

# TRA-8 anti-DR5 monoclonal antibody and gemcitabine induce apoptosis and inhibit radiologically validated orthotopic pancreatic tumor growth

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## Abstract

**Purpose:** To evaluate agonistic TRA-8 monoclonal antibody to human death receptor 5 (DR5) and gemcitabine *in vitro* and in an orthotopic pancreatic cancer model. **Experimental Design:** Pancreatic cancer cell lines were screened for DR5 expression, cytotoxicity, and apoptosis induced by TRA-8, gemcitabine, or gemcitabine and TRA-8. An orthotopic model of pancreatic cancer was established in severe combined immunodeficient mice. Mice were treated with TRA-8, gemcitabine, or a combination for one or two cycles of therapy. Tumor growth (ultrasound) and survival were analyzed. **Results:** All five pancreatic cancer cell lines showed DR5 protein expression and varying sensitivity to TRA-8-mediated cytotoxicity. MIA PaCa-2 cells were very sensitive to TRA-8, moderately resistant to gemcitabine, with additive cytotoxicity to the combination. S2-VP10 cells were resistant to TRA-8 and sensitive to gemcitabine with synergistic sensitivity to the combination. Combination treatment *in vitro* produced enhanced caspase-3 and caspase-8 activation. A single cycle of therapy produced comparable efficacy for single-agent TRA-8 and the combination of TRA-8 and gemcitabine, with signifi-

cant reduction in tumor size and prolonged survival compared with gemcitabine alone or control animals. With two cycles of therapy, TRA-8 and combination therapy produced enhanced inhibition of tumor growth compared with single-agent gemcitabine or untreated animals. However, the combination regimen showed enhanced survival as compared with single-agent TRA-8. **Conclusions:** Pancreatic cancer cell lines express varying levels of DR5 and differ in their sensitivity to TRA-8 and gemcitabine-induced cytotoxicity. TRA-8 with two cycles of gemcitabine therapy produced the best overall survival. [Mol Cancer Ther 2007;6(12):3198–207]

## Introduction

Pancreatic adenocarcinoma remains a deadly cancer, with an estimated incidence of 37,170 cases causing 33,370 deaths in the United States in 2007 (1). Surgical resection provides the only chance for cure; however, 5-year actual survival rates following resection range from 10% to 19% (2–4). Furthermore, only 15% to 20% of patients are resectable at the time of presentation. Equally disappointing are outcomes following chemotherapy in the setting of metastatic disease. Currently, gemcitabine is the first-line agent in the treatment of metastatic pancreatic cancer. Multiple studies have evaluated the efficacy of gemcitabine as monotherapy or combination therapy in the treatment of unresectable and/or metastatic pancreatic cancer. An early study of gemcitabine in advanced pancreatic cancer showed a measurable response in 23.8% of patients with median survival of 5.7 months and only 18% survival at 12 months (5). Combination therapies, including gemcitabine, have been associated with minimal improvement when compared with gemcitabine alone (6–9). Given these outcomes with currently available chemotherapeutic agents, interest has shifted to novel molecularly targeted agents. For example, gemcitabine, in combination with bevacizumab, an antivascular endothelial growth factor monoclonal antibody, or erlotinib (a small-molecule tyrosine-kinase inhibitor) produced some enhancement of efficacy in pancreatic cancer patients (10, 11).

An alternative strategy could include death receptor (DR)-targeted therapy. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis in several cancer cell lines (12, 13) via tumor cell surface receptors that bind TRAIL. Two of these receptors, DR4 and DR5 trigger apoptosis when bound by TRAIL. These receptors contain a cytoplasmic death domain required for intracellular signal transduction (14–16).

Upon binding of TRAIL to either DR4 or DR5, a cascade of protein activation occurs, leading to initiator (caspase-8 and caspase-10) and effector (caspase-3) caspase activation

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**Note:** T. Zhou, A.F. LoBuglio, and D.J. Buchsbaum have intellectual property related to the TRA-8 anti-DR5 antibody.

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(16–20). Although this extrinsic, ligand-dependent pathway is sufficient for apoptosis in a variety of tumor cell lines, there is another pathway of apoptosis that is activated after exposure to chemotherapeutic agents or radiation. Stimulation of this intrinsic pathway causes the destabilization of the mitochondrial membrane with the release of cytochrome *c*, leading to caspase-3 activation (16, 21–24).

Analysis of normal human and cancer cell lines showed differential expression of DR4 and DR5 by cancer cells, with minimal expression in normal cell lines (25). This has been shown in pancreatic malignancies as well. DR5 mRNA expression was detected in five of five specimens of normal pancreatic tissue and 15 of 17 resected pancreatic ductal adenocarcinomas specimens (26). Ozawa et al. 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 (27). There have been several studies evaluating the combined effects of TRAIL and gemcitabine. Xu et al. (28) reported synergistic cytotoxic effects with TRAIL and gemcitabine in six pancreatic cell lines. Hylander et al. (29) showed that human pancreatic tumor xenografts in severe combined immunodeficient (SCID) mice had heterogeneous responses to TRAIL, but combination treatment with gemcitabine inhibited tumor growth to a greater extent than with either agent alone.

An alternative strategy is the use of agonistic monoclonal antibodies to DR4 or DR5 (30). Ichikawa et al. developed TRA-8, a mouse monoclonal antibody that specifically binds to DR5, which induced apoptosis of most tumor cell lines *in vitro*, with tumoricidal effects *in vivo* (25). Additional studies verified the selective binding of TRA-8 to DR5 without binding to DR4 or decoy receptors (25). This antibody has potent cytotoxicity in multiple tumor cell lines, and chemotherapy agents induce additive or synergistic enhancement of efficacy *in vitro* and *in vivo* (31).

The aim of this study was to investigate the effects of TRA-8 and the combination of TRA-8 plus gemcitabine on human pancreatic cancer cell lines *in vitro* and to evaluate efficacy in an orthotopic pancreatic cancer model that mimics biological properties of clinical pancreatic tumors (32–37).

