

# Antitumor Efficacy of TRA-8 Anti-DR5 Monoclonal Antibody Alone or in Combination with Chemotherapy and/or Radiation Therapy in a Human Breast Cancer Model<sup>1</sup>

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## ABSTRACT

**Purpose:** A monoclonal antibody (TRA-8) has been developed that binds to death receptor 5 (DR5), one of two death receptors bound by tumor necrosis factor-related apoptosis-inducing ligand. The purpose of this study was to evaluate *in vitro* the binding and cytotoxicity of TRA-8 to human breast cancer cell lines. The antitumor efficacy of TRA-8 was evaluated in a xenograft human breast cancer murine model, as a single agent and in combination with chemotherapy or radiation therapy.

**Experimental Design:** The binding of TRA-8 to a panel of nine human breast cancer cell lines was evaluated by indirect immunofluorescence and flow cytometry. Cytotoxicity of TRA-8 alone and in the presence of Adriamycin or paclitaxel was measured *in vitro* using the ATP-lite assay. Antitumor efficacy was determined by treatment of nude mice bearing well-established s.c. DR5-positive 2LMP human breast cancer xenografts with TRA-8 alone or in combination with Adriamycin or paclitaxel. Tumor size and regression rates were determined. In addition, a study was carried out with TRA-8 and Adriamycin in combination with 3 Gy <sup>60</sup>Co irradiation of 2LMP xenografts on days 9 and 17.

**Results:** All nine human breast cancer cell lines expressed DR5 with TRA-8 reactivity varying from strongly to weakly positive. Four cell lines were sensitive to TRA-8 cytotoxicity with IC<sub>50</sub> of 17–299 ng/ml, whereas other cell lines had weak cytotoxicity or were resistant. *In vivo* studies demonstrated significant inhibition of growth of 2LMP xe-

nografts by TRA-8 treatment alone. The combination of TRA-8 + Adriamycin or paclitaxel produced significant inhibition of tumor growth as compared with controls or either agent alone. An aggregate analysis of all 166 animals studied demonstrated that TRA-8 alone or in combination with Adriamycin, paclitaxel, or radiation produced a significant increase in tumor doubling time compared with any modality alone with mean doubling time in days of 12 (untreated), 14 (radiation), 17 (Adriamycin), 25 (paclitaxel), 39 (Adriamycin + radiation), 47 (TRA-8), 65 (TRA-8 + radiation), 71 (TRA-8 + paclitaxel), 81 (TRA-8 + Adriamycin), and >140 (TRA-8 + Adriamycin and radiation). Complete tumor regressions occurred in 1 of 42 untreated animals, 1 of 54 animals receiving chemotherapy and/or radiation, and 28 of 68 animals receiving TRA-8 alone or TRA-8 combination regimens. Fourteen of those 28 complete regressions did not relapse over periods of follow-up between 99 and 171 days, with a mean of 146 ± 24 days.

**Conclusions:** The TRA-8 anti-DR5 antibody alone or in combination with chemotherapy and/or radiation has striking antitumor efficacy in breast cancer xenograft models. Additional studies with other tumor types and chemotherapy agents are warranted. These studies support the generation of a humanized TRA-8 for introduction into early clinical trials.

## INTRODUCTION

TRAIL,<sup>3</sup> which was identified independently by two groups and is also known as Apo-2L, is a member of the TNF superfamily, which includes TNF-α and Fas ligand (1, 2). Although all three of these proteins are potent inducers of apoptosis, TRAIL has been of particular interest in the development of cancer therapeutics because it preferentially induces apoptosis of tumor cells (3, 4). However, concern had been raised as regards TRAIL toxicity to normal human hepatocytes (5, 6), although this may reflect the molecular design of the TRAIL reagents (7). Five receptors for TRAIL have been identified, two of which, DR4 (TRAIL-R1) and DR5 (TRAIL-R2; Refs. 8–10), are capable of transducing the apoptosis signal, whereas the others, DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (3, 11–13), presumably serve as decoy receptors to block or modulate TRAIL-mediated apoptosis. The intracellular segments of DR4 and DR5 contain a death domain, which trans-

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<sup>3</sup> The abbreviations used are: Apo-2L, apoptosis-2 ligand; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; TNF, tumor necrosis factor; FBS, fetal bovine serum; PE, phycoerythrin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

duces an apoptosis signal through a Fas-associated death domain- and caspase 8-dependent pathway (9, 10, 14–18). Most human cancer cell lines express DR4, DR5, or both, and many are susceptible to TRAIL-induced apoptosis (6, 19–22). Most normal cells appear to be resistant to TRAIL because of a variety of mechanisms (21, 23, 24). Ashkenazi *et al.* (19, 21, 25) reported that lung fibroblasts, prostatic epithelial cells, and colon smooth muscle cells were resistant to apoptosis induction by TRAIL. Administration of soluble TRAIL in experimental animals with human colon, prostate, breast, glioma, and lung cancer xenografts induces significant tumor regression without systemic toxicity (21, 26). The potential for TRAIL-mediated apoptosis in anticancer therapy has been further demonstrated by greatly enhanced efficacy of chemotherapy and radiotherapy in combination with TRAIL (19, 20, 22, 25, 27–32).

We have reported previously on the development of a murine mAb called TRA-8, which binds specifically to DR5 and has agonistic characteristics, including the ability to induce signal transduction and apoptosis without the need for cross-linking agents or surface adherence (33, 34). This antibody does not induce apoptosis or cytotoxicity to normal cells, including hepatocytes *in vitro*.

The strategy of using apoptosis-inducing antibodies to specifically induce tumor apoptosis as antitumor therapy or enhance the antitumor efficacy of chemotherapy or radiation therapy is just emerging. Chuntharapai *et al.* (35) reported that a mAb that binds to the DR4 receptor had substantial antitumor efficacy in a COLO 205 human colon cancer xenograft model and somewhat less efficacy with the HCT 116 model.

The aims of this study were to determine the DR5 expression profile of human breast cancer cell lines and their *in vitro* sensitivity to cytotoxicity by TRA-8 anti-DR5 alone and in combination with chemotherapy agents. *In vivo* studies using a human breast cancer xenograft in nude mice examined the therapeutic efficacy of TRA-8 therapy alone or in combination with chemotherapy and/or radiation. This work may lead to a potential therapy for treatment of breast cancer using this novel death receptor antibody.

