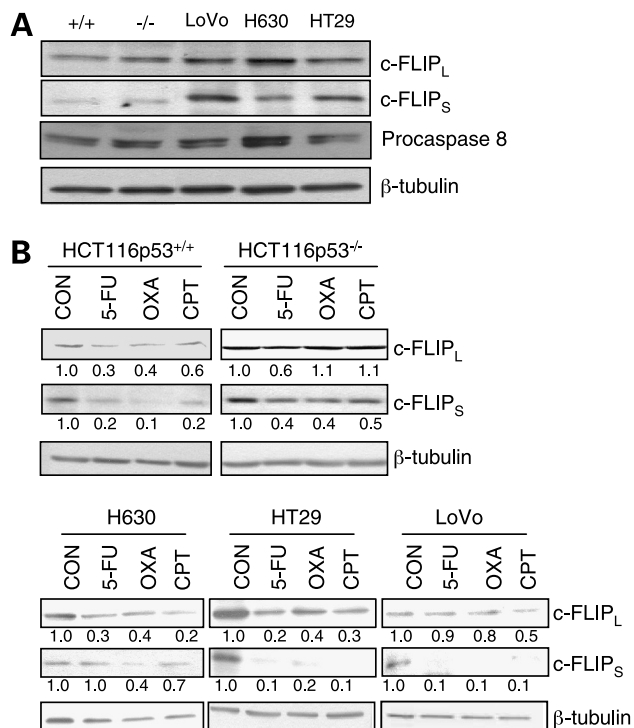


Figure 5. c-FLIP expression in colon cancer cell lines. **A**, constitutive c-FLIP protein and procaspase-8 expression in a panel of colon cancer cell lines as measured by Western blot. **B**, effect of 5-FU, oxaliplatin, or CPT-11 treatment on c-FLIP expression in a panel of colon cancer cell lines. Cells were treated with IC₅₀ (72 h) doses of each drug for 48 h, and c-FLIP_L and c-FLIP_S protein expression was assessed by Western blot. For each cell line, the intensity of c-FLIP_L and c-FLIP_S relative to the control lane was determined by densitometry.

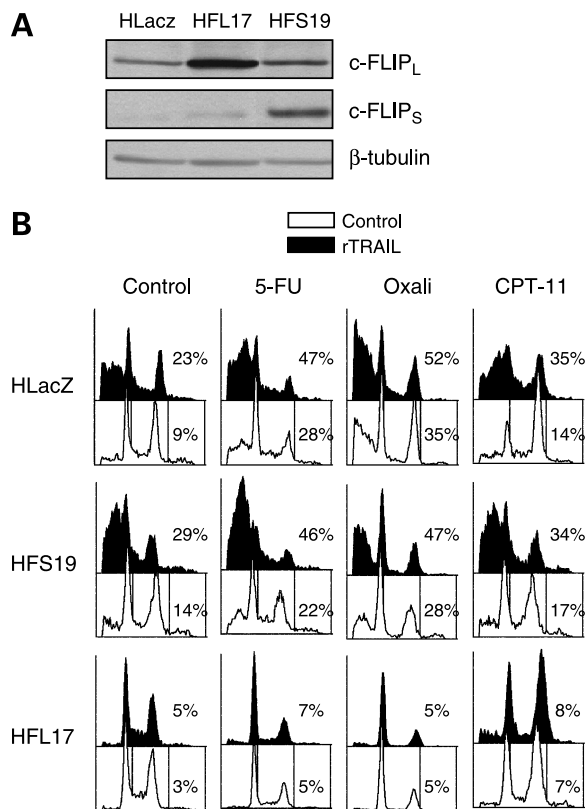


Figure 6. Overexpression of c-FLIP_L inhibits chemotherapy/rTRAIL-induced apoptosis. **A**, expression of c-FLIP_L and c-FLIP_S in c-FLIP_L-overexpressing HCT116 p53^{+/+} cell lines. **B**, flow cytometric analysis of apoptosis in c-FLIP_L-overexpressing (HFL17), c-FLIP_S-overexpressing (HFS19), and control (HLacZ) cell lines pretreated with 5 μmol/L 5-FU, 1 μmol/L oxaliplatin, or 5 μmol/L CPT-11 for 24 h followed by rTRAIL for a further 24 h in comparison with cells treated with each agent alone. The DNA content of propidium iodide-stained cells was assessed. Representative of at least three separate experiments.

similar levels of DR5 and c-FLIP as the parental line (Fig. 8A and B) and was equally sensitive to rTRAIL-induced apoptosis (Fig. 8C). Interestingly, despite this cell line being resistant to 5-FU, treatment with 5-FU still significantly sensitized these cells to rTRAIL (Fig. 8D). Furthermore, sensitization of the 5-FU-resistant cells to rTRAIL correlated with a decrease in c-FLIP expression following 5-FU treatment (Fig. 8E). These results suggest that chemotherapy-resistant cells may still be sensitized to rTRAIL by chemotherapy if the chemotherapy treatment results in down-regulation of c-FLIP expression.