## Materials and Methods

### Human Pancreatic Cell Lines and Reagents

MIA PaCa-2 and BxPC-3 were obtained from the American Type Culture Collection. Panc 2.03, was a gift from Dr. E. Jaffee (Johns Hopkins University, Baltimore, MD; ref. 38). S2-013 and S2-VP10 were a gift from Dr. M. Hollingsworth (University of Nebraska, Omaha, NE; ref. 39). MIA PaCa-2, BxPC-3, S2-013, and S2-VP10 cells were cultured in DMEM (Mediatech Inc.) with 10% fetal bovine serum (FBS, Hyclone). Panc 2.03 was grown in RPMI (Mediatech) supplemented with 0.1 mmol/L nonessential amino acids, 1 mmol/L

sodium pyruvate, 10 µg/mL human insulin, and 15% FBS. Murine monoclonal TRA-8 antibody was prepared by Dr. Tong Zhou (University of Alabama at Birmingham, Birmingham, AL) or by Sankyo Co. Ltd. (Tokyo, Japan). Gemcitabine (Eli Lilly and Company) was purchased from the University of Alabama at Birmingham Hospital Pharmacy.

### Expression of DR5 on Human Pancreatic Cancer Cell Lines and Tumors

Pancreatic cancer cells in exponential growth phase were washed with Dulbecco's PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> deficient) and harvested with Cell Stripper (Mediatech) for 15 to 20 min at 37°C. Cells were washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 1% bovine serum albumin and 0.01% sodium azide), resuspended in FACS buffer containing 1 mg/mL collagenase 11 (Sigma Chemical Co.), and incubated at 37°C for 15 min to obtain single cell suspensions. Cells were washed once, resuspended in FACS buffer (1 × 10<sup>5</sup> cells per reaction), and then incubated with TRA-8 (5 µg/mL) or an immunoglobulin G<sub>1</sub> (IgG<sub>1</sub>) isotype control antibody (Southern Biotechnology Associates) for 60 min at 4°C with shaking. Cells were washed once then incubated with phycoerythrin-conjugated goat anti-mouse IgG<sub>1</sub> (Southern Biotechnology Associates) for 20 min at 4°C with shaking. Cells were washed once, fixed with 1% paraformaldehyde for 15 min on ice, and examined using flow cytometry and Cell Quest software (FACScan, Becton Dickinson) to analyze DR5 expression. To investigate the effect of gemcitabine treatment on DR5 expression, cells were plated and treated 24 h later with 30 or 300 nmol/L gemcitabine for 24 h. Cells were harvested, and flow cytometry analysis was done. For immunocytochemistry, pancreatic cancer cells were harvested with trypsin/EDTA, plated on glass coverslips, and incubated for 24 h at 37°C. Cells were rinsed with PBS, then fixed in Pen-Fix for 15 min, washed with 70% ethanol, and stored in ethanol at 4°C. Four cases of pancreatic adenocarcinoma in paraffin sections and the pancreatic cell lines were stained for DR5 using TRA-8 at 5 µg/mL for cells and 10 µg/mL for tumors as previously described (40–42).

### *In vitro* Cell Viability Assay

Cell viability assays were done as described previously (43) using TRA-8 alone and in combination with gemcitabine. Briefly, cells were trypsinized, plated, incubated overnight at 37°C, then treated with TRA-8 (0–1,000 ng/mL). ATP levels were determined 24 h after adding TRA-8 using the ATPLite Luminescence Assay (Perkin-Elmer). ATP-based bioluminescence assays have been validated as a tool to evaluate cell viability following drug administration (44, 45). Viability data are expressed as a percentage of untreated control cells ±SE. All data points are the mean of two independent experiments, and each experiment included 12 replicates at each TRA-8 dose, and each untreated control included 24 replicates. MIA PaCa-2 and S2-VP10 cell lines were selected as representative cell lines for combination cytotoxicity studies based on TRA-8 response (MIA PaCa-2 sensitive, S2-VP10 resistant). Cells were

treated with gemcitabine (0-300 nmol/L) followed 24 h later by TRA-8 (0-1,000 ng/mL) for 24 h, and then ATP levels were determined. Data points are the mean of two independent experiments, and each experiment included quadruplicate wells at each combination.

A nonlinear model:  $y = \min + (\max - \min)/(1 + \text{dose}/\beta)^\alpha$  was applied to calculate IC<sub>50</sub>, where  $y$  is the response, the variable  $\beta$  represents IC<sub>50</sub>, the variable  $\alpha$  is used to scale concentration for proper transformation, and min and max represent the minimum and the maximum of response (46, 47). 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 relationship was modeled using a second-order response surface model with linear, quadratic, and interaction terms (48).

#### Western Blot Analysis of Intracellular Protein Changes and Immunoprecipitation of Death-Inducing Signaling Complex (DISC) Using Mouse Anti-DR5 Antibody

MIA PaCa-2 and S2-VP10 cells were plated at  $5 \times 10^5$  cells per well in DMEM/10% FBS. Cells were incubated overnight and then treated with 300 nmol/L of gemcitabine for 24 h. TRA-8 at different concentrations was added, and cells were incubated for 3 h. Cell lysates were prepared using radioimmunoprecipitation assay buffer with 150 mmol/L NaCl, 50 mmol/L Tris (pH, 7.4), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mmol/L sodium orthovanadate, and 1:100 Protease Inhibitor Cocktail (Sigma), sonicated on ice for 15 s twice, centrifuged at 14,000 rpm for 2 min, and assayed for protein content (Lowry detergent-compatible protein assay, Bio-Rad). Fifteen micrograms of samples were analyzed using polyvinylidene difluoride membranes (Millipore), probed with antibodies to caspase-3, caspase-8, poly(ADP-ribose)-polymerase (PARP; BD PharMingen), X-chromosome-linked inhibitor of apoptosis protein (XIAP), Bid (Cell Signaling Technology), actin (Sigma), and horseradish peroxidase-conjugated goat anti-mouse IgG<sub>1</sub> (Southern Biotechnology), goat anti-mouse IgG, or anti-rabbit IgG antibodies (Bio-Rad).

For DISC analysis, confluent cells in 100-mm Petri dishes were treated with 300 ng/mL TRA-8, 300 nmol/L gemcitabine, or the combination for 24 h. Untreated cells were used as controls. Floating and attached cells were collected by scraping, washed with PBS, resuspended in RIPA buffer, and incubated on ice for 30 min. Supernatants were collected after centrifugation, and 2 mg protein in 1 mL of lysis buffer from each sample were mixed with 1  $\mu$ g of TRA-8 and incubated overnight at 4°C. Samples were incubated with protein G beads for 2 h with rotation, and then bound proteins were washed thrice with lysis buffer, boiled for 5 min, and analyzed by Western blot using mouse monoclonal antibody against human FADD (BD Biosciences). Membranes were then treated with 7 mol/L guanidine hydrochloride to remove bound antibodies and probed again with caspase-8 antibody.