## MATERIALS AND METHODS

**Cell Lines and Reagents.** The 2LMP subclone of the human breast cancer cell line MDA-MB-231, the LCC6 subclone of MDA-MB-435, and the DY36T2 subclone of MDA-MB-361 were obtained from Dr. Marc Lippman (Georgetown University, Washington, DC) and maintained in improved MEM supplemented with 10% FBS (Hyclone, Logan, UT). The MDA-MB-231, MDA-MB-453, MDA-MB-468, BT-474, SK-BR-3, and ZR-75-1 human breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were grown in DMEM supplemented with MEM vitamins, MEM nonessential amino acids, 1 mM sodium pyruvate, and 10% FBS. BT-474 cells were grown in RPMI 1640 supplemented with 10  $\mu$ g/ml insulin, 4.5 grams/liter glucose, 10 mM HEPES, 1 mM sodium pyruvate, and 10% FBS. SK-BR-3 cells were grown in McCoy's medium with 15% FBS. ZR-75-1 cells were grown in Ham's F12K medium with 20% FBS. All cell lines were maintained in antibiotic-free medium at 37°C in a 5%

CO<sub>2</sub> atmosphere and routinely screened for *Mycoplasma* contamination.

Purified TRA-8 (IgG1) mAb was provided by Sankyo Co., Ltd. (Tokyo, Japan). Two lots of TRA-8 were used in these studies. They contained 4.1 and 9.2% aggregates determined by gel filtration analysis. The remainder of each preparation was monomeric TRA-8. The amount of endotoxin was below the detectable level (<5 pg/ml). PE-conjugated goat antimouse IgG1 and isotype-specific IgG1 control antibody were obtained from Southern Biotechnology Associates (Birmingham, AL). Adriamycin and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO) and prepared as 10 mM stock solutions in distilled H<sub>2</sub>O or DMSO, respectively. For animal studies, the clinical formulation of paclitaxel (Bristol-Myers Squibb Co., Princeton, NJ) was obtained from the University of Alabama at Birmingham Hospital Pharmacy (Birmingham, AL). This preparation was diluted 1:5 in PBS immediately before use.

**Indirect Immunofluorescence and Flow Cytometry Analysis of DR5 Expression.** Cells in exponential growth phase were washed once with Dulbecco's PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> deficient) and harvested with 4 mM EDTA/0.5% KCl at 37°C. Cells were collected by centrifugation at 4°C for 5 min at 1000 rpm, washed once, and resuspended in PBS containing 1% BSA and 0.01% sodium azide (FACS buffer) at 4°C. Cells were incubated with 10  $\mu$ g/ml purified TRA-8 or an isotype-specific IgG1 control antibody for 60 min at 4°C, washed once with buffer, then incubated with 10  $\mu$ g/ml PE-conjugated goat antimouse IgG1 for 20 min at 4°C. After antibody staining, cells were washed once with FACS buffer and fixed in 1% paraformaldehyde for 15 min on ice. Samples were analyzed on a Becton Dickinson FACScan (San Jose, CA), and data were analyzed using CellQuest software.

**Cell Viability Assays Using ATPLite.** Cells were trypsinized and resuspended in complete culture medium. One thousand cells per well were plated in optically clear 96-well black plates (Costar #3904, Corning, NY) and incubated overnight at 37°C before initiating treatments. Drugs and antibody were diluted in culture medium immediately before use, and the final concentration of DMSO was always  $\leq$ 0.001%. Cell viability was assessed after 24-h exposure to TRA-8 alone. For combination treatments with cytotoxic drugs, cells were pretreated with the drug for 24 h before adding antibody and incubated for an additional 24 h before assessing cell viability by measurement of cellular ATP levels using the ATPLite luminescence-based assay (Packard Instruments, Meriden, CT). The manufacturer's recommended protocol was followed with the exception that all reaction volumes (culture medium and reagents) were reduced by one-half. All samples were assayed in triplicate and are reported as the mean  $\pm$  SE from a minimum of three independent experiments.

**TRA-8 Therapy Studies Alone or in Combination with Chemotherapy or Radiation in Athymic Nude Mice Bearing Breast Cancer Xenografts.** Athymic nude mice were injected s.c. with  $3 \times 10^7$  2LMP cells. At 7 days after tumor cell injection, 200 or 600  $\mu$ g (10 or 30 mg/kg) TRA-8 were administered i.p. followed by five additional injections on days 10, 14, 17, 21, and 24. The growth of tumors was monitored over time. In subsequent studies, animals bearing well-established 2LMP s.c. tumors were injected i.p. with 200  $\mu$ g of TRA-8 on days 7,

10, 14, 17, 21, and 24 alone or in combination with Adriamycin (6 mg/kg i.v., days 8, 12, and 16) or paclitaxel (20 mg/kg i.p., on days 8, 12, 16, 20, and 24). The Adriamycin and paclitaxel regimens were established separately and represent the maximum tolerated dose for each drug. Tumor size and regression rates were determined. In addition, a study was carried out with TRA-8 and Adriamycin using the same regimen described above in combination with 3 Gy  $^{60}\text{Co}$  irradiation of 2LMP xenografts on days 9 and 17. The mean  $\pm$  SD baseline tumor size (surface area equal to product of two largest diameters) for all studies at the start of treatment was  $61 \pm 16 \text{ mm}^2$ .

**Analysis of Apoptosis in Xenografts.** Athymic nude mice injected s.c. with  $3 \times 10^7$  2LMP cells on day 0 received 100  $\mu\text{g}$  of TRA-8 i.p. on days 7 and 10. Groups of two mice each received Adriamycin (3 mg/kg) on days 8 and 11, paclitaxel (10 mg/kg) on days 8 and 11, or the combination of TRA-8 and Adriamycin or paclitaxel with the same dose and schedule. One group of mice was untreated. The xenografts were dissected for the study of apoptosis on day 14 after tumor cell injection. The reason for the substantial reduction in treatment intensity compared with our standard treatment protocol was to allow adequate tumor tissue for analysis on day 14. TUNEL assay for apoptosis in tumor xenografts was performed as follows. Five- $\mu\text{m}$  paraffin sections of tissue were mounted on Superfrost/Plus slides and heated at  $58^\circ\text{C}$  for 1 h. Tissue sections were deparaffinized in three changes of xylene and rehydrated with one change of absolute ethanol, 95% ethanol, and 70% ethanol, each in 5-min increments. Then, the sections were placed in Tris-buffered saline [0.5 M Tris base, 0.15 M NaCl, and 0.0002% Triton X-100 (pH 7.6)]. Apoptotic nuclei were detected using an Apop Tag Peroxidase kit (Intergen, Purchase, NY). Proteinase K (20  $\mu\text{g}/\text{ml}$  in distilled deionized  $\text{H}_2\text{O}$ ) was added to the tissue specimens and incubated at room temperature for 15 min. Endogenous peroxidases were quenched with an aqueous solution of 3% hydrogen peroxide for 5 min. Sections were treated with an equilibration buffer for 30 min and then incubated with the TdT/enzyme (diluted in labeling reaction mix) for 1 h at  $37^\circ\text{C}$  using parafilm covers. During this incubation, the TdT enzyme binds the 3'-OH ends of DNA fragments and catalyzes the addition of digoxigenin-labeled and unlabeled deoxynucleotides. Negative controls were incubated with distilled  $\text{H}_2\text{O}$  (diluted in labeling reaction mix) instead of the TdT enzyme. A stop buffer was added for 10 min at room temperature to terminate the labeling reaction. An antidigoxigenin conjugate was added to each slide for 30 min. The chromagen 3,3'-diaminobenzidine was used to visualize the labeled 3' OH end of DNA fragments. The slides were then rinsed in deionized water and lightly counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and coverslipped using Permount. Approximately 10 random fields were evaluated for percentage of TUNEL stained and percentage of intensely stained apoptotic bodies throughout the tissue.