Discussion

The initial aim of this study was to investigate the apoptotic potential of TRAIL in combination with chemotherapy in *in vitro* models of colon cancer and, more specifically, to examine the effect of p53 status on potential drug interactions. The p53 WT HCT116 cell line was more sensitive to rTRAIL [IC₅₀ (72 hours), 12 ng/mL] than the p53-null HCT116 daughter cell line [IC₅₀ (72 hours),

25 ng/mL], suggesting that p53 status plays a role in determining the sensitivity of colon cancer cells to TRAIL-mediated apoptosis. However, both p53 mutant HT29 and p53 WT LoVo cell lines were highly resistant to rTRAIL as a single agent [IC₅₀ (72 hours), >200 ng/mL], whereas the p53 mutant H630 cell line was sensitive to rTRAIL [IC₅₀ (72 hours), 29 ng/mL]. Therefore, p53 status is clearly not the sole determining factor of sensitivity TRAIL in colon cancer cells. We also examined cell surface expression of the TRAIL receptors DR4, DR5, DcR1, and DcR2. The three TRAIL-sensitive cell lines (HCT116 p53^{+/+}, HCT116 p53^{-/-}, and H630) expressed a relatively high level of DR5, whereas DR4 was only detected on the cell surface of H630 cells and was expressed at a lower level than DR5. In

addition, the H630 cell line was the only one found to express a TRAIL decoy receptor, DcR1, on its cell surface. Interestingly, the p53 WT and null HCT116 cell lines constitutively expressed almost identical levels of cell surface DR5, a reported p53 target gene (30, 31). The TRAIL-resistant line, LoVo, was found to express significantly lower levels of cell surface DR5 than the other cell lines, an observation that may explain their resistance to rTRAIL as a single agent (these cells did not express significant cell surface levels of DR4 or the decoy receptors). Interestingly, the TRAIL-resistant HT29 cell line expressed relatively high levels of DR5 and did not express significant cell surface levels of either decoy receptor. Our findings are consistent with recent evidence that suggests that DR5 may be more significant than DR4 in TRAIL-mediated apoptotic signaling (32). However, expression of DR5 is clearly not the sole determinant of TRAIL sensitivity in colon cancer cells, whereas decoy receptor expression does not seem to play a role in regulating TRAIL sensitivity in colon cancer cell lines. Although procaspase-8 expression was ~2-fold higher in the rTRAIL-sensitive H630 cell line, the levels of procaspase-8 expression were similar in both rTRAIL-sensitive HCT116 cell lines and rTRAIL-resistant LoVo and HT29 lines. This suggests that procaspase-8 expression levels do not determine the relative sensitivity of these cell lines to rTRAIL.

Recent studies have shown that a variety of chemotherapeutic drugs, including cisplatin, etoposide, and doxorubicin, can sensitize a wide range of cancer cells to TRAIL-induced apoptosis (33, 34). Although DR4 and DR5 have been described as p53 target genes, the importance of functional p53 expression in chemotherapy-mediated sensitization to TRAIL-induced apoptosis remains unclear (35). We examined whether chemotherapies used in the treatment of colon cancer (5-FU, oxaliplatin, and CPT-11) augmented the apoptotic effects of rTRAIL in isogenic p53 WT and null HCT116 cell lines. We found that treatment with each chemotherapeutic agent sensitized both p53 WT and null HCT116 cells to rTRAIL-induced apoptosis; however, the p53 WT cell line was the more sensitive to combined rTRAIL/chemotherapy treatment. These results again suggest that p53 plays a role in regulating sensitivity to rTRAIL. HCT116 cells have been reported to be "type II cells" (36), meaning that extrinsic death receptor-mediated apoptotic signal is dependent on amplification through the intrinsic mitochondrial-mediated apoptotic pathway. It is possible that p53-mediated regulation of proapoptotic and antiapoptotic regulators of the intrinsic pathway, such as Bcl-2 and Bax, will increase the sensitivity of HCT116 p53^{+/+} cells to rTRAIL and combined rTRAIL/chemotherapy treatment compared with the p53-null cell line. Indeed, the proapoptotic Bcl-2 family member Bid, which links the extrinsic and intrinsic apoptotic pathways, has been reported to be a p53 target gene (37). Similarly, the relative sensitivity to rTRAIL and chemotherapy cotreatment of the other colon cancer cell lines examined may also depend on whether they are type I (mitochondria-independent) or type II cells. In the p53 mutant H630 cell line, pretreatment