#### Orthotopic Treatment Model

Female SCID mice (National Cancer Institute-Frederick Animal Production Program) were anesthetized using i.p. injection of xylazine and lidocaine. The abdomens were shaved and prepped with a betadine solution. 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  $2.5 \times 10^6$  MIA PaCa-2 cells in 40  $\mu$ L 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.

#### High-Frequency Ultrasound Imaging of Tumor Size

Serial ultrasound imaging studies were conducted with a Vevo 660 high-frequency, high-resolution ultrasound system with version 1.3.8 software (Visualsonics). All mice were imaged initially (before therapy) with a 40-MHz probe (Model RMV 40B) that focused at a depth of 6 mm. A 30-MHz probe (RMV 30C) that focused at a depth of 12.6 mm was used at later times in mice where tumors exceeded 10 mm in diameter. The animals were anesthetized using isoflurane gas anesthesia, and abdominal hair was removed with depilatory cream. Animals were immobilized on a heated table in the supine position before copious amount of prewarmed ultrasound gel was applied. Dynamic B-Mode imaging (30 frames/s; 20 mm field of view) with the hand-held probe identified the tumors in the pancreatic region, and the probe was subsequently oriented to locate the largest tumor cross-section before moving back and forth over the tumor; the final 300 images (10 s) of the B-mode study was saved. A second B-Mode study was collected in a perpendicular orientation to the first study. For analyses, the first B-Mode study was reviewed to identify the maximum tumor cross-section, and the length diameter was taken as the maximum diameter of the transverse slice, whereas the width diameter was perpendicular to this line. The tumor area was calculated as the length multiplied by the width. In the case of multiple tumors, each tumor was measured by the same method, and tumors areas were added together. Animals were randomized to the treatment groups based on tumor area. By ANOVA analyses, there was no statistically significant difference among the treatment groups initially.

#### Orthotopic Tumor Growth Inhibition and Survival Studies

In the first therapy study, MIA PaCa-2 tumors were established using the above-described protocol. Twenty-one days following tumor cell injection, each mouse underwent abdominal ultrasound. The treatment groups ( $n = 10$  mice per group) were untreated, gemcitabine alone



(120 mg/kg i.p. on days 23 and 30 post-implant), TRA-8 alone (200 µg i.p. on days 22, 26, 29, and 33 post-implant), or combination therapy of gemcitabine and TRA-8 using the above doses and schedule. Mice were subjected to additional abdominal ultrasound examinations, and tumor areas were calculated on post-implant days 41 and 83.

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 untreated, gemcitabine alone (120 mg/kg i.p. on days 26, 33, 54, and 61 post-implant), TRA-8 alone (200 µg i.p. on days 25, 28, 32, 35, 53, 56, 60, and 63 post-implant), or combination therapy with TRA-8 and gemcitabine with the same doses and regimens for each agent alone. Mice underwent additional abdominal ultrasound examination to establish tumor areas on days 72 and 83.

Tumor areas were calculated for all mice in the treatment groups in each therapy study and compared with those from the initial (pre-therapy) ultrasound examination. A Kruskal-Wallis test was used to determine differences between the groups in terms of changes in tumor area following 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 the 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 compare survival between the groups. The Kaplan-Meier method was used to generate the survival curves.

#### Analysis of Xenografts for *In vivo* Apoptosis

SCID mice injected in the pancreas with  $2.5 \times 10^6$  MIA PaCa-2 cells on day 0 received 200 µg TRA-8 i.p. on days 34 and 44, and 120 mg/kg gemcitabine i.p. on days 35 and 45. One group of mice was untreated. The xenografts were dissected for the study of apoptosis and caspase-3 activation on day 48 after tumor cell injection. The reason for the substantial reduction in treatment intensity compared with the treatment protocol described above was to allow adequate tumor tissue for analysis. Tissue for Western blot analysis was minced into 2- to 3-mm pieces on ice and homogenized in 1 mL ice-cold RIPA buffer with protease and phosphatase inhibitors using a Tissue Tearor (Biospec Products Inc.). Homogenates were centrifuged at  $200 \times g$  for 5 min at 4°C. Supernatants were transferred to clean tubes and incubated on ice for 30 min. Samples were then centrifuged at  $16,000 \times g$  for 10 min at 4°C and processed for Western blot analysis as described above for treated cells.

Tumor xenografts were analyzed using the terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay for apoptosis and immunohistochemistry for activated caspase-3. Five-micrometer paraffin sections

of tissue were mounted on Superfrost/Plus slides, heated at 58°C for 1 h, then deparaffinized in three changes of xylene and rehydrated with one change of absolute ethanol, followed by 95% ethanol and 70% ethanol, each for 5 min. The sections were then placed in TBS [500 mmol/L Tris, 150 mmol/L NaCl, 0.0002% Triton X-100, (pH, 7.6)]. Apoptotic nuclei were detected using an ApopTag Peroxidase *In situ* Apoptosis Detection kit (Serologicals S7100). Immunostaining for cleaved caspase-3 was done following antigen retrieval for 5 min in 10 mmol/L EDTA (pH, 8) using a pressure cooker. Sections were incubated with rabbit anti-cleaved caspase-3 (Cell Signaling Technology), followed by biotinylated goat anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories) for 20 min and then Signet streptavidin labeled with horseradish peroxidase for 20 min. The chromagen was 3,3'-diaminobenzidine from BioGenex. The tissues were washed with Tris buffer between each step.