### Statistical Analysis

**Analysis of TRA-8 Interaction with Drug Cytotoxicity *in Vitro*.** 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 nine cell lines (36), as recommended by Gennings (37). 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 Adriamycin or TRA-8 and paclitaxel would be considered additive, provided the additive terms were significant.

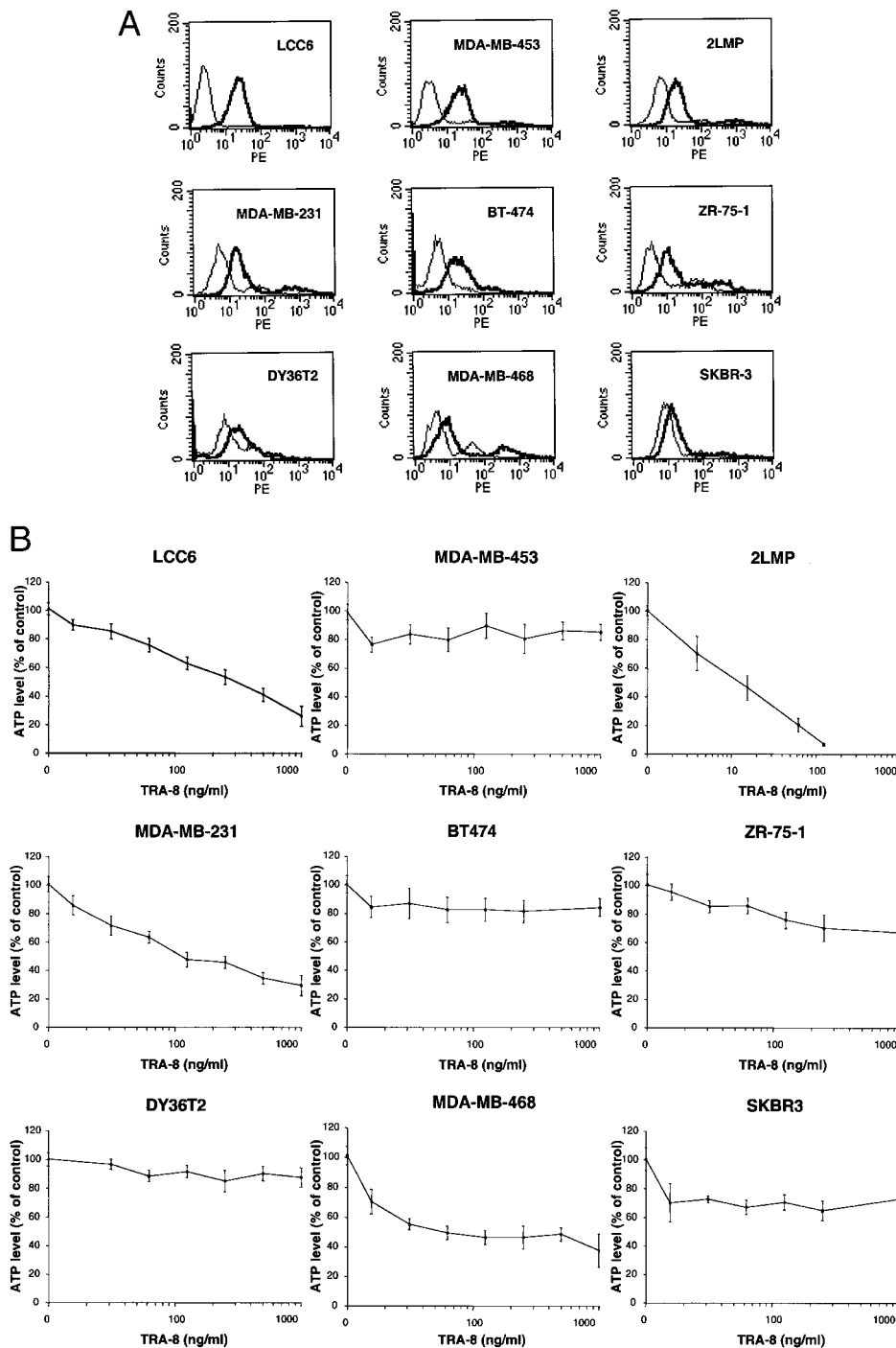
**Analysis of TRA-8, Chemotherapy, Radiation, and Combination Therapy of Individual Animal Experiments.** Data from six independent experiments were analyzed by individual experiments. Treatment combinations were compared with respect to *in vivo* antitumor efficacy, *i.e.*, inhibition of tumor growth, which was measured as three end points, extension of tumor doubling times, percentage of tumor regressions, and growth rates over time. The actual number of days at which the tumor doubled in surface area (product of two diameters) relative to baseline on day 7 after tumor cell injection was used in the doubling time analysis. The nonparametric Kruskal-Wallis test was used for median tumor doubling time comparisons between treatments. Fisher's exact test was used to compare the proportions of tumor regressions and relapse-free regressions across treatment groups. To determine whether any combination therapy produced significant synergistic inhibition of tumor growth, *i.e.*, more than additive, the growth curves from the serial area measurements were compared using a linear mixed model approach over the first 3 weeks after the start of therapy (38). To test for synergistic effects of the combination therapies, an interaction term was included in the model. If the interaction term was significant and the effect was inhibition of growth at a rate greater than additive, then the interaction was considered synergistic.

**Aggregate Analysis of Therapy Effects.** A total of 166 animals, 10 treatment groups, and 6 independent experiments was included in the aggregate analysis. Treatment combinations were compared with respect to *in vivo* antitumor efficacy. The median tumor doubling times were analyzed using the Kruskal-Wallis test, and Fisher's exact test was used to compare the proportions of tumor regressions and relapse-free regressions across treatment groups.

All statistical analyses were conducted using SAS<sup>®</sup> (39).

