Combination Treatment with TRA-8 Anti–Death Receptor 5 Antibody and CPT-11 Induces Tumor Regression in an Orthotopic Model of Pancreatic Cancer

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Abstract

**Purpose:** Evaluate the response of human pancreatic cancer cell lines and orthotopic tumors to TRA-8, an agonistic antibody to death receptor 5, in combination with irinotecan (CPT-11).

**Experimental Design:** MIAPaCa-2 and S2VP10 cells were treated with TRA-8 and/or CPT-11. Cell viability was determined by ATP assay. JC-1 mitochondrial depolarization and Annexin V assays confirmed cell death by apoptosis. Immunoblotting was used to evaluate protein changes. MIAPaCa-2 cells were injected into the pancreas of severe combined immunodeficient mice. Mice underwent abdominal ultrasound to quantitate tumor size before and after treatment with twice weekly injections of 200 μg TRA-8 and/or 25 mg/kg CPT-11 for one or two treatment cycles, each lasting 2 weeks.

**Results:** MIAPaCa-2 cells were more sensitive to TRA-8 and showed additive cytotoxicity, whereas S2VP10 cells showed synergistic cytotoxicity when treated with TRA-8 and CPT-11. Cell death occurred via apoptosis with increased cleavage of caspase-3, caspase-8, and caspase-9 and proapoptotic proteins Bid and poly(ADP)ribose polymerase after combination treatment compared with either agent alone. XIAP and Bcl-XL inhibitors of apoptosis were down-regulated. After a single cycle of *in vivo* combination therapy, tumor sizes had diminished significantly (*P* < 0.001) at 8 days posttreatment compared with no treatment, CPT-11, and TRA-8, and there was a 50-day increase in survival with combination treatment over untreated controls (*P* = 0.0002), 30 days over TRA-8, and a 36-day increase over CPT-11 monotherapy (*P* = 0.0003). With two cycles of TRA-8/CPT-11 treatment, mean survival time increased significantly (*P* < 0.001) to 169 days versus untreated controls, TRA-8 or CPT-11 (76, 121, or 108 days, respectively).

**Conclusions:** Combination TRA-8 and CPT-11 therapy produced enhanced cytotoxicity and survival in the MIAPaCa-2 orthotopic model of pancreatic cancer.

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Adenocarcinoma of the pancreas remains a deadly disease. Although it is the 10th most common cancer with 33,730 cases expected in 2006, it is the fourth most deadly cancer with 32,300 expected deaths (1). Based on these statistics, it is clear that new therapies for pancreatic cancer are needed. Gemcitabine has become the primary agent for pancreatic cancer. Although showing an improvement in clinical benefit response compared with 5-fluorouracil, the increase in median survival was small, although significant (5.65 versus 4.41 months) as was the increase in 12-month survival (18% versus 2%; ref. 2, 3). There have been many trials comparing combination therapy of gemcitabine and other traditional chemotherapeutic agents and biologicals to gemcitabine alone with minimal improvement in survival, if any (4–7). One of the agents that has received significant attention is irinotecan (CPT-11 or Camptosar), a camptothecin derivative that produces antitumor effects by DNA topoisomerase I inhibition. Early trials of CPT-11 in the 1990s in patients with advanced pancreatic cancer showed a median survival of 5.2 months (8). Differing mechanisms of action and reports of synergistic responses *in vitro* led to several studies examining combination therapy of gemcitabine and CPT-11 (9). As gemcitabine was established as the primary agent for pancreatic cancer, there have been trials examining combination therapy of CPT-11 and gemcitabine versus gemcitabine alone, but not those examining single-agent CPT-11 therapy. A phase II trial of CPT-11 in combination with gemcitabine produced a median survival of 7 months with an objective response rate of 25% (9). However, a phase III randomized multicenter study evaluating gemcitabine monotherapy compared with gemcitabine/CPT-11 combination therapy (IRINOGENM) failed to produce an increase in survival (6.6 versus 6.3 months,
respectively), although tumor response rates were improved (4.4% versus 16.1%; ref. 5). Based on these studies, there has been interest in using CPT-11 in combination with other agents in the treatment of pancreatic cancer.

