Treatment of Human Colon Cancer Xenografts with TRA-8 Anti-death Receptor 5 Antibody Alone or in Combination with CPT-11

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Abstract

Purpose: This study was designed to evaluate the in vitro cytotoxicity and in vivo efficacy of TRA-8, a mouse monoclonal antibody that binds to the DR5 death receptor for tumor necrosis factor–related apoptosis-inducing ligand (also called Apo2L), alone and in combination with CPT-11, against human colon cancer cells and xenografts.

Experimental Design: DR5 expression was assessed on human colon cancer cell lines using flow cytometry, and cellular cytotoxicity after TRA-8 treatment, alone and in combination with SN-38, was determined by measuring cellular ATP levels. Tumor growth inhibition and regression rates of well-established subcutaneous COLO 205, SW948, HCT116, and HT-29 colon cancer xenografts in athymic nude mice treated with TRA-8 or CPT-11 alone and in combination were determined. 99mTc-TRA-8 was used to examine tumor localization of TRA-8 in animals bearing each of the four xenografts. In addition, whole-body biodistribution and imaging was carried out in COLO 205–bearing animals using in vivo single-photon emission computed tomography imaging and tissue counting.

Results: DR5 expression was highest on HCT116, intermediate on SW948 and COLO 205 cells, and lowest on HT-29 cells. COLO 205 cells were the most sensitive to TRA-8–induced cytotoxicity in vitro, SW948 and HCT116 cell lines were moderately sensitive, and HT-29 cells were resistant. Combination treatment with TRA-8 and SN-38 produced additive to synergistic cytotoxicity against all cell lines compared with either single agent. The levels of apoptosis in all cell lines, including HT-29, were increased by combination treatment with SN-38. In vivo, combination therapy with TRA-8 and CPT-11 was superior to either single-agent regimen for three of the xenografts: COLO 205, SW948, and HCT116. COLO 205 tumors were most responsive to therapy with 73% complete regressions after combination therapy. HT-29 cells derived no antitumor efficacy from TRA-8 therapy. Tumor xenografts established from the four colon cancer cell lines had comparable specific localization of 99mTc-TRA-8.

Conclusions: In vitro and in vivo effects of TRA-8 anti-DR5 monoclonal antibody on four different colon cancer cell lines and xenografts were quite variable. The HT-29 cell line had low surface DR5 expression and was resistant to TRA-8 both in vitro and in vivo. Three cell lines (COLO 205, SW948, and HCT116) exhibited moderate to high sensitivity to TRA-8–mediated cytotoxicity which was further enhanced by the addition of SN-38, the active metabolite of CPT-11. In vivo, the combination of TRA-8 and CPT-11 treatment produced the highest antitumor efficacy against xenografts established from the three TRA-8–sensitive tumor cell lines. All four colon cancer xenografts had comparable localization of 99mTc-TRA-8. These studies support the strategy of TRA-8/CPT-11 combined treatment in human colon cancer clinical trials.

The discovery of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; also called as Apo-2L) and the finding that TRAIL induces apoptosis in human cancer cells to a greater extent than in normal cells (1–6) generated considerable interest in the potential therapeutic use of this cytokine. Five receptors for TRAIL have been identified: DR4 (TRAIL-R1) and DR5 (TRAIL-R2), which contain cytoplasmic death domains capable of transmitting apoptosis-inducing signals, and decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin, which are incapable of inducing apoptosis (4, 5). Early concerns about potential hepatotoxicity of TRAIL (1, 7) and its pharmacokinetics led to the development of agonistic antibodies specific for DR4 or DR5, which are capable of inducing apoptosis in human cancer cells (8–12).
Previously, we reported on the antitumor efficacy of an agonistic murine anti-DR5 monoclonal antibody (TRA-8) in a xenograft model of human breast cancer (10). TRA-8 bound to DR5 on the surface of most human breast cancer cell lines, but there was considerable variation in the sensitivity of these cell lines to TRA-8–induced apoptosis in vitro. In vivo studies using subcutaneous xenografts of 2LMP cells, an aggressive subclone of the MDA-MB-231 breast cancer cell line, showed significant enhancement of TRA-8 antitumor efficacy using combination chemotherapy with paclitaxel or Adriamycin with or without concurrent radiotherapy (10).

The purpose of the present study was to evaluate the antitumor efficacy of TRA-8 using in vitro cytotoxicity assays and xenograft models of human colon cancer. We and others have shown that DR5 is expressed in tumors of the colorectum (13–15). The cytotoxicity of TRA-8 alone or in combination with SN-38, the active metabolite of CPT-11, against human colon cancer cell lines of varying sensitivity to TRA-8 was investigated. Binding, cytotoxicity and mechanism studies were used to examine the relationship between in vitro sensitivity to TRA-8 and CPT-11, alterations in apoptotic signaling pathways and the ability to predict in vivo efficacy of TRA-8 and CPT-11 against xenograft models of colon cancer. We hypothesized that combination treatment with CPT-11 may increase TRA-8 signaling by engaging the intrinsic apoptotic pathway, through caspase-8 mediated Bid activation and down-regulation of antiapoptotic proteins of the Bcl-2 and IAP families. In vivo studies using subcutaneous colon cancer tumor models in athymic nude mice showed patterns of antitumor efficacy of TRA-8, CPT-11, and the combination which were unique for each cell line. This work provides a rationale for the investigation of TRA-8 and chemotherapy in patients with colon cancer.