## RESULTS

**DR5 Expression and TRA-8 Induced Cytotoxicity in Breast Cancer Cell Lines.** As illustrated in Fig. 1A, all nine breast cancer cell lines were DR5 positive with various degrees of expression from strongly positive (LCC6 and MDA-MB-453) to weakly positive (MDA-MB-468 and SK-BR-3). Fig. 1B illustrates the TRA-8-induced cytotoxicity of the nine cell lines. Four cell lines were sensitive to TRA-8-induced cytotoxicity with  $\text{IC}_{50}$  concentrations of 17–299 ng/ml (LCC6, 2LMP, MDA-MB-231, and MDA-MB-468), whereas others were quite resistant (DY36T2, BT-474, and MDA-MB-453). There was not a good correlation of DR5 expression and degree of TRA-8-induced cytotoxicity as illustrated by cell lines MDA-MB-453 and MDA-MB-468.

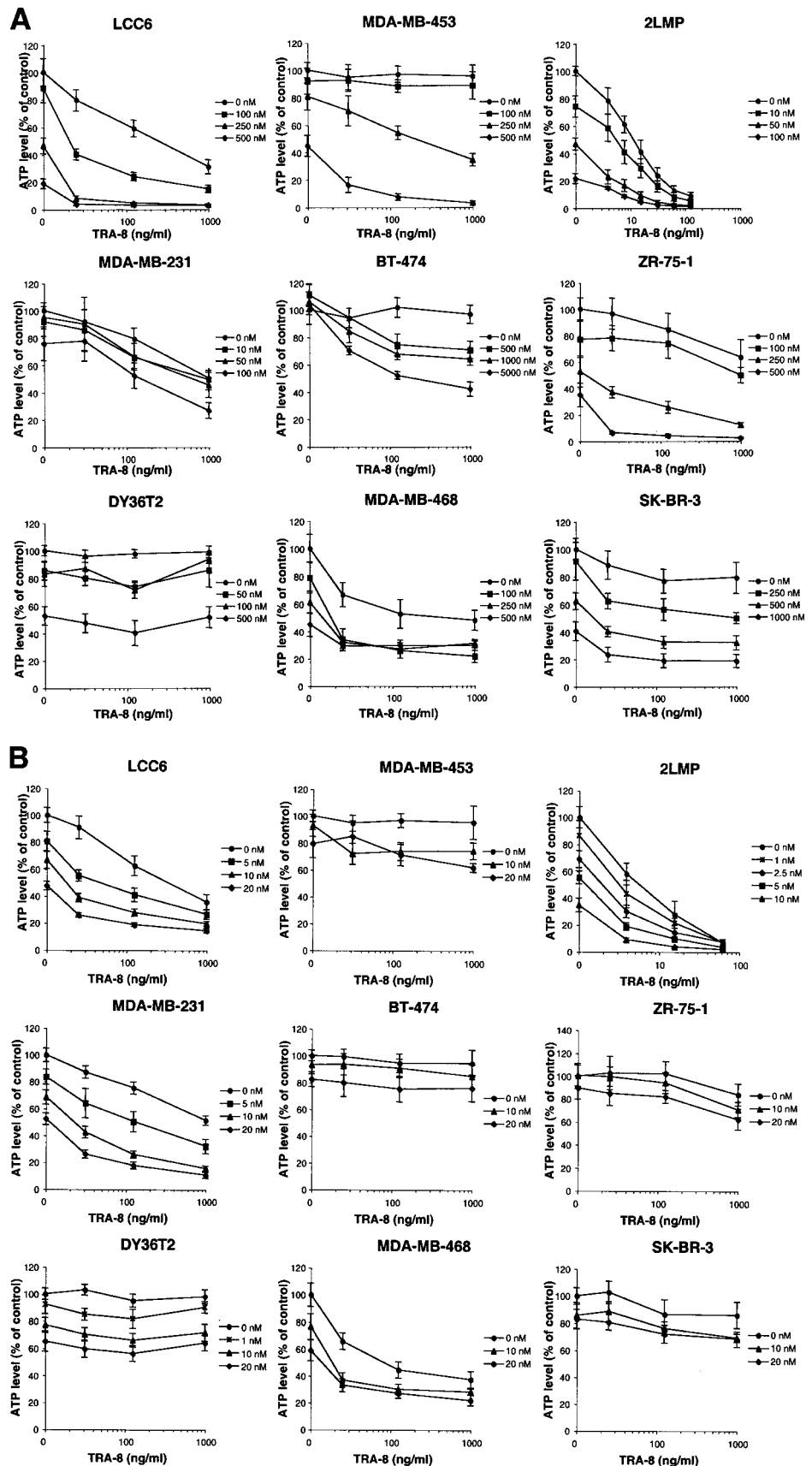


**Fig. 1** *A*, flow cytometry analysis of DR5 cell surface expression in a panel of human breast cancer cell lines. Breast cancer cells were harvested using EDTA and stained with 10  $\mu\text{g/ml}$  TRA-8 mAb for 1 h at 4°C followed by PE-conjugated goat antimouse IgG1, then analyzed using FACScan and CellQuest software. *Thick histograms*, TRA-8 staining; *thin histograms*, incubation with mouse IgG1 isotype control antibody. *B*, cytotoxicity of TRA-8 to human breast cancer cell lines. Cells were trypsinized and replated at a density of 1000 cells/well in a 96-well plate. TRA-8 antibody was added after plating cells and incubated for 24 h at 37°C. Cell viability was assessed 24 h after TRA-8 addition using the ATPLite assay. ATP levels are reported relative to untreated control cells as the mean and SE from two to three independent experiments, each done in triplicate.

TRA-8 effects on chemotherapy-induced cytotoxicity were then examined with Adriamycin (Fig. 2A) and paclitaxel (Fig. 2B). An analysis to test for interaction between antibody and drug effects (see “Materials and Methods”) is summarized in Table 1. There were no significant synergistic interactions between TRA-8 and paclitaxel, with most of the interactions being additive. Four of nine cell lines fulfilled criteria for a synergistic interaction between TRA-8 and Adriamycin. The cell line

2LMP demonstrated good sensitivity to TRA-8, as well as sensitivity to either Adriamycin or paclitaxel. This cell line was chosen to explore *in vivo* efficacy of antibody and/or drugs.

***In Vivo* Antitumor Effects of TRA-8 Alone or in Combination with Chemotherapy and/or Radiation.** TRA-8 at doses of 200 and 600  $\mu\text{g}$  twice a week for six doses produced a similar inhibition of tumor growth for well-established 2LMP s.c. tumors (Fig. 3). In three additional independent experi-



*Fig. 2* A, cytotoxicity of TRA-8 and Adriamycin combination treatment of human breast cancer cell lines. Cells (1000/well) were exposed to various concentrations of Adriamycin for 24 h at 37°C beginning 24 h after plating cells. TRA-8 was added 24 h after Adriamycin addition, and ATP levels were determined 24 h later. Values represent the mean and SE of triplicate determinations from two to four independent experiments each done in triplicate and are reported relative to untreated control cells. B, cytotoxicity of TRA-8 and paclitaxel combination treatment of human breast cancer cell lines. Cells (1000/well) were exposed to various concentrations of paclitaxel for 24 h at 37°C beginning 24 h after plating cells. TRA-8 was added 24 h after paclitaxel addition, and ATP levels were determined 24 h later. Values represent the mean and SE of triplicate determinations from two to four independent experiments each done in triplicate and are reported relative to untreated control cells.