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) has received considerable attention as a chemosensitizing agent, and there has been a large body of preclinical research describing its effects in many different cancer types. TRAIL has been shown to induce apoptosis in a variety of cell types through binding of death receptors (10–12). There are four cell membrane–bound receptors and one circulating receptor (osteoprotegerin) for TRAIL. Two of these receptors, death receptor 4 (DR4) and DR5, trigger apoptosis when bound by TRAIL. These receptors contain a cytoplasmic death domain required for intracellular signal transduction (13–15). There are two decoy receptors, decoy receptor-1 (DcR1), which lacks a cytoplasmic tail, and DcR2, which has a truncated intracellular death domain, neither of which induce apoptosis (13, 16–18). Decoy receptors are thought to provide resistance to TRAIL-mediated apoptosis either through competition for TRAIL binding or complexing with the death receptors (16, 17, 19). Osteoprotegerin is a fifth soluble receptor for TRAIL found to block TRAIL-mediated apoptosis in vivo (20).

Early recombinant forms of TRAIL were found to induce apoptosis in normal human hepatocytes, thus tempering the enthusiasm for TRAIL as an anticancer agent (21), although later forms of recombinant TRAIL avoided this toxicity (22, 23). There have been several reports of the development of apoptosis-inducing anti-DR4 and anti-DR5 agonistic monoclonal antibodies (24–26). Differential expression of DR5 or DR4 by tumor cells compared with normal cells has been an area of intense interest in TRAIL/death receptor–based therapy. Ozawa et al. (27) evaluated normal and malignant pancreatic tissue and found that DR4 mRNA transcripts were present only in pancreatic cancer cells. DR5 mRNA and protein expression were detected in both malignant and normal tissue, although the level in cancer tissue was increased 5.1-fold. Satoh et al. (28) identified DR5 receptors in 15 of 17 human pancreatic cancer samples and DR4 receptors in 14 of 17 samples.

Galligan et al. (29–32) showed enhanced p53-independent apoptosis in human colon cancer cell lines treated with CPT-11 and TRAIL, compared with either agent alone. Furthermore, other investigators showed enhanced antitumor effects after combination treatment with TRAIL and CPT-11 against colon cancer xenografts as well as in prostate cancer mouse models (31, 33). Naka et al. (34) reported that flank xenografts derived from human colon tumors showed tumor regression when treated with a combination of TRAIL and CPT-11. Based on these encouraging findings with a combination of TRAIL and CPT-11 in colon and prostate models, this study examines the effects of combination therapy with CPT-11 and an agonistic anti-DR5 monoclonal antibody both in vitro and in an in vivo orthotopic model of pancreatic cancer.

Our group previously described the in vitro and in vivo evaluation of TRA-8 anti-DR5 monoclonal antibody in combination with gemcitabine in the treatment of s.c. pancreatic cancer xenografts (35). TRA-8 has been shown to have specificity only for DR5 and induces apoptosis in a variety of tumor cell lines without in vitro hepatotoxicity (29). This study investigates combination treatment with CPT-11 and TRA-8 of two human pancreatic cancer cell lines, MIA PaCa-2 and S2VP10, in vitro and also in an ultrasound-verified orthotopic model of pancreatic cancer with monitoring of tumor size and survival of the study animals. The DR5 cell surface expression and response to TRA-8 treatment of the cell lines have been previously described (35). These cell lines have been chosen as representative TRA-8–sensitive (MIA PaCa-2) and TRA-8–resistant (S2VP10) cell lines. The use of ultrasound imaging allows for tumor verification before initiation of treatment, and quantification of tumor size before, during, and after treatment. Furthermore, the use of an orthotopic model provides a more accurate representation of human pancreatic cancer than a s.c. model.

Materials and Methods

**Cell lines and reagents.** The human pancreatic cancer cell line MIA PaCa-2 was obtained from the American Type Culture Collection. Human pancreatic cell line S2VP10 was a gift from Dr. M. Hollingsworth (University of Nebraska, Omaha, NE; ref. 36). MIA PaCa-2 and S2VP10 cells were cultured in DMEM (Mediatech, Inc.) with 10% fetal bovine serum (Hyclone). Mouse monoclonal antibody TRA-8 was prepared by Dr. Tong Zhou (University of Alabama, Birmingham, AL) or by Sankyo Co. Ltd. Iriotocan hydrochloride (Pharmacia and Upjohn Company) was purchased from the University of Alabama at Birmingham Hospital pharmacy. Radioimmunoprecipitation assay buffer was used to prepare cell lysates [150 mmol/L NaCl, 0.5 mol/L Tris (pH 7.4), 1 g/mL sodium deoxycholate, 1% Triton X-100, and 0.1% SDS].