Materials and Methods

Cell lines and reagents. All cell lines were obtained from the American Type Culture Collection and grown in RPMI 1640 supplemented with 4.5 g/L glucose, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 10% fetal bovine serum (COLO 205 and HT-29). DME/F12 with 10% fetal bovine serum (SW498), or McCoy’s medium with 10% fetal bovine serum (HCT116). All cell lines were maintained in antibiotic-free medium at 37°C in a 5% CO2 atmosphere and routinely screened for Mycoplasma contamination.

Purified TRA-8 (IgG1) monoclonal antibody used for in vitro studies was produced and purified as previously described (9), whereas Sankyo Co., Ltd. provided the preparations used for in vivo studies. Isotype-specific IgG1 control antibody and phycoerythrin-conjugated goat anti-mouse IgG1 were obtained from Southern Biotechnology Associates. CPT-11 (irinotecan hydrochloride, Camptosar; Pharmacia and Upjohn), oxaliplatin (Eloxatin; Sanofi Aventis), topotecan (Hycamtin; SmithKline Beecham Pharmaceuticals), and docietaxel (Taxotere; Aventis Pharmaceuticals, Inc.) were obtained from the University of Alabama at Birmingham Hospital Pharmacy and diluted in 0.9% sterile saline (in vivo studies) immediately before use. SN-38 was obtained from Toronto Chemical Co. Cell Striper was from Mediatech. Collagenase type I and protease inhibitor cocktail were from Sigma Chemical Co. Lowry detergent-compatible protein assay reagents and horseradish peroxidase–conjugated goat anti-mouse IgG and anti-rabbit IgG were from Bio-Rad. Antibodies for Western blot analysis were obtained from the following vendors: caspase-3, caspase-8, and poly(ADP-ribose) polymerase (PARP) from BD Pharmingen; Bax from Southern Biotechnology Associates; caspase-9, Bid, Bcl-xl, survivin, and Akt from Cell Signaling Technologies; FLIP and p53 from Calbiochem; and XIAP from Stressgen; actin from Sigma Chemical Co. Enhanced chemiluminescence reagents were from GE Healthcare.

Indirect immunofluorescence and flow cytometry analysis of DR5 expression. DR5 expression on colon cancer cells was analyzed as described previously (16) using FACScan and Cell Quest software (Becton Dickinson). To examine the effect of SN-38 on DR5 cell surface expression, colon cancer cell lines were treated with SN-38 for 24 h at concentrations selected from their SN-38 dose–response curves and then analyzed for DR5 expression as described above.

Cell viability assays using ATP-Lite. Cell cultures were trypsinized, replated in complete culture medium, and incubated overnight at 37°C before addition of drugs and/or antibody. For combination treatments, cells were pretreated with chemotherapy drugs for 24 h before adding TRA-8 antibody for an additional 24 h. Other studies examined the efficacy of 24 h concurrent treatment with TRA-8 and SN-38 (Supplementary Fig. S1). Cell viability was assessed after 24 h exposure to TRA-8 by measurement of cellular ATP levels using the ATP-Lite luminescence assay (Perkin-Elmer Biosciences). All samples were assayed in quadruplicate and are reported as the mean ± SD from a minimum of two independent experiments.

We screened a number of chemotherapy agents in terms of their ability to enhance TRA-8–mediated cytotoxicity to colon cancer cell lines. In general, 5-fluorouracil and gemcitabine produced inferior enhancement compared with CPT-11. Topotecan, docietaxel, oxaliplatin, and Adriamycin produced similar enhancement as CPT-11 on colon cancer cell lines, including the TRA-8 resistant cell line HT-29 (Supplementary Fig. S2). However, their combination with TRA-8 in vivo produced little or no enhancement above the antitumor effects of chemotherapy drug alone (examples in Supplementary Fig. S3). Because CPT-11 is commonly used for treatment in colorectal cancer and is superior to or comparable with other agents, we used this agent to characterize chemotherapy—TRA-8 combination therapy in the studies described below.

Statistical analysis of in vitro cytotoxicity of TRA-8 and chemotherapy drugs. A nonlinear model $y = \text{Min} + (\text{Max} - \text{Min}) / (1 + \text{dose} / \text{IC50})^a$; refs. 17, 18, was applied to calculate IC50, wherein y is the response, the variable $b$ represents IC50, the variable $x$ is used to scale concentration for proper transformation, and Min and Max represent the minimum and the maximum of response, respectively. A SAS procedure NLIN was used for the computation (SAS Institute, Inc.). The cytotoxicity data were evaluated to assess whether the combination cytotoxic effects were additive, less than additive (antagonistic), or greater than additive (synergistic). The dose-response relationships for the agents alone and in combination were modeled using a second-order response surface model with linear, quadratic, and interaction terms for each of the four cell lines (19). A significant interaction term was classed as either synergistic or antagonistic, depending on whether the interaction term was negative with more than additive cytotoxicity or positive with less than additive cytotoxicity. If the interaction term was not significant, then the relationship between TRA-8 and CPT-11 would be considered additive, provided the additive terms were significant.