## Results

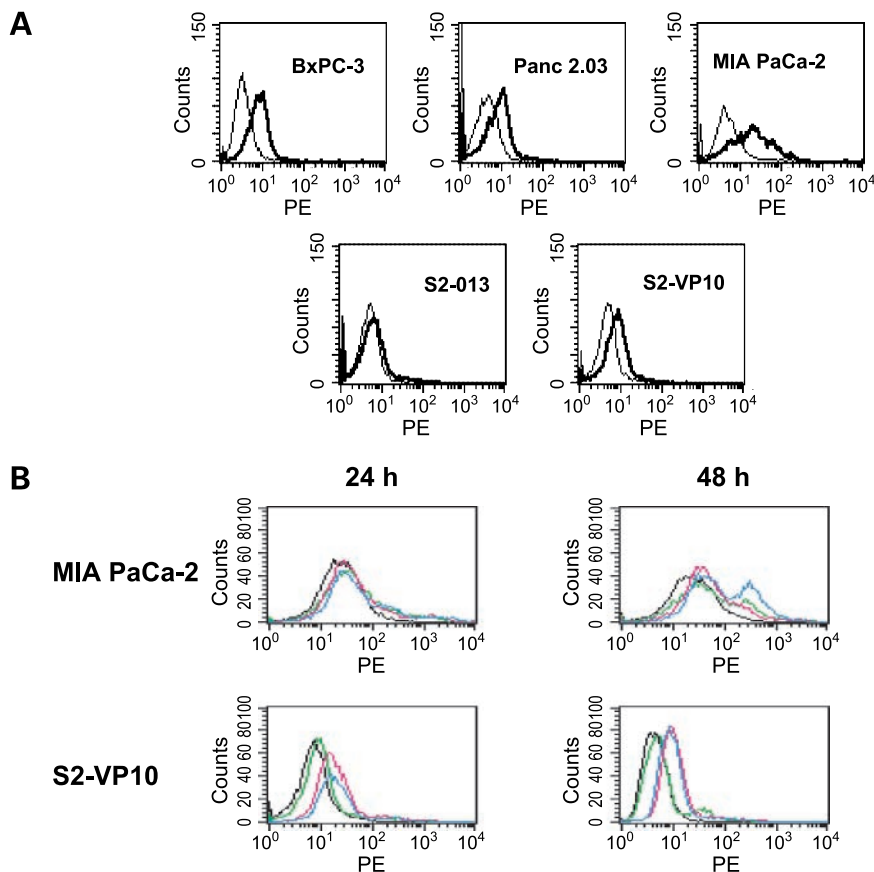
### Expression of DR5 in Established Human Pancreatic Cancer Cell Lines and Primary Tumor Specimens

DR5 cell surface expression was evaluated by flow cytometry analysis on a panel of human pancreatic cancer cell lines (BxPC-3, MIA PaCa-2, Panc 2.03, S2-013, and S2-VP10). As shown in Fig. 1, all human pancreatic cancer cell lines expressed DR5. Baseline cell surface expression of DR5 was highest in MIA PaCa-2 cells, intermediate in BxPC-3, Panc 2.03, and S2-VP10 cells, and lowest in S2-013 cells. The expression of DR5 in pancreatic cancer cell lines was also examined using immunohistochemistry, with specific features highlighted in Supplementary Fig. S1 and Supplementary Table S1.<sup>5</sup> The effect of 24 and 48 h gemcitabine treatment on DR5 cell surface expression was investigated in MIA PaCa-2 and S2-VP10 cells, as shown in Fig. 1B. DR5 levels in MIA PaCa-2 cells increased after 48 h treatment with 30 or 300 nmol/L gemcitabine, whereas 24 h treatment produced little change. In comparison, DR5 levels increased in S2-VP10 cells after 24 or 48 h gemcitabine treatment (Fig. 1B). Similar results were obtained by Western blot analysis (data not shown). In addition, all four cases of primary pancreatic adenocarcinoma expressed DR5 by immunohistochemistry (Supplementary Fig. S2).<sup>5</sup>

### Tumor Cell Cytotoxicity

Sensitivity to TRA-8-induced cytotoxicity varied among cell lines tested (Fig. 2). MIA PaCa-2 and BxPC-3 cells showed the highest sensitivity to TRA-8 ( $IC_{50} = 7.1$  and  $6.4$  ng/mL, respectively). S2-VP10 was the most resistant cell line ( $IC_{50} > 1,000$  ng/mL). Panc 2.03 and S2-013 were intermediate in sensitivity ( $IC_{50}$  of 42.4 and 686.9 ng/mL, respectively). TRA-8-induced cell killing was confirmed by microscopic evaluation of morphologic alterations

<sup>5</sup> Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).



**Figure 1.** Analysis of DR5 expression in human pancreatic cancer cell lines. **A**, cells were incubated with 5  $\mu\text{g}/\text{mL}$  TRA-8 followed by goat anti-mouse IgG conjugated with phycoerythrin fluorochrome. Cells were analyzed using FACScan and CellQuest software. *Thin lines*, isotype control antibody; *thick lines*, TRA-8 antibody binding to DR5. **B**, effect of gemcitabine on DR5 cell surface expression. MIA PaCa-2 and S2-VP10 cells were incubated with medium alone or gemcitabine for 24 or 48 h, and then DR5 levels were analyzed. *Black line*, mouse IgG<sub>1</sub> isotype control antibody; *green line*, DR5 on untreated cells. *Pink and blue lines*, cells incubated with 30 or 300 nmol/L gemcitabine, respectively.

(data not shown). With this limited number of cell lines examined, there was a correlation between DR5 expression (Fig. 1) and TRA-8 cytotoxicity (Fig. 2). MIA PaCa-2 and S2-VP10 cells were selected as representative of TRA-8-sensitive and TRA-8-resistant cell lines, respectively, for combination cytotoxicity studies with gemcitabine. MIA PaCa-2 cells were moderately resistant to gemcitabine (100% viable at 3 nmol/L and 78% viable at 30 nmol/L), which produced additive cytotoxicity when combined with 5 or 25 ng/mL TRA-8 ( $P = 0.75$ ). S2-VP10 cells were sensitive to gemcitabine (77% viable at 3 nmol/L and 33% viable at 30 nmol/L), and treatment with 30 or 300 nmol/L gemcitabine produced synergistic ( $P = 0.0035$ ) cytotoxicity when combined with 5 ng/mL TRA-8 (Fig. 3).