**In vitro cell viability assay.** To examine the effects of TRA-8 alone and in combination with CPT-11, MIA PaCa-2 and S2VP10 cells were trypsinized and plated at 1,000 per well in Costar 96-well culture plates, in their respective medium with 100 IU penicillin and 100 μg/mL streptomycin (Mediatech). Cells were incubated overnight at 37°C then treated with CPT-11 (0-30 μmol/L) for 24 h followed by TRA-8 (0-1,000 ng/mL) for 24 h. Cell viability was determined 24 h after adding TRA-8 using the ATPlite Luminescence Assay System (PerkinElmer) and luminescence was read on a TopCount plate reader (Packard Instruments). ATP-based bioluminescence assays have been validated as a tool to evaluate cell viability after drug administration (37, 38). Data points are the mean of 6 to 11 independent experiments, and each experiment included quadruplicate wells for each combination. Data are expressed as a percentage of untreated controls ± SE. Isotype control IgG1 antibody (Southern Biotechnology Associates) had no effect on cell viability.

A nonlinear model, \( y = \min + \frac{\max - \min}{1 + \left(\frac{A}{x}\right)^{\beta}} \), was applied to calculate IC_{50} (39, 40), where \( y \) is the response, the variable \( A \) represents IC_{50}, the variable \( x \) is used to scale concentration for proper transformation, and \( \min \) and \( \max \) represent the minimum and the maximum of response. A SAS procedure NLIN was used for the computation (SAS Institute, Inc.). The in vitro cytotoxicity data were assessed to determine if the treatment combinations had additive, less than additive (antagonistic), or greater than additive (synergistic) cytotoxic effects. The dose-response relationships were modeled using a second-order response surface model with linear, quadratic, and interaction terms (41).
control, was added 24 h after CPT-11 addition. Two hours after TRA-8 addition, cells were stained with Annexin V–FITC and PI using Annexin V–FITC Apoptosis Detection kit (BioVision, Inc.) according to the manufacturer’s protocol. Cells were analyzed using flow cytometry (FACScan, Becton Dickinson) and CellQuest software (Becton Dickinson). Gating was selected using untreated cells to separate the majority of double-negative cells from minor populations of PI or Annexin V–FITC–positive cells. Cells that were FITC+/PI were considered viable, FITC+/PI− cells were considered early apoptotic, and FITC−/PI+ cells were considered nonviable. Data are presented as a percentage of gated events ± SD. ANOVA was applied to compare apoptosis between treatment groups at a significance level of 0.05. Student-Newman-Keuls test was used for subcomparisons to control the type I experiment-wise error rate.

Analysis of mitochondrial membrane destabilization. Intact mitochondrial membranes allow accumulation of JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrachlorobenzimidazolylcarboxylic acid iodide) dye in the mitochondria where it will fluoresce red (42, 43) when a critical concentration is achieved. As loss of mitochondrial membrane potential (ΔΨm) occurs, JC-1 dye cannot accumulate in the mitochondria and will remain as a monomer in the cytosol and fluoresce green. MIA PaCa-2 and S2VP10 cells were plated at 3 × 105 per well in DMEM/10% fetal bovine serum, incubated overnight at 37°C, and then treated with 30 μmol/L CPT-11 or medium alone as a negative control. After 24 h of drug treatment, 300 ng/mL TRA-8 was added. Twenty-four hours after TRA-8 addition, cells were harvested and aliquots of 5 × 105 cells were stained using JC-1 Mitochondrial Membrane Potential Detection Kit (Cell Technology) according to the manufacturer’s protocol and analyzed using flow cytometry as described above.

Western blot analysis of apoptosis regulatory proteins. MIA PaCa-2 and S2VP10 cells were plated at 5 × 105 per well in DMEM/10% fetal bovine serum and incubated overnight. Cells were then treated with CPT-11 (30 μmol/L for MIA PaCa-2 and 10 μmol/L for S2VP10) or 30 nmol/L genistein for 24 h. TRA-8 (25 mg/mL) was added and cells were incubated for an additional 3 h. Cells were then lysed using radioimmunoprecipitation assay buffer with 10 mmol/L sodium orthovanadate and 1:100 Protease Inhibitor Cocktail (Sigma) and sonicated on ice for 15 s × 2, then centrifuged at 14,000 rpm for 2 min. Protein concentrations were determined using a Lowry detergent compatible assay (Bio-Rad). Fifteen microgram of samples were analyzed by Western blotting using 0.45 μmol/L polyvinylidene difluoride membranes (Millipore), and antibodies to caspase-3, caspase-8, caspase-9, poly(ADP)ribose polymerase (BD PharMingen), XIAP, Bid (Cell Signaling Technologies), and actin (Sigma).