Western blot analysis of colon cancer cells treated with TRA-8 and SN-38. Cell lines were trypsinized, replated, and incubated overnight at 37°C before starting treatments. Cells were treated with SN-38 (0.1 or 1 mmol/L) for 2 h at 37°C, and then TRA-8 was added. After 2.5 h (COLO 205, SW498, and HCT116) or 4 h (HT-29) treatment with TRA-8, whole-cell lysates were prepared in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 50 mmol/L Tris (pH 7.4), 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1% protease inhibitor cocktail, 10 mmol/L sodium orthovanadate]. Proteins (15-25 μg) were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore), and probed with primary antibodies followed by horseradish peroxidase–conjugated secondary antibodies. Actin levels were compared with control for protein loading.
Fig. 1. DR5 expression and TRA-8 cytotoxicity against colon cancer cell lines. 

A, flow cytometry analysis of DR5 cell surface expression in human colon cancer cell lines. Colon cancer cells were harvested and stained with 5 µg/mL TRA-8 for 1 h at 4°C followed by PE-conjugated goat anti-mouse IgG1 and then analyzed using FACS® and CellQuest® software. Thick histograms, TRA-8 staining; thin histograms, cells stained with mouse IgG1 isotype control antibody. 

B, cytotoxicity of TRA-8 alone and in combination with SN-38 treatment of human colon cancer cell lines. Cells (1,000 per well) were treated at 37°C for 24 h with TRA-8 at concentrations ranging from 1 to 1,000 ng/mL. Other cells were pretreated with SN-38 for 24 h followed by 24 h concurrent treatment with TRA-8 and SN-38 or were treated with SN-38 alone for 48 h. Cell viability was assessed by measuring ATP levels. Points, mean derived from three to four independent experiments, each done in quadruplicate, and are reported relative to untreated control cells; bars, SD. 

C, effect of SN-38 on DR5 expression on colon cancer cells. Cells were treated for 24 h with 0 to 10 µmol/L SN-38, then cells were harvested and DR5 levels were analyzed using TRA-8 binding and flow cytometry. Thick histograms, TRA-8 staining; thin histograms, cells stained with mouse IgG1 isotype control antibody.
Radiolabeling and binding of TRA-8. A fresh 1.8 mmol/L solution of succinimidyl 6-hydrazinonicotinate (courtesy Dr. Gary Bridger, AnorMED, Inc.) in dimethylformamide was prepared. Forty picomoles were transferred to glass vials, followed by freezing at -90°C, then the solutions were vacuum dried using an Advantage Benchtop Freeze Dryer (Virtis Co., Inc.) with the shelf temperature at -75°C and trap at -80°C until use. Each vial was reconstituted with 1 mg of TRA-8 (6-hydrazinonicotinate/TRA-8 molar ratio, 6) in 1 mL sodium phosphate buffer [0.15 mol/L (pH 7.8); ref. 20]. After a 3-h incubation at room temperature, the mixture was dialyzed (10,000 MW cutoff) against PBS (pH 7.4) overnight at 4°C. The 6-hydrazinonicotinate–modified TRA-8 was labeled with 99mTc using SnCl2/tricine as the transfer ligand (21), and unbound 99mTc was removed by G-25 Sephadex size-exclusion chromatography. Protein concentrations were measured by Lowry assay. The level of 99mTc binding to TRA-8 was always >96%, as measured by TLC using separate strips eluted with saturated saline and methyl ethyl ketone. The isotype control antibody (IgG1k, eBioscience, Inc.) was iodinated with 125I using the Iodogen method. The average specific activity of 99mTc-TRA-8 and 125I-isotype control antibody were 348.6 and 34.2 kBq/μg, respectively.

TRA-8, CPT-11, or combination therapy in athymic nude mice bearing colon cancer xenografts. Athymic nude mice were injected s.c. with 1 or 2 × 10^7 COLO 205, SW948, HCT116, or HT-29 colon cancer cells. Tumors were measured in the two largest dimensions, and the tumor surface area (length × width, in mm^2) was calculated. Treatment was initiated when tumors reached ~6 to 8 mm in diameter (8-14 d after tumor cell injection for COLO 205, 14 to 19 d with SW948, 10 d with HCT116, and 14 d for HT-29), and the growth of tumors was monitored over time. A dose-response study of single agent TRA-8 in COLO 205 tumor-bearing animals showed similar regression rates with 60 or 200 μg doses given twice per week × 3 wk. We chose the 200-μg dose schedule because the other cell lines were less sensitive to TRA-8 in vitro. In subsequent studies, animals bearing subcutaneous colon tumors were injected i.v. with 200 μg TRA-8 beginning on treatment day 1 followed by five additional injections given twice weekly for 3 wk. For animals receiving CPT-11 therapy (33 mg/kg), either alone or in combination with TRA-8, the drug was given by i.v. injection on treatment day 2 followed by five additional injections given every 3 to 4 d for 3 wk. Animals receiving combination treatment with TRA-8 and CPT-11 were injected with the drug 24 h after each TRA-8 administration. We used a twice weekly schedule to assure a continuous circulating level of TRA-8 (plasma T_{1/2} of ~5 d) which would limit any pretreatment effects of CPT-11. Tumors were measured thrice weekly with Vernier calipers, and tumor growth and regression rates were determined. We next investigated whether a second cycle of treatment with TRA-8 + CPT-11 (33 mg/kg, twice weekly × 3 wk for each cycle) initiated 10 d after the first cycle of treatment would enhance the antitumor effect against SW948 tumor xenografts. Two cycles of TRA-8 + CPT-11 were more effective (three of eight complete regressions) than a single cycle of TRA-8 + CPT-11 (0 of eight complete regressions), as shown in Supplementary Fig. S4. Animal studies were approved by the Institutional Animal Care and Use Committee.