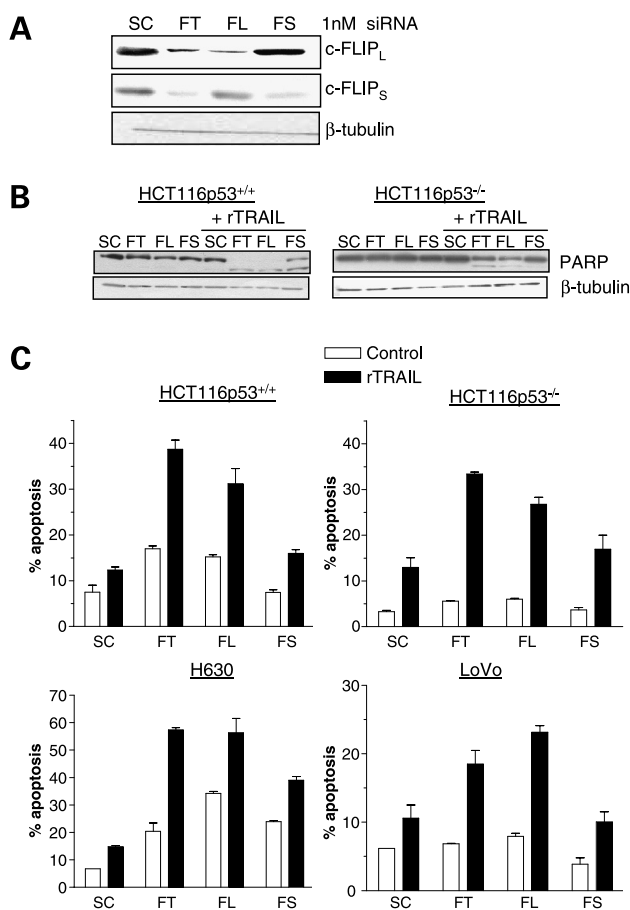


Figure 7. Down-regulating c-FLIP sensitizes colon cancer cells to rTRAIL. **A**, HCT116 p53^{+/+} cells were transfected with a nonsilencing control siRNA (SC), a siRNA that targets both long and short c-FLIP isoforms (FT), a c-FLIP_L-specific (FL) siRNA, or a c-FLIP_S-specific siRNA (FS). All siRNAs were transfected at a final concentration of 1 nmol/L, and the down-regulation of c-FLIP expression was confirmed by Western blot analysis. **B**, Western blot analysis of poly(ADP-ribose) polymerase cleavage in the HCT116 p53^{+/+} and p53^{-/-} cells transfected with 1 nmol/L siRNA and cotreated with 2.5 ng/mL rTRAIL for 24 h. **C**, flow cytometric analysis of apoptosis in HCT116 p53^{+/+}, HCT116 p53^{-/-}, H630, and LoVo cells transfected with 1 nmol/L siRNA and cotreated with 2.5 ng/mL rTRAIL (HCT116 and H630 cell lines) or 10 ng/mL rTRAIL (LoVo cell line) for 24 h. Representative of at least three separate experiments.