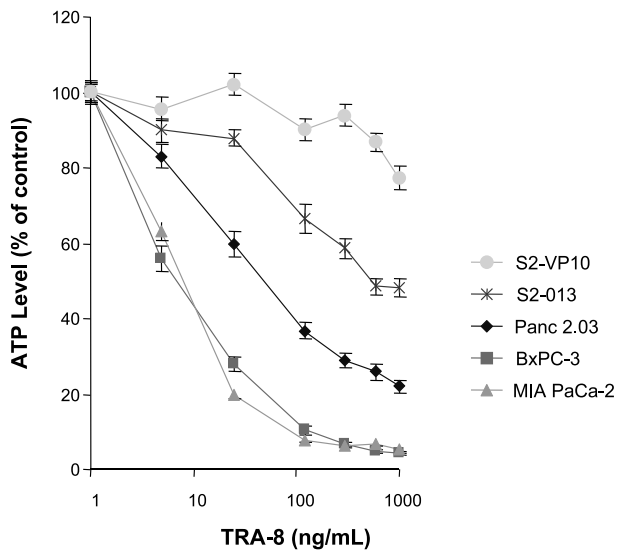
#### Western Blot Analysis of Intracellular Protein Changes

Combination treatment of MIA PaCa-2 cells with 300 nmol/L gemcitabine and 125 ng/mL TRA-8 increased caspase-8 and caspase-3 cleavage as compared with either treatment alone (Supplementary Fig. S3A).<sup>5</sup> In S2-VP10 cells, combination therapy with 300 nmol/L gemcitabine and 1,000 ng/mL TRA-8 enhanced caspase-8 and caspase-3 activation relative to either single treatment (Supplementary Fig. S3B).<sup>5</sup> Reduced XIAP levels were detected in S2-VP10 cells treated with gemcitabine and TRA-8 as compared with either treat-

ment alone (Supplementary Fig. S3B),<sup>5</sup> whereas XIAP levels in MIA PaCa-2 cells were unchanged by treatment with 300 ng/mL TRA-8, 300 nmol/L gemcitabine, or the combination (Supplementary Fig. S3A).<sup>5</sup> Shorter intervals were used for TRA-8 treatments for Western blot analysis compared with cytotoxicity assays to examine early events induced by TRA-8 and gemcitabine treatment. FADD was detected using TRA-8 immunoprecipitation of DISC complexes from MIA PaCa-2 and S2-VP10 cells treated with TRA-8 and combination therapy, as shown in Supplementary Fig. S3C.<sup>5</sup> Cleaved caspase-8 was detected in DISC complexes from MIA PaCa-2 cells treated with TRA-8 and combination therapy. Of note, cleaved caspase-8 was not found in DISC complexes from S2-VP10 cells despite the presence of FADD. This correlates with their increased resistance to TRA-8 treatment (Fig. 3B).

#### *In vivo* Tumor Growth and Survival: One Cycle of Therapy

To evaluate the *in vivo* efficacy of single-agent TRA-8, gemcitabine, or combination therapy, we evaluated tumor growth and survival in SCID mice with orthotopic MIA PaCa-2 tumors treated with a single cycle of therapy. Treatment was initiated on post-implantation day 22 and was completed on day 33. To verify tumor presence and evaluate baseline tumor size, all mice underwent ultrasound tumor measurement on post-implant day 21



**Figure 2.** TRA-8 cytotoxicity against pancreatic cancer cell lines. Pancreatic cancer cells were treated for 24 h with varying doses of TRA-8, and cell viability was determined using a luminescence-based ATP assay.

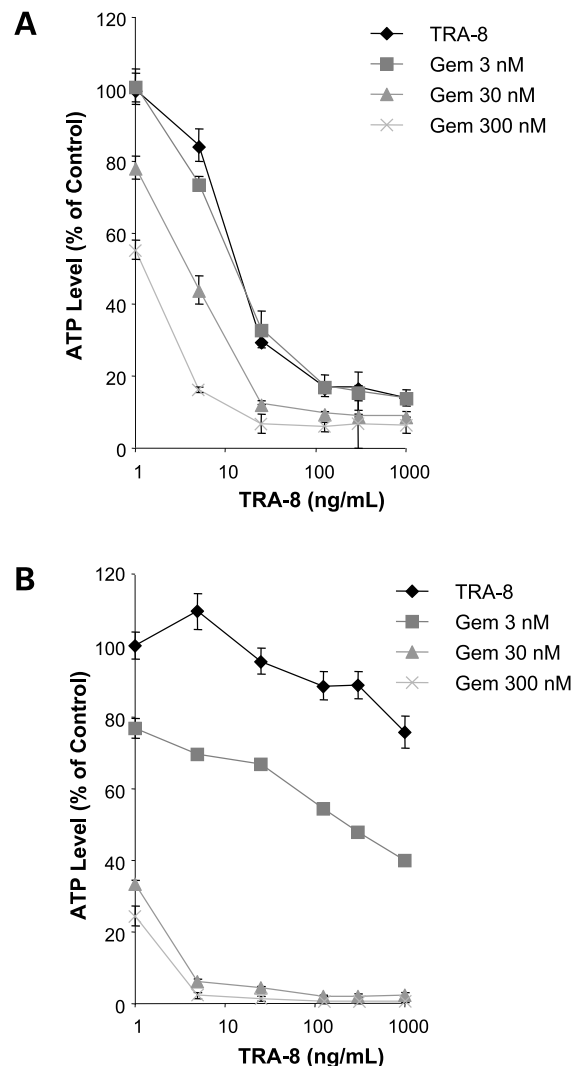
(Fig. 4A). The mean pretreatment tumor areas for untreated ( $32.3 \pm 5.7 \text{ mm}^2$ ), TRA-8 alone ( $39.1 \pm 6.5 \text{ mm}^2$ ), gemcitabine alone ( $42.0 \pm 9.5 \text{ mm}^2$ ), and TRA-8 + gemcitabine ( $34.7 \pm 6.7 \text{ mm}^2$ ) treatment groups were similar ( $P = 0.86$ ). Eight days after completion of treatment (day 41), mice underwent a second abdominal ultrasound examination to evaluate tumor size. As shown in Fig. 4B, mean tumor size of untreated animals increased from 32.3 to  $141.3 \text{ mm}^2$ , an increase of 338%. Gemcitabine-treated animals had tumors that increased from 42.0 to  $106.6 \text{ mm}^2$ , an increase of 154%. Animals treated with single-agent TRA-8 or combination therapy had complete inhibition of MIA PaCa-2 tumor growth. TRA-8 and combination treatments did not significantly differ from each other ( $P = 0.78$ ), and both were significantly different than control ( $P = 0.0048$  and  $P = 0.0036$ ) or single-agent gemcitabine ( $P = 0.0006$  and  $P = 0.0004$ ) on day 41. Tumor size in animals treated with gemcitabine alone was not significantly different than untreated control tumors ( $P = 0.08$ ) on day 41. We repeated the tumor ultrasound measurements on day 83 (50 days post-therapy). As shown in Fig. 4B, the orthotopic tumors in animals treated with TRA-8 or combination therapy had reestablished growth, whereas the majority of animals in the control- or gemcitabine-treated groups were dead of disease.