Fig. 2. Western blot protein analysis of colon cancer cell lines treated in vitro with TRA-8 alone and in combination with SN-38. COLO 205, SW948, and HCT116 cells were treated with 0.1 μmol/L SN-38 for 24 h followed by treatment with TRA-8 plus SN-38 for an additional 2.5 h or were exposed to TRA-8 alone for 2.5 h. HT-29 cells were treated with 0.1 or 1 μmol/L SN-38 for 24 h followed by treatment with TRA-8 plus SN-38 for an additional 4 h or were exposed to TRA-8 alone for 4 h. Whole-cell lysates were then prepared, and equivalent amounts of protein were analyzed by Western blot using antibodies specific for caspase-3, caspase-8, caspase-9, PARP, Bid, Bcl-xl, Bax, p53, XIAP, survivin, FLIP, and Akt. Actin levels were compared with control for protein loading.
Table 1. Effect of TRA-8, CPT-11, and the combination on colon xenograft tumor doubling time and complete regressions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Mean tumor doubling time, days</th>
<th>Complete regressions</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Total (%)</td>
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<td>COLO 205</td>
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<td></td>
<td></td>
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<tr>
<td>No treatment</td>
<td>39</td>
<td>13 ± 14</td>
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<td>42</td>
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<tr>
<td>CPT-11</td>
<td>19</td>
<td>64 ± 16</td>
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<td>TRA-8 + CPT-11</td>
<td>33</td>
<td>138 ± 79</td>
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<tr>
<td>SW948</td>
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<td></td>
<td></td>
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<tr>
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<td>25</td>
<td>13 ± 26</td>
<td>0 (0%)</td>
</tr>
<tr>
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<td>24</td>
<td>10 ± 5</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CPT-11</td>
<td>24</td>
<td>37 ± 33</td>
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<td>67 ± 39</td>
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<td>21 ± 1</td>
<td>0 (0%)</td>
</tr>
<tr>
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<tr>
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<td>0 (0%)</td>
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<tr>
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<td>7</td>
<td>78 ± 10</td>
<td>0 (0%)</td>
</tr>
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<td>HT-29</td>
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<tr>
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<td>0 (0%)</td>
</tr>
<tr>
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<td>8</td>
<td>26 ± 5</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>TRA-8 + CPT-11</td>
<td>8</td>
<td>26 ± 5</td>
<td>0 (0%)</td>
</tr>
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</table>

Animal imaging experiments. Two imaging experiments were conducted with athymic nude mice (Frederick Cancer Research Facility) implanted subcutaneously in the right thigh with 1 × 10^6 COLO 205 cells. In vivo tumor imaging and biodistribution studies were done 5 wk later. Mice were injected i.v. with 99mTc-TRA-8 (Experiment 1) or a mixture of 99mTc-TRA-8 and 125I-labeled isotype control antibody (Experiment 2). The average weight of the tumors was 0.7 g for both experiments. The mean dose of 99mTc-TRA-8 used in experiments 1 and 2 was 9.25 MBq (15 μCi) and 11.10 MBq (29 μCi), respectively, whereas that of 125I-isotype control antibody was 0.67 MBq (16 μCi). Doses of radioactivity were confirmed by measuring the syringes before and after dosing with a dose calibrator (AtomLab 100, Biodex Medical Systems, Inc.).

Images of mice bearing COLO 205 xenografts were acquired using X-SPECT, a single-photon emission computed tomography/CT dual-modality imaging instrument (Gamma Medica, Inc.) to monitor detailed 99mTc-TRA-8 distribution in the tumor in vivo. For single-photon emission computed tomography imaging, the focal length was 41 mm with 0.7 mm spacing between slices. A total of 64 projections were acquired with a 40-s acquisition time per projection at 20 h after dose injection, using a pinhole collimator with a 1-mm pinhole insert. The mice were induced and maintained with isoflurane gas anesthesia and monitored continuously to allow the lowest dose (typically 1-1.5%) to prevent movement. The energy window to collect photons from 99mTc was 126 to 161 keV and, therefore, excluded any contribution from 125I.

Bioluminescence imaging, using the Auto-gamma 5000 series Gamma Counter, Packard Instrument Company). The percentage of injected dose per gram for each tissue was averaged for all mice in each group.