Table 1 *In vitro* interaction effects for combination treatments

Cell line	TRA-8 + Adriamycin		TRA-8 + Paclitaxel	
	Interaction	$P^a$	Interaction	$P^a$
LCC6	Synergistic	<0.001	Additive	0.624
MDA-MB-453	Synergistic	<0.001	No response <sup>b</sup>	0.615
2LMP	Additive	0.153	Additive	0.937
MDA-MB-231	Additive	0.663	Additive	0.064
BT-474	Synergistic	<0.001	ND <sup>c</sup>	0.992
ZR-75-1	Synergistic	0.013	Additive	0.172
DY36T2	ND <sup>c</sup>	0.808	ND <sup>c</sup>	0.798
MDA-MB-468	Additive	0.184	Additive	0.724
SK-BR-3	Additive	0.361	No response <sup>b</sup>	0.871

<sup>a</sup>  $P$  refers to the significance of the synergistic interaction term. If both TRA-8 and drug effects were significant and the interaction term was significant, then the combination effects were considered synergistic. If the interaction  $P$  is not <0.05, then the combination effects were considered additive.

<sup>b</sup> There was no significant dose response for either agent.

<sup>c</sup> Not determined because the TRA-8 effect was not significant, but the Adriamycin/paclitaxel effect was significant.

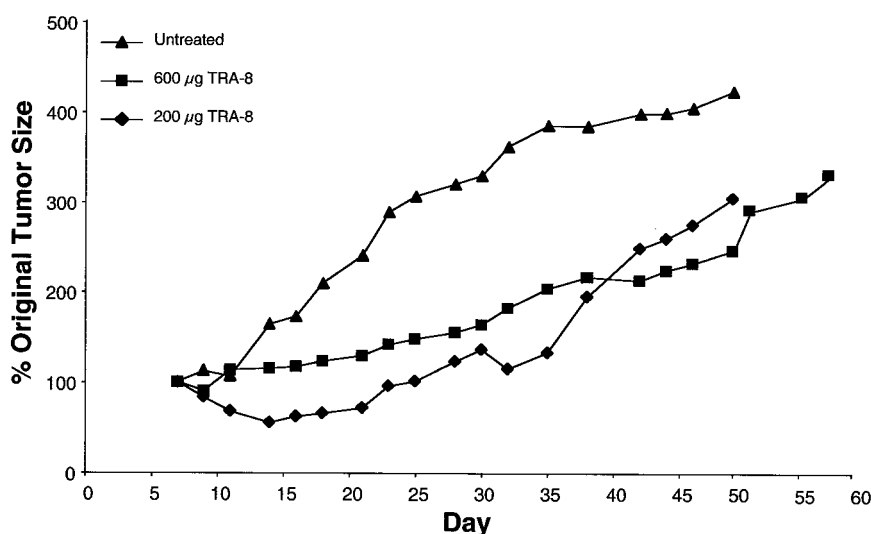


Fig. 3 The effect of TRA-8 on tumor growth in athymic nude mice bearing established 2LMP human breast cancer xenografts. 2LMP cells ( $3 \times 10^7$ ) were injected s.c. on day 0. Two groups of mice were injected i.p. with 200 or 600 µg of TRA-8 on days 7, 10, 14, 17, 21, and 24. One group of mice received no antibody. The data represent the average change in tumor size (product of two diameters) relative to size on day 7 ( $n = 8$  mice/group).

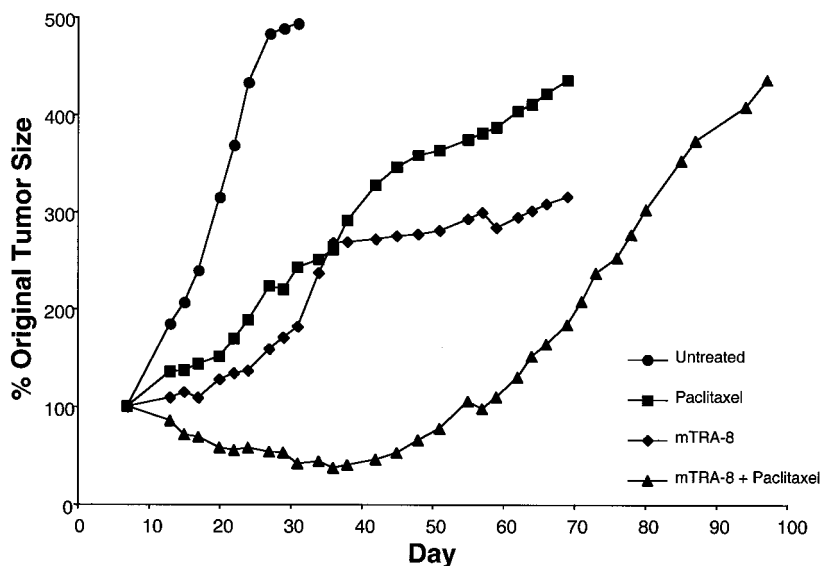
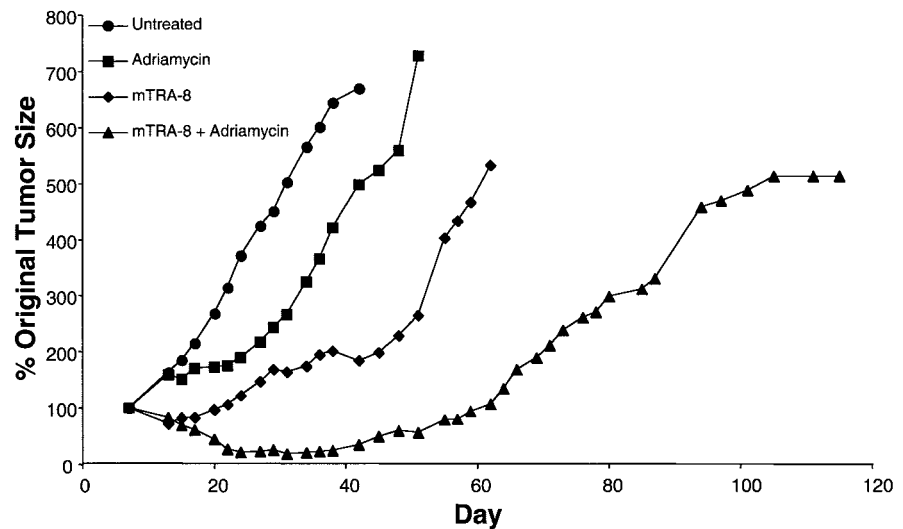
ments, the 200-µg dose/schedule produced statistically significant inhibition of tumor growth ( $P < 0.004$ ; Kruskal-Wallis test on tumor doubling times) compared with untreated controls, and this dose and schedule were selected for additional studies. Fig. 4 illustrates the effects of TRA-8, Adriamycin, or a combination of TRA-8 and Adriamycin on antitumor efficacy. As compared with untreated controls, therapy with TRA-8 alone or TRA-8 plus Adriamycin produced significant inhibition of tumor growth ( $P = 0.002$ ; Kruskal-Wallis test), whereas Adriamycin did not differ from controls. The combination of TRA-8 plus Adriamycin produced greater growth inhibition than either agent alone ( $P = 0.002$ ), as well as significantly more complete regressions of tumor (four) than either agent alone where no complete regressions were seen ( $P < 0.001$ ; Fisher's exact test). *In vivo* TRA-8 and Adriamycin synergism were evaluated using an early growth curve analysis (as described in "Materials and Methods"). The interaction term was significant ( $P < 0.001$ ) and synergistic. The synergistic interaction was corroborated in a second independent experiment.