Figure 4C depicts the survival curves for each group of animals. The mean survival times for untreated control, gemcitabine, TRA-8, and combination treatment groups were  $70 \pm 6$ ,  $66 \pm 3$ ,  $90 \pm 8$ , and  $99 \pm 4$  days post-implant, respectively. Animals treated with single-agent gemcitabine had survival curves that did not differ from control animals ( $P = 0.23$ ). Survival of animals treated with single-agent TRA-8 therapy was marginally better

than untreated controls ( $P = 0.08$ ). Survival with combination therapy was better than control ( $P = 0.0035$ ) or single-agent gemcitabine therapy ( $P < 0.0001$ ). Survival with TRA-8 or combination therapy were not different from each other ( $P = 0.75$ ).

#### Western Blot and Immunohistochemistry Analysis of Orthotopically Grown MIA PaCa-2 Tumors

Western blot analysis of tumor samples from control and various treatment groups are illustrated in Fig. 5A. Animals were treated with reduced doses of TRA-8 and gemcitabine to ensure sufficient viable tissue for analysis. Caspase-8 activation was very modest with all three treatment regimens (TRA-8, gemcitabine, and combination therapy), whereas caspase-3 activation occurred with TRA-8 and combination therapy. Similarly, Bid and PARP cleavage



**Figure 3.** TRA-8 and gemcitabine cytotoxicity against pancreatic cancer cell lines. MIA PaCa-2 (A) and S2-VP10 (B) pancreatic cancer cells were treated with gemcitabine for 24 h, followed by TRA-8 and gemcitabine for 24 h, and then cell viability was determined using a luminescence-based ATP assay.

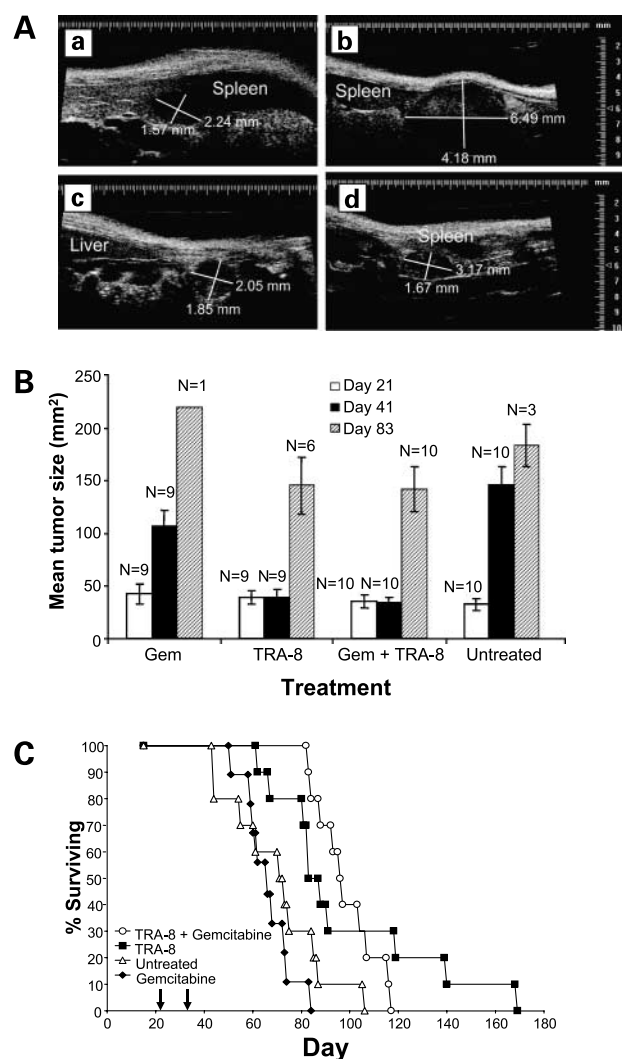
and reduced expression of Bax were primarily seen with TRA-8 and combination therapy. There were no changes noted in Bcl-2, Bcl-xL, and XIAP tumor levels. Immunohistochemistry of the TRA-8 + gemcitabine-treated tumors revealed the induction of apoptosis by TUNEL analysis and caspase-3 activation (Fig. 5B).

#### **In vivo Tumor Growth and Survival: Two Cycles of Therapy**

In the second therapy study, SCID mice with MIA PaCa-2 intrapancreatic tumors were treated with two cycles of TRA-8, gemcitabine, or combination therapy. Mice underwent ultrasound imaging on day 24 (pre-therapy) and were assigned to groups with similar mean tumor sizes for untreated control ( $47.7 \pm 5.5 \text{ mm}^2$ ), TRA-8 alone ( $48.0 \pm 6.4 \text{ mm}^2$ ), gemcitabine alone ( $47.5 \pm 7.0 \text{ mm}^2$ ), and TRA-8 + gemcitabine ( $47.8 \pm 5.7 \text{ mm}^2$ ) treatments ( $P = 1.00$ ). 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. Ultrasound imaging results on days 73 (10 days post-therapy) and 83 (20 days post-therapy) are shown in Fig. 6A. Both TRA-8 and combination treatment resulted in significant tumor growth inhibition from days 24 to 73 compared with the untreated group ( $P = 0.0020$  and  $0.0014$ ) or the gemcitabine-treated group ( $P = 0.0015$  and  $P = 0.0008$ ). On day 83, most of the control animals were dead of disease. Ultrasound measurements on that day showed some tumor growth in the TRA-8 and combination therapy groups ( $83.1 \pm 30.6$  and  $63.9 \pm 31.3$ ), which were significantly different than the six animals surviving in the gemcitabine-treated group ( $P = 0.003$ ). Mean tumor size of the TRA-8 and combination regimens on days 73 and 83 were not significantly different and neither had any deaths. Mean survival times (Fig. 6B) were  $76 \pm 3$ ,  $79 \pm 5$ ,  $121 \pm 4$ , and  $142 \pm 7$  days in the untreated, gemcitabine, TRA-8, and TRA-8 + gemcitabine treatment groups, respectively. Both TRA-8 alone and combination therapy significantly enhanced survival compared with control ( $P < 0.0001$ ) or gemcitabine alone ( $P < 0.0001$ ). Survival of animals treated with combination therapy was superior to the TRA-8 alone group ( $P = 0.009$ ). Two cycles of combination therapy provided better survival than a single cycle ( $P < 0.0001$ ), whereas two cycles of single-agent TRA-8 was marginally better than a single cycle ( $P = 0.07$ ).