Statistical analysis. Fisher’s exact test was used to compare the regression rate between groups. For tumor growth analyses, linear regression was done on each animal, where the percentage of tumor size, compared with the original tumor size, was the dependent variable. Based on the linear regression, a tumor doubling time was calculated for each animal. For animals that did not have tumor doubled at the end of the study, the study period was assigned to them as the tumor doubling time. Wilcoxon rank-sum test based on normal approximation was used to compare the tumor doubling time between groups. All tests had a significance level at 0.01 based on Bonferroni’s adjustment. One-way ANOVA (22) was carried out using SAS, version 8.2 (SAS Institute, Inc.) to compare biodistribution of 99mTc-TRA-8 and 125I-isotype control antibody.

Results

DR5 expression and in vitro cytotoxicity of TRA-8 alone and in combination with chemotherapy. DR5 expression, as detected by TRA-8 binding on four human colon cancer cell lines, is depicted in Fig. 1A. DR5 was detected on all cell lines at expression levels that ranged from weakly positive (HT-29 cells) to strongly positive (HCT116 cells). TRA-8–mediated cytotoxicity against the four colon cancer cell lines produced varying degrees of cell killing, as shown in Fig. 1B. COLO 205 cells were very sensitive to TRA-8, with an IC50 value of 9.2 ng/mL. In contrast, HT-29 cells were relatively resistant to TRA-8 with 58% viable cells remaining at 1,000 ng/mL TRA-8. For these cell lines, TRA-8 resistance correlated with DR5 expression (HT-29), whereas the cell line with greatest DR5 expression was only moderately sensitive to TRA-8 cytotoxicity (HCT116).

The cytotoxic effects of a combination of SN-38 (active metabolite of CPT-11) and TRA-8 are also presented in Fig. 1B. Pretreatment of TRA-8-resistant HT-29 cells with SN-38,
followed by TRA-8 addition, produced synergistic cell killing compared with either single agent \( (P = 0.0012) \). Synergistic killing of SW948 cells was also observed with combination treatment \( (P = 0.0362) \) using 1 \( \mu \text{mol/L} \) SN-38, a concentration that had no effect on cell viability. Combination treatment resulted in additive cytotoxicity against HCT116 cells \( (P = 0.481) \). SN-38 enhanced TRA-8–mediated cytotoxicity to COLO 205 cells, but analysis for synergism was hindered by their extreme sensitivity to TRA-8. Concurrent treatment of COLO 205 and HT-29 cells with SN-38 and TRA-8 for 24 hours produced a stronger synergistic effect \( (P < 0.0001) \) as shown in Supplementary Fig. S1, but higher doses of SN-38 were required to achieve synergistic killing compared with the drug pretreatment schedule. The IC\(_{50}\) of cell lines for single agent SN-38 was 0.02 \( \mu \text{mol/L} \) (HCT116), 0.5 \( \mu \text{mol/L} \) (HT-29), 1 \( \mu \text{mol/L} \) (COLO 205), and >10 \( \mu \text{mol/L} \) (SW948), as shown in Fig. 1B. It was interesting that the SN-38–resistant cell line (SW948) underwent significant synergistic enhancement of SN-38–mediated cytotoxicity by the addition of small amounts of TRA-8 (5 ng/mL).

Combination treatment with TRA-8 and SN-38 produced enhanced cytotoxicity against other TRA-8–resistant colon cancer cell lines, including SW1116, LoVo, and T84 (data not shown). The effect of combination treatment with TRA-8 and other chemotherapeutic drugs, including topotecan, docetaxel, oxaliplatin, and Adriamycin, was also examined, as shown in Supplementary Fig. S2. All four drugs produced additive or greater than additive cytotoxicity against TRA-8–resistant HT-29 cells.

The effect of SN-38 on DR5 cell surface expression was investigated by flow cytometry. Increased DR5 levels were detected on HCT116 and COLO 205 cells after 24 h treatment with 0.01 \( \mu \text{mol/L} \) and 10 \( \mu \text{mol/L} \) SN-38, respectively, as shown in Fig. 1C. DR5 expression was unchanged in SW948 and HT-29 cells after SN-38 treatment. SN-38 treatment for 48 h did not further up-regulate DR5 expression in any of the cell lines (data not shown).

**Western blot analysis of colon cancer cell lines treated with TRA-8 and SN-38.** The four colon cancer cell lines were used to examine alterations in apoptosis-related proteins produced...
by in vitro treatment with TRA-8 alone, SN-38 alone, or the combination. As seen in Fig. 2A, all three cell lines that were sensitive to TRA-8–mediated cytotoxicity had TRA-8–induced cleavage of caspase-3, caspase-8, caspase-9, PARP, and Bid with minimal or no effects in HT-29 cells. These changes were not seen with SN-38–alone treatment. The combination treatment had a more pronounced but similar pattern as TRA-8 alone for TRA-8–sensitive cell lines. Combination treatment induced dose-dependent cleavage of caspase-3, caspase-8, caspase-9, PARP, and Bid in the TRA-8–resistant cell line (HT-29) at 0.1 and 1 μmol/L SN-38.