The effects of TRA-8 and paclitaxel were studied in this

same model with similar observations (Fig. 5). As compared with untreated controls, TRA-8 and the TRA-8 plus paclitaxel produced significant inhibition of tumor growth ( $P < 0.001$ ; Kruskal-Wallis test). Tumor growth in animals treated with TRA-8 plus paclitaxel was significantly different from paclitaxel alone ( $P = 0.008$ ) and produced three of eight complete regressions as compared with none for either agent alone. The effects of the combination of TRA-8 and paclitaxel were additive ( $P < 0.001$ ) but not synergistic ( $P = 0.063$ ).

Finally, we examined the effects of TRA-8, Adriamycin, and  $^{60}\text{Co}$  radiation as single agents and in various combinations as illustrated in Fig. 6. There were significant differences overall with respect to tumor doubling times ( $P < 0.001$ ), and multiple comparisons indicated that the triple therapy with TRA-8, Adriamycin, and  $^{60}\text{Co}$  produced tumor growth inhibition that was significantly different from all other treated groups, whereas both dual therapy groups (Adriamycin plus TRA-8 or  $^{60}\text{Co}$  plus TRA-8) were different from either single agent group ( $P < 0.001$ ). The  $^{60}\text{Co}$  animals treated with radiation alone did not differ from untreated controls ( $P = 0.926$ ). All two-way treat-

**Fig. 4** The effect of TRA-8 and Adriamycin on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells ( $3 \times 10^7$ ) were injected s.c. into athymic nude mice on day 0. Two groups of mice were injected i.p. with 200  $\mu\text{g}$  of TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received i.v. Adriamycin (6 mg/kg) on days 8, 12, and 16. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 ( $n = 6-8$  mice/group).



**Fig. 5** The effect of TRA-8 and paclitaxel in athymic nude mice bearing breast cancer xenografts. 2LMP cells ( $3 \times 10^7$ ) were injected s.c. into athymic nude mice on day 0. Two groups of mice were injected i.p. with 200  $\mu\text{g}$  of TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received i.v. paclitaxel (20 mg/kg) on days 8, 12, 16, 20, and 24. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 ( $n = 8$  mice/group).

ment combinations had significant synergistic effects ( $P < 0.001$ ). Complete regressions were seen in six of eight animals receiving triple therapy, and four animals did not have tumor recurrence over 180 days of follow-up.

**Aggregate Analysis of Therapy Effects.** The *in vivo* antitumor studies were comprised of 166 animals, and we analyzed the tumor doubling times and frequency of complete tumor regression for all animals in each treatment group (Table 2). ANOVA analysis for mean tumor doubling times indicated significant differences among treatment groups ( $P < 0.001$ ), with multiple comparisons yielding that TRA-8 + paclitaxel, TRA-8 + Adriamycin, and TRA-8 + Adriamycin +  $^{60}\text{Co}$  had significantly longer mean tumor doubling times than any treatment group lacking TRA-8. The addition of TRA-8 to any treatment modality produced a longer tumor doubling time than that modality alone. Similarly, Kruskal-Wallis test on median

time to tumor doubling yielded that the medians were significantly different overall ( $P < 0.001$ ). Pair-wise comparisons using Wilcoxon's signed rank test yielded similar patterns for median time to tumor doubling as the ANOVA multiple comparisons. This analysis underestimates the growth inhibition produced by the most effective treatments in that groups that did not reach a doubling of tumor size by the end of the experiment were assigned the experiment termination day. Table 2 also provides the frequency of complete regression of tumor and the frequency of persistence of that regression to the end of the experiment. There were no complete regressions of tumor seen in animals treated with either chemotherapy regimen or radiation attesting to the well-established tumor growth and tumor aggressiveness. From Fisher's exact test, there were significant differences in the frequency of tumor complete regressions between treatment groups ( $P < 0.001$ ). Thirty of 166 animals