## **Discussion**

We have previously characterized the effects of TRA-8, an agonistic DR5-specific monoclonal antibody on breast cancer cell lines and breast cancer xenografts (43). These studies showed single-agent antitumor efficacy, which was dramatically enhanced in combination with chemotherapy (Adriamycin or taxol). The current study was undertaken to characterize TRA-8 in combination with gemcitabine in pancreatic cancer, a more treatment-resistant form of cancer. All five pancreatic cancer cell



**Figure 4.** Ultrasound imaging and survival of SCID mice with intrapancreatic MIA PaCa-2 tumors and one course of therapy. **A**, representative ultrasound B-mode images (40 MHz) of orthotopic pancreatic tumors in an untreated mouse at (a) 21 d and (b) 41 d after implantation, together with a mouse treated with combined TRA-8 and gemcitabine at (c) 21 d and (d) 41 d after implantation. **B**, mice underwent ultrasound imaging with measurements of tumor size on days 21, 41, and 83. Mice received one course of treatment as follows: untreated, gemcitabine alone (120 mg/kg i.p. on days 23 and 30 post-implant), TRA-8 alone (200  $\mu\text{g}$  i.p. on days 22, 26, 29, and 33 post-implant), or combination therapy of gemcitabine and TRA-8 with the same dose and schedule as either agent alone. **C**, survival of the same groups of mice treated with one course of TRA-8, gemcitabine, or the combination of the two agents. Arrows, start and end of treatment.

lines reacted with TRA-8 to varying degrees by flow cytometry analysis as did four primary human pancreatic adenocarcinoma tumor tissues, consistent with prior reports of DR5 expression in pancreatic cell lines and primary tumors (26, 27). TRA-8-mediated cytotoxicity of pancreatic cell lines showed a wide range of sensitivity from highly sensitive MIA PaCa-2 and BxPC-3 to highly resistant S2-VP10 (Fig. 2). The degree of cytotoxicity



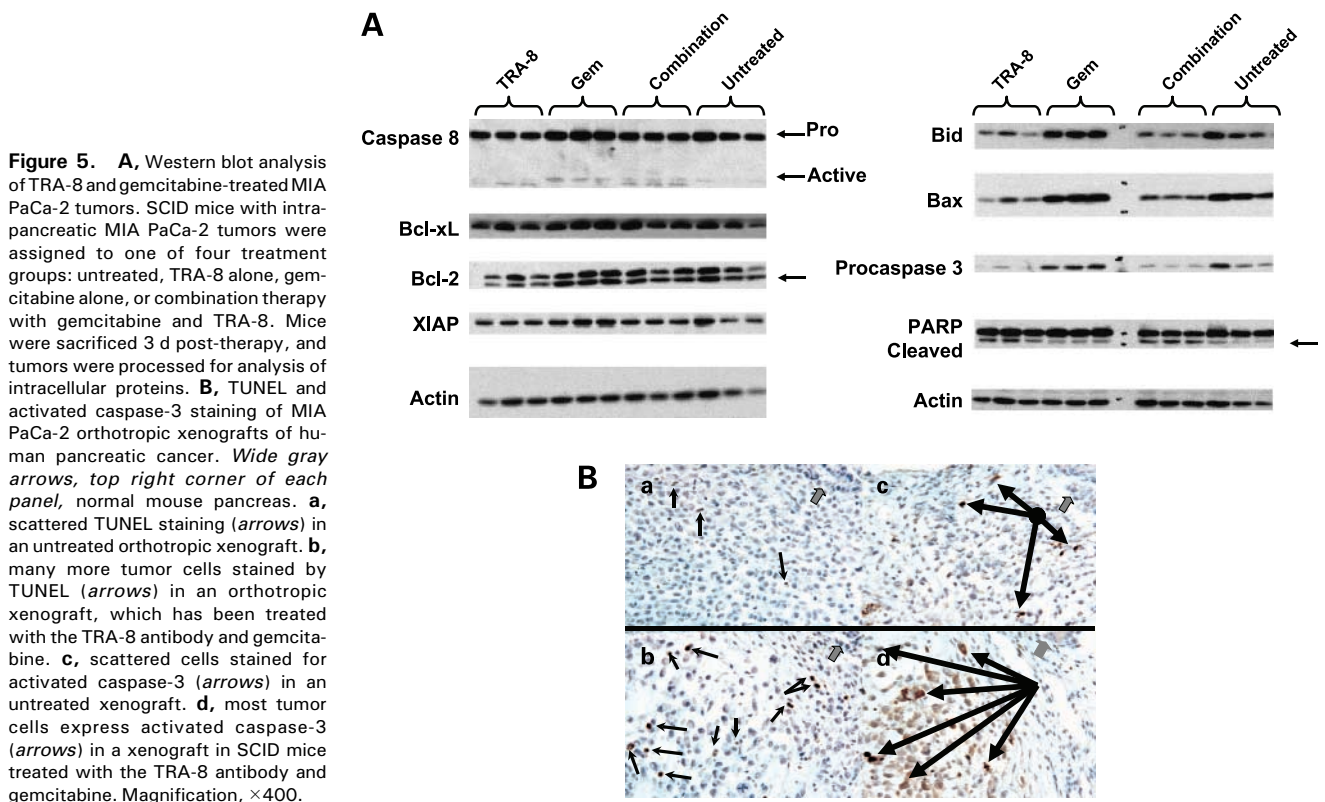
correlated with cellular expression of DR5. The lack of correlation of TRAIL-induced cytotoxicity with DR5 expression has been previously reported (28, 49, 50). These observations suggest that for certain cell lines, DR5 expression levels and/or intracellular mechanisms are responsible for varying degrees of tumor cell resistance or sensitivity, although the intracellular molecules or pathways responsible are yet to be clarified.

Enhanced tumor cell cytotoxicity has been noted with the addition of chemotherapy agents to TRAIL or DR4/DR5 agonistic antibodies (30, 43). We analyzed the effect of gemcitabine + TRA-8 on a TRA-8-sensitive (MIA PaCa-2) and TRA-8-resistant cell line (S2-VP10). The MIA PaCa-2 cells were moderately resistant to gemcitabine, and the combination produced additive cytotoxicity (Fig. 3A). The S2-VP10 cell line was more sensitive to gemcitabine, and the combination produced synergistic cytotoxicity (Fig. 3B). The synergistic effect may have reflected, in part, the enhanced DR5 expression seen with gemcitabine exposure of S2-VP10 cells, which was less effective in MIA PaCa-2 cells. We also characterized several apoptosis-related proteins in each cell line. Combination therapy produced a more dramatic activation of caspase-3 and reduction in XIAP levels in S2-VP10 cells than in MIA PaCa-2 cells.