As seen in Fig. 2B, the cell line most sensitive to SN-38 (HCT116) had a dramatic SN-38–mediated induction of p53 (wild-type), as well as the p53-regulated protein Bax. SN-38 induced a modest increase in p53 (mutated) and Bax in COLO 205, whereas the p53 and Bax levels were unaffected in SW948 (p53 absent) or HT-29 cells (high p53). As regards inhibitors of apoptosis, survivin levels increased in the presence of SN-38 and decreased with the addition of TRA-8 (COLO 205, SW948, and HCT116). HT-29 cells had a dramatic increase in survivin due to the presence of SN-38, which was not affected by the addition of TRA-8. XIAP levels were reduced by TRA-8 alone or in combination for all three TRA-8–sensitive cell lines but only reduced by TRA-8/SN-38 combination in HT-29 cells. Bcl-xl levels were high and unchanged by drug exposure except for HT-29, where high levels were modestly reduced by TRA-8, moderately reduced by SN-38, and dramatically reduced by the combination.

In vivo antitumor activity of TRA-8, CPT-11, or the combination against colon cancer xenografts. We examined the in vivo antitumor efficacy of TRA-8, CPT-11, or the combination in animals bearing COLO 205, SW948, HCT116, or HT-29 xenografts. Table 1 provides our aggregate experience in terms of tumor doubling time and complete tumor regressions with each treatment regimen. Single agent TRA-8 or CPT-11 produced moderate and significant inhibition of COLO 205 tumor growth ($P < 0.001$), whereas the combination produced dramatic inhibition of tumor growth compared with no treatment ($P < 0.001$), TRA-8 alone ($P = 0.0002$), or CPT-11 alone ($P = 0.009$). Combination therapy produced complete regression of COLO 205 tumors in 73% of animals, and half of these animals had no tumor recurrence over 93 to 270 days of observation. Single agent TRA-8 or CPT-11 produced 17% and 20% complete regressions, respectively, with about half of these animals having no recurrence. A typical experiment with COLO 205 is depicted in Fig. 3A. The animals were given multiple doses of CPT-11 and TRA-8, which circulates for several days, so that both agents are present throughout the treatment cycle and the potential benefit of treating with CPT-11 before TRA-8 is expected to be limited.

The SW948 pattern of antitumor efficacy was quite different than COLO 205 in that TRA-8 alone had no beneficial effect whereas single agent CPT-11 significantly inhibited tumor growth (tumor doubling time, 37 ± 33 days) compared with control ($P < 0.001$) or TRA-8–treated animals ($P < 0.001$). Although TRA-8 alone had no benefit, its combination with CPT-11 produced further inhibition of tumor growth (67 ± 39 days) compared with CPT-11 alone ($P = 0.0016$). The benefit of combination therapy was further supported by an incidence of 36% complete regression compared with 4% for CPT-11 alone and none in TRA-8–treated or control animals. A typical experiment is presented in Fig. 3B.

HCT116 tumor–bearing animals treated with TRA-8 alone had a modest inhibition of tumor growth (doubling time, 37 ± 24 days; $P = 0.002$). Treatment with CPT-11 alone produced a moderate inhibition of tumor growth (56 ± 4 days; $P = 0.002$ compared with untreated). Combination therapy produced a further inhibition of tumor growth (78 ± 10 days) compared with TRA-8 alone ($P = 0.002$) or CPT-11 alone ($P = 0.02$). There was only one complete regression which occurred in the TRA-8–alone treatment group. The experiment is depicted in Fig. 3C.

Finally, HT-29 tumor cells, which were very resistant to TRA-8–induced cytotoxicity in vitro, formed tumors that were not inhibited by TRA-8 therapy alone (doubling time, 33 days) compared with untreated. Combination therapy produced 17% complete regression in 73% of animals, and half of these animals had no tumor recurrence over 93 to 270 days of observation. Single agent TRA-8 or CPT-11 produced 17% and 20% complete regressions, respectively, with about half of these animals having no recurrence. A typical experiment with COLO 205 is depicted in Fig. 3A. The animals were given multiple doses of CPT-11 and TRA-8, which circulates for several days, so that both agents are present throughout the treatment cycle and the potential benefit of treating with CPT-11 before TRA-8 is expected to be limited.

The SW948 pattern of antitumor efficacy was quite different than COLO 205 in that TRA-8 alone had no beneficial effect whereas single agent CPT-11 significantly inhibited tumor growth (tumor doubling time, 37 ± 33 days) compared with control ($P < 0.001$) or TRA-8–treated animals ($P < 0.001$). Although TRA-8 alone had no benefit, its combination with CPT-11 produced further inhibition of tumor growth (67 ± 39 days) compared with CPT-11 alone ($P = 0.0016$). The benefit of combination therapy was further supported by an incidence of 36% complete regression compared with 4% for CPT-11 alone and none in TRA-8–treated or control animals. A typical experiment is presented in Fig. 3B.

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12 ± 2 days compared with untreated, 11 ± 3 days) but there was modest efficacy of CPT-11 alone (26 ± 5 days; \( P = 0.0009 \) compared with untreated). Furthermore, TRA-8, in combination with CPT-11, had no increase in efficacy compared with CPT-11 alone (\( P = 0.87 \)). The experiment is depicted in Fig. 3D. In vivo therapy studies were also conducted to examine the efficacy of TRA-8, in combination with topotecan or docetaxel against HT-29 xenografts, as shown in Supplementary Fig. S3. Little tumor growth inhibition occurred in mice treated with either drug alone, and combination therapy with TRA-8 and drug produced no benefit in this model compared with drug alone.