Orthotopic model of pancreatic cancer. Female severe combined immunodeficient (SCID) mice were anesthetized using i.p. injection of xylazine and lidocaine. The abdomens were shaved and prepped with xylazine and lidocaine. The abdomens were shaved and prepped with xylazine and lidocaine. A 1-cm incision was made in the left upper quadrant of the abdomen, and the pancreas was exposed by retraction of the spleen. A solution of 2.5 × 105 MIA PaCa-2 cells in 40 μL of DMEM was slowly injected into the pancreas using a 27-gauge needle. The needle was slowly removed and a sterile cotton tipped applicator was held over the injection site for 30 s to prevent leakage of the cell suspension into the abdomen. The spleen was then returned to the appropriate position in the abdomen, and the skin and peritoneum were closed in one layer with three interrupted 5-0 Prolene sutures. The animals were then placed on a warming blanket until they recovered from anesthesia. Once animals regained full mobility, they were placed in sterile cages and provided liquid acetaminophen for 24 h along with food and water ad libitum.

Orthotopic tumor growth inhibition and survival studies. MIA PaCa-2 orthotopic tumors were established in female SCID mice (National Cancer Institute-Frederick Animal Production Program, Frederick, MD) using the above-described protocol. Twenty-one days after tumor cell injection, each mouse underwent abdominal ultrasound examination using a VisualSonics VEVO 660 high-frequency, high-resolution ultrasound instrument with 40 and 30 mHz probes. The animals were anesthetized using isoflurane gas anesthesia and hair removal with depilatory cream. Animals were placed in the supine position for examination with brightness mode (B-Mode) imaging (30 frames/s; 20 nm field of view; ref. 44). The final 300 images (10 s) of the B-mode study was saved. The largest diameter was found in the anterior-posterior plane, and this diameter as well as a transverse diameter were measured to quantify tumor area (length × width). Animals were then stratified according to tumor size and assigned to study groups. In the first therapy study, animals were treated with one cycle of therapy lasting 12 days. The treatment groups (n = 10 mice per group) were as follows: untreated, CPT-11 alone (25 mg/kg i.v. on days 23, 27, 30, and 34), TRA-8 alone (200 μg i.p. on days 22, 26, 29, and 33 postimplant), or combination therapy of CPT-11 and TRA-8 using the same doses for each agent alone. Mice underwent repeat abdominal ultrasound examinations on postimplant days 41 and 83, and tumor measurements were made in two dimensions and surface areas were calculated. In the second therapy study, MIA PaCa-2 intrapancreatic tumors were established as described above and underwent two cycles of treatment. Twenty-four days after tumor cell injection, all mice underwent abdominal ultrasound imaging and were randomized according to tumor size. The treatment groups (n = 10 mice per group) were as follows: untreated, CPT-11 alone (25 mg/kg i.v. on days 26, 29, 33, 36 and 54, 57, 61, 64 postimplant), TRA-8 alone (200 μg i.p. on days 25, 28, 32, 35 and 53, 56, 60, 63 postimplant), or combination therapy with TRA-8 and CPT-11 with the same doses and regimens for each agent alone. Mice underwent additional abdominal ultrasound examinations on days 72 and 83.

Mean tumor areas were calculated for the four treatment groups in each therapy study and compared with those from the first ultrasound examination. A Kruskal-Wallis test was used to determine differences between the groups in terms of changes in tumor size after treatment. If the test was significant at the 0.05 level, the Wilcoxon rank sum test using normal approximation was applied for pairwise comparisons with the significance level adjusted for the multiple comparisons. Animals were followed for survival, with tumor being documented when mice died, or at time of sacrifice according to the University of Alabama at Birmingham Institutional Animal Care and Use Committee–approved protocol. A log rank test was used to determine differences in survival. The Kaplan-Meier method was used to generate the survival curves.