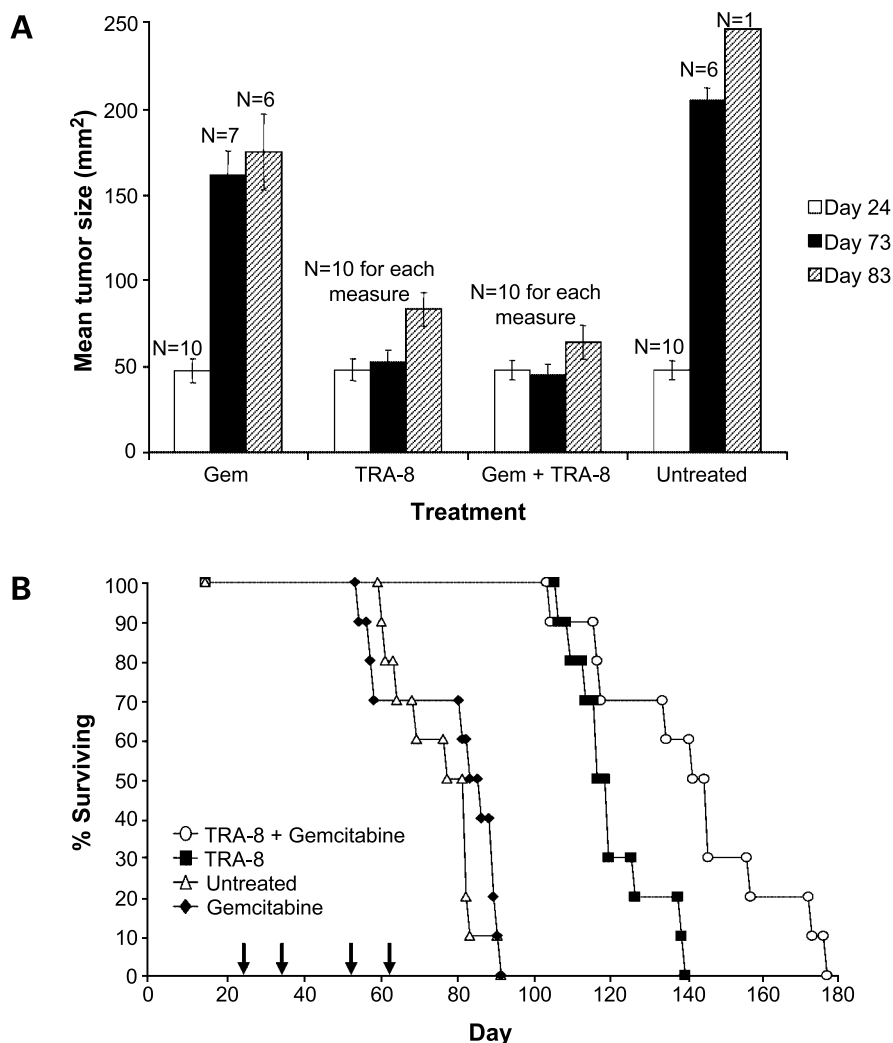
We selected the MIA PaCa-2 cell line for *in vivo* studies given its sensitivity to TRA-8 cytotoxicity *in vitro*. Our animal model studies used an orthotopic tumor site that

better reflects the human disease counterpart. In addition, we used ultrasound monitoring of orthotopic tumors so that we could assess tumor growth as a parameter in addition to survival. A further advantage is that the ultrasound assessment pre-therapy allows the distribution of tumor sizes to be equally distributed among control and treatment groups. In this model, TRA-8 had striking inhibition of tumor growth, whereas gemcitabine had a modest effect that was not statistically significant (Fig. 4B). Similarly, TRA-8 produced a significant prolongation of survival (Fig. 4C) compared with untreated animals ( $P = 0.0048$ ), whereas gemcitabine did not ( $P = 0.08$ ). The combination treatment was not superior to TRA-8 single-agent therapy, but was superior to gemcitabine or no treatment as regards the inhibition of tumor growth and survival. Analysis of tumor specimens showed reduced procaspase-3 levels, cleavage of PARP, a caspase-3 substrate, and reduced levels of Bid, a caspase-8 substrate, in both the TRA-8 single-agent and combination therapy animal groups.

To further assess the potential benefit of combination therapy, we evaluated the antitumor efficacy of two cycles of therapy (Fig. 6). The inhibition of tumor growth was more pronounced with two cycles of therapy (comparison of Figs. 4B and 6A) as seen in the day 83 tumor size and number of surviving animals. The survival curves (Fig. 6B) showed enhanced survival for the TRA-8 and combination treatment groups compared with either single-agent







**Figure 6.** Ultrasound imaging and survival of SCID mice with orthotopic MIA PaCa-2 tumors and two courses of therapy. **A**, mice underwent ultrasound imaging with measurements of tumor size on days 24, 73, and 83. They were treated with one course of 200  $\mu$ g TRA-8 i.p. on days 25, 28, 32, and 35 and a second course on days 53, 56, 60, and 63, one course of 120 mg/kg gemcitabine i.p. on days 26 and 33, and a second course on days 54 and 61, or a combination of TRA-8 and gemcitabine with the same dose and schedule as either agent alone. **B**, survival of the same groups of mice treated with two courses of TRA-8, gemcitabine, or the combination of the two agents. Arrows, start and end of each treatment course.

gemcitabine or untreated groups. However, the two cycles of therapy showed the superior survival of combination therapy over single-agent TRA-8-treated animals ( $P = 0.009$ ). Thus, the addition of gemcitabine to TRA-8 produced enhanced antitumor efficacy even in a tumor model with moderate gemcitabine resistance *in vitro* and lack of gemcitabine efficacy *in vivo*. This situation is similar to human pancreatic cancer where gemcitabine has modest effects on tumor shrinkage or survival prolongation. The addition of TRA-8 to gemcitabine in the therapy of human pancreatic cancer may also enhance the modest efficacy of gemcitabine. Phase I and phase I/II trials of agonistic anti-DR4 and anti-DR5 human monoclonal antibodies and TRAIL have been reported in a preliminary fashion (30) with limited evidence of efficacy to date.

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