Excessive toxicity was not observed using the present TRA-8 and CPT-11 dosing schedule, and mean weight loss was <15% in mice treated with CPT-11 alone or in combination with TRA-8. However, this treatment regimen approaches the MTD for CPT-11, because 12.6% of mice exhibited >15% weight loss during treatment and two deaths occurred out of 167 mice (1.2%) treated in preliminary studies and those reported here. Some animals were also subjected to a second treatment cycle with TRA-8 and CPT-11, which produced an increase in the time to tumor doubling in the SW948 xenograft model (Supplementary Fig. S4) with no enhancement of toxicity compared with one cycle of treatment.

In summary, xenografts of three colon cancer cell lines, which had high or intermediate sensitivity to TRA-8–mediated cytotoxicity in vitro, achieved optimal antitumor efficacy with a combination of TRA-8 and CPT-11, whereas the single cell line that was TRA-8–resistant derived no benefit from TRA-8 alone or when added to CPT-11, topotecan, or docetaxel. It is possible that the levels of TRA-8 binding to HT-29 cells may not reach the threshold required to induce apoptosis in vivo.

**Discussion**

We and others have examined the sensitivity of various human tumor cell lines to TRAIL and death receptor agonistic antibodies as single agents and in combination with chemotherapy agents (4, 5, 10). In this study, we compared the sensitivity of four different colon cancer cell lines to an agonistic anti-DR5 monoclonal antibody (TRA-8), either alone or in combination with SN-38 (active metabolite of irinotecan), both in vitro and in vivo. We also examined the response of TRA-8–resistant HT-29 cells and xenografts to TRA-8 in combination with additional chemotherapeutic drugs. These observations illustrate both similarities and substantial differences in the biological responses of these cell lines after exposure to TRA-8.

Using flow cytometry analysis, all four cell lines expressed DR5 on their tumor cell membranes, although the weakest expression was observed on the HT-29 cells (Fig. 1A). All four cell lines exhibited TRA-8–mediated cytotoxicity, but their sensitivity or resistance to cytotoxicity was widely disparate with COLO 205 having a high sensitivity \( \left( IC_{50} 2.4 \text{ ng/mL} \right) \) and HT-29 being relatively resistant \( \left( IC_{50} \geq 1,000 \text{ ng/mL} \right) \). SW948 and HCT116 had intermediate sensitivity with \( IC_{50} \) values of 9.2 and 27.2 ng/mL, respectively (Fig. 1B). The three cell lines which were sensitive to TRA-8–mediated cytotoxicity were found to exhibit tumor cell cleavage of caspase-3, caspase-8, caspase-9, PARP, and Bid at cytotoxicity-inducing dose levels, reflecting their apoptotic mechanism of cell death (Fig. 2A). Similar evidence for apoptosis has been presented for a variety of cell lines treated with TRAIL or death receptor antibodies (9, 11, 12, 23, 24). HT-29 cells had a minimal level of caspase cleavage even with exposure to 1,000 ng/mL of TRA-8.

Sensitivity of the individual cell lines to single agent SN-38 cytotoxicity varied greatly and was different than their sensitivity to TRA-8 (Fig. 1B). The \( IC_{50} \) values were 0.02 \( \mu \text{mol/L} \) (HCT116), 0.5 \( \mu \text{mol/L} \) (HT-29), 1 \( \mu \text{mol/L} \) (COLO 205), and >10 \( \mu \text{mol/L} \) (SW948). When cells were exposed to the combination of TRA-8 and SN-38, all four cell lines had enhanced cytotoxicity compared with either agent alone (Fig. 1B). Interestingly, the TRA-8–resistant cell line (HT-29) exhibited synergistic cytotoxicity with the addition of low doses of SN-38, whereas the SN-38–resistant cell line (SW948) also had synergistic cytotoxicity when as little as 5 ng/mL of TRA-6 was added. Thus, combination of TRA-8 and SN-38 achieved optimal in vitro cytotoxicity with all four colon cancer cell lines. This was reflected in the enhanced cleavage of caspases, PARP, and Bid elicited by combination therapy (Fig. 2A). The chemotherapy enhancement of death receptor–mediated apoptosis has been reported with a variety of cell lines and drugs (3, 10, 11, 23–30). As also illustrated in Fig. 2A, SN-38 as a single agent did not trigger apoptosis as evidenced by the absence of cleaved caspases, PARP, or Bid.

We attempted to explore potential mechanisms playing a role in the enhanced in vitro effects of combination therapy. Other studies have suggested that chemotherapy agents can enhance death receptor–mediated cytotoxicity by enhancing expression of tumor DR5 expression (3, 23, 26, 27). Our analysis of SN-38 effects on tumor cell DR5 expression at drug levels used in the cytotoxicity assays were unimpressive (Fig. 1C). HT-29 cells, which were TRA-8 resistant and had synergistic cytotoxicity with addition of SN-38, had no change in their minimal level of DR5.
expression with 24 or 48 h of drug exposure. Similarly, SW948, which exhibited synergistic cytotoxicity with combination therapy, had no significant change in DR5 expression. The two cell lines that showed either a modest increase (COLO 205) or moderate increase (HCT116) in DR5 expression (Fig. 1C) did not have synergistic effects from combination therapy.