Results

Cell viability in vitro after treatment with CPT-11 and TRA-8. As shown in Fig. 1, MIA PaCa-2 cells showed sensitivity to TRA-8 with an IC50 <20 ng/mL. Pretreatment of cells with CPT-11 before TRA-8 addition yielded a greater than additive reduction in cell viability. For example, cells treated with 10 μmol/L CPT-11 alone showed a 26.2 ± 4.0% reduction in cell viability, whereas treatment with 5 ng/mL TRA-8 produced a 35.3 ± 7.0% reduction in cell viability. Combination treatment with 10 μmol/L CPT-11 and 5 ng/mL TRA-8 yielded a 75.7 ± 5.1% reduction in cell viability. The combination therapy provided an additive effect (P = 0.06). S2VP10, a TRA-8–resistant cell line (IC50 >1,000 ng/mL), as shown in Fig. 1, showed synergistic reduction (P < 0.0001) in cell viability with combination treatment. Treatment with 3 μmol/L CPT-11 produced a 43.3 ± 3.2% reduction in cell viability, whereas 25 ng/mL TRA-8 produced a 10.6 ± 3.5% reduction in cell viability. Combination therapy with these doses provided a 73.8 ± 7.5% reduction in cell viability, which was greater than the sum of the two agents alone (73.8% versus 53.9%). Similar results were obtained with S2VP10 cells using 10 μmol/L CPT-11, whereas
1 μmol/L CPT-11 produced slightly greater than additive killing in combination with 125 or 1,000 ng/mL TRA-8.

**Annexin V–FITC analysis of apoptosis in cells treated with CPT-11 and TRA-8.** Combination treatment of MIA PaCa-2 cells with CPT-11 and TRA-8 induced apoptosis to a greater extent than treatment with either agent alone. Treatment with 30 μmol/L CPT-11 and 300 ng/mL TRA-8 resulted in 82.2 ± 12.5% of cells staining FITC+/PI− (apoptotic) compared with CPT-11 alone (60.4 ± 13.7%, P < 0.01) or TRA-8 alone (29.3 ± 9.2%, P < 0.05) as shown in Fig. 2. Treatment of S2VP10 cells with 10 μmol/L CPT-11 and 125 ng/mL TRA-8 produced enhanced apoptosis greater than either agent alone. Combination therapy yielded 67.8 ± 3.8% of cells staining FITC+/PI− (apoptotic) compared with TRA-8 alone (17.9 ± 1.3%, P < 0.001) or CPT-11 alone (10.2 ± 0.13%, P < 0.001).

**Effect of CPT-11 and TRA-8 on mitochondrial membrane destabilization.** JC-1 assay results showed that combination treatment reduced the mitochondrial membrane potential of MIA PaCa-2 cells compared with either agent alone. As shown in Fig. 3, combination treatment enhanced JC-1 green fluorescence, indicating loss of mitochondrial membrane potential, compared with either single agent. Similar results were found in S2VP10 cells.

**Western blot analysis of apoptosis regulatory proteins.** As shown in Fig. 4, caspase-3, caspase-8, and caspase-9 cleavage was greater in MIA PaCa-2 and S2VP10 cells treated with a combination of CPT-11 and TRA-8 than in cells treated with CPT-11 or TRA-8 alone. Consistent with these results, Bid, a caspase-8 substrate, and poly(ADP)ribose polymerase, a caspase-3 substrate, also showed enhanced cleavage with combination.
Combination treatment with CPT-11 and TRA-8 produced a greater reduction in XIAP in S2VP10 cells receiving combination therapy than with either agent alone. There was a greater reduction in XIAP in S2VP10 cells treated with CPT-11 but not in cells treated with TRA-8. There was a greater reduction in XIAP in S2VP10 cells treated with TRA-8–treated MIA PaCa-2 cells, although to a lesser extent in cells treated with CPT-11 or combination treatment. No appreciable change was observed in Bcl-XL in S2VP10 cells.