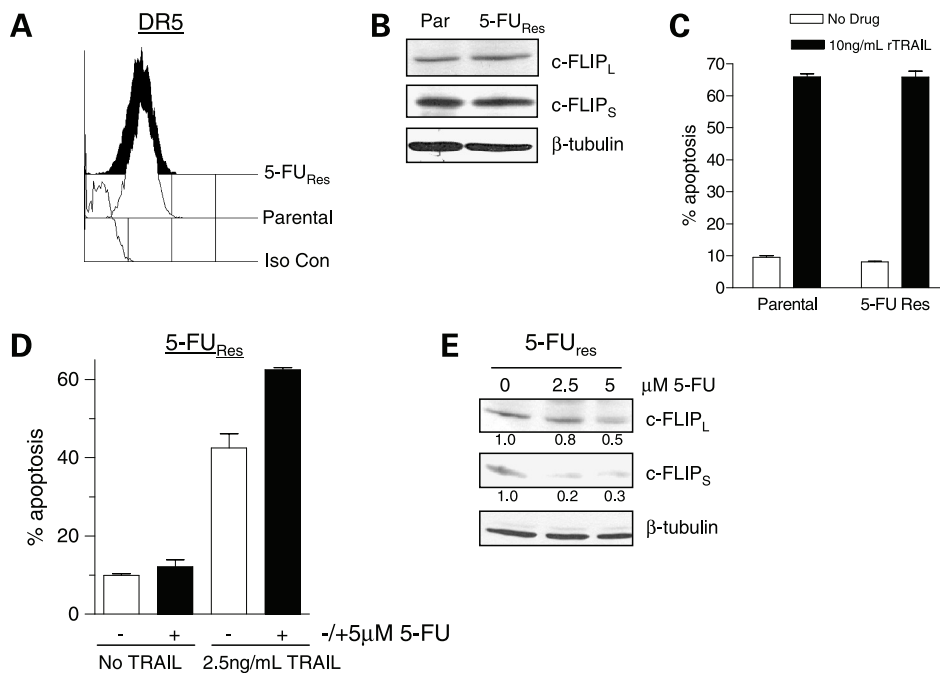


Figure 8. Sensitivity of 5-FU-resistant cells to rTRAIL. **A**, cell surface DR5 expression in HCT116 p53^{+/+} parental and 5-FU-resistant (5-FU_{Res}) cells. **B**, Western blot analysis of c-FLIP expression in HCT116 p53^{+/+} parental (Par) and 5-FU-resistant cells. **C**, flow cytometric analysis of apoptosis in HCT116 p53^{+/+} parental and 5-FU-resistant cells treated with 10 ng/mL rTRAIL for 24 h. **D**, flow cytometric analysis of apoptosis in HCT116 p53^{+/+} parental and 5-FU-resistant cells treated with 5 μmol/L 5-FU for 24 h followed by 2.5 ng/mL rTRAIL for 24 h. **E**, Western blot analysis of c-FLIP expression in HCT116 p53^{+/+} parental and 5-FU-resistant cells treated with 2.5 and 5 μmol/L 5-FU for 48 h. The intensity of c-FLIP_L and c-FLIP_S relative to the control lane was determined by densitometry.

with chemotherapy was found to superadditively enhance rTRAIL-induced apoptosis. Furthermore, chemotherapy pretreatment was found to enhance rTRAIL-induced apoptosis in the two TRAIL-resistant cell lines, HT29 (p53 mutant) and LoVo (p53 WT). As chemotherapy enhanced rTRAIL-induced apoptosis in p53 WT, null, and mutant cell lines, mutant p53 expression, which is commonly observed in colon cancer, may not limit the potential of rTRAIL as a therapeutic agent in the treatment of this disease.

It has been shown previously that various chemotherapeutic agents can induce up-regulation of DR4 and DR5 expression (28, 31, 38). However, we found that chemotherapy treatment had little effect on DR4 and DR5 mRNA levels in either HCT116 p53^{+/+} and p53^{-/-} cell line, and more significantly, we found that 5-FU, oxaliplatin, or CPT-11 did not modulate DR4 or DR5 cell surface expression following drug treatment in any of the cell lines examined. Furthermore, expression of the TRAIL decoy receptors DcR1 and DcR2 was not affected by chemotherapy treatment. We conclude that modulation of TRAIL receptor expression in response to chemotherapy is not the mechanism by which these colon cancer cell lines are sensitized to rTRAIL.