A number of prior studies have proposed that the enhancement of cytotoxicity observed with combinations of chemotherapy and death receptor signaling is due to chemotherapeutic modulation of molecules that either enhance or inhibit the apoptotic pathway (4, 5). We surveyed a number of such regulatory molecules in each of the four colon cancer cell lines at baseline and after TRA-8 and SN-38 exposure (Fig. 2B). The results of this survey were quite variable among the four cell lines. In all four cell lines, the combination treatment seemed to reduce levels of XIAP from baseline. This is interesting because a recent report described the ability of a small molecule inhibitor of XIAP to dramatically enhance cytotoxicity by TRAIL (31). All four cell lines had induction of survivin on exposure to single agent SN-38, especially HT-29 cells. This would presumably inhibit apoptosis, and yet SN-38 produced synergistic cytotoxicity when combined with TRA-8 in HT-29 cells. Bcl-xl was prominent baseline in all four cell lines, and levels were decreased in HT-29 by the combination of TRA-8 and SN-38. These studies provided no consistent mechanism for SN-38 enhancement of TRA-8-mediated cytotoxicity. Each cell line seems to have its own baseline and treatment-related pattern of response of these factors.

We then examined the effects of TRA-8 and CPT-11 on xenografts of each of these four colon cancer cell lines, as depicted in Table 1 and Fig. 3. Each of the cell lines had its own pattern of TRA-8 and CPT-11 antitumor efficacy. The TRA-8–sensitive COLO 205 tumors had substantial efficacy from single agent TRA-8 or CPT-11 (P < 0.001), including 17% and 20% complete regressions. The combination of TRA-8 and CPT-11 had impressive efficacy greater than single agent TRA-8 (P = 0.0002) or CPT-11 (P = 0.009), with 73% complete regressions and 36% of animals who had no relapse. This high degree of efficacy in COLO 205 tumors may reflect their in vitro sensitivity to SN-38, modest enhancement of DR5 expression, and moderate inhibition of XIAP and survivin by combination therapy.

In contrast, the TRA-8 resistant HT-29 tumors derived no benefit from treatment with TRA-8. Animals treated with single agent CPT-11 had a modest prolongation of tumor doubling time (26 ± 5 versus 11 ± 3 days), which was significant (P = 0.0009). Combination therapy was no better than single agent CPT-11, suggesting that the synergistic effects of the combination in vitro did not translate to the in vivo setting. This finding was also true for HT-29 xenografts treated with TRA-8 and topotecan or docetaxel, despite the enhanced in vitro cytotoxicity produced by combination treatments with these drugs (Supplementary Figs. S2 and S4). The low cell membrane DR5 expression may have been a contributing factor to the poor effects of TRA-8 in this model.

The results of therapy in SW948 xenografts were unexpected. This cell line had an appreciable sensitivity to TRA-8 in vitro (IC50, 9.2 ng/mL) and was resistant to SN-38–mediated cytotoxicity (IC50 >10 μmol/L). However, SW948 tumors were unresponsive to TRA-8 treatment, and CPT-11 had moderate efficacy (P < 0.001). Despite lack of efficacy by TRA-8, the combination of TRA-8 and CPT-11 was superior to CPT-11 alone (P = 0.0016), including 36% complete regressions. These observations reflect the discrepancies often noted in correlating in vitro with in vivo therapeutic strategies.

In vitro, HCT116 cells had moderate sensitivity to TRA-8 (IC50, 27.2 ng/mL) and high sensitivity to SN-38 (IC50 0.02 μmol/L). In vivo, HCT116 tumors had a moderate response to TRA-8 with a doubling time of 37 ± 24 days (P = 0.002 compared with 21 ± 1 days with no therapy) and an even better response to single agent CPT-11 (doubling time, 56 ± 4 days with P = 0.002 versus TRA-8 alone). Combination therapy (doubling time, 78 ± 10 days) was more effective than single agent TRA-8 (P = 0.002) or CPT-11 (P = 0.02).

These in vivo observations correlated reasonably well with the in vitro studies. We also examined TRA-8 localization to xenograft tumors of each of these cell lines as an additional variable in the treatment strategy. Radiolabeled TRA-8 was found to have specific and comparable radiolocalization to each of the cell line tumors, including HT-29, despite its modest tumor cell membrane expression of DR5. These studies illustrate the considerable in vitro and in vivo variation observed in tumor cell line responsiveness to an anti-DR5 agonistic monoclonal antibody (TRA-8) alone, CPT-11 alone, or the combination. However, it is clear that combination therapy with TRA-8 and CPT-11 was superior to either single agent alone in three of the four tumor cell lines. These three cell lines shared a high or moderate sensitivity to TRA-8 in vitro, moderate to high cell surface expression of DR5, and inhibition of XIAP and survivin after in vitro exposure to the combination of TRA-8 and CPT-11. The poorly responsive HT-29 cell line was resistant to TRA-8 in vitro, had minimal surface membrane expression of DR5, and no inhibition of survivin with in vitro exposure to the combination of TRA-8 and CPT-11. These studies further emphasize the need to use the combination of chemotherapy with death receptor targeting in future clinical trials.

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