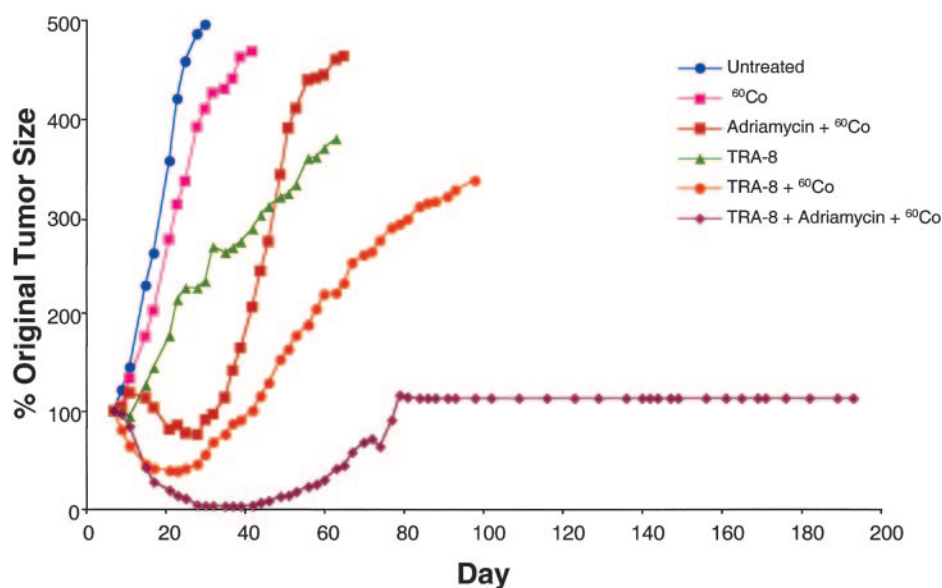


Fig. 6 The effect of TRA-8, Adriamycin, and  $^{60}\text{Co}$  radiation on tumor growth in athymic nude mice bearing breast cancer xenografts. 2LMP cells ( $3 \times 10^7$ ) were injected s.c. into athymic nude mice on day 0. Three groups of mice were injected i.p. with 200  $\mu\text{g}$  of TRA-8 on days 7, 10, 14, 17, 21, and 24. Two groups of mice received i.v. Adriamycin (6 mg/kg) on days 8, 12, and 16. Four groups of mice received 3 Gy  $^{60}\text{Co}$  radiation on days 9 and 17. One group of mice received no antibody. Data are expressed as the average change in tumor size (product of two diameters) relative to size on day 7 ( $n = 8$  mice/group).

Table 2 Aggregate results of doubling time and complete regression of 2LMP tumors

Treatment	No. of animals	Tumor doubling time (days) (mean/median)	Complete regressions		
			Total (%)	No relapse (%)	Mean observation period (days)
Untreated Controls	44 (42) <sup>a</sup>	12/8	1 (2%)	1 (2%)	177
$^{60}\text{Co}$	8 (7)	14/10	0	0	186
Adriamycin	31 (28)	17/18	0	0	197
Paclitaxel	7 (5)	25/20	0	0	
Adriamycin + $^{60}\text{Co}$	8 (8)	39/36	1 (13%)	0	197
TRA-8	30 (26)	47/23	6 (20%)	5 (17%)	159
TRA-8 + $^{60}\text{Co}$	8 (8)	65/50	3 (38%)	1 (13%)	186
TRA-8 + Paclitaxel	8 (8)	71/62	3 (38%)	1 (13%)	148
TRA-8 + Adriamycin	14 (12)	81/64	10 (71%)	3 (21%)	185
TRA-8 + Adriamycin + $^{60}\text{Co}$	8 (6)	>140/179	6 (75%)	4 (50%)	192

<sup>a</sup> The numbers in parentheses are the number of uncensored animals.

achieved complete regression, and 28 of these received TRA-8 alone or in combination with other modalities. Complete regression occurred in 1 of 42 control animals; 1 of 54 animals receiving chemotherapy, radiation, or a combination; and 28 of 68 of TRA-8 alone or TRA-8 combination regimens. The TRA-8-treated groups had a significantly ( $P < 0.001$ ) greater frequency of complete regression. Similarly, 14 of 68 animals receiving TRA-8 or TRA-8 combinations did not have tumor regrowth compared with 1 of 42 controls and 0 of 52 animals treated with chemotherapy and/or radiation. The relapse-free regressions had observation periods of 99–171 days ( $146 \pm 24$  days).

**Apoptosis in Treated Tumors.** The induction of apoptosis in 2LMP xenografts after treatment with TRA-8, Adriamycin, paclitaxel, TRA-8 + Adriamycin, and TRA-8 + paclitaxel was assessed using the TUNEL technique (Fig. 7). In untreated animals, tumors had 4% stained cells (1% intense), whereas treatment with Adriamycin or paclitaxel had 8% (6% intense) and 7% (2% intense) stained cells. Animals treated with TRA-8 alone had striking apoptosis with 25% (15% intense)

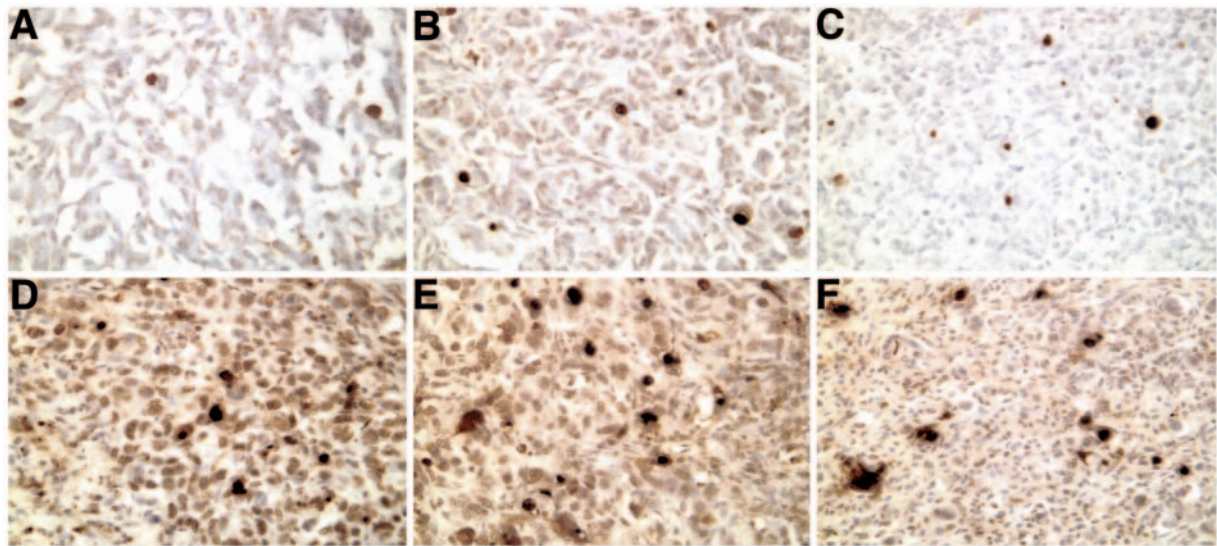
stained cells. TRA-8 plus Adriamycin had 28% (22% intense) and TRA-8 plus paclitaxel had 26% (12% intense) stained cells.

## DISCUSSION

The past decade has witnessed the clinical development of mAbs, including Food and Drug Administration approval for malignant and nonmalignant indications (40–42). These efforts have delineated a clear path of drug development, taking advantage of the *in vivo* kinetics and biodistribution of antibody molecules, their specificity to biologically important targets, and their intrinsic lack of toxicity. Our research group has begun to explore the use of mAbs to the family of cell surface TNF receptors as therapeutics for immune-mediated (33) and malignant disease (33, 34, 43).