**Effect of CPT-11 and TRA-8 on orthotopic tumor growth and survival.** In the first study with a single cycle of therapy, SCID mice were assigned to treatment groups after stratification based on ultrasound measurements of intrapancreatic tumors on day 21 posttumor cell injection. The mean pretreatment tumor sizes were untreated 32.3 ± 18.1 mm², TRA-8 39.1 ± 19.4 mm², CPT-11 34.7 ± 20.5 mm², and combination treatment 37.8 ± 16.7 mm². There was no statistical difference between mean pretreatment tumor sizes \((P = 0.89)\) among the different treatment groups. The second ultrasound measurement done 41 days postimplantation (8 days after completion of treatment) showed tumor growth in both untreated and CPT-11–treated mice [an average of 74.2% increase in untreated animals and 47% increase \((P = 0.0022)\) compared with untreated] in CPT-11–treated animals. The increase in mean tumor size is shown in Fig. 5. The TRA-8–treated group showed a 3.7% reduction \((P = 0.0029)\) compared with untreated) in mean tumor size, whereas the group receiving combination treatment showed a 90% reduction \((P = 0.0002, 0.0003, \text{and } 0.0019)\) compared with untreated, CPT-11, and TRA-8, respectively) in tumor size. The third abdominal ultrasound examination on day 83 showed growth in all treatment groups, although the mean tumor size in the combination treatment group was substantially lower than mean size in the other treatment groups (Fig. 5). Notably, all mice in the combination therapy group were alive at day 83, whereas only 3 of 10 mice were alive in the untreated group and 4 of 10 mice were alive in the individual CPT-11 or TRA-8 treatment groups. Survival was also followed among the treatment groups as shown in Fig. 6. Mean survival was 70.1 ± 6.2 days in untreated mice, 84.3 ± 3.8 days in mice treated with CPT-11 alone, 90.4 ± 8.2 days in mice treated with TRA-8, and 120.6 ± 6.6 days in those receiving combination therapy. There was no significant difference in survival between CPT-11 alone and untreated groups \((P = 0.310)\); however, combination therapy produced an increase in survival compared with untreated controls \((P = 0.0002)\). Combination therapy increased survival compared with CPT-11 only \((P = 0.0003)\); however, this increase in survival was not significant compared with TRA-8 alone \((P = 0.1132)\).

In the second therapy study, SCID mice with MIA PaCa-2 intrapancreatic tumors were treated with two cycles of TRA-8, CPT-11, or combination therapy. Mice underwent ultrasound imaging on posttumor cell injection day 24 (Fig. 7). The mean pretreatment tumor sizes on day 24 were as follows: untreated 47.7 ± 17.3 mm², TRA-8 alone 48.0 ± 20.3 mm², CPT-11 alone 47.9 ± 17.7 mm², and TRA-8 + CPT-11 47.8 ± 17.3 mm², which were similar among treatment groups \((P = 1.00)\). The first cycle of treatment was initiated on day 25 and was completed on day 35, and the second cycle of treatment started on day 53 and was completed on day 63. The treatments were 200 µg TRA-8 or 25 mg/kg CPT-11, both given twice weekly for 2 weeks per cycle, or a combination of TRA-8 and CPT-11. Mice underwent abdominal ultrasound imaging on postimplant days 24, 73, and 83 to evaluate orthotopic tumor response to treatment. The mean size of tumors on the days of imaging are shown in Fig. 7. The untreated tumors increased from 47.7 mm² on day 25 to 204.6 and 246.0 mm² on days 73 and 83, respectively. TRA-8–treated tumors showed a corresponding increase from 48.0 ± 3.4, 83.1 mm², whereas CPT-11–treated tumors increased from 47.9 to 102.1 and 121.3 mm². Animals treated with combination therapy had tumors that changed from 47.8 mm² on day 25 to 9.8 and 20.9 mm² on days 73 and 83, respectively. Thus, combination treatment produced a significant reduction in tumor growth compared with the other treatment groups \((P = 0.0014, 0.0002, \text{and } 0.0013)\) compared with untreated, CPT-11, and TRA-8, respectively, for percentage change from day 25 to day 73; and \(P = 0.0002\) and 0.0008 compared with CPT-11 and TRA-8, respectively, for percentage change from day 25 to day 83. Mean survival times (Fig. 8) were 76.1 ± 3.4, 108.4 ± 4.2, 121.1 ± 3.5, and 169.2 ± 4.2 days in the untreated, CPT-11, TRA-8, and TRA-8 + CPT-11 treatment groups, respectively. There was a significant difference in survival between CPT-11 and TRA-8 alone and untreated groups of mice \((P < 0.001)\), whereas combination therapy produced an increase in
in survival compared with CPT-11 or TRA-8 alone \( (P < 0.0001) \). The difference between CPT-11 and TRA-8 was not significant \( (P = 0.0525) \).