c-FLIP is a well-documented negative regulator of death receptor-induced apoptosis, the expression of which has been shown to correlate with TRAIL resistance in several types of cancer (17, 20, 39). We found that expression of c-FLIP was lowest in the most TRAIL-sensitive cell line (HCT116 p53^{+/+}) and that c-FLIP_S expression was highest in the two most TRAIL-resistant cell lines (LoVo and HT29). These findings suggested that c-FLIP expression may play a role in determining TRAIL sensitivity in colon cancer cells, so we examined the effects of chemotherapy

treatment on c-FLIP expression. We found that chemotherapy treatment down-regulated c-FLIP_L and/or c-FLIP_S protein expression in each cell line. The antiapoptotic role of c-FLIP in regulating TRAIL-mediated apoptosis in colon cancer cells was clearly shown using siRNA methodology. Specific down-regulation of c-FLIP_L significantly enhanced the sensitivity of the colon cancer cell lines to rTRAIL, whereas specific down-regulation of c-FLIP_S was less effective in sensitizing these cells to rTRAIL. However, with the exception of H630 cells, the levels of apoptosis in cells cotreated with c-FLIP-targeted siRNA and rTRAIL did not reach the levels observed in chemotherapy/rTRAIL-treated cells, suggesting that chemotherapy treatment can also enhance rTRAIL sensitivity by mechanisms other than c-FLIP down-regulation. Our results are similar to those of Sharp et al., who found that down-regulating c-FLIP with siRNA sensitized several cancer cell lines to TRAIL-mediated cell death (40). In their study, one cell line (A549 lung cancer) was more highly sensitized to TRAIL when c-FLIP_L was knocked down, whereas others (U2OS osteosarcoma and human 293 embryonic kidney cells) were more highly sensitized to TRAIL when c-FLIP_S was knocked down and others (HeLa cervical carcinoma and H460 lung cancer) were equally sensitized to TRAIL by c-FLIP_L and c-FLIP_S down-regulation. In our study, down-regulation of c-FLIP_L consistently resulted in more potent sensitization to rTRAIL than down-regulation of c-FLIP_S, suggesting that c-FLIP_L is the more important regulator of TRAIL-mediated apoptosis in colon cancer cells. This was further supported by data from c-FLIP-overexpressing cell lines, as overexpression of c-FLIP_L, but not c-FLIP_S, abolished the sensitivity of the cells to both rTRAIL alone and TRAIL in combination with chemotherapy. Of note,

the level of c-FLIP_L overexpression in the HFL17 cell line (~4-fold) is similar to the level of overexpression observed by Ryu et al. in colonic tumors (21). Collectively, these studies suggest that c-FLIP_L is an important regulator of colon cancer cell death in response to rTRAIL alone and in combination with chemotherapy.

Our results suggest that down-regulation of c-FLIP expression in response to chemotherapy is at least partially responsible for the increased sensitivity of colon cancer cells to rTRAIL-mediated apoptosis. Interestingly, we found that a 5-FU-resistant cell line was also sensitized to rTRAIL by 5-FU, and this correlated with 5-FU treatment causing down-regulation of c-FLIP expression in the resistant cell line. This suggests that chemotherapy-resistant colon cancer cells may still be sensitized to TRAIL-mediated apoptosis by chemotherapy. Given the problems of drug resistance in cancer therapy, this result is highly clinically relevant.

Among the death ligands, TRAIL is showing the most promise as a novel cancer therapy mainly due to its apparent tumor specificity and lack of liver toxicity (41). Agonistic antibodies targeting either DR4 or DR5 are also in development and are showing promise as potential therapeutic agents in the treatment of cancer (42). However, many cell lines derived from a variety of tumor types show resistance to TRAIL-mediated apoptosis, thus limiting the efficacy of TRAIL-based treatment regimens. To date, the basis of TRAIL resistance is not fully elucidated. In this study, we have shown that the differential sensitivity to rTRAIL as a single agent in our panel of colon carcinoma cell lines did not correlate with p53 status. Furthermore, cotreatment with chemotherapy was found to enhance rTRAIL-induced apoptosis in p53 WT, null, and mutant colon cancer cell lines, and this was not due to changes in TRAIL receptor expression following chemotherapy. However, chemotherapy was found to down-regulate expression of the caspase-8 inhibitor c-FLIP. Furthermore, we showed that overexpression of c-FLIP_L protected colon cancer cells against apoptosis induced by rTRAIL alone and in the presence of chemotherapy. This is an important observation given in the study by Ryu et al., who found that c-FLIP_L is commonly overexpressed in colon cancer (21). Most significantly, down-regulation of c-FLIP_L sensitized colon cancer cells to rTRAIL-induced apoptosis. In conclusion, we present evidence that agents that directly or indirectly inhibit or down-regulate c-FLIP_L may be important accessory agents for the optimal use of TRAIL and TRAIL receptor-targeted antibodies as therapeutic agents in colorectal cancer.

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