The extensive literature on TRAIL, its cell surface death receptors (DR4 and DR5), and its apparent specific cytotoxicity to malignant tumor cells provides a rationale to select DR5 and DR4 as biologically relevant targets. We have developed mAbs to both of these targets and have selected mAbs that appear





**Fig. 7** A, an apparently viable area of a 2LMP tumor xenograft from an untreated nude mouse; B, a viable area of a 2LMP xenograft tumor from an animal treated with Adriamycin only. Like the untreated tumor, few cells exhibited apoptosis (dark brown); C, a viable area of a 2LMP xenograft tumor from an animal treated with paclitaxel only. Like the untreated tumor, few cells exhibited apoptosis (dark brown); D, a characteristic area of tumors from animals treated only with TRA-8 antibody at a dose of 100  $\mu\text{g}$  administered twice with a 3-day interval between injections. A high percentage of cells demonstrates apoptosis; E, a characteristic area of tumors from animals treated with the TRA-8 antibody plus Adriamycin. Just as in tumors treated only with TRA-8, a high percentage of cells are undergoing apoptosis; F, a characteristic area of tumors from animals treated with the TRA-8 antibody plus paclitaxel. Just as in tumors treated only with TRA-8, a high percentage of cells are undergoing apoptosis. Magnification:  $\times 400$ .

capable of mediating apoptosis *in vitro* without the need for cross-linking (33). This report provides the first description of *in vitro* and *in vivo* antitumor activity of the murine anti-DR5 reagent, TRA-8. A previous study described the activity of a second anti-DR4 antibody (35).

TRA-8 was found to react with all nine breast cancer cell lines examined, and these cell lines were found to have a range of *in vitro* sensitivity to antibody-mediated cytotoxicity similar to the variability that has been reported with TRAIL (19, 27, 31, 44–49). Breast cancer lines were not unique regarding TRA-8 antibody-mediated cytotoxicity in that we have observed that cell lines from brain, colon, prostate, pancreas, and cervix tumors have shown similar responses.<sup>4</sup>

Coincubation of TRA-8 and Adriamycin or paclitaxel produced enhancement of cytotoxicity compared with either agent alone in TRA-8 sensitive breast cancer cell lines (Figs. 2, A and B). The enhancement was synergistic in certain cell lines and additive in others (Table 1), although we did not carry out extensive optimization of *in vitro* conditions in lieu of moving to *in vivo* studies.

For the *in vivo* studies, we used the 2LMP breast cancer cell line that was developed as a more aggressive subclone derived from MDA-MB-231 (50). This cell line had moderate expression of DR5 (Fig. 1A) and was sensitive to TRA-8-induced cytotoxicity *in vitro* (Fig. 1B). The 2LMP cell line had a dose-dependent cytotoxicity with either Adriamycin (Fig. 2A)

or paclitaxel (Fig. 2B), and the combination of TRA-8 and Adriamycin or paclitaxel produced additive enhancement of cytotoxicity (Fig. 2, A and B and Table 1). The study design used well-established s.c. 7-day tumors that were  $\sim 60 \text{ mm}^2$  in size and exhibited modest chemotherapy or radiation antitumor efficacy (Table 2), reflecting the aggressive nature of the *in vivo* model.

Our initial studies with TRA-8 as a single agent demonstrated significant inhibition of tumor growth in three independent experiments ( $P < 0.004$ ) at a dose of 200  $\mu\text{g}$  (8 mg/kg) given six times over 3 weeks. A dose of 600  $\mu\text{g} \times 6$  (24 mg/kg) was no better (Fig. 3), and lower doses had less efficacy.<sup>5</sup>

We then carried out chemotherapy and TRA-8 single agent and combination therapies. Neither Adriamycin nor paclitaxel produced significant growth inhibition compared with controls while producing striking tumor inhibition and tumor regression when combined with TRA-8 (Figs. 4 and 5). The combination of Adriamycin and TRA-8 fulfilled the criteria for *in vivo* synergism ( $P < 0.001$ ) and produced four of eight complete regressions of tumor. The combination of paclitaxel and TRA-8 produced similar effects, although the interaction met criteria for additive effects and included three of eight complete regressions. These effects are similar to or greater than reports of TRAIL combinations with chemotherapy agents, although such comparisons are difficult, given different drugs, schedules, and tumor models. We have similar observations with COLO 205, a

<sup>4</sup> D. J. Buchsbaum and P. G. Oliver, unpublished observations.

<sup>5</sup> D. J. Buchsbaum and C. J. Hammond, unpublished data.

human colon cancer cell line, indicating that these effects are not specific for 2LMP (51).

The aggregate analysis in 166 experimental animals confirms the individual experiment observations (Table 2). We were impressed with the frequency of complete regressions in this well-established and chemotherapy-resistant model and, therefore, carried out extended follow-up of all animals for  $\geq 148$  days (Table 2). There was 1 of 42 control animals that had a complete regression, and this animal did not have tumor relapse; 1 animal of 54 who received chemotherapy and/or radiation had a transient complete regression. In contrast, 41% of animals receiving TRA-8 alone or in combination with chemotherapy and/or radiation had complete regressions (28 of 68 animals). Furthermore, 21% of these animals had no evidence of tumor recurrence over 148–192 days of observation.

Animals with complete regressions who later relapsed generally had time to recurrence of 2–4 weeks, and no relapses occurred beyond 101 days of remission. The TRA-8 regimens complete regression rate, rate of recurrence-free complete regressions, and effects on tumor doubling time were all statistically different from single agents or non-TRA-8 combinations. It is difficult to compare these results with studies of TRAIL and TRAIL combinations, given the lack of complete regression data and/or long-term experimental designs.

It is clear that TRAIL and monoclonal antibodies to death receptors are going to be studied in clinical trials as prospective therapeutic agents. The DR5 receptor has been reported to be expressed in a broad array of normal tissues and tumor cells (4, 52). In preliminary fashion, we have noted TRA-8 cell membrane and cytoplasmic staining in 22 of 22 breast cancer tumor specimens and similar binding in normal breast epithelial cells.<sup>6</sup> The TRAIL studies will be unique in that such a molecule has not been given to humans previously, and characteristics like kinetics, biodistribution, and dose-dependent tumor accessibility are unknowns. Monoclonal antibodies have a background in clinical studies to address these variables, whereas both agents will need to address toxicity and efficacy issues. The data in this report support the development of TRA-8 and other death receptor antibodies as potential therapeutic agents, either as single agents or in combination with chemotherapy and/or radiation. TRA-8 is a murine antibody and as such will require a “humanized” version that is currently in production.<sup>7</sup> In addition, Human Genome Sciences has announced initiation (53) of a Phase I trial of human anti-DR4 mAb as well. TRAIL and death receptor monoclonal antibodies are attractive candidates as molecular targeted strategies for cancer therapy.

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<sup>6</sup> W. E. Grizzle, unpublished observations.

<sup>7</sup> Sankyo Co., Ltd., personal communication, 2003.

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# Clinical Cancer Research

## Antitumor Efficacy of TRA-8 Anti-DR5 Monoclonal Antibody Alone or in Combination with Chemotherapy and/or Radiation Therapy in a Human Breast Cancer Model

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