**Discussion**

Although not advantageous in combination with current gemcitabine therapy, CPT-11 offers intriguing potential for combination with other drugs in the pursuit of more effective pancreatic cancer treatments. One therapeutic mechanism augmented by CPT-11 treatment is apoptosis (45). We have shown that the TRA-8 anti-DR5 antibody induces apoptosis in a variety of human tumor cells (46–48). In the present study, pretreatment of pancreatic cancer cells in vitro with CPT-11 enhanced apoptosis in combination with TRA-8 treatment. The additive combination cytotoxicity in MIA PaCa-2 cells (TRA-8 sensitive) and synergistic effect in S2VP10 cells (TRA-8 resistant) suggest that CPT-11 not only enhances the apoptotic effect of TRA-8 but is also active in overcoming TRA-8 resistance in pancreatic cancer cell lines. Additionally, JC-1 and Annexin V data confirm that this tumor cell killing occurred via apoptosis with loss of mitochondrial membrane potential.

Two main pathways of TRAIL-mediated apoptosis have been described (47). The intrinsic pathway is initiated when a DNA-damaging cellular event, such as chemotherapy, activates the caspase cascade with cytochrome c release from mitochondria. The extrinsic or extracellular pathway begins with the extracellular binding of ligands to one of the tumor necrosis factor family of cytokine receptors. Signal transduction is then relayed to an intracellular sequence of upstream caspases (caspase-8, caspase-9, caspase-10, and caspase-2) triggering the activation of downstream caspases (caspase-3, caspase-6, and caspase-7) responsible for the final execution of the apoptotic signal (49). In the current study, Western blot analysis of TRA-8 and CPT-11 combination therapy in both MIA PaCa-2 and S2VP10 cell lines confirmed activation of the apoptosis cascade resulting in cleavage of caspase-3 and poly(ADP)ribose polymerase. The extrinsic pathway was also clearly activated as shown by caspase-8 cleavage. Combination therapy also showed evidence of intrinsic apoptosis with caspase-9 and Bid cleavage. Prior studies have shown that Bid causes oligomerization of Bax and Bak stimulating release of cytochrome c and Smac/DIABLO, which activates the intrinsic pathway via caspase-9 (50, 51).

![Fig. 4. Western blot analysis of apoptosis regulatory proteins in MIA PaCa-2 and S2VP10 cells treated with CPT-11, gemcitabine, TRA-8, or the combination of TRA-8 and the chemotherapy drugs. Cells were treated with CPT-11 (10 or 300 \( \mu \)mol/L for S2VP10 and MIA PaCa-2, respectively) or 30 \( \mu \)mol/L gemcitabine for 24 h. Cells were then treated with 24 \( ng/mL \) TRA-8 for 3 h. Untreated cells were used as controls. Equivalent amounts of protein were analyzed by Western blot and actin levels were compared with control for protein loading (not shown). Combination treatment produced enhanced caspase-3, caspase-8, and caspase-9 activation as shown by cleavage of their proforms. Bid, a caspase-8 substrate, and poly(ADP)ribose polymerase, a caspase-3 substrate, also showed enhanced cleavage. Bcl-XL was down-regulated in MIA PaCa-2 cells receiving CPT-11 and combination treatment. XIAP was down-regulated in S2VP10 cells receiving combination treatment with TRA-8 and CPT-11.](image-url)
In this study, TRA-8 and CPT-11 combination treatment enhanced apoptosis in conjunction with down-regulation of the antiapoptotic proteins Bcl-XL and XIAP. Bcl-XL is a Bcl-2 family inhibitor of apoptosis that functions to sequester its proapoptotic Bcl-2 family relatives Bax and Bak (52–55). Both Bcl-XL and XIAP have been previously shown to be inhibited by CPT-11 through the signal transducers and activators of transcription/Janus-activated kinase-dependent pathway in colon cancer cells (31). Bcl-XL was weakly down-regulated by TRA-8 and reduced to a greater extent by CPT-11 or combination therapy in the TRA-8-sensitive MIA PaCa-2 cell line. Little change in Bcl-XL occurred in TRA-8-resistant S2VP10 cells. XIAP inhibits caspases when up-regulated by signal transducers and activators

**Fig. 5.** Intrapancreatic MIA PaCa-2 tumor growth measured by ultrasound imaging. The mean tumor size in each treatment group were equivalent before initiation of treatment (day 21). Combination therapy with one cycle of CPT-11 and TRA-8 produced a 90.2% mean decrease in tumor size at day 41, whereas there was no change in mean tumor size in the group treated with TRA-8 alone. At day 83 after tumor cell injection, the group treated with CPT-11 and TRA-8 showed the least amount of tumor growth.

**Fig. 6.** Survival study comparing SCID mice with intrapancreatic MIA PaCa-2 tumors treated with one cycle of therapy with CPT-11 (25 mg/kg i.p. on days 15, 19, 22, and 26 postimplant), TRA-8 (200 μg i.p. on days 14, 18, 21, and 25 postimplant), or the combination of CPT-11 and TRA-8. Mean survival was significantly increased in the combination therapy group. Arrows, start and end of treatment.

**Fig. 7.** Ultrasound imaging of SCID mice with orthotopic MIA PaCa-2 tumors and two cycles of therapy. Mice underwent ultrasound imaging with measurements of tumor size on days 24, 73, and 83. They were treated with one cycle of 200 μg TRA-8 i.p. on days 25, 28, 32, and 35 and a second cycle on days 53, 56, 60, and 63; one cycle of 25 mg/kg CPT-11 i.p. on days 26, 29, 33, 36 and a second cycle on days 54, 57, 61, and 64; or a combination of TRA-8 and CPT-11 with the same dose and schedule as either agent alone.
of transcription 3/5 (52–55, 56–60). XIAP down-regulation in the TRA-8–resistant cell line S2VP10 occurred with combination therapy, indicating that CPT-11 may be important in overcoming this antiapoptotic signal and decreasing TRA-8 resistance. Significant XIAP down-regulation did not occur in the TRA-8–sensitive cell line MIA-PaCa-2.

Combination therapy showed dramatic initial tumor regression and delayed tumor growth in the MIA PaCa-2 orthotopic model when compared with untreated controls and monotherapy. Prior studies have shown a similar response to human DR4 monoclonal antibody (HGS-ETRI) and CPT-11 in the SU.86.86 athymic nude mouse flank model (61) and TRAIL/CPT-11 combination treatment in colon cancer athymic nude mouse flank models (31). However, the orthotopic model is a more accurate physiologic model for parenchymal and metastatic tumor invasion as well as pharmacokinetic drug delivery (62–66). Our previous work showed a significant improvement with combination TRA-8 and gemcitabine treatment of s.c. MIA PaCa-2 xenografts versus monotherapy for single-cycle treatment (35, 67). However, to more closely resemble the clinical efficacy and toxicity of multiple cycles of CPT-11 (35, 67), two 10-day cycles of combination treatment were used in this study. Mean survival time increased substantially versus monotherapy after two cycles of combination treatment.

Several limitations must be acknowledged. First, TRA-8 is not a human antibody and thus has theoretical potential for immunogenicity seen with other murine antibodies when used in humans. However, it has been humanized and is being investigated in an ongoing clinical trial. Second, whereas the TRA-8–resistant cell line S2VP10 showed increased sensitivity to TRA-8 when pretreated with CPT-11, \textit{in vivo} studies (data not shown) have not produced a substantial survival benefit with this cell line (35, 67). Third, complete profiling of these cell lines was not done with DNA microarray or two-dimensional gels to determine whether multiple other resistance mechanisms were present.

In conclusion, \textit{in vitro} combination treatment with CPT-11 and TRA-8 produced additive cell killing and enhanced apoptosis in the TRA-8–sensitive cell line MIA-PaCa-2. In the TRA-8–resistant cell line S2VP10, synergistic killing occurred using combination treatment, and down-regulation of XIAP may have contributed to the reversal of TRA-8 resistance. Furthermore, \textit{in vivo} data showed a dramatic reduction in MIA PaCa-2 tumor burden and increase in mean survival time for those mice treated with combination therapy. This method could be applied to individual patient tumors to profile TRA-8 sensitivity with the intent of customizing patient specific, cost-effective, and optimally efficacious combination therapy.

References

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Combination Treatment with TRA-8 Anti–Death Receptor 5 Antibody and CPT-11 Induces Tumor Regression in an Orthotopic Model of Pancreatic Cancer

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